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List of publications

Articles

Liu M.C., Pirrello J., Mila I., Roustan J.P., Li Z.G., Latch A., Pech J.C., Bouzayen M., and Regad F. (2013) A dominant repressor version of the tomato *Sl-ERF.B3* gene confers ethylene hypersensitivity via feedback regulation of ethylene signaling and response components. *Plant J*, 76, 406-419.

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Deng W., Yan F., **Liu M.C.**, Wang X.Y., Li Z.G. (2012) Down-regulation of SlIAA15 in tomato altered stem xylem development and production of volatile compounds in leaf exudates. *Plant Signal Behav*, 7(8).

Tang L., Li J., Khalil R., Yang Y.W., Fan J., **Liu M.C.**, Li Z.G. (2012) Cloning and functional analysis of *CDS_CCI2*: A *Tanacetum cinerariaefolium* chrysanthemyl diphosphate synthase gene. *Plant Growth Regul*, 67, 161-169.

Oral communication

Liu M.C., Pirrello J., Mila I., Bouzayen M., Regad F. (2012). The transcriptional repressor *ERF.B3-SRDX* alters ethylene sensitivity and modulates developmental processes in tomato. French Society of Plant Biology 7th Congress for Young Scientists. July 4th-6th, 2012, Grenoble, France.

Poster

Liu M.C., Pirrello J., Mila I., Bouzayen M., Regad F. (2012). The role of tomato *Sl-ERF.B3* in mediating ethylene responses uncovered by a chimeric repressor construct. La journée de l'école doctorale SEVAB, 2012, Toulouse, France.

Résumé

Les derniers acteurs de la voie de signalisation à l'éthylène sont des facteurs de transcription appelés ERF (Ethylene Response Factors). La connaissance de leur rôle spécifique dans la régulation des processus développementaux dépendant de l'éthylène reste limitée. Les travaux présentés dans la thèse concernent la caractérisation fonctionnelle du gène *Sl-ERF.B3*, un membre de cette grande famille de régulateurs transcriptonnels dans la tomate (*Solanum lycopersicum*). Utilisant une stratégie répresseur dominant ; il est montré en particulier que ce gène intervient dans la mise en place de la réponse à l'éthylène et dans le contrôle de la maturation du fruit. L'expression d'une construction ERF.B3-SRDX, une version chimérique de Sl-ERF.B3 fusionné à un domaine répresseur de type EAR, entraine des phénotypes pléotropiques aussi bien dans la signalisation de l'éthylène que dans le développement des parties végétatives et des organes reproducteurs. Ainsi, une altération de la triple réponse à l'éthylène est constatée chez les lignées transgéniques et au stade adulte, les plantes présentent des phénotypes d'épinastie des feuilles, de sénescence prématurée des fleurs et d'abscission accélérée des fruits. L'ensemble de ces observations est corrélée avec une modification de l'expression de gènes impliqués dans la biosynthèse et la réponse à l'éthylène. Ces données suggèrent que ERF.B3 intervient dans un mécanisme de rétro-control de la réponse à l'éthylène en agissant à la fois sur les gènes de biosynthèse et de signalisation de l'hormone. Au niveau du fruit, la sur-expression d'ERF.B3-SRDX entraine une modification du processus de maturation avec un retard notable de l'avènement de l'acquisition de la compétence à murir. Cependant, une fois la maturation initiée, elle s'accompagne d'une forte production d'éthylène et d'une accélération du ramollissement du fruit. A l'inverse, l'accumulation de pigment est inhibée par altération de la voie de biosynthèse des caroténoïdes. Ces données phénotypiques sont corrélées avec le niveau d'expression des gènes clés impliqués dans ces processus. Les résultats indiquent que dans les lignées transgéniques, il y a découplage de certaines caractéristiques de la maturation du fruit et permettent de mettre en lumière le rôle d'ERF.B3 dans la régulation des processus de développement dépendant de l'éthylène chez la tomate.

Abstract

Ethylene Response Factors (ERFs) are known to be the last transcription factors of the ethylene transduction pathway. Their specific role in ethylene-dependent developmental processes remains poorly understood. This work demonstrated a specific role of *Sl-ERF.B3*, a member of the ERF gene family in tomato (*Solanum lycopersicum*), in mediating ethylene response and fruit ripening through a dominant repressor strategy. *ERF.B3-SRDX* dominant repressor etiolated seedlings displayed partial constitutive ethylene-response in the absence of ethylene and adult plants exhibited typical ethylene-related alterations such as leaf epinasty, premature flower senescence and accelerated fruit abscission. The multiple symptoms related to enhanced ethylene sensitivity correlate with the altered expression of ethylene biosynthesis and signaling genes, suggesting the involvement of *Sl-ERF.B3* in a feedback mechanism regulating components of ethylene production and response. In addition, over-expression of *ERF.B3-SRDX* in tomato results in alterations in both fruit morphology and ripening process. The attainment of competence to ripen is dramatically delayed in *ERF.B3-SRDX* fruits but once ripening proceeds it is associated with high climacteric ethylene production and enhanced fruit softening while pigment accumulation is strongly reduced. Moreover, a number of genes involved in the fruit ripening process showed expression pattern deviating from that of wild type. These data suggest a putative role of *Sl-ERF.B3* in the transcriptional network underlying the ripening process and uncover a mean for uncoupling some of the main features of fruit ripening such as fruit softening and pigment accumulation. Overall, the study highlighted the importance of an ERF gene in ethylene-mediated developmental processes such as plant growth and fruit ripening.

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List of abbreviations

1-MCP: 1-Methyl Cyclopropane

ABA: Abscissic Acid

ACC: Acide 1-AminoCyclopropane-1-Carboxilique

ACO: Acide 1-AminoCyclopropane-1-Carboxilique Oxydase

ACS: Acide 1-AminoCyclopropane-1-Carboxilique Synthase

AP2: Apetala2

ARC: Age-Related Changes

ARR: Age Related Resistance

CBF: C-repeat Binding Factor

CNR: Colorless Non Ripening

CTR: Constitutive Triple Response

CRES-T: Chimeric Repressor Silencing Technology

DAB: Delayed Floral Organ Abscission

DPA: Day Post Anthesis

EAR: ERF Amphiphilic Repression domain

EBF: E3-Binding F-box protein

EBS: Ethylene Binding Site

EIN: Ethylene insensitive

ER: Ethylene Regulated

EREBP: Ethylene Response Element Binding Protein

ERF: Ethylene Response Factor

ERN: ERF Required for Nodulation

ERS: Ethylene Response Sensor

EST: Express Sequence Tag

ETR: Ethylene Triple Response

GA: Gibberellic Acid

GR: Green Ripe

GUS: ß-glucuronidase

HR: Hypersensitive Response

HEG: Homing Endonuclease Genes

ISR: Induced systemic Resistance

JA: Jasmonic Acid

JERF: Jasmonate Ethylene Response Factor

KO: Knock Out

LOX: Lipoxigenase

MAP: Mitogen-Activated Protein

MAPK: Mitogen-Activated Kinase

MAPKKK: Mitogen-Activated Kinase Kinase Kinase

1-MCP: 1-methyl cyclo propane

NOR: Non-Ripening

NPA: Naphthylphthalamic Acid

NR: Never Ripe

IAA: Indol Acetic Acid

PAG: Photosynthesis Associated Genes

PERE: Primary Ethylene Response

PG: Polygalacturonase

PR: Pathogenesis Related

RIN: Ripening Inhibitor

QTL: Quantitative Trait Loci

SA: Salicylic Acid

SAG: Senescence Associated Genes

SAR: Systemic aquired Resistance

SCF: Skp1-Cullin-F-box

SRDX: SUPERMAN Repression Domain X

TERF: Tomato Ethylene Response Factor

VWRE: Vascular system specific and Wound Responsive *cis*-Element

WT: Wild Type

Main components of the thesis

The plant hormone ethylene is involved in many developmental processes and plays a critical role in a wide range of physiological responses, including seed germination, cell elongation, flowering, fruit ripening, organ senescence, abscission, root nodulation, programmed cell death, and response to abiotic stresses and pathogen attacks. After its biosynthesis, ethylene is perceived by a receptor which generates a signal leading to the activation of a transduction machinery that triggers specific biological responses. To improve our understanding of how ethylene is able to mediate plant growth and fruit ripening it is important to study specific function of the molecular components involved in ethylene signaling pathway. Ethylene Response Factors (ERFs) are the last known downstream components of the ethylene signal transduction pathway. Being encoded by one of the largest plant families of transcription factors, ERF proteins are the suited step where the diversity and specificity of ethylene responses may originate. Using tomato (*Solanum lycopersicum*) as a model plant, my Ph.D research project aimed at deciphering the role of *Sl-ERF.B3*, a tomato Ethylene Response Factor, during plant growth and fruit development using advanced reverse genetics and genomics methodologies.

The body of this thesis consists of four main chapters. The first chapter is a bibliographic review on ethylene. In this chapter, up-to-date knowledge regarding ethylene biosynthesis, perception and signal transduction is presented. The main roles of ethylene in regulating different plant growth and development processes and the interaction of this hormone with other phytohormones were described. The advantage of using tomato as plant model in my study dealing with ethylene-mediated fruit development processes is emphasized.

Chapter II focuses on addressing the physiological significance of *Sl-ERF.B3* and its potential role in mediating ethylene responses. This chapter is presented on the form of an article which has been recently accepted for publication in *The Plant Journal*. Using a dominant repressor strategy, it is demonstrated in this work that *Sl-ERF.B3* gene controls ethylene sensitivity via feedback regulation of ethylene signaling and response components. It is shown that the expression of a dominant repressor version of *Sl-ERF.B3* (*ERF.B3-SRDX*) in the tomato results in pleiotropic ethylene responses and vegetative and reproductive growth phenotypes. The multiple symptoms related to

enhanced ethylene sensitivity correlate with the altered expression of ethylene biosynthesis and signaling genes, suggesting the involvement of *Sl-ERF.B3* in a feedback mechanism regulating components of ethylene production and response. Moreover, Sl-ERF.B3 is shown to modulate the transcription of a set of *ERFs* revealing the existence of a complex network interconnecting different *ERF* genes.

Chapter III mainly describes the putative role of *ERF* family genes in controlling fruit maturation and ripening in tomato and more particularly the role of *Sl-ERF.B3* in fruit development and ripening. In this chapter, it is shown that most of *ERF* family genes display a ripening-associated expression pattern suggesting their involvement in the ripening process. Specific roles for *ERF* gene family members in fruit development and ripening is further revealed by the functional characterization of *Sl-ERF.B3*, a member tomato *ERF* genes. *Sl-ERF.B3* displays a pattern of expression that is quite distinctive from other *ERF* genes, its transcript accumulation being induced at the breaker stage and maintained at a high level in all stages of fruit ripening, suggesting that its expression is continuously required for the modulation of the ripening-regulated genes all along the ripening process. The study indicate that over-expression of a chimeric repressor construct of *Sl-ERF.B3* (*ERF.B3-SRDX*) in tomato results in alterations in fruit shape and size, abnormal seed morphology, orange ripe fruits, and accelerated fruit senescence. Moreover, genes involved in different metabolic pathways, such as carotenoid biosynthesis, ethylene synthesis, and cell wall metabolism exhibit altered mRNA accumulation patterns in transgenic lines during fruit ripening process. Further characterization at the molecular level of the dominant repressor lines, indicated that *Sl-ERF.B3* impacts the ripening process through mediating ripening-associated genes.

The last chapter presents general conclusions and perspectives of the work performed in this thesis.

Objectives of the study

Ethylene mediates diverse developmental and physiological processes throughout the entire life cycle of plants. After its synthesis, ethylene is perceived and its signal transduced through a transduction machinery that triggers specific biological responses. Significant progress has been made in our understanding of how plants perceive and transduce the ethylene signal (Benavente and Alonso, 2006; Ju *et al.,* 2012). Studies on components of ethylene signaling have revealed a linear transduction pathway that leads to the activation of transcriptional regulators belonging to the Ethylene Response Factor (ERF) type. ERF proteins which are responsible for regulating the transcription of primary ethylene-responsive genes are the last known downstream components of ethylene transduction pathway. Since the upstream components of the ethylene transduction pathway are common to all ethylene responses, the apparent simplicity of the ethylene signaling pathway cannot account for the wide diversity of ethylene responses. A tempting hypothesis is that differential responses to ethylene are directed at the transcriptional levels. Being encoded by one of the largest family of plant transcription factors, ERF proteins are the most suited step of the ethylene signaling pathway where the diversity and specificity of ethylene responses may originate.

In an attempt to understand the molecular basis of ethylene-regulated plant growth and fruit development, 28 tomato ERF genes have been isolated in the laboratory of Genomics and Biotechnology of the Fruit (GBF) and these genes have been shown to fall into 9 subclasses defined by distinct structural features. Previous studies on these genes have provided some molecular clues on how ERFs can contribute to the specificity and selectivity of ethylene responses through (i) the differential expression of gene family members, (ii) the ability to negatively or positively impact transcriptional activity and, (iii) the capacity to select with specificity target genes based on the nucleotide environment of the GCC-box (Pirrello *et al.,* 2012). The diversity of their transcriptional activity and expression patterns suggest that ERFs possess the necessary features for channeling ethylene signaling to a selected set of genes required for the appropriate developmental responses or the desired responses to environmental cues. To date, however, most of the members of the ERF family have yet to be studied, the specific role of individual ERF in

controlling ethylene responses and plant developmental processes remains poorly understood.

The aim of this study was to unravel the molecular mechanism underlying the specificity of ethylene responses during plant development and fruit ripening. The function significance of ERF genes is addressed in the tomato using advanced reverse genetics and genomics methodologies, with a special focus given to the role of ERFs in fruit development and ripening. My own project was dedicated to the functional characterization of *Sl-ERF.B3*, a tomato ethylene-inducible *ERF* gene previously shown to display a strong binding affinity to GCC-box-containing promoters. Specifically, the study addresses two main questions: (i) Is *Sl-ERF.B3* involved in mediating ethylene responses and if so by which molecular mechanism it performs this function? (ii) Does *Sl-ERF.B3* impact ethylene-dependent developmental processes such as fruit ripening?

Chapter I

Bibliographic review

Ethylene, the simplest olefin, has been recognized as a plant hormone for over a century. The discovery of the biological activity of ethylene came about in the 19th century as leaks in illuminating gas caused premature senescence and defoliation of plants in the greenhouse and of trees near gas lines (Abeles *et al.*, 1992). In 1901, the Russian physiologist Dimitri Neljubow demonstrated that ethylene causes the "triple response" in dark-grown pea seedlings with inhibited epicotyl elongation, radial swelling, and a horizontal growth habit. The first evidence that ethylene was produced by plants was the observation that the ripening of bananas was promoted by gases produced from oranges. Definitive chemical proof that ethylene is produced by plants was reported by the English scientist Gane in 1934. It is now known that ethylene is produced by all cells during plant development with the highest rates being associated with meristematic, stressed, or ripening tissues (Abeles *et al.,* 1992).

In the plant life cycle, ethylene is involved in a wide range of plant growth and developmental processes, including seed germination, cell elongation, flowering, fruit ripening, organ senescence, abscission, root nodulation, programmed cell death, and response to abiotic stresses and pathogen attacks (Johnson and Ecker, 1998; Bleecker and Kende, 2000; Lin *et al.*, 2009). To better understand the roles of ethylene in plant functions, it is important to know how this gaseous hormone is synthesized, transduced and able to regulate so many plant development processes.

1. Ethylene biosynthesis

Ethylene biosynthesis has been intensively studied in plants and the establishment of S-adenosyl-L-methionine (*S*-Ado Met) and ACC as the biological precursors of ethylene is thought to be the main breakthroughs in the ethylene biosynthesis pathway. As it is shown in Figure 1, ethylene is synthesized from the amino acid methionine. In the ethylene biosynthesis pathway, methionine is converted to *S*-AdoMet by *S*-AdoMet synthetase and *S*-AdoMet is then converted to 1-aminocyclopropane-1-carboxylic acid (ACC) and 5'-deoxy-5 methylthioadenosine (MTA) by the enzyme 1-aminocyclopropane-1-carboxylase synthase (ACS) (Adams and Yang, 1979). Through the Yang cycle, MTA can be recycled to methionine, which allows high rates of ethylene

production without depletion of the endogenous methionine pool (Miyazaki and Yang, 1987). At the last step, ACC is further converted to ethylene by ACC oxidase (ACO), with CO2 and cyanide as by-products. In ethylene biosynthesis pathway, the rate-limiting step of ethylene synthesis is the conversion of *S*-AdoMet to ACC by ACC synthase, but there are situations where ACO is absent and ACS and ACO are induced, for example by wounding and the ripening stimulus (Alexander and Grierson, 2002a; Lin *et al.*, 2009). In most of the species investigated, including *Arabidopsis*, ACS and ACO are members of large and small multigene families, respectively (De Paepe and Van der Straeten, 2005). Both positive and negative feedback regulation of ethylene biosynthesis have been reported in different plant species (Wang *et al.*, 2002).

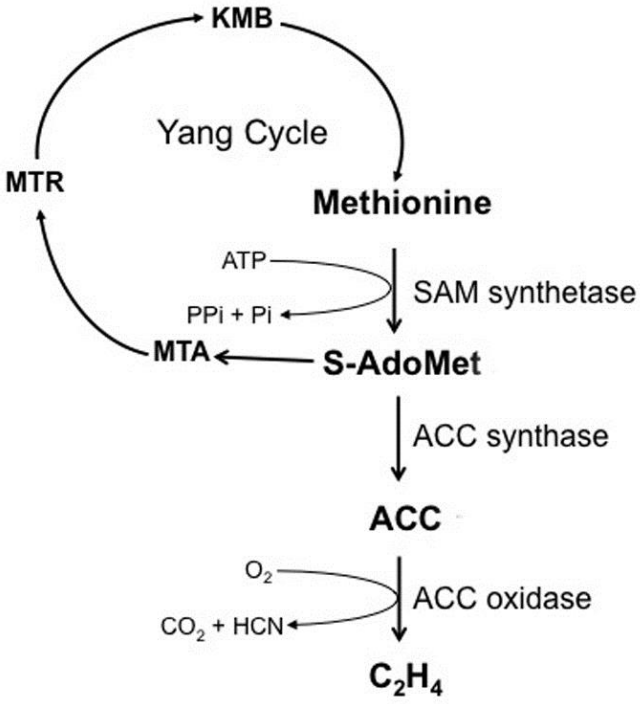

Figure 1. Main steps of ethylene biosynthesis pathway. *S*-adenosyl-methionine (*S*-AdoMet) is synthesized from the methionine by the *S*-adenosyl-methionine synthetase (SAM synthetase) with one ATP molecule expensed per *S*-AdoMet synthesized. *S*-AdoMet is then converted to 1-aminocyclopropane-1-carboxylicacid (ACC) by ACC synthase (ACS), 5'-methylthioadenosine (MTA) being a by-product. MTA is recycled to methionine by successive enzymatic reactions involving various intermediates (MTR, 5-methylthioribose; KMB, 2-keto-4-methylthiobutyrate), which constitute the methionine (Yang) cycle. Ethylene production is catalyzed by the ACC oxidase using ACC as substrate, and generates carbon dioxide and hydrogen cyanide (Arc et al., 2013).

2. Ethylene signaling pathway

After its synthesis, ethylene is perceived and its signal transduced through transduction machinery to trigger specific biological responses. Insight into the ethylene signaling pathway has been mainly provided by molecular genetic studies in *Arabidopsis thaliana*. Dissection of the ethylene signaling pathway began with the isolation of ethylene-

response mutants, using genetic screens that are based on the "triple response" of ethylene-treated seedlings (Bleecker *et al.*, 1988; Guzmán and Ecker, 1990). The triple response is a striking morphology adopted by germinating seedlings exposed to ethylene in the dark. In Arabidopsis, the triple response consists of shortening and thickening of the hypocotyl and root, exaggeration of the apical hook curvature and proliferation of root hairs (Figure 2). Decades of scientific research devoted to deciphering how plants are able to sense and respond to ethylene have culminated in the establishment of one of the best characterized signal transduction pathways in Arabidopsis (Bleecker *et al.*, 1988; Guzmán and Ecker, 1990; Chang *et al.*, 1993; Roman *et al.*, 1995; Chao *et al.*, 1997; Sakai *et al.*, 1998; Alonso *et al.*, 1999). In the currently accepted model, ethylene signaling pathway starts with the ethylene perception by their specific receptors, which have been shown to activate the hormone transduction pathway through releasing the block exerted by CTR1 on EIN2 (Solano and Ecker, 1998; Ju *et al.*, 2012).

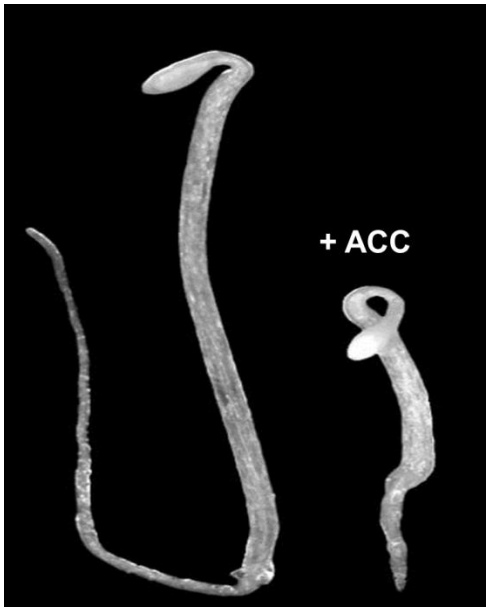

Figure 2. Phenotypes of dark-grown three-day-old seedlings of Arabidopsis thaliana. The plant on the left was grown without hormonal treatment, whereas the plant on the right was exposed to 10 μM ethylene precursor ACC and thus shows a typical triple response (Benavente and Alonso, 2006).

The release of EIN2 then activates EIN3/EIL1 primary transcription factors, resulting in the expression of secondary transcription factors, namely ERFs, which regulate the expression of downstream ethylene-responsive genes (Solano *et al.*, 1998; Alonso *et al.*, 2003). Through using a combination of genetic, biochemical and molecular approaches, a

pathway that transduces the ethylene signal from the endoplasmic reticulum membrane to the nucleus has been uncovered (Figure 3).

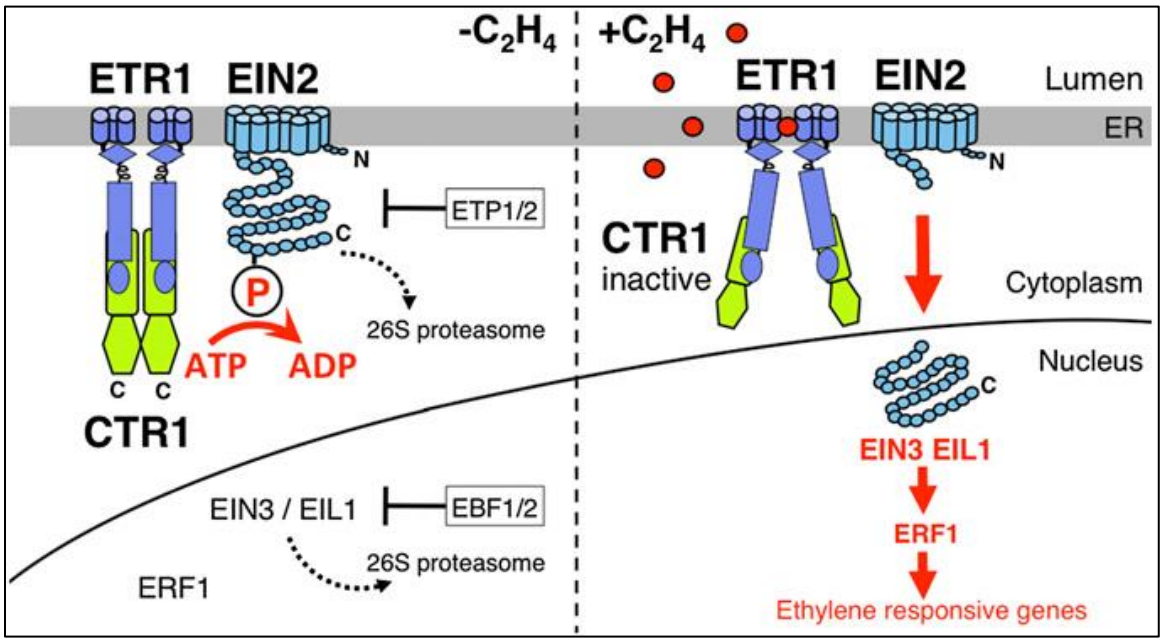

Figure 3. Model of ethylene signaling. In the absence of ethylene (Left), the ethylene receptors (e.g., ETR1) at the ER membrane activate the CTR1 protein kinase, a dimer, which phosphorylates the C-terminal domain of EIN2, preventing its nuclear localization. Without ethylene, EIN2 is targeted for 26S proteasomal degradation by F-box proteins ETP1/2. Transcription factors EIN3/EIL1 are also targeted for degradation by F-box proteins EBF1/2. In the presence of ethylene (Right), the receptors are inactivated and therefore the CTR1 kinase is no longer active. The absence of phosphorylation on EIN2 results in EIN2 C terminus being cleaved and localizing to the nucleus where it can activate the downstream transcriptional cascade (Ju *et al.*, 2012).

2.1 Ethylene perception is mediated by a small family of receptors

Perception of ethylene in plants is achieved by several related membrane-bound histidine kinases. In Arabidopsis, ethylene is perceived by a family of five receptors (ETR1, ETR2, ERS1, ERS2 and EIN4) that share similarity with bacterial two-component regulators (Chang *et al.*, 1993; Hua *et al.*, 1995; Sakai *et al.*, 1998; Hua and Meyerowitz, 1998; Chang and Stadler, 2001). All of the initial receptor mutants identified where gain-of-function mutants exhibiting dominant ethylene insensitivity.

Single loss-of-function receptor mutants in Arabidopsis show no visible phenotypes likely due to functional redundancy. The presence of triple, quadruple or *etr1/ers1* double mutants results in a constitutive ethylene response in the absence of increased ethylene production (Wang *et al.*, 2003). This evidence is consistent with a model where the receptors are negative regulators of ethylene response and that ethylene receptors actively suppress the response in the absence of ethylene (Hua and Meyerowitz, 1998). Ethylene was found to bind receptor through a transition metal copper co-factor. Ethylene binding results to a modification of the coordination chemistry of the copper in the N-terminal region. This modification is transmitted to the C-terminal region (Rodriguez et al., 1999) and initiates the ethylene response. On the basis of structural similarities, the receptor family can be divided into two subfamilies (Figure 4). Subfamily 1, consisting of ETR1 and ERS1, features three hydrophobic transmembrane domains in the N-terminal region, where ethylene binding occurs (Schaller and Bleecker, 1995; Hall *et al.*, 2000), and a well conserved histidine kinase domain in C-terminal region. Subfamily 2, which includes ETR2, ERS2, and EIN4, has four hydrophobic domains in the N-terminal region and a non-conserved His-kinase domain, in which some consensus amino acid residues essential for His-kinase activity are lacking (Moussatche and Klee, 2004; Xie *et al.*, 2006). The fact that the subfamily 2 receptors ETR2, ERS2 and EIN4 have Ser/Thr kinase activity in vitro (Gamble *et al.*, 1998; Moussatche and Klee, 2004) supports the notion that the subfamily 2 of receptors may function not as histidine kinases but possibly as serine/threonine kinases. In addition, ETR1, ETR2 and EIN4 possess a C-terminal receiver (Figure 4).

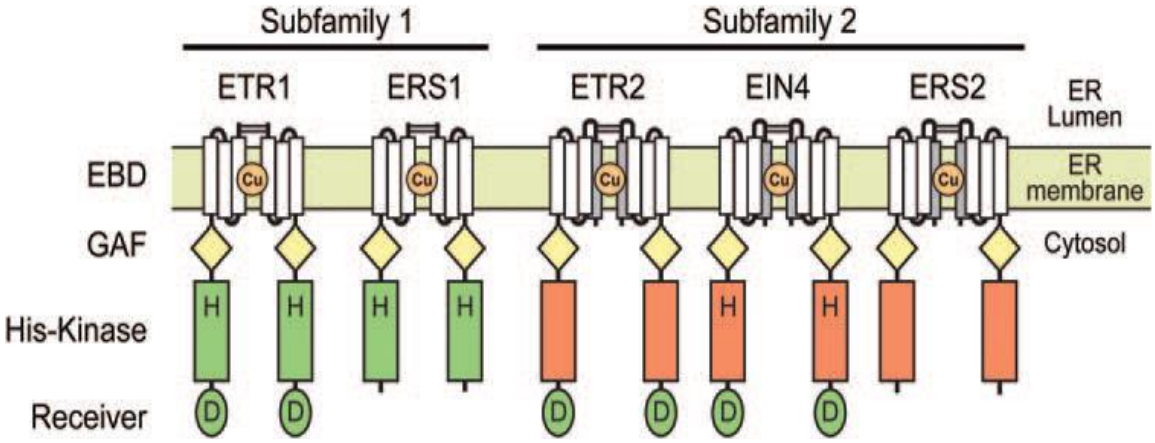

Figure. 4 Ethylene receptor family of Arabidopsis. The ethylene receptor family of Arabidopsis is divided into subfamilies 1 and 2 based on phylogenetic analysis and structural features. Receptors are shown as homo-dimers. The ethylene-binding domain (EBD) is found within the conserved transmembrane domains (white rectangles), and includes a copper cofactor (Cu); subfamily 2 receptors have an additional predicted transmembrane domain (grey rectangle) that may function as a signal sequence. All five members of the ethylene receptor family have a GAF domain (yellow diamond) implicated in protein–protein interactions. His kinase domains are indicated by green or red rectangles, green indicating a functional His kinase domain and red indicating a diverged His kinase domain. The receiver domains (ovals) have the conserved residues required for function and are therefore colored green. Conserved His (H) and Asp (D) phosphorylation sites are indicated if present (Shakeel *et al.*, 2013).

The C-terminal domains of the ethylene receptors show sequence homology to bacterial two-component system histidine kinases. These systems are generally constituted of a sensor molecule containing an histidine kinase domain which autophosphorylates itself in reaction to a stimuli, and a response regulator containing a receiver domain which accept, the residue phosphate from the histidine sensor (Pirrung, 1999). The histidine kinase domain of the ethylene receptors has been shown to be important for the association of the receptors with CTR1 (Clark *et al.*, 1998; Gao *et al.*, 2003; Zhong *et al.*, 2008). It was demonstrated that a truncated ETR1 lacking the histidine kinase and receiver domain failed to rescue a *etr1-6;etr2-3;ein4-4* triple loss-of-function mutant, while the truncated ETR1 lacking only the receiver domain was able to restore normal growth of the triple mutant in air, and the transgenic plants show ethylene hypersensitivity (Qu and Schaller, 2004). These results demonstrate that the kinase domain is necessary for signal transmission by the receptor and that the receiver domain was not essential for restoring

ethylene responsiveness. However, while ethylene may inhibit His kinase activity in ETR1 (Voet-van-Vormizeele and Groth, 2008), it seems that His kinase activity is not needed for signaling (Wang *et al.*, 2003; Qu and Schaller, 2004) but does have a role in growth recovery after ethylene removal (Binder *et al.*, 2004) and in regulation of growth (Qu and Schaller, 2004; Cho and Yoo, 2007). Kim *et al.*, (2011) also showed that ETR1 histidine kinase activity and phosphotransfer through the receiver domain are not required to rescue ethylene-mediated nutations. Hall *et al.*, (2012) demonstrated that the histidine kinase activity of ETR1 is not required for but plays a modulating role in the regulation of ethylene responses. By comparing the dominant-negative effect of ETR1-1 (1–349) in a two receptor double LOF mutant background: etr1-7;ers1-2 and etr1-7;ers1-3, Xie *et al.*, (2006) showed that the truncated ETR1 without the histidine kinase domain must rely on the remaining ERS1, which has the histidine kinase activity, to repress ethylene signaling. Indeed, up till now, the exact function of the receptor histidine kinase domains and the role of the receptor heterodimer interaction in ethylene signaling are still open questions. In tomato, there are six ethylene receptors (LeETR1-2, NR, and LeETR4-6) which can be broken down into two subfamilies. The predicted structures of these tomato receptors are very similar to those in Arabidopsis (Klee and Tieman, 2002). LeETR1, 2 and NR are members of Subfamily I which contain three transmembrane domains and all of the conserved residues of known histidine kinases. Subfamily II, consisting of LeETR4-6, contains four transmembrane domains and degenerate histidine kinase domains with LeETR5 containing none of the conserved residues. Moreover, NR is the only member of the family that does not contain the carboxy-terminal receiver domain whose function in ethylene signaling is still unknown. In contrast to the Arabidopsis ethylene receptors that have been considered to be functionally redundant, the tomato ethylene receptors NR, LeETR4, and LeETR6 are preferentially expressed in fruit and have been suggested to have unique roles during ripening (Tieman *et al.*, 2000; Kevany *et al.*, 2007).

2.2 CTR1 acts as a negative regulator of ethylene signaling pathway

In the ethylene linear signaling pathway, acting directly downstream of the ethylene receptors is the mitogen-activated protein kinase kinase kinase (MAPKKK) constitutive triple-response1 (CTR1) protein. CTR1 was identified by mutants that displayed the triple response morphology in the absence of exogenous ethylene. Loss-of-function *ctr1* mutations result in the constitutive activation of the ethylene response pathway in seedlings and adult plants, which indicates that the encoded protein acts as a negative regulator of ethylene signaling (Kieber *et al.*, 1993). The binding of ethylene to the receptors results in the inactivation of CTR1 and in turn activation of the downstream components of the pathway, thereby leading to ethylene responses (Kieber *et al.,* 1993; Huang *et al.,* 2003; Figure 5).

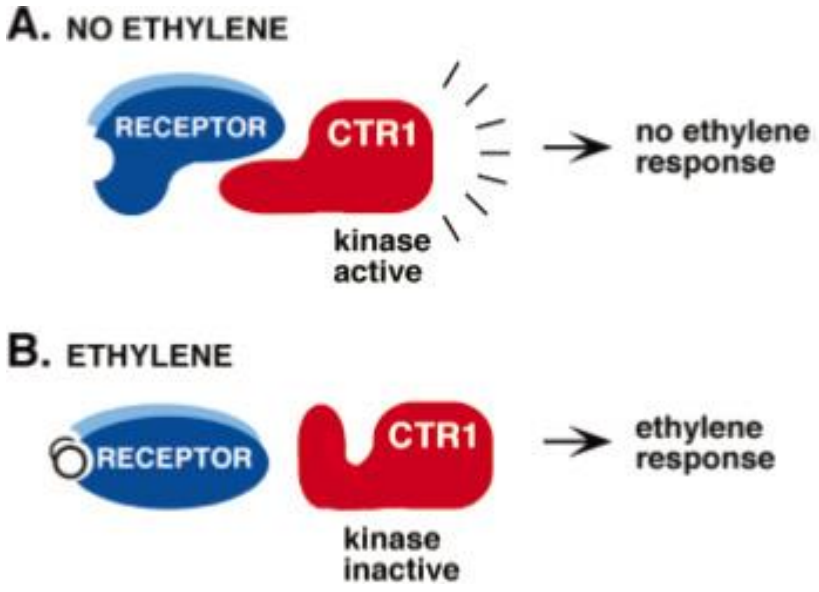

Figure 5. The binding of ethylene to the receptors results in ethylene response through inactivation of CTR1. When ethylene is not bound (A), the receptors interact and activate CTR1, which results in the inhibition of ethylene responses. When ethylene is bound (B), the receptors are inactive with respect to activating the downstream CTR1 protein (Chang and Stadler 2001).

CTR1 consists of a unique N-terminal regulatory domain and a C-terminal serine/threonine kinase domain. Although CTR1 contains no predicted transmembrane domains (Kieber *et al.*, 1993; Huang *et al.*, 2003), CTR1 is found at the ER membrane

due to its association with the ethylene receptors (Clark *et al.*, 1998; Huang *et al.*, 2003; Gao *et al.*, 2003; Zhong *et al.*, 2008). It has been demonstrated that the N-terminal regulatory domain of CTR1 can interact directly with the subfamily I ethylene receptors (ETR1 and ERS1) in the yeast two-hybrid assay (Clark *et al.*, 1998). Zhong *et al.*, (2008) also showed that ethylene receptors recruit tomato LeCTR proteins to the ER membrane through direct protein-protein interaction. Moreover, Mayerhofer *et al.*, (2012) proposed that the interaction of CTR1 dimers with the ethylene receptor dimers reinforces the receptor complex by promoting associations between neighboring ethylene receptors (Figure 6).

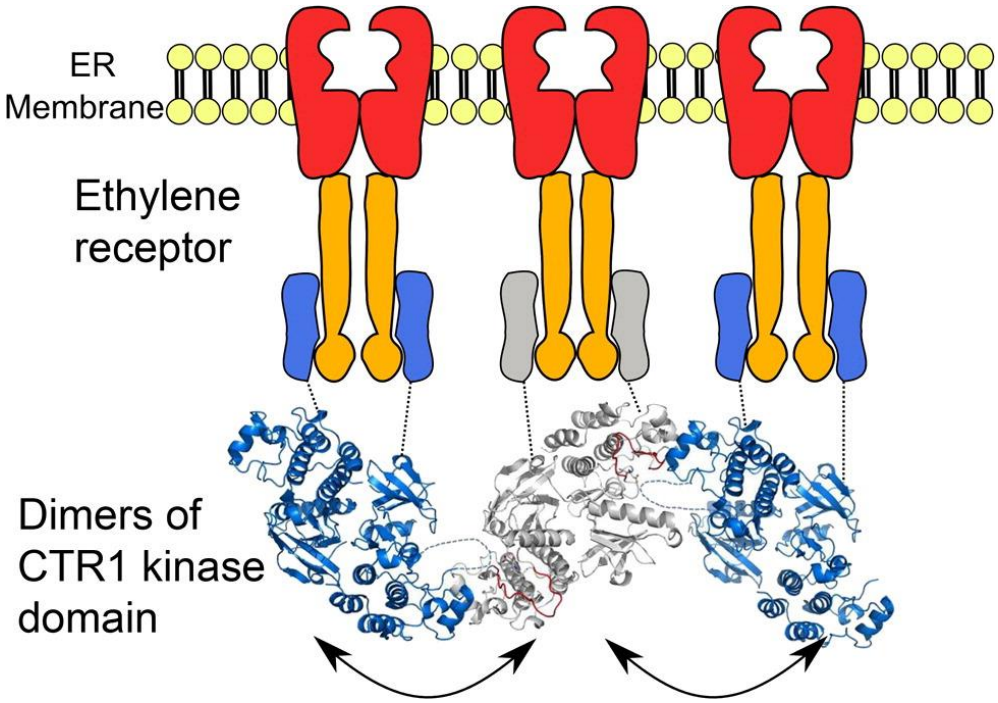

Figure 6. **Model of CTR1-mediated receptor oligomerization and cross talk.** Ethylene receptor dimers are shown as cartoons with the endoplasmic-reticulum-membrane embedded domains in red and the cytosolic domains in orange. Also shown as cartoons are the N-terminal domains of CTR1 (gray and blue), which interact with the ethylene receptors. The connection between the N-terminal CTR1 domains and their C-terminal kinase domains are indicated as dotted lines. Three consecutive CTR1 kinase dimers are depicted as ribbons as they form across the back-to-back and front-to-front interfaces in the crystal. The back-to-back interface dimer was placed at a receptor dimer and the activation interface connects neighboring receptors. Active CTR1-kd is depicted in gray and activation loops are in red. Inactive dimers of CTR1-D676N (colored blue) are positioned across the front-to-front interface, as observed in the CTR1-D676N crystals. The disordered activation loop is indicated by a dotted line (Mayerhofer *et al.*, 2012).

Although the serine/threonine kinase activity of CTR1 was demonstrated *in vitro* and shown to be essential for proper functioning of the receptors/CTR1 signaling complex, as kinase-inactive alleles of CTR1 also resulted in a constitutive response phenotype (Huang *et al.*, 2003), the molecular mechanism by which the receptors control CTR1 kinase activity remains unclear. The ethylene receptor-CTR1 association represents a novel combination of proteins that do not fit the existing paradigms for either the Raf-like CTR1 or the two-component receptors (Wang *et al.*, 2003; Wellbrock *et al.*, 2004; Schaller *et al.*, 2011). A likely mechanism for CTR1 activation could be that the receptors interact with CTR1 in an active conformation in the absence of ethylene. When the receptors bind ethylene and presumably undergo a conformational change, there could be a concomitant alteration in the conformation of CTR1 that turns off the CTR1 kinase activity (Gao *et al.*, 2003; Ju and Chang, 2012). It is conceivable that the histidine autophosphorylation induced by ethylene binding, as suggested by Hall *et al.,* (2012), plays a role in the conformational change that terminates CTR1 activation. Because structural studies have shown that the CTR1 kinase domain is a dimer when active (Mayerhofer *et al.*, 2012), a conformational change causing monomerization of CTR1 could be a possible mechanism for inactivation of CTR1.

While the main ethylene signaling pathway involves CTR1, it is worth pointing out that subtle effects of ethylene receptor signaling might occur via the two-component system's phosphotransfer proteins and response regulators in *Arabidopsis* (known as AHPs and ARRs, respectively). This is based on evidence that the ethylene receptors can interact with AHP proteins (Urao *et al.*, 2000; Scharein *et al.*, 2008) and that a response regulator, ARR2, might have a role as a positive regulator in modulating ethylene responses downstream of ETR1 (Hass *et al.*, 2004; Mason *et al.*, 2005). Thus, ethylene receptor signaling through AHPs and ARRs might represent an ethylene response pathway that bypasses CTR1 (Ju and Chang, 2012).

2.3 EIN2 is a central component of the signaling pathway and positively regulates ethylene responses

Based on genetic analyses, downstream of the receptors/CTR1 complexes there acts a positive regulator of ethylene responses, ETHYLENE INSENSITIVE2 (EIN2). EIN2 is required for all ethylene responses studied and constitutes a critical step in the signal transduction (Alonso *et al.*, 1999). EIN2 consists of an N-terminal integral membrane domain of 12 predicted transmembrane helices (residues 1–461) with sequence similarity to Nramp metal ion transporters, followed by a hydrophilic C-terminal domain (residues 462–1294) believed to be cytosolic (Alonso *et al.*, 1999; Ju *et al.,* 2012; Figure 7). Qiao *et al.,* (2009) reported that EIN2 can be stabilized by ethylene at the protein level, which protects it from proteasomal degradation mediated by two F-box proteins ETP1/2. EIN2 was also shown to reside at the ER membrane in tobacco leaves and is capable of interacting with the kinase domain of ethylene receptors (Bisson *et al.*, 2009; Bisson and Groth, 2010). Moreover, EIN2 was found to be required for the ethylene-induced EIN3/EIL1 protein stabilization (Guo and Ecker, 2003), by promoting the proteasomal degradation of EBF1/EBF2 in the nucleus (An et al., 2010).

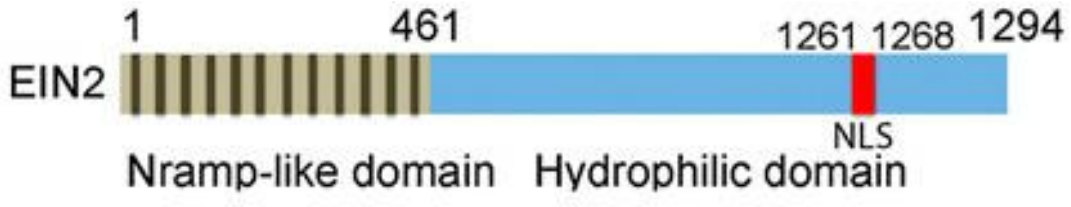

Figure 7. Cartoon of EIN2 protein domain structure. EIN2 consists of an N-terminal integral membrane domain of 12 transmembrane helices followed by a hydrophilic C-terminal domain containing a conserved nuclear localization signal (NLS; Ju *et al.,* 2012).

For more than a decade, although it has been well deciphered by genetic and double-mutant analyses that EIN2 is a central and most critical element of the ethylene signaling pathway and acts between the soluble serine/threonine kinase CTR1 and the EIN3/EILs transcription factors, two key mysteries (i) how is the ethylene signal from CTR1 transmitted to EIN2, (ii) how is the signal transmitted from the ER-localized EIN2 to the

nuclear-localized transcription factors remain unknown. Recent research progress by three groups (Ju *et al.,* 2012; Qiao *et al.,* 2012; Wen *et al.,* 2012) has shown that CTR1, instead of operating through an intermediary MAPK cascade, directly phosphorylates EIN2 to inhibit its activity. The most significant sites of EIN2 phosphorylation are on Ser645 and Ser924 (Chen *et al.*, 2011; Qiao *et al.*, 2012; Ju *et al.*, 2012), with Ser924 playing a predominant role in EIN2 regulation (Ju *et al.*, 2012). Following ethylene binding to the receptors, CTR1 becomes inactivated, resulting in the dephosphorylation and proteolytic cleavage of EIN2, the cleaved C-terminal portion (CEND) of EIN2 then translocating to the nucleus to regulate transcriptional events (Ju *et al.*, 2012; Qiao *et al.*, 2012). These findings uncover a mechanism of subcellular communication whereby ethylene stimulates phosphorylation-dependent cleavage and nuclear movement of the EIN2-C' peptide, linking hormone perception and signaling components in the ER with nuclear-localized transcriptional regulators (Figure 8).

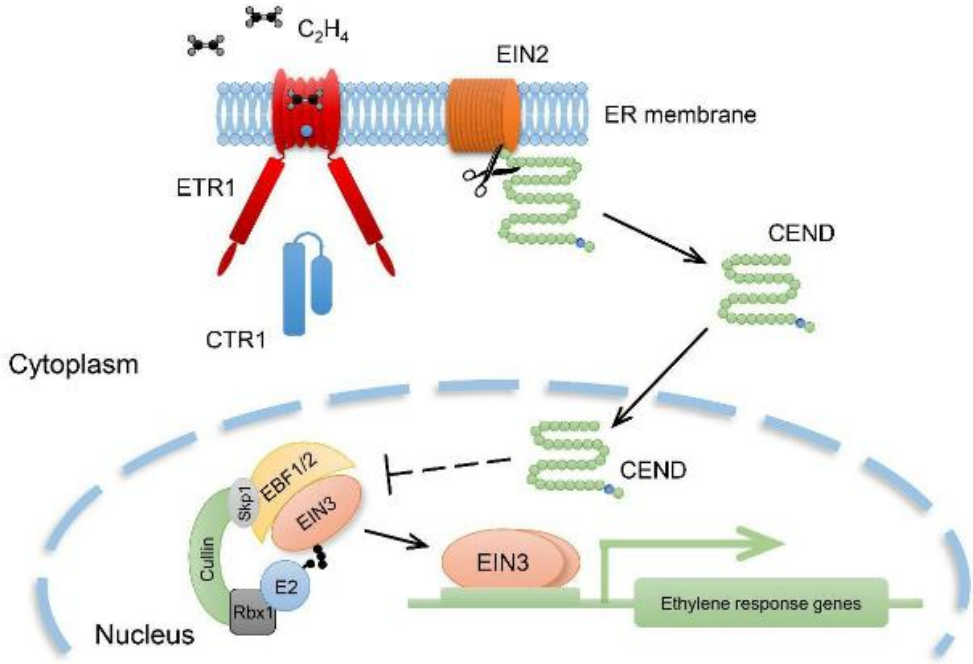

Figure 8. Model of EIN2 action in the ethylene signaling pathway. Ethylene is perceived by ER-located receptors, and the signal acts to promote the cleavage of EIN2 in an unknown mechanism. The cleaved C-terminal fragment (CEND) can be transported into the nucleus, preventing EIN3 from EBF1/2-mediated proteasomal degradation, and consequently leading to the activation of downstream ethylene response (Wen *et al.,* 2012).

2.4 A transcriptional cascade modulates the expression of ethylene-responsive genes

Genetically acting downstream of EIN2 are several nuclear-localized transcription factors (EIN3/EILs and ERFs) that mediate ethylene response at transcriptional levels (Chao *et al.*, 1997; Solano *et al.*, 1998; An *et al.*, 2010). After translocating to the nucleus, EIN2 either directly or indirectly activates the EIN3 family of transcription factors to initiate the transcriptional response to ethylene (Solano *et al.*, 1998; Alonso *et al.*, 2003; An *et al.*, 2010). EIN3 belongs to a small gene family that in Arabidopsis also includes five EIN3-LIKE (EIL) proteins (Alonso *et al.*, 2003). EIN3/EILs type of transcription factors are positive regulators of the ethylene signaling that function as trans-activating factors to trigger ethylene responses (Chao *et al.*, 1997; Solano *et al.*, 1998). Overexpression of *EIN3* or *EIL1* results in a constitutive ethylene phenotype in *Arabidopsis*, while *ein3 eil1* double LOF mutants show complete ethylene insensitivity in all known ethylene responses (Chao *et al.*, 1997; Alonso *et al.*, 2003).

None of *EIN3/EIL* genes identified to date is transcriptionally regulated in response to ethylene (Chao *et al.*, 1997; Tieman *et al.*, 2001; Rieu *et al.*, 2003), suggesting that the activities of these genes are regulated by ethylene through a posttranscriptional mechanism. Studies have demonstrated the involvement of the SCF/26S proteasome to regulate the level of EIN3/EILs (Guo and Ecker, 2003; Potuschak *et al.*, 2003; Gagne *et al.*, 2004; An *et al.*, 2010; Figure 9). In the absence of ethylene, EIN3/EILs are targeted for ubiquitination by the SCF complex containing one of the two F-box proteins, EBF1 and EBF2. The ubiquitinated form of EIN3/EIL proteins is thus recruited by the 26S proteasome for degradation. whereas, in the presence of ethylene, EIN3/EIL proteins accumulate in the nucleus and bind to EIN3 binding site (EBS) located in target gene promoters leading to the activation of the expression of downstream genes (Guo and Ecker, 2003; Potuschak *et al.*, 2003; Binder *et al.*, 2007). Indeed, EIN3/EIL proteins were shown to bind in a sequence specific manner to the primary ethylene-response element (PERE) of the ERF genes which are the last known actors of ethylene transduction pathway(Solano *et al.*, 1998; Chang *et al.*, 2013). This binding triggers the primary ethylene response through a transcriptional cascade that first includes the activation of target ERF genes which in turn through binding to GCC box *cis*-element in the promoter

of target genes modulate the expression of ethylene-responsive genes.

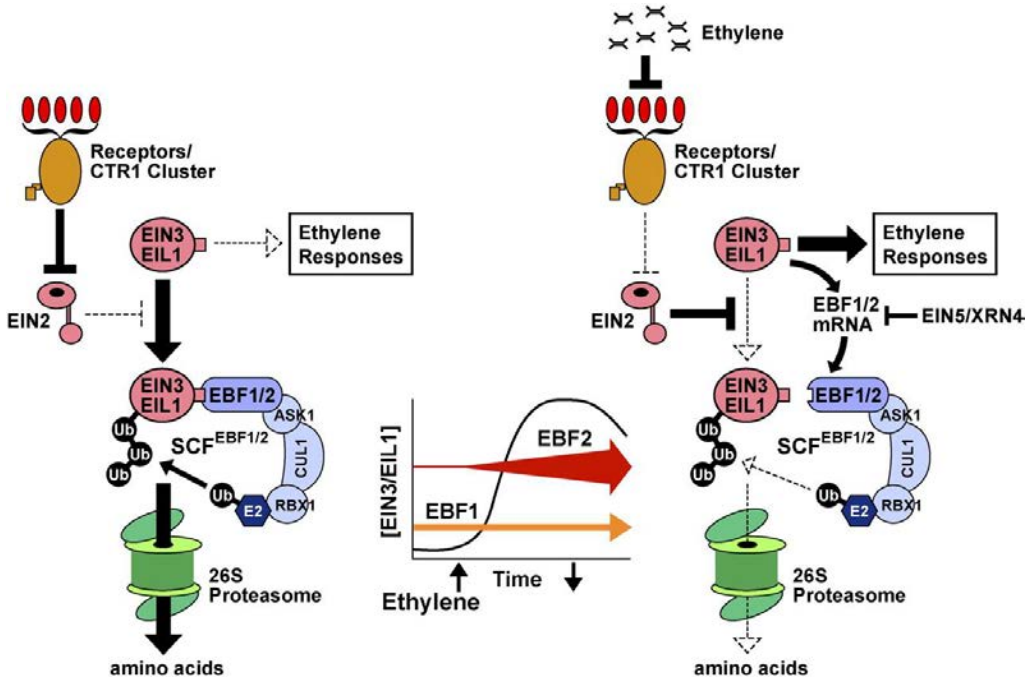

Figure 9. Model for the regulation of SCF/26S proteasome to the level of EIN3/EIL1.
In the absence of ethylene, the receptor family activates the negative regulator CTR1, which leads to inhibition of EIN2. EIN3 and EIL1 levels are kept low by selective ubiquitination of the proteins by SCF^{EBF1} and SCF^{EBF1}, which induces their subsequent breakdown by the 26S proteasome. In the presence of ethylene, the receptors are inhibited, thus reducing the output of CTR1 and its subsequent inhibition of EIN2. EIN2 acts in part to directly or indirectly block the interaction of EIN3 and EIL1 with the SCF E3s containing EBF1 and EBF2. The reduction in ubiquitination allows EIN3 and EIL1 levels to rise to mediate ethylene responses. (Binder *et al.,* 2007).

2.5 Ethylene Response Factors (ERFs)

In the linear ethylene signal transduction pathway, ERFs are shown to be the last known downstream components which are responsible for modulating the transcription of early ethylene-regulated genes in plants. Being encoded by one of the largest family of plant transcription factors, ERF proteins are the most suited step of ethylene signaling where the diversity and specificity of ethylene responses may originate.

ERFs are part of AP2 (APETALA2)/ERF super-family which also contains AP2 and RAV family genes (Riechmann *et al.*, 2000). The AP2/ERF superfamily is characterized

by the presence of the AP2/ERF domain (Riechmann and Meyerowitz, 1998; Sakuma *et al.*, 2002), which consists of about 59-60 amino acids and is involved in DNA binding. ERF family proteins contain only one AP2/ERF domain, whereas, AP2 family genes have two such domains. RAV family proteins contain an additional B3 DNA binding domain along with AP2/ERF domain. The AP2 domain was first identified as a repeated motif within the *Arabidopsis* AP2 protein, which is involved in flower development (Jofuku *et al.*, 1994). The ERF domain was first identified as a conserved motif in four DNA-binding proteins from tobacco (*Nicotiana tabacum*), namely, EREBP1, 2, 3, and 4 (currently renamed ERF1, 2, 3, and 4), and was shown to specifically bind to a GCC box, which is a DNA sequence involved in the ethylene-responsive transcription of genes (Ohme-Takagi and Shinshi, 1995). In the case of the RAV family, RAV1 and RAV2 were first identified as full-length cDNAs encoding proteins that contain a B3-like domain and an AP2/ERF domain in *Arabidopsis* (Kagaya *et al.*, 1999). Using hetero-nuclear multidimensional Nuclear Magnetic Resonance, Allen *et al.*, (1998) described the three dimensional structure of AP2/ERF domain from AtERF1. It consists of three anti-parallel β-sheets and an α-helix. In the DNA-TF complex, tryptophan and arginine residues present in the b-sheets have been found to make contact with the DNA in its major groove. Based upon the binding of ERF domain to DNA sequence element, ERF family has been further divided into two subfamilies, i.e., ERF and CBF/DREB (C-repeat binding factor/dehydration responsive element binding factor). ERF subfamily is characterized by the presence of an alanine and aspartic acid respectively at position 14 and 19 in the AP2 domain, whereas valine and glutamic acid are conserved in the corresponding positions for CBF/DREB (Sakuma *et al.*, 2002). ERFs have been shown to bind the GCC-box sequence (AGCCGCC) found in ethylene-responsive genes and DREBs to the DRE/CRT cis-regulatory element (A/GCCGAC) (Ohme-Takagi and Shinshi, 1995; Stockinger *et al.*, 1997; Hao *et al.*, 1998; Hao *et al.*, 2002; Oñate-Sánchez *et al.*, 2007).

In *Arabidopsis*, it was shown that the ERF subfamily contains 65 members and is divided into 5 subclasses based on the conservation of the AP2/ERF domain (Nakano *et al.*, 2006). Recently, genome-wide study also showed that the tomato *ERF* gene family comprises 9 subclasses (Figure 10) defined by distinct structural features and this work

proposed a new nomenclature for tomato ERFs (Pirrello *et al.*, 2012) which complies with the most complete classification available in *Arabidopsis* and clarifies the correspondence between ERF subclasses in different species (Nakano *et al.*, 2006). Based on functional analysis of 28 tomato ERFs and through testing their ability to activate or repress transcriptional activity of target genes, suggested that functional activity is conserved among ERF proteins sharing the same structural features (Pirrello *et al.*, 2012). It was reported that ERF subgroups which are characterized by the presence of conserved domain enriched in acidic amino acids, such as, glutamine, and proline are putative transcriptional activators (Liu *et al.*, 1999). More recently, a novel short motif termed EDLL was described by (Tiwari *et al.*, 2012). This motif is present in AtERF98/TDR1 and other clade members from the same AP2 sub-family. It has a unique arrangement of acidic amino acids and hydrophobic leucines, and functions as a strong activation domain, even to heterologous DNA binding proteins. These results suggest that most of ERF transcription factors identified so far are activators (Zhou *et al.*, 1997; Fujimoto *et al.*, 2000; Ohta *et al.*, 2000; Oñate-Sánchez and Singh, 2002; Nakano *et al.*, 2006; Oñate-Sánchez *et al.*, 2007; Wu *et al.*, 2007; Pirrello *et al.*, 2012; Tiwari *et al.*, 2012). However, a few of them, such as some members in group VIII, act as repressors (Fujimoto *et al.*, 2000; Ohta *et al.*, 2001; Kazan, 2006; Nakano *et al.*, 2006; Pirrello *et al.*, 2012). In contrast to the ERF activators, the ERF repressors contain a conserved (L/F)DLN(L/F)xP sequence, also called the ERF-associated amphiphilic repression (EAR) motif, in their C-terminal regions (Ohta *et al.*, 2001). Indeed tobacco *ERF3*, *Arabidopsis AtERF3*, *AtERF4* and *AtERF7* were shown to repress the expression of a GCC-box-containing reporter gene (Fujimoto *et al.*, 2000; Ohta *et al.*, 2000; Ohta *et al.*, 2001; Song *et al.*, 2005; Yang *et al.*, 2005).

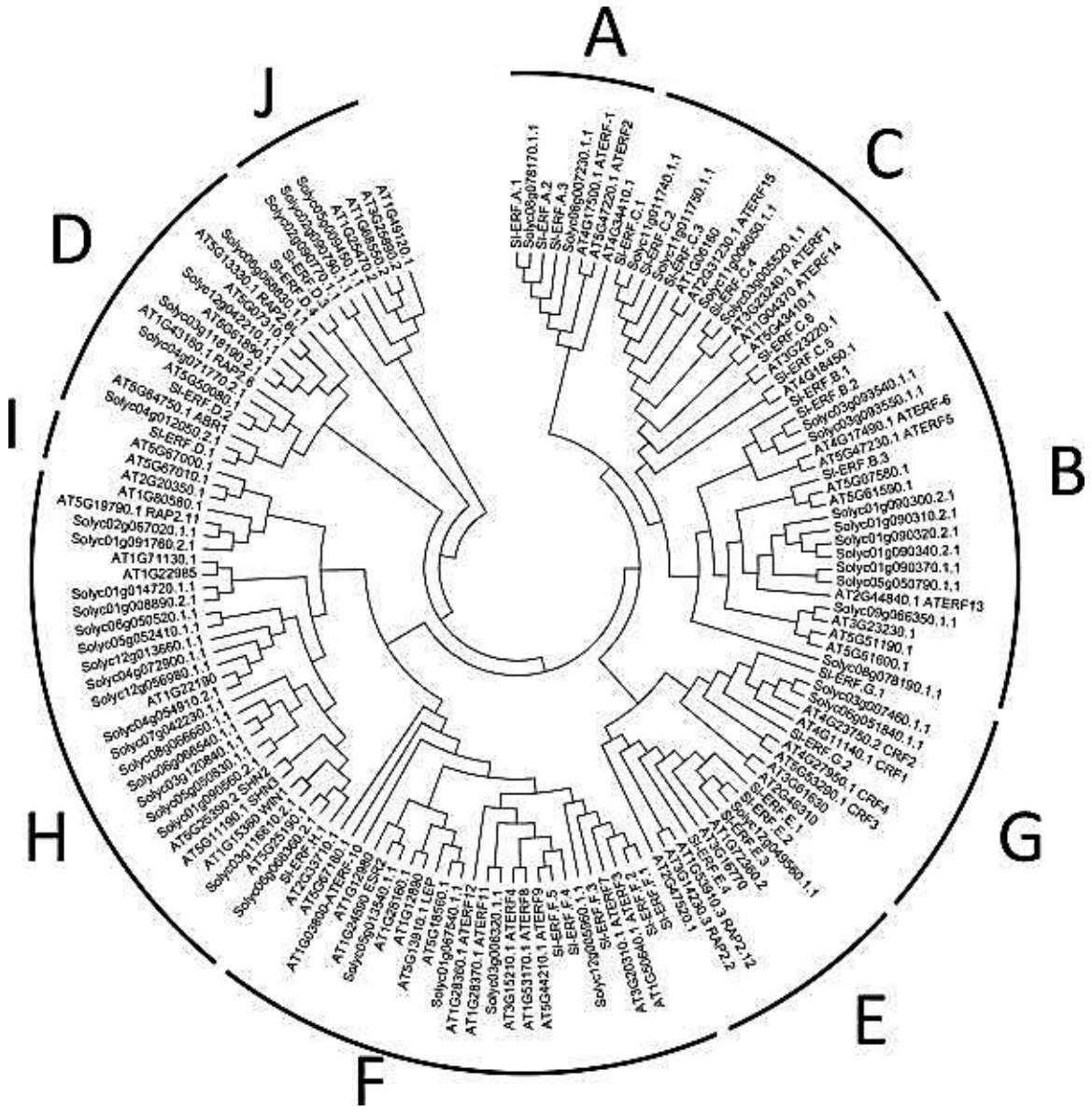

Figure 10. Phylogenetic tree of Arabidopsis and Tomato ERFs. Different subclasses are named by letters (A to J). Tomato genes for which the corresponding cDNA has been successfully isolated and that were subjected to functional analysis in this paper are named using the Sl-ERF nomenclature while other tomato ERFs are named using International Tomato Annotation Genome (ITAG 2.3) nomenclature. Phylogenetic trees were constructed with the whole protein sequences using neighbor joining method (Pirrello *et al.,* 2012).

ERF proteins are ubiquitous in plant kingdom. It has been demonstrated that the expression of many ERF genes can be regulated by plant hormones such as ethylene (ET), salicylic acid (SA) and jasmonic acid (JA), as well as by biotic and abiotic stresses (Fujimoto *et al.*, 2000; Park *et al.*, 2001; Gu *et al.*, 2002; Chen *et al.*, 2002; Oñate-

Sánchez and Singh, 2002; Brown *et al.*, 2003; Cheong *et al.*, 2003; Lorenzo *et al.*, 2003; McGrath *et al.*, 2005; Yang *et al.*, 2005; Jin and Liu, 2008; Trujillo *et al.*, 2008; Liu *et al.*, 2010; Pan *et al.*, 2010; Pan *et al.*, 2012; Pirrello *et al.*, 2012; Figure 11) suggesting their putative roles in these responses. Indeed, in different plant species, the ERF proteins have been shown to be involved in the transcriptional regulation of a wide range of processes including response to biotic and abiotic stresses, hormonal signal transduction, regulation of metabolism, and in developmental processes (Ohme-Takagi and Shinshi, 1995; van der Fits and Memelink, 2000; van der Graaff *et al.*, 2000; Banno *et al.*, 2001; Chuck *et al.*, 2002; Broun *et al.*, 2004; Pirrello *et al.*, 2006; Li *et al.*, 2007; Hu *et al.*, 2008; Zhang *et al.*, 2009; Pan *et al.*, 2010; Lee *et al.*, 2012). Constitutive expression of *ERF1* increases the resistance of Arabidopsis to *B. cinerea* and *P. cucumerina* and induces the expression of several defense-related genes, including *PLANT DEFENSIN1.2* (*PDF1.2*) and *BASIC CHITINASE* (Berrocal-Lobo *et al.*, 2002; Lorenzo *et al.*, 2003). Pré *et al.*, (2008) also demonstrated that ORA59, a prominent representative of the ERF group IX in *Arabidopsis*, acts as an essential integrator of the JA and ethylene signal transduction pathways and overexpression of *ORA59* causes increased resistance against the fungus *Botrytis cinerea*, whereas *ORA59*-silenced plants were more susceptible. Moreover, it was reported that a transcriptional activator, AtERF14, has a prominent role in the plant defense response. *AtERF14* loss-of-function mutants showed impaired induction of defense genes and increased susceptibility to *Fusarium oxysporum* (Oñate-Sánchez *et al.*, 2007). In tomato, the first ERFs which have been isolated are *Pti4, 5* and *Pti6* and the expression of these *ERFs* are induced by *pseudomonas syringae* (Zhou *et al.*, 1997; Thara *et al.*, 1999; Gu *et al.*, 2000). Overexpression of *Pti5* or *Pti5*-VP16, a translational fusion with a constitutive transcriptional activation domain, in tomato accelerated pathogen-induced expression of *GluB* and *Catalase* and enhanced resistance to *Pseudomonas syringae* pv. *tomato* (He *et al.*, 2001).

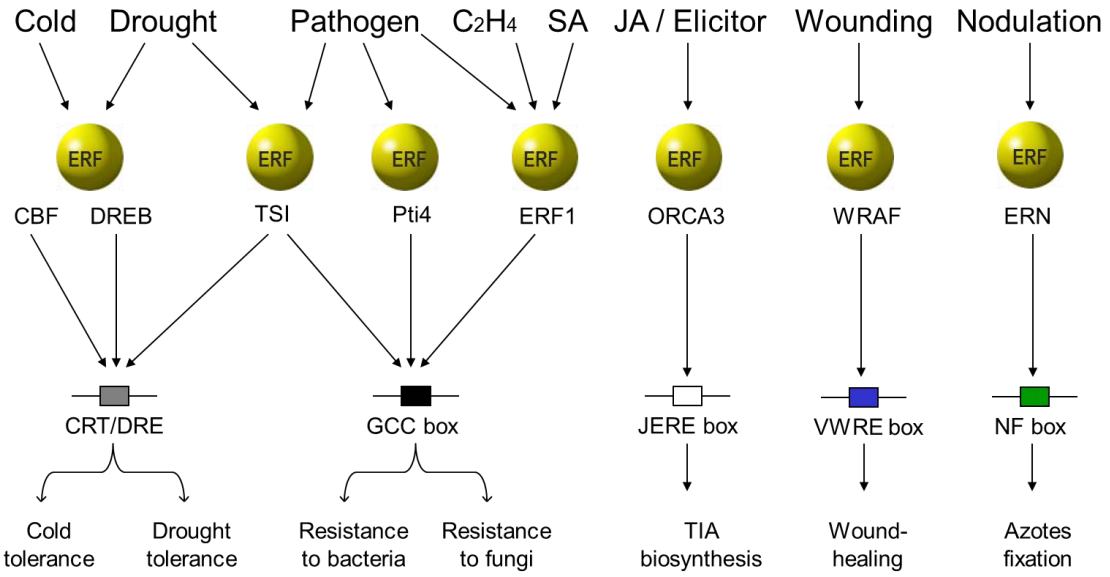

Figure 11: Outline of some of the stress responses and/or signals linked to ERF transcription factors. The promoter elements that they bind to and the effects of their over expression in plants are shown.

Studies have also shown that ERF proteins play important roles in the response to environmental stresses such as high salinity, drought and low temperature conditions via regulation of stress responsive genes (van der Fits and Memelink, 2000; Park *et al.*, 2001; Aharoni *et al.*, 2004; Huang *et al.*, 2004; Taketa *et al.*, 2008; Zhang *et al.*, 2009; De Boer *et al.*, 2011; Fukao *et al.*, 2011; Wan *et al.*, 2011). It was shown that the tomato ethylene responsive factor 1 (*TERF1*) was induced by both ethylene and NaCl treatment (Huang *et al.*, 2004). Overexpression of *TERF1* in tobaccos activates constitutive expression of PR genes like *Prb-1b*, *GLA*, *osmotin* and *CHN50* (Huang *et al.*, 2004) and activates genes involved in ABA/osmotic stress known to be involved in response to ABA, cold-, drought-, salt-stress. Transgenic tobacco plants constitutive expressing TERF1 displayed typical ethylene triple response and enhanced both salt and drought tolerance (Huang *et al.*, 2004; Zhang *et al.*, 2005). These results suggest that TERF1 may make the link between ethylene and salt response and it may also integrate different signaling pathway. Gao *et al.*, (2008) also reported that expression of *TERF1* gene in rice induces expression of stress responsive genes and enhances tolerance to drought and high-salinity. In tomato and tobacco, it was demonstrated that overexpression of *LeERF2/TERF2* regulates the expression of genes involved in ethylene synthesis and resulted in increased ethylene

synthesis and increased tolerance to cold (Zhang *et al.*, 2009; Zhang and Huang, 2010). The tomato JERF1 mRNA was rapidly accumulated within 10 min, and peaked after 1 h, 40 min, 8 h or 4 h under ethylene, MeJA, ABA or high salt treatment, respectively (Zhang *et al.*, 2004) suggesting that JERF1 as a transcriptional factor may play important roles in the regulation of plant stress and defense responses through different signaling pathways. Constitutive expression of *JERF1* in tobacco caused an increase in the transcript levels of GCC box-containing PR genes such as *osmotin*, *GLA*, *Prb-1b* and *CHN50,* and subsequently resulted in enhanced tolerance to salt stress during germination. This suggests that JERF1 modulates osmotic tolerance by activation of downstream gene expression via interaction with the GCC-box cis-elements. Moreover, it was also shown that JERF1 also enhances tolerance to drought, salinity and cold in tobacco by modulating the expression of an abscisic acid (ABA) biosynthesis-related gene (Wu *et al.*, 2007). Recently, in *Arabidopsis*, an ethylene response factor, *RAP2.2*, which functions in an ethylene-controlled signal transduction pathway, was reported to involve in plant survival under hypoxia (low-oxygen) stress (Hinz *et al.*, 2010). It was also demonstrated that *AtERF98* regulates the response to salt stress in Arabidopsis by increasing ascorbic acid (AsA) synthesis (Zhang *et al.*, 2012).

In addition to the functions in response to biotic and abiotic stresses, ERF proteins have also been shown to play an important role in plant development and fruit ripening. It was shown that expression of an ERF gene, *TINY,* impacted plant height, hypocotyl elongation, and fertility in *Arabidopsis* and resulted in a “tiny” phenotype (Wilson *et al.*, 1996). Results of (Banno *et al.*, 2001) indicated that the ERF gene, *ESR1,* specifically regulates the induction of shoot regeneration after the acquisition of competence for organogenesis in *Arabidopsis*. Moreover, transgenic plant overexpressing *Sl-ERF2* shows an early germinating phenotype, probably due to an over expression of *mannanase* genes involved in the radicle protrusion (Pirrello *et al.*, 2006). Recent studies demonstrated that *SlERF36*, an EAR-motif-containing ERF gene from tomato, alters stomatal density and modulates plant growth, flowering time and senescence in tobacco (Upadhyay *et al.*, 2013). In tomato, it was shown that LeERF1 is involved in leaf morphology, fruit ripening and softening (Li *et al.*, 2007). Furthermore, SlERF6 was also reported to play

an important role in fruit ripening by integrating the ethylene and carotenoid synthesis pathways in tomato (Lee *et al.*, 2012).

3. Role of ethylene

The gaseous phytohormone ethylene plays multiple roles in regulating plant growth and development. Alone or in content with the other phytohormones, ethylene regulates a wide range of plant activities, including seed germination, root growth and development, flowering, fruit ripening, organ senescence and abscission, and response to abiotic stresses and pathogen attacks (Johnson and Ecker, 1998; Bleecker and Kende, 2000; Lin *et al.*, 2009).

3.1 Seed germination

Seed germination is a complex physiological process under the control of plant hormones that play important and manifold roles (Bewley, 1997). Among the phytohormones ethylene is regarded as one of the key regulators in the process of seed germination. The addition of ethylene in the germination medium to *Arabidopsis* seeds results in accelerated germination, while adding norbornadiene, an inhibitor of ethylene action, delays germination. Analysis of mutant lines altered in ethylene biosynthesis or signaling pathway also demonstrated the involvement of ethylene in regulating seed germination. In *Arabidopsis*, constitutive ethylene insensitive mutant, *etr1-1*, show a delayed germination phenotype (Bleecker *et al.*, 1988). Mutation in *ETHYLENE INSENSITIVE2* (*EIN2*) gene results in poor germination and deeper dormancy, in contrast constitutive triple response 1 (*ctr1*) seeds germinate slightly faster compared to wild type (Leubner-Metzger *et al.*, 1998; Subbiah and Reddy, 2010). Ethylene response factor (*ERF)* genes which act as the last known components of ethylene signaling pathway play a key role in seed germination regulation (Leubner-Metzger *et al.*, 1998; Song *et al.*, 2005; Pirrello *et al.*, 2006). The ABA-insensitive Arabidopsis mutant *abi4* affected in seed germination displays altered expression of seed-specific genes (Finkelstein *et al.*, 1998) and the *abi4* mutation is caused by a single pair deletion within an AP2 family

gene. It was reported that *AtERF7* acts as a transcriptional repressor of the ABA response and that transgenic *Arabidopsis* lines expressing an RNAi construct targeted to down-regulate the *AtERF7* gene are more sensitive to ABA and germinate later than the wild-type seeds (Song *et al.*, 2005). Beechnut *FsERF1* is almost undetectable in dormant seeds incubated under high temperature conditions that maintain dormancy, or in the presence of germination inhibitors, either ABA or AOA, an inhibitor of ethylene biosynthesis, but increases during moist chilling that progressively breaks dormancy (Arc *et al.*, 2013). Pirrello *et al.*, (2006) also demonstrated that *SlERF2* transcript accumulation is higher in germinating seeds than in non-germinating ones and overexpression of this transcription factor in transgenic tomato lines results in enhanced ethylene sensitivity and premature seed germination. In seeds of *Arabidopsis* and other species, ethylene has a demonstrated antagonism to ABA, a hormone that inhibits germination (Arc *et al.*, 2013).

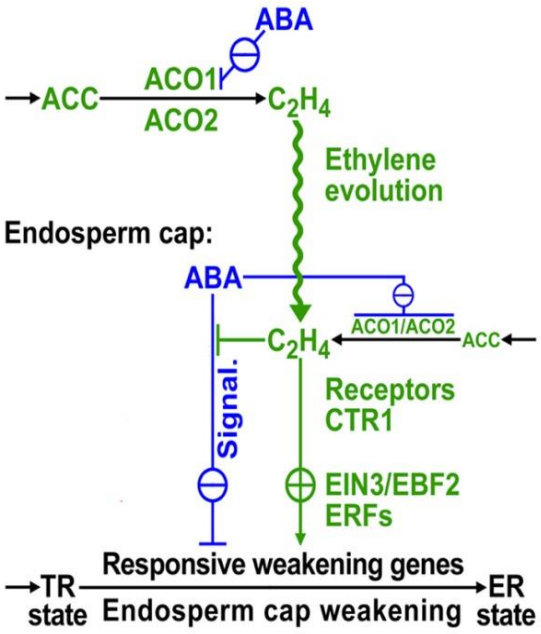

Figure 12. Model for the regulation of ethylene and ABA in endosperm cap weakening and rupture. ABA delays ACO activity in the radicle and inhibits ACO1 transcript accumulation, but not ACO2 transcript accumulation. The later increase in ACO activity in the radicle of ABA-treated seeds is therefore due to ACO2, and the ethylene produced promotes endosperm cap weakening by antagonizing the ABA inhibition. In the endosperm cap, ABA inhibits ACO2 and ACO1 transcript accumulation. Ethylene does not affect the seed ABA levels and therefore must counteract the ABA-induced inhibition of endosperm rupture by interfering with ABA signaling (Linkies *et al.*, 2009).

In *Arabidopsis,* ethylene counteracts the inhibitory effects of ABA on endosperm cap weakening and endosperm rupture (Linkies *et al.*, 2009). ABA also increases the ethylene requirement to release primary and secondary dormancies. Mutants with enhanced response to ABA were found to be ethylene insensitive alleles in known genes of the ethylene pathway (Beaudoin *et al.*, 2000; Ghassemian *et al.*, 2000). Therefore, ethylene's effects in regulating germination may be explained, at least partially, by the ABA antagonism (Figure 12).

3.2 Root development

In recent years, studies of the effect of ethylene on root growth and development has achieved substantial progress, with identification of both inhibitory effects of ethylene on root elongation and lateral root development and stimulatory effects of ethylene on root hair initiation. The inhibition of root elongation in the presence of ethylene or its precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), has been well known in different species (Abeles *et al.,* 1992; Negi *et al.,* 2010; Figure 13). Ethylene-insensitive mutants, including *etr1*, *ein2*, *ein3*, and *eil1* in *Arabidopsis* and *never ripe* (*nr*) and *green ripe* (*gr*) in tomato, have elevated primary root growth rates compared to the wild type (Růzicka *et al.*, 2007; Stepanova *et al.*, 2007; Negi *et al.*, 2010). By contrast, roots of seedlings with elevated ethylene signaling or synthesis, *ctr1* and *eto1*, respectively, display reductions in the rate of root elongation (Kieber *et al.*, 1993). These results clearly indicate the inhibitory effect of ethylene on root growth.

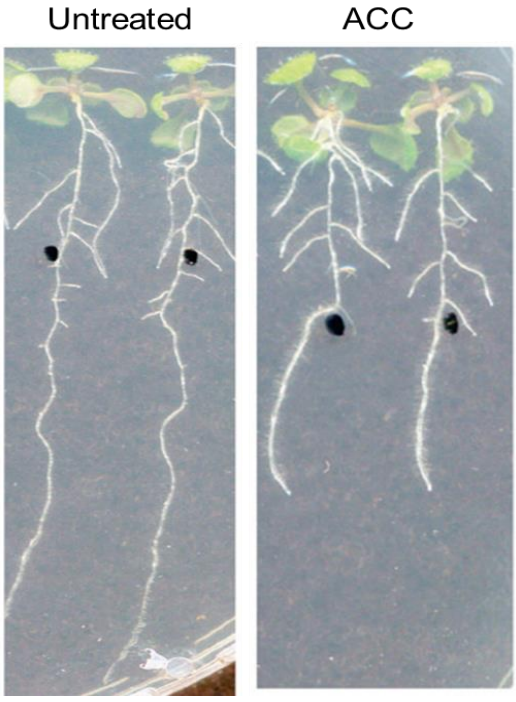

Figure 13. Ethylene inhibits root elongation and lateral root development. Five-day-old Arabidopsis seedlings were transferred to medium containing 1 μM ACC and the tip of the roots at the time of transfer was marked by a black dot. When their roots were imaged five days later, ethylene had decreased the rate of root elongation relative to an untreated control, as judged by the length of root that had formed below the black dots. By contrast, ethylene treatment prevented lateral root formation in the region formed after transfer (Muday *et al.,* 2012)

Ethylene has been shown to play a negative role in lateral root formation through the studies in Arabidopsis and tomato using the diversity of mutants with altered ethylene signaling or synthesis (Ivanchenko *et al.*, 2008; Negi *et al.*, 2008; Negi *et al.*, 2010). Treatments or mutations to elevate ethylene levels inhibit lateral root formation. *Arabidopsis* wild type Columbia seedlings show a dose dependent decrease in lateral root numbers when grown on the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) and this is reversed by the treatment with ethylene antagonist, silver nitrate (Negi *et al.,* 2008). The *ctr1* mutant, with enhanced ethylene signaling (Kieber *et al.,* 1993; Huang *et al.,* 2003), and the *eto1* mutant, with enhanced ethylene synthesis (Kieber *et al.,* 1993), exhibited significant reductions in lateral root numbers compared with wild-type seedlings. In contrast the *Arabidopsis* and tomato ethylene-insensitive mutants exhibit elevated numbers of lateral roots (Negi *et al.*, 2008; Negi *et al.*, 2010; Strader *et al.*, 2010). The ethylene-insensitive mutants *etr1*, which has a dominant negative receptor mutation (Hua *et al.*, 1998; Sakai *et al.*, 1998), and *ein2*, which has a defect in an ethylene signaling protein (Kendrick and Chang, 2008), showed enhanced lateral root formation and was insensitive to the inhibitory effect of ACC on lateral root numbers (Negi *et al.,* 2008). Interestingly, although most of these studies examined root growth on agar medium, the increase in lateral root formation seen in *nr* is even more striking in seedlings grown in soil for several weeks (Negi *et al.,* 2010), suggesting that ethylene may have even more profound effects on roots during standard cultivation. These effects of ethylene are on the earliest stages of lateral root initiation (Ivanchenko *et al.*, 2008) and alter auxin transport, suggesting that crosstalk with auxin should be a critical component of the activity of ethylene in lateral root development (Negi *et al.,* 2008).

The role of ethylene in the formation of adventitious roots has been examined in a variety of plant species, but the results have been contradictory. In tomato, elevated exogenous or endogenous ethylene levels increase adventitious root formation, while ethylene-insensitive *nr* produces fewer adventitious roots (Clark *et al.*, 1999; Kim *et al.*, 2008; Negi *et al.*, 2010). In contrast in Arabidopsis, ACC treatment, as well as the *eto1* and *ctr1* mutations, results in reduced adventitious root formation (Sukumar, 2010). These contradictory findings may be due to variation in the different tissues, growth conditions, and methods of quantifying adventitious root formation.

In contrast to the negative effects of ethylene on root elongation and lateral root formation, root hair development is positive regulated by ethylene (Tanimoto *et al.*, 1995; Rahman *et al.*, 2002). In *Arabidopsis*, the ethylene precursor ACC induces ectopic root hair formation (Tanimoto *et al.*, 1995; Pitts *et al.*, 1998), whereas application of an ethylene biosynthesis inhibitor, aminoethoxyvinylglycine (AVG) and Ag^{+} (an ethylene action inhibitor) reduce root hairs (Masucci and Schiefelbein, 1994; Tanimoto *et al.*, 1995). The initiation and elongation of root hairs may synergistically induced by ethylene and auxin. Since auxin is able to rescue root hair elongation defects in ethylene-insensitive mutants, and inhibition of auxin influx exacerbates the *ein2* root hair phenotype (Rahman *et al.*, 2002). Application of ACC or IAA to the root hair-deficient mutant root hair defective 6 (rhd6) can restore root hair initiation (Masucci and Schiefelbein, 1994). Moreover, auxin-insensitive mutants that also show ethylene insensitivity, such as axr2, axr3 and aux1, exhibit reduced root hair initiation (Pickett *et al.*, 1990; Wilson *et al.*, 1990; Leyser *et al.*, 1996). Interestingly, ethylene also affects the positioning of root hairs and ethylene-insensitive mutants having apically shifted root hairs (Fischer *et al.*, 2007).

3.3 Flowering

The transition from vegetative growth to flowering is the most drastic change in plant development. Ethylene has been known to be involved in flowering process for a long time (Abeles *et al.,* 1992). Experiments applying ethylene and chemical inhibitors of its biosynthesis and function have demonstrated that ethylene differentially regulates flowering in different plant species. Ethylene plays a role in floral promotion in pineapple, mango, lychee and *Plumbago indica*, and in floral inhibition in short-day plants such as cocklebur, Japanese morning glory, chrysanthemum, tobacco and *Chenopodium* (Abeles *et al.,* 1992). It has been reported that ethylene induced flowering in pineapple (Ananas comosus) and that silencing of the *AcACC* synthase gene caused delayed flowering in pineapple (Trusov and Botella, 2006). In rice, overexpression of *ETR2* leads to reduced ethylene sensitivity and late flowering, whereas T-DNA insertion mutant *etr2* showed enhanced ethylene sensitivity and early flowering (Wuriyanghan *et al.*, 2009). It is

showed that ethylene represses flowering in *Arabidopsis* and inactivation of specific ACS gene products enhances flowering time (Tsuchisaka *et al.*, 2009). In *Arabidopsis*, it is found that wild-type plants grown in the presence of the ethylene precursor ACC, or in an ethylene-rich atmosphere, flowered late and the constitutive ethylene response *Arabidopsis* mutant *ctr1-1* showed a late flowering phenotype (Achard *et al.*, 2007). Moreover, enhanced ethylene response mutant *eer2* also displayed a delay in bolting and flowering time compared with wild type plants (De Paepe *et al.*, 2005). Based on the data obtained in *acs* mutants, it was proposed that ethylene exerts its effect on flowering by regulating the expression of the FLC which acts as a rheostat to repress flowering through repression of the floral pathway integrators FT and SOC1. Interestingly, on the other hand, based on the observation that constitutively active ethylene signaling in the *ctr1* mutant reduces GA levels, and the late flowering phenotype of the *ctr1* mutant is partially rescued by loss-of-function mutations in DELLA genes, Achard *et al.,* (2007) showed that ethylene delays flowering via modulating DELLA activity. The induced DELLA accumulation by ethylene in turn delays flowering via repression of the floral meristem-identity genes LEAFY (LFY) and SOC1 (Achard *et al.,* 2007). A different flowering pathway operates in the *acs* mutants and in the *ctr1* mutant could be a possible explanation for this difference findings. In tomato, overexpression of *SlTPR1* gene, a tomato tetratricopeptide repeat protein, results in enhanced ethylene response and delayed flowering time (Lin *et al.*, 2008). Further studies should disclose the mechanism by which ethylene differentially regulates flowering in different plant species.

3.4 Fruit ripening

Fruit ripening is a developmentally regulated process unique to plants during which the majority of the sensory quality attributes are elaborated including aroma, flavor, texture and nutritional compounds (Carrari and Fernie, 2006). Biochemical and physiological changes that occur during fruit ripening are driven by a cascade of molecular events leading to the stimulation of specific transcriptional regulators responsible for the coordinated expression of fruit ripening-related genes directly involved in the biochemical processes (Giovannoni, 2004). The requirement for ethylene

in the ripening of climacteric fruit has long been recognized (Abeles *et al.*, 1992) and discrimination between climacteric and non-climacteric fruits has been made on the basis of the presence or absence of the climacteric rise in respiration and of autocatalytic ethylene production. In climacteric fruit, the plant hormone ethylene is considered to be the major signaling molecule that controls most aspects of fruit ripening. By contrast, in non-climacteric fruit, ethylene is not the trigger of the ripening process, which appears to depend on signals not yet elucidated. It should be noted that, however, these distinctions are not absolute, as closely related melon and capsicum species can be both climacteric and non-climacteric and some non-climacteric fruits show enhanced ripening phenotypes in response to exogenous ethylene (Lelièvre *et al.*, 1997; Alexander and Grierson, 2002; Mailhac and Chervin, 2006). Nevertheless, enhanced ethylene synthesis at the onset of ripening is required for the normal ripening of many fruits.

Two systems of ethylene biosynthesis have been proposed in climacteric plants (McMurchie *et al.*, 1972). System 1 functions during normal vegetative growth, is ethylene autoinhibitory and is responsible for producing basal ethylene levels that are detected in all tissues including those of non-climacteric fruit. System 2 operates during the ripening of climacteric fruit when ethylene production is autocatalytic. Through a combination of ethylene and inhibitor studies together with expression analysis of ethylene biosynthesis genes in ripening tomato fruits and various ripening mutants, the molecular mechanisms of autocatalytic ethylene production were investigated in tomato (Barry *et al.*, 2000). Expression analysis has revealed that at least four *ACS* (*LEACS1A*, *LEACS2*, *LEACS4*, and *LEACS6*) and three *ACO* (*LEACO1*, *LEACO3*, and *LEACO4*) genes are differentially expressed in tomato fruit (Rottmann *et al.*, 1991; Barry *et al.*, 1996; Nakatsuka *et al.*, 1998; Barry *et al.*, 2000). It was shown that system 1 ethylene is regulated by the expression of *LeACS1A* and *LeACS6* with the two genes being negatively regulated by ethylene. Subsequently, the up-regulation of *LeACS2* and *LeACS4* through positive feedback by ethylene is responsible for the activation of System 2 (Nakatsuka *et al.*, 1998; Barry *et al.*, 2000). *LeACO1*, *LeACO3*, and *LeACO4* are all expressed at low levels in green fruit that are in a system 1 mode of ethylene synthesis, but the transcripts of each increase at the climacteric peak as the fruit ethylene production transition to system 2. Moreover, *LEACO1* and *LEACO4* are sustained in expression

during fruit ripening, whereas the increase in *LEACO3* expression is transient (Barry *et al.*, 1996; Nakatsuka *et al.*, 1998). In the case of *LEACO1* and *LEACO4*, ripening-related increases in transcript abundance can be largely blocked by 1-MCP treatment, indicating that these genes are positively regulated by ethylene. The main changes in *ACS* and *ACO* gene expression associated with the ethylene synthesis from system 1 to system 2 during tomato fruit development and ripening are shown in Figure 14. It should be point out that because the expression of ethylene biosynthesis genes were shown to be also regulated by some regulator factors, such as, through binding to their promoters, RIN and LeHB-1 regulate the expression of *LeACS2* and *LeACO1*, respectively (Ito *et al.*, 2008; Lin *et al.*, 2009), it is possible that the autocatalytic regulation is not the only mechanism of the system 2 ethylene synthesis.

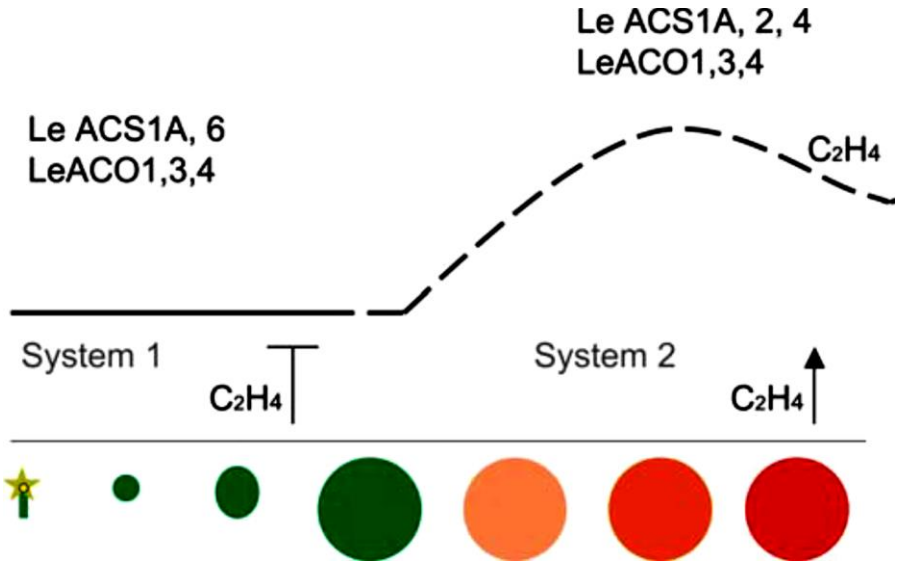

Figure 14. Differential expression of ACS and ACO genes associated with system 1 and system 2 ethylene synthesis during fruit development and ripening in tomato. Auto-inhibition of ethylene synthesis during system 1 ethylene production is mediated by a reduced expression of *LeACS1A* and 6. Autocatalytic ethylene synthesis at the onset of fruit ripening is mediated through ethylene-stimulated expression of LeACS2 and 4 and LeACO1 and 4 (Barry and Giovannoni, 2007).

Inhibition or delay in fruit ripening in tomato by antisense expression of tomato *ACS2* and *ACO1* genes was the first direct evidence that ethylene biosynthesis is essential for climacteric fruit ripening (Oeller *et al.*, 1991). Furthermore, effects of ethylene perception and signal transduction on fruit ripening were also well known. It was shown

that the *Never-ripe* (*Nr*) mutant of tomato corresponding to a mutation in the ethylene receptor conferred ethylene insensitivity and thus produced non-ripening fruit (Wilkinson *et al.*, 1995). Moreover, antisense inhibition of production of the mutant mRNA in the *Nr* mutant resulted in failure to synthesize the mutant receptor protein, and partially or completely restored fruit ripening (Hackett *et al.*, 2000) supporting the evidence that the ethylene receptors act as negative regulators of ethylene action. Another mutant, *Green-ripe* (*Gr*), a dominant ripening mutation that occurs in a gene encoding another component of ethylene signaling failed to fully ripen as a consequence of inhibition of ethylene responsiveness (Barry *et al.*, 2005). The GR protein which corresponds to the *REVERSION TO ETHYLENE SENSITIVITY1* (*RTE1*) in *Arabidopsis* was proposed to mediate ethylene response via interacting with and regulating the ethylene receptor(s) (Barry and Giovannoni, 2006; Resnick *et al.*, 2006; Zhou *et al.*, 2007). In addition, it was demonstrated that receptor level, during fruit development, determines the timing of ripening (Kevany *et al.*, 2007) and fruit-specific suppression of *LeETR4* resulted in early-ripening fruit in tomato (Kevany *et al.*, 2008).

The ethylene transduction pathway leads to the regulation of fruit ripening through a transcriptional cascade including primary (*ETHYLENE-INSENSITIVE3* (*EIN3*) and *EIN3-like* (*EIL*)) and secondary response factors (*ETHYLENE RESPONSE FACTORS* (*ERF*)). It was shown that transgenic tomato plants with reduction in expression of multiple tomato *LeEIL* genes significantly reduced ethylene sensitivity and thus affected fruit ripening (Tieman *et al.*, 2001). Chen *et al.*, (2004) also demonstrated that overexpression of *LeEIL1* in the *Nr* mutant partially restored normal fruit ripening and stimulated the expression of some ethylene-responsive genes. In Kiwifruit, *AdEIL* genes were constitutively expressed during fruit ripening and the transcription factors AdEIL2 and AdEIL3 can activate transcription of the ripening-related genes *AdACO1* suggesting a role of *EIL* genes in fruit ripening via regulating ethylene biosynthesis genes. In addition, silencing of *SlEBF1* and *SlEBF2* which negatively regulate ethylene signaling by mediating the degradation of EIN3/EIL proteins resulted in a constitutive ethylene response phenotype and accelerated fruit ripening in tomato (Yang *et al.*, 2010). These results indicated that ethylene-mediated climacteric fruit ripening is also controlled at the transcriptional levels.

The identification of several key ripening regulatory genes from tomato, such as MADS-box *Ripening-Inhibitor* (*RIN*) (Vrebalov *et al.*, 2002), SBP-box *Colourless Non-Ripening* (*CNR*) (Manning *et al.*, 2006), MADS-box *AGAMOUS-LIKE1* (*TAGL1*) (Itkin *et al.*, 2009; Vrebalov *et al.*, 2009), and leucine zipper homeobox *LeHB-1* (Lin *et al.*, 2008), has led to new insights into understanding of ethylene and ripening control mechanisms. The *RIN* gene encodes a putative MADS box transcription factor that controls tomato fruit ripening, with its mutated version (*rin*) conferring a non-ripening character (Vrebalov *et al.*, 2002). Molecular and biochemical studies have shown that RIN participates in ethylene production by inducing many of the ethylene synthesis/signaling genes (e.g., *ACS2* and *ACS4*), by upregulating *NOR* and *CNR* and by downregulating *HB-1* (Ito *et al.*, 2008; Fujisawa *et al.*, 2011; Fujisawa *et al.*, 2012; Martel *et al.*, 2011; Qin *et al.*, 2012; Zhou *et al.*, 2012). Cnr is 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*, and several other ripening-related genes (Thompson *et al.*, 1999). Tomato *AGAMOUS-LIKE1* (*TAGL1*) gene whose down-regulation results in yellow fruit with reduced carotenoids and thin pericarp, has been shown to control fruit expansion and ripening (Itkin *et al.*, 2009; Vrebalov *et al.*, 2009). Furthermore, *TAGL1*-suppressed fruit produce lower amounts of ethylene with a reduced expression of *LeACS2* suggesting that TAGL1 may be another important regulator of ripening-related ethylene production. The transcription factor encoded by the *LeHB-1* gene belonging to class-I HD-Zip proteins can bind the promoter of *LeACO1* (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. The putative transcription factor, *Sl-AP2a*, a member of the AP2/ERF superfamily gene was also recently described as a negative regulator of fruit ripening and of ethylene production (Chung *et al.*, 2010; Karlova *et al.*, 2011). The characterization of these transcriptional regulators indicates that transcription factors play key roles in relaying ripening-inducing signals and controlling ethylene biosynthesis. Thus, the regulation of transcriptional regulators by acting upstream of ethylene synthesis should be an important mechanism for controlling fruit ripening (Figure 15).

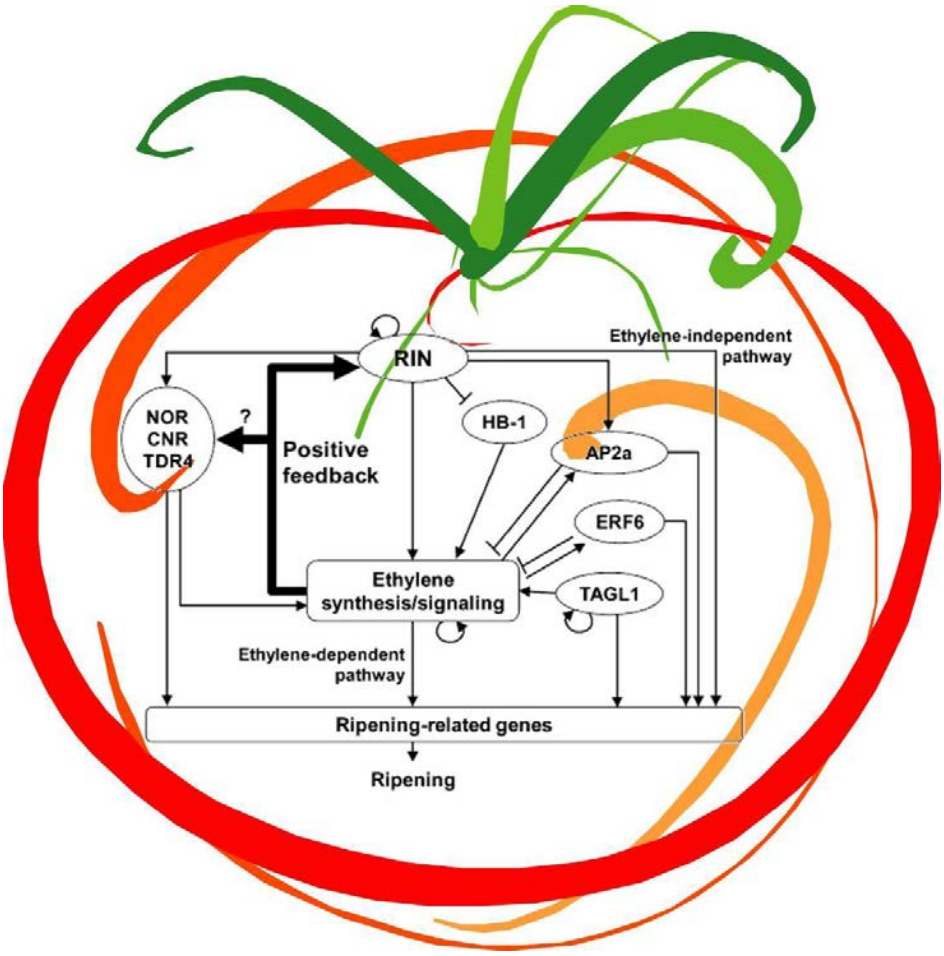

Figure 15. A schematic representation of the proposed model for a regulatory mechanism of tomato fruit ripening. Bold line arrows indicate an ethylene-mediated positive feedback loop that enhances RIN expression. It is unclear whether the loop regulates the expression of the other ripening regulators (such as NOR and TDR4) affected by ethylene during ripening directly or indirectly (via RIN). Arrows indicate the direction of the transcriptional regulatory pathways. Blunt-ended lines indicate repression. Circle arrows on RIN and TAGL1 indicate auto-regulation and on ethylene indicate autocatalytic ethylene production (Fujisawa *et al.*, 2013).

Ethylene Response Factors (ERFs) are the last identified downstream components of the ethylene signal transduction pathway known to regulate early ethylene-responsive genes. Recently, accumulating studies have shown that ERF proteins play an important role in fruit ripening (Klee and Giovannoni, 2011). Most of the *ERF* genes identified in tomato were ethylene inducible and showed ripening-related expression pattern (Pirrello *et al.*, 2012). Tomato *LeERF1* was reported to mediate ethylene response and thus control fruit ripening (Li *et al.,* 2007). Overexpression of *LeERF1* in tomato resulted in constitutive

ethylene response and accelerated fruit ripening and softening (Li *et al.,* 2007). A ripening-related pattern of expression has also been shown for *LeERF2* and *LeERF3b* in tomato fruit (Tournier *et al.*, 2003; Chen *et al.*, 2008). *LeERF2* is induced by ethylene and suppressed in ripening-inhibited mutants (Wu *et al.,* 2002; Tournier *et al.,* 2003). Moreover, LeERF2 regulates ethylene response in tomato by modulating ethylene biosynthesis genes (Pirrello *et al.,* 2006; Zhang *et al.,* 2009), transcriptional regulation being achieved by interaction of LeERF2 with promoter of LeACO3 (Zhang *et al.,* 2009). Recently, *SlERF6* was reported to play an important role in fruit ripening by integrating the ethylene and carotenoid synthesis pathways in tomato (Lee *et al.,* 2012). In banana, ERFs are also demonstrated to be involved in fruit ripening through their interactions with ethylene biosynthesis genes (Xiao *et al.*, 2013). Nevertheless, so far only one ERF has been identified as direct regulator of ripening-associated genes *via* binding a cis-element present in the promoter of E4 (Montgomery *et al.*, 1993), a ripening-regulated gene (Lincoln and Fischer, 1988) encoding proteins of unknown function, and therefore the specific role of each *ERF* in ethylene response and the ripening process is still far from being well understood.

Although ripening control in non-climacteric fruit was thought to be independent of ethylene, some studies do show an increase in ethylene production in non-climacteric fruit which suggesting a role of ethylene in ripening. Indeed, the effect of ethylene in inducing color changes in the flavedo tissue of citrus fruit, a non-climacteric fruit, has long been known (Goldschmidt *et al.*, 1993; Goldschmidt, 1997). In strawberry, which is generally considered as non-climacteric fruit, an increase in ethylene production associated with a raise in respiration has been observed when the fruit reaches the red-ripe stage (Iannetta *et al.*, 2006). It was reported that, in grapes, a small increase in ethylene production occurs at the veraison stage when berries reach the onset of color changes and treatment of grape berries with 1-MCP, an inhibitor of ethylene perception, affected anthocyanin accumulation and berry swelling and caused a decrease in acidity (Chervin *et al.*, 2008) suggesting that ethylene might be required for the full accomplishment of the ripening process. Overall, these data suggest a putative involvement of ethylene in at least some aspects of the ripening process in non-climacteric fruit.

3.5 Organ senescence and abscission

Senescence is a vitally important developmental step in the life cycle of a plant or a plant organ that determines yield and reproductive success. The most prominent symptom of leaf senescence is the visible yellowing, which correlates with physiological and biochemical changes such as dismantling of chloroplasts, drop of chlorophyll content and photosynthetic activities, and degradation of RNA and proteins (Jing *et al.*, 2003). Senescence is a complex developmental phase involving the actions of a complex network consisting of multiple pathways. In many species, and in different plant organs, ethylene has long been considered a key hormone in regulating the onset of leaf senescence (Bleecker *et al.*, 1988; Zacarias and Reid, 1990). In *Arabidopsis*, ethylene treatment advances the visible yellowing and senescence-associated genes (*SAGs*) induction in leaves that are primed to senesce (Grbic and Bleecker, 1995). Ethylene insensitive mutants, such as *etr1* and *ein2/ore3* display delayed leaf senescence (Oh *et al.*, 1997). It was reported that the lifespan of e*tr1-1* leaves is 30% longer than wild-type leaves and this delay is accompanied by a delayed induction of senescence associated genes (*SAGs*) which are used as molecular markers of leaf senescence (Hensel *et al.,* 1993) and higher expression of photosynthesis-associated genes (PAGs) (Grbic and Bleecker, 1995). In tomato, reduced ethylene production due to antisense suppression of the genes involved in ethylene biosynthesis also resulted in a temporal delay in the onset of foliar senescence (Jone *et al.*, 1995). Recently, Chen *et al.*, (2011) showed that a MADS box gene, FOREVER YOUNG FLOWER (*FYF*) acts as a repressor of organ senescence and abscission through suppressing ethylene response.

It was demonstrated that ethylene can induce senescence only when developmental changes controlled by leaf age are present and before senescence can be initiated, some age-related changes (ARCs) must have taken place in the leaf (Hensel *et al.*, 1993; Jing *et al.*, 2002; Jing *et al.*, 2005). For example, the oldest leaves showed the greatest increase in SAG transcripts after ethylene treatment, and little or no effect of ethylene was observed in the youngest leaves (Grbic and Bleecker, 1995). These results strongly suggest that ethylene can induce leaf senescence only within specific age window. Indeed, at early leaf growth, ethylene does not induce leaf senescence, and this is the

never senescence phase. This phase could be controlled by developmental signals or homeostatic genes such as so-called age-related factors. Only after a defined stage a leaf switches to the second phase, which allows the action of ethylene to promote leaf senescence. This promoting effect operates within a defined time span, marking the ethylene-dependent senescence phase (Grbic and Bleecker, 1995; Jing *et al.*, 2002; Jing *et al.*, 2005).

Abscission is a physiological process that involves the programmed separation of entire organs, such as leaves, petals, flowers, and fruit. It is the mechanism for the removal of senescing or damaged organs but also for the release of the fruit when it is ripe (Bleecker and Patterson, 1997). The first demonstration that ethylene can promote abscission was documented by Wehmer in 1917 (Abeles *et al.,* 1992). Application of exogenous ethylene hastens abscission by inducing expression of cellulase and polygalacturonase genes in different species and different plant organs (Bonghi *et al.*, 1992; Kalaitzis *et al.*, 1995; del Campillo and Bennett, 1996). Inhibitors of ethylene action such as silver and 2, 5-norbornadiene have been found to block abscission and inhibitors of ethylene synthesis such as AVG have also been shown to retard abscission of leaves, flowers, and fruit (Kushad and Poovaiah, 1984; Abeles *et al.,* 1992). The first genetic evidence to substantiate an involvement of the ethylene signaling pathway in organ abscission was when the *ETHYLENE RECEPTOR1* (*ETR1*) was identified (Bleecker *et al.*, 1988). Ethylene insensitive *etr1* mutant displayed a delayed capacity to undergo floral organ abscission (Bleecker *et al.*, 1988; Bleecker and Patterson, 1997). Thanks to the identification of ethylene-signaling mutants in tomato, such as *never ripe* (*nr*), *nr2*, *green ripe* (*gr*), *eil1*, *2* and *3*, our understanding of the effect of ethylene in mediating organ abscission has increased. The *nr* mutant which has a mutation in the ethylene receptor gene *NR* was shown to exhibit delayed pedicel abscission (Lanahan *et al.*, 1994; Wilkinson *et al.*, 1995b; Lashbrook *et al.*, 1998; Hackett *et al.*, 2000). Moreover, both *nr2* and *gr* dominant mutants which show reduced sensitivity to ethylene display delayed flower abscission even after exposure to exogenous ethylene (Barry *et al.*, 2005). By contrast, overexpressing the ethylene biosynthesis gene ACC synthase in tomato plants resulted in premature flower abscission (Whitelaw *et al.*, 2002).

Up till now, several *Arabidopsis* mutants which show defects in organ abscission have been identified, including *inflorescence deficient in abscission* (Butenko *et al.*, 2003), *delayed floral organ abscission1, 2* and *3* (Patterson and Bleecker, 2004), *hawaiian skirt* (González-Carranza *et al.*, 2007), and the *haesa (hae) haesa-like2 (hsl2)* double mutant (Cho *et al.*, 2008; Stenvik *et al.*, 2008). All of these mutants display normal ethylene sensitivity. Moreover, exogenous ethylene, which promotes the cell separation phase of abscission in wild-type plants, does not alter the abscission defective phenotype in the *MKK4-MKK5RAi* and *hae hsl2* plants (Cho *et al.*, 2008). These results indicate that even if ethylene accelerates the abscission process, the perception of ethylene is not the unique process.

3.6 Pathogen resistance

Plants have evolved sophisticated detection and defense systems to protect themselves from pathogen invasion. When plants perceive a pathogen attack, an increase of transcription of ethylene response genes is generally observed. This over-production of ethylene is usually associated with induction of defence reaction. However, depending on the type of pathogen and plant species, the role of ethylene can be dramatically different, as ethylene has been demonstrated to stimulate, as well as to counteract disease development. Ethylene can enhance resistance against various pathogens (Thomma *et al.*, 2001; Díaz *et al.*, 2002), but it can also increase disease severity, probably by promoting chlorosis, senescence, and cell necrosis (Abeles *et al.,* 1992). Taking advantage of the availability of plant mutants and transgenic lines that are affected in their response to ethylene, the reactions of these mutant and transgenic plants to different types of attackers were compared, either enhanced or reduced disease development were observed (van Loon *et al.*, 2006; Table 1). It is shown that as a result of increased symptom severity in non-responsive mutants, ethylene was found to reduce diseases caused by several fungi and bacteria that kill their hosts (necrotrophs), or have a mixed biotrophic-necrotrophic lifestyle (in which they start exploiting the living host before killing it). By contrast, the occurrence of less severe symptoms indicated that ethylene stimulated diseases caused by various other fungi and bacteria with varying lifestyles, as well as

infection by a cyst nematode and insect attack. Bent *et al.*, (1992) showed that the ethylene-insensitive *Arabidopsis* mutant *ein2-1* was resistant to *X. campestris* pv. *campestris*, whereas the *etr1* and *etr2* mutants were reported to display more severe symptoms, indicating an enhanced susceptibility (O'Donnell *et al.*, 2003). Moreover, tomato plants treated with 1-methylcyclopropene (MCP), an inhibitor of ethylene perception, were show to display enhanced susceptibility to *B. cinerea* (Díaz *et al.*, 2002). Nevertheless, the ethylene-insensitive tomato mutant *Never ripe* (*Nr*) seemed to be as susceptible as wild-type plants to *B. cinerea* (Díaz *et al.*, 2002), and even less susceptible to the vascular wilt fungus *Fusarium oxysporum* f.sp. *lycopersici* (Lund *et al.*, 1998). Ethylene-insensitive soybean mutants displayed increased disease severity after infection with the brown-spot fungus *Septoria glycines* or the root rot fungus *Rhizoctonia solani*, but less severe symptoms upon inoculation with the root and crown rot-causing oomycete *Phytophthora sojae*. By contrast, it was shown that inoculation of ethylene-insensitive soybean with the bacterial blight pathogen *P. syringae* pv. *glycinea* led to reduced disease severity compared to wild-type plants (Hoffman *et al.*, 1999). These results indicate that altered ethylene sensitivity can result in more or less severe disease, reflecting reduced or increased pathogen resistance, respectively, depending on the plant-pathogen combination. Indeed, after systematic testing of several pathogenic fungi and bacteria on different accessions and various mutants of *Arabidopsis* the conclusion that, in general, ethylene contributes to resistance against necrotrophic, but not biotrophic pathogens was proposed (Thomma *et al.*, 2001; Ton *et al.*, 2002).

Table 1. Ethylene-related mutant and transgenic plants with altered sensitivity to pathogens (van Loon *et al.,* 2006).

Plant species	Mutant or transgenic	Pathogen	Lifestyle	Disease severity[a]
Arabidopsis	*ein2-1*	*Botrytis cinerea*	Necrotrophic	+
Arabidopsis	*ein2-5, ein3-1*	*Botrytis cinerea*	Necrotrophic	+
Arabidopsis	*etr1-1, ein2-1*	*Chalara elegans*	Necrotrophic	+
Arabidopsis	*ein2-1*	*Erwinia carotovora pv. carotovora*	Necrotrophic	+
Arabidopsis	*ein2-5*	*Fusarium oxysporum f.sp. conglutinans*	Necrotrophic	+
Arabidopsis	*ein2-5*	*Fusarium oxysporum f.sp. conglutinans*	Necrotrophic	+
Arabidopsis	*etr1-1, ein2-1*	*Fusarium oxysporum f.sp. matthiolae*	Necrotrophic	+
Arabidopsis	*etr1-1, ein2-1*	*Fusarium oxysporum f.sp. raphani*	Mixed	-
Arabidopsis	*eto1 – eto3*	*Heterodera schachtii*	Biotrophic	+
Arabidopsis	*etr1-1, ein2-1*	*Heterodera schachtii*	Biotrophic	-
Arabidopsis	*ein2-5*	*Plectosphaerella cucumerina*	Necrotrophic	+
Arabidopsis	*ein2-1*	*Pseudomonas syringae pv. maculicola*	Mixed	-
Arabidopsis	*ein2-1,-3,-4,-5*	*Pseudomonas syringae pv. tomato*	Mixed	-
Arabidopsis	*etr1-1, ein2-1*	*Pythium spp.*	Necrotrophic	+
Arabidopsis	*ein2-1, eto3*	*Ralstonia solanacearum*	Necrotrophic	-
Arabidopsis	*etr1*	*Spodoptera exigua*	Herbivore	-
Arabidopsis	*ein2-1, hls1-1*	*Spodoptera littoralis*	Herbivore	-
Arabidopsis	*etr1-1*	*Verticillium dahliae*	Necrotrophic	-
Arabidopsis	*etr1-1, etr2-1*	*Xanthomonas campestris pv. campestris*	Mixed	+
Arabidopsis	*ein2-1*	*Xanthomonas campestris pv. campestris*	Mixed	-
Arabidopsis	*eto1-1*	*Xanthomonas campestris pv. campestris*	Mixed	+
Tomato	*ACD*	*Botrytis cinerea*	Necrotrophic	+
Tomato	*Epi*	*Botrytis cinerea*	Necrotrophic	-
Tomato	*ACD*	*Verticillium dahliae*	Necrotrophic	- (tolerant)
Tomato	*ACD*	*Xanthomonas campestris pv. vesicatoria*	Mixed	- (tolerant)
Tomato	*NR, Nr*	*Xanthomonas campestris pv. vesicatoria*	Mixed	- (tolerant)
Tomato	*Nr*	*Fusarium oxysporum f.sp. lycopersici*	Necrotrophic	-
Tomato	*Nr*	*Pseudomonas syringae pv. tomato*	Mixed	- (tolerant)
Tomato	*Nr*	*Xanthomonas campestris pv. vesicatoria*	Mixed	- (tolerant)
Tomato	*Atetr1-1-LeEtr3*	*Xanthomonas campestris pv. vesicatoria*	Mixed	-

[a]Disease severity: + increased; -, decreased.

In response to pathogen attack in plant, ethylene can induce certain types of pathogenesis-related (PR) proteins or phytoalexins, and, through stimulation of the phenylpropanoid pathway, can rigidify cell walls in various plant species (Abeles *et al.,* 1992). Pathogenesis-related (PR) proteins which are constituted by a broad class of inducible defense-related proteins expressed either locally or systemically in response to pathogen stress are the most extensively studied set of defense molecules in relation to ethylene. The extensive role of ethylene in the regulation of expression of different classes of *PR* genes, such as *PR–2* (*β-1,3-glucanases*), *PR-3* (*basic-chitinases*), *PR-4* (*hevein-like*), and *PR-12* (*plant defensins, PDFs*) have been well demonstrated (Broglie *et al.*, 1989; Samac *et al.*, 1990; Penninckx *et al.*, 1996; Penninckx *et al.*, 1998; Thomma *et al.*, 1998; Thomma *et al.*, 1999; Thomma *et al.*, 2001; van Loon *et al.*, 2006). Analysis of the promoters of the *PR* genes led to the identification of several *cis*-elements required for ethylene regulation, including GCC-box and the dehydration responsive element/C-repeat element (DRE/CRT). The GCC-box (11-bp sequence TAAGAGCCGCC), was shown to be necessary, and in some cases sufficient, for the regulation by ethylene of *PR* genes in different plant species (Ohme-Takagi and Shinshi, 1995; Solano *et al.*, 1998; Fujimoto *et al.*, 2000; Gu *et al.*, 2000; Brown *et al.*, 2003; Chakravarthy *et al.*, 2003; Oñate-Sánchez *et al.*, 2007; Zhou *et al.*, 2008; Anderson *et al.*, 2010). The Ethylene Response Factors (ERFs) which involves in the ethylene signal cascade have been shown to regulate the expression of *PR* genes via directly binding to the GCC-box or the dehydration responsive element/C-repeat element (DRE/CRT) located in the promoters of various pathogenesis-related (PR) genes (Park *et al.*, 2001; Hao *et al.*, 2002; Gutterson and Reuber, 2004; Moffat *et al.*, 2012). Indeed, Plant ERF transcription factors are widely involved in biotic stress responses and particularly in pathogen resistance. Overexpression of ERF genes, such as *Pti4*, *ERF1*, *OPBP1*, *TSRF*1, *AtERF14*, *ORA59*, confers resistance to fungal and bacterial pathogens in transgenic plants (Berrocal-Lobo *et al.*, 2002; Gu *et al.*, 2002; Oñate-Sánchez and Singh, 2002; Guo *et al.*, 2004; Oñate-Sánchez *et al.*, 2007; Pré *et al.*, 2008; Zhang *et al.*, 2008). The functionality of ERF subfamily members in different species has suggested their involvement in ethylene signaling and ethylene-related defenses and the complexity of the regulation of repressor

and activator-types of ERFs during pathogen challenge may explain the different role of ethylene in mediating pathogen stress.

4. Crosstalk between ethylene and other phytohormones

Ethylene regulates many aspects of plant developmental processes, and it is no doubt that the diversity of ethylene functions is achieved, at least in part, by its interactions with other hormones. The interactions between ethylene and other phytohormones are discussed below.

4.1 Ethylene and auxin

Ethylene and auxin interact at both the physiological and molecular levels in plant growth and development with either synergistic or antagonistic effects. Ethylene and auxin are able to regulate the synthesis of each other. Elevated levels of auxin lead to increased ethylene synthesis via increased transcription of the genes that drive ethylene synthesis, including specific members of the *ACC synthase* (*ACS*) family, which catalyze the rate-limiting step in ethylene synthesis (Abel *et al.,* 1995; Wang *et al.*, 2002; Tsuchisaka and Theologis, 2004; Stepanova *et al.*, 2007). Reciprocally, ethylene regulates the expression of *WEI2/ASA1* and *WEI7/ASB1*, the subunits of an anthranilate synthase that catalyzes the first step in tryptophan biosynthesis, the principal precursor of auxin biosynthesis (Stepanova *et al.*, 2005).

At root level, ethylene and auxin affect synergistically in the processes of root elongation and root hair formation, while in other processes, such as lateral root formation, they act antagonistically (Muday *et al.*, 2012). The earliest genetic evidence that ethylene and auxin may act through convergent pathways to regulate root growth came from the identification of ethylene-insensitive mutants with defects in auxin transporters: *aux1* and *ethylene insensitive root 1/pinformed 2* (*eir1/pin2*) (Pickett *et al.*, 1990; Roman *et al.*, 1995; Luschnig *et al.*, 1998). Kinematic analyses of root growth inhibition by ethylene and auxin by high temporal and spatial resolution revealed that ethylene and auxin reduce the expansion rate of the cells in the central elongation zone (Rahman *et al.*, 2007;

Swarup *et al.*, 2007). Mutants with enhanced ethylene or auxin synthesis have reduced root elongation and wild-type plants treated with exogenous ethylene or auxin also show reduction in root elongation (Kieber *et al.*, 1993; Delarue *et al.*, 1998; Zhao *et al.*, 2001; Rahman *et al.*, 2007). Moreover, like in ethylene-insensitive mutants, auxin-induced root growth inhibition is lost or substantially reduced in auxin-resistant mutants such as *tir1*, *axr2*, *axr3*, and *solitary-root* (*slr*) (Timpte *et al.*, 1994; Leyser *et al.*, 1996; Fukaki *et al.*, 2002; Biswas *et al.*, 2007). These results indicate that auxin and ethylene have similar effects on root elongation. Indeed, more and more evidences proved that ethylene inhibits root growth via modulation of auxin signaling, transport and synthesis is one of the mechanisms by which ethylene and auxin synergistically inhibit root elongation (Stepanova *et al.*, 2005; Stepanova *et al.*, 2007).

In contrast to root elongation, which is synergistically inhibited by auxin and ethylene, these two hormones act antagonistically on lateral root initiation. Treatment with ethylene or ACC reduces lateral root initiation in both *Arabidopsis* and tomato. Dominant negative *etr1* and *Nr* ethylene receptor mutants, as well as the ethylene-insensitive *ein2* and *Gr* mutants have an enhanced number of lateral roots (Negi *et al.*, 2008; Negi *et al.*, 2010). By contrast, auxin stimulates lateral root formation and elongation, mutants and inhibitors that reduce auxin transport reduce lateral root initiation and emergence (Reed *et al.*, 1998; Casimiro *et al.*, 2001; Ivanchenko *et al.*, 2008; Péret *et al.*, 2009). Recently, it was demonstrated that ethylene inhibits lateral root development by blocking changes in the abundance of local auxin transport protein needed to form local auxin maxima that drive lateral root formation (Lewis *et al.*, 2011).

Pharmacological and genetic studies have revealed that ethylene and auxin promote the processes of root hair initiation (Tanimoto *et al.*, 1995; Rahman *et al.*, 2002). Application of ethylene or auxin to the root hair-deficient mutant *root hair defective 6* (*rhd6*) was found can restore the root hair initiation (Masucci and Schiefelbein, 1994b). Moreover, auxin-insensitive mutants that also show ethylene insensitivity, such as *aux1*, *axr2* and *axr3*, display reduced root hair initiation (Pickett *et al.*, 1990; Wilson *et al.*, 1990; Leyser *et al.*, 1996). It was predicted that root hair initiation is directly linked to the amount of auxin and auxin signaling, and the effect of ethylene is less direct and likely to occur through intracellular auxin levels (Muday *et al.,* 2012).

The induction of apical hook formation in *Arabidopsis* represents one of the best described examples of ethylene-auxin interaction in plants (Lehman *et al.*, 1996; Raz and Ecker, 1999). Various studies indicate that ethylene affects auxin transport, synthesis, and, perhaps, signaling to regulate the differential growth leading to the apical hook. Combination of all of these studies, Muday *et al.,* (2012) proposed a working model of ethylene-auxin crosstalk in apical hook formation (Figure 16). In this model, ethylene causes enhanced apical hook formation by both increasing the levels of components important for auxin signaling and increasing auxin levels on the concave side of the apical hook.

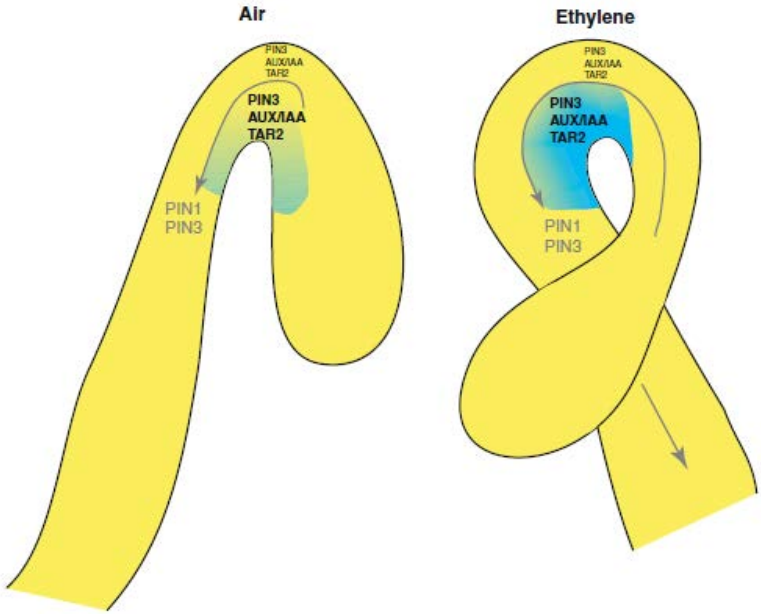

Figure 16. Model of the control of apical hook curvature by auxin and ethylene. Auxin is transported in at rootward direction from the cotyledons predominantly through the action of PIN1 and PIN3. The apical hook is formed because of the asymmetric distribution of auxin (shown in blue) that arises through differential auxin synthesis, auxin transport and auxin signaling. This is reflected by an asymmetrical distribution of proteins regulating these processes in the region of the apical hook. When ethylene is added (right-hand image), auxin levels rise on the concave side of the hook to cause an exaggerated curvature due to increases in PIN3, AUX1, IAA3, IAA12, IAA13 and TAR2 on the concave side of the hook (Muday *et al.,* 2012).

The interplay between ethylene and auxin in flower and fruit abscission is also well established (Abeles and Rubinstein, 1964; Roberts *et al.*, 2002). The generally accepted

model is that a basipetal auxin flux through the abscission zone (AZ) prevents abscission by rendering the AZ insensitive to ethylene.

In addition, ethylene and auxin crosstalk is necessary to determine normal fruit ripening. Indeed, the levels of auxin must decrease prior to the onset of ripening in both climacteric and non-climacteric fruits.

4.2 Ethylene and gibberellins (GAs)

Ethylene and gibberellins (GAs) control similar developmental processes in plants. The crosstalk between GA and ethylene has been demonstrated (Achard *et al.*, 2003; Vriezen *et al.*, 2004; Achard *et al.*, 2007; De Grauwe *et al.*, 2007). DELLA proteins, which act as nuclear repressors of GA signaling, appear to be key integrators in the ethylene-GA crosstalk. It was shown that ethylene controls the maintenance and exaggeration of the apical hook via modifying DELLA degradation (Achard *et al*., 2003; Vriezen *et al*., 2004). In addition, Achard *et al.,* (2007) reported that ethylene controls floral transition via DELLA-dependent regulation of floral meristem identity genes. Enhanced ethylene response reduces bioactive GA levels, thus promoting the accumulation of DELLA proteins. DELLA accumulation in turn slows the plant life cycle and delays flowering via repression of floral meristem identity *LFY* and *SOC1* genes (Achard *et al.,* 2007; Figure 17).

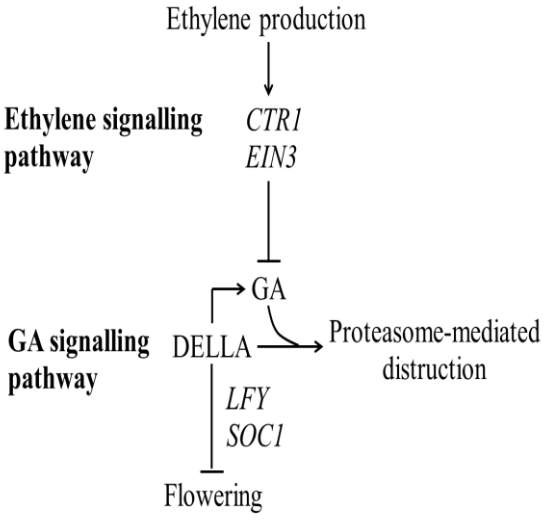

Figure 17. Model for integration of the ethylene and GA-DELLA signaling pathways in the regulation of floral transition. Activation of ethylene signaling reduces bioactive GA levels, thus promoting the accumulation of DELLAs. DELLA accumulation in turn slows the plant life cycle and delays flowering. Accumulation of DELLAs delays floral transition (via regulation of *LFY* and *SOC1* transcript levels) and increases the abundance of GA-biosynthesis gene transcripts via a negative feedback loop (Achard *et al.,* 2007).

It was reported that regulatory crosstalk involving ethylene and GA affects the transition from seed dormancy to germination in common beech (*Fagus sylvatica* L.) seeds where a drastic increase in *FsACO1* expression when seeds were treated with GA3 or ethephon, but the stimulatory effect of ethephon could be reversed by paclobutrazol, a GA biosynthesis inhibitor, suggesting that GA positively regulates the expression of *FsACO1* gene (Calvo *et al.*, 2004). De Grauwe *et al.,* (2007) also demonstrated that the absence of an active GA-signaling cascade suppresses the higher ethylene biosynthesis observed in *eto2-1* while the responsiveness to ethylene is slightly enhanced. The suppression of ethylene biosynthesis in the double mutant suggests that the absence of active GA signaling may affect the stability of ethylene-biosynthesis enzymes in a negative feedback mechanism. The enhanced sensitivity to GA in the *gai eto2-1* double mutant suggests a reciprocal influence of the two pathways on one another and this also was corroborated by earlier data demonstrating that ACC enhances the activity of the GA-biosynthesis gene *GA1* (Vriezen *et al*., 2004).

Pierik *et al.*, (2004) reported that the involvement of ethylene in phytochrome-mediated shade avoidance responses can at least partly be attributed to interactions between ethylene and GA action, and it is likely that GA acts downstream of ethylene in regulating shade avoidance responses. In addition, Dubois *et al.*, (2013) also reported that upon exposure to osmotic stress, ACC accumulates in the actively growing leaves, where it is converted to ethylene. Ethylene further activates the signaling pathway involving MPK3 and MPK6. These kinases phosphorylate the basal amount of ERF5 and ERF6 proteins present in the cell prior to stress exposure. The activated ERF5 and ERF6 then activation of leaf growth inhibition via the transcriptional activation of the gene encoding the GA-inactivating enzyme GA2-OX6, thereby decreasing the bioactive GA concentration and stabilizing the DELLA proteins (Dubois *et al.,* 2013). Recently, in tomato, it was found that dominant repression of an ethylene response factor, *Sl-ERF.B3*, confers ethylene hypersensitivity with reduced plant size and delayed flowering time. The reduced expression of *GA oxidase* genes in the transgenic lines sustains the idea of altered GA metabolism and suggests that ERFs may represent a potential molecular link between ethylene and GA (Liu *et al.*, 2013).

4.3 Ethylene and abscisic acid (ABA)

Abscisic acid (ABA) is a classic phytohormone that plays an important role in various aspects of plant growth and development. It has been shown that a subset of the functions of ABA overlaps with those of ethylene including in seed germination and early seedling establishment, albeit with antagonistic effects (Zhou *et al.*, 1998). In *Arabidopsis,* ethylene counteracts the inhibitory effects of ABA on endosperm cap weakening and endosperm rupture (Linkies *et al.*, 2009). ABA increases the ethylene requirement to release primary and secondary dormancies. Inhibition of seed germination by ABA was shown to be associated with a reduction in ethylene synthesis (reviewed in Arc *et al.,* 2013). In *Arabidopsis*, ABA inhibited the accumulation of *ACO1* transcripts in both the embryo and endosperm during seed germination and the high levels of *ACO1* transcripts in ABA-insensitive mutants also suggests the regulation of *ACO* expression by ABA (Penfield *et al.*, 2006; Linkies *et al.*, 2009). Interestingly, it was also reported that ABA-deficient mutants of *Arabidopsis aba2* and tomato *flacca* and *notabilis* reveal inhibition of shoot growth, largely because of high ethylene production in these mutants (Sharp *et al.*, 2000; LeNoble *et al.*, 2004).

The intertwining nature of ethylene and ABA biosynthesis and signaling pathways in germination has been well studied (Beaudoin *et al.*, 2000; Ghassemian *et al.*, 2000; Cutler *et al.*, 2010). Beaudoin *et al.,* (2000) reported that genetic screening of the enhancer and repressor of ABA-insensitive germination of the *abi1-1* mutant were allelic to *ctr1* and *ein2*, respectively. Ghassemian *et al.,* (2000) also found that the *enhanced response to ABA3* (*era3*) mutant was a new allele of *ein2* that shows hypersensitivity to ABA in seed germination. Seeds of *Arabidopsis* ethylene-insensitive mutants, *etr1* and *ein2*, exhibit higher ABA content than wild type and consistently germinate more slowly (Chiwocha *et al.*, 2005; Wang *et al.*, 2007). Moreover, Cheng *et al.*, (2009) showed that the expression of *9-CIS-EP-OXYCAROTENOID DIOXYGENASE 3* (*NCED3*), which encodes the key enzyme in ABA biosynthesis, is up-regulated in the *ein2-1* mutant, and *CYP707A2*, a cytochrome P450 gene which encodes the key component of ABA catabolism, is down-regulated in *etr1-1*, suggesting that when ethylene signaling is impaired, ABA biosynthesis may be enhanced. Mutations that reduce ethylene

sensitivity, such as *etr1*, *ein2*, and *ein6*, result in an increase in ABA sensitivity, while increased ethylene sensitivity in *ctr1* and *eto1* reduces ABA sensitivity (Beaudoin *et al.,* 2000; Ghassemian *et al.,* 2000; Chiwocha *et al.,* 2005; Linkies *et al.,* 2009; Subbiah and Reddy, 2010). These results suggest that ethylene not only acts on ABA metabolism to reduce ABA levels, but also negatively regulates ABA signaling.

The interactions between ethylene and ABA are known not only in developmental processes but also in adaptive stress responses of plants. Exogenous ABA suppresses ethylene-responsive defense genes such as *PDF1.2* and *b-CHI*, while mutations in the ABA biosynthesis pathway have the opposite effect. Accordingly, *aba2-1* mutants with enhanced levels of these PR proteins exhibited improved resistance against *F. oxysporum*. An ethylene response factor gene, *AtERF4*, has been shown to modulate the antagonistic ethylene-ABA crosstalk (Yang *et al.,* 2005). Moreover, it was reported that ethylene biosynthesis gene *ACS7* acts as a negative regulator of ABA sensitivity and accumulation under stress and appears as a node in the cross-talk between ethylene and ABA (Dong *et al.*, 2011).

Based on the timing of ABA accumulation, changes to ethylene production and the expression of ABA and ethylene biosynthesis genes, (Zhang *et al.*, 2009) concluded that the two hormones may also play a coordinating role in tomato ripening. Treatment of fruit with ABA increased the expression of three ethylene biosynthetic genes, promoting ethylene synthesis and ripening, while inhibitors of ABA synthesis prevented this increase.

4.4 Ethylene and jasmonates (JAs)

Interactions between ethylene and JAs have been shown to contribute to a variety of responses of plants to biotic and abiotic stresses or developmental cues. Studies have indicated that ethylene- and JA-signaling often operate synergistically to induce the expression of a number of defense related genes including *PR1b*, *PR5* (osmitin), *PDF1.2*, the basic chitinase gene *CHI-B*, a hevein-like protein gene, and proteinase inhibitors (*PIN*) genes after pathogen inoculation (Xu *et al.*, 1994; O'Donnell *et al.*, 1996; Penninckx *et al.*, 1998; Ellis and Turner, 2001; Thomma *et al.*, 2001). Moreover, the *Arabidopsis cev1*

mutant, that is defective in the cellulose synthase gene *CesA3*, displays constitutively active ethylene and JA responses indicating that CEV1 acts as a negative regulator of ethylene and JA signaling in Arabidopsis (Ellis *et al.*, 2002). A convergence point between ethylene and JAs pathways was represented by the transcriptional activation of *ETHYLENE TRANSCRIPTION FACTOR1* (*ERF1*), a transcription factor that regulates the expression of pathogen response genes that prevent disease progression (Lorenzo *et al.,* 2003). The expression of ERF1 was induced rapidly by ethylene or JAs and could be activated synergistically by both hormones. Moreover, constitutive expression of *ERF1* could rescue the defense response defects of *coi1* (coronatine insensitive1) and *ein2* (ethylene insensitive2) by restoring PR gene expression, suggesting that ERF1 is a key downstream element of both ethylene and JAs signaling pathways for the regulation of defense response genes (Lorenzo *et al.,* 2003; Figure 18). Indeed, several members of ERF family have been shown to play important role in mediating defense responses in *Arabidopsis* (McGrath *et al.,* 2005). The *Arabidopsis* transcription factor MYC2 has also been shown to regulate the crosstalk between ethylene- and JA-mediated defense signaling (Lorenzo and Solano, 2005; Dombrecht *et al.*, 2007).

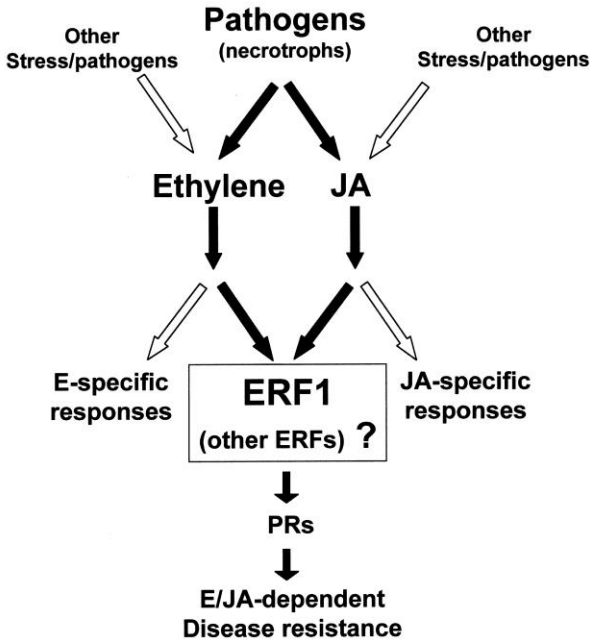

Figure 18. Ethylene/Jasmonate-dependent pathway of the Arabidopsis response to pathogens. Infection by some types of pathogens induces the synthesis and subsequent activation of the ethylene and jasmonate pathways simultaneously (black arrows). As a consequence, *ERF1* is transcriptionally activated; in turn, it activates the expression of defense-related genes that prevent disease progression. Other types of stress or pathogens (white arrows) induce the activation of only one of these signaling pathways and, therefore, ethylene- or jasmonate-specific responses (Lorenzo *et al.,* 2003).

In the wound response, the oligosaccharide-mediated repression of the JA-dependent signaling pathway was exerted through the production and perception of ethylene in the locally damaged tissue. This negative interaction between ethylene and JA allows the establishment of the correct spatial pattern of systemically induced genes in plants reacting to injury (Rojo *et al.*, 1999). Furthermore, by using JA-deficient (*asLOX3*), ethylene-insensitive (*mETR1*) *Nicotiana attenuata* plants, and their genetic cross, it was proposed that in *N. attenuata*, the crosstalk between ethylene and JA restrains local cell expansion and growth after herbivore attack, allowing more resources to be allocated to induced defenses against herbivores (Onkokesung *et al.*, 2010).

It was reported that the effects of JAs on root hair development were abolished in the ethylene-insensitive mutants *etr1-1* and *etr1-3*, or by ethylene action (Ag^{+}) or biosynthesis inhibitors (AVG). Moreover, it was found that JA biosynthesis inhibitors, ibuprofen and SHAM, also repressed ACC-driven or *eto1-1*-induced root hair formation (Zhu *et al.*, 2006). These results support a role for the interaction between ethylene and JAs in the regulation of root hair development. In addition, the triple-response that includes an exaggerated apical hook of *Arabidopsis* seedlings germinated in the dark in the presence of ethylene can be suppressed by JA in a *COI1*-dependent manner is an example of interaction of ethylene and JAs in development process (Ellis and Turner, 2001).

4.5 Ethylene and brassinosteroids (BRs)

Brassinosteroids (BRs) are a family of poly-hydroxylated steroid hormones that are involved in many aspects of plant growth and development. Studies have shown that BRs and ethylene have overlapping functions in hypocotyl elongation and apical hook formation (De Grauwe *et al.*, 2005). It was suggested that ethylene controls the biosynthesis of BRs and establishes a gradient of BR in the apical hook region that contributes to the hook formation. Furthermore, Gendron *et al.*, (2008) reported that ethylene functions partly through BR to regulate both hypocotyl length and apical hook formation, and it is likely that ethylene functions through the BES1 dependent branch of the BR signaling pathway to control hypocotyl elongation.

BRs are known for a long time to stimulate the production of ethylene in shoots and roots (Yi *et al.*, 1999; Arteca and Arteca, 2001), and this ethylene probably plays an important role in the many effects of BR on plant growth and development. In mung bean (*Vigna radiata*), the brassinosteroid (BR) 2, 4-epibrassinolide has been shown to specifically enhance the expression of *VrACS7* in hypocotyls, and BR also synergistically increased the IAA-induced *VRACS6* and *VrACS7* transcript levels (Yi *et al.,* 1999). Müssig *et al.*, (2003) also reported that BR has a positive effect on genes involved in ethylene biosynthesis and ethylene response based on the expression data. Interestingly, it was found that brassinopride (BRP), an inhibitor of BR biosynthesis, also causes exaggerated apical hooks in dark-grown seedlings, an effect similar to that of ethylene (Gendron *et al.,* 2008). Physiological experiments using ethylene mutants and treatment with ethylene (ACC) and an ethylene perception inhibitor suggest that BRP promotes ethylene action at a step of or upstream of ethylene perception, possibly ethylene synthesis.

It was shown that ACS5 protein was stabilized in response to BR (Hansen *et al.*, 2009), suggesting that BR increases ethylene synthesis by regulation of ethylene biosynthesis gene at post-transcriptional levels. However, studies in mung bean have indicated that *VrACS7* is regulated transcriptionally by BR (Yi *et al.*, 1999; Zimmermann *et al.*, 2004). Moreover, transcriptome data indicated that both *ACS5* and *ACS6* transcripts are elevated in response to BR (Hansen *et al.,* 2009), and in etiolated seedlings, BR treatment also resulted in increase of *ACS5* transcript levels. Taken together, BRs have an effect on the transcription of *ACS* genes, but also act by increasing ACS protein stability.

5. Tomato as model plant

The Solanaceae family comprises many agriculturally valuable crops, including eggplant, potato, pepper, tobacco, and tomato. Among them, tomato is one of the most important crops in the fresh vegetable market and the food-processing industry (Matsukura *et al.*, 2008). Tomato (*Solanum lycopersicum*) originated in South America and was brought to Europe in the early 16th century. After its introduction in Europe the tomato has gone a long way. Dedicated breeding has resulted in numerous cultivars grown all over the world, differing in all kind of aspects such as yield, shape, resistance, taste and quality.

Up to now, there are over 7500 tomato varieties cultivated throughout the world presenting a huge variability in fruit color or size. Although original tomatoes were small fruit, now most cultivars produce large red fruit, a number of cultivars with yellow, orange, pink, purple, green, black, or white fruit are also available. Tomato fruit size varies from 5mm of diameter in cherry tomatoes to more than 10 centimeters in beefsteak tomatoes (http://en.wikipedia.org/wiki/Tomato). In addition, tomatoes are loaded with phytochemicals, plant-derived chemical compounds, which work in concert with the body to protect against cancer, clogged arteries and skin ailments. The lycopene in tomatoes is one of the most powerful anti-oxidants and helps in the fight against cancerous cell formation.

In addition to its agronomical and economic importance, tomato is an excellent model plant for genomic research of solanaceous plants, as well as for studying the development, ripening, and metabolism of fruit. Indeed, tomato has been recognized as a model system for studying the molecular basis of fleshy fruit development and unravelling the role of ethylene in controlling the ripening of climacteric fruit since the early 1980s. For genetic and genomic studies, tomato has many advantages over other species of agronomical interest, such as simple diploid genetics (n = 12), a relatively compact genome (900 Mb) that has recently been sequenced, numerous mapped traits, developed DNA markers, rich collections of germplasm and mutants, available RNAseq data, highly efficient transformation protocols and a relatively short reproductive cycle (3-4 generation per year) (Tanksley *et al.*, 1992; Van der Hoeven *et al.*, 2002; Tomato Genome Consortium, 2012). Moreover, the tomato plant has many interesting features such as fleshy fruit, a sympodial shoot, compound leaves, photoperiod-independent sympodial flowering, glandular trichomes and the formation of fleshy climacteric fruits, which other model plants (such as *Arabidopsis* and rice) do not have. Most of these traits are agronomically important and cannot be studied using other model plant systems. These advantages have made tomato an excellent model organism for investigating fruit development, ripening processes, sugar metabolism, carotenoid biosynthesis, quantitative trait locus (QTL) analyses, and plant-pathogen interactions (Robinson *et al.*, 1988; Wilkinson *et al.*, 1995; Frary *et al.*, 2000; Giovannoni, 2001; Bramley, 2002; Pedley and Martin, 2003; Carrari *et al.*, 2006; Giovannoni, 2007). Indeed, the adaptation of a range of technological tools (e.g.

microarray) and the generation of new biological resources on the tomato (e.g. EST database, TILLING resources, genetic and physical maps) have led to a step forward on the understanding of the molecular mechanisms underlying plant development and fruit ripening. Since the genome structures of most of the solanaceous plants are relatively well conserved, the genomic and molecular studies of tomato can serve as a reference to understanding of other Solanaceae species, which then allows researchers to investigate molecular mechanisms underlying fruit development and ripening in different species.

Fleshy fruits are important worldwide crops because they are important sources of useful and functional compounds for human diet. Tomato has proved to be an excellent model system for the research on fleshy fruit development and ripening. Fruit development starts after the ovules in the ovary have been successfully fertilized and the ovary begins to develop into the fruit. Generally, fruit development can be divided into essentially three stages which are depicted in Figure 19. These are (i) a period of intensive cell division that begins at anthesis and continues approximately for 2 weeks after fertilization; (ii) a period of rapid cell expansion that begins toward the end of the cell division stage and continues until one week before the onset of ripening; (iii) a ripening phase that initiates after growth has ceased and involves rapid chemical and structural changes that determine fruit aroma, color, texture and biochemical composition but not fruit size and shape.

Figure 19. **Overview of tomato fruit development.** Fruit set is the initiation of fruit growth after the flower has been successfully pollinated and fertilized. After fertilization, cell division takes place, which lasts up to 12 d. This period is followed cell expansion, during which the volume of the fruit rapidly increases. Once the fruit has reached its final size it starts to ripen (Mounet *et al.*, 2007).

Ripening in the cultivated tomato comprises a series of biochemical and physiological events, including softening, pigment change, development of flavor components, autocatalytic ethylene production, and climacteric respiratory behavior, which together make ripe fruits. Several naturally occurring ripening mutations have been characterized. These include for example *ripening-inhibitor* (*rin,* Vrebalov *et al.,* 2002), *non-ripening* (*nor,* Giovannoni, 2004), *Colorless non-ripening* (*Cnr,* Manning *et al.,* 2006), *Green-ripe* (*Gr,* Barry and Giovannoni, 2006), *Never-ripe* (*Nr,* Wilkinson *et al.,* 1995), *high-pigment 1* (Liu *et al.*, 2004) and *Never-ripe 2* (*Nr-2, Barry et al., 2005*). The availability of these well characterized ripening mutants is indeed an important reason for tomato being taken as a model for study fruit ripening (Figure 20).

Through investigation of these tomato mutants in fruit ripening, many of the underlying genes control the ripening processes were isolated. The *RIN*, *CNR*, and *NOR* genes have been shown to encode transcriptional regulators and act to regulate the expression of other genes responsible for fruit ripening processes, including ethylene biosynthesis (Vrebalov *et al.,* 2002; Giovannoni, 2004; Manning *et al.,* 2006). The *Gr* gene encodes a still poorly defined component of ethylene signal transduction while *Nr* encodes an ethylene receptor important for fruit and additional non-fruit ethylene responses (Lanahan *et al.,* 1994; Barry and Giovannoni, 2006). Moreover, other ripening transcriptional regulators have also been demonstrated via functional studies in transgenic plants, including *LeHB1* which directly regulates ACC oxidase expression (Lin *et al.,* 2008) and TAGL1, a MADS box transcription factor, which links early fruit fleshy expansion with downstream ripening (Lin *et al.,* 2008; Itkin *et al.,* 2009; Vrebalov *et al.,* 2009; Pan *et al.,* 2010). The putative transcription factor, *Sl-AP2a*, a member of the AP2/ERF superfamily gene was also recently described as a negative regulator of fruit ripening and of ethylene production (Chung *et al.,* 2010; Karlova *et al.,* 2011). These discoveries have further facilitated the demonstration of regulatory mechanisms for fruit ripening.

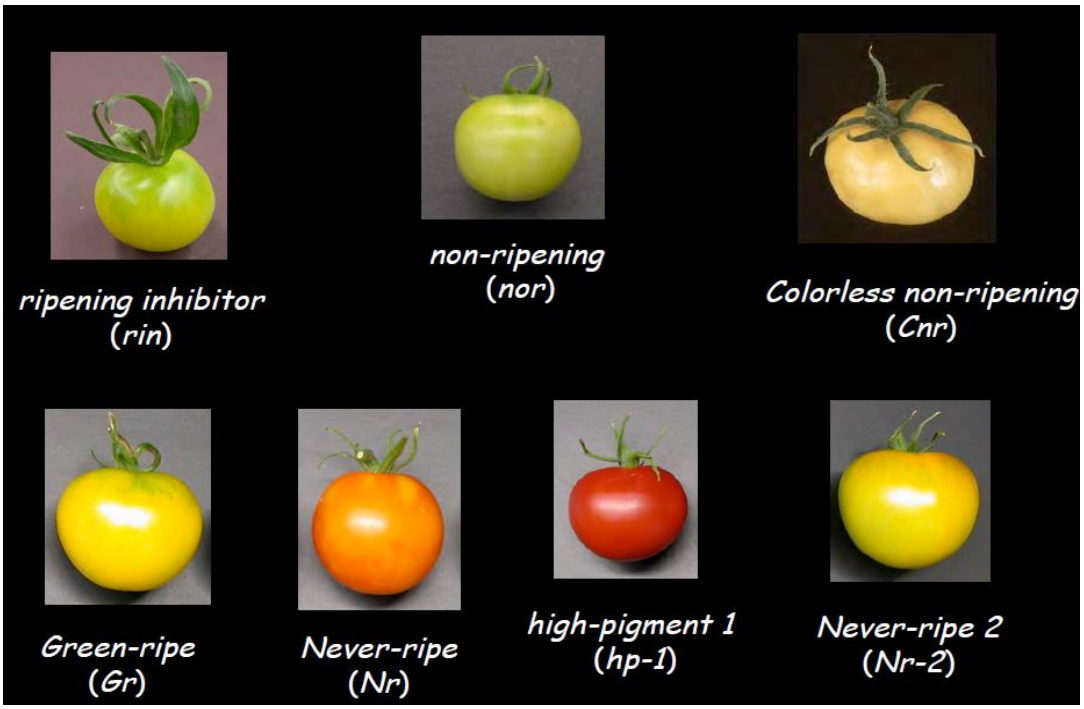

Figure 20. Tomato ripening mutants (Giovannoni, 2004; Barry et al., 2005; Giovannoni, 2007).

Like *Arabidopsis*, the convenient small size and amenability to large-scale cultivation are also found in tomato. Micro-Tom (MT), a dwarf cultivar of tomato, has been proposed as a preferred variety to carry out molecular research in tomato. MT was initially created for ornamental purposes by crossing Florida Basket and Ohio 4013-3 cultivars (Martí *et al.*, 2006). MT cultivar displays a very dwarf phenotype with a bushy appearance and its leaves are small, with deformed leaflets, and a deep green color compared with other commonly used wild-type cultivars (Figure 21). It was confirmed that Micro-Tom phenotype results from mutations in the *SELF PRUNING (SP)* and *DWARF (D)* genes (Martí *et al.*, 2006). SP belongs to the CETS family of regulatory genes encoding modulator proteins that determine the potential for continuous growth of the shoot apical meristem, while, the *DWARF* (D) gene encodes a P450 protein involved in brassinosteroid (BR) biosynthesis (Bishop *et al.*, 1996; Pnueli *et al.*, 2001). Regardless of the presence of mutations that cause the MT's dwarf size, it has been proven to be suitable as a standard genotype in tomato research, including the study of novel hormonal interactions (Wang *et al.*, 2009; Campos *et al.*, 2010; Serrani *et al.*, 2010). Due to its

small size, rapid life cycle, high-throughput capabilities and easy transformation, Micro-Tom was chosen as the main tomato cultivar during my Ph.D. studies.

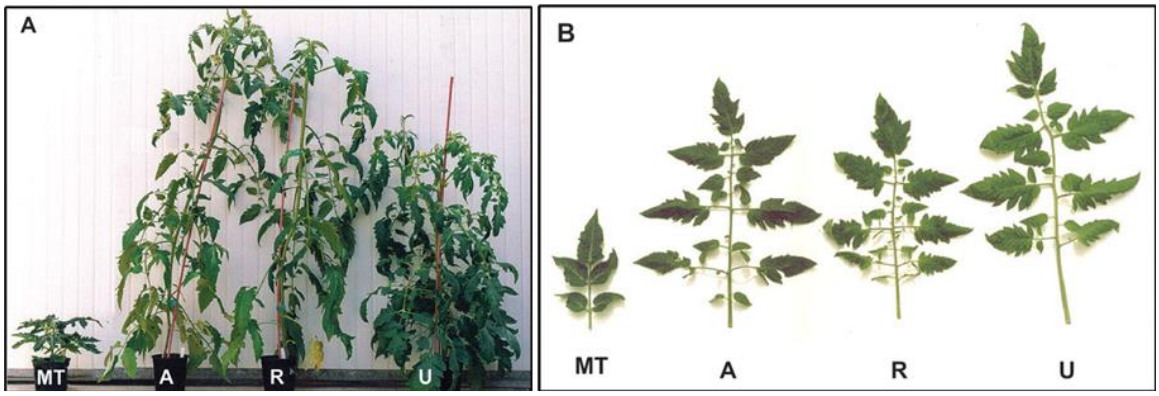

Figure 21. **Plants of Micro-Tom, Ailsa Craig, Rutgers, and UC-82.** (A) Entire plants at the time of flowering (~ 2 months old). (B) Fifth leaf from the base. MT, Micro-Tom; A, Ailsa-Craig; R, Rutgers, U, UC-82 (Martí *et al.*, 2006).

Chapter II

A dominant repressor version of the tomato *Sl-ERF.B3* gene confers ethylene hypersensitivity via feedback regulation of ethylene signaling and response components

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A dominant repressor version of the tomato *Sl-ERF.B3* gene confers ethylene hypersensitivity via feedback regulation of ethylene signaling and response components

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Running title: *Sl-ERF.B3*-mediated Ethylene Responses

Keywords: tomato (*Solanum lycopersicum*), ethylene, hormone signaling, ethylene response factor, dominant repressor

ACCESSION NUMBERS

Gene ID data for the genes described in this article are listed in Table S4.

SUMMARY

Ethylene Response Factors (ERFs) are downstream components of the ethylene signal transduction pathway although their role in ethylene-dependent developmental processes remains poorly understood. Since the ethylene-inducible tomato *Sl-ERF.B3* has been previously shown to display a strong binding affinity to GCC-box-containing promoters, its physiological significance was addressed here by reverse genetics approach. However, classical up- and down-regulation strategies failed to give clear clue on its roles *in planta,* likely owing to functional redundancy among ERF family members. Expression of a dominant repressor *ERF.B3-SRDX* version of *Sl-ERF.B3* in the tomato resulted in pleiotropic ethylene responses and vegetative and reproductive growth phenotypes. The dominant repressor etiolated seedlings displayed partial *constitutive ethylene-response* in the absence of ethylene and adult plants exhibited typical ethylene-related alterations such as leaf epinasty, premature flower senescence and accelerated fruit abscission. The multiple symptoms related to enhanced ethylene sensitivity correlate with the altered expression of ethylene biosynthesis and signaling genes, suggesting the involvement of *Sl-ERF.B3* in a feedback mechanism regulating components of ethylene production and response. Moreover, Sl-ERF.B3 is shown to modulate the transcription of a set of *ERFs* revealing the existence of a complex network interconnecting different *ERF* genes. Overall, the study indicates that Sl-ERF.B3 has a critical role in regulating multiple genes and identifies a number of *ERFs* among its primary targets, consistent with the pleiotropic phenotypes displayed by the dominant repression lines.

INTRODUCTION

The plant hormone ethylene is involved in many developmental processes and plays a critical role in a wide range of physiological responses, including seed germination, cell elongation, flowering, fruit ripening, organ senescence, abscission, root nodulation, programmed cell death, and response to abiotic stresses and pathogen attacks (Johnson and Ecker, 1998; Bleecker and Kende, 2000; Lin *et al.*, 2009). Ethylene Response Factors (ERFs) are known to be the last downstream components of the ethylene transduction pathway and signal transmission cascade has been linked to the transcriptional activation of some *ERF* genes (Solano *et al.*, 1998; Benavente and Alonso, 2006). According to the currently accepted model, ethylene is perceived by specific receptors, which have been shown to activate the hormone transduction pathway through releasing the block exerted by CTR1 on EIN2 (Solano and Ecker, 1998; Ju *et al.*, 2012). The release of EIN2 then activates EIN3/EIL1 primary transcription factors, resulting in the expression of secondary transcription factors, namely ERFs, which regulate the expression of downstream ethylene-responsive genes (Solano *et al.*, 1998; Alonso *et al.*, 2003). The receptors act as redundant negative regulators of ethylene signaling to suppress ethylene responses (Hua and Meyerowitz, 1998; Hall and Bleecker, 2003). In the absence of the hormone, the receptor actively suppresses ethylene responses and ethylene binding removes this suppression. EIN3/EILs type of transcription factors are positive regulators of the ethylene signaling that function as trans-activating factors to trigger ethylene responses (Chao *et al.*, 1997; Solano *et al.*, 1998). In *Arabidopsis*, overexpression of *EIN3* or *EIL1* results in a constitutive ethylene phenotype and reduced expression of multiple *LeEIL* genes in the tomato results in decreased ethylene sensitivity (Chao *et al.*, 1997; Tieman *et al.*, 2001).

ERFs are plant specific transcription factors, belonging to the large AP2/ERF multi-gene family (Riechmann *et al.*, 2000). Proteins encoded by this gene family have a highly conserved DNA-binding domain known as AP2 domain made of 58-59 amino acids involved in the binding to the target DNA sequences (Allen *et al.*, 1998). ERFs from different plant species have been reported to be involved in a variety of processes such as responses to biotic and abiotic stresses, metabolic pathways, fruit ripening and ethylene

response (Fujimoto *et al.*, 2000; van der Fits and Memelink, 2000; Li *et al.*, 2007; Trujillo *et al.*, 2008; Lee *et al.*, 2012). ERF proteins are known to interact with multiple *cis*-acting elements found in the promoter regions of ethylene-responsive genes, including the GCC box and DRE/CRT dehydration-responsive element/C-repeat (Ohme-Takagi and Shinshi, 1995; Hao *et al.*, 2002; Oñate-Sánchez *et al.*, 2007). It was also shown that *Pti4*, an ERF type transcription factor, regulates gene expression by directly interacting with a non-GCC element (Chakravarthy *et al.*, 2003). Moreover, in addition to regulating the expression of ethylene-responsive genes, ERFs can regulate jasmonic acid and salicylic acid-responsive genes (Gu *et al.*, 2000; Brown *et al.*, 2003). ERFs can also bind the Vascular Wounding Responsive Element (VWRE) in tobacco (Sasaki *et al.*, 2007) further demonstrating their capacity to bind a wide range of *cis*-regulatory elements beside the GCC and DRE/CRT boxes.

ERFs have been associated with ethylene-regulated growth control, with either a positive or a negative regulatory function (Alonso *et al.*, 2003; Nakano *et al.*, 2006; Pirrello *et al.*, 2012). Strikingly, in *Arabidopsis* little has been reported (McGrath *et al.*, 2005) on ethylene-responsive phenotypes caused by silencing, mutation, or knockout of ERFs probably due to the high level of functional redundancy among family members. Indeed, the ERF family is composed of up to 65 members in *Arabidopsis* (Nakano *et al.*, 2006), many of which are regulated by the same stimuli and can potentially bind the same target promoter. **C**himeric **R**epressor **S**ilencing **T**echnology (CRES-T), consisting in the expression of a dominant repressor version of a transcription factor encoding gene proved to be an efficient mean to overcome experimental limitations caused by functional redundancy and this strategy has been developed to study the consequences of silencing target genes of single transcription factors (Hiratsu *et al.*, 2003; Matsui *et al.*, 2005; Heyl *et al.*, 2008). Fusing the so-called SRDX repression domain to a transcription factor suppresses the expression of its target genes dominantly over the activity of endogenous and functionally redundant transcription factors and as a result, the transgenic plants expressing the chimeric repressor version exhibit phenotypes similar to loss-of-function of the alleles of the gene encoding the transcription factor (Hiratsu *et al.*, 2003; Matsui and Ohme-Takagi, 2010).

Genome-wide study recently showed that the tomato *ERF* gene family comprises 9 subclasses defined by distinct structural features and a new nomenclature for tomato ERFs was proposed (Pirrello *et al.*, 2012) which complies with the most complete classification available in *Arabidopsis* and clarifies the correspondence between ERF subclasses in different species (Nakano *et al.*, 2006). In the tomato, only few ERF genes have been functionally characterized so far, most of these have been shown to participate in stress and/or hormonal responses (Gu *et al.*, 2002; Pirrello *et al.*, 2006; Li *et al.*, 2007; Zhang *et al.*, 2009; Lee *et al.*, 2012; Pan *et al.*, 2012). The tomato *Sl-ERF.B3* is related to *Arabidopsis* factors *ERF106* and *ERF107*, which are members of group IX according to Nakano *et al.,* (2006). This group has been implicated in the regulation of defense responses and knock-out analysis of *ORA59* (Pré *et al.,* 2008) and *AtERF14* (Oñate-Sánchez *et al.*, 2007), prominent representatives of group IX, has revealed disease susceptibility phenotypes. Consistently, overexpression of *ERF1* another member of the group has led to enhanced resistance to necrotrophic pathogens (Berrocal-Lobo *et al.*, 2002).

Sl-ERF.B3 was previously shown to act as strong transcriptional activator on GCC-box-containing promoters and its transcripts accumulate upon ethylene treatment, suggesting a putative involvement in ethylene-regulated processes (Tournier *et al.*, 2003; Pirrello *et al.*, 2012). Because overexpressing and down-regulated lines failed to reveal the functional significance of *Sl-ERF.B3*, a dominant chimeric repressor version was used which resulted in phenotypes consistent with Sl-ERF.B3 being involved in both ethylene biosynthesis and signaling pathway. The *ERF.B3-SRDX* lines displayed *constitutive ethylene-responses* in the absence of ethylene and the data identified a set of *ERFs* among the target genes regulated by Sl-ERF.B3 supporting the idea that the alteration of such a high number of *ERFs* may account for the pleiotropic phenotypes displayed by the transgenic lines.

RESULTS

Classical down- and up-regulation approaches failed to provide clear clues on *Sl-ERF.B3* functional significance

To address the physiological significance of *Sl-ERF.B3* and its potential role in mediating ethylene responses, tomato lines under- and over-expressing *Sl-ERF.B3* gene were generated by stably transforming tomato plants with either sense or antisense constructs under the control of the constitutive 35S promoter. A number of homozygous transgenic lines corresponding to independent transformation events were obtained for both antisense and sense construct. Overall, 10 antisense and 12 sense independent lines were examined and the evidence for the expression of the transgene and for its ability to alter the levels of endogenous *Sl-ERF.B3* transcripts in the transgenic lines was provided by qRT-PCR analysis (Figure S1a). No consistent phenotypes could be revealed in antisense lines whereas close examination of *Sl-ERF.B3* over-expressing plants revealed slightly but significantly higher plants at early development stages (4-week-old) though the plant size returned to normal at 8-week-old plants (Figure S1b). No other consistent growth or reproductive phenotypes could be detected in these *Sl-ERF.B3* over-expressing lines.

ERF.B3-SRDX suppresses the transactivation capacity of Sl-ERF.B3

In an attempt to overcome the experimental limitations likely owing to functional redundancy among members of the ERF gene family, we generated a dominant repressor version of *Sl-ERF.B3* (*ERF.B3-SRDX*) using the Chimeric Repressor Silencing Technology (CRES-T). The *Sl-ERF.B3* coding sequence lacking the Stop Codon was fused to the SRDX repression domain LDLDLELRLGFA, known as the EAR motif (Mitsuda *et al.*, 2006) and cloned downstream of the Cauliflower Mosaic Virus 35S promoter. The capacity of the ERF.B3-SRDX chimeric protein to function as a transcriptional repressor on ethylene-responsive genes was assessed in a transient transformation assay via co-transfection of protoplasts with reporter and effector constructs. The reporter construct was obtained by fusing the GFP coding sequence either

to a synthetic promoter containing the ethylene inducible GCC box, or to a native osmotin promoter containing the canonical GCC *cis*-acting element. The effector constructs allow the expression of either the Sl-ERF.B3 protein or its repressor version fused to the SRDX motif (ERF.B3-SRDX). Transactivation assays indicated that Sl-ERF.B3 enhances the expression of the reporter gene driven by both the synthetic and native promoter, clearly indicating that Sl-ERF.B3 acts as a transcriptional activator of GCC-box containing promoters (Figure 1). By contrast, co-transfection of the reporter constructs with the ERF.B3-SRDX results in 8-fold and 15-fold suppression of the activity of the synthetic and the native ethylene-responsive promoters, respectively (Figure 1). These data confirm that ERF.B3-SRDX retains the capacity to bind the same target promoters than Sl-ERF.B3 and to dominantly repress its transcriptional activity. These data support the hypothesis that the ERF.B3-SRDX chimeric protein can potentially be used as transcriptional repressor of Sl-ERF.B3 target genes *in planta*.

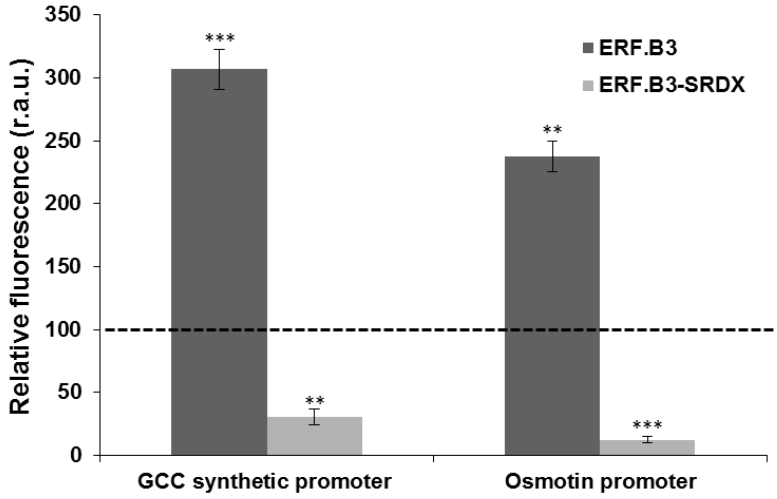

Figure 1. Transactivation assay in a single cell system. Protoplasts were co-transfected with a reporter construct consisting of the GFP gene driven by a GCC-rich synthetic promoter or a native osmotin GCC-containing promoter and an effector plasmid expressing either ERF.B3 or ERF.B3-SRDX protein. The basal fluorescence obtained in the assay transfected with the reporter construct and an empty effector construct was standardized to 100 and is taken as reference. Values are means ± SD of three independent biological replicates.

Dark-grown 35S:*ERF.B3-SRDX* seedlings display enhanced triple response

To gain insight on the physiological function of *Sl-ERF.B3*, transgenic tomato lines (*Microtom cv*) expressing the *ERF.B3-SRDX* dominant repressor construct were produced. Ten independent homozygous *35S:ERF.B3-SRDX* lines were generated, all of them displayed similar pleiotropic alterations. Three representative lines, *SR1, SR2* and *SR3*, showing a characteristic phenotype with different expressivity, were selected for further studies. The relative expression level of *ERF.B3-SRDX* transcript in these three lines was assessed using primers specific for *ERF.B3-SRDX* (Figure S2). The accumulation of the endogenous *Sl-ERF.B3* assessed by qRT-PCR was similar in the transformed and non-transformed plants ruling out the eventuality of a feedback regulation of *Sl-ERF.B3* in the transgenic lines (Figure S2).

Dark-grown *ERF.B3-SRDX* seedlings exhibited exaggerated apical hook formation and inhibited hypocotyl elongation in the absence of exogenous ethylene treatment (Figure 2a). Hypocotyl length of 7-day-old etiolated seedlings was 50% lower in *ERF.B3-SRDX* lines compared to wild type (Figure 2b). Interestingly, application of 1-MCP, the ethylene perception inhibitor, reversed the triple response phenotype of *ERF.B3-SRDX* dominant repressor lines (Figure 2a) leading to a complete loss of the exaggerated apical hook and a recovery of hypocotyl length similar to that of wild type (Figure 2a, b). Treatment with 10 $\mu L\ L^{-1}$ ethylene resulted in a more pronounced ethylene triple response in *ERF.B3-SRDX* lines than in wild type (Figure 2a, b), suggesting a higher sensitivity to the hormone for the transgenic lines.

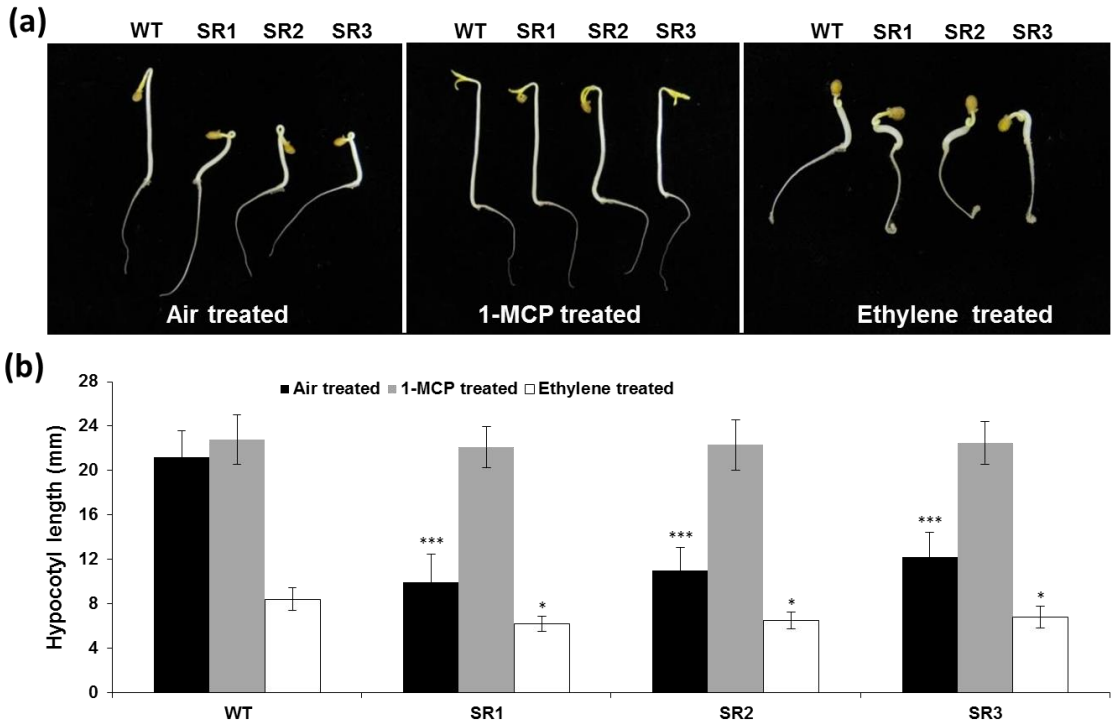

Figure 2. Ethylene hypersensitivity of *35S:ERF.B3-SRDX* lines.

(a) Etiolated *35S:ERF.B3-SRDX* seedlings display partial constitutive ethylene response in the absence of exogenous ethylene that can be removed by 1-MCP application (1.0 mg L^{-1}) or exaggerated upon exogenous ethylene (10 µL L^{-1}) treatment.

(b) Hypocotyl elongation in *ERF.B3-SRDX* etiolated seedlings and WT treated or untreated with ethylene and 1-MCP. Values are means ± SD (n ≥ 30) of three replicates. *, $0.01 < P < 0.05$, ***, $P < 0.001$ (Student's test). *SR1, SR2* and *SR3* are three independent *35S:ERF.B3-SRDX* lines.

Because *Sl-ERF.B3* over-expressing plants displayed some, though very mild, growth phenotype at early stages (4-week-old) of plant development, these lines have been tested for the ethylene response phenotype. While the over-expressing lines cannot be discriminated from wild-type plants when dark-grown in air, upon exogenous ethylene treatment some of the transgenic lines show a slightly lower reduction in hypocotyl length than in wild type thus suggesting a reduced response to the hormone (Figure S3).

35S:ERF.B3-SRDX plants show a suite of ethylene hypersensitive phenotypes

Several developmental processes known to be regulated by ethylene were altered in the dominant repressor lines among which leaf and petiole epinasty (Figure 3). Additional ethylene-related phenotypes displayed by *ERF.B3-SRDX* plants included premature flower senescence and early fruit abscission (Figure 3). The majority of flowers in *ERF.B3-SRDX* plants undergo premature senescence and abscission before full opening of the petals (Figure 3). Moreover, the *ERF.B3-SRDX* fruits display early abscission compared to wild-type fruit (Figure 3). Approximately two weeks after the breaker stage, the fruit abscission zone starts to dehisce in the *ERF.B3-SRDX* lines, whereas this occurs at later stages in wild-type lines (Figure 3). Collectively, these ethylene-related phenotypes are consistent with an ethylene hypersensitivity of the *ERF.B3-SRDX* dominant repressor lines.

Figure 3. Ethylene hypersensitive phenotypes of adult *35S:ERF.B3-SRDX* plants showing petioles and leaves epinasty (upper panel) enhanced premature flower senescence (middle panel) and accelerated fruit abscission (lower panel). The white arrows point to the abscission zone.

Dominant repressor plants display pleiotropic vegetative and reproductive phenotypes

35S:ERF.B3-SRDX plants showed a stunted phenotype from early developmental stages and the size of adult plants was severely reduced (Figure 4a) with an average height being less than one third of that of wild-type plants after 80 days (Figure 4b). Noteworthy, the transcript level of two GA oxidase biosynthetic genes, *Sl-GA20ox1* and *Sl-GA20ox2*, was found to be significantly lower than the transgenic plants (Figure 4c). A reduced GA synthesis may therefore account for the dramatic dwarf phenotype displayed by *ERF.B3-SRDX* plants. Consistent with this hypothesis, application of GA_3 to 10-day-old transgenic plants partially rescued the dwarf phenotype (Figure 4d). Nevertheless, *in silico* analysis of the promoter region of the two GA biosynthesis genes did not reveal the presence of any canonical ethylene-response elements.

Leaf morphology is remarkably altered in the transgenic lines (Figure S4a) with a severe reduction in leaflet size, ranging from 51% to 32% in length and 47% to 22% in width (Figure S4b). The leaf margins of the *ERF.B3-SRDX* plants are twisted and the lamina is often wrinkled (Figure S4a). Scanning electron microscopy revealed smaller epidermal cells in the transgenic leaves (Figure S4c) with the strongest *ERF.B3-SRDX* expressing line showing epidermal cell size less than one third of that in wild type (Figure S4d).

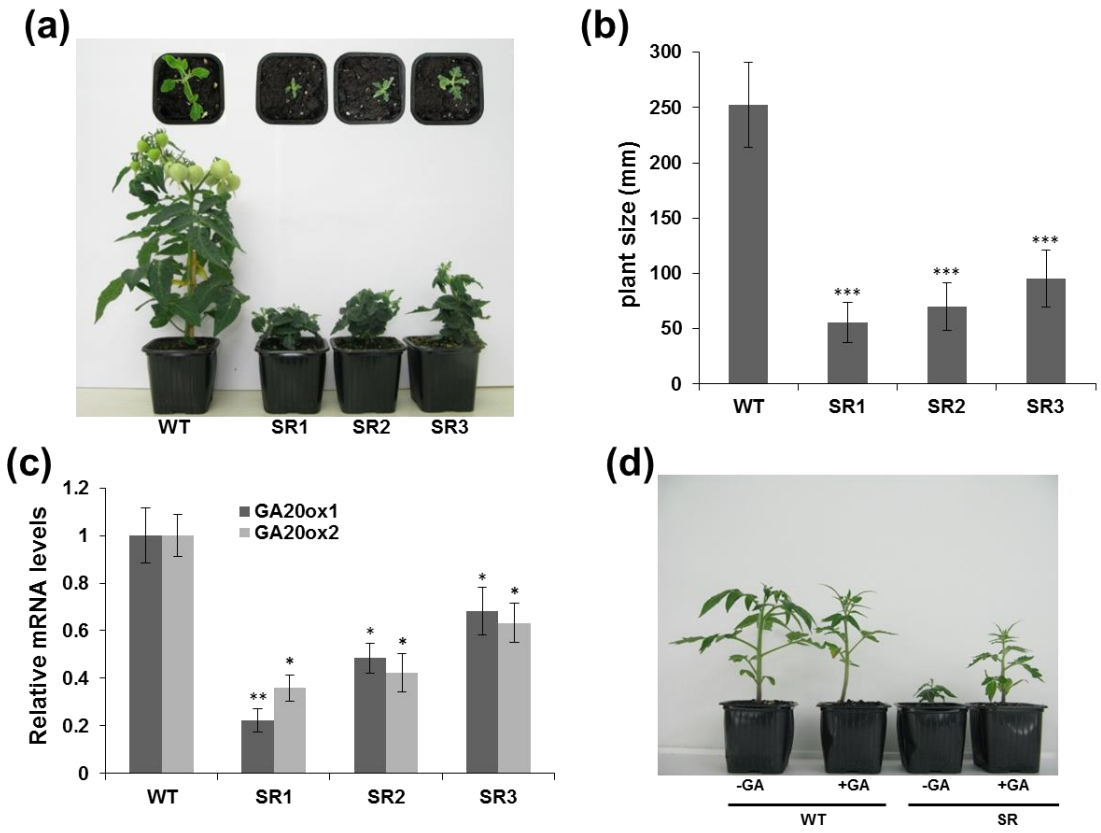

Figure 4. Dwarf phenotype of *35S:ERF.B3-SRDX* plants.

(a) Dwarf phenotype of *35S:ERF.B3-SRDX* plants. Photographs were taken at 7 days (upper panel) and 80 days (lower panel) after germination.

(b) Reduced plant size of 80-day-old *ERF.B3-SRDX* plants. Values are means ± SD (n ≥ 15) of three replicates.

(c) Relative mRNA levels of two *GA oxidase* genes in wild-type and *ERF.B3-SRDX* lines assessed by qRT-PCR. The relative mRNA levels of each gene in the wild type were standardized to 1.0, referring to *Sl-Actin* gene as internal control.

(d) *ERF.B3-SRDX* dwarfism partially rescued by exogenous gibberellic acid (GA) application. Ten-day-old wild-type and *ERF.B3-SRDX* plants were sprayed with GA (10^{-5} M) twice a week for three weeks.

*, $0.01 < P < 0.05$, **, $0.001 < P < 0.01$, ***, $P < 0.001$ (Student's test). *SR1, SR2* and *SR3* are three independent *35S:ERF.B3-SRDX* lines.

ERF.B3-SRDX plants also showed severely delayed reproductive growth (Figure 5a). The time from germination to flower bud setting was delayed by 14 to 20 days in transgenic lines compared to the reference wild-type lines (Figure 5b). Likewise, flower anthesis in *ERF.B3-SRDX* plants occurred 29 to 34 days later than in WT (Figure 5b). Moreover, compared to wild type, transgenic plants produced significantly smaller flowers (Figure S5a) with up to 30% reduction in anther length. A reduction in fruit size was also observed in the *ERF.B3* dominant repressor lines which produced heart-like shaped fruit (Figure S5b) and small seeds with aberrant shape (Figure S5c). The *ERF.B3-SRDX* lines also displayed dramatic reduction in fruit set, leading to markedly lower fruit number per plant at maturity (Figure 5d). Up to 91% of successful fruit set was achieved in wild type while in the same growing condition, the fruit set rate reached 10-18% in the *ERF.B3-SRDX* lines (Figure 5d). Cross-fertilization assay was performed to examine fertility of transgenic flower. Using wild-type flowers as female recipient and *ERF.B3-SRDX* plants as pollen donor, 87% of successful fruit set was achieved. Notably, all the developed fruits were seeded, and when germinated, all the seeds were hygromycin resistant (Table 1) indicating that *ERF.B3-SRDX* pollen is viable and fertile. Using wild type as pollen donor, pollinated *ERF.B3-SRDX* flowers also showed 80% success of fruit set (Table 1). The reciprocal crossing indicated that both ovule and pollen are fertile in the *ERF.B3-SRDX* dominant repressor lines (Table 1). Pollen viability of transgenic lines was further confirmed by Alexander's staining assay (Figure S5d). A closer examination of the flower organ structure revealed that *ERF.B3-SRDX* flowers display exerted stigma positioned beyond the tip of the anther cone, in contrast to wild-type flowers where the *stigma* is slightly *inserted* within *the anther cone* (Figure 5c). The stigma to anther length ratio is significantly higher in the transgenic lines (Figure 5e) which may consequently prevent efficient self-pollination thus resulting in poor fruit set.

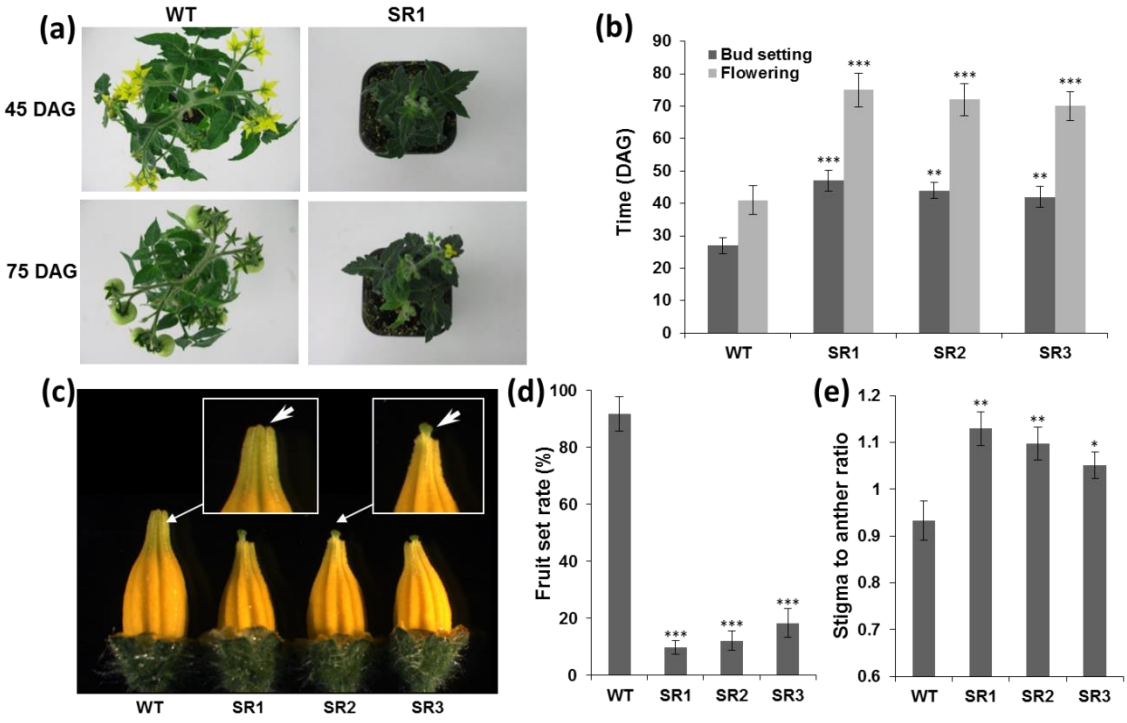

Figure 5. Delayed reproductive development and reduced fruit set in *35S:ERF.B3-SRDX* plants.

(a) Late flower bud setting and flowering time in *ERF.B3-SRDX* plants compared to WT. DAG, Day After Germination.

(b) Assessing the time of flower bud setting and flower opening in *ERF.B3-SRDX* and WT plants.

(c) Abnormal flowers with short anther and exerted stigma in *ERF.B3-SRDX* lines.

(d) Reduced fruit set rate in *ERF.B3-SRDX* lines.

(e) Stigma to anther length ratio in *ERF.B3-SRDX* lines compared to WT.

Values are means ± SD ($n \geq 30$) of three replicates. *, $0.01 < P < 0.05$, **, $0.001 < P < 0.01$, ***, $P < 0.001$ (Student's test). *SR1, SR2* and *SR3* are three independent *35S:ERF.B3-SRDX* lines.

Table 1 Cross-Fertilization Assay. Emasculated wild-type flowers were fertilized with *ERF.B3-SRDX* pollen and the number of fruit was assessed at the ripe stage. Conversely, tomato pollen from wild-type flowers was used to fertilize emasculated *ERF.B3-SRDX* flowers. In the control assay, wild-type emasculated flowers were fertilized with wild-type pollen. For each cross-fertilization assay, the capacity of the F1 seeds to grow on hygromycin-containing medium was assessed. Results are representative of data from three independent *ERF.B3-SRDX* lines (*SR1*, *SR2*, and *SR3*).

Female recipient	Pollen donor	Fruit set /crossed flowers	Fruit set (%)	F1 Hygromycin Resistance (%)
Wild type	ERF.B3-SRDX	39/45	87	100
ERF.B3-SRDX	Wild type	36/45	80	100
Wild type	Wild type	41/45	91	0

Expression of *ERF.B3-SRDX* leads to reduced ethylene production

To investigate the role of Sl-ERF.B3 in regulating ethylene biosynthesis, the level of ethylene production was assessed in etiolated seedlings revealing that *ERF.B3-SRDX* seedlings produce significantly less ethylene than wild type (Figure 6a). Accordingly the dominant repressor lines displayed reduced accumulation of transcripts corresponding to *Sl-ACS* and *Sl-ACO* ethylene biosynthesis genes (Figure 6b) which account for the decreased ethylene production in the *ERF.B3-SRDX* lines. *In silico* analysis of the promoter regions of *Sl-ACS* and *Sl-ACO* genes using three software packages (PLACE, PlantCARE and PlantPAN) revealed the presence of *cis*-acting elements that can serve as putative targets for ERFs, including a GCC box (GCCGCC) and DRE/CRT (CCGAC) in *Sl-ACO3* promoter and a conserved DRE/CRT (CCGAC) motif in *Sl-ACS1* promoter (Table S1).

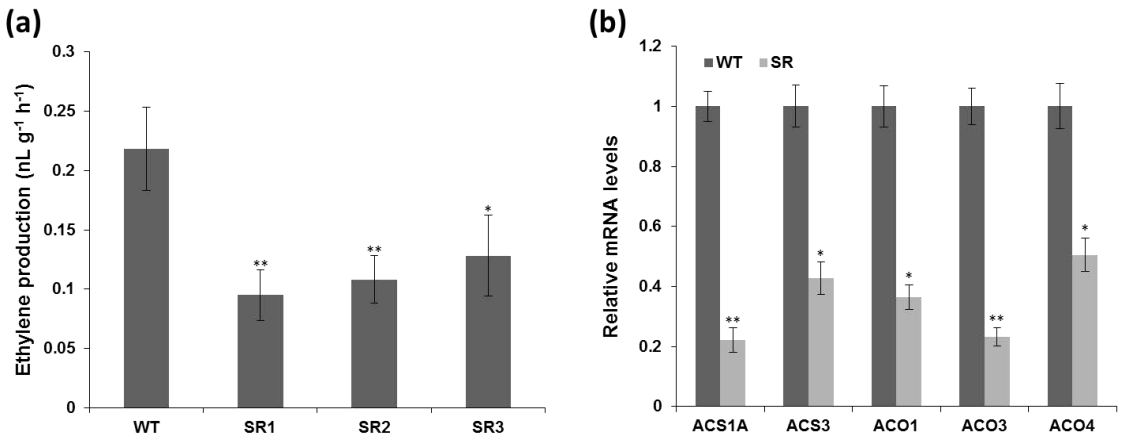

Figure 6. Down-regulation of ethylene production and ethylene biosynthesis genes in *35S:ERF.B3-SRDX* plants.

(a) Ethylene production of etiolated seedlings in WT and *ERF.B3-SRDX* lines.

(b) *ACS* and *ACO* transcript accumulation in WT and *ERF.B3-SRDX* plants assessed by qRT-PCR. The relative mRNA levels of each gene in the wild type were standardized to 1.0, referring to *Sl-Actin* gene as internal control.

Values are means ± SD of three replicates. *, $0.01 < P < 0.05$, **, $0.001 < P < 0.01$ (Student's test). *SR1, SR2* and *SR3* are three independent *35S:ERF.B3-SRDX* lines.

Ethylene receptor levels are down-regulated in *ERF.B3-SRDX* plants

In order to determine whether the expression of ethylene receptor genes may contribute to the ethylene hypersensitivity of the *35S:ERF.B3-SRDX* lines, we assessed the transcript accumulation of six tomato ethylene receptor genes in the leaves of transgenic plants. While no significant change was found for the expression of *Sl-ETR1* and *Sl-ETR4*, the four remaining ethylene receptor genes (*Sl-ETR2, Sl-ETR5, Sl-ETR6* and *NR)* were substantially down-regulated in the *ERF.B3-SRDX* lines (Figure 7a). Notably, the expression of *Sl-ETR5* was decreased by 84% in the strongest *ERF.B3-SRDX* line (Figure 7a). The expression of *Sl-ETR2* was reduced by 52-65% in three independent lines (Figure 7a) while that of *NR* was decreased by 46-61% (Figure 7a). The transcript levels of *Sl-ETR6* showed 35-50% reduction compared to wild type (Figure 7a). *In silico* search revealed the absence of conserved GCC-box in the promoter regions of all four ethylene receptor genes displaying altered expression in the transgenic lines (Table S1), in contrast

to *NR* and *Sl-ETR5* promoters which contain GCC-box-like and DRE/CRT consensus sequences. However, because Sl-ETR6 receptor has been shown to play a prominent role in regulating ethylene response (Tieman *et al.*, 2000; Kevany *et al.*, 2007b), the ability of the native Sl-ERF.B3 and the chimeric ERF.B3-SRDX proteins to regulate the *Sl-ETR6* promoter activity was tested. Transactivation assays show that Sl-ERF.B3 induced more than 2-fold increase of the *Sl-ETR6* promoter activity whereas ERF.B3-SRDX strongly suppressed this activity (Figure 7b) indicating that Sl-ERF.B3 and its dominant repressor version can both regulate the expression of *Sl-ETR6* in despite of the absence of a typical ethylene-responsive element in its promoter region. Given that ERF.B3-SRDX down-regulates the expression of the ethylene receptor genes *in vivo* and that both Sl-ERF.B3 and its repressor version strongly impact the transcriptional activity of *Sl-ETR6* in the transactivation assay, we then looked at the expression of ethylene receptor genes in tomato over-expressing lines. Among all six receptor genes present in the tomato genome, *ETR1*, *NR* and *ETR6* are up-regulated in the *Sl-ERF.B3* over-expressing lines consistent with the activator function of the Sl-ERF.B3 protein (Figure 7c).

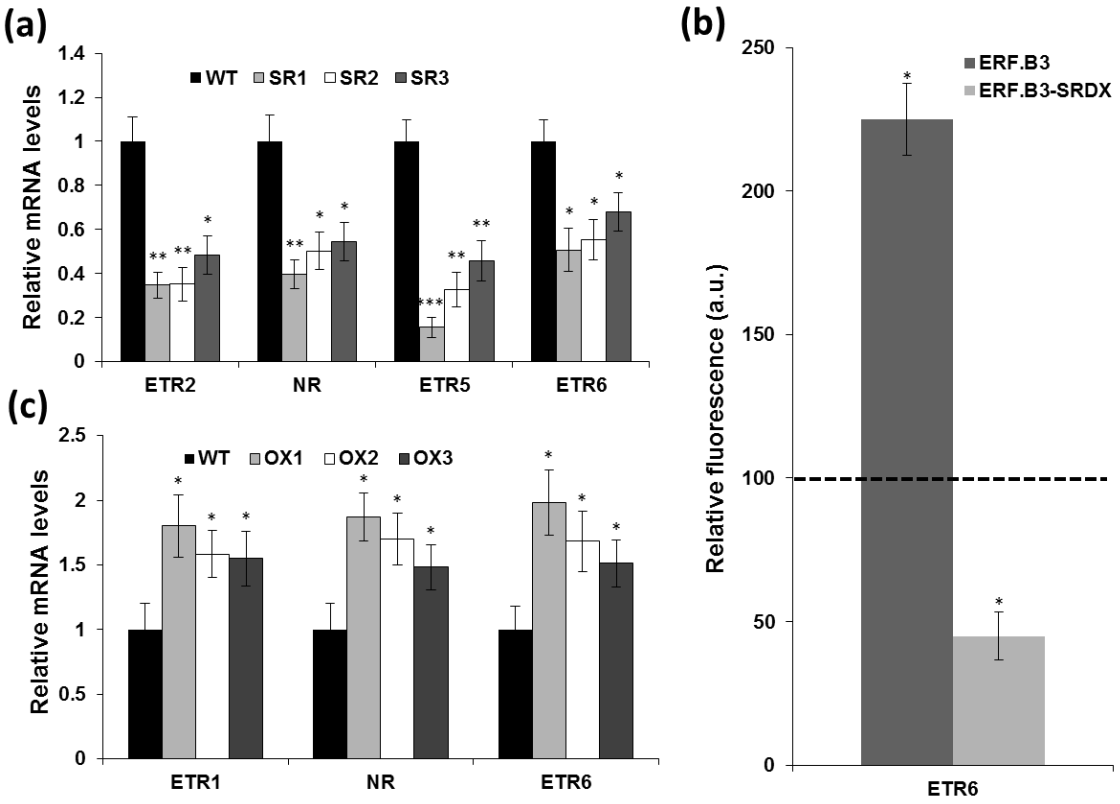

Figure 7. Expression of ethylene receptor genes in *35:ERF.B3-SRDX and ERF.B3* overexpression lines.

(a) Relative mRNA levels of *ETR2*, *NR*, *ETR5* and *ETR6* receptor genes assessed by qRT-PCR in 4-week-old WT and *ERF.B3-SRDX* lines.

(b) The transcriptional activity of *ETR6* promoter is regulated by both ERF.B3 and ERF.B3-SRDX in a protoplast transactivation assay. Protoplasts were co-transfected with GFP reporters fused to the *ETR6* promoter and with an effector plasmid expressing either ERF.B3 or ERF.B3-SRDX proteins.

(c) Relative mRNA levels of *ETR1*, *NR*, and *ETR6* assessed by qRT-PCR in 4-week-old WT and *ERF.B3* overexpression lines.

*, $0.01 < P < 0.05$, **, $0.001 < P < 0.01$, ***, $P < 0.001$ (Student's test). *SR1, SR2* and *SR3* are three independent *ERF.B3-SRDX* lines. *OX1, OX2* and *OX3* are three independent *Sl-ERF.B3* overexpressing lines.

EIN3-Like genes are up-regulated in ERF.B3-SRDX transgenic plants

EIN3/EILs are positive regulators of ethylene signaling by acting as transactivation factors to trigger ethylene responses. The expression of the four *EIN3-like* genes (*Sl-EIL1, 2, 3 and 4)* present in the tomato genome was examined at the transcript level

showing a two-fold increase in transcript accumulation for all four *Sl-EIL* genes in the *ERF.B3-SRDX* lines (Figure 8). However, none of the *EIN*3-*like* genes gather a consensus ethylene-response element in the promoter. Transactivation assays performed revealed that neither Sl-ERF.B3 nor ERF.B3-SRDX proteins are capable to modulate transcription driven by any of the four *Sl-EILs* promoters (Figure S6) suggesting that *Sl-EILs* do not serve as direct target genes for Sl-ERF.B3.

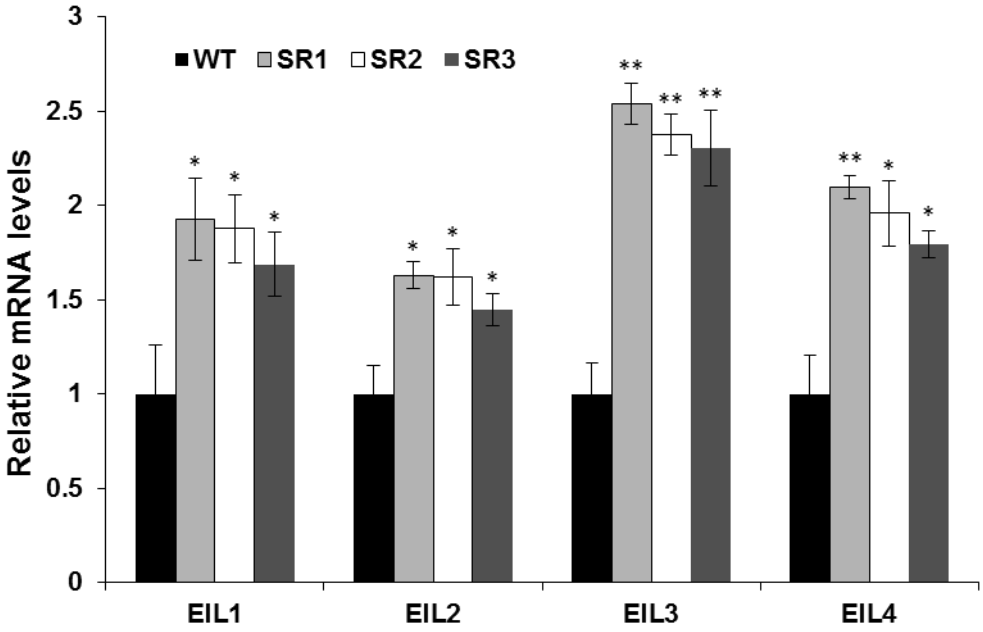

Figure 8. Expression of *EIN3*-like genes in *35S:ERF.B3-SRDX* lines.

Relative mRNA levels of *Sl-EIL1*, *Sl-EIL2*, *Sl-EIL3*, *Sl-EIL4* in WT and *ERF.B3-SRDX* lines were assessed by qRT-PCR in 4-week-old plants. The relative mRNA level of each gene in wild type was standardized to 1.0, referring to the internal control of *Sl-Actin*.

Values are means ± SD of three replicates. *, $0.01 < P < 0.05$, **, $0.001 < P < 0.01$ (Student's test). *SR1, SR2* and *SR3* are three independent *35S:ERF.B3-SRDX* lines.

Sl-ERFs are among the target genes of Sl-ERF.B3

Considering the putative role of ERFs in mediating ethylene responses, we examined the transcript levels of *Sl-ERF* genes in both wild-type and the *ERF.B3-SRDX* lines. A dramatic change in the transcript levels for a number of *ERF* genes was revealed in the dominant repressor lines (Figure 9a). That is, among the 19 *Sl-ERF*s that showed

detectable transcript accumulation, 14 were significantly down-regulated in the *ERF.B3-SRDX* dominant repressor lines while 4 *Sl-ERFs* displayed similar expression in transgenic and wild-type lines. Notably, the expression of *Sl-ERF.G1* displayed dramatic up-regulation in transgenic lines (Figure 9a). To gain further insight on the mechanisms underlying the regulation of *Sl-ERF* genes in the transgenic lines, the promoters of down- and up-regulated *ERFs* genes were cloned to examine the ability of Sl-ERF.B3 and ERF.B3-SRDX proteins to regulate their activity in a single cell system. The data indicate that Sl-ERF.B3 protein acts as activator on *Sl-ERF.C3, Sl-ERF.D2, Sl-ERF.F5* and *Sl-ERF.F4* promoters while it is inactive on *Sl-ERF.G1*. The ERF.B3-SRDX repressor version retains the capacity to recognize the same target genes than Sl-ERF.B3 as demonstrated by its repressing activity on the promoters activated by Sl-ERF.B3 (Figure 9b). By contrast, neither Sl-ERF.B3 nor ERF.B3-SRDX proteins were able to modulate the activity of the *Sl-ERF.G1* promoter. Taking advantage of the available *Sl-ERF.B3* up-regulated lines we also examined the expression level of *Sl-ERF* genes in these over-expressing lines. Opposite to the situation prevailing in the *ERF.B3-SRDX* lines most *ERF* genes are up-regulated in the *Sl-ERF.B3* over-expressing lines (Figure 9c) with the most significant up-regulation found in the lines displaying a reduced ethylene response (Figure S3). Of particular note, *Sl-ERF* genes (*Sl-ERF.C3*, *Sl-ERF.D2*, *Sl-ERF.F5 and Sl-ERF.F4*) shown to be direct target for Sl-ERF.B3 in the transactivation assay are all up-regulated in the *Sl-ERF.B3* over-expressing lines. Moreover, *ERF* genes that show regulation by Sl-ERF.B3 in the single cell system (Figure 9b) harbor *cis*-acting elements (GCC-box and DRE/CRT) known to be putative binding site for ERFs whereas the *Sl-ERF.G1* promoter lacks any of these typical *cis*-elements (Figure 9d and Table S2).

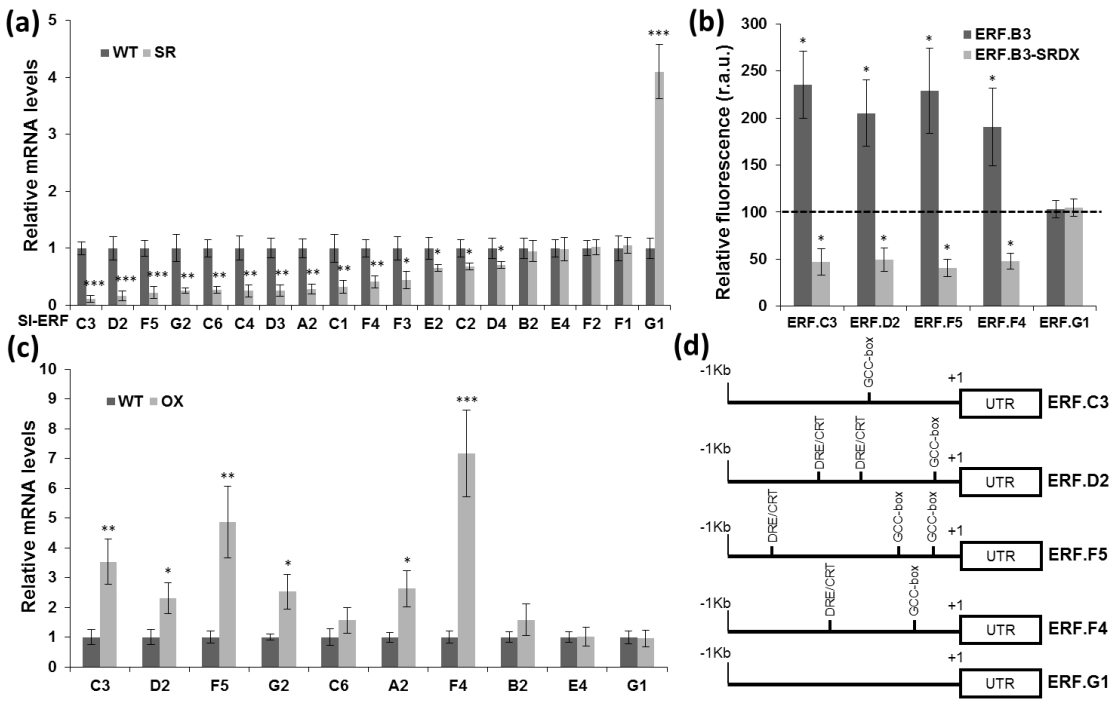

Figure 9. *Sl-ERFs* are among the target genes of ERF.B3 and ERF.B3-SRDX.

(a) Accumulation of *Sl-ERFs* transcripts in WT and *ERF.B3-SRDX* lines assessed by qRT-PCR in 4-week-old plants. The relative mRNA level of each gene in WT was standardized to 1.0, referring to *Sl-Actin* as internal control.

(b) Transactivation of *Sl-ERF* promoters by ERF.B3 and ERF.B3-SRDX. Protoplasts were co-transfected with GFP reporter fused to the promoters of *Sl-ERFs* (*ERF.C3, ERF.D2, ERF.F4, ERF.F5* and *ERF.G1*) and an effector plasmid expressing ERF.B3 or ERF.B3-SRDX.

(c) *Sl-ERFs* transcript levels in *ERF.B3* overexpression lines assessed by qRT-PCR in 4-week-old plants. The relative mRNA level of each gene in WT was standardized to 1.0, referring to *Sl-Actin* as internal control.

(d) The presence of putative ERF binding sites in the promoters of *Sl-ERFs* genes. The *cis*-acting elements identified are represented by black bars.

Values are means ± SD of three replicates *, $0.01 < P < 0.05$, **, $0.001 < P < 0.01$, ***, $P < 0.001$ (Student's test).

DISCUSSION

Although ERFs are generally considered as important components of the ethylene response mechanism, direct evidences for the involvement of these transcription factors in this process are still scarce. So far, classical approaches of forward and reverse

genetics aiming at up- or down-regulating the expression of *ERF* genes failed to provide sufficient clues on the physiological significance of different members of this gene family likely owing to functional redundancy among family members. In the present study, the ectopic expression of a dominant repressor form of the Sl-ERF.B3 protein provided a mean towards altering the activity of the native Sl-ERF.B3 protein. This strategy allowed revealing vegetative and reproductive growth phenotypes that could not be uncovered by the expression of neither sense nor antisense constructs of *Sl-ERF.B3*. Notably, the *ERF.B3-SRDX* plants display enhanced ethylene responses that tend to phenocopy the Arabidopsis *ctr1* mutant as well as the transgenic tomato lines deficient in receptors, exhibiting all of the hallmarks of exposure to ethylene (Kieber *et al.*, 1993; Tieman *et al.*, 2000). Although, the opposite effect would have been intuitively expected from blocking the action of an ERF, the physiological and molecular characterization clearly indicated that the phenotypes are consistent with enhanced ethylene sensitivity due to depletion of ethylene receptor pools but not to ethylene over-production.

The *35S:ERF.B3-SRDX* lines displayed enhanced ethylene responses and *pleiotropic ethylene-related alterations,* likely resulting from the transcriptional repression of ethylene-responsive genes that are natural targets of the native protein. Indeed, Sl-ERF.B3 and ERF.B3-SRDX are shown to modulate the activity of the same promoters harboring ethylene-responsive elements, indicating that ERF.B3-SRDX has the ability to interfere with the regulation of Sl-ERF.B3 target genes. ERF.B3-SRDX fusion protein is a strong repressor of both synthetic and native ethylene-responsive promoters whereas the native Sl-ERF.B3 protein enhances the activity of these promoters. The eventuality that the pleiotropic phenotypes displayed by the *ERF.B3-SRDX* dominant suppressor plants may arise from a co-suppression of the endogenous *Sl-ERF.B3* is ruled out since the levels of *Sl-ERF.B3* transcripts are not altered in the transgenic lines. Notably, the higher the *ERF.B3-SRDX* transgene expression the more severe was the phenotypic abnormality, indicating that the phenotypic effects were directly related to the expression levels of the *ERF.B3-SRDX* transgene. *Therefore,* the *ERF.B3-SRDX* tomato lines proved to be a valuable tool to uncover at least some of the processes controlled by Sl-ERF.B3 and to reveal roles for ERF genes that have not been described previously.

Dark-grown *ERF.B3-SRDX* seedlings displayed a constitutive ethylene response-like phenotype with inhibited hypocotyl elongation and exaggerated apical hook formation in the absence of exogenous ethylene. Moreover, adult plants show typical constitutive ethylene responses including leaf epinasty, premature flower senescence and accelerated fruit abscission. These phenotypes may arise from: (i) a constitutive ethylene response, (ii) an increased sensitivity to endogenous ethylene, or (iii) an ethylene overproduction. Noteworthy, the ethylene response phenotypes displayed by *ERF.B3-SRDX* etiolated seedlings can be reversed by the inhibition of ethylene perception (Figure 2a) and treatment with exogenous ethylene resulted in a more pronounced ethylene triple response compared to wild type. Taken together with the reduced ethylene production, these results indicate that the ethylene response phenotypes displayed by *ERF.B3-SRDX* lines are not due to constitutive activation of ethylene signaling pathway but rather to enhanced ethylene sensitivity. It is well accepted that ethylene receptors act as negative regulators and function redundantly in ethylene signaling with a decreased expression of ethylene receptor genes resulting in increased sensitivity to the hormone (Hua and Meyerowitz, 1998; Kevany and Klee, 2007). The reduced transcript levels of the receptors and the ethylene hypersensitivity of *ERF.B3-SRDX* lines are consistent with this model. In tomato, although gene-specific antisense reductions in *Sl-ETR1, Sl-ETR2, NR or Sl-ETR5* do not affect ethylene sensitivity, transgenic lines with single reduction in *Sl-ETR4* or *Sl-ETR6* expression display phenotypes consistent with enhanced ethylene response (Tieman *et al.*, 2000; Kevany *et al.*, 2007) indicating these two receptors may act as a special component in regulating ethylene response. The down-regulation of *Sl-ETR6* in the *ERF.B3-SRDX* lines may therefore account for the increased ethylene sensitivity. Interestingly, opposite to its down-regulation in the dominant repressor lines, *ETR6* shows a net up-regulation in the *Sl-ERF.B3* over-expressing plants suggesting that this receptor gene may represent a direct target for Sl-ERF.B3 protein *in vivo*.

The increased expression of transcription factors belonging to the *EIN3* gene family may also contribute to enhanced ethylene responses. Over-expression of *EIN3* or *EIL1*confers constitutive ethylene phenotypes in *Arabidopsis*, while reduced *Sl-EILs* expression in transgenic tomato decreases ethylene sensitivity (Chao *et al.*, 1997; Tieman *et al.*, 2001). Four *EIN3-like* genes were isolated in tomato and designed as *Sl-EIL1*, *Sl-EIL2*, *Sl-EIL3*

and *Sl-EIL4* (Tieman *et al.*, 2001; Yokotani *et al.*, 2003). Since it is well documented that EIN3/EIL proteins act as transactivation factors to trigger ethylene responses, up-regulation of all four *Sl-EIL* genes in the *ERF.B3-SRDX* plants may contribute to their ethylene hypersensitivity. However, because the promoter of *EIN-like* genes are devoid of consensus ethylene-response elements and since transactivation assays indicated that Sl-ERF.B3 and ERF.B3-SRDX proteins are unable to modulate transcription driven by any of the four *Sl-EILs* promoters, it is likely that the up-regulation of *Sl-EIL* genes in the dominant repressor lines is due to intermediate factor(s) whose expression/activation is regulated by ERF.B3-SRDX.

Previous studies have already shown that ERF proteins are involved in a feedback regulation of ethylene production by modulating the expression of ethylene biosynthesis genes (Zhang *et al.*, 2009; Lee *et al.*, 2012). Our data show that ectopic expression of the *ERF.B3*-SRDX dominant repressor results in reduced ethylene production associated with the down-regulation of *ACS* and *ACO* ethylene biosynthesis genes. The presence of conserved GCC box and DRE/CRT motifs in *ACS* and *ACO* promoters that can serve as binding sites for ERF proteins supports the hypothesis that these ethylene biosynthesis genes can directly be regulated by Sl-ERF.B3. Together, the reduced ethylene production and enhanced ethylene sensitivity in the *ERF.B3-SRDX* lines suggest the presence of a feedback loop regulating both ethylene biosynthesis and signal transduction pathway and involving ERF proteins.

Strikingly, the expression of a considerable number of *Sl-ERF* genes, 15 out of 19 monitored in our study, was found to be markedly altered in *ERF.B3-SRDX* tomato lines suggesting intense inter-regulation among *ERF* family members. Consistent with the dominant repressor function of the ERF.B3-SRDX protein, most of the *ERF* genes were down-regulated while solely *Sl-ERF.G1* displayed higher transcript levels in the dominant repressor lines. By contrast, in *Sl-ERF.B3* over-expressing lines, most *ERF* genes tested displayed enhanced transcript levels. In particular, *Sl-ERF.C3*, *Sl-ERF.D2*, *Sl-ERF.F5 and Sl-ERF.F4,* shown to be direct target for Sl-ERF.B3 in the transactivation assay, display enhanced expression in the *Sl-ERF.B3* sense lines. While these data support the idea that these *ERFs* can serve as direct target for both the native and chimeric Sl-ERF.B3 proteins, the up-regulation of *Sl-ERF.G1* in the dominant repressor

lines likely requires an additional mediating factor. *In silico* search revealed that all *ERF* genes down-regulated in the transgenic lines harbor *cis*-acting elements known to be putative binding targets for ERFs. The down-regulation of such a high number of *Sl-ERFs* supports a model implying that a single ERF can impact the expression of other members of the gene family. This inter-connected regulation among *ERF* genes may therefore account for the pleiotropic alterations in the *ERF.B3-SRDX* lines and for the diversity of responses displayed by the dominant repressor lines.

Phenotypes such as stunted plant development, reduced leaf size and late flowering time are reminiscent not only of constitutive ethylene-response mutants but also of GA deficient *Arabidopsis* plants (Kieber *et al.*, 1993; Hua and Meyerowitz, 1998; Hall and Bleecker, 2003; Magome *et al.*, 2004; Qu *et al.*, 2007). The partial rescue of the dwarf phenotype in the *ERF.B3-SRDX* lines by exogenous application of GA suggests that these alterations are partly due to GA deficiency. In line with the model supporting that ethylene regulates plant growth and floral organ differentiation via modulating GA levels (Achard *et al.*, 2007), ethylene hypersensitivity in the *ERF.B3-SRDX* dominant suppressor lines is associated with reduced plant size and substantially delayed flowering time. The reduced expression of *GA oxidase* genes in the transgenic lines sustains the idea of altered GA metabolism and suggests that ERFs may represent a potential molecular link between ethylene and GA. In agreement with this, it has been recently reported that transcriptional activation of some genes involved in GA metabolism is mediated by ERF6 in Arabidopsis leaves (Dubois *et al*., 2013). Because the study has been carried out with Micro-Tom, a dwarf genotype, it is important to mention that the dwarfing mutations in this genotype do not seem to impact the phenotype displayed by *ERF.B3-SRDX* plants since the dwarf phenotype is well reproduced in Ailsa Craig tomato, a non-dwarf variety (data not shown). Altogether, the data suggest that ethylene hypersensitivity is likely to be the fundamental cause of the severe dwarf and late-flowering phenotypes in the *ERF.B3-SRDX* plants.

Since ectopic expression of transcription factors might influence target genes that are normally not under the control of this regulator, it cannot be totally ruled out that at least part of gene regulations caused by ERF.B3-SRDX are off-target effects due to interference with other related transcription factors. However, the data support the idea

that Sl-ERF.B3 is part of an intricate web of regulation in which multiple transcription factors are competing for promoters to control the expression of genes that are essential for a wide range of plant responses to ethylene. As depicted in the tentative regulation model presented in Figure 10, Sl-ERF.B3 is shown to modulate ethylene responses at four different levels: (i) ethylene biosynthesis, (ii) ethylene receptor, (iii) primary ethylene transcription factors (*EIL* genes), and (iv) downstream *ERF* genes. The high number of ERF genes regulated by Sl-ERF.B3 is consistent with the pleiotropic phenotypes displayed by the dominant repressor lines and suggests that ERFs form a complex network with a subset of the family members functioning in an interconnected manner. Such level of complexity matches the high level of plasticity needed for the implementation of plant growth and developmental processes which require continuous fine-tuning through the integration of different cues and signaling pathways.

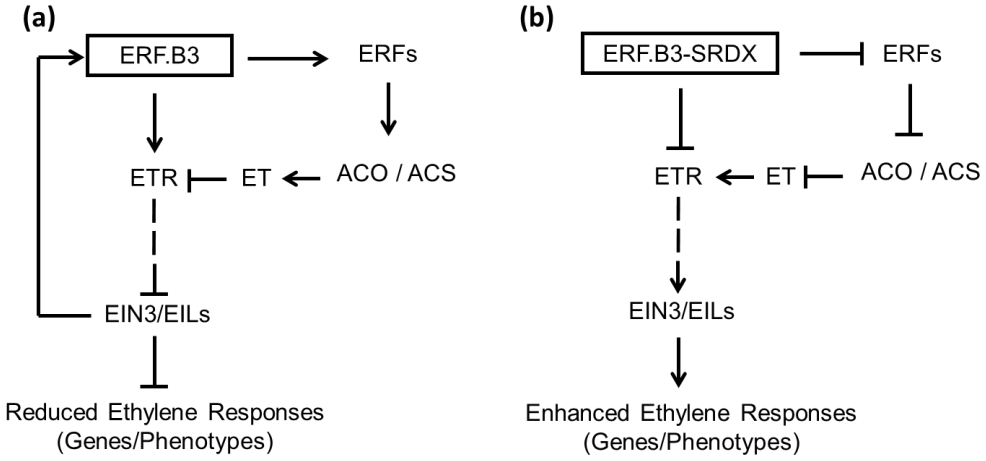

Figure 10. Tentative model proposing the involvement of Sl-ERF.B3 in the control of ethylene responses.

Sl-ERF.B3 modulates ethylene responses at different levels including ethylene biosynthesis (ACO/ACS), receptors, and *ERF* genes. ERF.B3-mediated ethylene response occurs partly via direct transcriptional regulation of specific ethylene receptor genes (*ETR6*) and selected members of the *ERF* gene family (*ERF.C3*, *ERF.D2*, *ERF.F4* and *ERF.F5*). Ectopic expression of Sl-ERF.B3 decreases ethylene responses in vegetative tissues through up-regulation of ethylene receptor genes and down-regulation of EIN3-like genes (panel **a**). By contrast, ectopic expression of Sl-ERF.B3-SRDX repressor version, leads to enhanced ethylene responses via down-regulation of receptor genes and repression of some *ERF* genes (panel **b**). This scheme is validated by transactivation assays showing direct regulation of the target *ERFs* and *ETR6* genes by the native form of Sl-ERF.B3 protein and by the enhanced transcript levels of these target genes in the Sl-ERF.B3 over-expressing lines.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Tomato plants (Solanum lycopersicum cv. Micro Tom) were grown under standard greenhouse culture conditions. The culture chamber rooms were set as follows: 14 h-day/10 h-night cycle, 25/20°C day/night temperature, 80% hygrometry, 250 µmol m-2 s-1 intense luminosity.

Constructs and plant transformation

To generate the chimeric repressor transgene, the coding sequence of *Sl-ERF.B3* without the stop codon was cloned via blunt-end ligation into the SmaI site of p35SSRDXG in frame to the SRDX repression domain (LDLDLELRLGFA) from SUPERMAN (Hiratsu *et al.*, 2003; Mitsuda *et al.*, 2006). *Agrobacterium tumefaciens*-mediated transformation of tomato plants was carried out according to (Wang *et al.*, 2005) and transformed lines were selected on a hygromycin-containing medium. All experiments were carried out using homozygous lines from F3 or later generations.

Transient expression using a single cell system

Protoplasts used for transfection were isolated from suspension-cultured tobacco (*Nicotiana tabacum*) BY-2 cells according to (Leclercq *et al.*, 2005). The synthetic reporter construct (4xGCC-GFP) was generated by fusing the synthetic GCC-box promoter to the coding region of the GFP (Pirrello *et al.*, 2012). Reporter constructs were also generated with native promoters, *Sl-osmotin* (C08HBa0235H18.1) and *Sl-ERFs* (*ERF.C3, ERF.D2, ERF.F4, ERF.F5 and ERF.G1*), fused to GFP. Protoplast co-transfection assays was performed using the reporter plasmids and effector vectors carrying 35S:ERF.B3 or 35S:ERFB3-SRDX. GFP expression was analyzed and quantified by flow cytometry (FACS Calibur II instrument, BD Biosciences) 16 hours following protoplast transfection. For each sample, 100-1000 protoplasts were gated on forward light scatter and the 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 Cell Quest software and were normalized using an experiment with

protoplasts transformed with the reporter vector in combination with the vector used as effector but lacking the Sl-ERF.B3 coding sequence.

RNA isolation and qRT-PCR

Total RNA from 4-week-old plants was extracted using a Plant RNA Purification Reagent (Invitrogen, Cat. No. 12322-012). Total RNA was DNase-treated (Invitrogen, Cat. No. AM1906) and first-strand cDNA was reverse transcribed from 2 μg of total RNA using an Omniscript Reverse Transcription kit (Qiagen, Cat. No. 74904). Gene-specific primers were designed by Primer Express software (PE-Applied Biosystems) and were further checked using BLAST against all tomato unigenes (Tomato unigene database). qRT-PCR analyses were performed as described previously (Pirrello *et al.*, 2006). The primer sequences used in this study are listed in Table S3.

Gibberellin treatment

For application of gibberellin to young plants growing on soil, 10^{-5} M of Gibberellic Acid (GA_3) was sprayed twice a week starting on the 10th day post-germination. After 2 weeks of treatment, the treated plants were compared with the control ones.

Triple-response assay

Sterilized seeds were first put on MS/2 medium plates and placed at 4°C for 3 days and then transferred to 25°C for germination in the dark for another 5 days. The seedling triple response was scored by assessing hypocotyl length and apical curvature. At least 50 seedlings were scored for each measurement. For ethylene treatment, Petri dishes were enclosed in wide mouth Mason jars sealed with a lid containing a rubber syringe cap. Ethylene (10 μL L^{-1}) was then injected into the Mason jars using a syringe. For 1-MCP treatment, 1 μL L^{-1} was applied into the Mason jars and kept in the dark for one week. At least 50 seedlings were used for each experiment and three independent biological replicates were performed.

Ethylene production

Ethylene production was assayed on 7 day-old dark-grown seedlings for 12 h by withdrawing 1-mL gas samples from sealed jars. Gas samples were analyzed via gas chromatography (7820A GC system Agilent Technologies). Ethylene was identified via co-migration with an ethylene standard and quantified with reference to a standard curve for ethylene concentration.

Supplemental data for chapter II

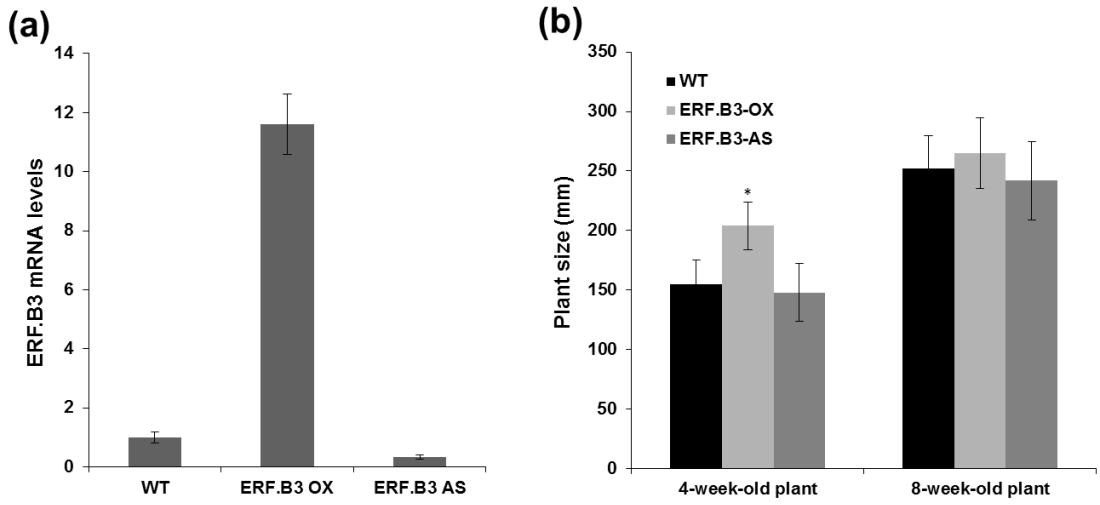

Figure S1. Impact of *Sl-ERF.B3* up- and down-regulation on vegetative growth.

(a) *ERF.B3* transcripts accumulation assessed by qRT-PCR in *ERF.B3* OX and AS lines on 4-week-old plants.

(b) Plant growth assessed by plant size in wild type (WT), *ERF.B3* overexpression (OX) and antisense (AS) tomato lines at 4-week-old and 8-week-old stages.

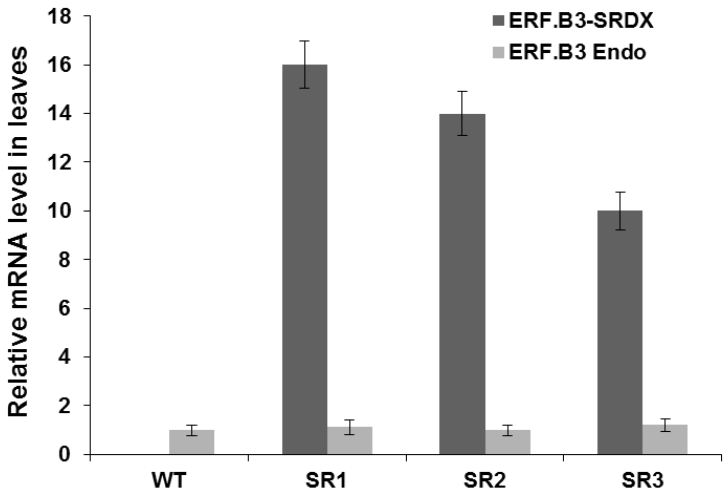

Figure S2. Transcript accumulation of the chimeric *ERF.B3-SRDX* and the endogenous *ERF.B3* genes in transgenic lines. Transcript levels were assessed by qRT-PCR in 4-week-old plants. The relative mRNA levels of *Sl-ERF.B3* endogenous (Endo) in the wild type were standardized to 1.0, referring to *Sl-Actin* gene as internal control. Values are means ± SD of three replicates. *SR1, SR2* and *SR3* are three independent *35S:ERF.B3-SRDX* lines.

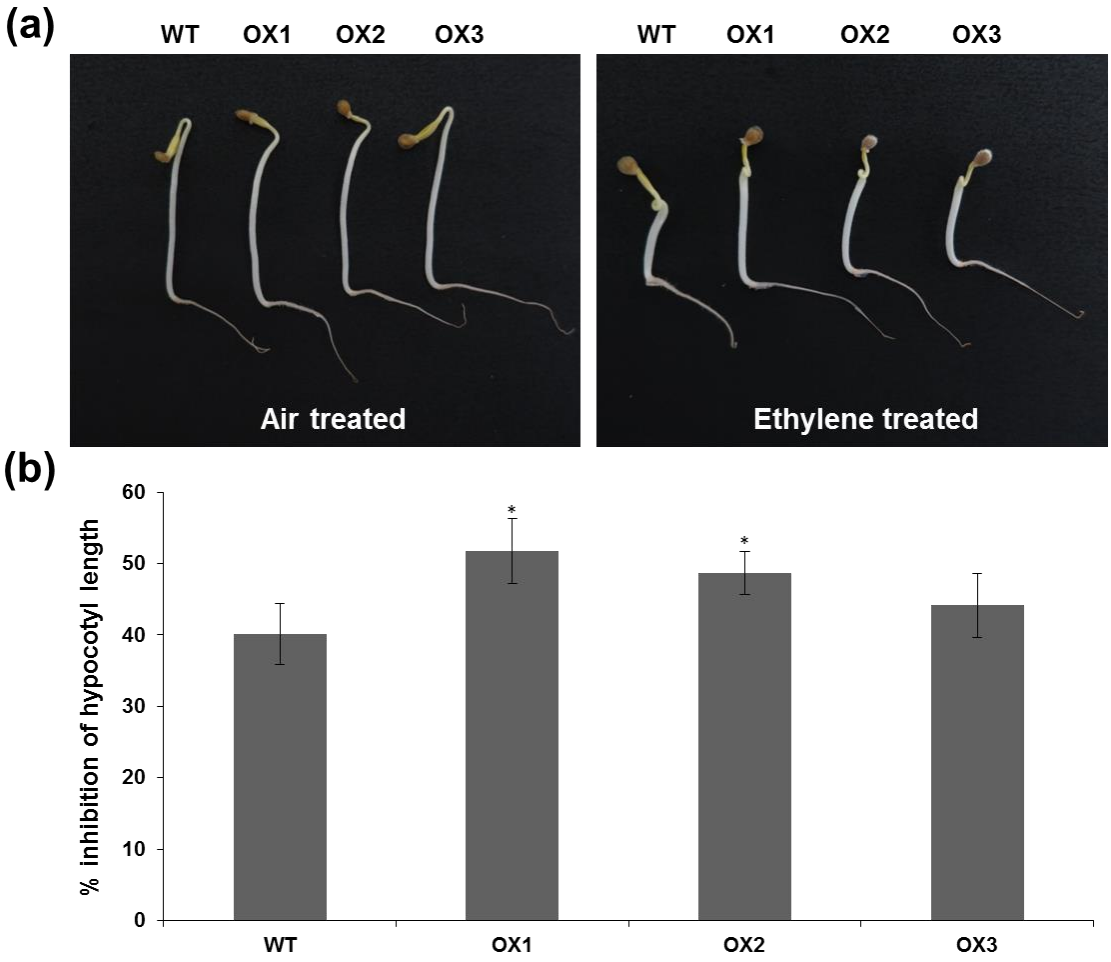

Figure S3. Triple response phenotype of *Sl-ERF.B3* overexpression lines.

(a) Responsiveness of dark-grown WT and *Sl-ERF.B3* overexpressing lines treated with exogenous ethylene (10 µL L^{-1}).

(b) Inhibition of hypocotyl length in WT and *ERF.B3* overexpressing lines treated with ethylene (10 µL L^{-1}). Values are expressed as % of the initial hypocotyl length prior to hormone treatment of dark-grown seedlings. Values are means ± SD ($n \geq 30$) of three replicates. *, $0.01 < P < 0.05$ (Student's test). *OX1, OX2* and *OX3* are three independent *Sl-ERF.B3* overexpression lines.

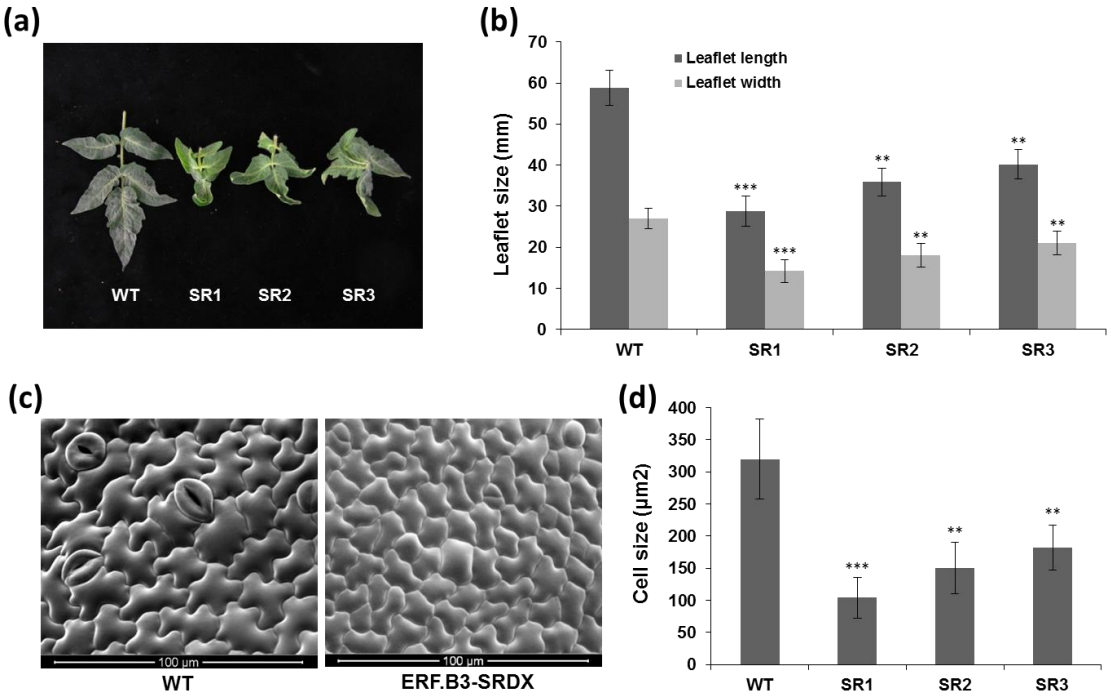

Figure S4. Altered leaf morphology in *ERF.B3-SRDX* plants.

(a) Leaf margins are twisted and the lamina is often wrinkled.

(b) Leaflet length and width assessed in 30-day-old *ERF.B3-SRDX* and WT plants. Values are means ± SD ($n \geq 15$) of three replicates.

(c) Scanning electron microscopy observation of leaf epidermal cells in WT and *ERF.B3-SRDX* lines.

(d) Monitoring epidermal cell size in WT and *ERF.B3-SRDX* leaves.

**, $0.001 < P < 0.01$, ***, $P < 0.001$ (Student's test). *SR1, SR2* and *SR3* are three independent *35S:ERF.B3-SRDX* lines.

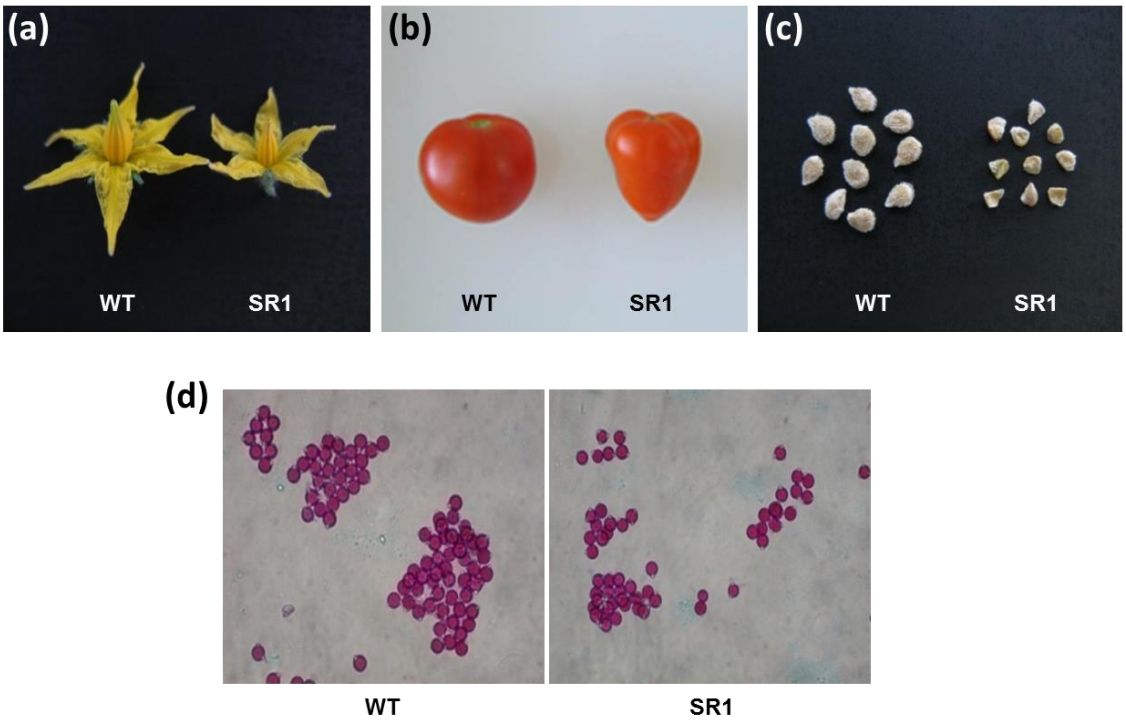

Figure S5. Phenotypes of reproductive organs

(a) *ERF.B3-SRDX* plants show reduced flower size.

(b) Reduced fruit size with altered fruit shape in *ERF.B3-SRDX* plants.

(c) Reduced seed size in *ERF.B3-SRDX* lines.

(d) *ERF.B3-SRDX* pollen viability is similar to that of WT as revealed by Alexander's staining test.

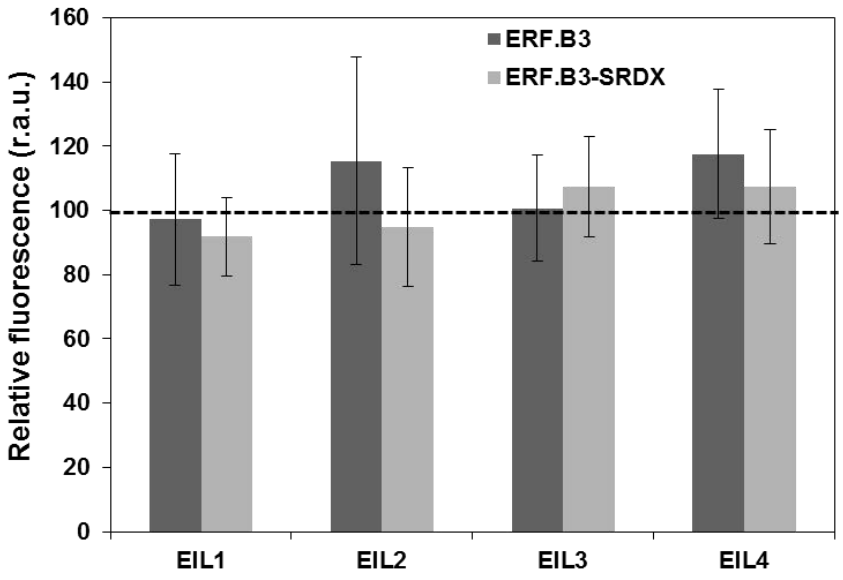

Figure S6. ERF.B3 and ERF.B3-SRDX are unable to modulate the transcriptional activity of *Sl-EIL* promoters in a protoplast transactivation assay

Protoplasts were co-transfected with a reporter construct consisting of the GFP gene driven by the promoters of *Sl-EILs* (*EIL1, EIL2, EIL3* and *EIL4*) and an effector plasmid expressing either ERF.B3 or ERF.B3-SRDX. GFP fluorescence was measured by flow cytometry 16 h after transfection. The basal fluorescence obtained by co-transformation with the promoter fused to the reporter gene and the empty vector was standardized to 100 and is taken as reference. The results are mean of 3 independent biological repetitions.

Table S1. Putative ERF binding *cis*-elements present in the promoter regions of ethylene receptor and ethylene biosynthesis genes. For each gene, the genomic sequence corresponding to 2.0 kb upstream of the predicted translation start codon (ATG) was analyzed for the presence of known ethylene response *cis*-acting elements using three different softwares: (i) PLACE (http://www.dna.affrc.go.jp/ PLACE/), (ii) PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html), and (iii) PlantPAN (http://plantpan.mbc.nctu.edu.tw/seq_analysis.php).

Gene	***Motif***	***Position***	***Sequence***
Ethylene receptor genes			
Sl-ETR2	None	-	-
NR	DRE/CRT	-700	CCGAC
	DRE/CRT	-1503	CCGAC
Sl-ETR5	GCC-box-like	-1558(-)	TCCGCC
	DRE/CRT	-1707(-)	CCGAC
Sl-ETR6	None	-	-
Ethylene biosynthesis genes			
Sl-ACS1	GCC-box-like	-1685	TCCGCC
	DRE/CRT	-999	CCGAC
	DRE/CRT	-1188	CCGAC
	DRE/CRT	-1752	CCGAC
Sl-ACS3	GCC-box-like	-1455	TCCGCC
	GCC-box-like	-1586	CCCGCC
	DRE/CRT	-718	CCGAC
	DRE/CRT	-804	CCGAC
Sl-ACO1	None	-	-
Sl-ACO3	GCC-box	-1851	GCCGCC
	DRE/CRT	-541	CCGAC
	DRE/CRT	-505	CCGAC
	DRE/CRT	-810	TCGAC
Sl-ACO4	DRE/CRT	-1136	CCGAC
	GCC-box	-1218(-)	GCCGCC
	GCC-box -like	-1215(-)	CCCGCC

Table S2. Putative *cis*-acting ethylene-response elements present in the promoter regions of *Sl-ERF* genes. The genomic sequence corresponding to 2.0 kb upstream of the predicted translation start codon (ATG) was analyzed for the presence of known ethylene response *cis*-acting elements using three softwares, PLACE (http://www.dna.affrc.go.jp/ PLACE/), PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) and PlantPAN (http://plantpan.mbc.nctu.edu.tw/seq_analysis.php).

Gene	Motif	Position	Sequence
Down regulated genes			
Sl-ERF.C3	GCC-box	-458	GCCGCC
Sl-ERF.D2	DRE/CRT	-475	CCGAC
	GCC-box	-70(-)	GCCGCC
	DRE/CRT	-388(-)	CCGAC
Sl-ERF.F5	GCC-box	-173	GCCGCC
	DRE/CRT	-969	CCGAC
	DRE/CRT	-1068	CCGAC
	GCC-box	-87(-)	GCCGCC
Sl-ERF.F4	DRE/CRT	-431	CCGAC
	GCC-box	-139(-)	GCCGCC
Up regulated genes			
Sl-ERF.G1	None	-	-

Table S3. List of the primers used in the study.

Gene Name	Primer Sequence
Sl-Actin	F 5'-TGTCCCTATTTACGAGGGTTATGC-3'
	R 5'-CAGTTAAATCACGACCAGCAAGAT-3'
Sl-ERF.B3	F 5'-CGGAGATAAGAGATCCAAGTCGAA-3'
	R 5'-CTTAAACGCTGCACAATCATAAGC-3'
Sl-ERF.B3-endo	F 5'-TTCACAGAGACATAAACACAAACACCT-3'
	R 5'-TGTTGTCGTATGAGTTCTAATGTTAATCCT-3'
Sl-ERF.B3-SRDX	F 5'-GGAAAATCTGGTGCTCCGG-3'
	R 5'-CTCGTCGACTTAAGCGAAAC-3'
Sl-GA20ox-1	F 5'-CAACTACTATCCACCATGCCAG-3'
	R 5'-CACCAACACAATCTTGATGGAG-3'
Sl-GA20ox-2	F 5'-ACGATTCTTCTCTACTTGGCT-3'
	R 5'-GCTAAGGTCTTGATCTACATTGG-3'
Sl-ACS1	F 5'-TCGTTTCGAAGATTGGATGA-3'
	R 5'-CAACAACAACAAATCTAAGCCATT-3'
Sl-ACS2	F 5'-TGTTAGCGTATGTATTGACAACTGG-3'
	R 5'-TCATAACATAACTTCACTTTTGCATTC-3'
Sl-ACS3	F 5'-CCCTTGTCCACAAATCCAGA-3'
	R 5'-ACAGAGTGCACCCTCTAACATTT-3'
Sl-ACS4	F 5'-CTCCTCAAATGGGGAGTACG-3'
	R 5'-TTTTGTTTGCTCGCACTACG-3'
Sl-ACS6	F 5'-CTCCTATGGTCCAAGCAAGG-3'
	R 5'-CGACATGTCCATAATTGAACG-3'
Sl-ACO1	F 5'-GCCAAAGAGCCAAGATTTGA-3'
	R 5'-TTTTTAATTGAATTGGGATCTAAGC-3'
Sl-ACO2	F 5'- TTTATTACAAAGTGTGCGTCCCTA-3'
	R 5'- CTCATTTTTGGGTATTAAAATATGTGT-3'
Sl-ACO3	F 5'-TGATCAAATTGCAAGTGCTTAAA-3'
	R 5'-ACCACACAACAATCACACACA-3'
Sl-ACO4	F 5'-GGAGCCTAGGTTTGAAGCAA-3'
	R 5'-AAACAAATTCCCCCTTGAAAA-3'
Sl-EIL1	F 5'-CCTCAACAATATGTCCAGCCA-3'
	R 5'-TCATCCTTTGCCCATCTTCAG-3'
Sl-EIL2	F 5'-TGAAGATGGAAGTCTGTAAGG-3'
	R 5'-CCACTCCCTGAGATTATCCGA-3'
Sl-EIL3	F 5'-ACAGGACTTCAAGAAACAACCA-3'

	R 5'-GTGTTGTGCTCATAGTTGATCTG-3'
Sl-EIL4	F 5'-TATACCCTGATCGTTGTCCAC-3'
	R 5'-TTACACTCATCTTGAGCACCA-3'
Sl-ETR1	F 5'-GGAAGAACATTGGCATTGGAAG-3'
	R 5'-CCAACTGGATTTTGGTGTCGT-3'
Sl-ETR2	F 5'-TTGGAGGAATCAATGAGGGC-3'
	R 5'-TCATTACGCGCACGAACAG-3'
NR	F 5'-TGCTGTTCGTGTACCGCTTT-3'
	R 5'-TCATCGGGAGAACCAGAACC-3'
Sl-ETR4	F 5'-ATGGCTGTCGTTCTTGGGC-3'
	R 5'-TGGAGGAGTGAGTGTGGATGC-3'
Sl-ETR5	F 5'-GTGCTCTGGGCCCTTCACTA-3'
	R 5'-GAACTTACGCACCCTCAATGC-3'
Sl-ETR6	F 5'-TCAAAAAGCCGGTGATCTCG-3'
	R 5'-GCACCCATTTGAACGGAAAA-3'
Pro*Sl-ETR6*	F 5'-TTGTAGTTAAAAGATTTGCTTC-3'
	R 5'-ATCCAATAGAACTACTCTTGTT-3'
Sl-ERF.A2	F 5'-CGGTATCATCAGCTTCGGAAA-3'
	R 5'-TCTCAACTTCTAATTCGGCTTGCT-3'
Sl-ERF.B2	F 5'-AGTTTGCAGCGGAGATTCGT-3'
	R 5'-TGCCCTGTCATATGCCTTTG-3'
Sl-ERF.C1	F 5'-TTCTTCGTGTCGAAAATACTAAGTTCAGT-3'
	R 5'-ACTCTAAATTCTTCAAGAAATCCAGAACA-3'
Sl-ERF.C2	F 5'-ATCATTACCATGGAATGATCAACATT-3'
	R 5'-CCGTCTATAACTTTCTTTCGAGGTTAA-3'
Sl-ERF.C3	F 5'-CAAGAAGTTTCCTCAATCTCTCATGTAT-3'
	R 5'-CCGAGATGAATAATCCATTTGATTT-3'
Sl-ERF.C4	F 5'-CAACGTTGACAACATCTTTGCA-3'
	R 5'-AACTTGGGAAGATATTCTCAATGGAA-3'
Sl-ERF.C6	F 5'-GGGAAATACGCTGCGGAAA-3'
	R 5'-TTTCGAACGTACCTAGCCATACTCT-3'
Sl-ERF.D2	F 5'-ACACAAGTAGCACCAGCACCACTA-3'
	R 5'-ACCCCAAAAAAAGCAAGAAAATT-3'
Sl-ERF.D3	F 5'-ATTCATTTTCGGGTTGTGCAGTA-3'
	R 5'-CGACTATAATGATTTCTGCCGAACT-3'
Sl-ERF.D4	F 5'-GTTGCTGCTTTAACCAATGTGATTAT-3'
	R 5'-CTTCCGGTACGCGAAACAAG-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.F1	F 5'-ACGAGCTTTCTTCTTTTCTCTCTCTAAA-3'
	R 5'-GAAACTCGATATCCTTCTGTAAAATCTTC-3'
Sl-ERF.F2	F 5'-TTGATACCACTGCTTACCTAGTTTTTCT-3'
	R 5'-TATCTTCTATGGCTCCTTCCTCTTCT-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.G1	F 5'-GAAGAAAGCGATCGATTTGAAGA-3'
	R 5'-TTTTCCCCATGGCCTCTGT-3'
Sl-ERF.G2	F 5'-CGGTGGAGATAAAAGCGAAAAC-3'
	R 5'-CCACTTCGCAGAACCCTAGATT-3'
Pro*Sl-ERF.C3*	F 5'-ACAATCATCACCATCAACCA-3'
	R 5'-GGAAACTTCTTGCTTAACAGG-3'
Pro*Sl-ERF.D2*	F 5'-GGCTTCCCGCTATAATAAGG-3'
	R 5'-CGAATAATCAAACTGACCACC-3'
Pro*Sl-ERF.F5*	F 5'-ACACTTACCAGTTATCTGCCAC-3'
	R 5'-AAATGGAGAAAGGGTGAAGAG-3'
Pro*Sl-ERF.F4*	F 5'-CTGCTGAACCTAGTGTCTC-3'
	R 5'-ATGAATGAAGAGTATGCGGT-3'
Pro*Sl-ERF.G1*	F 5'-CTAAGACGAATCATAGAGTAGGAC-3'
	R 5'-AGGAAGAACAAGTCTTGATGAG-3'

Table S4. Gene names used in the study and corresponding gene ID.

Gene Name	Gene ID
Sl-ERF.B3	Solyc05g052030
Sl-ERF.C3	Solyc09g066360
Sl-ERF.D2	Solyc12g056590
Sl-ERF.F5	Solyc10g009110
Sl-ERF.G2	Solyc06g082590
Sl-ERF.C6	Solyc03g093560
Sl-ERF.C4	Solyc03g123500
Sl-ERF.D3	Solyc01g108240
Sl-ERF.A2	Solyc03g093610
Sl-ERF.F4	Solyc07g053740
Sl-ERF.F3	Solyc07g049490
Sl-ERF.E2	Solyc09g089930
Sl-ERF.C2	Solyc04g014530
Sl-ERF.D4	Solyc10g050970
Sl-ERF.B2	Solyc02g077360
Sl-ERF.E4	Solyc01g065980
Sl-ERF.F2	Solyc07g064890
Sl-ERF.F1	Solyc10g006130
Sl-ERF.G1	Solyc01g095500
Sl-GA20ox1	Solyc03g006880
Sl-GA20ox2	Solyc06g035530
Sl-ACS1A	Solyc08g081540
Sl-ACS3	Solyc02g091990
Sl-ACO1	Solyc07g049530
Sl-ACO3	Solyc07g049550
Sl-ACO4	Solyc02g081190
Sl-EIL1	Solyc06g073720
Sl-EIL2	Solyc01g009170
Sl-EIL3	Solyc01g096810
Sl-EIL4	Solyc06g073730
Sl-ETR2	Solyc07g056580
NR	Solyc09g075440
Sl-ETR5	Solyc11g006180
Sl-ETR6	Solyc09g089610

Chapter III

The chimeric repressor version of *ERF.B3* shows contrasting effects on tomato fruit ripening

(*Manuscript in preparation*)

The chimeric repressor version of *Sl-ERF.B3* shows contrasting effects on tomato fruit ripening

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ABSTRACT

Fruit ripening in tomato is a genetically regulated process involving a complex interplay between ethylene and ripening-associated transcriptional regulators. Ethylene Response Factors (ERFs) are downstream components of the ethylene signal transduction pathway known to mediate ethylene action through the regulation of ethylene-responsive genes. Our previous work has demonstrated the involvement of the tomato *Sl-ERF.B3* in a feedback control of ethylene action in vegetative growth. Here, we show the role of *Sl-ERF.B3* in controlling fruit maturation and ripening. Over-expression of a chimeric repressor construct of this *ERF* gene (*ERF.B3-SRDX*) results in altered fruit shape and size, seed morphology, orange ripe fruits, and accelerated fruit senescence. The attainment of competence to ripen is dramatically delayed in *ERF.B3-SRDX* fruits but once ripening proceeds it is associated with high climacteric ethylene production and enhanced fruit softening, while pigment accumulation is decreased. Consistently genes involved in ethylene biosynthesis, perception and in cell wall degradation are up-regulated whereas those involved in lycopene biosynthesis are down-regulated. Moreover, the expression of ripening regulator genes such as *RIN, CNR* and *HB-1* is stimulated in *ERF.B3-SRDX* dominant repressor fruits. Notably, a number of *ERF* genes show altered expression patterns in *ERF.B3-SRDX* ripening fruits, suggesting the existence of a complex network enabling interconnection between different *ERF* genes and accounting for the pleiotropic alterations in fruit maturation and ripening. Altogether, the data suggest a central role of *Sl-ERF.B3* in the transcriptional network controlling the ripening process and reveal a means for uncoupling some of the main processes underlying fruit ripening, such as fruit softening and pigment accumulation.

INTRODUCTION

The maturation and ripening of fleshy fruits is a developmental process unique to plants. Although specific fruit-ripening characteristics vary among species, fruit ripening can be generally described as a complex, genetically programmed process that culminates in dramatic changes in color, texture, flavor, aroma and nutritional characteristics (Carrari and Fernie, 2006). In the case of fleshy fruits these changes not only make fruit attractive for seed dispersal organisms, but also provide essential vitamins, minerals and antioxidants (phenolics, folate, lycopene, and β-carotene) for human diet (Seymour *et al.*, 1993; Fraser *et al.*, 2009; Chung *et al.*, 2010).

Fruits have been classically categorized into climacteric and non-climacteric based on increased ethylene synthesis and a concomitant rise in the rate of respiration during ripening. Climacteric fruits display a burst in respiration at the onset of ripening, in contrast to non-climacteric ones. Climacteric fruits, such as tomatoes, bananas and apples, also show increased biosynthesis of the gaseous hormone ethylene, which is a fundamental signal in climacteric fruit ripening (Vrebalov *et al.*, 2002; Alba *et al.*, 2005). In the ethylene biosynthetic pathway, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO) catalyze the conversion of S-adenosyl-L-methionine (SAM) to ACC and of ACC to ethylene, respectively (Adams and Yang, 1979; Bleecker and Kende, 2000). Autocatalytic ethylene synthesis at the onset of tomato fruit ripening is mainly mediated through ethylene-stimulated expression of *ACS2*, *ACS4*, *ACO1* and *ACO4* (Barry and Giovannoni, 2007). Unraveling the regulation of the ethylene signaling pathway is important to understanding the processes of fruit ripening.

Tomato possesses many favorable genetic characteristics such as simple diploid genetics, relatively small genome size, short generation time, efficient genetic transformation and distinct ripening phenotypes, making it a primary model system for studying the molecular basis of fleshy fruit development and the role of ethylene in climacteric fruit ripening. The adaptation of a range of technological tools and the generation of new biological resources on the tomato (e.g. EST database, TILLING resources, genetic and physical maps) have led to a large progress in our understanding of the molecular mechanisms underlying the ripening process through the identification of the associated

key regulatory genes (Pirrello *et al.*, 2009). Moreover, the ripening process of tomato has been well characterized in terms of metabolic changes impacting softening, accumulation of sugars and acids, chlorophyll degradation, lycopene accumulation, and dramatic increases in ethylene and flavor volatiles (Chung *et al.*, 2010; Karlova *et al.*, 2011).

Through investigation of tomato mutants affected in fruit development and ripening (mainly ripening-deficient mutants), such as *ripening inhibitor* (*rin*; Vrebalov *et al.*, 2002), *Colorless non-ripening* (*Cnr*; Manning *et al.*, 2006), *non-ripening* (*nor*; Giovannoni, 2004), *Green-ripe* (*Gr*; Barry and Giovannoni, 2006), and *Never-ripe* (*Nr*; Wilkinson *et al.*, 1995), many genes were isolated and shown to act upstream of ethylene in the ripening cascade, determining the competence of the fruit to ripen (Barry and Giovannoni, 2007). The *RIN*, *CNR*, and *NOR* genes encode transcriptional regulators regulating the expression of other genes responsible for fruit ripening processes, including ethylene and carotenoid biosynthesis (Vrebalov *et al.*, 2002; Giovannoni, 2004; Manning *et al.*, 2006; Martel *et al.*, 2011; Fujisawa *et al.*, 2013). The *Gr* gene encodes a still poorly defined component of ethylene signal transduction, while *Nr* encodes an ethylene receptor important for both fruit and non-fruit ethylene responses (Lanahan *et al.*, 1994; Barry and Giovannoni, 2006). Other ripening transcriptional regulators have recently been characterized *via* functional studies in transgenic plants, including LeHB1, which directly regulates ACC oxidase expression (Lin *et al.*, 2008) and TAGL1, a MADS box transcription factor, which links early fruit fleshy expansion with downstream ripening (Itkin *et al.*, 2009; Vrebalov *et al.*, 2009). The putative transcription factor, Sl-AP2a, a member of the AP2/ERF superfamily was also recently described as a negative regulator of fruit ripening and of ethylene production (Chung *et al.,* 2010; Karlova *et al.,* 2011). Unraveling the transcriptional networks that regulate fruit ripening is crucial for the understanding of this complex process. The present study describes the critical roles of *Sl-ERF.B3* in fruit ripening, a member of *Sl-ERF* multi-family genes.

ERFs are plant specific transcription factors, belonging to the large AP2/ERF superfamily (Riechmann *et al.*, 2000). Proteins encoded by this family have a highly conserved DNA-binding domain known as the AP2 domain, containing 58-59 amino acids involved in the high affinity binding to target DNA sequences (Allen *et al.*, 1998). A growing number of investigations suggest that through interacting with multiple *cis*-acting elements found in

the promoter regions of ethylene-responsive genes, including the GCC box and dehydration-responsive element/C-repeat (DRE/CRT), ERF proteins play a critical role during plant development and adaptation to stress conditions (Ohme-Takagi and Shinshi, 1995; Wu *et al.*, 2002; Wan *et al.*, 2011). Generally, in different plant species ERFs have been shown to be involved in hormonal signaling, responses to biotic and abiotic stresses, developmental processes, metabolic regulation, ethylene biosynthesis and fruit ripening (Ohme-Takagi and Shinshi, 1995; Fujimoto *et al.*, 2000; van der Fits and Memelink, 2000; Wu *et al.*, 2002; Dubouzet *et al.*, 2003; Zhang *et al.*, 2009; Lee *et al.*, 2012). Although Sl-ERF6 was reported to play an important role in fruit ripening by integrating ethylene and carotenoid pathways in tomato (Lee *et al.,* 2012), the role of most ERF proteins in the ripening process awaits elucidation.

To date, no *ERF-like* mutants have been identified in tomato. As we described in our previous article (Liu *et al.*, 2013), classical reverse genetics approach based on down- and up-regulation of *ERF.B3* failed to provide a clue regarding its functional significance. In an attempt to overcome the experimental limitations due to the functional redundancy among members of the ERF gene family, we generated a dominant repressor version of *ERF.B3* (*ERF.B3-SRDX*) using the Chimeric Repressor Silencing Technology (*CRES-T*). Through the *CRES-T* strategy, we show here that *ERF.B3* plays a critical role in fruit ripening. This gene was previously described as an important regulator of ethylene response and plant development (Liu *et al.,* 2013). We found here that *Sl-ERF.B3* also function in fruit development and plays a critical role in the fruit ripening process, In addition, we show that *Sl-ERF.B3* primarily regulates genes involved in both carotenoid and ethylene biosynthesis. Moreover, by the analysis of the expression levels of the tomato ripening regulators including *RIN, CNR, NOR*, *HB-1*, *TAGL1* and *AP2a* during fruit ripening process in the *ERF.B3-SRDX* lines, we demonstrated that *Sl-ERF.B3* is a new regulator involved in the tomato regulatory network controlling ripening.

RESULTS

Expression patterns of tomato ERF genes during fruit ripening

Ethylene is known to play a critical role in fruit development and ripening, and Ethylene Response Factors (ERFs) are considered as the primary actors in mediating responses to the hormone. To gain further insight on the expression of members of the tomato ERF gene family during the ripening process, the accumulation of *Sl-ERFs* transcripts was assessed by quantitative RT-PCR at different stages of fruit ripening. The data indicate (Figure 1) that out of the 25 *ERF* genes tested, eleven (*Sl-ERF.A2*, *A3*, *B1*, *B2*, *C1*, *C3*, *D1*, *D2*, *F3*, *F5* and *G2*) show an increase in their expression peaking 3 days post-breaker (Br+3) and then decline at later ripening stages. The expression of 5 genes (*Sl-ERF.B3*, *C2*, *E2*, *E4* and *F2*) peaks at the breaker stage (Br) and then decreases. A distinct expression pattern is displayed by a group of *ERF* genes (*Sl-ERF.A1*, *C6*, *D3*, *D4*, *E1*, *F1*, *F4* and *H1*) that undergo steady increase in transcript accumulation throughout ripening. *Sl-ERF.E3*, is the only gene showing no ripening-induced expression but rather a decline of its transcript levels from breaker to late ripening stages. While the expression dynamics of most *ERF* genes suggests their involvement in the ripening process, no link was established between their repressor or activator function and their pattern of expression.

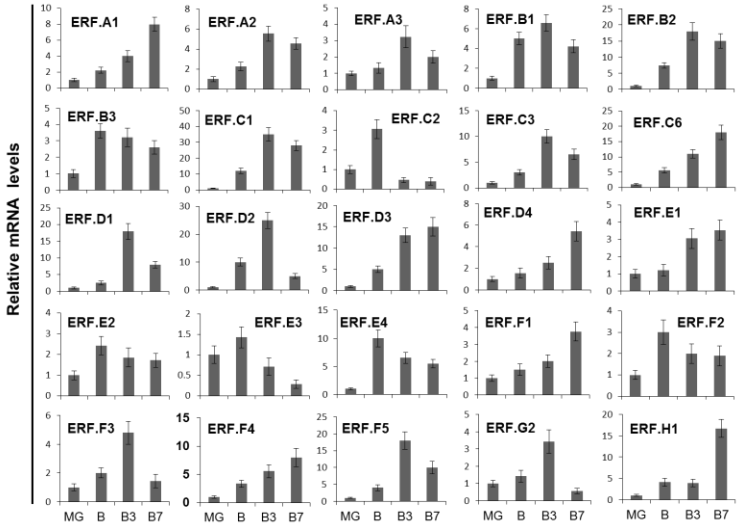

Figure 1. Relative expression profiles of tomato ERF genes in different ripening stages obtained by quantitative RT-PCR. MG, mature green fruit; Br, breaker stage fruit; Br+3, 3 days after breaker stage; Br+7, 7 days after breaker stage. Values represent means of three biological replicates, and vertical bars represent SD of the means.

Sl-ERF.B3 shows fruit development- and ripening-related expression pattern

The *Sl-ERF.B3* transcript is induced at the breaker stage and maintained a high levels at all later stages of ripening, suggesting that its expression might be continuously required along the ripening process. This observation motivated the further assessment of *Sl-ERF.B3* transcript accumulation in vegetative and reproductive tissues by quantitative RT-PCR (Figure 2A). Analysis of stem, root, leaf, flower and in a series of fruit developmental stages, indicated that the accumulation of *Sl-ERF.B3* transcripts is relatively high in both vegetative and reproductive tissues (Figure 2A).

To address the functional significance of this tomato ERF family member, we first attempted to alter its expression using antisense or overexpression strategies. However, both approaches failed to provide significant clues on the physiological role of *Sl-ERF.B3,* which prompted the use of a dominant repressor version of the gene (*ERF.B3-SRDX*), relying on the so-called Chimeric Repressor Silencing Technology (CRES-T). This technology has been developed to study the consequences of silencing of the target genes of single transcription factors and has also been used to overcome the experimental limitations caused by functional redundancy of transcription factor families (Hiratsu *et al.*, 2003). Three out of ten transgenic *ERF.B3-SRDX* lines (*SR1, SR2* and *SR3*) showed a characteristic phenotype with different expressivity and were selected for further studies. The relative expression levels of the *ERF.B3-SRDX* transcript in fruit tissues of the three independent lines was assessed by qRT-PCR using primers specific for the transgene (Figure 2B).

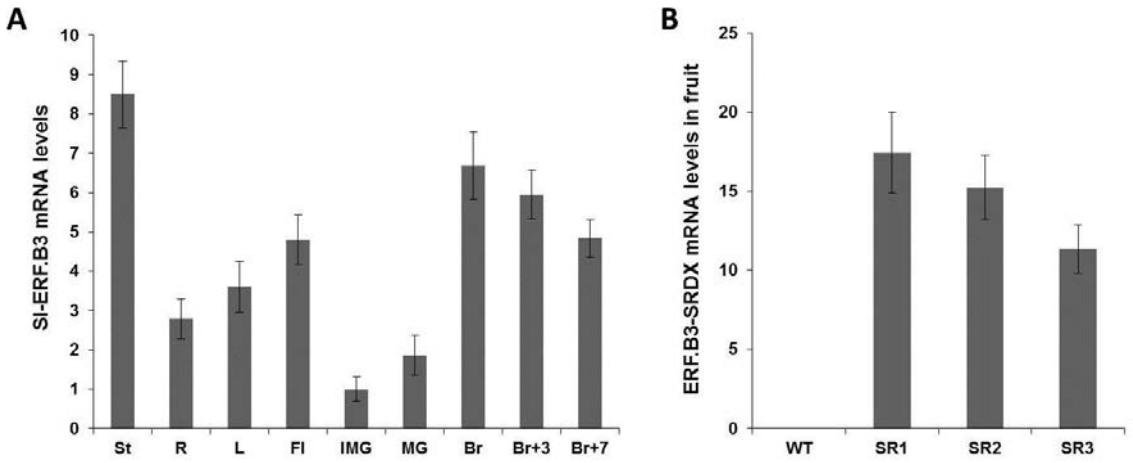

Figure 2. *Sl-ERF.B3* gene expression during development and in *ERF.B3-SRDX* dominant repressor lines. A, Total RNA was extracted from different developmental stages; St (stem), R (root), L (leaf), Fl (flower), IMG (immature fruit), MG (mature green fruit), Br (breaker stage fruit), Br+3 (3 days after breaker stage), Br+7 (7 days after breaker stage). The relative mRNA levels of *Sl-ERF.B3* at immature green stage were standardized to 1.0, referring to *Sl-Actin* gene as internal control. Values are means ± SD of three biological replicates. B, Transcript accumulation of the chimeric *ERF.B3-SRDX* gene in wild-type and transgenic lines (*SR1*, *SR2* and *SR3*) in ripening fruit. Total RNA was extracted from fruit at 3 days after breaker stage (Br+3).

Altered fruit development in *ERF.B3-SRDX* dominant repressor lines

One of the most evident phenotypes displayed by *ERF.B3-SRDX* transgenic lines is the altered fruit shape and reduced size (Figure 3A). Wild-type tomato fruits (Figure 3A) are round shaped in contrast to the *ERF.B3-SRDX* fruits which heart shaped with bumpy areas present intermittently on the surface of the fruit (Figure 3A). Changes in fruit anatomy also include thicker pericarp and decreased jelly formation with enhanced pericarp to total fruit volume ratio (Figure 3A, lower panel). As a consequence of the smaller size, the mean weight of *ERF.B3-SRDX* fruits is significantly reduced (Figure 3B). The number of seeds is dramatically reduced in *ERF.B3-SRDX* fruits compared to wild type and the average seed number drops from 25 per fruit in wild-type to 6 in *ERF.B3-SRDX* lines (Figure S1A). In addition to their reduced number, the seeds show reduced size in dominant repressor lines (Figure S1B). In the *ERF.B3-SRDX* line showing

the strongest phenotype, seed weight is less than half of the wild type (Figure S1C). *ERF.B3-SRDX* lines produced hairless seed with altered morphology (Figure S1B).

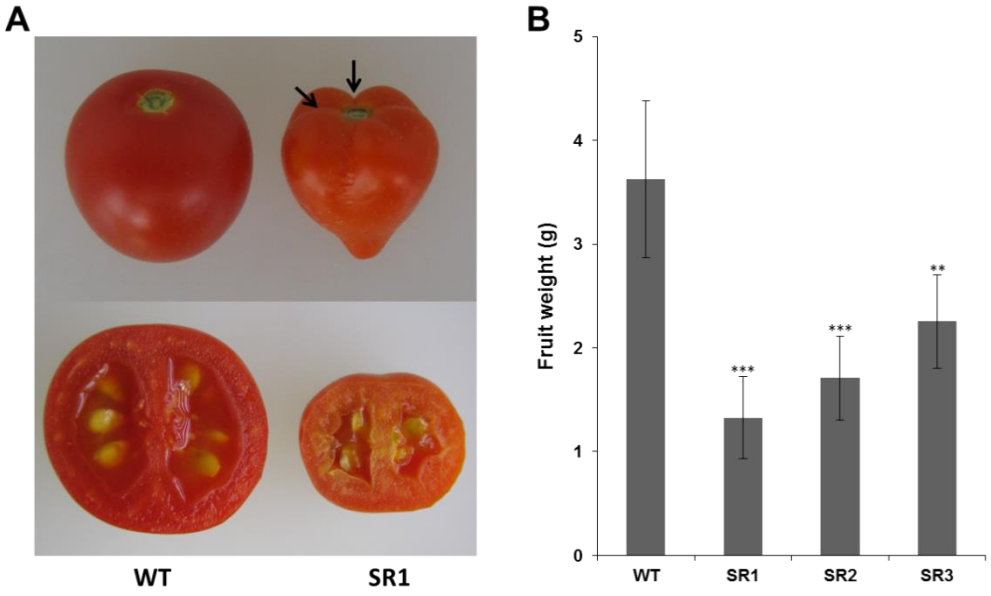

Figure 3. Fruit morphology in wild-type and ERF.B3-SRDX lines. A, Altered fruit shape and size in ERF.B3-SRDX fruits. B, Fruit weight is significantly reduced in ERF.B3-SRDX lines compared with wild type. 50 fruits were used for each measurement and values shown are the means ± SD. **, $0.001 < P < 0.01$, ***, $P < 0.001$ (Student's test). SR1, SR2 and SR3 are three independent ERF.B3-SRDX lines.

ERF.B3-SRDX fruits fail to display a red-ripe phenotype

In addition to the altered fruit shape and size, *ERF.B3-SRDX* lines exhibited distinct ripening changes. The ripening-related phenotypes were investigated in wild-type and *ERF.B3-SRDX* lines using fruits at different development stages sampled from the same truss. Dramatic changes were revealed in the transgenic lines with regard to both the time at which the ripening process starts and the speed at which it proceeds. The onset of ripening occurs with at least two weeks delay in *ERF.B3-SRDX* lines (57 days post-anthesis) than in wild type (41 days post anthesis) suggesting that the attainment of competence to ripen is dramatically delayed in the transgenic lines (Table 1).

Table 1 Days from anthesis to breaker stage for control and *ERF.B3-SRDX* lines.

Lines	Days
Wild type	41.49 ± 2.49
SR1	59.34 ± 3.27 ***
SR2	56.18 ± 2.19 **
SR3	54.82 ± 3.46 **

Values represent means ± SD for at least 15 fruit for each line. **, $0.001 < P < 0.01$, ***, $P < 0.001$ (Student's test).

Moreover, once the ripening process starts at breaker stage, when a visible color change just begins to occur, the ripening of *ERF.B3-SRDX* fruits seems much slower than wild type (Figure 4A). In contrast to wild type which reaches the red-ripe stage 5 days post-breaker (Br+5), *ERF.B3-SRDX* fruits remain orange at breaker+10 stage (Br+10; Figure 4A). The assessment of color change via measuring the evolution of hue angle values, indicative of color saturation, further emphasized the difference between wild type and dominant repressor lines throughout the ripening process (Figure 4B). The value of hue angle is even higher for *ERF.B3-SRDX* fruit at Br+10 than for wild type at Br+5, thus confirming the orange-ripe phenotype observed visually (Figure 4A, 4B).

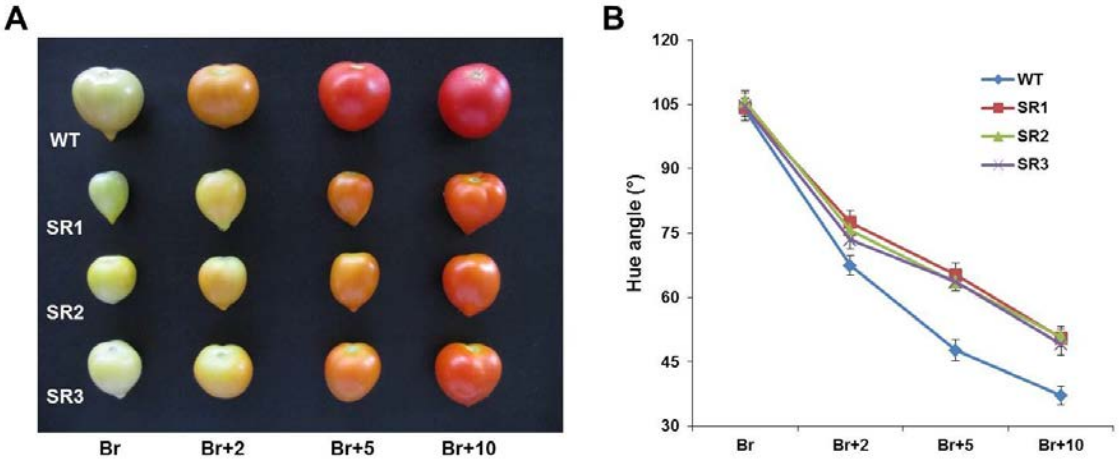

Figure. 4 Fruit ripening in wild-type and *ERF.B3-SRDX* lines. A, Different stages of fruit ripening for wild-type (WT) and *ERF.B3-SRDX* lines. Fruits from independent transformant lines are shown, which are delayed in color development, never developing full red color. Br, breaker stage , Br+2, tuning stage (2 days after breaker stage), Br+5, pink stage (5 days after breaker stage), and Br+10, red ripe stage (10 days after breaker stage). B, Changes in hue angle in WT and *ERF.B3-SRDX* lines during different ripening stages. *SR1, SR2* and *SR3* are three independent *ERF.B3-SRDX* lines.

Decreased lycopene and increased beta-carotene levels are responsible for the orange-ripe phenotype in *ERF.B3-SRDX* fruits

To investigate the cause of the altered pigmentation in *ERF.B3-SRDX* fruits, LC-PDA-MS analysis of carotenoid levels was performed on wild-type and *ERF.B3-SRDX* fruits at both breaker and breaker+7 stages. Notably, levels of lycopene and its precursors phytoene, phytofluene, ζ-carotene and neurosporene were significantly decreased in the *ERF.B3-SRDX* fruits at the breaker stage (Figure 5). At the ripe stage (Br+7), lycopene levels and its precursors were dramatically decreased, and, a sharp increase in *β*-carotene content was also observed in the *ERF.B3-SRDX* lines (Figure 5) in keeping with the orange-ripe phenotype.

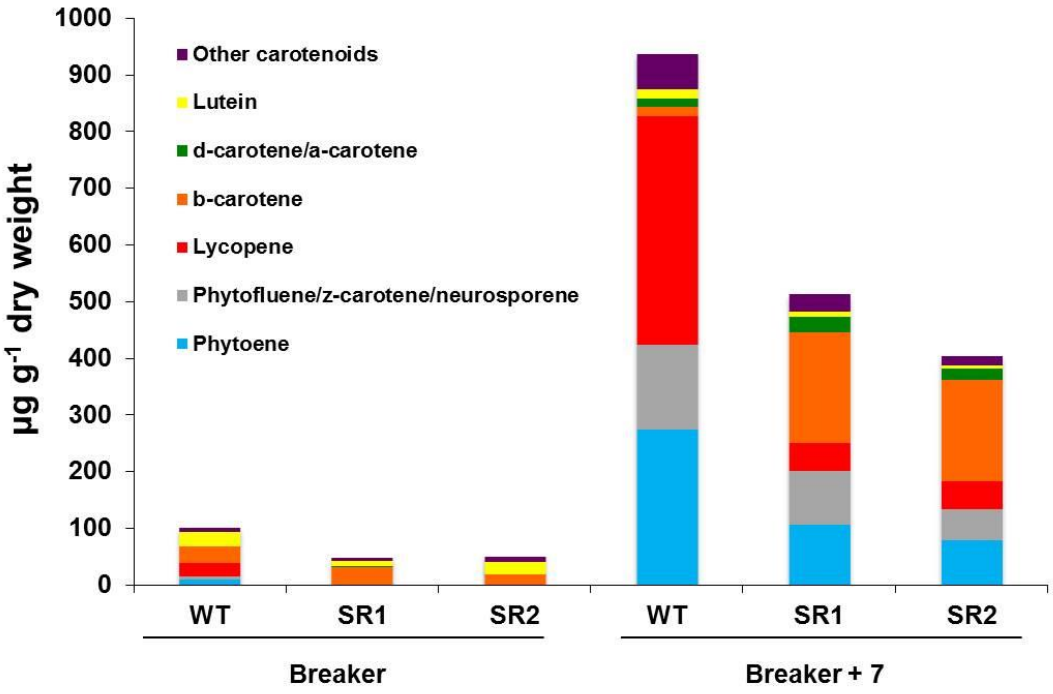

Figure 5. Carotenoid composition of wild-type and *ERF.B3-SRDX* fruits at breaker and breaker+7 stages. Amounts of the different carotenoid species in wild-type and *ERF.B3-SRDX* fruits, plotted as stacked bars.

To uncover the molecular basis of the altered carotenoid composition in *ERF.B3-SRDX* lines, we examined the transcript levels of genes involved in carotenoid biosynthesis pathway at different stages of fruit ripening by quantitative RT-PCR (Figure 6). Even

though transcript of phytoene synthase (*PSY1*) in *ERF.B3-SRDX* lines showed similar ripening-regulated accumulation pattern than in wild type, *PSY1* levels were dramatically reduced in *ERF.B3-SRDX* fruits at the breaker stages (Figure 6). *PSY1* is a key regulator of flux through the carotenoid pathway and its repression is consistent with the reduction of lycopene and total carotenoids at the breaker stage fruits (Figure 5). A decrease in phytoene desaturase (*PDS*) expression levels was also observed in *ERF.B3-SRDX* fruits (Figure. 6). It is also noteworthy that transcript accumulation of all three lycopene beta cyclases (*β-LCY1, β-LCY2, CYC-β*) was markedly elevated in *ERF.B3-SRDX* fruits compared to wild-type (Figure 6) accounting for the significantly increased *β*-carotene content in *ERF.B3-SRDX* lines (Figure 5). The data indicate that the dominant repressor version of *ERF.B3* leads to decreased expression of *PSY1* and *PDS* and increased expression of lycopene beta cyclases, thus resulting in a modified lycopene to *β*-carotene ratio.

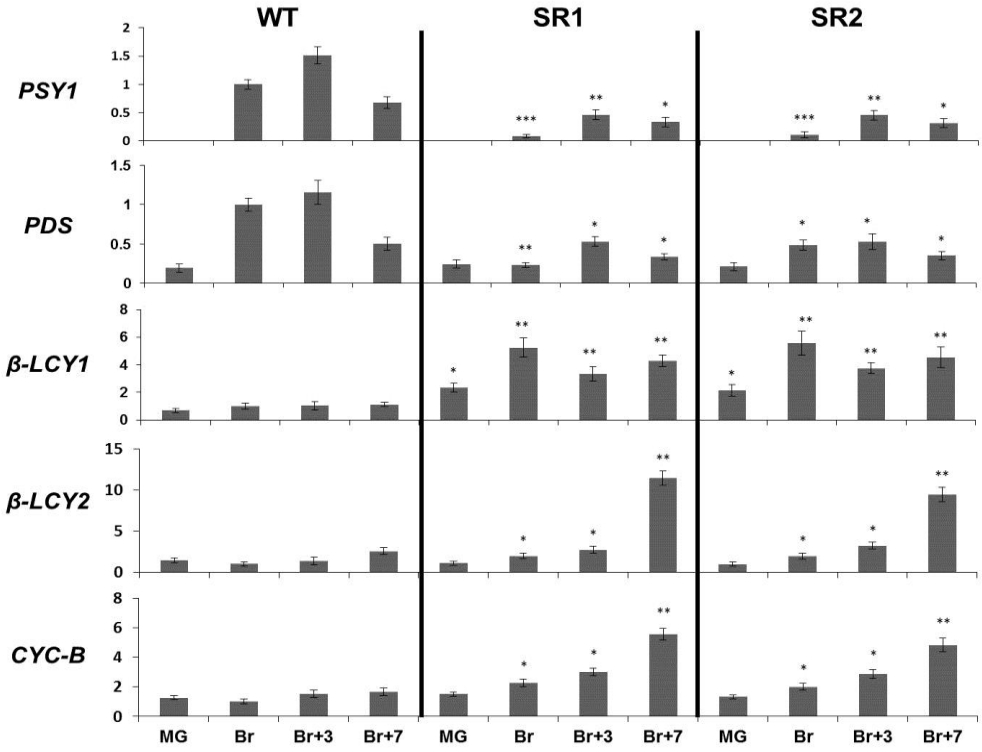

Figure 6. Expression of carotenoid biosynthesis genes in wild-type and *ERF.B3-SRDX* lines. Total RNA was extracted from the indicated developmental stages of fruit (MG, Br, Br+3 and Br+7). The relative mRNA levels of each gene at breaker (Br) stage were standardized to 1.0, referring to *Sl-Actin* gene as internal control. Values are means ± SD of three biological replicates. *, $0.01 < P < 0.05$, **, $0.001 < P < 0.01$, ***, $P < 0.001$ (Student's test). *SR1* and *SR2* are two independent *ERF.B3-SRDX* lines.

ERF.B3-SRDX fruits show fast softening and elevated ethylene production

To uncover whether the failure to reach the red-ripe stage in dominant repressor fruits results from the incapacity to ripen, other major ripening-associated features, like softening and climacteric rise of ethylene production, were investigated. The evolution of firmness determined from breaker stage to 20 days post-breaker (Br+20) showed that *ERF.B3-SRDX* transgenic fruits undergo significantly faster softening than control fruit (Figure 7A). Given that fruit softening is highly regulated by ethylene, we therefore assessed the production of ripening-associated ethylene in the *ERF.B3-SRDX* fruits. As shown in Figure 7B, the accelerated softening observed in transgenic fruits was associated with a dramatic increase in climacteric ethylene production (Figure 7B) which reached a maximum 3 to 4 times higher than in wild type fruit. Altogether these data indicate that once triggered, the ripening process is accelerated in the *ERF.B3-SRDX* repressor lines.

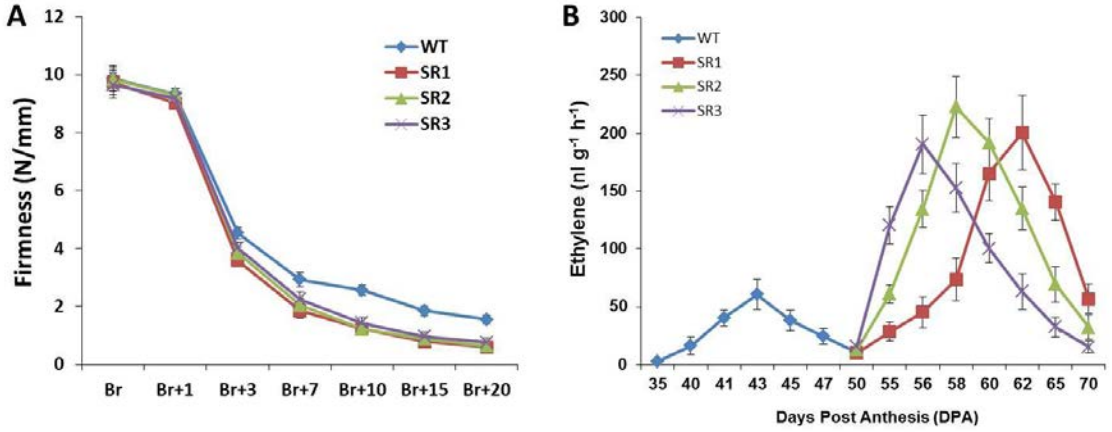

Figure 7. Firmness and ethylene production in wild-type and *ERF.B3-SRDX* fruits. A, Firmness analysis of control and *ERF.B3-SRDX* fruits. Fruits were harvested at breaker stage, kept at room temperate and measured for firmness at different stages (Br, Be+3, Br+7, Br+10, Br+15 and Br+20 as defined in the methods). 15 fruit were used for each measurement and values shown are the means ± SD. B, Production of ethylene in control and *ERF.B3-SRDX* lines. Fruits of different ripening stages representative by Days Post Anthesis (DPA). Values represent means of at least ten individual fruits. Vertical bars represent SD. 35 DPA represents mature green (MG) stage in WT; 40 DPA, breaker (Br) stage in WT. 50 DPA, mature green (MG) stage in *ERF.B3-SRDX* lines; 55 DPA, breaker (Br) stage in transgenic lines. *SR1, SR2* and *SR3* are three independent *ERF.B3-SRDX* lines.

Ethylene and ripening-related genes are highly induced in *ERF.B3-SRDX*-expressing fruits

To gain more insight on the regulation of fruit ripening in *ERF.B3-SRDX* lines, we examined transcript accumulation of a set of ripening-related genes. The expression of ethylene biosynthesis genes, such as *ACS2*, *ACS4* and *ACO1,* was significantly higher in *ERF.B3-SRDX* expressing fruits than in wild type (Figure 8). Transcript accumulation of these genes was similarly low in transgenic and control fruit at mature green stage, but was more strongly induced after the breaker stage in the dominant repressor lines, concomitant to the rise in ethylene production. In addition, mRNA accumulation of ethylene-inducible genes, like E4 and E8 was also increased in *ERF.B3-SRDX*, consistent with the elevated ethylene production (Figure 8). The transcript accumulation of a major fruit polygalacturonase gene, *PG2A*, involved in ripening-related cell wall metabolism, was significantly induced in *ERF.B3-SRDX* fruits (Figure 8) in line with the enhanced softening phenotype.

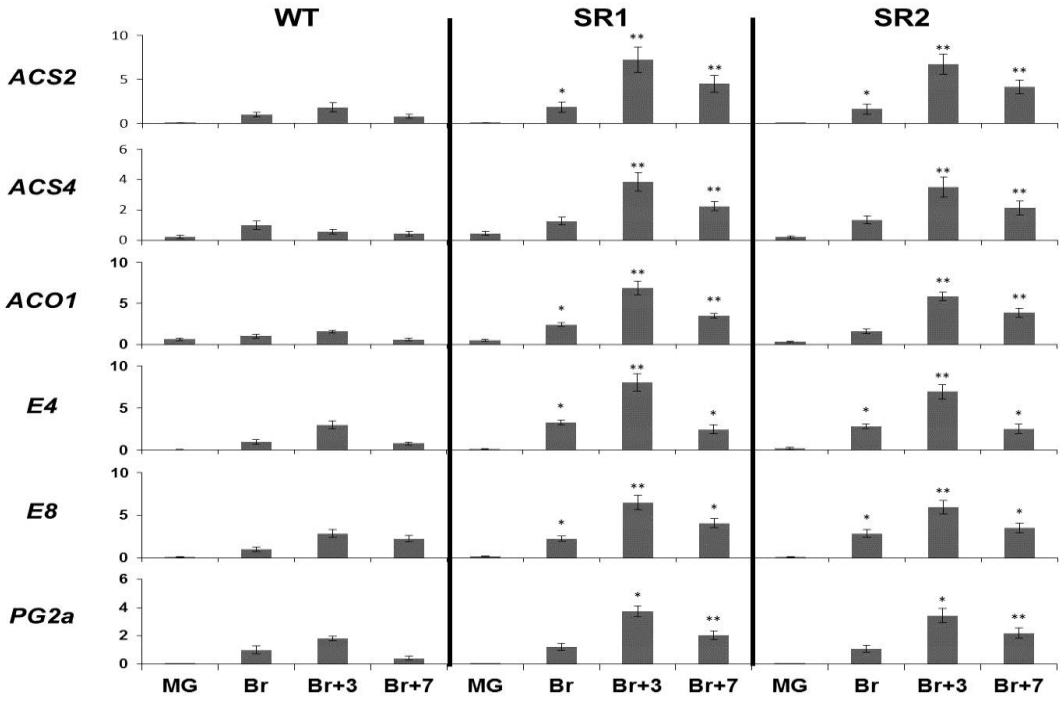

Figure 8. Ripening-related gene expression in wild-type and *ERF.B3-SRDX* lines during fruit ripening. Total RNA was extracted from the indicated developmental stages of fruit (MG, Br, Br+3 and Br+7). Values are means ± SD of three biological replicates. *, $0.01< P < 0.05$, **, $0.001 < P < 0.01$ (Student's test). *SR1 and SR2* are two independent *ERF.B3-SRDX* lines.

The expression of key regulatory genes of the ripening process like *RIN*, *NOR* and *CNR* was increased at post-breaker stages compared to wild type, even though their induction took place later than in control fruit (Figure 9). The altered expression pattern of these genes in the *ERF.B3-SRDX* fruits is consistent with the dramatically delayed attainment of competence to ripen in transgenic fruits. Moreover, the mRNA levels of *LeHB-1*, another ripening regulator gene, were higher in *ERF.B3-SRDX* lines at all ripening stages (Figure 9). By contrast, the expression of *TAGL1*, a tomato SHATTERPROOF gene, and *AP2a*, an *AP2/ERF* family gene acting as a negative regulator of fruit ripening did not display significant changes in *ERF.B3-SRDX* dominant repressor fruits compared to wild type (Figure 9).

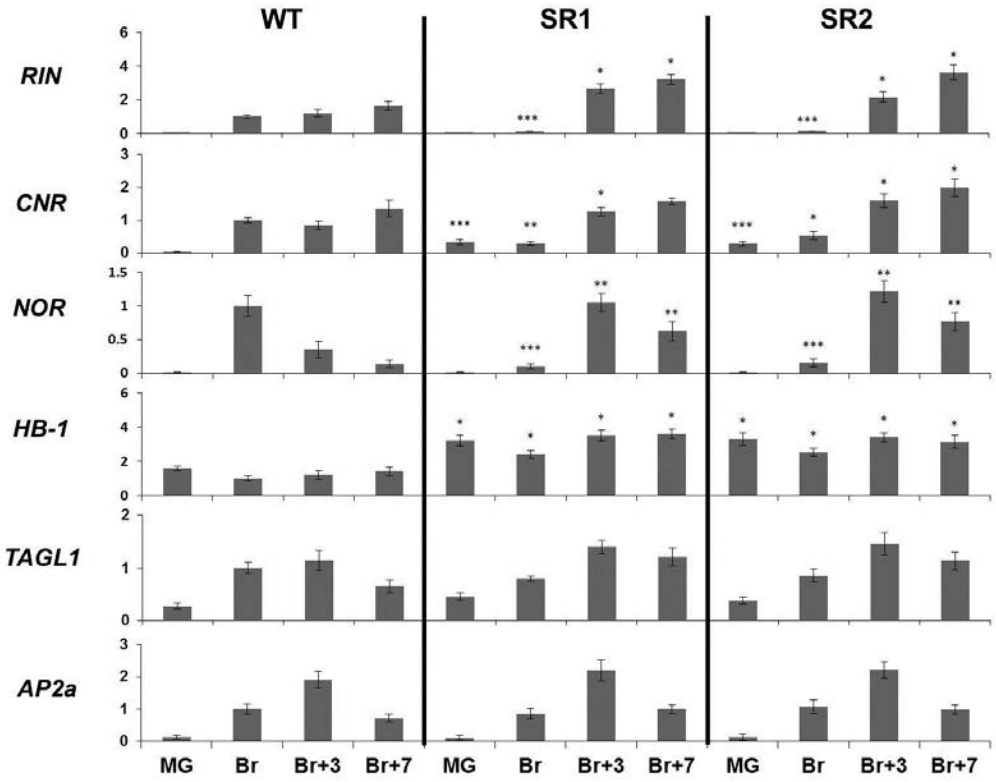

Figure 9. Ripening regulator genes expression in wild-type and *ERF.B3-SRDX* lines during fruit ripening. Total RNA was extracted from the indicated developmental stages of fruit (MG, Br, Br+3 and Br+7) as defined in the methods. The relative mRNA levels of each gene at breaker (Br) stage were standardized to 1.0, referring to *Sl-Actin* gene as internal control. Values are means ± SD of three biological replicates. *, 0.01< P < 0.05, **, 0.001 < P < 0.01, ***, P < 0.001 (Student's test). *SR1* and *SR2* are two independent *ERF.B3-SRDX* lines.

A number of ERF gene family members show altered expression in the *ERF.B3-SRDX* lines

Considering the putative role of ERFs in mediating ethylene responses, and given the role of ethylene in regulating the ripening process, we examined the transcript levels of *Sl-ERF* genes in both wild-type and the *ERF.B3-SRDX* fruits. A dramatic change in the transcript levels for a number of *ERF* genes was revealed in the dominant repressor lines (Figure 10). That is, among the 25 *Sl-ERF*s that showed detectable transcript accumulation, 10 were significantly down-regulated in the *ERF.B3-SRDX* dominant repressor lines while 8 *Sl-ERFs* displayed up-regulation in transgenic lines (Figure 10). Of particular note, accumulation of transcripts corresponding to *Sl-ERF.A1,* whose expression is strongly induced during ripening (Figure 1), was dramatically enhanced in the *ERF.B3-SRDX* expressing lines.

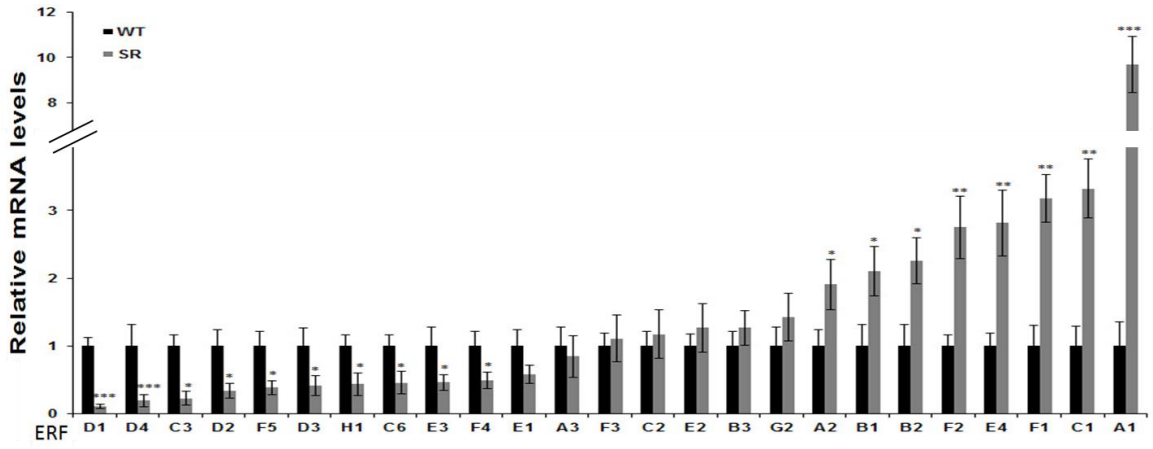

Figure 10. Accumulation of *Sl-ERFs* transcripts in WT and *ERF.B3-SRDX* lines assessed by qRT-PCR in fruits at Br+3 stage. The relative mRNA level of each gene in WT was standardized to 1.0, referring to *Sl-Actin* as internal control. *, $0.01 < P < 0.05$, **, $0.001 < P < 0.01$, ***, $P < 0.001$ (Student's test). *SR* is representative of data from three independent *ERF.B3-SRDX* lines (*SR1*, *SR2*, and *SR3*).

DISCUSSION

Our previous study has demonstrated that tomato *Sl-ERF.B3* plays an important role in controlling pleiotropic ethylene responses in tomato via feedback regulation of ethylene

signaling and *ERF* genes (Liu *et al.,* 2013). Here we show that dominant repression of the *Sl-ERF.B3* transcription factor in tomato also broadly impacts fruit development and ripening. Indeed, ERF genes have been shown to be involved in fruit ripening in tomato (Li *et al.*, 2007; Lee *et al.*, 2012). The highly induced mRNA accumulation of the tomato *Sl-ERF.B3* gene in fruit at the breaker stage suggested a putative role of this gene in fruit ripening. The dramatically delayed time from anthesis to the breaker stage was an apparent effect of dominant repression of *Sl-ERF.B3* on early fruit development. The time from anthesis to the breaker stage was delayed by approximately 16 days in the dominant repressor (*ERF.B3-SRDX*) lines compared with the wild type (Table 1), indicating a strong influence of dominant repression of *Sl-ERF.B3* on tomato early fruit development. Another obvious effect of dominant repression of *Sl-ERF.B3* on fruit development is the significantly reduced fruit size with a bumpy appearance from the young green fruit stage, which is probably caused by a reduction in epidermal cell size and a defect in the normal or coordinated expansion of the pericarp. The difference in color development between wild-type and the *ERF.B3-SRDX* fruits became obvious after the breaker stage and the *ERF.B3-SRDX* fruits retained its orange color, failing to turn red as in wild type at the final ripening stage. Data from the time course for fruit firmness also showed an early fruit softening phenotype in the *ERF.B3-SRDX* lines. Moreover, ripening fruit anatomy showed that *ERF.B3-SRDX* fruits have a thicker pericarp, a smaller volume of jelly, a dry and crumbly appearance of the pericarp, suggesting a defect in the expansion or elasticity of the epidermis. Since fruit ripening is a complex process with dramatic changes in color, texture, flavor, and aroma of the fruit flesh (Seymour *et al.*, 1993; Alexander & Grierson, 2002; Carrari & Fernie, 2006), the changes in the *ERF.B3-SRDX* lines related to fruit ripening indicate the involvement of *Sl-ERF.B3* gene in regulating fruit ripening in tomato.

The dramatic development of red pigmentation of ripening fruits is one of the most notable features of tomato and the principal carotenoids that accumulate in ripening tomato are lycopene and *β*-carotene, which confer the red and orange colors to ripe fruits, respectively (Fraser *et al.*, 1994; Burns *et al.*, 2003; Alba *et al.*, 2005). Changes in carotenoid accumulation have been demonstrated to correspond with alterations in expression of genes encoding the carotenoid pathway enzymes (Giuliano *et al.*, 1993;

Ronen *et al.*, 2000; Galpaz *et al.*, 2006; Chung *et al.*, 2010; Lee *et al.*, 2012; Luo *et al.*, 2013). Dominant repression of *Sl-ERF.B3* in tomato resulted in orange fruit color with decreased levels of lycopene and elevated accumulation of *β*-carotene. This suggests a role of *Sl-ERF.B3* in regulating carotenoid biosynthesis in tomato. The altered carotenoid levels in *ERF.B3-SRDX* lines are tightly correlated with the altered mRNA accumulation of the carotenoid biosynthesis genes. The transcript accumulation of *PSY1* which acts as a major regulator of metabolic flux toward downstream carotenoids during fruit maturation (Fray & Grierson, 1993), was markedly reduced and its repression is consistent with the substantial reduction in total carotenoid levels at the breaker stage (due to a precipitous decline in lycopene) observed in the *ERF.B3-SRDX* dominant repressor lines. Moreover, *PDS*, another carotenoid biosynthesis gene which encodes phytoene desaturase catalyzing the conversion of phytoene to ζ-carotene upstream of lycopene synthesis (Pecker *et al.*, 1992), was also down regulated in the *ERF.B3-SRDX* fruits during ripening and thus may also contribute to the decreased carotenoid levels. In addition, a significant induction of all three lycopene beta cyclases (*β-LCY1, β-LCY2, CYC-β*) genes was observed in *ERF.B3-SRDX* lines, most probably accounting for the elevated levels of β-carotene. In line with this hypothesis, overexpression of *β-LCY* and *CYC-β* has been previously shown to cause β-carotene accumulation in fruits (Rosati *et al.*, 2000; Ronen *et al.,* 2000). In addition, positive correlations between *β-LCY* and *CYC-β* expression and β-carotene levels were revealed by correlation analysis of fruit metabolome and transcriptome data from S. *pennellii* x *S. lycopersicum* introgression lines (Lee *et al.,* 2012). Ethylene, light and some transcription factors are known putative regulators of carotenoid accumulation (Mustilli *et al.*, 1999; Vrebalov *et al.*, 2002, 2009; Giovannoni, 2004; Liu *et al.*, 2004; Alba *et al.*, 2005). It is known that ethylene regulates carotenoid accumulation during fruit ripening by regulating the expression of carotenoid biosynthesis genes controlling final carotenoid profiles such as *PSY1*, *β-LCY* and *CYC- β* (Fraser *et al.,* 1994; Ronen *et al.,* 2000; Alba *et al.,* 2005). It is noteworthy that dominant repression of *Sl-ERF.B3* in tomato resulted in decreased total carotenoid levels and elevated ethylene production. This phenotype at least partially resembles that of the *SlAP2a* repressed lines, in which significantly elevated ethylene levels are associated with altered total carotenoids and a shift to β-carotene rather than lycopene (Chung *et al.,* 2010; Karlova *et al.,* 2011).

Moreover, the phenotype also recalls that of the tomato *never ripe* mutant, in which lycopene biosynthesis and *PDS* gene expression are repressed and ethylene production is increased (Alba *et al.,* 2005). Furthermore, assessing the relative mRNA accumulation of *SlAP2a* failed to show significant difference in its expression levels in *ERF.B3-SRDX* fruits compared to wild type, suggesting that the regulation of dominant repression of *Sl-ERF.B3* in fruit ripening is likely to be independent of *SlAP2a*. It is possible that dominant repression of *Sl-ERF.B3* in tomato results in complex alterations in carotenoid accumulation network and impacts carotenoid biosynthesis genes through mechanisms beyond the influence of ethylene. Transcription factors impacting carotenoid accumulation in tomato include *RIN* (Vrebalov *et al.,* 2002), *CNR* (Manning *et al.,* 2006), *TAGL1* (Vrebalov *et al.,* 2009), *SlAP2a* (Chung *et al.,* 2010; Karlova *et al.,* 2011), *SlERF6* (Lee *et al.,* 2012) and SlMADS1 (Dong *et al.*, 2013). The expression data during fruit ripening revealed altered expression of *RIN*, *CNR* and *SlERF6* (*Sl-ERF.E4*) genes in *ERF.B3-SRDX* lines compared with wild-type controls, suggesting that these transcription factors may be involved in the regulation networks of carotenoid accumulation in the *ERF.B3-SRDX* lines. Interestingly, an *Arabidopsis* ERF transcription factor, RAP2.2, via binding to the ATCTA *cis*-element in the promoter regions of *PSY* and *PDS*, regulates the expression of carotenoid biosynthesis genes (Welsch *et al.*, 2007). Given that the *Sl-ERF.B3* fused to the SRDX motif strongly suppresses the expression of the putative target genes (Liu *et al.,* 2013) together with the presence of the ATCTA motif in the promoter regions of both tomato *PSY1* and *PDS* genes*,* it is possible that ERF.B3-SRDX represses the expression of *PSY1* and *PDS* in *ERF.B3-SRDX* lines by directly binding to their promoters.

A hallmark of climacteric fruit ripening such as tomato is the dramatically induced respiration and ethylene production at the onset of ripening. Dominant repression of *Sl-ERF.B3* in tomato resulted in substantially elevated levels of ethylene production (Figure. 7B), indicating that altered fruit ripening in *ERF.B3-SRDX* dominant repressor lines was at least partly through influencing ethylene synthesis. *ERF.B3-SRDX* fruits produced up to four-fold more ethylene than the wild-type fruits during ripening with correspondingly elevated transcript accumulation of ethylene biosynthesis genes, including *ACS2*, *ACS4* and *ACO1* (Figure. 8). Ethylene biosynthesis is tightly controlled by *ACS* and *ACO*

multigene families during fruit development and ripening (Nakatsuka *et al.*, 1998; Barry *et al.*, 2000; Barry & Giovannoni, 2007). Based on the level of ethylene production during fruit development, two systems of ethylene regulation have been proposed (McMurchie *et al.*, 1972). System 1 represents the basal level of ethylene in immature fruit and vegetative tissues, whereas system 2 represents a high level of ethylene production associated with fruit ripening (Oetiker & Yang, 1995). Tomato *ACS1* and *ACS6* have been shown to mediate the system 1 ethylene production in immature fruit in tomato, and the autocatalytic ethylene biosynthesis in system 2 is mediated through ethylene-stimulated expression of *ACS2*, *ACS4*, *ACO1* and *ACO4* genes (Nakatsuka *et al.,* 1998; Barry *et al.,* 2000; Barry & Giovannoni, 2007). The mRNA accumulation of *ACS2* is induced at the onset of ripening and this induction is ethylene-dependent but is independent of *rin* (Nakatsuka *et al.,* 1998; Barry *et al.,* 2000). Moreover, repression of the *ACS2* gene could block fruit ripening in tomato (Oeller *et al.*, 1991). *ACS4* is also induced during ripening in a *rin*-dependent fashion. Indeed, the tomato *ACS2* and *ACS4* are the predominant *ACS* mRNAs in ripening fruit and both genes are under additional developmental controls (Barry *et al.*, 2000; Yokotani *et al.*, 2004). The transcript of *ACO1* increases at the onset of ripening and is sustained in high expression during tomato fruit ripening (Nakatsuka *et al.,* 1998), suggesting a role of this gene in controlling ethylene synthesis in fruit ripening. Since *ACS* and *ACO* catalyze the rate limiting and final steps in ethylene biosynthesis, the significantly elevated mRNA levels of *ACS2*, *ACS4* and *ACO1* are probably responsible for the elevated ethylene levels in the *ERF.B3-SRDX* dominant repressor lines. Dominant repression of *Sl-ERF.B3* results in high levels of ethylene, suggesting that regulators (either directly or indirectly regulated by ERF.B3-SRDX) for regulation of these key ethylene synthesis genes may lie upstream of ethylene synthesis control and remain to be discovered. It is noteworthy that dominant repression of *Sl-ERF.B3* leads to reduced ethylene production in dark-grown seedlings with reduced mRNA accumulation of *ACS1A* and *ACO1* genes (Liu *et al.,* 2013) indicating that the mechanisms of controlling ethylene production in *ERF.B3-SRDX* lines by the regulation of ethylene biosynthesis genes in vegetative tissue and ripening fruits are distinct.

Fruit ripening is a complex developmental process and is genetically controlled by intricate transcriptional cascades through ethylene-and non-ethylene-mediated regulation.

The MADS-box transcription factor RIN has been regarded as a key regulator responsible for the onset of ripening by acting upstream of both ethylene- and non-ethylene-mediated controls in tomato (Vrebalov *et al.*, 2002; Ito *et al.*, 2008; Fujisawa *et al.*, 2011; Martel *et al.,* 2011; Fujisawa *et al.,* 2013). Using a chromatin immunoprecipitation (CHIP) approach, RIN was proved to be a master regulator of ripening that directly influences many ripening-associated processes in a developmental specific pattern (Fujisawa *et al.,* 2011; Martel *et al.,* 2011; Fujisawa *et al.,* 2013). Indeed, RIN interacts with the promoters (CArG motif) of genes involved in the major pathways associated with observed and well-studied ripening phenotypes and phenomena, including the transcriptional control network involved in overall ripening regulation (*CNR, NOR* and *HB1*), ethylene biosynthesis (*ACS2* and *ACS4*), downstream ethylene response (*E4* and *E8*), cell wall metabolism (*PG2a*), and carotenoid biosynthesis (*PSY1*) (Fujisawa *et al.,* 2011; Martel *et al.,* 2011; Fujisawa *et al.,* 2013). ACO1 is influenced by RIN via the homebox protein HB1, which interacts with the promoter of ACO1 (Lin *et al.,* 2008; Martel *et al.,* 2011). In *ERF.B3-SRDX* mutants, although the induction of *RIN* is delayed at the breaker stage, the transcript accumulation is significantly increased compared to the wild-type controls during the later development stages (Br+3 and Br+7). *CNR* and *NOR* show the same transcript accumulation patterns with *RIN* in *ERF.B3-SRDX* mutants during fruit ripening in consistent with the regulation of RIN to *CNR* and *NOR*. The transcript of *HB1* also shows higher accumulation in *ERF.B3-SRDX* lines during fruit ripening. Moreover, genes involving in ethylene biosynthesis, ethylene downstream response and cell wall metabolism including *ACS2, ACS4, ACO1, E4, E8* and *PG2a* display significant induction in *ERF.B3-SRDX* mutants during fruit ripening process. These data are in line with the model that RIN acts as a master regulator of the ripening cascade by influencing numerous molecular pathways. However, since ERF.B3-SRDX is a dominant repressor, the regulation mechanism between ERF.B3 or its chimeric protein and RIN remains to be revealed.

The expression dynamics of most *Sl-ERF* genes during fruit ripening suggests their putative involvement in the ripening process. Moreover, the expression of a number of *Sl-ERF* genes was found to be markedly altered in *ERF.B3-SRDX* fruit at the breaker + 3 stage. The alteration of *Sl-ERF* transcript levels in *ERF.B3-SRDX* ripening fruit may

account for diversity of ripening phenotype displayed by the dominant repressor fruits. Notably, *ERF* genes (*Sl-ERF. C3*, *Sl-ERF. D2*, *Sl-ERF. F5* and *Sl-ERF. F4*) which shown to be the putative target of Sl-ERF.B3 and ERF.B3-SRDX (Liu *et al.,* 2013) are down-regulated in *ERF.B3-SRDX* fruits, further supports the model that a single ERF can impact the expression of other members of the gene family and this inter-connected regulation among *ERF* genes may therefore account for the pleiotropic alterations in the *ERF.B3-SRDX* lines.

MATERIAL AND METHODS

Plant materials and growth conditions

Tomato (*Solanum lycopersicum* cv. *MicroTom*) plants were transferred to soil and grown under standard greenhouse conditions. Conditions in the culture chamber room were set as follows: 14h-day/10h-night cycle, 25/20°C day/ night temperature, 80% hygrometry, 250µmol.m^{-2}.s^{-1} intense luminosity. For measuring time to ripening, flowers were tagged at anthesis and number of days from anthesis to breaker stage was counted. More than 15 fruits of each genotype were used for this analysis.

Plant transformation

To generate the *ERF.B3-SRDX* transgenic plants, the coding sequence of *Sl-ERF.B3* missing the stop codon was amplified by PCR from a tomato fruit cDNA library. This coding region was cloned via blunt-end ligation into the SmaI site of p35SSRDXG in frame to the region that encodes the SRDX repression domain (LDLDLELRLGFA) from SUPERMAN (Hiratsu *et al.*, 2003; Mitsuda *et al.*, 2006). The transgene cassette was transferred into the destination vector pBCKH, which was derived from the plant transformation vector pBIG-HYG (Becker, 1990) using the gateway LR reaction (Invitrogen Corp.) Agrobacterium tumefaciens-mediated transformation of tomato plants was carried out according to (Wang *et al.*, 2005), and transformed lines were selected on a hygromycin-containing medium. All experiments were carried out using homozygous lines from F3 or later generations.

RNA isolation and quantitative RT-PCR

Fruits 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 in this article was extracted using a 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) to remove contaminating genomic DNA. First-strand cDNA was reverse transcribed from 2 μg of total RNA using an 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 all tomato unigenes (Tomato unigene database). Quantitative real-time PCR analyses were performed as previously described (Pirrello *et al.*, 2006). The primer sequences used in this study are listed in Supplemental Table S2.

LC-MS analysis of fruit carotenoids

Carotenoid extractions have been performed as previously described (Fantini *et al.*, 2013). Briefly, 5 mg of ground lyophilized tomato fruit powder were extracted with chloroform (spiked with 50 mg l^{-1} DL-α-tocopherol acetate as internal standard) and methanol (2:1 by volume); subsequently, 1 volume of 50 mM Tris buffer (pH 7.5, containing 1 M NaCl) was added and samples were kept 20 minutes on ice. After centrifugation (15,000 g for 10 minutes at 4°C), the organic hypophase was collected and the aqueous phase was re-extracted with the same amount of spiked chloroform. Combined organic phases were then dried by speedvac and resuspended in 100 μl of ethyl acetate. For each genotype, at least five independent extractions were performed. LC-MS analyses were carried out using a Discovery LTQ-Orbitrap mass spectrometry system (Thermo Fischer Scientific) operating in positive mode-atmospheric pressure chemical ionization (APCI), coupled to an Accela U-HPLC system (Thermo Fischer Scientific, Waltham, MA). LC separations were performed using a C30 reverse-phase column (100 x 3.0 mm) purchased from YMC (YMC Europe GmbH, Schermbeck, Germany). The mobile phases used were methanol (A), water/methanol (20/80 by volume), containing 0.2% ammonium acetate (B), and tert-methyl butyl ether (C). The

gradient was: 95%A:5%B for 1.3 minutes, followed by 80%A:5%B:15%C for 2.0 minutes and by a linear gradient to 30%A:5%B:65%C over 9.2 minutes. UV-Vis detection was performed continuously from 220 to 700 nm with an online Accela Surveyor photodiode array detector (PDA, Thermo Fischer Scientific, Waltham, MA). All solvents used were LC-MS grade quality (CHROMASOLV® from Sigma-Aldrich). Carotenoids were quantified on the basis of the internal standard amounts, obtained by through comparison with peak areas of known amounts of external standard LC-MS runs; data were then normalized on spectrophotometric chlorophyll/carotenoid contents. For APCI-MS ionization of xanthophylls (0-6 minutes of LC-MS run), nitrogen was used as sheath and auxiliary gas which were set to 40 and 20 units, respectively while the vaporizer temperature was 300 °C, the capillary temperature was 250 °C, the discharge current was set to 4.0 µA, the capillary voltage and tube lens settings were 27 V and 90 V, respectively. APCI-MS ionization of carotenes (6-14 minutes of LC-MS runs) was performed with the following parameters: 30 and 10 unites of, respectively, nitrogen sheath and auxiliary gas; 300 °C and 250 °C for, respectively, vaporizer and capillary temperatures, 5.0 µA as discharge current, 0 and 95 V as, respectively, capillary voltage and tube lens settings. Identification was performed as previously reported (Fantini *et al.,* 2013), and on the basis of the m/z accurate masses, as reported on Pubchem database (http://pubchem.ncbi.nlm.nih.gov/) for monoisotopic masses identification, or on Metabolomics Fiehn Lab Mass Spectrometry Adduct Calculator (http://fiehnlab.ucdavis.edu/staff/kind/Metabolomics/MS-Adduct-Calculator/) in case of adduct ion detection.

Color measurement

L, a and b values were measured with a Minolta chromameter (CR-200, 78903131) on fruit at different stages during fruit ripening. The chromameter was calibrated against a standard white tile. Hue angle values were calculated according to the following equation: Hue angle = $\tan^{-1}$ (b/a) if a>0 or 180+ $\tan^{-1}$ (b/a) if a<0.

Fruit firmness

Fifteen fruits from each line were harvested at the breaker stage and the firmness was assessed using Harpenden calipers (British Indicators Ltd) as described by Ecarnot *et al.* (2013).

Ethylene measurements

Fruits at each developmental stage were harvested and placed in open 120-ml jars for 2 h to minimize the effect of wound ethylene caused by picking. Jars were then sealed and incubated at room temperate for 35 min, and 1 ml of headspace gas was injected into an Agilent 7820A gas chromatograph equipped with a flame ionization detector. Samples were compared with reagent grade ethylene standards of known concentration and normalized for fruit weight.

Supplemental data for chapter III

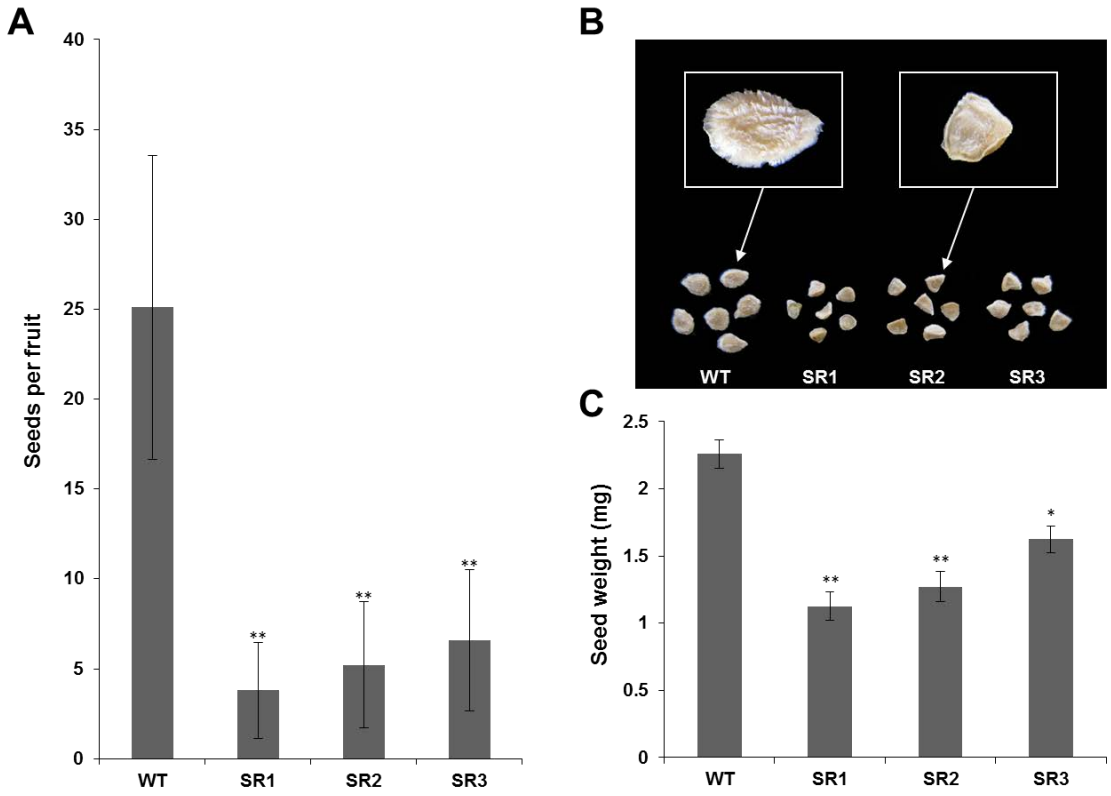

Figure S1. Reduced seed production and altered seed morphology from wild-type and *ERF.B3-SRDX* transgenic lines. A, Significant reduced seed number of *ERF.B3-SRDX* lines. B, The seed morphology is altered relative to the wild type. C, Significantly lower weight of ERF.B3-SRDX seeds compared with wild type. *, $0.01 < P < 0.05$, **, $0.001 < P < 0.01$ (Student's test). *SR1, SR2* and *SR3* are three independent *ERF.B3-SRDX* lines.

Table S1. List of the primers used in the study

Gene Name	Primer Sequence
Sl-Actin	F 5'-TGTCCCTATTTACGAGGGTTATGC-3'
	R 5'-CAGTTAAATCACGACCAGCAAGAT-3'
Sl-ERF.B3	F 5'-CGGAGATAAGAGATCCAAGTCGAA-3'
	R 5'-CTTAAACGCTGCACAATCATAAGC-3'
Sl-ERF.B3-endo	F 5'-TTCACAGAGACATAAACACAAACACCT-3'
	R 5'-TGTTGTCGTATGAGTTCTAATGTTAATCCT-3'
Sl-ERF.B3-SRDX	F 5'-GGAAAATCTGGTGCTCCGG-3'
	R 5'-CTCGTCGACTTAAGCGAAAC-3'
Sl-ERF.A1	F 5'-ACCGGATCCTGTTAGAGTTGGA-3'
	R 5'-CGACGCCGATGAACAATG-3'
Sl-ERF.A2	F 5'-CGGTATCATCAGCTTCGGAAA-3'
	R 5'-TCTCAACTTCTAATTCGGCTTGCT-3'
Sl-ERF.A3	F 5'-GCGAAATGGATCAACAGTTACCA-3'
	R 5'-ATTAGACGACTGAAGCTTGAATTCC-3'
Sl-ERF.B1	F 5'-GAATGATGACGGAATTGTAATGAAGA-3'
	R 5'-TTCCACAATCCCAAATTGAAGA-3'
Sl-ERF.B2	F 5'-AGTTTGCAGCGGAGATTCGT-3'
	R 5'-TGCCCTGTCATATGCCTTTG-3'
Sl-ERF.C1	F 5'-TTCTTCGTGTCGAAAATACTAAGTTCAGT-3'
	R 5'-ACTCTAAATTCTTCAAGAAATCCAGAACA-3'
Sl-ERF.C2	F 5'-ATCATTACCATGGAATGATCAACATT-3'
	R 5'-CCGTCTATAACTTTCTTTCGAGGTTAA-3'
Sl-ERF.C3	F 5'-CAAGAAGTTTCCTCAATCTCTCATGTAT-3'
	R 5'-CCGAGATGAATAATCCATTTGATTT-3'
Sl-ERF.C6	F 5'-GGGAAATACGCTGCGGAAA-3'
	R 5'-TTTCGAACGTACCTAGCCATACTCT-3'
Sl-ERF.D1	F 5'-GGCAGCTGAAATAAGAGATCCATATAA-3'
	R 5'-CTAGCAGCCCCTTCAGCAGTAT-3'
Sl-ERF.D2	F 5'-ACACAAGTAGCACCAGCACCACTA-3'
	R 5'-ACCCCAAAAAAAGCAAGAAAATT-3'
Sl-ERF.D3	F 5'-ATTCATTTTCGGGTTGTGCAGTA-3'
	R 5'-CGACTATAATGATTTCTGCCGAACT-3'
Sl-ERF.D4	F 5'-GTTGCTGCTTTAACCAATGTGATTAT-3'
	R 5'-CTTCCGGTACGCGAAACAAG-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.E3	F 5'-GCATTTGCGATCTGAAGTTGTT-3'
	R 5'-CAAATGGCTTGACATCGACTTG-3'
Sl-ERF.E4	F 5'-AGGCCAAGGAAGAACAAGTACAGA-3'
	R 5'-CCAAGCCAAACGCGTACAC-3'
Sl-ERF.F1	F 5'-ACGAGCTTTCTTCTTTTCTCTCTCTAAA-3'
	R 5'-GAAACTCGATATCCTTCTGTAAAATCTTC-3'
Sl-ERF.F2	F 5'-TTGATACCACTGCTTACCTAGTTTTTCT-3'
	R 5'-TATCTTCTATGGCTCCTTCCTCTTCT-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.G1	F 5'-GAAGAAAGCGATCGATTTGAAGA-3'
	R 5'-TTTTCCCCATGGCCTCTGT-3'
Sl-ERF.G2	F 5'-CGGTGGAGATAAAAGCGAAAAC-3'
	R 5'-CCACTTCGCAGAACCCTAGATT-3'
Sl-ERF.H1	F 5'-AGATGCAGCAAGAGCATATGATG-3'
	R 5'-TTGGGTTGTATGGGAAATTAGTTCT-3'
PSY1	F 5'-GGAAAGCAAACTAATAATGGACGG-3'
	R 5'-CCACATCATAGACCATCTGTTCC-3'
PDS	F 5'-GGTCACAAACCGATACTGCT-3'
	R 5'-AAACCAGTCTCGTACCAATCTC-3'
ZDS	F 5'-AGTGGTTTCTGTCTAAAGGTGG-3'
	R 5'-ACCGAGCACTCATGTTATCAC-3'
β-LCY1	F 5'-GTCCACTTCCAGTATTACCTCAG-3'
	R 5'-TGTCCTTGCCACCATATAACC-3'
Sl-ACS2	F 5'-TGTTAGCGTATGTATTGACAACTGG-3'
	R 5'-TCATAACATAACTTCACTTTTGCATTC-3'
Sl-ACS4	F 5'-CTCCTCAAATGGGGAGTACG-3'
	R 5'-TTTTGTTTGCTCGCACTACG-3'
Sl-ACO1	F 5'-GCCAAAGAGCCAAGATTTGA-3'
	R 5'-TTTTTAATTGAATTGGGATCTAAGC-3'

E4	F 5'-GACCACTCTAAATCGCCAGG-3'
	R 5'-TTCCTGAGCGGTATTGCTTT-3'
E8	F 5'-TGGCTCCGAATCCTCCCAGTCT-3'
	R 5'-GTCCGCCTCTGCCACTGAGC-3'
PG2a	F 5'-TCAAGGGCACAAGTGCAACAAAGG-3'
	R 5'-TGCACGTAGCCTCTGATGGTTT-3'
RIN	F 5'-ATGCAGCACCATCAACACAT-3'
	R 5'-CTCCAAATTCAAAGCATCCA-3'
CNR	F 5'-GCCAAATCAAGCAATGATGA-3'
	R 5'-TCGCAACCATACAGACCATT-3'
NOR	F 5'-AGAGAACGATGCATGGAGGTTTGT-3'
	R 5'-ACTGGCTCAGGAAATTGGCAATGG-3'
HB-1	F 5'-CAATCGGAGGAAGATGATGG-3'
	R 5'-TGTTCATGGTGCTGCTCTTC-3'
TAGL1	F 5'-ACTTTCTGTTCTTTGTGATGCT-3'
	R 5'-TTGGATGCTTCTTGCTGGTAG-3'
AP2a	F 5'-AACGGACCACAATCTTGAC-3'
	R 5'-CTGCTCGGAGTCTGAACC-3'

Chapter IV

General conclusions and perspectives

The gaseous phytohormone ethylene plays a critical role in a wide range of developmental processes, including germination, flower and leaf senescence, fruit ripening, leaf abscission, root nodulation, programmed cell death, and responses to biotic and abiotic stresses. After its synthesis, the perception and signaling of ethylene rely on the cooperative action of several components among which Ethylene Response Factors (ERFs) play a poorly understood role. However, being encoded by one of the largest family of plant transcription factors, ERF proteins are the most suited step of ethylene signaling where the diversity and specificity of ethylene responses may originate. My Ph.D project mainly dealt with the functional characterization of ERF genes during plant growth and fruit development using tomato as the plant model. More particularly, my work aimed to decipher the role of *Sl-ERF.B.3*, a member of ERF family gene, in mediating ethylene response and tomato plant development and fruit ripening using advanced reverse genetics and genomics methodologies.

Sl-ERF.B3 was previously shown to act as strong transcriptional activator on GCC-box-containing promoters and its transcripts accumulate upon ethylene treatment, suggesting a putative involvement in ethylene-regulated processes. To address the physiological significance of *Sl-ERF.B3* and its potential role in mediating ethylene responses, tomato lines under- and over-expressing *Sl-ERF.B3* gene were generated by stably transforming tomato plants with either sense or antisense constructs under the control of the constitutive 35S promoter. Since classical down- and up-regulation approaches failed to provide clear clues on *Sl-ERF.B3* functional significance in tomato, we generated a dominant repressor version of *Sl-ERF.B3* (*ERF.B3-SRDX*) using the Chimeric Repressor Silencing Technology (CRES-T) to overcome the functional redundancy among ERF family members. The capacity of ERF.B3-SRDX retaining to bind the same target genes than Sl-ERF.B3 protein and to dominantly repress its transcriptional activity was confirmed by a transient transformation assay. This indicates the usefulness of this strategy in functional studies by overcoming redundancy among members of a multigene family. Using the dominant repression version of Sl-ERF.B3 (ERF.B3-SRDX), the involvement of Sl-ERF.B3 in ethylene response and a wide range of development processes was demonstrated. Expression of a dominant repressor *ERF.B3-SRDX* version of *Sl-ERF.B3* in the tomato resulted in pleiotropic ethylene responses and vegetative and

reproductive growth phenotypes. The dominant repressor etiolated seedlings displayed partial constitutive ethylene-response in the absence of ethylene and adult plants exhibited typical ethylene-related alterations such as leaf epinasty, premature flower senescence and accelerated fruit abscission. The multiple symptoms related to enhanced ethylene sensitivity correlate with the altered expression of ethylene biosynthesis and signaling genes, suggesting the involvement of *Sl-ERF.B3* in a feedback mechanism regulating components of ethylene production and response. Moreover, Sl-ERF.B3 is shown to modulate the transcription of a set of *ERFs* revealing the existence of a complex network in which multiple transcription factors are competing for promoters to control the expression of genes that are essential for a wide range of plant responses to ethylene. Overall, Sl-ERF.B3 is shown to modulate ethylene responses at four different levels: (i) ethylene biosynthesis, (ii) ethylene receptor, (iii) primary ethylene transcription factors (*EIL* genes), and (iv) downstream *ERF* genes.

Expression dynamics of most *ERF* genes also suggest their involvement in the ripening process, although no link was established between their repressor or activator function and their pattern of expression. The transcript accumulation of *Sl-ERF.B3* is induced at the breaker stage and maintained a high level all stages along the ripening process, suggesting that its expression might be continuously required for the modulation of the ripening-regulated genes all along the ripening process. Indeed, over-expression of *Sl-ERF.B3* as a chimeric repressor (*ERF.B3-SRDX*) in tomato results in alterations in both fruit morphology and ripening process. Transgenic lines produce significantly smaller fruit with heart shape and raised bumpy areas present intermittently on the epidermal surface. In addition to the altered fruit morphology, *ERF.B3-SRDX* lines exhibited a distinct ripening process. The attainment of competence to ripen is dramatically delayed in the transgenic lines, however, once the ripening process starts at breaker stage, when a visible color change just begins to occur, the softening of *ERF.B3-SRDX* fruits is actually much faster than wild type. These results indicate the involvement of Sl-ERF.B3 in fruit development and ripening. Although after the breaker stage, the *ERF.B3-SRDX* fruit soften faster with a significantly higher ethylene production, these transgenic fruits fail to display a red-ripe phenotype with a shift of carotenoid accumulation from lycopene to β-carotene at the ripening stages. Moreover, genes involved in different metabolic

pathways, such as carotenoid biosynthesis pathway, ethylene synthesis, and cell wall metabolism exhibit altered mRNA accumulation patterns in transgenic lines during fruit ripening process, indicating that *Sl-ERF.B3* impacts fruit ripening through mediating fruit ripening-associated genes.

In this study, the ectopic expression of a dominant repressor form of the Sl-ERF.B3 protein provided a mean towards altering the activity of the native Sl-ERF.B3 protein. This **C**himeric **R**epressor **S**ilencing **T**echnology (CRES-T) strategy allowed revealing vegetative and reproductive growth phenotypes that could not be uncovered by the expression of neither sense nor antisense constructs of *Sl-ERF.B3*. The eventuality that the pleiotropic phenotypes displayed by the *ERF.B3-SRDX* dominant suppressor plants may arise from a co-suppression of the endogenous *Sl-ERF.B3* is ruled out since the levels of *Sl-ERF.B3* transcripts are not altered in the transgenic lines. Therefore, the *ERF.B3-SRDX* tomato lines proved to be a valuable tool to uncover at least some of the processes controlled by Sl-ERF.B3 and to reveal roles for ERF genes that have not been described previously. Moreover, since the study has been carried out with Micro-Tom, a dwarf genotype, it is important to mention that the dwarfing mutations in this genotype do not seem to impact the phenotype displayed by *ERF.B3-SRDX* plants since the dwarf phenotype is well reproduced in Ailsa Craig tomato, a non-dwarf variety.

Sl-ERF.B3 is positively regulated by both ethylene and auxin. This suggests that *Sl-ERF.B3* could a suitable candidate gene to analyze the cross-talk between ethylene and auxin during plant growth and fruit development. Indeed, *ERF.B3-SRDX* transgenic lines show several auxin-related phenotypes, such as altered auxin sensitivity with modified root development and reduced auxin responsiveness. These results indicate that *Sl-ERF.B3* may act as a regulator at the crossroads between ethylene and auxin signaling. Moreover, exogenous application of GA partially rescued the dwarf phenotype of the transgenic lines together with the significantly decreased transcript levels of GA biosynthesis genes (*Sl-GA20ox1* and *Sl-GA20ox2*) also indicates a crosstalk between ethylene and GA. Future work will study the putative role of Sl-ERF.B3 in the crosstalk network between ethylene and other hormones.

The tomato *Sl-ERF.B3* is related to *Arabidopsis* factors *ERF106* and *ERF107*, which are members of group IX according to Nakano *et al.,* (2006). This group has been implicated

in the regulation of defense responses and knock-out analysis of *ORA59* (Pré *et al.,* 2008) and *AtERF14* (Oñate-Sánchez *et al.*, 2007), prominent representatives of group IX, has revealed disease susceptibility phenotypes. Consistently, overexpression of *ERF1,* another member of the group, has led to enhanced resistance to necrotrophic pathogens (Berrocal-Lobo *et al.*, 2002). As one member of this group, Sl-ERF.B3 may also play an important role in plant response to various stresses. In the future, we will focus on the investigation of the specific role of Sl-ERF.B3 in plant immunity.

ERFs act as the last known downstream components of ethylene signal pathway, the investigation of their roles in ethylene-dependent processes is important for better understanding the distinct regulation mechanisms of the ethylene responses. The demonstration of the specific role of *Sl-ERF.B3*, a member of ERF gene family in tomato, in ethylene-mediated developmental processes such as plant growth and fruit ripening provided a clue of the functional significance of ERF genes in ethylene response processes. The data showing that most of *ERF* genes display altered expression patterns in the *ERF.B3-SRDX* dominant repressor lines which resulted in pleiotropic ethylene responses and vegetative and reproductive growth phenotypes further, stress the importance of *ERF* genes in a wide range of ethylene-dependent developmental processes. To further decipher the function of *ERF* genes in both ethylene-dependent and ethylene-independent processes, it is important to continue to generate mutant lines altered in the expression of these genes. For better understanding of the mechanisms by which these transcription factors control plant developmental processes, it is crucial to identify the target genes of ERFs. This can be achieved by comparative transcriptomic profiling of the lines altered in specific ERFs or by a ChIP-seq (Chromatin Immunoprecipitation coupled to deep sequencing) approach. Both strategies are being carried out within the GBF lab.

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