HAL
open science

A dominant repressor version of the tomatoSl-ERF.B3gene confers ethylene hypersensitivity via feedback regulation of ethylene signaling and response components

Mingchun Liu, Julien Pirrello, Ravi Kesari, Isabelle Mila, Jean-Paul Roustan, Zhengguo Li, Alain Latché, Jean-Claude Pech, Mondher Bouzayen, Farid Regad

To cite this version:

Mingchun Liu, Julien Pirrello, Ravi Kesari, Isabelle Mila, Jean-Paul Roustan, et al.. A dominant repressor version of the tomatoSl-ERF.B3gene confers ethylene hypersensitivity via feedback regulation of ethylene signaling and response components. The Plant Journal, 2013, Vol. 73 (3), pp. 406-419. 10.1111/tpj.12305 . hal-00906407

HAL Id: hal-00906407

https://hal.science/hal-00906407v1

Submitted on 19 Nov 2013

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Open Archive TOULOUSE Archive Ouverte (OATAO)
OATAO is an open access repository that collects the work of Toulouse researchers and makes it freely available over the web where possible.

This is an author-deposited version published in : http://oatao.univ-toulouse.fr/
Eprints ID : 9358

To link to this article : DOI : 10.1111/tpj.12305
URL : http://dx.doi.org/10.1111/tpj.12305

To cite this version : Liu, Mingchun and Pirrello, Julien and Kesari, Ravi and Mila, Isabelle and Roustan, Jean-Paul and Li, Zhengguo and Latché, Alain and Pech, Jean-Claude and Bouzayen, Mondher and Regad, Farid *A dominant repressor version of the tomatoSl-ERF.B3gene confers ethylene hypersensitivity via feedback regulation of ethylene signaling and response components*. (2013) The Plant Journal, Vol. 73 (n° 3). pp. 406-419. ISSN 0960-7412

Any correspondance concerning this service should be sent to the repository administrator: staff-oatao@listes-diff.inp-toulouse.fr

doi: 10.1111/tpj.12305

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

Mingchun Liu[1,2,†], Julien Pirrello[1,2,†], Ravi Kesari[1,2], Isabelle Mila[1,2], Jean-Paul Roustan[1,2], Zhengguo Li[3], Alain Latché[1,2], Jean-Claude Pech[1,2], Mondher Bouzayen[1,2,*] and Farid Regad[1,2]

[1]*Université de Toulouse, INP-ENSA Toulouse, Génomique et Biotechnologie des Fruits, Avenue de l'Agrobiopole BP 32607, Castanet-Tolosan F-31326, France,*

[2]*INRA, Génomique et Biotechnologie des Fruits, Chemin de Borde Rouge, Castanet-Tolosan F-31326, France, and*

[3]*School of Life Sciences, Chongqing University, Chongqing 400044, China*

*For correspondence (e-mail bouzayen@ensat.fr).

[†]These authors contributed equally to this work.

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. As the ethylene-inducible tomato *Sl-ERF.B3* has been shown previously to display a strong binding affinity to GCC-box-containing promoters, its physiological significance was addressed here by a reverse genetics approach. However, classical up- and down-regulation strategies failed to give clear clues to its roles *in planta*, probably due 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 correlated with the altered expression of ethylene biosynthesis and signaling genes and suggested the involvement of *Sl-ERF.B3* in a feedback mechanism that regulates components of ethylene production and response. Moreover, Sl-ERF.B3 was shown to modulate the transcription of a set of *ERF*s and revealed the existence of a complex network interconnecting different *ERF* genes. Overall, the study indicated that Sl-ERF.B3 had a critical role in the regulation of multiple genes and identified a number of *ERF*s among its primary targets, consistent with the pleiotropic phenotypes displayed by the dominant repression lines.

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

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 the 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 release of 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. The 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, over-expression 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).

Ethylene Response Factors are plant specific transcription factors and belong 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 the AP2 domain made of 58 or 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 (van der Fits and Memelink, 2000; Fujimoto *et al.*, 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 has also been shown that Pti4, an ERF type transcription factor, regulates gene expression by interacting directly 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.

Ethylene Response Factors 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 information has been reported (McGrath *et al.*, 2005) on ethylene-responsive phenotypes caused by silencing, mutation, or knock-out 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. Chimeric Repressor Silencing Technology (CRES-T), consisting in the expression of a dominant repressor version of a transcription factor encoding gene proved to be an efficient means 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 that express the chimeric repressor version exhibit phenotypes similar to loss-of-function of the alleles of the gene that encodes the transcription factor (Hiratsu *et al.*, 2003; Heyl *et al.*, 2008; Matsui and Ohme-Takagi, 2010).

Genome-wide study recently showed that the tomato *ERF* gene family comprises nine subclasses defined by distinct structural features and a new nomenclature for tomato ERFs was proposed (Pirrello *et al.*, 2012) that 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 a few ERF genes have been characterized functionally so far, most of these genes 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, over-expression of *ERF1*, another member of the group, has led to enhanced resistance to necrotrophic pathogens (Berrocal-Lobo *et al.*, 2002).

Sl-ERF.B3 has been shown previously 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 over-expressing and down-regulated lines failed to reveal the functional significance of *Sl-ERF.B3*, a dominant chimeric repressor version was used that resulted in phenotypes that were consistent with Sl-ERF.B3 involvement 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 *ERF*s among the target genes regulated by Sl-ERF.B3, and supported the idea that the alteration of such a high number of *ERF*s 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. Several homozygous transgenic lines that corresponded 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 probably due 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). Trans-activation 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*.

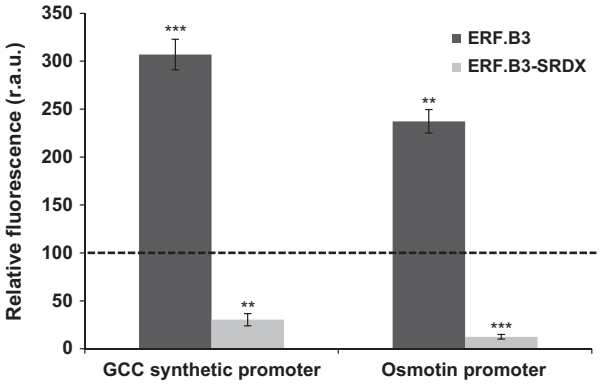

Figure 1. Transactivation assay in a single cell system.
Protoplasts were co-transfected with a reporter construct consisting of the *GFP* gene driven by a GCC-rich synthetic promoter or a native osmotin GCC-containing promoter and an effector plasmid expressing either ERF.B3 or ERF.B3-SRDX protein. 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 ± standard deviation (SD) of three independent biological replicates. $^{**}0.001 < P < 0.01$, $^{***}P < 0.001$ (Student's *t*-test).

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*) that expressed 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*, that showed 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 that were specific for *ERF.B3-SRDX* (Figure S2). Accumulation of the endogenous *Sl-ERF.B3* assessed by qRT-PCR was similar in the transformed and non-transformed plants and ruled 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 with wild-type (WT; Figure 2b). Interestingly, application of 1-MCP, the ethylene perception inhibitor, reversed the triple-response phenotype of *ERF.B3-SRDX* dominant repressor

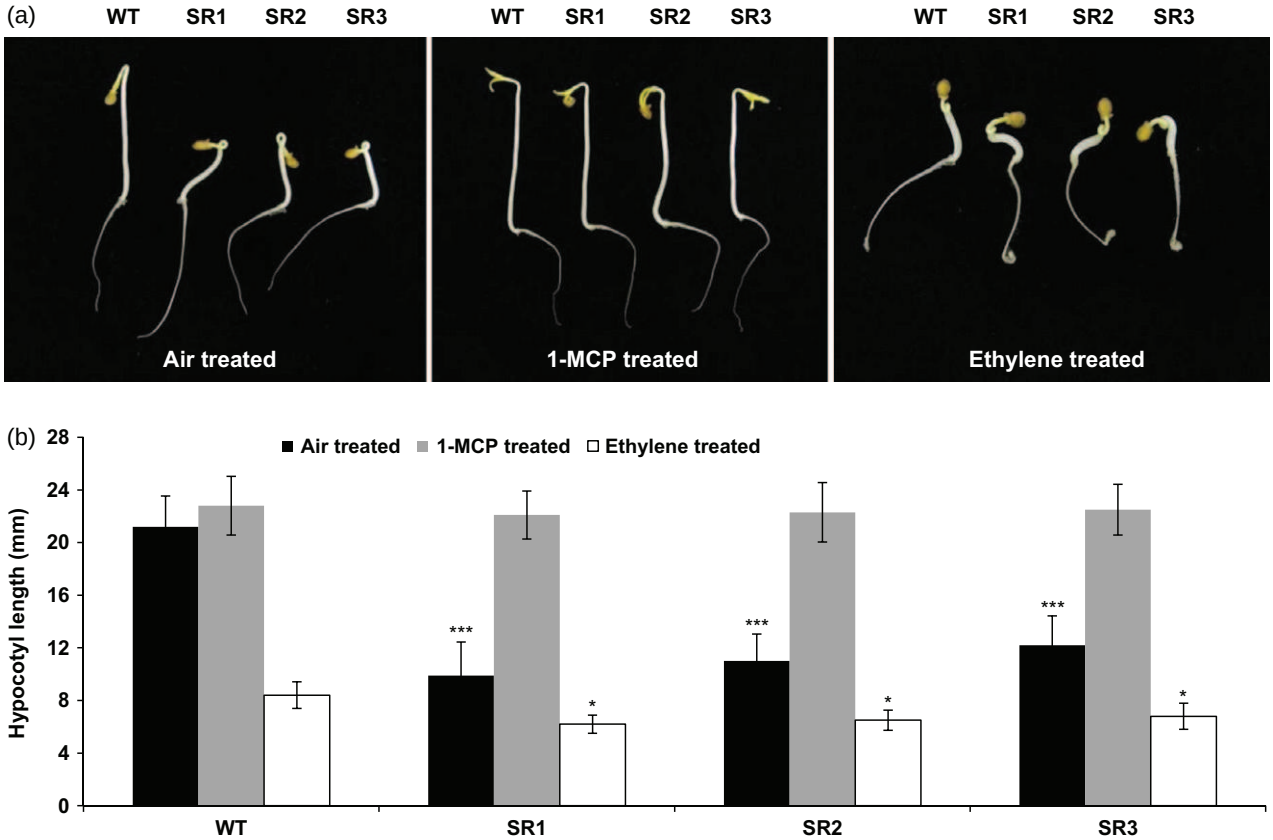

Figure 2. Ethylene hypersensitivity of *35S:ERF.B3-SRDX* lines.
(a) Etiolated *35S:ERF.B3-SRDX* seedlings display partial constitutive ethylene response in the absence of exogenous ethylene that can be removed by 1-MCP application (1.0 mg L^{-1}) or exaggerated upon exogenous ethylene (10 $\mu l\ l^{-1}$) treatment.
(b) Hypocotyl elongation in *35S:ERF.B3-SRDX* etiolated seedlings and wild-type (WT) treated or untreated with ethylene and 1-MCP. Values are means ± standard deviation (SD) ($n \geq 30$) of three replicates. *$0.01 < P < 0.05$, ***$P < 0.001$ (Student's *t*-test). *SR1*, *SR2* and *SR3* are three independent *35S:ERF.B3-SRDX* lines.

lines (Figure 2a) and led to a complete loss of the exaggerated apical hook and recovery of hypocotyl length similar to that of WT (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 WT (Figure 2a,b), suggesting a higher sensitivity to the hormone for the transgenic lines.

Because *Sl-ERF.B3* over-expressing plants displayed some, although 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 WT 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 the WT, 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 were 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). Most 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 with WT fruit (Figure 3). Approximately 2 weeks after the breaker stage, the fruit abscission zone starts to dehisce in the *ERF.B3-SRDX* lines, whereas this event occurs at later stages in wild-type lines (Figure 3). Collectively, these ethylene-related phenotypes are consistent with ethylene hypersensitivity of the *ERF.B3-SRDX* dominant repressor lines.

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 the average

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.

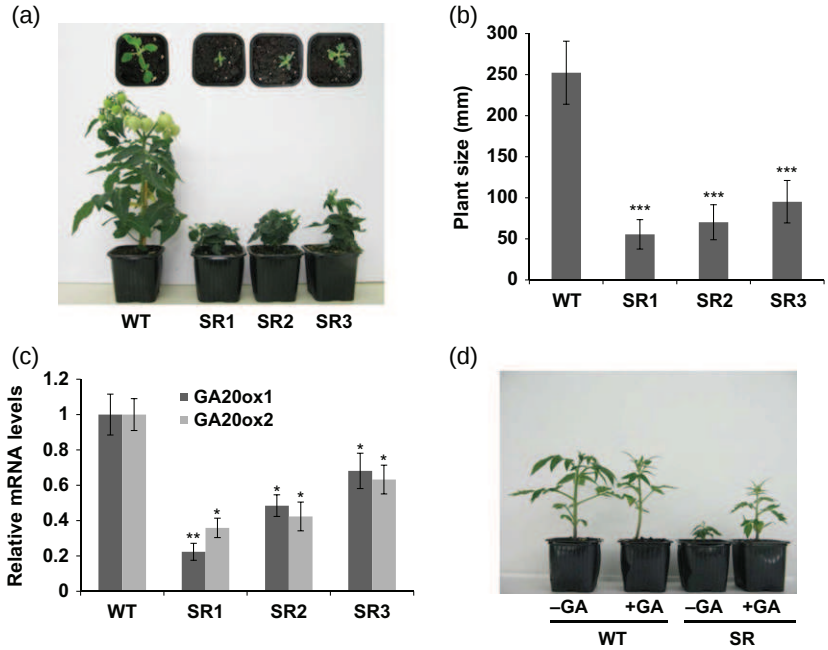

Figure 4. Dwarf phenotype of *35S:ERF.B3-SRDX* plants.

(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 ± standard deviation (SD) ($n \geq 15$) 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 3 weeks.

*$0.01 < P < 0.05$, **$0.001 < P < 0.01$, ***$P < 0.001$ (Student's *t*-test). *SR1*, *SR2* and *SR3* are three independent *35S:ERF.B3-SRDX* lines.

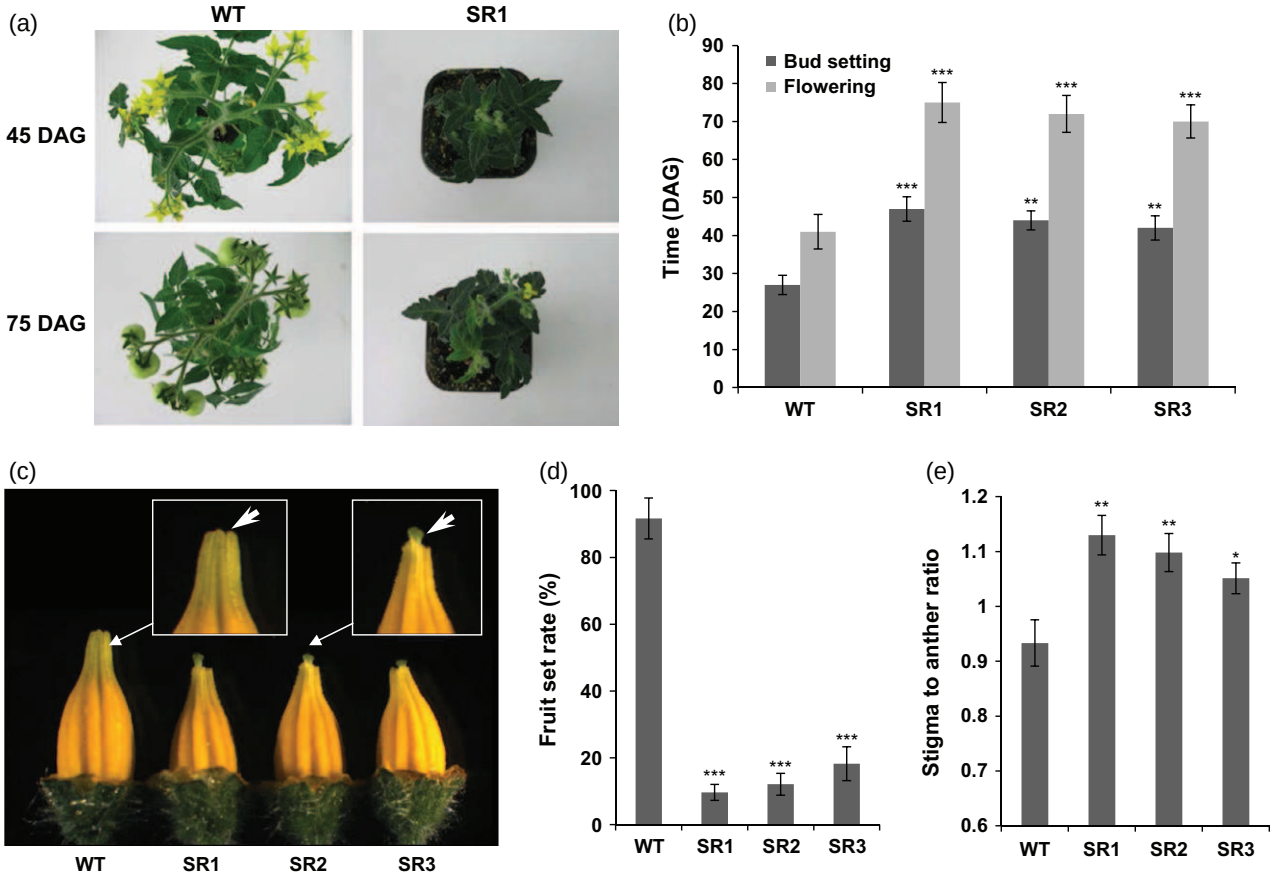

Figure 5. Delayed reproductive development and reduced fruit set in *35S:ERF.B3-SRDX* plants.
(a) Late flower bud setting and flowering time in *ERF.B3-SRDX* plants compared with wild type (WT). DAG, day after germination.
(b) Assessing the time of flower bud setting and flower opening in *ERF.B3-SRDX* 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 with WT.
Values are means ± standard deviation (SD) ($n \geq 30$) of three replicates.
*$0.01 < P < 0.05$, **$0.001 < P < 0.01$, ***$P < 0.001$ (Student's *t*-test). *SR1*, *SR2* and *SR3* are three independent *35S:ERF.B3-SRDX* lines.

height being less than one-third of that of WT plants after 80 days (Figure 4b). Noteworthy, the transcript level of two gibberellic acid (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); the strongest *ERF.B3-SRDX* expressing line showed epidermal cell size less than one-third of that in wild-type (Figure S4d).

ERF.B3-SRDX plants also showed severely delayed reproductive growth (Figure 5a). The time from germination to flower bud setting was delayed by 14–20 days in transgenic lines compared with the reference WT lines (Figure 5b). Likewise, flower anthesis in *ERF.B3-SRDX* plants occurred 29–34 days later than in WT (Figure 5b). Moreover, compared with WT, 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

Table 1 Cross-fertilization assay

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

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*).

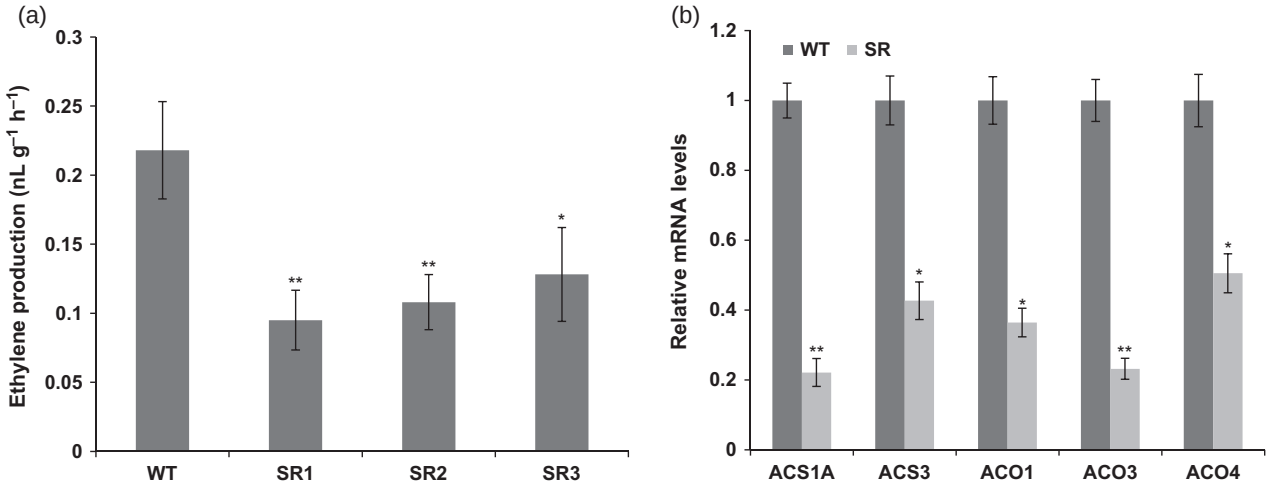

Figure 6. Down-regulation of ethylene production and ethylene biosynthesis genes in *35S:ERF.B3-SRDX* plants.
(a) Ethylene production of etiolated seedlings in wild-type (WT) and *ERF.B3-SRDX* lines.
(b) *ACS* and *ACO* transcript accumulation in WT and *ERF.B3-SRDX* plants assessed by qRT-PCR. The relative 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 ± standard deviation (SD) of three replicates.
*$0.01 < P < 0.05$, **$0.001 < P < 0.01$ (Student's *t*-test). *SR1*, *SR2* and *SR3* are three independent *35S:ERF.B3-SRDX* lines.

achieved in WT, 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 WT 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), a finding that indicated that *ERF.B3-SRDX* pollen is viable and fertile. Using WT 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 WT 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.

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 accounted 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 (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) 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).

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 with wild-type (Figure 7a). An *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 with *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.*, 2007), 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 a 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).

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 2-fold increase in transcript accumulation for all four *Sl-EIL* genes in the *ERF.B3-SRDX* lines (Figure 8). However, none of the *EIN3-like* genes gathers 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-EIL*s promoters (Figure S6), suggesting that *Sl-EIL*s do not serve as direct target genes for Sl-ERF.B3.

*Sl-ERF*s 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*

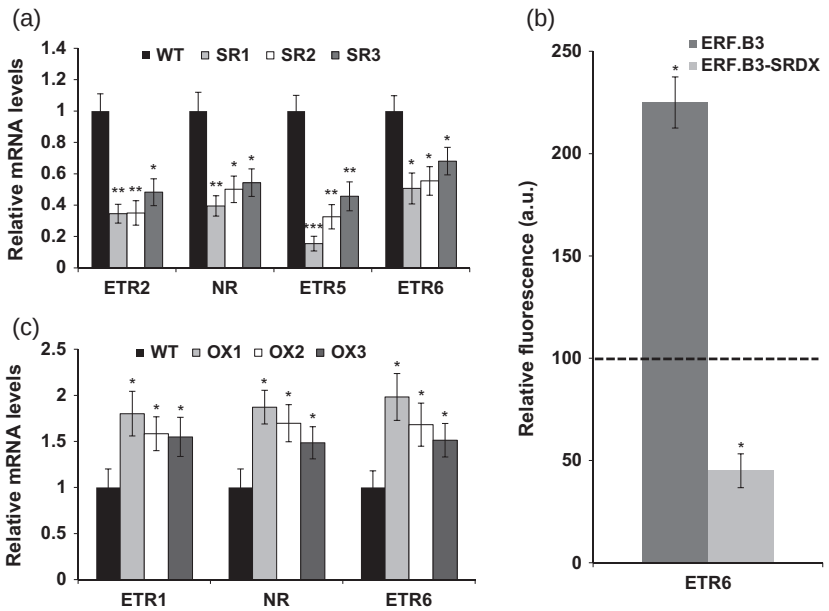

Figure 7. Expression of ethylene receptor genes in *35:ERF.B3-SRDX* and *ERF.B3* over-expression lines.
(a) Relative mRNA levels of *ETR2, NR, ETR5* and *ETR6* receptor genes assessed by qRT-PCR in 4-week-old wild-type (WT) and *ERF.B3-SRDX* lines.
(b) The transcriptional activity of *ETR6* promoter 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* over-expression lines.
*$0.01 < P < 0.05$, **$0.001 < P < 0.01$, ***$P < 0.001$ (Student's *t*-test). *SR1*, *SR2* and *SR3* are three independent *ERF.B3-SRDX* lines.

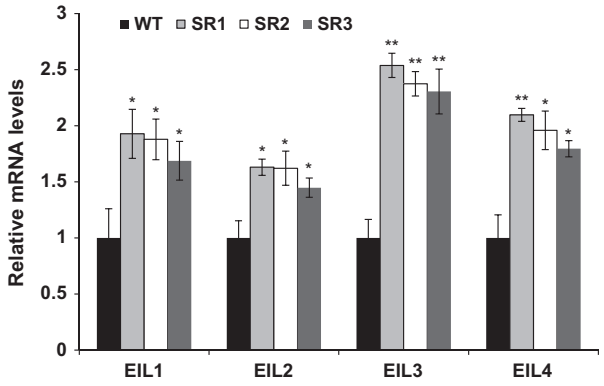

Figure 8. Expression of *EIN3*-like genes in *35S:ERF.B3-SRDX* lines.
Relative mRNA levels of *Sl-EIL1*, *Sl-EIL2*, *Sl-EIL3*, *Sl-EIL4* in wild-type (WT) and *ERF.B3-SRDX* lines assessed by qRT-PCR in 4-week-old plants. The relative mRNA level of each gene in WT was standardized to 1.0, referring to the internal control of *Sl-Actin*. Values are means ± standard deviation (SD) of three replicates. *0.01 < P < 0.05, **0.001 < P < 0.01 (Student's *t*-test). *SR1*, *SR2* and *SR3* are three independent *35S:ERF.B3-SRDX* lines.

genes in both WT 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-ERF*s displayed similar expression in transgenic and WT 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 *ERF*s 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

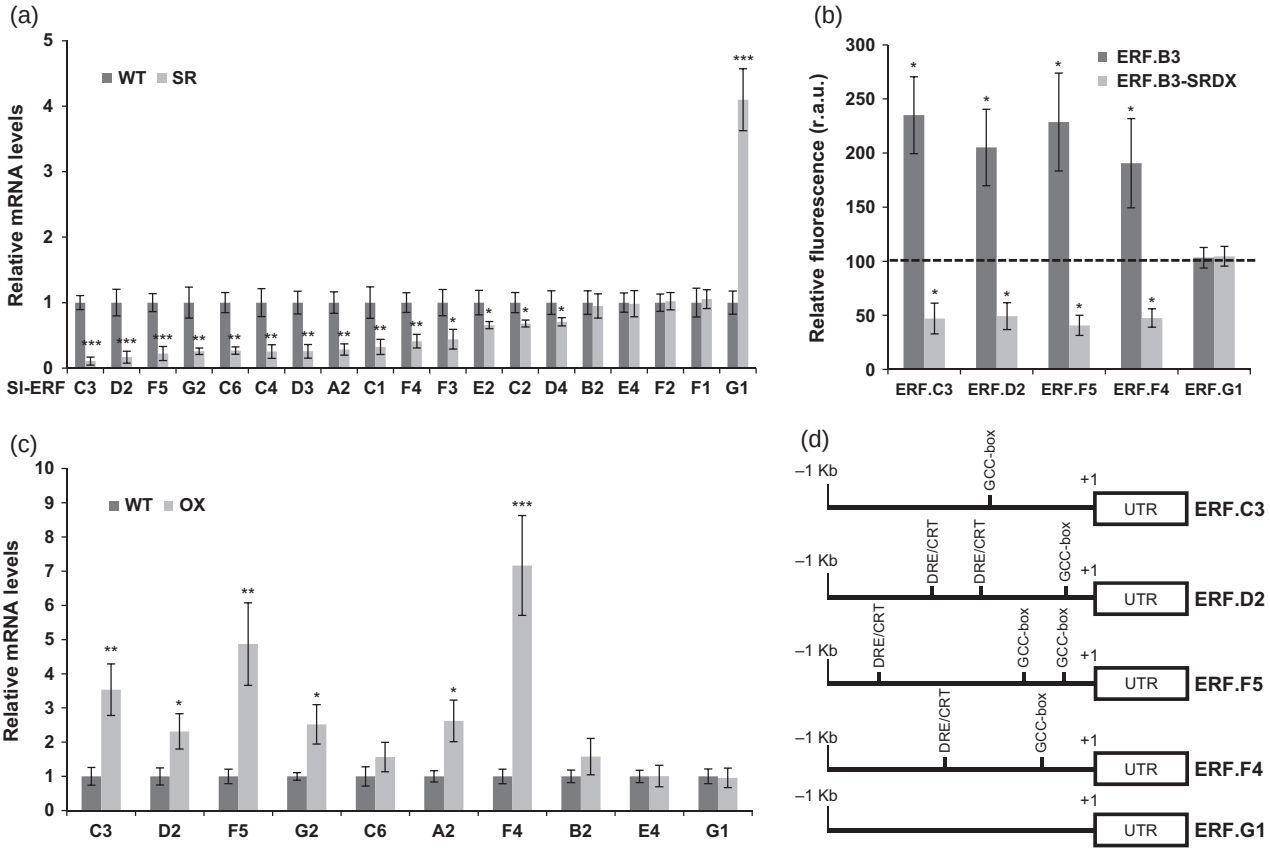

Figure 9. *Sl-ERF*s are among the target genes of ERF.B3 and ERF.B3-SRDX.
(a) Accumulation of *Sl-ERF*s transcripts in wild-type (WT) and *ERF.B3-SRDX* lines assessed by qRT-PCR in 4-week-old plants. The relative mRNA level of each gene in WT was standardized to 1.0, referring to *Sl-Actin* as internal control.
(b) Trans-activation of *Sl-ERF* promoters by ERF.B3 and ERF.B3-SRDX. Protoplasts were co-transfected with GFP reporter fused to the promoters of *Sl-ERF*s (*ERF.C3*, *ERF.D2*, *ERF.F4*, *ERF.F5* and *ERF.G1*) and an effector plasmid expressing ERF.B3 or ERF.B3-SRDX.
(c) *Sl-ERF*s transcript levels in *ERF.B3* over-expression 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-ERF*s genes. The *cis*-acting elements identified are represented by black bars.
Values are means ± standard deviation (SD) of three replicates *0.01 < P < 0.05, **0.001 < P < 0.01, ***P < 0.001 (Student's *t*-test).

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 that displayed 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).

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 probably due 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 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, probably 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 over-production. 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 with 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. This hypothesis is further supported by the reversion of the ethylene-response phenotype upon treatment with 1-MCP, a potent inhibitor of ethylene receptors. That is, endogenous ethylene levels inactivate the residual amounts of the receptors; blocking ethylene receptors by 1-MCP reverts the phenotype. 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 that results in increased sensitivity to the hormone (Hua and Meyerowitz, 1998; Kevany and Klee, 2007; Wuriyanghan *et al.*, 2009). 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) – a finding that indicates that 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 and suggests 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-EIL*s 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). As 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 as transactivation assays indicated that Sl-ERF.B3 and ERF.B3-SRDX proteins are unable to modulate transcription driven by any of the four *Sl-EIL*s 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 a 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, a finding that suggested intense inter-regulation among *ERF* family members. Consistent with the dominant repressor function of the ERF.B3-SRDX protein, most *ERF* genes were down-regulated while *Sl-ERF.G1* alone 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, displayed enhanced expression in the *Sl-ERF.B3* sense lines. While these data support the idea that these *ERF*s 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 probably requires an additional mediating factor. An *in silico* search revealed that all *ERF* genes down-regulated in the transgenic lines harbor *cis*-acting elements that are known to be putative binding targets for ERFs. The down-regulation of such a high number of *Sl-ERF*s supports a model that implies that a single ERF can affect 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). 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 idea 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 idea, 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, as the dwarf phenotype is well reproduced in Ailsa Craig tomato, a non-dwarf variety. 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.

As 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 regulation caused by ERF.B3-SRDX are off-target effects due to interference with other related

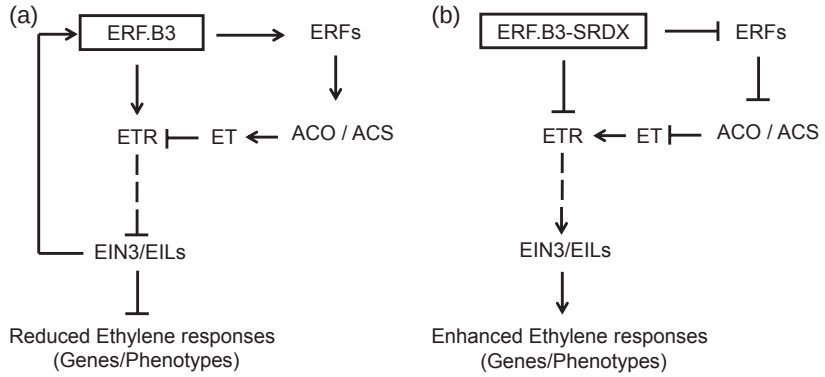

Figure 10. Tentative model proposing the involvement of Sl-ERF.B3 in the control of ethylene responses.
Sl-ERF.B3 modulates ethylene responses at different levels including ethylene biosynthesis (ACO/ACS), receptors, and *ERF* genes. ERF.B3-mediated ethylene response occurs partly via direct transcriptional 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 *ERF*s 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.

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 that function in an inter-connected manner. Such level of complexity matches the high level of plasticity needed for the implementation of plant growth and developmental processes that require continuous fine-tuning through the integration of different cues and signaling pathways.

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} sec^{-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 *Sma*I site of p35SSRDXG in frame with 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 in accordance with 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 in accordance with (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-ERF*s (*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, San Jose, CA, USA, http://www.bdbiosciences.com/eu/index.jsp) 16 h following protoplast transfection. For each sample, 100–1000 protoplasts were gated on forward light scatter; GFP fluorescence per population of cells corresponds to the average fluorescence intensity of the population of cells above the background. The data were analyzed using CELL QUEST software (BD Biosciences) 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, Carlsbad, CA, USA, http://www.lifetechnologies.com/fr/fr/home.html). 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, Valencia, CA, USA, http://www.qiagen.com/). Gene-specific primers were designed by PRIMER EXPRESS software (PE-Applied Biosystems, Foster City, CA, USA, http://www.appliedbiosystems.com/absite/us/en/home.html) 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 on the plants 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 $\mu l\ l^{-1}$) was then injected into the Mason jars using a syringe. For 1-MCP treatment, 1 $\mu l\ l^{-1}$ was applied into the Mason jars and kept in the dark for 1 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, Santa Clara, CA, USA, http://www.chem.agilent.com/en-US/products-services/Instruments-Systems/Gas-Chromatography/7820A-GC/Pages/default.aspx). Ethylene was identified via co-migration with an ethylene standard and quantified with reference to a standard curve for ethylene concentration.

ACCESSION NUMBERS

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

ACKNOWLEDGEMENTS

This work forms part of the requirement for the degree of Ph.D. for M.C Liu supported by the China Scholarship Council. The authors are grateful to L. Lemonnier, D. Saint-Martin, O. Berseille and L. Gress for genetic transformation, culture and phenotyping of tomato plants. This research was supported by funds from the 'Laboratoire d'Excellence' (LABEX) entitled TULIP (ANR-10-LABX-41) and benefited from the networking activities within the European COST Action FA1106.

SUPPORTING INFORMATION

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

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

Figure S2. Transcript accumulation of the chimeric *ERF.B3-SRDX* and the endogenous *ERF.B3* genes in transgenic lines.

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

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

Figure S5. Phenotypes of reproductive organs.

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

Table S1. Putative ERF binding *cis*-elements present in the promoter regions of ethylene receptors and ethylene biosynthesis genes.

Table S2. Putative *cis*-acting ethylene-response elements present in the promoter regions of *Sl-ERF* genes.

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

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

REFERENCES

Achard, P., Baghour, M., Chapple, A., Hedden, P., Van Der Straeten, D., Genschik, P., Moritz, T. and Harberd, N.P. (2007) The plant stress hormone ethylene controls floral transition via DELLA-dependent regulation of floral meristem-identity genes. *Proc. Natl Acad. Sci. USA*, **104**, 6484–6489.

Allen, M.D., Yamasaki, K., Ohme-Takagi, M., Tateno, M. and Suzuki, M. (1998) A novel mode of DNA recognition by a beta-sheet revealed by the solution structure of the GCC-box binding domain in complex with DNA. *EMBO J.* **17**, 5484–5496.

Alonso, J.M., Stepanova, A.N., Leisse, T.J. *et al.* (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science*, **301**, 653–657.

Benavente, L.M. and Alonso, J.M. (2006) Molecular mechanisms of ethylene signaling in Arabidopsis. *Mol. BioSyst.* **2**, 165–173.

Berrocal-Lobo, M., Molina, A. and Solano, R. (2002) Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in Arabidopsis confers resistance to several necrotrophic fungi. *Plant J.* **29**, 23–32.

Bleecker, A.B. and Kende, H. (2000) Ethylene: a gaseous signal molecule in plants. *Annu. Rev. Cell Dev. Biol.* **16**, 1–18.

Brown, R.L., Kazan, K., McGrath, K.C., Maclean, D.J. and Manners, J.M. (2003) A role for the GCC-box in jasmonate-mediated activation of the PDF1.2 gene of Arabidopsis. *Plant Physiol.* **132**, 1020–1032.

Chakravarthy, S., Tuori, R.P., D'Ascenzo, M.D., Fobert, P.R., Despres, C. and Martin, G.B. (2003) The tomato transcription factor Pti4 regulates defense-related gene expression via GCC box and non-GCC box cis elements. *Plant Cell*, **15**, 3033–3050.

Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W. and Ecker, J.R. (1997) Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. *Cell*, **89**, 1133–1144.

Dubois, M., Skirycz, A., Claeys, H. *et al.* (2013) Ethylene Response Factor 6 acts as a central regulator of leaf growth under water-limiting conditions in Arabidopsis. *Plant Physiol.* **162**, 319–332.

van der Fits, L. and Memelink, J. (2000) ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. *Science*, **289**, 295–297.

Fujimoto, S.Y., Ohta, M., Usui, A., Shinshi, H. and Ohme-Takagi, M. (2000) Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. *Plant Cell*, **12**, 393–404.

Gu, Y.Q., Yang, C., Thara, V.K., Zhou, J. and Martin, G.B. (2000) Pti4 is induced by ethylene and salicylic acid, and its product is phosphorylated by the Pto kinase. *Plant Cell*, **12**, 771–786.

Gu, Y.Q., Wildermuth, M.C., Chakravarthy, S., Loh, Y.T., Yang, C., He, X., Han, Y. and Martin, G.B. (2002) Tomato transcription factors pti4, pti5, and pti6 activate defense responses when expressed in Arabidopsis. *Plant Cell*, **14**, 817–831.

Hall, A.E. and Bleecker, A.B. (2003) Analysis of combinatorial loss-of-function mutants in the Arabidopsis ethylene receptors reveals that the ers1 etr1 double mutant has severe developmental defects that are EIN2 dependent. *Plant Cell*, **15**, 2032–2041.

Hao, D., Yamasaki, K., Sarai, A. and Ohme-Takagi, M. (2002) Determinants in the sequence specific binding of two plant transcription factors, CBF1 and NtERF2, to the DRE and GCC motifs. *Biochemistry*, **41**, 4202–4208.

Heyl, A., Ramireddy, E., Brenner, W.G., Riefler, M., Allemeersch, J. and Schmülling, T. (2008) The transcriptional repressor ARR1-SRDX sup-

presses pleiotropic cytokinin activities in Arabidopsis. *Plant Physiol.* **147**, 1380–1395.

Hiratsu, K., Matsui, K., Koyama, T. and Ohme-Takagi, M. (2003) Dominant repression of target genes by chimeric repressors that include the EAR motif, a repression domain, in Arabidopsis. *Plant J.* **34**, 733–739.

Hua, J. and Meyerowitz, E.M. (1998) Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. *Cell*, **94**, 261–271.

Johnson, P.R. and Ecker, J.R. (1998) The ethylene gas signal transduction pathway: a molecular perspective. *Annu. Rev. Genet.* **32**, 227–254.

Ju, C., Yoon, G.M., Shemansky, J.M. *et al.* (2012) CTR1 phosphorylates the central regulator EIN2 to control ethylene hormone signaling from the ER membrane to the nucleus in Arabidopsis. *Proc. Natl Acad. Sci. USA*, **109**, 19486–19491.

Kevany, B. and Klee, H. (2007) Changes in ethylene sensitivity by regulated expression of the tomato ethylene receptor family. In *Advances in Plant Ethylene Research* (Ramina, A., Chang, C., Giovannoni, J., Klee, H., Perata, P. and Woltering, E., eds). Netherlands: Springer, pp. 123–128.

Kevany, B.M., Tieman, D.M., Taylor, M.G., Cin, V.D. and Klee, H.J. (2007) Ethylene receptor degradation controls the timing of ripening in tomato fruit. *Plant J.* **51**, 458–467.

Kieber, J.J., Rothenberg, M., Roman, G., Feldmann, K.A. and Ecker, J.R. (1993) CTR1, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the raf family of protein kinases. *Cell*, **72**, 427–441.

Leclercq, J., Ranty, B., Sanchez-Ballesta, M.-T. *et al.* (2005) Molecular and biochemical characterization of LeCRK1, a ripening-associated tomato CDPK-related kinase. *J. Exp. Bot.* **56**, 25–35.

Lee, J.M., Joung, J.-G., McQuinn, R., Chung, M.-Y., Fei, Z., Tieman, D., Klee, H. and Giovannoni, J. (2012) Combined transcriptome, genetic diversity and metabolite profiling in tomato fruit reveals that the ethylene response factor SlERF6 plays an important role in ripening and carotenoid accumulation. *Plant J.* **70**, 191–204.

Li, Y., Zhu, B., Xu, W., Zhu, H., Chen, A., Xie, Y., Shao, Y. and Luo, Y. (2007) LeERF1 positively modulated ethylene triple response on etiolated seedling, plant development and fruit ripening and softening in tomato. *Plant Cell Rep.* **26**, 1999–2008.

Lin, Z., Zhong, S. and Grierson, D. (2009) Recent advances in ethylene research. *J. Exp. Bot.* **60**, 3311–3336.

Magome, H., Yamaguchi, S., Hanada, A., Kamiya, Y. and Oda, K. (2004) Dwarf and delayed-flowering 1, a novel Arabidopsis mutant deficient in gibberellin biosynthesis because of over-expression of a putative AP2 transcription factor. *Plant J.* **37**, 720–729.

Matsui, K. and Ohme-Takagi, M. (2010) Detection of protein-protein interactions in plants using the transrepressive activity of the EAR motif repression domain. *Plant J.* **61**, 570–578.

Matsui, K., Hiratsu, K., Koyama, T., Tanaka, H. and Ohme-Takagi, M. (2005) A chimeric AtMYB23 repressor induces hairy roots, elongation of leaves and stems, and inhibition of the deposition of mucilage on seed coats in Arabidopsis. *Plant Cell Physiol.* **46**, 147–155.

McGrath, K.C., Dombrecht, B., Manners, J.M., Schenk, P.M., Edgar, C.I., Maclean, D.J., Scheible, W.-R., Udvardi, M.K. and Kazan, K. (2005) Repressor- and activator-type ethylene response factors functioning in jasmonate signaling and disease resistance identified via a genome-wide screen of Arabidopsis transcription factor gene expression. *Plant Physiol.* **139**, 949–959.

Mitsuda, N., Hiratsu, K., Todaka, D., Nakashima, K., Yamaguchi-Shinozaki, K. and Ohme-Takagi, M. (2006) Efficient production of male and female sterile plants by expression of a chimeric repressor in Arabidopsis and rice. *Plant Biotechnol. J.* **4**, 325–332.

Nakano, T., Suzuki, K., Fujimura, T. and Shinshi, H. (2006) Genome-wide analysis of the ERF gene family in Arabidopsis and rice. *Plant Physiol.* **140**, 411–432.

Ohme-Takagi, M. and Shinshi, H. (1995) Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. *Plant Cell*, **7**, 173–182.

Oñate-Sánchez, L., Anderson, J.P., Young, J. and Singh, K.B. (2007) AtERF14, a member of the ERF family of transcription factors, plays a nonredundant role in plant defense. *Plant Physiol.* **143**, 400–409.

Pan, Y., Seymour, G.B., Lu, C., Hu, Z., Chen, X. and Chen, G. (2012) An ethylene response factor (ERF5) promoting adaptation to drought and salt tolerance in tomato. *Plant Cell Rep.* **31**, 349–360.

Pirrello, J., Jaimes-Miranda, F., Sanchez-Ballesta, M.T., Tournier, B., Khalil-Ahmad, Q., Regad, F., Latché, A., Pech, J.C. and Bouzayen, M. (2006) Sl-ERF2, a tomato ethylene response factor involved in ethylene response and seed germination. *Plant Cell Physiol.* **47**, 1195–1205.

Pirrello, J., Prasad, B.C.N., Zhang, W. *et al.* (2012) Functional analysis and binding affinity of tomato ethylene response factors provide insight on the molecular bases of plant differential responses to ethylene. *BMC Plant Biol.* **12**, 190.

Pré, M., Atallah, M., Champion, A., Vos, M.D., Pieterse, C.M.J. and Memelink, J. (2008) The AP2/ERF domain transcription factor ORA59 integrates jasmonic acid and ethylene signals in plant defense. *Plant Physiol.* **147**, 1347–1357.

Riechmann, J.L., Heard, J., Martin, G. *et al.* (2000) Arabidopsis transcription factors: genome-wide comparative analysis among eukaryotes. *Science*, **290**, 2105–2110.

Sasaki, K., Mitsuhara, I., Seo, S., Ito, H., Matsui, H. and Ohashi, Y. (2007) Two novel AP2/ERF domain proteins interact with cis-element VWRE for wound-induced expression of the Tobacco tpoxN1 gene. *Plant J.* **50**, 1079–1092.

Solano, R. and Ecker, J.R. (1998) Ethylene gas: perception, signaling and response. *Curr. Opin. Plant Biol.* **1**, 393–398.

Solano, R., Stepanova, A., Chao, Q. and Ecker, J.R. (1998) Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. *Genes Dev.* **12**, 3703–3714.

Tieman, D.M., Taylor, M.G., Ciardi, J.A. and Klee, H.J. (2000) The tomato ethylene receptors NR and LeETR4 are negative regulators of ethylene response and exhibit functional compensation within a multigene family. *Proc. Natl Acad. Sci. USA*, **97**, 5663–5668.

Tieman, D.M., Ciardi, J.A., Taylor, M.G. and Klee, H.J. (2001) Members of the tomato LeEIL (EIN3-like) gene family are functionally redundant and regulate ethylene responses throughout plant development. *Plant J.* **26**, 47–58.

Tournier, B., Sanchez-Ballesta, M.T., Jones, B., Pesquet, E., Regad, F., Latché, A., Pech, J.-C. and Bouzayen, M. (2003) New members of the tomato ERF family show specific expression pattern and diverse DNA-binding capacity to the GCC box element. *FEBS Lett.* **550**, 149–154.

Trujillo, L.E., Sotolongo, M., Menéndez, C. *et al.* (2008) SodERF3, a novel sugarcane ethylene responsive factor (ERF), enhances salt and drought tolerance when overexpressed in tobacco plants. *Plant Cell Physiol.* **49**, 512–525.

Wang, H., Jones, B., Li, Z. *et al.* (2005) The tomato Aux/IAA transcription factor IAA9 is involved in fruit development and leaf morphogenesis. *Plant Cell*, **17**, 2676–2692.

Wuriyanghan, H., Zhang, B., Cao, W.H. *et al.* (2009) The ethylene receptor ETR2 delays floral transition and affects starch accumulation in rice. *Plant Cell*, **21**, 1473–1494.

Yokotani, N., Tamura, S., Nakano, R., Inaba, A. and Kubo, Y. (2003) Characterization of a novel tomato EIN3-like gene (LeEIL4). *J. Exp. Bot.* **54**, 2775–2776.

Zhang, Z., Zhang, H., Quan, R., Wang, X.-C. and Huang, R. (2009) Transcriptional regulation of the ethylene response factor LeERF2 in the expression of ethylene biosynthesis genes controls ethylene production in tomato and tobacco. *Plant Physiol.* **150**, 365–377.