

RESEARCH PAPER

Silencing *SI-EBF1* and *SI-EBF2* expression causes constitutive ethylene response phenotype, accelerated plant senescence, and fruit ripening in tomato

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Abstract

The hormone ethylene regulates a wide range of plant developmental processes and EBF (EIN3-binding F-box) proteins were shown to negatively regulate the ethylene signalling pathway via mediating the degradation of EIN3/EIL proteins. The present study reports on the identification of two tomato F-box genes, *SI-EBF1* and *SI-EBF2* from the EBF subfamily. The two genes display contrasting expression patterns in reproductive and vegetative tissues and in response to ethylene and auxin treatment. *SI-EBF1* and *SI-EBF2* genes are actively regulated at crucial stages in the development of the reproductive organs. Their dynamic expression in flowers during bud-to-anthesis and anthesis-to-post-anthesis transitions, and at the onset of fruit ripening, suggests their role in situations where ethylene is required for stimulating flower opening and triggering fruit ripening. VIGS-mediated silencing of a single tomato *EBF* gene uncovered a compensation mechanism that tends to maintain a threshold level of *SI-EBF* expression via enhancing the expression of the second *SI-EBF* gene. In line with this compensation, tomato plants silenced for either of the *SI-EBF* genes were indistinguishable from control plants, indicating functional redundancy among *SI-EBF* genes. By contrast, co-silencing of both *SI-EBFs* resulted in ethylene-associated phenotypes. While reports on *EBF* genes to date have focused on their role in modulating ethylene responses in *Arabidopsis*, the present study uncovered their role in regulating crucial stages of flower and fruit development in tomato. The data support the hypothesis that protein degradation via the ubiquitin/26S proteasome pathway is a control point of fruit ripening and open new leads for engineering fruit quality.

Key words: EIN3-binding F-box protein, ethylene signalling, fruit, gene silencing, tomato.

Introduction

Ethylene is an important plant hormone involved in a wide range of plant developmental processes, including seed germination, plant growth, leaf expansion, root hair formation, fruit ripening, timing of vegetative senescence, and responses to abiotic stresses and pathogen attack (Johnson and Ecker, 1998; Wang *et al.*, 2002; Potuschak *et al.*, 2003). The ethylene signalling pathway, uncovered through the extensive characterization of *Arabidopsis* mutants altered in ethylene responses (Wang *et al.*, 2002), is defined in its

upstream part as a linear pathway. Ethylene signal transduction initiates with ethylene binding at ethylene receptors (ETR1, ETR2, EIN4, ERS1, and ERS2) and terminates in a transcription cascade involving the EIN3/EILs (EIN3-like proteins) and ERF (ethylene response factor) families (Wang *et al.*, 2002). Briefly, ethylene is perceived by the ethylene receptor and the hormone binding to the receptor represses its activity (Chang *et al.*, 1993; Hua *et al.*, 1998). In the absence of ethylene, the receptors are in

an active state and constitutively activate CTR1, a mitogen-activating protein kinase kinase kinase (MAPKKK) that negatively regulates the downstream component in the pathway, EIN2, a member of the N-Ramp family of metal-transporters (Kieber *et al.*, 1993). Therefore, binding of ethylene to the receptor inactivates CTR1 thus allowing EIN2 to promote ethylene responses via activating the downstream EIN3/EILs transcription factors (Chao *et al.*, 1997), which are vital transcription factors for mediating ethylene-regulated gene expression and associated morphological responses (Chao *et al.*, 1997; Solano *et al.*, 1998; Guo and Ecker, 2003). Subsequently, EIN3/EIL proteins activate the transcription of ethylene response factors (ERFs), another type of transcription factor, which regulates the expression of genes involved in the response to ethylene (Potuschak *et al.*, 2003).

Studies using the *Arabidopsis* model plant, revealed that the ubiquitin/26S proteasome pathway negatively regulates ethylene responses by targeting EIN3 for degradation (Guo and Ecker, 2003; Potuschak *et al.*, 2003; Gagne *et al.*, 2004). The ubiquitin/26S proteasome pathway is an important post-transcriptional regulatory mechanism present in all eukaryotes. This protein degradation process involved in the removal of abnormal polypeptides, is also operating for the degradation of naturally short-lived regulators thus allowing cells to respond rapidly to signal molecules and changes in environmental conditions (Hershko and Ciechanover, 1998; Gagne *et al.*, 2004).

Consequently, the ubiquitin/proteasome pathway plays an important role in various plant hormone signal transduction pathways through positive or negative regulatory mechanisms. Substrate recognition and ubiquitination are mediated by E3 type ubiquitin–protein ligases that catalyse the transfer of activated ubiquitin to free lysyl ϵ -amino groups on appropriate targets (Gagne *et al.*, 2004; Smalle and Vierstra, 2004). One major E3 type is the SCF ubiquitin–ligase complex, which is composed in *Saccharomyces cerevisiae* of four primary subunits: Skp1, Cullin (CDC53), RBX1, and F-box protein (Deshaies, 1999; Potuschak *et al.*, 2003). The F-box protein performs the crucial role of delivering appropriate targets to the complex for ubiquitin-mediated proteolysis (Deshaies, 1999; Kipreos and Pagano, 2000). It contains a conserved F-box motif at the N-terminus made of 40–50 amino acid residues necessary for interacting with the Skp1 subunit, and a highly variable protein–protein interaction domain of tandem leucine-rich repeats (LRRs) at the C-terminus that allows substrate recognition for ubiquitination (Xiao and Jang, 2000; Gagne *et al.*, 2002). Most plant hormone signalling pathways are subjected to F-box protein-dependent regulation, including auxin, ethylene, gibberellin acid (GA), jasmonic acid (JA), abscisic acid (ABA), salicylic acid (SA), cytokinin, and brassinosteroid (reviewed by Frugis and Chua, 2002; Guo and Ecker, 2003; Vierstra, 2003). Interestingly, the F-box proteins TIR1 (Ruegger *et al.*, 1998), CO11 (Xie *et al.*, 1998), and GID2 (Sasaki *et al.*, 2003) positively regulate auxin, JA, and GA signalling pathways by targeting negative regu-

lators for degradation. In this case, the hormone acts to promote the repressors' degradation. By contrast, EBF1 and 2 (EIN3-binding F-box proteins 1 and 2) negatively regulate the ethylene signalling pathway by targeting EIN3 (and possibly the related EILs) for degradation, and ethylene can stabilize EIN3 protein by preventing its degradation (Guo and Ecker, 2003; Potuschak *et al.*, 2003; Binder *et al.*, 2007). Similarly, recent study revealed another two F-box proteins ETP1 and 2 (EIN2 targeting proteins 1 and 2) that also negatively regulate the ethylene signalling pathway by negatively regulating EIN2 protein stability (Qiao *et al.*, 2009). It was reported that the levels of ethylene receptors in ripening fruit are also regulated by the 26S proteasome pathway and that the degradation of the receptor modulates ethylene responses (Kevany *et al.*, 2007). Together, these data indicate that protein degradation is instrumental to the control of ethylene responses in plants.

Two *Arabidopsis* F-box proteins, EBF1 and 2, were shown to play an important role in the ethylene signalling pathway through directing EIN3 for degradation by the ubiquitin/26S proteasome pathway (Guo *et al.*, 2003; Potuschak *et al.*, 2003; Gagne *et al.*, 2004; Binder *et al.*, 2007). 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 2. The ubiquitinated form of EIN3/EIL proteins is thus recruited by the 26S proteasome for degradation. However, 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 the corresponding genes. While it is well established that EBF1 and 2 play an important role in regulating ethylene responses in the plant model *Arabidopsis*, little is known about their role in other plant species and their impact on plant growth and development.

Tomato (*Solanum lycopersicum*) is the model system for studying the biological bases of fleshy fruit development and ripening. In tomato, the fruit developmental process includes active cell division and expansion at the early stages and dramatic changes in texture and carotenoid, sugar, and acid content during the ripening stage (Giovannoni, 2004). Since ethylene is the main trigger of climacteric fruit ripening, it is important to uncover whether EBF1 and/or EBF2 play a role in controlling plant growth and fruit ripening in the tomato. In the present study, two tomato F-box genes, *Sl-EBF1* and *Sl-EBF2*, were identified and their expression profile was established in different tomato tissues and at various stages of flower and fruit development. *Sl-EBF1* and *Sl-EBF2* expression is regulated by both ethylene and auxin and silencing of *Sl-EBF1* and *Sl-EBF2* expression caused a constitutive ethylene response phenotype, fertility defect, strong growth arrest, accelerated plant senescence, and fruit ripening. These data indicate that the co-ordinated regulation of *Sl-EBF1* and *Sl-EBF2* is instrumental to tomato plant growth and that the dynamic regulation of these genes is essential for proper flower development and fruit ripening.

Materials and methods

Plant materials and growth conditions

Tomato (*Solanum lycopersicum* cv. MicroTom) plants were grown in a culture chamber under the following conditions: 14/10 h day/night cycle, 25/20 °C day/night temperature (for VIGS plants, 20/18 °C day/night), 80% humidity, and 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity. The root, stem, leaf, flower, and fruit tissues were collected from 10-week-old water-cultured tomato plants. Samples taken from different parts of the flower (ovary, stamen, petal, and sepal) were harvested at bud (–2 dpa; days post anthesis), anthesis (0 dpa), and post-anthesis (4 dpa) stages. The developmental stages of tomato fruit investigated in this study are 8 dpa, mature green, breaker, and ripening.

Ethylene and auxin treatment

To perform phytohormone treatment, plants were germinated and grown in Murashige and Skoog (MS) culture medium as described by Wang *et al.* (2005). The 21-d-old light-grown tomato seedlings were treated with 50 $\mu\text{l l}^{-1}$ ethylene for 1 h or incubated in 50% MS buffer containing 20 μM IAA for 3 h. The corresponding control experiments (mock treatment) were run concomitantly. Treated tissues were then immediately frozen in liquid nitrogen and stored at –80 °C until RNA extraction. Each treatment was performed in replicate.

Sequence analysis

Amino acid sequence alignments were performed using ClustalX 2.0.10 assisted by manual adjustment. Phylogenetic analyses were performed with Phylip (version 3.68) and the tree was shown using Treeview 1.6.6. The F-box domains and leucine-rich repeats (LRRs) motifs were analysed using the SMART tool (<http://smart.embl-heidelberg.de>) as described previously (Schultz *et al.*, 1998; Letunic *et al.*, 2009). GenBank accession numbers for the sequences analysed are as follows: *Arabidopsis thaliana* AtEBF1 (NP_565597), AtEBF2 (NP_197917), AtCOI1 (NP_565919), AtFBL4 (NP_567467), AtFKF1 (AAF32298), AtSKP2 (NP_565147), AtTIR1 (NP_567135), AtZTL (NP_568855), *Brassica oleracea* BoF-box (ACB59221), *Danio rerio* DrSLY1 (AAN87034), *Gossypium hirsutum* GhTIR1 (ABG46343), *Glycine max* GmCOI1 (AAZ66745), GmFKF1 (ABD28287), *Hevea brasiliensis* HbCOI1 (ABV72393), *Ipomoea nil* InZTL (ABC25060), *Mesembryanthemum crystallinum* McFKF1 (AAQ73528), McZTL (AAQ73527), *Oryza sativa* OsCOI1 (AAO38719), OsFBL2 (BAD35544), OsF-box (BAD15849), OsTIR1 (ABY87942), *Populus trichocarpa* PtEBF3 (EEE92188), PtEBF4 (EEE92505), PtF-box (EEF03786), PtTIR1 (AAK16647), *Saccharomyces cerevisiae* ScSLY1 (CAA38221), *Solanum lycopersicum* SICOI1 (AAR82926), SIEBF1 (ACS44349), SIEBF2 (ACS44350), *Schizosaccharomyces pombe* SpSLY1 (NP_588374), *Triticum aestivum* TaFKF1 (ABL11478), *Zea mays* ZmEBF1 (ACG17917).

Gene expression analysis

Total RNA samples were isolated using Trizol (Invitrogen) according to the manufacturer's instructions, and were treated with the DNA-free™ Kit (Ambion) for 30 min at 25 °C and purified following the handbook description. The first-strand cDNA synthesis was performed using 2 μg of total RNA by Omniscript® Reverse Transcription (QiaGen). Quantitative PCR (Q-PCR) was performed using cDNAs corresponding to 2.5 ng of total RNA in a 10 μl reaction volume using SYBR GREEN PCR Master Mix (PE-Applied Biosystems) on an ABI PRISM 7900HT sequence-detection system. *Slactin-51* (GenBank accession number Q96483) was used as a reference gene with constitutive expression in various tissues. Forward (F) and reverse (R) primers used for Q-PCR amplification are the following:

F 5'-ATTGCCATCACTGACATAGC-3' and R 5'-AGTTA-TAGCAAGCGACCTC-3' for *Sl-EBF1*, F 5'-ATGTGATGGAT-ACCTTACCAG-3' and R 5'-CCGACATTAGTAATACCACGA-3' for *Sl-EBF2*, F 5'-TGTCCCTATTACGAGGGTTATGC-3' and R 5'-CAGTTAAATCAGACCAGCAAGAT-3' for *SlActin-51*. For *Sl-EBF1* and *Sl-EBF2*, primers that anneal outside the region targeted for silencing were used to ensure that only the endogenous gene was being tested (Rotenberg *et al.*, 2006). Q-PCR reactions were performed as follow: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min and one cycle of 95 °C for 15 s and 60 °C for 15 s. For all Q-PCR experiments, at least three biological replicates were performed and each reaction was run in triplicate. For each sample, a threshold cycle (*Ct*) value was calculated from the amplification curves by selecting the optimal *Rn* (emission of reporter dye over starting background fluorescence) in the exponential portion of the amplification plot. Relative fold differences were calculated based on the comparative *Ct* method using the *SlActin-51* as an internal standard. To determine relative fold differences for each sample in each experiment, the *Ct* value for the transcripts *Sl-EBF1* and *Sl-EBF2* was normalized to the *Ct* value for *SlActin-51* and was calculated relative to a calibrator using the formula $2^{-\Delta\Delta C_t}$.

VIGS vector construction

The TRV VIGS vectors and pTRV2-SIPDS (described in Liu *et al.*, 2002) were kindly offered by Dr Dinesh-Kumar (Yale University). A 483 bp fragment of *Sl-EBF1* and a 482 bp fragment of *Sl-EBF2* were PCR-amplified from tomato cDNA using the following primers: F 5'-CCGGAATTCATCCTGTCAGATA-ATGGCTTG-3' and R 5'-CCGGAATTCGTATCGACACTCG-TCAACAT-3' with an *EcoRI* restriction site for *Sl-EBF1*, and F 5'-CGCGTCTAGATTACTAATGTCGGTCTATCT-3' with an *XbaI* restriction site and R 5'-CTTCGAGCTCTCCCTTCT-GACTCACATTACG-3' with a *SacI* restriction site for *Sl-EBF2*. The PCR products corresponding to *Sl-EBF1* and *Sl-EBF2* fragments were cloned into pTRV2 and named pTRV2-SIEBF1 and pTRV2-SIEBF2, respectively. To generate the construct intended to silence both *Sl-EBF1* and *Sl-EBF2* genes, the PCR product of *Sl-EBF1* was cloned into *EcoRI*-cut pTRV2-SIEBF2 vector to generate pTRV2-SIEBF1-SIEBF2.

Virus infection by Agrobacterium-mediated infiltration

Virus infection was performed as described by Liu *et al.* (2002). Briefly, a 1 ml culture of *A. tumefaciens* strain GV3101 containing each TRV derivative was grown for 8–10 h at 28 °C in the Luria-Bertani (LB) medium containing the appropriate antibiotics. The culture was inoculated into 20 ml LB medium containing antibiotics, 10 mM MES, and 20 μM acetosyringone and was shaken overnight at 28 °C. *Agrobacterium* cell pellets were washed, resuspended in infiltration buffer (10 mM MgCl_2 , 10 mM MES, 200 μM acetosyringone), adjusted to an OD of 2.0 and left at room temperature for 3 h. Plants were infected when the first pair of leaves had emerged using a needleless 1 ml syringe and were left covered overnight.

Results

Sl-EBF1 and *Sl-EBF2* belong to a distinct subfamily of the F-box protein family

The partial sequences of *Sl-EBF1* and *Sl-EBF2* were obtained by a computational identification approach. Briefly, TBLASTN analysis against the Solanaceae Genome Network tomato expression database (<http://sgn.cornell.edu>) with At-EBF1 and At-EBF2 identified two

tomato clones, SGN-U316405 and SGN-U315243, encoding putative proteins that displayed conservation with their *Arabidopsis* counterparts. When analysed by a translation tool (<http://www.expasy.org/tools/dna.html>), the SGN-U316405 (1995 bases) and SGN-U315243 (1911 bases) clones are predicted to encode two proteins of 665 and 637 amino acids corresponding to the complete coding sequences of SI-EBF1 and SI-EBF2, respectively. Subsequently, the full-length *SI-EBF1* and *SI-EBF2* cDNA clones were isolated using RACE PCR (Takara, Japan) and the corresponding sequences deposited in GenBank database (accession numbers GQ144955 and GQ144956, respectively). The two predicted tomato proteins share 58.99% amino acid sequence identity (Table 1). Moreover, SI-EBF1 shares 59.13% and 55.56% amino acid identity with At-EBF1 and At-EBF2, respectively, whereas SI-EBF2 shares 56.59% and 56.17% identity with the corresponding *Arabidopsis* genes (Table 1). Both SI-EBF1 and SI-EBF2 contain a well-conserved F-box domain made of 49 amino acids at the N-terminus and 13 tandem leucine-rich repeats (LRRs) at the C-terminal moiety, consistent with the corresponding domains of At-EBF1, At-EBF2, Pt-EBF3, and Pt-EBF4 (Fig. 1). Phylogenetic analysis was performed to uncover the position of SI-EBF1 and SI-EBF2 among other related F-box protein subfamilies from plant, animal, and yeast organisms including EBF, TIR1, COI1, SLY1, ZTL, and FKF1. The phylogenetic tree presented in Fig. 2 clearly shows that SI-EBF1 and SI-EBF2 belong to the EBF branch of the F-box protein super-family.

Expression patterns of SI-EBF1 and SI-EBF2 in different tomato organs

Knowing the tissue-specific and developmentally-regulated patterns of expression of a particular gene can sometime provide important clues about its physiological function. To assist with the determination of the function of SI-EBF1 and SI-EBF2 in ethylene-regulated developmental processes, such as tomato fruit development and ripening, the expression patterns of *SI-EBF1* and *SI-EBF2* were examined in different plant organs and at various stages of fruit and flower developmental. Expression analysis performed by Quantitative RT-PCR (Fig. 3) indicated that *SI-EBF1* and *SI-EBF2* display similar expression patterns in leaf, flower, and fruit (Fig. 3A, B). However, the two genes exhibit different expression profiles in root and stem where *SI-EBF1* transcripts show enormously higher accumulation than that of *SI-EBF2* whose transcripts are barely detect-

able in the root tissue and almost below detection levels in the stem (Fig. 3A, B). These expression profiles suggest that both SI-EBF1 and SI-EBF2 are operating in leaf, flower, and fruit whereas SI-EBF1 alone is being active in root and stem tissues.

The expression profiles of *SI-EBF1* and *SI-EBF2* were then examined in different parts of the flower and at three contrasting stages of flower development. Transcripts of both genes were detected in all the parts of the flower at bud and anthesis stages (Fig. 3C, D). Generally, both *SI-EBF1* and *SI-EBF2* exhibit moderate expression at the bud stage, higher expression at the anthesis stage, and is markedly down-regulated at the post-anthesis stage. From bud to anthesis, *SI-EBF1* expression increases remarkably in the stamen, whereas *SI-EBF2* displays significant up-regulation in all parts of the flower except in the ovary (Fig. 3C, D). From anthesis to post-anthesis when fruit set is expected to occur, both *SI-EBF1* and *SI-EBF2* are sharply down-regulated in the ovary and sepals. This dynamic expression pattern suggests that SI-EBF1 and SI-EBF2 may play a critical role during flower development in tomato and particularly during the flower-to-fruit transition triggered upon pollination.

Given the established role devoted to ethylene in tomato fruit ripening, the expression of *SI-EBF1* and *SI-EBF2* was analysed throughout fruit development and ripening (Fig. 3E, F). *SI-EBF1* and *SI-EBF2* exhibit similar variation in transcript accumulation during fruit development and ripening. Both *SI-EBF1* and *SI-EBF2* have moderate expression at the very early stages of fruit development (8 dpa) and only background expression levels at the mature green stage (MG, about 40 dpa). Subsequently, both genes display a sharp increase in expression at the breaker stage (Br, 42 dpa) and maintain a high level of expression at the ripening stage (Ri, 50 dpa). These data suggest that both SI-EBF1 and SI-EBF2 might play an active role in tuning ethylene responses during fruit development and particularly at the onset of ripening.

SI-EBF1 and SI-EBF2 expression is positively regulated by ethylene and negatively regulated by auxin

To determine whether *SI-EBF1* and *SI-EBF2* are under ethylene regulation, Q-PCR was used to test their relative mRNA accumulation upon short-time exogenous ethylene treatment. In light-grown seedlings, both *SI-EBF1* and *SI-EBF2* show clear responsiveness to ethylene (Fig. 4A). *SI-EBF2* mRNA levels display a dramatic increase (73-fold) in treated seedlings while, comparatively, *SI-EBF1* show only a modest increase (4-fold) in the same conditions. The regulation of tomato *EBF* genes in the flower during the transition from anthesis to post-anthesis prompted us to test their potential responsiveness to auxin, a key plant hormone controlling fruit set. The expression of both *SI-EBF1* and *SI-EBF2* genes was found to be negatively regulated upon exogenous treatment by IAA, the major auxin compound (Fig. 4B). However, opposite to ethylene treatment for which *SI-EBF2* was the most responsive,

Table 1. Comparative analysis of SI-EBFs amino acid sequences with its closest homologues in *Arabidopsis* and poplar

	Identity (%)				
	SI-EBF2	At-EBF1	At-EBF2	Pt-EBF3	Pt-EBF4
SI-EBF1	58.99	59.13	55.56	64.48	59.78
SI-EBF2	–	56.59	56.17	64.62	71.43

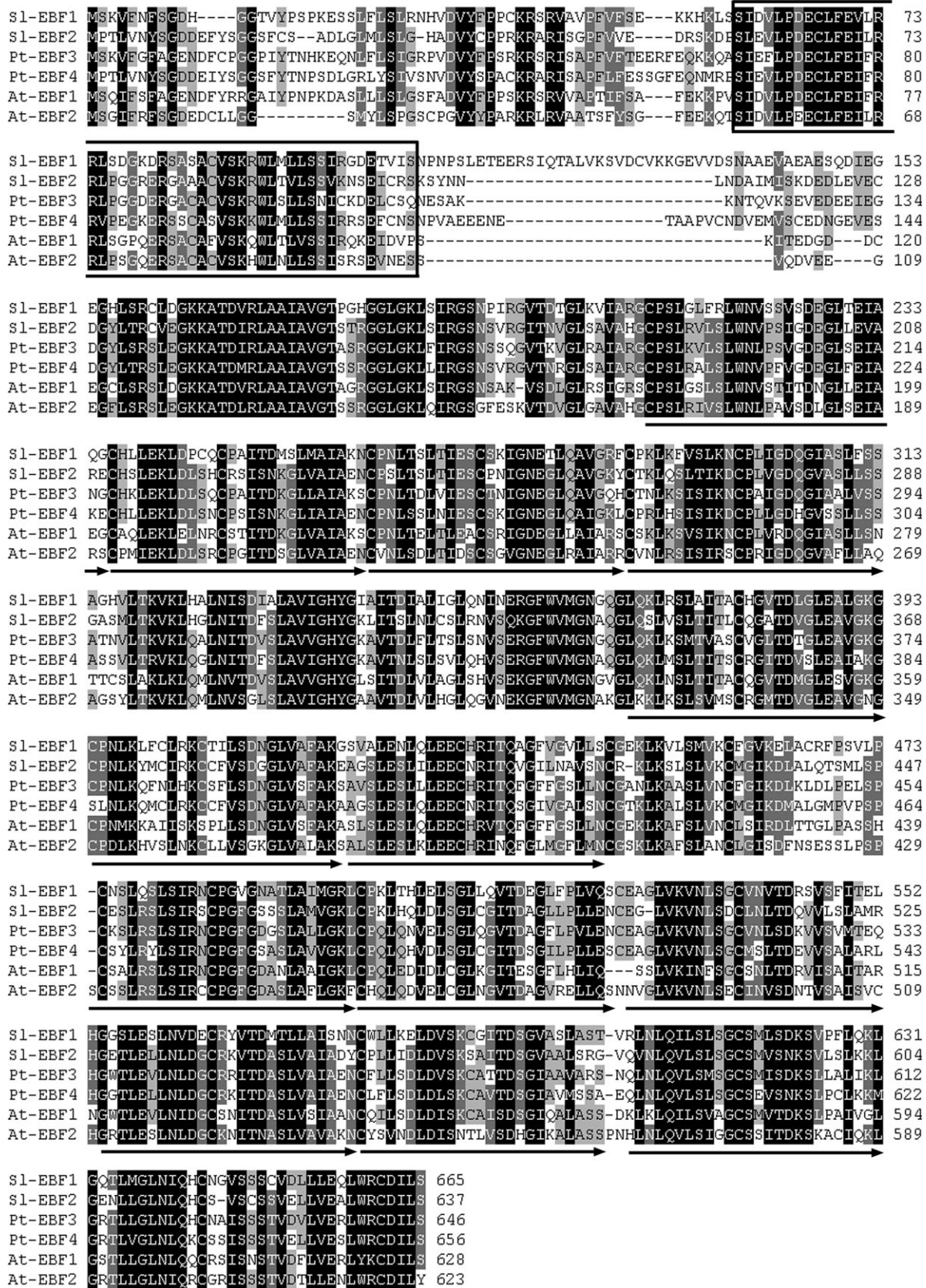


Fig. 1. Sequence analysis of Sl-EBF1 and Sl-EBF2. The amino acid sequences of tomato Sl-EBF1 and Sl-EBF2, *Arabidopsis* At-EBF1 and At-EBF2, and poplar Pt-EBF3 and Pt-EBF4 were aligned using the ClustalX (2.0.10) program. Numbers show the positions of amino acid residues. Conserved residues are shaded in black, dark grey shading indicates similar residues in at least five out of the six sequences, and light grey shading indicates similar residues in three to four out of the six sequences. The putative F-box motif sequences are boxed, and the 13 deduced leucine-rich repeats (LRRs) are indicated by arrows under the sequences.

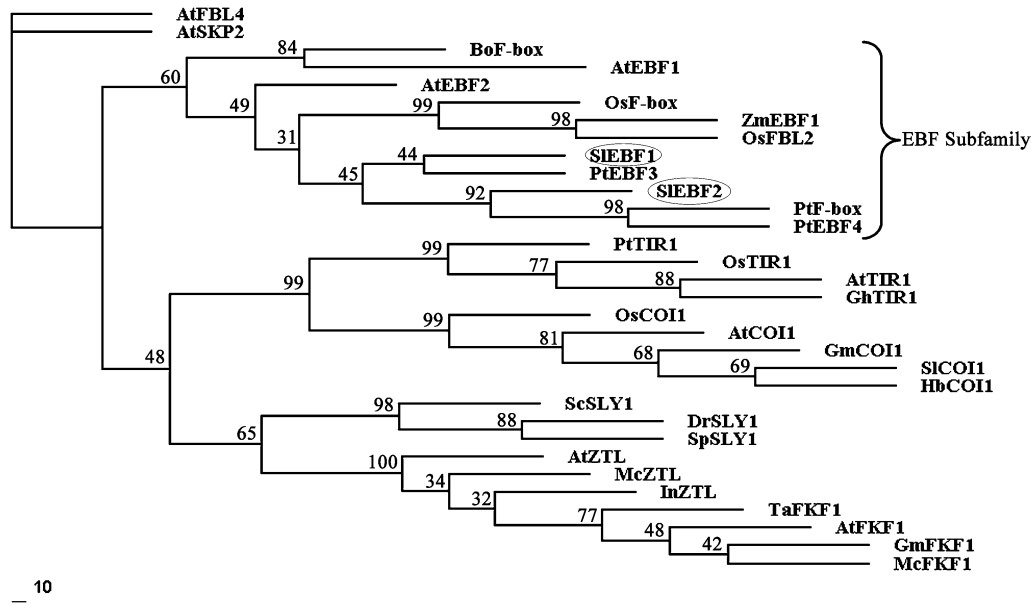


Fig. 2. SI-EBF1 and SI-EBF2 belong to a distinct subfamily of the F-box protein family. The phylogenetic tree was obtained using the Neighbor-Joining approach by Phylip 3.68. AtFBL4 and AtSKP2 were used as outgroups because of their relative isolation on preliminary calculations. Values above the branches are bootstrap percentages (1000 replicates). The phylogenetic tree was constructed with gene sequences from the following species: *Arabidopsis thaliana* AtEBF1, AtEBF2, AtCOI1, AtFBL4, AtFKF1, AtSKP2, AtTIR1, and AtZTL; *Brassica oleracea* BoF-box; *Danio rerio* DrSLY1; *Gossypium hirsutum* GhTIR1; *Glycine max* GmCOI1 and GmFKF1; *Hevea brasiliensis* HbCOI1; *Ipomoea nil* InZTL; *Mesembryanthemum crystallinum* McFKF1 and McZTL; *Oryza sativa* OsCOI1, OsFBL2, OsF-box, and OsTIR1; *Populus trichocarpa* PtEBF3, PtEBF4, PtF-box, and PtTIR1; *Saccharomyces cerevisiae* ScSLY1; *Solanum lycopersicum* SICOI1, SIEBF1, and SIEBF2; *Schizosaccharomyces pombe* SpSLY1; *Triticum aestivum* TaFKF1; *Zea mays* ZmEBF1.

Sl-EBF1 displayed a substantially stronger response to auxin. Treatment of tomato seedlings with IAA for 3 h resulted in a 5-fold decrease of *Sl-EBF1* transcript accumulation compared to the 2-fold decrease in *Sl-EBF2* transcripts.

Silencing Sl-EBF1 and Sl-EBF2 expression reduces fertility and accelerates plant senescence and fruit ripening

To characterize *Sl-EBF1* and *Sl-EBF2* functionally, a loss-of-function approach was implemented using the tobacco rattle virus (TRV)-mediated gene silencing (VIGS) strategy that has been optimized for tomato plants (Liu *et al.*, 2002; Fu *et al.*, 2005). Two *Agrobacterium* expression vectors (pTRV1 and pTRV2) carrying the bipartite genome of TRV were used. Following known requirements for efficient gene silencing (Burch-Smith *et al.*, 2004), the constructs for either single gene silencing or co-silencing of *Sl-EBF1* and *Sl-EBF2* were designed. To ensure that the dedicated VIGS constructs target *Sl-EBF1* and *Sl-EBF2* separately, or both genes, the specificity of the inserted fragments was analysed by BLAST against tomato expressed sequence tags (ESTs) and the unigene database (<http://sgn.cornell.edu>). The failure to detect any tomato *EBF* gene related to EBF1 and EBF2 in the available comprehensive tomato EST databases and the existence of only two *EBF* genes in *Arabidopsis* suggest that it is unlikely that additional *EBF* genes exist in this species. To validate the efficiency of the

VIGS strategy, the pTRV2-*SIPDS* construct targeting the *Phytoene Desaturase (PDS)* gene and the pTRV2 empty vector were also used for tomato plant transfection. *PDS* silencing in tomato causes the plants to exhibit a photo-bleached phenotype (Liu *et al.*, 2002) and was therefore used as a positive control for successful VIGS silencing.

Three to four weeks after TRV infection when *PDS*-silenced plants exhibited a visible photo-bleaching phenotype, total RNA samples were isolated from leaf tissue collected from the upper part of each silenced plant. To test whether the target genes were effectively silenced, the relative abundance of transcripts for the targeted gene was determined by quantitative RT-PCR in gene-silenced plants and empty pTRV2-infected control plants (Fig. 5A). Transcript accumulation was carried out using primers that anneal outside the gene region of *Sl-EBF1* and *Sl-EBF2* targeted for silencing. Comparing with control plants, mRNA accumulation of *Sl-EBF1* and *Sl-EBF2* was significantly reduced in the corresponding silenced plants whereas both genes were co-silenced in TRV2-*SIEBF1/2*-infiltrated plants (Fig. 5A). Interestingly, the expression of the *Sl-EBF1* gene was enhanced in *Sl-EBF2* single gene-silenced plants and, conversely, the *Sl-EBF2* gene was up-regulated in *Sl-EBF1* single gene-silenced plants (Fig. 5A). These data are suggestive of a compensation mechanism, implying that when one of the two *EBF* genes is down-regulated, the expression of the other gene is concomitantly enhanced.

The growth behaviour of single gene-silenced plants for either *Sl-EBF1* or *Sl-EBF2* were indistinguishable from

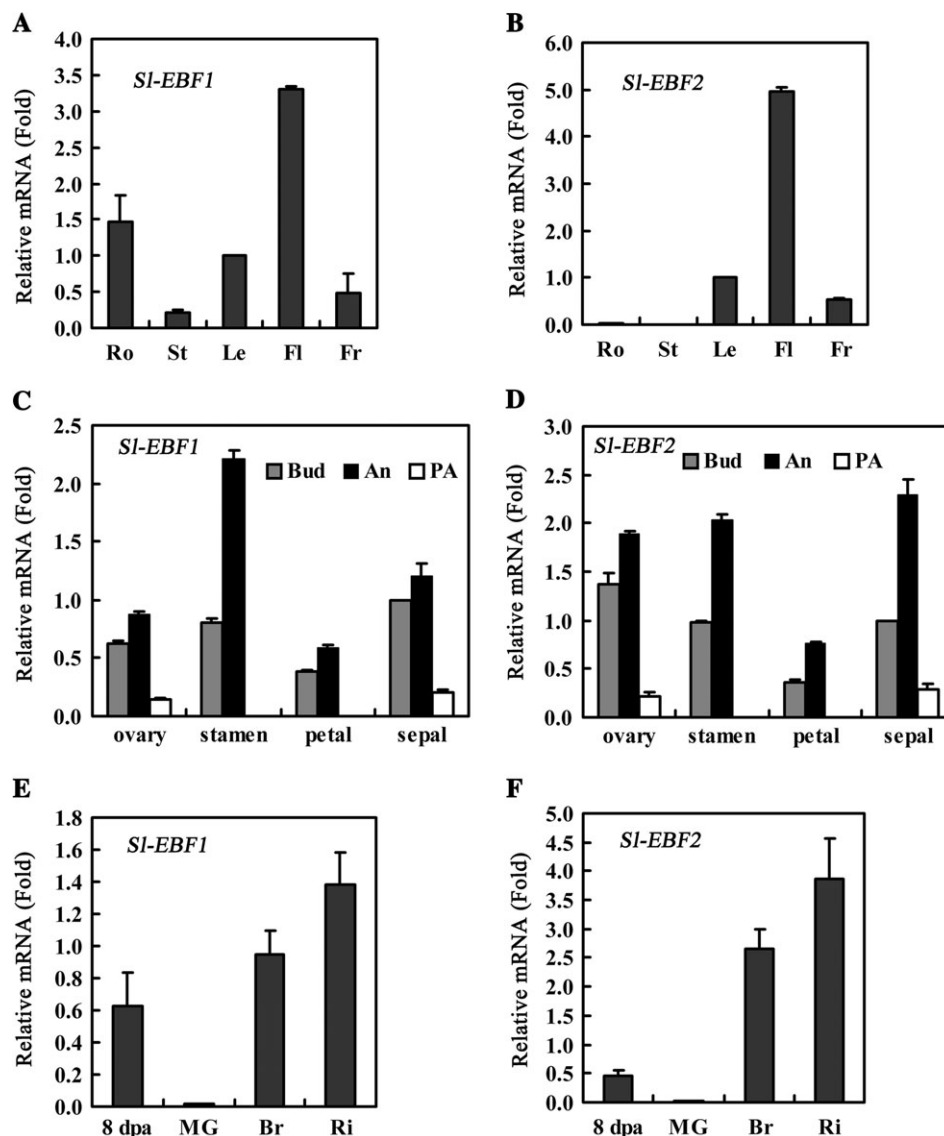


Fig. 3. Expression patterns of *SI-EBF1* and *SI-EBF2* in tomato. Expression analysis of *SI-EBF1* and *SI-EBF2* was performed in different tissues (A, B), in different parts of flower at different developmental stages (bud, anthesis, post-anthesis) (C, D), and in fruits at different developmental stages (E, F) by Q-PCR. Stamens and petals have been shed at the post-anthesis stage, so no data were shown at this stage in the two parts. Data are expressed as relative values, based on the values of leaf in (A, B, E, F) and sepal in (C, D) taken as reference sample set to 1. Each value represents mean \pm standard error of three replicates. Ro, root; St, stem; Le, leaf; Fl, flower; Fr, fruit; An, anthesis; PA, post-anthesis; dpa, days post-anthesis; MG, mature green; Br, breaker; Ri, ripening.

control plants, while co-silenced plants displayed strong visible growth phenotypes (Fig. 5B, C). Among the *SI-EBF1/2* co-silenced plants, 10 lines displayed a marked constitutive ethylene response phenotype including petiole and leaf epinasty and curly leaves (Fig. 5B). Noteworthy, the growth of these co-silenced plants was arrested once the silencing became active, as assessed by the appearance the photo-bleaching phenotype in *PDS*-silenced plants (Figs 5C, 6B). In the most severely co-silenced plants, pale green spots appeared and spread rapidly along the main stem and branches leading to full senescence and, ultimately, the plants perished after 35 dpi (days post-infiltration) whereas control plants continued to grow normally and entered the full flowering stage (Fig. 5C). Six co-silenced plants with

a relatively mild ethylene response phenotype remained alive, flowered, and set fruit that displayed the visible ethylene response phenotype with droop of fruit stems and sepals (Fig. 6A). Based on colour change, fruits appeared to undergo premature ripening with the breaker stage occurring about 10 d earlier than in control plants under normal growth conditions (Table 2). The co-silenced plants also exhibited a fertility defect, with reduced fresh blossom buds emergence after the appearance of the silencing phenotype (Table 2). The co-silenced plants were severely dwarfed with reduced fertility, and senescence and fruit ripening were accelerated compared with non-silenced plants (Fig. 6B). Although the single gene-silenced plants for either *SI-EBF1* or *SI-EBF2* were indistinguishable from the control plants

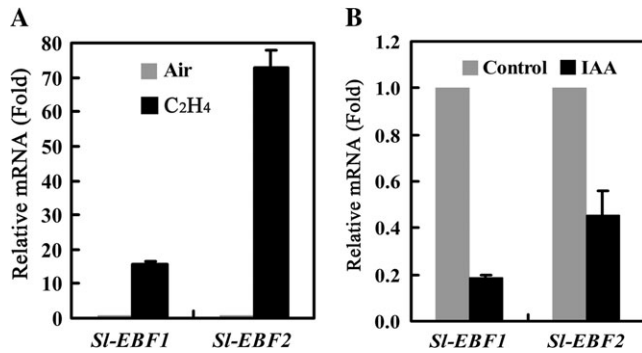


Fig. 4. *SI-EBF1* and *SI-EBF2* are regulated by ethylene and auxin. Light-grown tomato seedlings were treated with 50 $\mu\text{l l}^{-1}$ ethylene for 1 h (A) or 20 μM IAA for 3 h (B). Relative mRNA accumulation of *SI-EBF1* and *SI-EBF2* in response to ethylene and auxin treatment was tested by Q-PCR. Data are expressed as relative values, based on the values of control taken as reference sample set to 1. Each value represents mean \pm standard error of three replicates.

with regard to the growth phenotype, they displayed accelerated fruit ripening under normal growth conditions and exhibited the fertility defect but milder than in co-silenced plants (Table 2; Fig. 6B).

Discussion

F-box type proteins are key regulators of plant hormone signalling and, as such, they play an active role in mediating various aspects of plant growth and development. The present work reports on the isolation of two tomato F-box genes, *SI-EBF1* and *SI-EBF2* belonging to the EBF subfamily and bearing strong sequence and structural similarities with their respective *Arabidopsis* orthologues *At-EBF1* and *At-EBF2*. The existence of more than two tomato *EBF* genes seems unlikely since the mining of available sequences in the comprehensive tomato EST databases only identified two EBF-type genes and only two *EBF* genes are found in the *Arabidopsis* genome. However, the existence of putative additional *EBF* genes still remains a possibility that cannot be absolutely ruled out until the complete tomato genome sequence becomes available.

The data presented indicate that the encoded proteins are integral components of ethylene-regulated developmental processes such as epinasty, premature senescence, and accelerated fruit ripening. It was previously shown that *Arabidopsis* F-box proteins At-EBF1 and 2 regulate ethylene signalling through directing EIN3 type transcription factors for degradation via the ubiquitin/26S proteasome pathway (Guo *et al.*, 2003; Potuschak *et al.*, 2003; Gagne *et al.*, 2004; Binder *et al.*, 2007). Both tomato *SI-EBF1* and *SI-EBF2* genes encode proteins with the typical F-box domain at the N-terminus and the tandem leucine-rich repeats (LRRs) at the C-terminus (Xiao and Jang, 2000) which are required for EIN3 binding (Guo and Ecker, 2003). The strong sequence similarity and domain identity among *SI-EBF1*, *SI-EBF2*, *At-EBF1*, *At-EBF2*, *Pt-EBF3*,

and *Pt-EBF4*, as well as the phenotypes of silenced plants strongly suggest that *SI-EBF1* and *SI-EBF2* encode two functional F-box proteins belonging to the EBF subfamily. In line with these data, phylogenetic analysis clearly indicated that among all F-box-related proteins across eukaryote organisms, *SI-EBF1* and *SI-EBF2* cluster within the EBF branch of the F-box protein super-family.

Phenotypes of single and co-silenced plants revealed functional redundancy among *SI-EBF1* and *SI-EBF2* proteins and suggest that the two F-box proteins work synergistically in the tomato. This is first supported by the growth phenotypes of single gene-silenced plants for either *SI-EBF1* or *SI-EBF2* that were indistinguishable from control plants. Functional complementation of the two *EBF* genes is also sustained by the strong growth phenotypes displayed by co-silenced plants down-regulated in the expression of both *SI-EBF1* and *SI-EBF2* genes. It has been similarly shown in *Arabidopsis* that two F-box proteins work synergistically in ethylene signalling transduction (Gagne *et al.*, 2004). In addition to functional redundancy, the data reveal the presence of a compensation mechanism that allows single gene-silenced plants to up-regulate the expression of the second *EBF* gene. That is, *SI-EBF2* transcript accumulation is enhanced in *SI-EBF1*-silenced plants compared with control plants and, likewise, the level of *SI-EBF1* transcripts in *SI-EBF2*-silenced lines is higher than in non-silenced plants. In single gene-silenced tomato lines the compensation mechanism may therefore be essential to maintain a threshold level of *EBF* transcripts similar to that in wild-type plants. The adjustment of *SI-EBF1/2* transcript levels may operate through a negative feedback loop. The negative feedback hypothesis is in agreement with the data showing that over-expression of *At-EBF1* in *Arabidopsis* results in the down-regulation of endogenous *At-EBF1* and *At-EBF2* (Potuschak *et al.*, 2003). Nevertheless, even though functional redundancy is likely to be responsible for the absence of strong visible growth phenotypes in single gene-silenced plants, the presence of mild phenotypes in these lines such as lower flowering capacity, premature fruit ripening, and fertility defect are indicative of partial functional redundancy among the two tomato EBF proteins. Taken together, these data suggest that both *SI-EBF1* and *SI-EBF2* are necessary for controlling normal tomato growth, especially, for regulating senescence, floescence, fertility, and fruit ripening. The combined importance of both *SI-EBF1* and *SI-EBF2* in ethylene action, plant growth, and fruit ripening was strikingly evident in co-silencing plants, which showed severely dwarfed growth, curled leaves, a pale green stem, reduced fertility, early senescence, and accelerated fruit ripening (Figs 5, 6).

While the role of *SI-EBF1* and *SI-EBF2* in controlling tomato plant growth and development was mainly inferred from the phenotypes of co-silenced lines, their expression patterns clearly hints at their involvement in reproductive organs with *SI-EBF2* displaying, however, the most dynamic pattern of expression during crucial phases of flower and fruit development. The expression of *SI-EBF1*

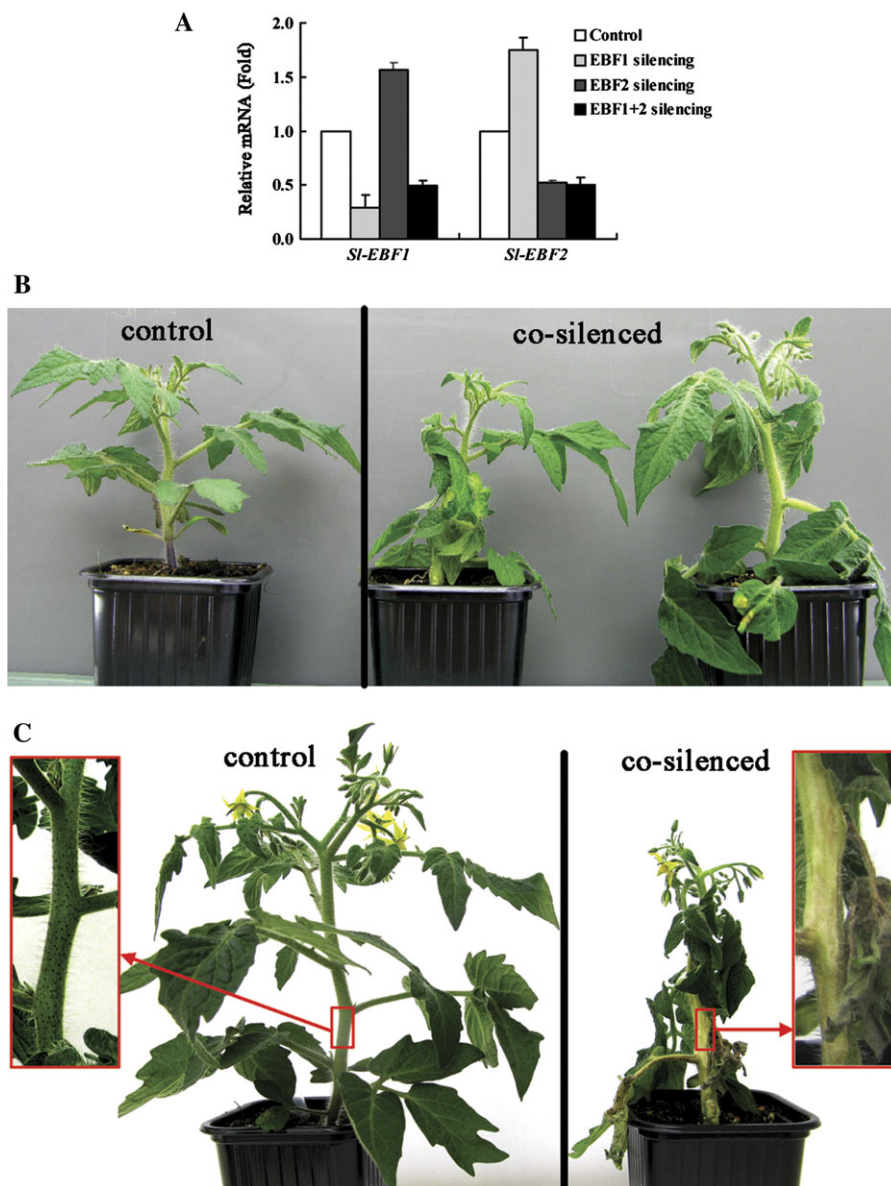


Fig. 5. Ethylene-related phenotypes associated with silencing of the *SI-EBF1* and *SI-EBF2* genes. Gene silencing was confirmed at the molecular level by Q-PCR (A). Ethylene-associated phenotypes in *SI-EBF1* and *SI-EBF2* co-silenced (right) and control non-silenced (left) tomato plants (B, C). Control non-silenced and EBF-silenced plants were generated via infiltration with pTRV2 empty and pTRV2-SIEBF1-SIEBF2 vectors, respectively. Data of Q-PCR are expressed as relative values, based on the values of the control taken as the reference sample set to 1. Each value represents mean \pm standard error of three replicates.

and *SI-EBF2* (Fig. 3C, D) is up-regulated during the transition from bud to anthesis and then decreases dramatically at the post-anthesis stage, coinciding with the initiation of fruit set. The expression of the two genes was also sharply enhanced at the onset of fruit ripening (Fig. 3E, F), especially that of *SI-EBF2*, suggesting that tomato *EBF* genes are key components in modulating ethylene responses in tissues and organs where this hormone is needed, such as for stimulating flower opening and fruit ripening. To get a better insight into the mechanism by which EBF proteins regulate ethylene signalling, it is important to discover whether EBF1 and EBF2 have preferential EIL targets. However, this will require the use of specific antibodies against different members of the tomato EIL protein family

that are not yet available. It was reported recently that the ethylene signal transduction pathway in *Arabidopsis* is controlled by a negative feedback regulation between EBF2 and EIN3, where EIN3 targets the promoter of *EBF2* to modulate its expression level thus allowing fine-tuning of ethylene responses (Binder *et al.*, 2007; Konishi and Yanagisawa, 2008). In this model, an ethylene signal elevates the levels of EIN3 protein, and the resulting accumulation of EIN3 induces the expression of EBF2. Then EBF2 promotes the degradation of EIN3 and hence down-regulates ethylene signalling, allowing for a rapid recovery after ethylene removal (Konishi and Yanagisawa, 2008). Both *SI-EBF1* and *SI-EBF2* are induced by exogenous ethylene in tomato seedlings with *SI-EBF2* being by

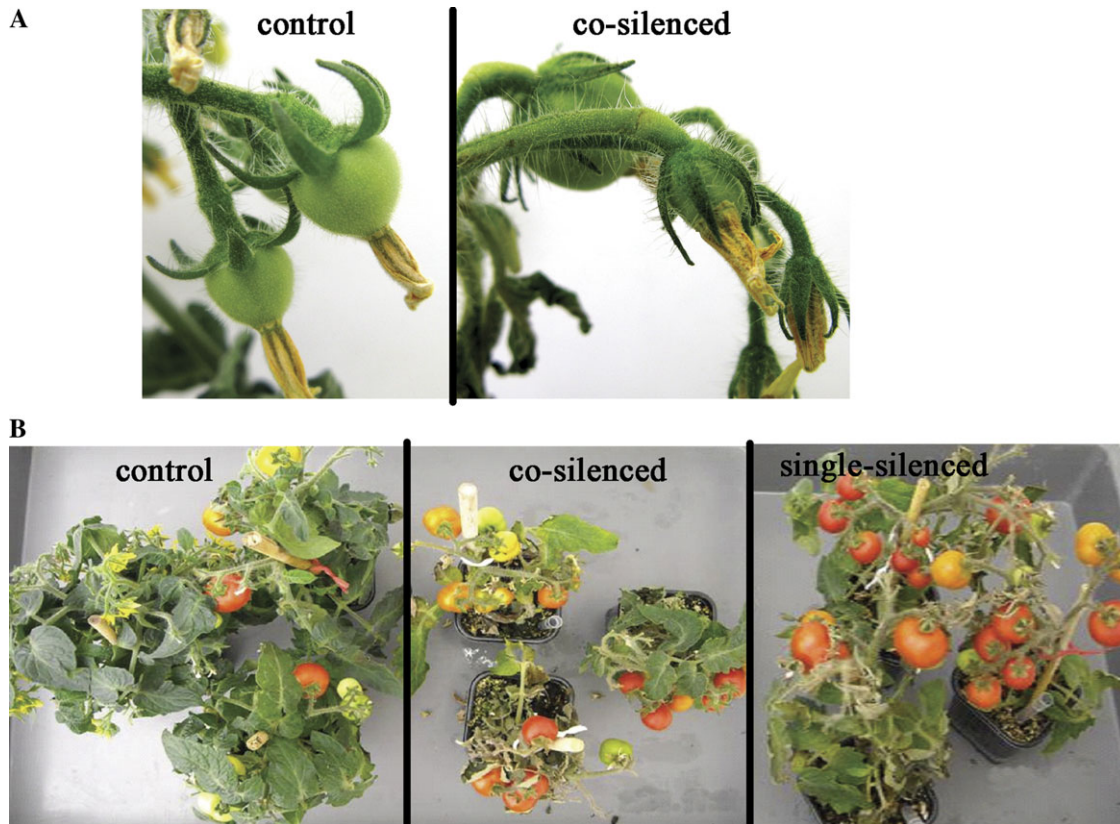


Fig. 6. Phenotypes affecting fruit development and ripening in *SI-EBF1* and *SI-EBF2* silenced plants. Droop of fruit phenotype in *SI-EBF1* and *SI-EBF2* co-silenced (right) and control non-silenced (left) tomato plants (A). Accelerated fruit ripening and dwarf phenotype of EBF silenced lines (B).

far the most strongly up-regulated upon hormone treatment (Fig. 4A). Differential responsiveness to ethylene of *Arabidopsis EBF* genes was also reported, leading to the hypothesis that EBF1 plays the main role in the baseline ubiquitination, while EBF2 is more important once ethylene signalling is engaged and during recovery after hormone withdrawal (Potuschak *et al.*, 2003; Gagne *et al.*, 2004; Binder *et al.*, 2007).

Cross-talk between ethylene and auxin has been reported to be important for the regulation of several biological processes, such as hypocotyls elongation (Smalle *et al.*, 1997), root growth (Růžička *et al.*, 2007), root hair growth and differentiation (Pitts *et al.*, 1998), and differential growth (Chaabouni *et al.*, 2009a, b). However, only a few molecular actors involved in the interaction between these two signalling pathways have been identified so far. In addition to acting independently on the same target genes, ethylene and auxin can also regulate each other's biosynthesis and response pathways. Ethylene can regulate auxin biosynthesis through the activation of anthranilase synthase subunits catalysing the first step in tryptophan biosynthesis (Stepanova *et al.*, 2005; Chilly *et al.*, 2006; Swarup *et al.*, 2007) and, reciprocally, auxin controls ethylene biosynthesis through the activation of ACC synthase genes (Stepanova *et al.*, 2007). More recently, it was reported that *SI-IAA3*, a typical auxin transcriptional regulator, is an integral regulator of auxin and ethylene

Table 2. Reduced flower formation and accelerated fruit ripening in EBF-silenced tomato plants

The total flower number included bud, flower, and fruit and was counted at the full flowering stage of control non-silenced plants transfected with the pTRV empty vector. The data are means \pm standard error of three replicates with at least six plants for assessing flower number and 15 fruits for the calculation of days from pollination to breaker in each replicate.

	Flower number	Days from pollination to breaker of fruits
Control	30 \pm 6	42 \pm 2
<i>SI-EBF1</i> silenced	20 \pm 4	33 \pm 3
<i>SI-EBF2</i> silenced	18 \pm 5	33 \pm 4
Co-silenced	9 \pm 3	30 \pm 3

responses in tomato plants and that its down-regulation in the tomato results in both auxin and ethylene-associated phenotypes (Chaabouni *et al.*, 2009a). The sharp regulation of both *SI-EBF1* and *SI-EBF2* by auxin reported here (Fig. 4B), may define a new potential molecular site for the interaction between ethylene and auxin. While, so far, auxin has been shown to impact ethylene responses mainly by controlling components of ethylene biosynthesis, the present data suggest that *EBF* genes might represent a target component of the ethylene signalling pathway that

integrates both hormone signalling pathways. Generation of stable tomato mutants altered in the expression of *EBF* genes will provide dedicated biological resources for validating and better defining the auxin-dependent developmental responses requiring *SI-EBF* genes.

While most studies devoted so far to *EBF* genes have focused on their role in regulating ethylene responses in the plant model *Arabidopsis*, the present study uncovered the role of two tomato *EBF* genes in regulating crucial stages of flower and fleshy fruit development. Moreover, the data strongly suggest that protein degradation via the ubiquitin/26S proteasome pathway is a control point of fruit ripening, thus adding a new layer to the well-documented regulation of fruit ripening at the genetic and transcriptional levels (Giovannoni, 2007; Seymour *et al.*, 2008), and hence opens new leads for engineering fruit ripening.

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