



A conserved phosphorylation site regulates the transcriptional function of ETHYLENE-INSENSITIVE3-like1 in tomato

Ji Li, Zhengguo Li, Ling Tang, Yingwu Yang, Mohamed Zouine, Mondher Bouzayen

► To cite this version:

Ji Li, Zhengguo Li, Ling Tang, Yingwu Yang, Mohamed Zouine, et al.. A conserved phosphorylation site regulates the transcriptional function of ETHYLENE-INSENSITIVE3-like1 in tomato. *Journal of Experimental Botany*, 2011, Vol. 63 (1), pp. 427-439. 10.1093/jxb/err289 . hal-00793397

HAL Id: hal-00793397

<https://hal.science/hal-00793397>

Submitted on 22 Feb 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: 8061

To link to this article: DOI: 10.1093/jxb/err289

URL: <http://dx.doi.org/10.1093/jxb/err289>

To cite this version: Li, Ji and Li, Zhengguo and Tang, Ling and Yang, Yingwu and Zouine, Mohamed and Bouzayen, Mondher *A conserved phosphorylation site regulates the transcriptional function of ETHYLENE-INSENSITIVE3-like1 in tomato*. (2011) Journal of Experimental Botany, Vol. 63 (n° 1). pp. 427-439. ISSN 0022-0957

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

A conserved phosphorylation site regulates the transcriptional function of ETHYLENE-INSENSITIVE3-like1 in tomato

Ji Li¹, Zhengguo Li^{1,*}, Ling Tang¹, Yingwu Yang¹, Mohamed Zouine^{2,3} and Mondher Bouzayen^{2,3,*}

¹ Genetic Engineering Research Center, College of Bioengineering, Chongqing University, Chongqing 400044, PR China

² Université de Toulouse, INP-ENSA Toulouse, Génomique et Biotechnologie des Fruits, Avenue de l'Agrobiopole BP 32607, Castanet-Tolosan F-31326, France

³ INRA, UMR990 Génomique et Biotechnologie des Fruits, Chemin de Borde Rouge, Castanet-Tolosan F-31326, France

* To whom correspondence should be addressed. E-mail: zhengguoli@cqu.edu.cn or bouzayen@ensat.fr

Abstract

ETHYLENE-INSENSITIVE3/ETHYLENE-INSENSITIVE3-like (EIN3/EIL) transcription factors are important downstream components of the ethylene transduction pathway known to regulate the transcription of early ethylene-responsive genes in plants. Previous studies have shown that phosphorylation can repress their transcriptional activity by promoting protein degradation. The present study identifies a new phosphorylation region named EPR1 (EIN3/EIL phosphorylation region 1) in tomato EIL1 proteins. The functional significance of EPR1 was tested by introducing mutations in this region of the *Sl-EIL1* gene and by expressing these mutated versions in transgenic tomato plants. Transient expression data and phenotypic analysis of the transgenic lines indicated that EPR1 is essential for the transcriptional activity of *Sl-EIL1*. Moreover, mutation in the EPR1 site that prevents phosphorylation abolishes ethylene constitutive responses normally displayed by the *Sl-EIL1*-overexpressing lines. Bimolecular fluorescence complementation (BiFC) studies showed that the presence of a functional phosphorylation site within EPR1 is instrumental in the dimerization of *Sl-EIL1* proteins. The results illuminate a new molecular mechanism for the control of EIN3/EIL activity and propose a model where phosphorylation within the EPR1 promotes the dimerization process allowing the initiation of EIL-mediated transcription of early ethylene-regulated genes.

Key words: EIN3/EIL, EPR1, ethylene signalling, phosphorylation, transcriptional function.

Introduction

Ethylene is an important plant hormone known to regulate a wide range of plant developmental processes, including seed germination, seedling growth, lateral root development, leaf and flower abscission, fruit ripening, organ senescence, and responses to biotic and abiotic stresses (Brown, 1997; Lelievre *et al.*, 1997; Morgan and Drew, 1997; Smalle and Van Der Straeten, 1997). Most components of the ethylene transduction pathway have been uncovered primarily in the model plant *Arabidopsis thaliana* (Guo and Ecker, 2004), revealing a linear cascade of events

that leads to the activation of transcription factors belonging to the ETHYLENE INSENSITIVE3/EIN3-like (EIN3/EIL) family (Chao *et al.*, 1997; Solano *et al.*, 1998). EIN3/EILs are positive regulators of the ethylene signalling pathway that act as transactivating factors to trigger ethylene responses mainly via the regulation of ethylene response factor (ERF) genes known to be their downstream targets. *Arabidopsis ein3* and *eil* loss-of-function mutants show severe ethylene-insensitive phenotypes (Roman *et al.*, 1995), while *EIN3* overexpression confers ethylene

constitutive response in *Arabidopsis* (Chao *et al.*, 1997). Following the isolation of four *EIN3/EIL* genes in *Arabidopsis* (Chao *et al.*, 1997), cDNAs encoding EIL proteins have been cloned from various species, revealing the ubiquitous nature of these transcriptional regulators in the plant kingdom. In tomato, four EIL genes were cloned and designed as *Sl-EIL1*, *Sl-EIL2*, *Sl-EIL3* (Tieman *et al.*, 2001), and *Sl-EIL4* (Yokotani *et al.*, 2003).

As with other plant hormones, responses to ethylene vary widely and can be in some cases opposite, depending on the tissue and on the developmental stages taken into consideration (Chang *et al.*, 1993; Kieber *et al.*, 1993; Lehman *et al.*, 1996; Chao *et al.*, 1997; Alonso *et al.*, 1999). It has been suggested that temporal and spatial specificity of ethylene responses arise downstream of *EIN3/EILs* at the level of ERFs based on the important size of the ERF gene family and the distinctive expression patterns of its members (Riechmann and Meyerowitz, 1998; Sharma *et al.*, 2011). *EIN3/EIL* proteins were shown to bind in a sequence-specific manner to the primary ethylene-response element (PERE) of the *ERF* genes. This binding triggers the primary ethylene response through a transcriptional cascade that first includes the activation of target ERF genes which in turn modulate the expression of ethylene-responsive genes (Solano *et al.*, 1998). The *EIN3/EIL* genes are functionally redundant and even though their expression is not regulated by ethylene at the transcriptional level (Tieman *et al.*, 2001), it is largely accepted that *EIN3/EIL* proteins are the first triggers of the *ERF* super gene family that are responsible for most of the ethylene responses (Riechmann *et al.*, 2000; Zhuang *et al.*, 2008).

Functional domains within *EIN3/EIL* proteins have been identified, revealing that the DNA-binding domains (DBDs) of At-*EIN3* (*Arabidopsis thaliana* *EIN3*) and T-*EIL* (tobacco *EIN3*-like) are well conserved, showing structural and functional similarities (Kosugi and Ohashi, 2000; Yamasaki *et al.*, 2005). This conserved DBD is also found in all *EIN3* homologues examined so far in other plant species. The DBD of *EIN3/EIL* specifically binds to the *EIN3*-binding site (EBS) located in the promoter region of ethylene-regulated genes. Moreover, experimental evidence supports the ability of *EIN3/EIL* proteins to form a homodimer consistent with the presence of a dimerization domain residing between amino acids 113 and 257 within the At-*EIN3* protein (Solano *et al.*, 1998; Yamasaki *et al.*, 2005). However, it was shown that the interaction of *EIN3* with the target DNA is not required for protein dimerization (Solano *et al.*, 1998).

Protein phosphorylation is an important and widely conserved mechanism underlying the regulation of a variety of biological processes including the ethylene signalling pathway. Indeed, ethylene transduction is activated in its upstream part through a phosphorylation cascade involving specific mitogen-activated protein kinase (MAPK) kinases (Raz and Fluhr, 1993; Mishra *et al.*, 2006; Yoo and Sheen, 2008; Stepanova and Alonso, 2009). Phosphorylation-dependent regulation of ethylene responses has also been demonstrated at the level of *EIN3/EIL* (Yamamoto *et al.*,

1999; Yoo *et al.*, 2008). Phosphorylation of two different threonine residues has opposite effects on *EIN3* stability, with phosphorylation of the T174 residue, mediated by the MKK9 cascade, leading to protein stabilization, whereas the phosphorylation occurring at the T592 residue, mediated by a CTR1/MAPK pathway, promotes protein degradation (Yoo *et al.*, 2008). These data highlight the importance of protein phosphorylation in regulating the activity of transcription factors.

Extending understanding on the role of phosphorylation in regulating the activity of *Sl-EIL1*, the present study uncovers a new phosphorylation site located in the region encompassing amino acid residues 92–95 of the tomato *EIL1* protein (*Sl-EIL1*) that is essential for the transcriptional activity. Experimental evidence is provided showing that mutation in this functional domain, named *EPR1* (*EIN3/EIL* phosphorylation region 1), causes a complete loss of the ability of *Sl-EIL1* to activate transcription of target genes. The requirement for a functional *EPR1* phosphorylation domain is further emphasized by revealing that *EPR1* is necessary for the dimerization of *Sl-EIL1* proteins.

Materials and methods

Plant materials and growth conditions

Tomato plants (*Solanum lycopersicum*, Micro-Tom) were grown in a greenhouse under the following conditions: 14 /10 h light/dark cycle, 25/20 °C day/night temperature, 80% humidity, 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity. Seedlings were grown in a culture chamber in 50% Murashige and Skoog (MS) culture medium with 0.8% agar under the same conditions as above. Leaves were collected from 8-week-old tomatoes, and 5-day-old seedlings were harvested for quantitative RT-PCR (qRT-PCR).

Sequence analysis

Amino acid sequence alignments were performed using the DNAMAN (v5.2.2) program assisted by manual adjustment. The conserved phosphorylation sites were analysed by the Group-based Prediction system (GPS 2.1) program (Zhou *et al.*, 2004) and ExPaSy online prediction tools (<http://www.expasy.ch/prosite/>) (Sigrist *et al.*, 2010). GenBank accession numbers for the sequences analysed are as follows: *S. lycopersicum* *Sl-EIL1* (AAK58857.1), *S. lycopersicum* *Sl-EIL2* (AAK58858.1), *S. lycopersicum* *Sl-EIL3* (AAK58859.1), *S. lycopersicum* *Sl-EIL4* (BAC99307.1), *A. thaliana* At-*EIN3* (AAC49749.1), *Vigna radiate* Vr-*EIL1* (AAL76272.1), *Nicotiana tabacum* T-*EIL* (BAA74714.1), *Oryza sativa* Os-*EIL1* (AAZ78349.1), *Musa acuminata* Ma-*EIL2* (BAF44108.1), and *Dianthus caryophyllus* Dc-*EIL1* (AAF69017.1).

H-89 treatment

To perform H-89 treatment, seedlings were grown in 50% MS culture medium under dark conditions with 50 μM H-89 (Beyotime, China) dissolved with dimethylsulphoxide (DMSO). The control seedlings were grown concomitantly in 50% MS to which was added the same volume of DMSO as for the seedlings treated with H-89. After 7 d, the developing seedlings were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. All the treatments were repeated three times in three independent experiments.

Gene expression analysis

Total RNAs were isolated using Trizol (Invitrogen, USA) according to the manufacturer's instruction, and were treated with DNase I (Fermentas, UK) for 15 min at 37 °C and purified following the description in the handbook. The first-strand cDNA synthesis was performed using a First Strand cDNA Sythesis Kit (Fermentas). qRT-PCR was performed as described previously (Yang *et al.*, 2010) using SYBR GREEN PCR Master Mix (Fermentas) on an ABI PRISM 7900HT sequence detection system (ABI, USA). *SL-actin-51* (GenBank accession no. Q96483) was used as a reference gene with constitutive expression in various tissues. To determine relative fold differences for each sample in each experiment, the Ct value for the *SL-EIL1*, *SL-ERF1*, *SL-ERF2*, *SL-EBF1*, and *SL-EBF2* transcripts was normalized to the Ct value for *SL-Actin-51* and was calculated relative to a calibrator using the formula $2^{-\Delta\Delta C_t}$. The primer sequences used are listed in Table 1.

Site-specific mutagenesis

Mutagenesis was performed using megaprimer PCR (Kammann *et al.*, 1989). The megaprimers were synthesized using Primer-STAR DNA polymerase (Takara, China) in a 25 µl reaction under the following reaction conditions: 98 °C for 15 s, 68 °C for 30 s, for a total of 25 cycles. Tomato cDNA was used as template. The megaprimers were purified by a DNA Gel Extraction Kit (Omega) according to the manufacturer's instructions. A 500 ng aliquot of each megaprimer was used for synthesis of the mutated version of genes in a 50 µl reaction under the following reaction conditions: 98 °C for 15 s, 60 °C for 30 s, 72 °C for 2 min, for a total of 30 cycles using PrimerSTAR DNA polymerase (Takara). The primer sequences used are listed in Table 1.

Plant transformation

To generate transgenic plants, *SL-EIL1*, *SL-EM1*, and *SL-EM2* were cloned into the modified binary vector pCambia1301 (kanamycin gene instead of the hygromycin gene) with the *Cauliflower*

mosaic virus (CaMV) 35S promoter. Transgenic plants were generated by *Agrobacterium tumefaciens*-mediated transformation (Wang *et al.*, 2005). Transformed lines were first selected on kanamycin (100 mg l⁻¹) and then analysed by PCR to verify the presence of T-DNA insertions in the various transgenic lines.

Protoplast isolation

Tobacco BY-2 cells were incubated for 1.5–2 h at 37 °C in solution containing 1% cetylase, 0.2% pectolyase Y-23, 1% bovine serum albumin (BSA), and 0.55 M D-mannitol, in an agitated water bath (30–40 rpm min⁻¹). Tobacco protoplasts were purified as described by Abel and Theologis (1994).

Transient gene expression assay and bimolecular fluorescence complementation (BiFC) assay

SL-EIL1, *SL-EM1*, and *SL-EM2* were fused with GFP (green fluorescent protein) into the pGreen vector (Hellens *et al.*, 2000) as a C-terminal fusion protein expressed under the control of the CaMV 35S promoter. The E4 promoter was cloned into a modified binary vector pGreen-RFP [the red fluorescent protein (RFP) gene instead of the GFP gene] and replaced the CaMV 35S promoter (Fig. 2C). The pGreen-RN159 and pGreen-RC160 plasmids were modified from the pGreen vector according to the RFP BiFC system (Fan *et al.*, 2008). RFP was split up into two fragments: an N-terminal fragment (RFP amino acids 1–159) and a C-terminal fragment (RFP amino acids 160–237). The BiFC constructs are shown in Fig. 4. The plasmids in pairs were co-transformed into protoplasts. Typically, 0.3 ml of tobacco protoplast suspension (0.3 × 10⁶ cells) was transfected with 50 µg of shared salmon sperm carrier DNA and 30 µg of each plasmid DNA. Transfected protoplasts were incubated at 25 °C for at least 16 h and were analysed for fluorescence by confocal microscopy (Zeiss LSM 510 META, Germany; Leica Tcs spz AoBs, Germany). The samples were illuminated with an argon ion laser. The GFP excitation wavelength was 488 nm and was detected at 500 nm; the RFP excitation wavelength was 558 nm and it was detected at 583 nm. All transient expression assays were repeated at least three times.

Fluorescence intensity assay

Fluorescence data from 100 infected protoplasts from each sample were collected. The mean fluorescence intensity was measured by image pro plus software (v6.0). RFP fluorescence was scanned by a fluorescence microscope (Olympus, Japan) and photographed by a colour CCD camera (Phenix, Japan) under the same conditions: magnification × 200 and exposure time 150 µs. Figure 3A shows part of these images.

Results

Identification of a new conserved phosphorylation site associated with the transcriptional activation domain of *SL-EIL1*

The search for putative phosphorylation sites within *SL-EIL1* protein performed with the GPS 2.1 program and ExPaSy online prediction tools identified two putative sites for protein kinase A (PKA) and six putative sites for MAPK (Table 2). Of particular interest, amino acid sequence alignments of EIN3/EIL proteins from seven plant species revealed a highly conserved region located close to the N-terminal DBD of the EIN3/EIL protein (Fig. 1A). This glutamine-rich (Q-rich) region contains a putative PKA phosphorylation site located at amino acids 92–95

Table 1. The primer sequences used in qRT-PCR to monitor transcript accumulation corresponding to *SL-EIL1* and selected ethylene-responsive genes

Primer	Primer sequence	Orientation	Use
ACT-F	TGTCCTATTTACGAGGGTTATGC	Sense	qRT-PCR
ACT-R	CAGTTAAATCACGACCAGCAAGAT	Antisense	qRT-PCR
EIL1-QF	CGGAAATCTACCTAG CATTGGATAC	Sense	qRT-PCR
EIL1-QR	ACTTCTGGCTGGCTAGTACAAA	Antisense	qRT-PCR
ERF1-QF	CGCCTAAGAGGAGGAGAAAG	Sense	qRT-PCR
ERF1-QR	GCTCGCCAACCTGGAACAT	Antisense	qRT-PCR
ERF2-QF	AAGGGGTTAGGGTTTGTTAGG	Sense	qRT-PCR
ERF2-QR	CAAGCAATGTTCAAGGGAGGG	Antisense	qRT-PCR
EBF1-QF	ATTGCCATCACTGACATAGC	Sense	qRT-PCR
EBF1-QR	AGTTATAGCAAGCGACCTC	Antisense	qRT-PCR
EBF2-QF	ATGTGATGGATACCTTACCAG	Sense	qRT-PCR
EBF2-QR	CCGACATTAGTAATACCACGA	Antisense	qRT-PCR
EIL1-F	TTTGATCCTCCTGTGGAAG ATGATGATG	Sense	Gene cloning
EIL1-R	TGTCAACAGACTTCTGGCTG	Antisense	Gene cloning
EM1-R	TGTGCCCTTCTCCTCTTAGAC CTCCTCG	Antisense	Mutagenesis
EM2-R	CCATCCTGTGCCCTAGCCATCT TCTTCC	Antisense	Mutagenesis

Table 2. Predicted phosphorylation sites in the SI-EIL1 protein

The phosphorylation sites are predicted by the Group-based Prediction system (GPS 2.1) program and ExPASy online prediction tools and are found in highly conserved regions. The position within the protein sequence is given by the numbers. EPR1 is underlined.

Highly conserved peptide	Position	Kinase family	Comment
RQSQEQA	85	PKA	PhosphAT prediction
<u>RKKMSRA</u>	95		PhosphAT prediction/ExPASy Prosit analysis
GASDNLR	133		PhosphAT prediction
AKYQAD	157	MAPK	PhosphAT prediction
GTPHTL	176		PhosphAT prediction/ reported (Yoo <i>et al.</i> , 2008)
QDTTLGS	187		PhosphAT prediction
ESATWLA	289	MAPK	PhosphAT prediction
RELYPD	305		PhosphAT prediction
GSPFNI	580		PhosphAT prediction/ reported (Yoo <i>et al.</i> , 2008)

and named here EPR1. The position of EPR1 within the vicinity of the transcriptional activation domain of SI-EIL1 (Gerber *et al.*, 1994; Fryer *et al.*, 2002; Kim *et al.*, 2007) raises the hypothesis of its potential contribution to the functional properties of the protein. Therefore, the functional significance of this phosphorylation site was addressed using combined cellular and reverse genetic approaches.

The EPR1 domain is essential for the transcriptional activity of SI-EIL1 protein

To gain insight on the function of EPR1, two individual mutant versions of the *SI-EIL1* gene were generated. *SI-EIL1-Mutant1* (*SI-EM1*) and *SI-EIL1-Mutant2* (*SI-EM2*) were constructed by site-directed mutagenesis to introduce nucleotide substitutions within the EPR1 coding sequence (nucleotides 276–285). The introduced nucleotide substitutions in *SI-EM1* resulted in the change of the native PKA phosphorylation site (KKMS) into a protein kinase C (PKC) phosphorylation site (SKRR) (Fig. 1B). In *SI-EM2*, the mutation of the same nucleotides resulted in a complete loss of the EPR1 phosphorylation site (amino acids KKMA) (Fig. 1B). The impact of these mutations on the subcellular targeting of SI-EIL1 protein was tested using transient expression experiments in a single cell system. The coding sequences of *SI-EIL1*, *SI-EM1*, and *SI-EM2* were fused in-frame with the GFP coding sequence and expressed in tobacco protoplasts under the control of the constitutive CaMV 35S promoter (Fig. 1C). The data clearly indicated that none of the mutations in the EPR1 domain of SI-EIL1 prevents or alters the nuclear localization of the protein, suggesting that this domain is not essential for the proper targeting of SI-EIL1 (Fig. 2A).

To assess whether the mutated versions of SI-EIL1 bearing a non-functional or a modified EPR1 domain retain the

ability to direct transcription of target genes, the promoter of the E4 ethylene-responsive gene (Montgomery *et al.*, 1993) was used as the native EBS. The E4 promoter was fused to the RFP coding sequence (Shaner *et al.*, 2004) and used in a transactivation assay as a reporter construct for assessing the transcription activities of SI-EIL1, SI-EM1, and SI-EM2. In control tobacco protoplasts transfected with the *E4::RFP* reporter construct alone, no RFP expression was detected (Fig. 2B, bottom panel), whereas co-transfection of this reporter construct with an effector construct carrying the *EIL1::GFP* fusion under the control of the constitutive CaMV 35S promoter resulted in a net activation of RFP expression (Fig. 2B, upper panel), showing that SI-EIL1 is capable of activating the E4 promoter. Likewise, the mutated SI-EM1 protein retains the capacity to induce the expression of the reporter gene under the control of the native E4 ethylene-responsive promoter (Fig. 2B, second panel from the top). In contrast, co-transfection with the SI-EM2 construct failed to induce any detectable expression of the *RFP* reporter gene (Fig. 2B, second panel from the bottom), indicating that this mutation in the EPR1 domain results in a complete loss of the transcriptional activity of SI-EIL1 protein. Interestingly, although SI-EM1 has been mutated in EPR1, the protein retained its transcriptional activation function, though the mean fluorescence intensity indicated that the SI-EM1-mediated RFP expression was significantly lower than that induced by SI-EIL1 (Fig. 3). These data strongly suggest that phosphorylation is pivotal to the transcriptional function of SI-EIL1. However, considering that in SI-EIL1 the EPR1 domain carries a PKA phosphorylation site while in SI-EM1 it contains a PKC phosphorylation site, it can be concluded that the transcriptional function of EIN3/EIL proteins can be activated via EPR1 phosphorylation whether this phosphorylation is performed by PKA or by PKC, even though PKA gives rise to more efficient transcriptional activity.

Phosphorylation of the EPR1 domain stimulates SI-EIL1 dimerization

It has been reported that EIN3/EIL proteins can dimerize (Solano *et al.*, 1998; Yamasaki *et al.*, 2005) and, coincidentally, EPR1 is located within the dimerization domain identified in *Arabidopsis* EIN3/EIL proteins (Fig. 1A). To investigate the putative role of the EPR1 domain in controlling the transcriptional function of SI-EIL1, its potential involvement in protein dimerization was explored. A BiFC approach was implemented to test how mutations in the EPR1 domain affect SI-EIL1 dimerization. Two BiFC plasmids were constructed (pG-RN159 and pG-RC160) carrying either the native (SI-EIL1) or mutated (SI-EM1 and SI-EM2) versions of the EIL1 coding sequence fused to the N- and C-terminal moieties of the RFP reporter protein (Fig. 4A). Following co-transfection into tobacco protoplasts, fluorescent analysis showed that both SI-EIL1 and SI-EM1 can form homodimers and are able to cross-interact and to form heterodimers (Fig. 4B), while SI-EM2 proteins failed to show any dimerization capability (data not shown). These data strongly support the

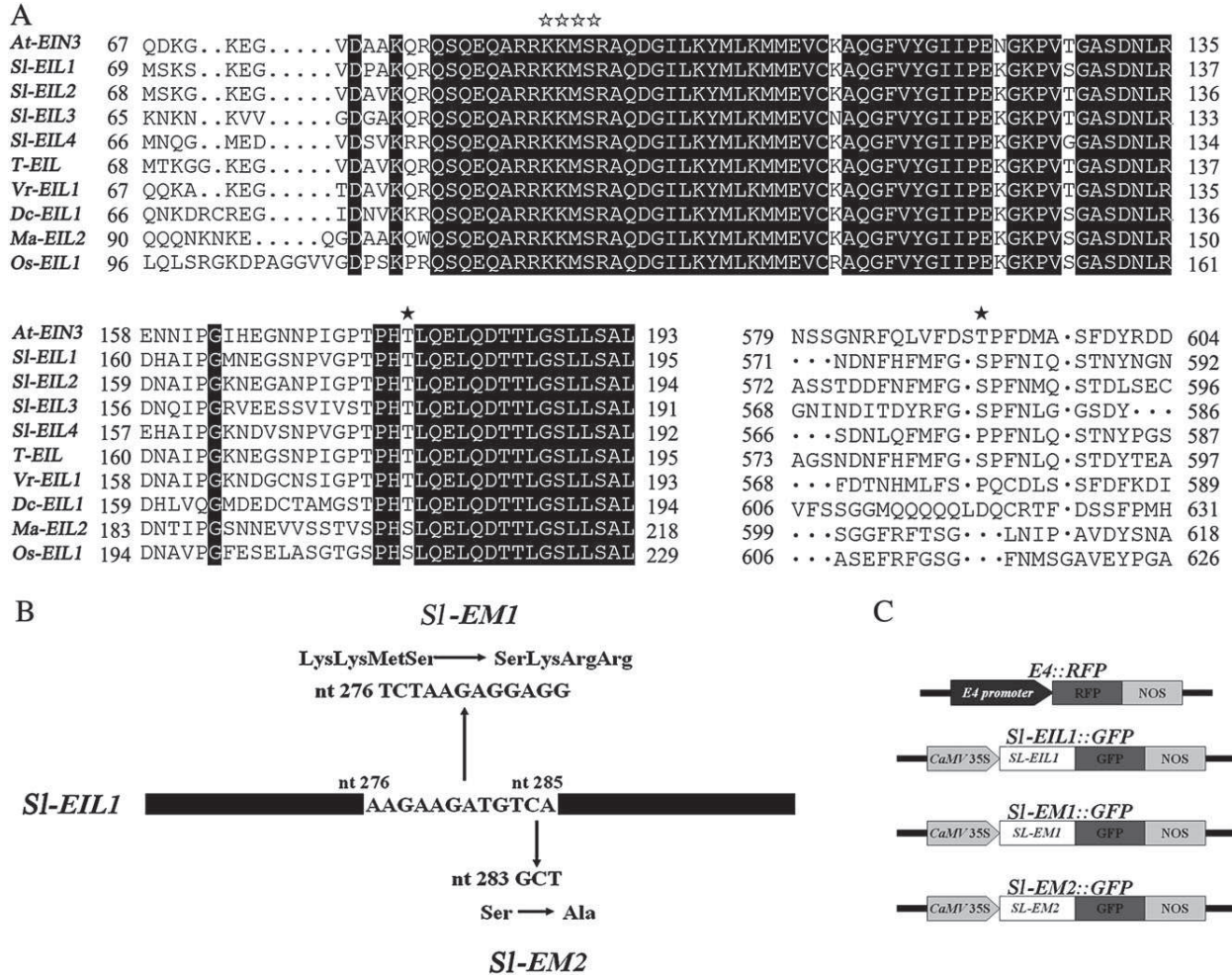


Fig. 1. Identification of a putative phosphorylation site within EIN3/EIL proteins and assessing its potential role in the nuclear targeting of *Sl-EIL1*. (A) The amino acid sequences of tomato *Sl-EIL1*, *Sl-EIL2*, *Sl-EIL4*, and *Sl-EIL4*, *Arabidopsis At-EIN3*, *tobacco T-EIL*, *Vigna radiata Vr-EIL1*, *Dianthus caryophyllus Dc-EIL1*, *Musa acuminata Ma-EIL2*, and *Oryza sativa Os-EIL1* were aligned using the DNAMAN (v5.2.2) program. Numbers show the positions of amino acid residues. Conserved residues are shaded in black. The amino acid residues KKMS marked by open stars define the putative PKA phosphorylation site predicted by the Group-based Prediction system (GPS 2.1) program and ExPaSy online prediction tools. This conserved region was named EPR1 for EIN3/EIL phosphorylation region 1. The filled stars mark amino acid residues serving as MAPK phosphorylation sites previously reported by Yoo *et al.* (2008). (B) Nucleotide substitutions leading to the loss or the modification of the phosphorylation site. The EPR1 coding sequence AAGAAGATGTCA corresponding to nucleotides 276–285 of *Sl-EIL1* was mutated to create the modified versions of *Sl-EIL1*. The introduced nucleotide substitutions in *Sl-EMI* resulted in changing the native PKA phosphorylation site into a PKC phosphorylation site (amino acids SKRR). In *Sl-EM2*, the mutation resulted in the complete loss of the EPR1 phosphorylation site (amino acids KKMA). (C) Construction of chimeric genes corresponding to the native or mutated *Sl-EIL1* coding sequences fused in-frame to the RFP reporter gene. All gene constructs are expressed under the control of the constitutive CaMV 35S promoter following cloning in the pGreen vector.

idea that a functional EPR1 is required for *Sl-EIL1* dimerization and that mutation resulting in a loss of the phosphorylation site within the EPR1 domain leads to a loss of dimerization capacity.

Phenotypes associated with the ectopic expression of native and mutated *Sl-EIL1* genes

Transgenic tomatoes (Micro-Tom) expressing either the native (*Sl-EIL1*) or the mutated (*Sl-EMI* and *Sl-EM2*) versions of the *Sl-EIL1* gene were generated and the

phenotypes related to ethylene-regulated processes were analysed. Both *EIL1*-overexpressing (*EIL1-OX*) and *EMI*-*OX* transgenic lines expressing *Sl-EIL1* and *Sl-EMI* genes, respectively, displayed constitutive ethylene response phenotypes (Fig. 5A) with dramatically enhanced flower senescence. The leaves of the *EIL1-OX* and *EMI-OX* transgenic lines presented a dark-green colour and smaller size than the wild type. Small leaves have also been reported for *Arabidopsis* EIN3-overexpressing lines (Chao *et al.*, 1997). Moreover, the *EIL1-OX* and *EMI-OX* lines showed premature fruit ripening and early senescence of sepals (Fig. 5B).

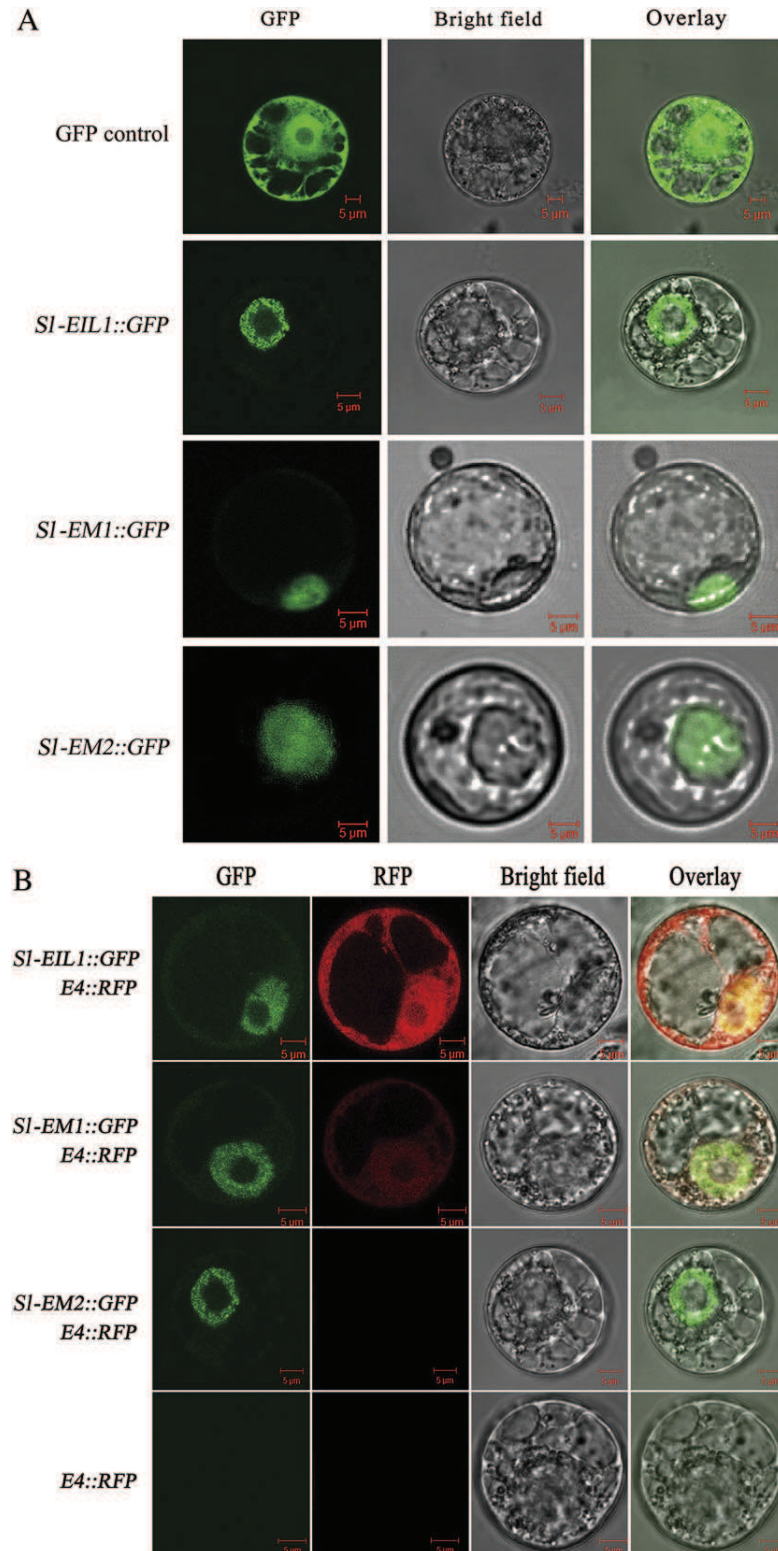


Fig. 2. Transactivation assays in a single cell system. (A) The nuclear localization of the SI-EIL1, SI-EM1, and SI-EM2 proteins fused to GFP was assessed by confocal laser scanning microscopy. The pGreen vector carrying GFP alone was used as a control. Light micrographs (middle panel) and fluorescence (left panel) images are merged (right panel) to illustrate the subcellular location of the proteins. (B) Tobacco protoplasts were transiently co-transformed with a reporter construct (*E4::RFP*) and an effector construct (*SI-EIL1::GFP*, *SI-EM1::GFP*, and *SI-EM2::GFP*). The RFP and GFP fusion proteins were analysed by confocal laser scanning microscopy. Light micrographs (the third column) and fluorescence (GFP, the first column; RFP, the second column) images are merged (the fourth column). Transient transformation with the *E4::RFP* alone was used to set the basal expression of the reporter construct.

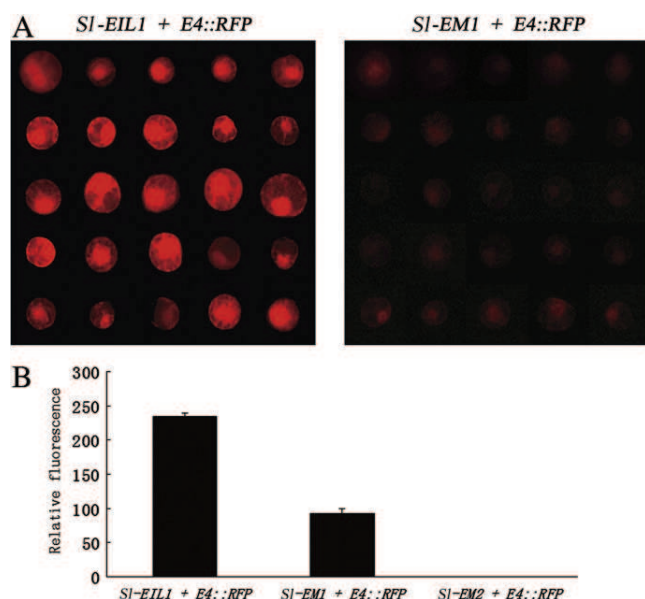


Fig. 3. Quantification of the transactivation activity of the native SI-EIL1 and mutated SI-EM1 proteins. (A) The fluorescent image was analysed by ordinary fluorescent microscopy and RFP expression was measured for individual cells expressing either SI-EIL1 (left panel) or SI-EM1 (right panel). (B) Relative fluorescent intensity was analysed and quantified by the Image-Pro-Plus (v6.0) program. The bar graph corresponds to the data obtained in A. No RFP expression was detectable with SI-EM2, so the relative fluorescence intensity in this case was estimated to be close to zero. The experiment was repeated four times.

In contrast, the *EM2-OX* lines did not show any of these ethylene-related phenotypes and are indistinguishable from wild-type tomato plants (Fig. 5A, B). Gene expression analysis performed by qRT-PCR showed that in contrast to wild-type plants, transgenic lines accumulate high levels of transcripts corresponding to *Sl-EIL1*, *Sl-EM1*, and *Sl-EM2*, attesting to the efficient ectopic expression of the transgenes (Fig. 5C). The expression of the ethylene-responsive genes *Sl-ERF1/2* and *Sl-EBF1/2* was up-regulated in *EIL1-OX* and *EM1-OX* lines but remained unaffected in *EM2-OX* lines (Fig. 5C). Taking together the phenotypes of the transgenic lines and the RT-PCR data strongly suggests that EPR1 is instrumental in the transcriptional function of SI-EIL1 and hence in the activation of ethylene response.

The constitutive ethylene phenotype of tomato SI-EIL1-overexpressing lines is released by inhibition of phosphorylation

To investigate further the physiological role of the EPR1 domain, the effect of the phosphorylation inhibitor H-89 was assessed on tomato seedlings corresponding to the wild type and to transgenic lines overexpressing either the native (*EIL1-OX*) or the mutated versions (*EM1-OX* and *EM2-OX*) of *Sl-EIL1*. The inhibitory effect of H-89 is due to its

competitive binding to the ATP pocket on the kinase catalytic subunit (Engh *et al.*, 1996). As expected, dark-grown tomato seedlings expressing the native *Sl-EIL1* gene or the EPR1-mutated version retaining a phosphorylation capacity (*EM1-OX*) showed a constitutive ethylene triple-response phenotype in the absence of ethylene treatment as attested by the strong reduction in hypocotyl and root growth and exaggerated hook curvature compared with the wild type (Fig. 6A, middle of left panel). In contrast, seedlings expressing the mutated *Sl-EIL1* gene with a non-functional EPR1 domain (*EM2-OX*) displayed a similar phenotype to wild-type seedlings (Fig. 6A, top and bottom of left panel). Treatment with H-89 resulted in enhanced root and hypocotyl growth in *EIL1-OX* and *EM1-OX* lines but not in wild-type and *EM2-OX* lines (Fig. 6A, B). These data indicate that H-89 can partially alleviate the severe constitutive ethylene-response phenotype in the *EIL1-OX* and *EM1-OX* lines, supporting the idea that phosphorylation of the EPR1 domain is required for EIL1-mediated ethylene signalling. The *EM2-OX* tomato seedlings showed the same phenotypes as the wild type whether treated or untreated by H-89 (Fig. 6A), and measurement of hypocotyl lengths showed that H-89 can repress the growth of the seedlings slightly (Fig. 6B).

To gain molecular insight into the H-89-mediated inhibition of ethylene responses, a comparative analysis of the transcriptional expression of some ethylene-responsive genes was performed in tomato seedlings expressing the native and mutated versions of the *Sl-EIL1* gene. qRT-PCR data clearly confirmed the accumulation of transcripts corresponding to *EIL-OX*, *EM1-OX*, and *EM2-OX* in transformed lines (Fig. 6C, upper panel). More interestingly, the data revealed that transcript accumulation of *Sl-ERF1* and *Sl-ERF2*, two ethylene-regulated genes, displays a dramatic increase in *EIL-OX* and *EM1-OX* lines but not in *EM2-OX* lines where the expression of *Sl-ERF1* and *Sl-ERF2* remained low and similar to that observed in wild-type lines. Likewise, *EIL-OX* and *EM1-OX* lines showed a net increase in *Sl-EBF1* and *Sl-EBF2* transcript accumulation that was not observed in *EM2-OX* lines. These data strongly indicated that EIL1 proteins lacking a functional EPR1 domain lose their ability to activate the transcription of ethylene-regulated genes. Moreover, the enhanced expression of *Sl-ERF1* and *Sl-ERF2* ethylene-responsive genes is significantly reduced upon H-89 treatment (Fig. 6C, middle and bottom panels), suggesting that SI-EIL1 might be the direct target of H-89.

Discussion

Plants must continuously adapt to changing environmental conditions in order to ensure optimal growth and development and, to this end, they rely greatly on hormonal cues to select and orchestrate the mechanism underlying the desired developmental process. The plant hormone ethylene provides, in this regard, a remarkable example illustrating the requirement for a sophisticated tuning of hormone

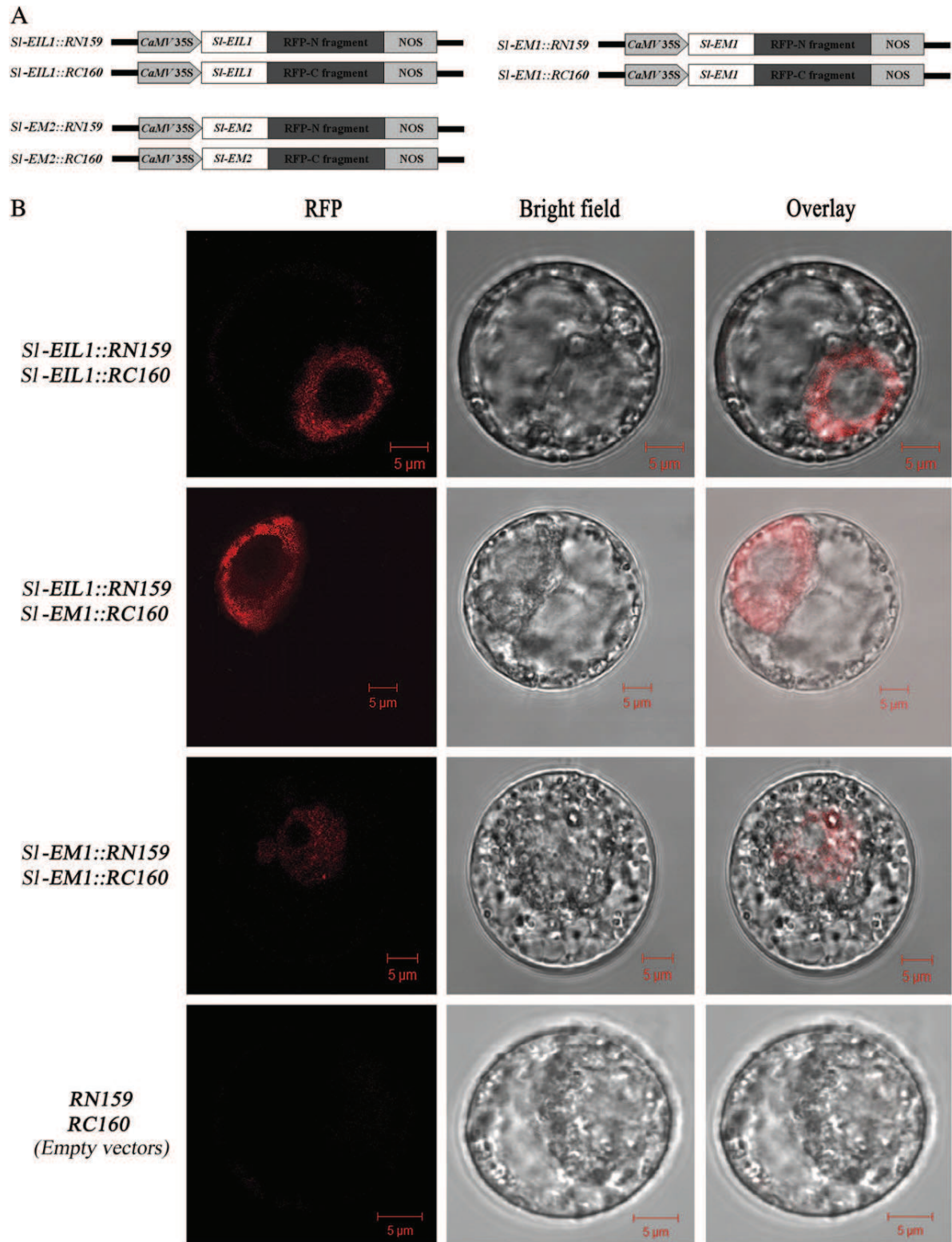


Fig. 4. Assessing the capacity of SI-EIL1, SI-EM1, and SI-EM2 proteins to form hetero- and homodimers. (A) Schematic view of the constructs used in the BiFC protein–protein interaction assays. The BiFC vectors carry one RFP moiety fused to the native or mutated versions of SI-EIL1 coding sequences. RFP was split into two moieties: the N-terminal fragment (amino acids 1–159) and the C-terminal fragment (amino acids 160–237). (B) RFP transient expression in tobacco protoplast. Cells transfected with the indicated constructs were analysed by confocal laser scanning microscopy. Homodimer formation by SI-EIL1 (top panel) and SI-EM1 (second panel from the bottom) localized in the nuclear compartment. Heterodimer formation between SI-EIL1 and SI-EM1 (second panel from the top). The empty BiFC vectors were co-transfected into protoplast as a negative control. The experiment was repeated three times.

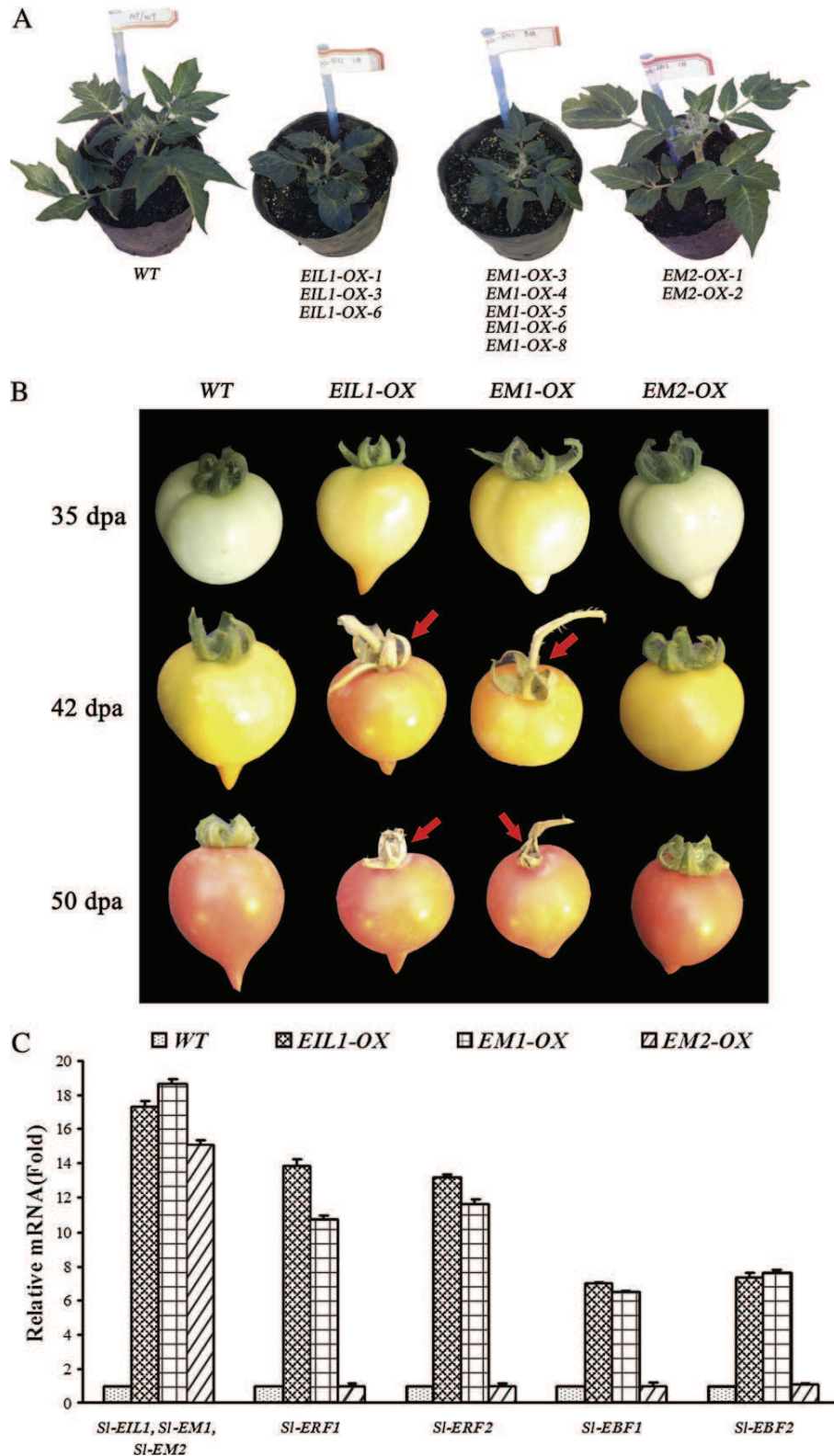


Fig. 5. Phenotyping and expression analysis of ethylene-responsive genes in transgenic tomatoes lines expressing SI-EIL1 carrying a native or mutated version of EPR1. (A) The phenotype of 1-month-old wild-type (WT), *EIL1-OX* (lines -1 and -3), *EM1-OX* (lines -3, -4, -5, -6, and -8), and *EM2-OX* (lines -1 and -2) tomato transgenic lines. (B) The fruit morphology of WT, *EIL1-OX*, *EM1-OX*, and *EM2-OX* tomato lines. Premature fruit ripening in *EIL1-OX* and *EM1-OX* transgenic lines compared with normal ripening in WT and *EM2-OX* tomato. Red arrows point to early senescence of sepals. (C) Quantitative RT-PCR analysis of transcript accumulation corresponding to *SI-EIL1*, *SI-EM1*, *SI-EM2*, and ethylene-related genes in transgenic tomatoes. Data are expressed as relative values, based on the values of the untreated WT. Each value represents the mean \pm SE of three replicates.

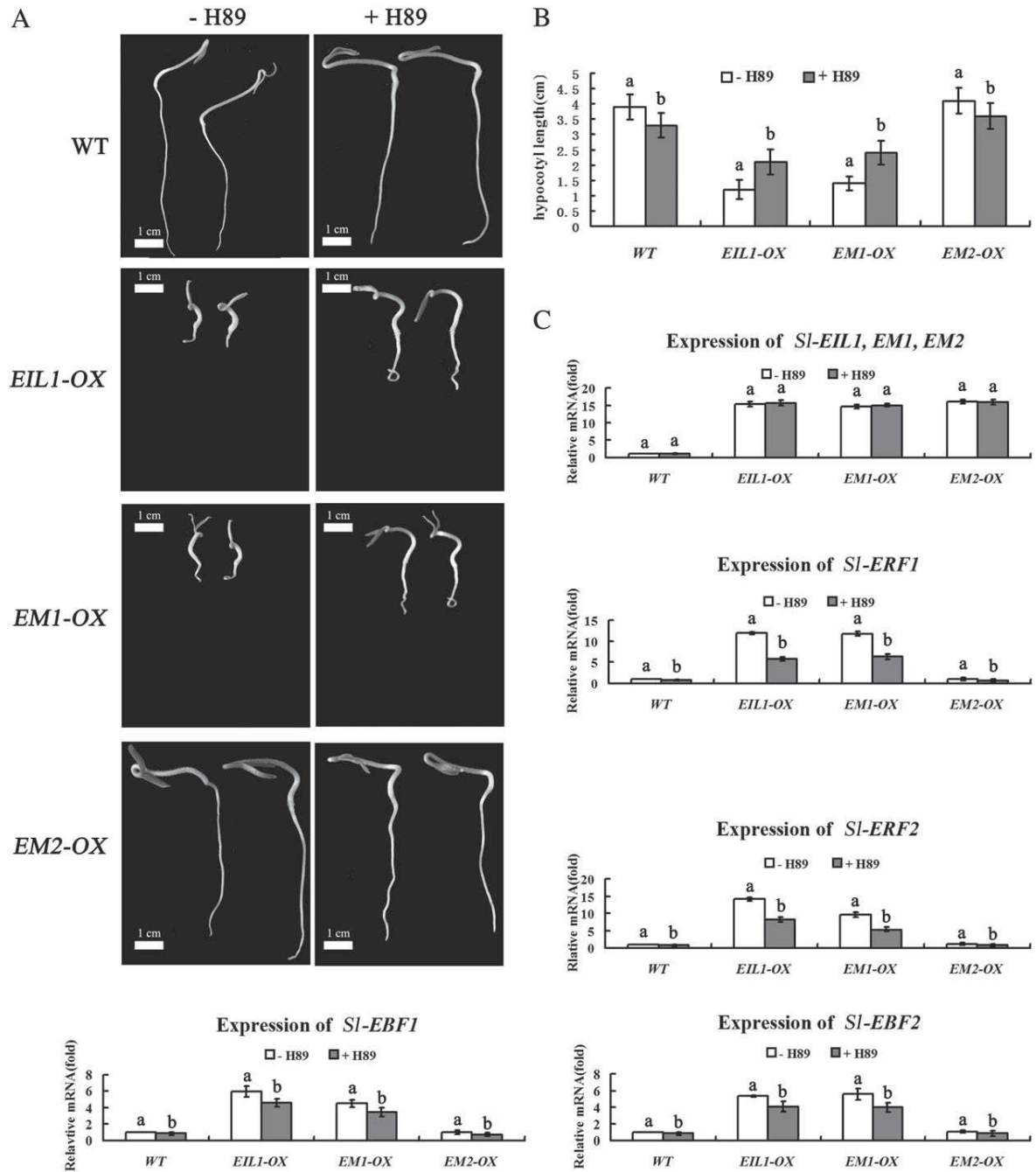


Fig. 6. Assessing the phenotypes and expression of ethylene-responsive genes in tomato seedlings treated with H-89, a phosphorylation inhibitor. (A) Tomato seedlings (7 d old) were cultured on 50% MS medium under dark conditions and the phenotypes of the etiolated seedlings untreated (right panel) or treated with H-89, a phosphorylation inhibitor (left panel), were analysed. Wild type (WT) and *Sl-EM2*-overexpressing (*EM2-OX*) lines (upper and lower panel, respectively) display a wild-type phenotype with regard to ethylene response, while *Sl-EIL1*- (*EIL1-OX*) and *Sl-EM1*- (*EM1-OX*) overexpressing lines (middle panels) show marked constitutive ethylene phenotypes that can be partly alleviated by H-89 treatment. (B) Hypocotyl length of wild-type and tomato *Sl-EIL1*-overexpressing seedlings treated and untreated with H-89. (C) Transcript accumulation of ethylene-regulated genes in wild-type and *Sl-EIL1*-expressing seedlings treated and untreated with H-89. The transcript levels were assessed by qRT-PCR for *Sl-EIL1*, *Sl-EM1*, and *Sl-EM2* (upper panel), *Sl-ERF1* and *Sl-ERF2* (middle panel), and *Sl-EBF1* and *Sl-EBF2* (lower panel). Data are expressed as relative values, based on the values of untreated WT samples. Each value represents the mean \pm SE of three replicates. Letters in B and C indicate differences between the treated and untreated seedlings with statistical significance at $P < 0.05$ (t -test). The same letter means not significantly different; different letters means significantly different.

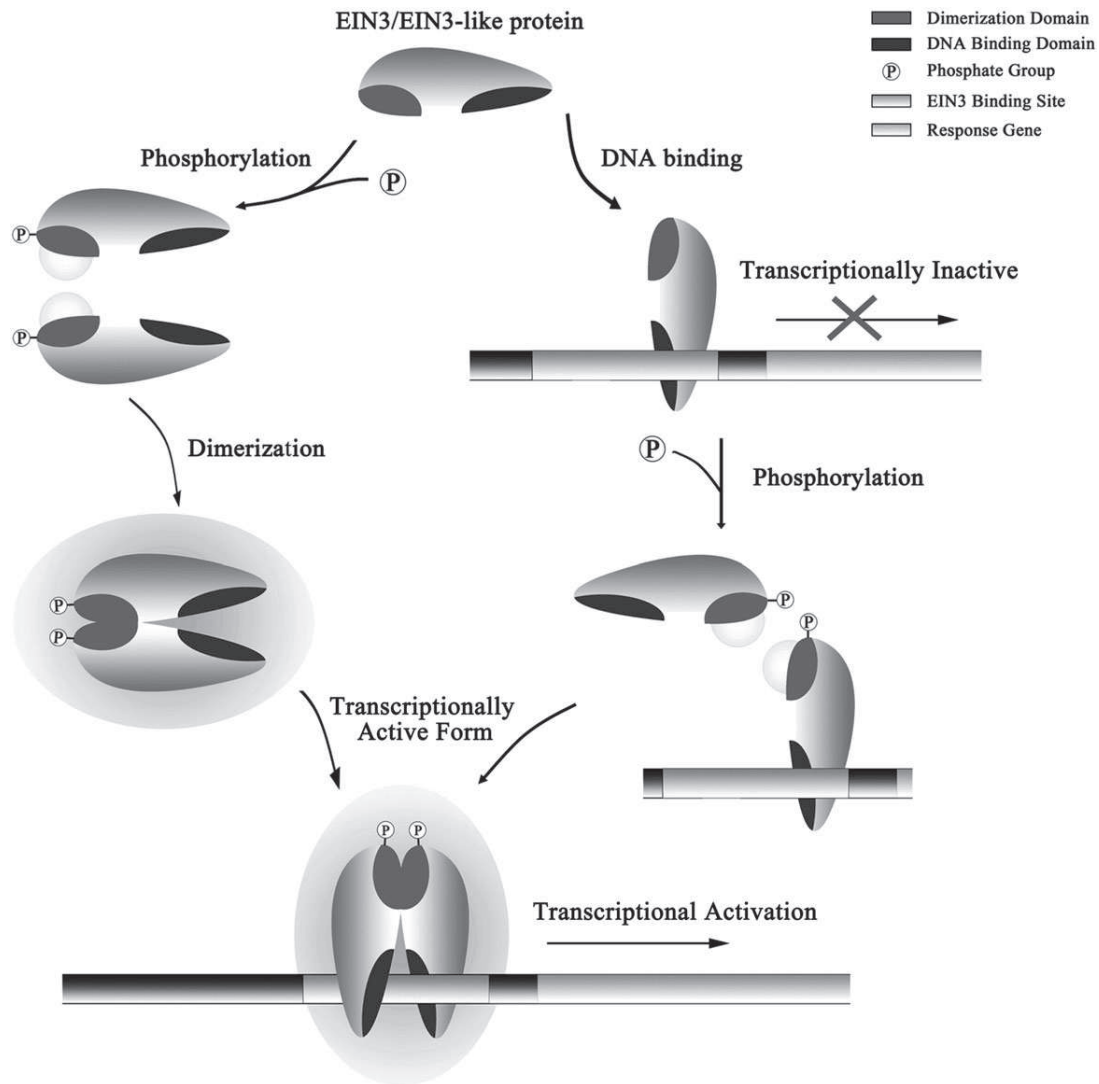


Fig. 7. Proposed model illustrating the mechanism by which the phosphorylation-dependent activation of EIN3/EIL promotes transcription of ethylene-regulated genes and ethylene responses. In the non-phosphorylated state, EIN3/EILs are unable to form dimers and to initiate transcription of target genes. Upon phosphorylation, EIN3/EIL forms an activated homodimer that binds to the EBS (EIN3/EIL-binding site) of ethylene response genes and then initiates transcription of these target genes (left panel). In an alternative pathway, EIN3/EIL first binds to EBS as a monomer then undergoes phosphorylation which triggers its dimerization with unbound phosphorylated EIN3/EIL proteins, thus allowing the transcription of target genes to proceed. In the absence of phosphorylation, EIN3/EIL is unable to initiate transcription of ethylene-regulated genes which leads to the lack of ethylene responses in plants expressing a mutated EPR1 (right panel).

responses required in different stages of the plant life (Brown, 1997; Lelievre *et al.*, 1997; Morgan and Drew, 1997; Smalle and Van Der Straeten, 1997). One of these tuning mechanisms lies in the downstream part of the ethylene signalling pathway at the level of EIN3/EIL transcription factors, the primary modulators of the expression of target ethylene-regulated genes (Chao *et al.*, 1997; Solano *et al.*, 1998). Although the EIN3/EIL genes have been cloned from various species and their function thoroughly investigated, there is still more to learn about the mechanisms by which EIN3/EIL modulates a wide variety of specific, and in some cases opposite, growth responses depending on the environmental condition.

Regulation of transcription factors via protein kinases and phosphatases is rather common in eukaryotes including plants, and phosphorylation can impact the activity of these transcriptional regulators by affecting their transcriptional function or their binding to target DNA (Klimczak *et al.*, 1995). While MAPK-mediated phosphorylation has been reported to play an important role in regulating EIN3/EIL protein stability in *Arabidopsis* (Yoo *et al.*, 2008), the data presented here uncover a putative phosphorylation domain, named EPR1 (EIN3/EIL phosphorylation region 1), in the tomato Sl-EIL protein that adds a new regulatory switch to the control mechanism underlying plant responses to ethylene. In direct support of the instrumental role of the EPR1

domain, the data show that mutation in the EPR1 phosphorylation site prevents the dimerization of Sl-EIL1 proteins and leads to their loss of ability to activate the transcription of target genes. The requirement for a functional EPR1 phosphorylation domain is further evidenced by the phenotypes of the tomato *Sl-EM2*-overexpressing lines lacking a functional EPR1 phosphorylation domain that fail to show any of the constitutive ethylene response phenotypes displayed by *Sl-EIL1*-expressing lines. In contrast, transgenic lines expressing the *Sl-EM1* mutant version of Sl-EIL1 that retains the capacity to be phosphorylated by PKC display a clear constitutive ethylene response as exemplified by premature fruit ripening and exaggerated triple response in the absence of exogenous ethylene treatment. This is not surprising since though EIN3/EIL proteins generally contain a PKA-dependent phosphorylation site, the EPR1 domain of Arabidopsis EIL2 (At-EIL2, GenBank accession no. AF004214.1) contains a PKC-dependent phosphorylation site (amino acids SKR), suggesting that the function of EIN3/EIL may be regulated either way by PKA and PKC.

Based on the present findings, a new model is proposed that takes into account the role of the EPR1 domain in promoting the ethylene-dependent transcriptional regulation (Fig. 7). In this model, EIN3/EIL proteins with a non-phosphorylated EPR1 domain are in the inactive state, and phosphorylation via PKA triggers dimerization of EIN3/EIL proteins which activates the transcriptional function of EIN3/EIL. Therefore, the emerging global picture is that ethylene responses are tuned by the interplay of protein kinases where PKA- and MAPK-dependent phosphorylation of Sl-EIL1 creates a dynamic equilibrium. On the one hand, MAPK phosphorylation indirectly reduces the Sl-EIL1 activity by promoting its degradation, and, on the other hand, the transcriptional activity of Sl-EIL1 is positively regulated by EPR1-dependent phosphorylation and subsequent dimerization.

To reconcile the fact that the temporal and spatial expression of ethylene-responsive genes is under the control of EIN3/EIL transcription factors (Chao *et al.*, 1997; Tieman *et al.*, 2001) while the expression of *EIN3/EIL* genes escapes the regulation at the transcriptional level by ethylene, it is proposed that the activation of EIN3/EIL proteins is induced by ethylene through the phosphorylation of the EPR1 domain. Adding to the previously described post-translational regulation of EIN3/EIL via MAPK-dependent phosphorylation (Yoo *et al.*, 2008), the discovery of the EPR1 phosphorylation site described here brings the mechanisms driving the control of ethylene responses in higher plants to a more sophisticated level of complexity. Using dedicated liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis, further studies will attempt to clarify whether the phosphorylation status of Sl-EIL1 at the EPR1 site is dependent on ethylene treatment. It will also be of interest to uncover whether the two phosphorylation events occur in a sequential or a randomized order and whether the PKA-dependent phosphorylation of EIL1 has a synergistic or antagonistic effect on the MAPK phosphorylation event, and vice versa.

Acknowledgements

We thank Professor Jean Claude Pech (University of Toulouse, France) for critical comments. This work was supported by the Projects of the National Natural Science Foundation of China (30972002, 31071798) and the Committee of Science and Technology of Chongqing (2011BA1024).

References

- Abel S, Theologis A.** 1994. Transient transformation of Arabidopsis leaf protoplasts: a versatile experimental system to study gene expression. *The Plant Journal* **5**, 421–427.
- Alonso JM, Hirayama T, Roman G, Nourizadeh S, Ecker JR.** 1999. EIN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis. *Science* **284**, 2148–2152.
- Brown KM.** 1997. Ethylene and abscission. *Physiologia Plantarum* **100**, 567–576.
- Chang C, Kwok SF, Bleecker AB, Meyerowitz EM.** 1993. Arabidopsis ethylene-response gene ETR1: similarity of product to two-component regulators. *Science* **262**, 539–544.
- Chao Q, Rothenberg M, Solano R, Roman G, Terzaghi W, Ecker JR.** 1997. Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. *Cell* **89**, 1133–1144.
- Engh RA, Girod A, Kinzel V, Huber R, Bossemeyer D.** 1996. Crystal structures of catalytic subunit of cAMP-dependent protein kinase in complex with isoquinolinesulfonyl protein kinase inhibitors H7, H8, and H89. *Journal of Biological Chemistry* **271**, 26157–26164.
- Fan JY, Cui ZQ, Wei HP, Zhang ZP, Zhou YF, Wang YP, Zhang XE.** 2008. Split mCherry as a new red bimolecular fluorescence complementation system for visualizing protein–protein interactions in living cells. *Biochemical and Biophysical Research Communications* **367**, 47–53.
- Fryer CJ, Lamar E, Turbachova I, Kintner C, Jones KA.** 2002. Mastermind mediates chromatin-specific transcription and turnover of the Notch enhancer complex. *Genes and Development* **16**, 1397–1411.
- Gerber HP, Seipel K, Georgiev O, Hofferer M, Hug M, Rusconi S, Schaffner W.** 1994. Transcriptional activation modulated by homopolymeric glutamine and proline stretches. *Science* **263**, 808–811.
- Guo H, Ecker JR.** 2004. The ethylene signaling pathway: new insights. *Current Opinion in Plant Biology* **7**, 40–49.
- Hellens RP, Edwards EA, Leyland NR, Bean S, Mullineaux PM.** 2000. pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. *Plant Molecular Biology* **42**, 819–832.
- Kamman M, Laufs J, Schell J, Gronenborn B.** 1989. Rapid insertional mutagenesis of DNA by polymerase chain reaction (PCR). *Nucleic Acids Research* **17**, 5404.
- Kieber JJ, Rothenberg M, Roman G, Feldmann KA, Ecker JR.** 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.

Kim KY, Cosano IC, Levin DE, Molina M, Martin H. 2007. Dissecting the transcriptional activation function of the cell wall integrity MAP kinase. *Yeast* **24**, 335–342.

Klimczak LJ, Collinge MA, Farini D, Giuliano G, Walker JC, Cashmore AR. 1995. Reconstitution of Arabidopsis casein kinase II from recombinant subunits and phosphorylation of transcription factor GBF1. *The Plant Cell* **7**, 105–115.

Kosugi S, Ohashi Y. 2000. Cloning and DNA-binding properties of a tobacco Ethylene-Insensitive3 (EIN3) homolog. *Nucleic Acids Research* **28**, 960–967.

Lehman A, Black R, Ecker JR. 1996. HOOKLESS1, an ethylene response gene, is required for differential cell elongation in the Arabidopsis hypocotyl. *Cell* **85**, 183–194.

Lelievre JM, Latche A, Jones B, Bouzayen M, Pech J-C. 1997. Ethylene and fruit ripening. *Physiologia Plantarum* **101**, 727–739.

Mishra NS, Tuteja R, Tuteja N. 2006. Signaling through MAP kinase networks in plants. *Archives of Biochemistry and Biophysics* **452**, 55–68.

Montgomery J, Goldman S, Deikman J, Margossian L, Fischer RL. 1993. Identification of an ethylene-responsive region in the promoter of a fruit ripening gene. *Proceedings of the National Academy of Sciences, USA* **90**, 5939–5943.

Morgan PW, Drew MC. 1997. Ethylene and plant responses to stress. *Physiologia Plantarum* **100**, 620–630.

Raz V, Fluhr R. 1993. Ethylene signal is transduced via protein phosphorylation events in plants. *The Plant Cell* **5**, 523–530.

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

Riechmann JL, Meyerowitz EM. 1998. The AP2/EREBP family of plant transcription factors. *Biological Chemistry* **379**, 633–646.

Roman G, Lubarsky B, Kieber JJ, Rothenberg M, Ecker JR. 1995. Genetic analysis of ethylene signal transduction in Arabidopsis thaliana: five novel mutant loci integrated into a stress response pathway. *Genetics* **139**, 1393–1409.

Shaner NC, Campbell RE, Steinbach PA, Giepmans BN, Palmer AE, Tsien RY. 2004. Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nature Biotechnology* **22**, 1567–1572.

Sharma MK, Kumar R, Solanke AU, Sharma R, Tyagi AK, Sharma AK. 2010. Identification, phylogeny, and transcript profiling of ERF family genes during development and abiotic stress treatments in tomato. *Molecular Genetics and Genomics* **284**, 455–475.

Sigrist CJ, Cerutti L, de Castro E, Langendijk-Genevaux PS, Bulliard V, Bairoch A, Hulo N. 2010. PROSITE, a protein domain

database for functional characterization and annotation. *Nucleic Acids Research* **38**, D161–D166.

Smalle J, Van Der Straeten D. 1997. Ethylene and vegetative development. *Physiologia Plantarum* **100**, 593–605.

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

Stepanova AN, Alonso JM. 2009. Ethylene signaling and response: where different regulatory modules meet. *Current Opinion in Plant Biology* **12**, 548–555.

Tieman DM, Ciardi JA, Taylor MG, Klee HJ. 2001. Members of the tomato LeEIL (EIN3-like) gene family are functionally redundant and regulate ethylene responses throughout plant development. *The Plant Journal* **26**, 47–58.

Wang H, Jones B, Li Z, Frasse P, Delalande C, Regad F, Chaabouni S, Latche A, Pech JC, Bouzayen M. 2005. The tomato Aux/IAA transcription factor IAA9 is involved in fruit development and leaf morphogenesis. *The Plant Cell* **17**, 2676–2692.

Yamamoto S, Suzuki K, Shinshi H. 1999. Elicitor-responsive, ethylene-independent activation of GCC box-mediated transcription that is regulated by both protein phosphorylation and dephosphorylation in cultured tobacco cells. *The Plant Journal* **20**, 571–579.

Yamasaki K, Kigawa T, Inoue M, et al. 2005. Solution structure of the major DNA-binding domain of Arabidopsis thaliana ethylene-insensitive3-like3. *Journal of Molecular Biology* **348**, 253–264.

Yang Y, Wu Y, Pirrello J, Regad F, Bouzayen M, Deng W, Li Z. 2010. Silencing Sl-EBF1 and Sl-EBF2 expression causes constitutive ethylene response phenotype, accelerated plant senescence, and fruit ripening in tomato. *Journal of Experimental Botany* **61**, 697–708.

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

Yoo SD, Cho YH, Tena G, Xiong Y, Sheen J. 2008. Dual control of nuclear EIN3 by bifurcate MAPK cascades in C2H4 signalling. *Nature* **451**, 789–795.

Yoo SD, Sheen J. 2008. MAPK signaling in plant hormone ethylene signal transduction. *Plant Signaling and Behavior* **3**, 848–849.

Zhou FF, Xue Y, Chen GL, Yao X. 2004. GPS: a novel group-based phosphorylation predicting and scoring method. *Biochemical and Biophysical Research Communications* **325**, 1443–1448.

Zhuang J, Cai B, Peng RH, et al. 2008. Genome-wide analysis of the AP2/ERF gene family in *Populus trichocarpa*. *Biochemical and Biophysical Research Communications* **371**, 468–474.