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

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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 (ERF) 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 Flurh, 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 M KK9 cascade, leading to protein stabilization, whereas the phosphorylation occurring at the T392 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 μmol m⁻² 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 in 50% Murashige and Skoog (MS) culture medium under dark conditions with 50 mol m⁻² 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 DNA- MAN (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 (AAC58857.1), 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 (BA47414.1), Oryza sativa Os-EIL1 (AAZ78349.1), Musa acuminate 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

To generate transgenic plants, a Cin gene instead of the hygromycin gene was used. Site-specific mutagenesis was performed using a First Strand cDNA Synthesis 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-EBF1, Sl-EBF2, and Sl-Actin transcripts was normalized to the Ct value for Sl-Actin-51 and was calculated relative to a calibrator using the formula 2^{-ΔΔCt}. The primer sequences used are listed in Table 1.

Site-specific mutagenesis

Mutagenesis was performed using megaprimer PCR (Kammann et al., 1989). The megaprimer sequences used in qRT-PCR to monitor expression of the Cin gene instead of the hygromycin gene were design according to the manufacturer’s instructions. A 500 ng aliquot of each megaprimer was used for synthesis of the mutated version of the Cin gene sequence.

Plant transformation

To generate transgenic plants, Sl-EIL1, Sl-EM1, and Sl-EM2 were cloned into the modified binary vector pCAMBIA1301 (kanamycin resistance 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% casamino acids, 0.2% peptone, 0.2% bovine serum albumin (BSA), and 0.55 M β-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

SI-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 pCreen-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 (0.3×10^6 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 A0Bs, 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 585 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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence</th>
<th>Orientation</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT-F</td>
<td>TGTCCCATATTTACAGGGGTGTTATGC</td>
<td>Sense</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>ACT-R</td>
<td>CAAGTTAATACAGCCGGCAAGGA</td>
<td>Antisense</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>EIL1-QF</td>
<td>CGGAAATCTACCTAGACT</td>
<td>Sense</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>EIL1-OR</td>
<td>ACTCTGCGCTGCTAGTACAA</td>
<td>Antisense</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>ERF1-QF</td>
<td>CGCGCTAAAGAGGGAGAAAG</td>
<td>Sense</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>ERF1-OR</td>
<td>CGTCGGCACTGGGCAACAT</td>
<td>Antisense</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>ERF2-QF</td>
<td>AAGGGGTTAGGGTGGTTAGG</td>
<td>Sense</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>ERF2-OR</td>
<td>CAAGGCAAATGTCAGGAG</td>
<td>Antisense</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>EBF1-QF</td>
<td>ATTCGATCATGACAGATGG</td>
<td>Sense</td>
<td>qRT-PCR</td>
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<td>EBF1-OR</td>
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<td>qRT-PCR</td>
</tr>
<tr>
<td>EBF2-QF</td>
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<td>Sense</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>EBF2-OR</td>
<td>CAGAACCACTTTTACACGAA</td>
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<td>qRT-PCR</td>
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<tr>
<td>EIL1-F</td>
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<td>Sense</td>
<td>Gene cloning</td>
</tr>
<tr>
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<td>ATGATGATG</td>
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<td>Gene cloning</td>
</tr>
<tr>
<td>EIL1-R</td>
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<td>Antisense</td>
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</tr>
<tr>
<td>EM1-R</td>
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<td>Sense</td>
<td>Mutagenesis</td>
</tr>
<tr>
<td>EM2-R</td>
<td>CCTCCCTG</td>
<td>Antisense</td>
<td>Mutagenesis</td>
</tr>
</tbody>
</table>

### Results

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 2. Predicted phosphorylation sites in SL-EIL1 protein

<table>
<thead>
<tr>
<th>Highly conserved peptide</th>
<th>Position</th>
<th>Kinase family</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>RQSEQGA</td>
<td>85</td>
<td>PhosphAT prediction</td>
<td></td>
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<tr>
<td>RKMSRA</td>
<td>96</td>
<td>PKA</td>
<td></td>
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<tr>
<td>GASDLNR</td>
<td>133</td>
<td>PhosphAT prediction</td>
<td></td>
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<tr>
<td>AKYQAD</td>
<td>157</td>
<td>PhosphAT prediction</td>
<td></td>
</tr>
<tr>
<td>GTPHTL</td>
<td>176</td>
<td>MAPK</td>
<td></td>
</tr>
<tr>
<td>QDGLSG</td>
<td>187</td>
<td>PhosphAT prediction</td>
<td></td>
</tr>
<tr>
<td>EATWLA</td>
<td>289</td>
<td>PhosphAT prediction</td>
<td></td>
</tr>
<tr>
<td>RELTPD</td>
<td>305</td>
<td>PhosphAT prediction</td>
<td></td>
</tr>
<tr>
<td>GSPFNI</td>
<td>580</td>
<td>MAPK</td>
<td></td>
</tr>
</tbody>
</table>

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.

and named here EPR1. The position of EPR1 within the vicinity of the transcriptional activation domain of SL-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 SL-EIL1 protein

To gain insight on the function of EPR1, two individual mutant versions of the SL-EIL1 gene were generated. SL-EIL1-Mutant1 (SL-EM1) and SL-EIL1-Mutant2 (SL-EM2) were constructed by site-directed mutagenesis to introduce nucleotide substitutions within the EPR1 coding sequence (nucleotides 276–285). The introduced nucleotide substitutions in SL-EM1 resulted in the change of the native PKA phosphorylation site (KKMS) into a protein kinase C (PKC) phosphorylation site (SKRR) (Fig. 1B). In SL-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 SL-EIL1 protein was tested using transient expression experiments in a single cell system. The coding sequences of SL-EIL1, SL-EM1, and SL-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). In SL-EM1, the data clearly indicated that none of the mutations in the EPR1 domain of SL-EIL1 prevents or alters the nuclear localization of the protein, suggesting that this domain is not essential for the proper targeting of SL-EIL1 (Fig. 2A).

To assess whether the mutated versions of SL-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 SL-EIL1, SL-EM1, and SL-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 SL-EIL1 is capable of activating the E4 promoter. Likewise, the mutated SL-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 SL-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 SL-EIL1 protein. Interestingly, although SL-EM1 has been mutated in EPR1, the protein retained its transcriptional activation function, though the mean fluorescence intensity indicated that the SL-EM1-mediated RFP expression was significantly lower than that induced by SL-EIL1 (Fig. 3). These data strongly suggest that phosphorylation is pivotal to the transcriptional function of SL-EIL1. However, considering that in SL-EIL1 the EPR1 domain carries a PKA phosphorylation site while in SL-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 SL-EIL1 dimerization

It has been reported that EIN3/EIL proteins can dimerize (Solano et al., 1998; Yamazaki et al., 2005) and, coincidently, 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 SL-EIL1, its potential involvement in protein dimerization was explored. A BiFC approach was implemented to test how mutations in the EPR1 domain affect SL-EIL1 dimerization. Two BiFC plasmids were constructed (pG-RN159 and pG-RC160) carrying either the native (SL-EIL1) or mutated (SL-EM1 and SL-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 SL-EIL1 and SL-EM1 can form homodimers and are able to cross-interact and to form heterodimers (Fig. 4B), while SL-EM2 proteins failed to show any dimerization capability (data not shown). These data strongly support the...
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-EM1 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 EM1-OX transgenic lines expressing Sl-EIL1 and Sl-EM1 genes, respectively, displayed constitutive ethylene response phenotypes (Fig. 5A) with dramatically enhanced flower senescence. The leaves of the EIL1-OX and EM1-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 EM1-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 Sl-EIL1, Sl-EM1, and Sl-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 (Sl-EIL1::GFP, Sl-EM1::GFP, and Sl-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.
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

The constitutive ethylene phenotype of tomato Sl-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 Sl-EIL1 might be the direct target of H-89.

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-ERF1/2 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 Sl-EIL1 and hence in the activation of ethylene response.

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
Assessing the capacity of Sl-EIL1, Sl-EM1, and Sl-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 Sl-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 Sl-EIL1 (top panel) and Sl-EM1 (second panel from the bottom) localized in the nuclear compartment. Heterodimer formation between Sl-EIL1 and Sl-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 ±SE of three replicates.
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 ± 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.
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

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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).
domain, the data show that mutation in the EPR1 phosphorylation site prevents the dimerization of SI-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). This model suggests that 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 picture is that ethylene responses are tuned by the interplay of protein kinases where PKA- and MAPK-dependent phosphorylation of SI-EIL1 creates a dynamic equilibrium. On the one hand, MAPK phosphorylation indirectly reduces the SI-EIL1 activity by promoting its degradation, and, on the other hand, the transcriptional activity of SI-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 SI-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.

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