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ORIGINAL PAPER

Functional analysis of tomato *LeEIL1* in an *Arabidopsis ein2* mutant

Yu Pan · Guoping Chen · Chungui Lu · Xuqing Chen · Zongli Hu

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Abstract The ETHYLENE-INSENSITIVE3 (EIN3)/EIN3-Like (EIL) EIN3/EILs, novel nuclear proteins, are located at the downstream position of the ethylene signal transduction pathway. LeEIL1, which is expressed in fruit throughout ripening, is key transcription factor in the ethylene signaling pathway in tomato. To reveal its function, the *LeEIL1* gene has been transformed into and expressed in the *ein2* mutant of *Arabidopsis*. The expression levels of the transgene in the single copy line, LeEIL1-ein2-b, were higher than those in the multiple-copy line, LeEIL1-ein2-a. The ethylene-insensitive phenotype of the *ein2* mutant plants has been partially recovered by expression of *LeEIL1*. The florescence of LeEIL1-ein2-a and LeEIL1-ein2-b exceeded that of the *ein2* mutant but was still less than that of wild type of *Arabidopsis*. The expression of four pathology-related genes (*AtPR3*, *4*, *AtPDF1.2* and *AtGST2*) has been analyzed in *LeEIL1* transgenic *ein2* mutant plants. The expression of AtPR3 and AtPR4, which was reduced in the *ein2* mutant, was enhanced in the two transgenic *Arabidopsis* plants. The expression of the *AtPDF1.2* gene was unaffected in the two transgenic *Arabidopsis* lines, the ein2 mutant and wild-type *Arabidopsis* plants. In addition, the expression level of *AtGST2* in transgenic *Arabidopsis* plants was lower even than that in ein2 mutant and wild-type *Arabidopsis* plants.

Keywords LeEIL1 · *ein2 mutant* · Ethylene · Signal transduction · *Lycopericon esculentum* · *Arabidopsis thaliana*

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The EMBL data library accession number for the EIL-like transcription factor cDNA nucleotide sequence is AF328784.

Y. Pan · G. Chen · Z. Hu (✉)
College of Bioengineering, Chongqing University, 400044 Chongqing, People's Republic of China
e-mail: huzongli@cqu.edu.cn

Y. Pan
e-mail: yu.pan82@yahoo.com

G. Chen
e-mail: chenguoping@cqu.edu.cn

C. Lu
Multidisciplinary Centre for Integrative Biology, School of Biosciences, University of Nottingham, Sutton Bonington, Leicestershire LE12 5RD, UK
e-mail: chungui.lu@nottingham.ac.uk

X. Chen
Beijing Agro-Biotechnology Research Center, 100089 Beijing, People's Republic of China
e-mail: chenxvqing@sina.com

Introduction

Ethylene, a gaseous phytohormone, plays an important role in many aspects of plant growth and development, and the responses of plants to biotic and abiotic stresses (Guo and Ecker 2004; Johnson and Ecker 1998; Yang and Hoffman 1984). It regulates many growth and developmental processes such as seed germination, growth, leaf and petal abscission, fruit ripening, organ senescence, and stress and pathogen responses (Bleecker and Kende 2000; Schaller and Kieber Joseph 2009; Stepanova et al. 2007).

In *Arabidopsis*, ethylene perception is mediated by a family of receptors that include five members: ethylene resistant/ethylene receptor ETRl, ETR2, ethylene response sensor ERS l, ERS2, and ethylene insensitive EIN4 (Hua et al. 1995, 1998; Sakai et al. 1998). The ethylene receptors activate the kinase activity of CTR1 in the absence of

ethylene. The active CTR1 suppresses the downstream responses, so that EIN2 and the downstream EIN3/EIL transcription factors remain inactive (Mao et al. 2006). The ethylene signal is propagated via a MAPK pathway to EIN2, which is a membrane protein with homology to Nramp metal-ion transporters (Alonso et al. 1999; Ouaked et al. 2003). EIN2 activates the EIN3/EIL transcription factors and consequently induces ethylene responses (Alonso and Stepanova 2004; Chen et al. 2005; Guo and Ecker 2004). EIN3, a positive regulator at the most downstream position of the ethylene signal transduction pathway, encodes a transcription factor that belongs to a small family that includes EIN3 and various EIN3-like (EIL) proteins in *Arabidopsis* (Chao et al. 1997). For example, EIN3 mutants (knock down) have reduced responses to ethylene, whereas over-expressed EIN3 results in ethylene hypersensitivity or a constitutive ethylene response (Chao et al. 1997; Roman et al. 1995). The EIN3/EIL family genes are involved in a regulatory cascade and activate other transcription factors such as ERF1 (ethylene response factor), which belongs to the ERF family (also called the EREBP family for ethylne response element-binding protein) (Alonso et al. 2003; Fujimoto et al. 2000; Solano et al. 1998). These transcription factors have been shown to act as activators or repressors of additional downstream ethylene-responsive genes (Solano et al. 1998; Huanga et al. 2010). More recently, evidence has been accumulating that the stability of the ethylene signaling protein EIN2 is modulated by two F-box proteins ETP1/2 which might facilitate the ubiquitination of EIN2, thereby regulating its degradation by the proteasome (Qiao et al. 2009). In addition to another regulator of ethylene response, EIN3, is also targeted by the F-box proteins EBF1/2, that mediate EIN3 or EIL degradation in an ethylene-dependent manner (Potuschak et al. 2003; Wang et al. 2009; Yang et al. 2010).

The *Arabidopsis ein2* mutant is a typical ethylene insensitive mutant which is selected by the “triple response”. The *ein2* mutant phenotype is a lack of three distinct morphological changes in the shape of the seedling: inhibition of stem elongation, radial swelling of the stem, and the absence of a normal geotropic response during the application of 10 μL/L ethylene. The rosette of *ein2-1* plants is larger compared with the wild-type rosette, and bolting is delayed (Hirsch et al. 2002). Mutant *ein2* blocks the ethylene signal transduction pathway, making the mutant completely insensitive to ethylene, both in seedlings and adults (Chen and Bleecker 1995; Roman et al. 1995). An ethylene-related signaling pathway from receptor to EIN3/EILs, then to ethylene responsive proteins, has been established in *Arabidopsis*. A similar route of ethylene signal transduction pathway needs to be elucidated in tomato, which is regarded as the model plant for fruit ripening. Several tomato (*Lycopersicon esculentum*) homologs of *Arabidopsis* ethylene signal transduction pathway components have been investigated in the past years, including six ethylene receptor genes, (*LeETR1*, *LeETR2*, *NR*, *LeETR4-6*)(Lashbrook et al. 1998; Tieman and Klee 1999; Wilkinson et al. 1995), the EIN2-homolog *LeEIN2* (Wang et al. 2007), four EIN3-Like genes (*LeEIL1*, LeEIL2, *LeEIL3* and *LeEIL4*) (Chen et al. 2004; Tieman et al. 2001; Yokotani et al. 2003), and ethylene response factors (*ERF*s)(Chen et al. 2008; Hongxing et al. 2005; Tournier et al. 2003).

In recent years, we have cloned the tomato homologs to the EIN3 genes in *Arabidopsis*, LeEILs. Down-regulated expression of these genes in transgenic tomato resulted in an ethylene-insensitive phenotype for all responses examined, including leaf epinasty, flower abscission and senescence, and fruit ripening (Chen et al. 2004). Constitutive over expression of *LeEIL1* can partially restore ripening in the ethylene-insensitive *Nr* tomato mutant (Chen et al. 2004). Martin’s group has demonstrated an in vivo function in defense responses for the transcription factors Pti4, Pti 5, and Pti 6 that were identified from tomato by expressing them in Arabidopsis plants (Gu et al. 2002). In this study, we used the cross species transformation method to over-express the *LeEIL1* gene in the *Arabidopsis ein2* mutant to investigate the role of *LeEIL1* and the EIN3 family in the ethylene signal transduction pathway. The reason for choosing the *Arabidopsis ein2* mutant as material to do the cross species gene transformation was that an *ein2* mutant has not been found in tomato. The expression of four pathology-related genes in *LeEIL1* transgenic *ein2* mutant plants and the functional role of *EIN3/EIL1* in ethylene signaling are also demonstrated.

Results

Transformation of *Arabidopsis ein2* mutants by pBIN-LeEIL1

A full-length cDNA clone for *LeEIL1* was isolated by PCR and confirmed by DNA sequencing. The *LeEIL1* cDNA was inserted in the sense orientation, downstream of the CaMV 35S promoter and upstream of the CaMV 35S terminator in the vector pBIN19, and then through *Agrobacterium*-mediated transfer into *Arabidopsis ein2* mutants. Two over-expressed *LeEIL1* transgene lines were selected by 50 mg l^{-1} kanamycin (Fig. 1). Copies of the transgene in each transformed line were analyzed by Southern blot using the *nptII* gene as a probe (Fig. 2). The Southern analysis shows that there were at least three copies of the transgene in the genome of the transgenic line LeEIL1-ein2-a and just one copy in transgenic line LeEIL1-ein2-b.

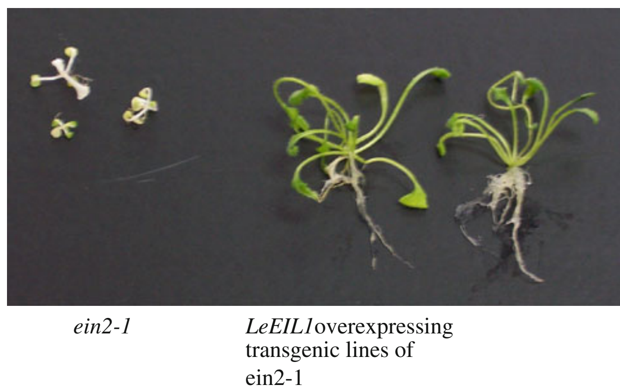

Fig. 1 Comparison of transformed seedlings selected by kanamycin and *ein2* mutants

Fig. 2 Southern blot of the transgenic plants probed with *nptII*. *1* LeEIL1-ein2-a transgenic plant, *2* LeEIL1-ein2-b transgenic plant, *3* *Arabidopsis ein2* mutant

Analysis of *LeEIL1* expression in the transgenic plants

The expression levels of *LeEIL1* in the transgenic lines were determined by northern analysis in seedlings and florescent plants. The *LeEIL1* gene is over-expressed in the transgenic plants, and there was no evident distinction between the expression levels in the seedlings and flowering period plants (Fig. 3). The expression levels between two transgenic lines had evident differences. The expression level of transgenic *LeEIL1* in line LeEIL1-ein2-b is much higher than that in line LeEIL1-ein2-a.

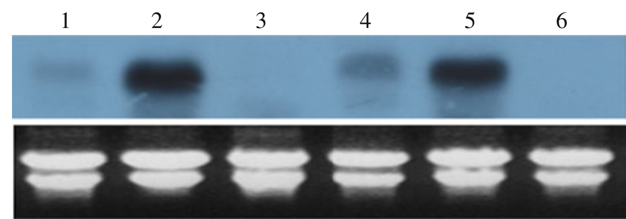

Fig. 3 Northern analysis of the *LeEIL1* transgenic plants. To detect the expression pattern of *LeEIL1* in transgenic *ein2* plants, seedlings and florescent plants of each transformed line, the wild type and the *ein2* mutant. *1* Seedling of LeEIL1-ein2-a, *2* seedling of LeEIL1-ein2-b, *3* seedling of *ein2*, *4* florescent plants of LeEIL1-ein2-a, *5* florescent plants of LeEIL1-ein2-b, *6* florescent plants of *ein2*

Expression of PR genes in transgenic plants

Ethylene, salicylic acid (SA), and jasmonic acid (JA) have been shown to be important components of defense response pathways (Gu et al. 2002). Many pathogenesis-related (*PR*) genes that are induced upon pathogen infection also are up regulated by one or more of these signaling pathways. The ethylene-related signaling pathway regulates the expression of vacuole localized basic PR proteins such as PR3, PR4, PDF1.2, and GST2. The ethylene-response factors (ERFs) family of transcription factors binds specifically to the GCC-box *cis* element present in the promoters of many (*PR*) genes. The PR family genes *PR3*, *PR4*, *PDF1.2*, and *AtGST2*, the expression of which is regulated by jasmonic acid- and ethylene-genes were affected differently by over-expression of each of the three tomato ERFs *Arabidopsis* plants (Gu et al. 2002).

To further elucidate the effect of *LeEIL1* on the expression of ethylene-regulated *PR* genes, RNA isolated from transgenic plants was probed by the *PR* genes which were reported previously (Gu et al. 2002).

The northern analysis shows that the expression level of *At-PR3* in wild-type *Arabidopsis* is stronger than that in the *ein2-1* mutant. The expression level of *At-PR3* in the two transgenic lines, LeEIL1-ein2-a and LeEIL1-ein2-b, is between that in the wild type and *ein2-1* mutant. Although the ethylene signal transduction is interrupted at the EIN2 in the *ein2-1* mutant, the over-expression of *LeEIL1* can partially restore the expression of the At-PR3 gene. The *LeEIL1* gene partially restores the ethylene signal transduction of the *ein2-1* mutant. The *LeEIL1* has the similar function of *AtEIN3* and *AtEILs. At-PR4* is has a similar expression pattern to *At-PR3* as describe in Fig. 4.

AtPDF1.2 is a plant defensin gene, whose expression is induced by ethylene and jasmonic acid (Gu et al. 2002). *AtPDF1.2* gene expression in *Arabidopsis* wild type (Col-0) and *ein2-1* mutants is similar in air. Ethylene can induce the expression of the *AtPDF1.2* gene in wild type, but has no effect in the *ein2-1* mutant (data is not shown). The

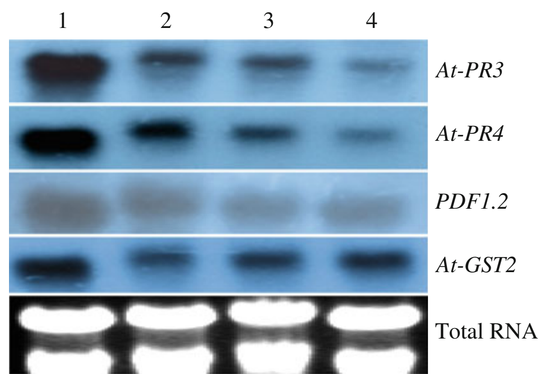

Fig. 4 The expression pattern of *At-PR3*, *At-PR4*, *PDF1.2* and *At-GST2* in *LeEIL1* transgenic *Arabidopsis ein2-1* plants. *1 Arabidopsis* (wild type) Col-0, *2* LeEIL1-ein2-a, *3* LeEIL1-ein2-b, *4 Arabidopsis ein2-1* mutant

expression level of *AtPDF1.2* in *ein2-1* mutant is similar to wild type in air and the expression pattern of *AtPDF1.2* shows no difference between the WT, *ein2-1* and the two transgenic lines. The over-expression of *LeEIL1* in the *ein2-1* mutant has no effect on the gene *AtPDF1.2*. That means *LeEIL1* is not directly regulating the *AtPDF1.2* gene. *At-GST2* is a glutathione *S*-transferase gene which is induced by ethylene (Alonso et al. 1999). Previous results indicated that *AtGST2* in an *ein2-5* mutant is obviously enhanced after transformation by the EIN2 gene, but the result of our experiment indicated that the *AtGST2* gene in the transgenic lines LeEIL1-ein2-a and LeEIL1-ein2-b had lower expression than the *ein2-1* and wild type. It is not clear whether these different results are a consequence of perturbations in transformation. How AtGST2 is involved in the ethylene signal transduction pathway also needs further analysis (Fig. 4).

LeEIL1 partially restores the ethylene-responsive phenotype in the *ein2-1* mutant

To evaluate the function of *LeEIL1* in the ethylene signaling pathway, *LeEIL1* transgenic plant lines were examined for the ethylene-responsive phenotype. This is characterized by a triple response in *Arabidopsis*, which includes inhibition of root and hypocotyl elongation, radial swelling of the hypocotyl and root, and exaggeration in the curvature of the apical hook (Chang and Shockey 1999; Ecker 1995). The hypocotyls of the etiolated transgenic seedlings were measured 72 h after germination (Table 1). In Fig. 5, the transgenic lines showed inhibition of hypocotyl elongation. The seedlings from the transgenic lines, which had a high *LeEIL1* expression level, displayed strong inhibition of hypocotyl elongation (LeEIL1-ein2-b). The seedlings from the transgenic line LeEIL1-ein2-a, which had a lower level of *LeEIL1* expression, showed weak

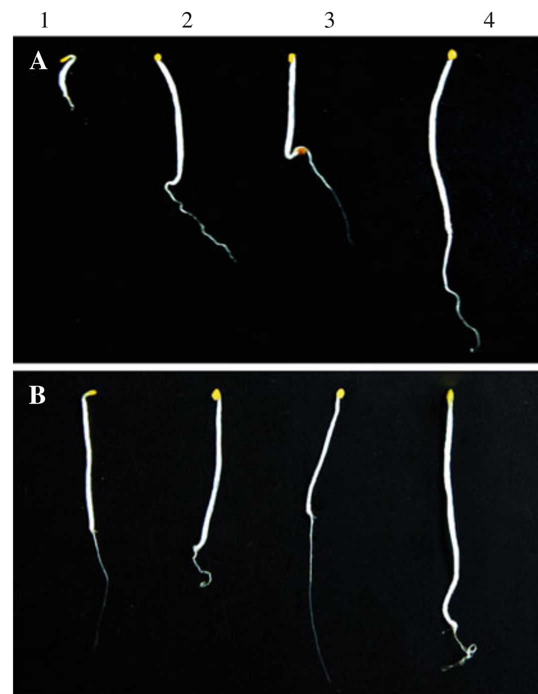

Fig. 5 Triple response of *LeEIL1* transgenic *Arabidopsis ein2-1* plants. **a** Ethylene 10 μM, **b** air. *1 Arabidopsis* (wild type) Clo-0, *2* LeEIL1-ein2-a, *3* LeEIL1-ein2-b, *4 Arabidopsis ein2-1* mutant

inhibition of hypocotyl elongation. These data indicated that there was a correlation between the *LeEIL1* expression and the inhibition of hypocotyl elongation. The adult plants from the transgenic lines displayed reduced size and early flowering compared with the *ein2-1* mutant plants (Fig. 6). It is similar to the overexpression of *Pti4* in *Arabidopsis*.

Discussion

Four genes, *LeEIL1*, *LeEIL2*, *LeEIL3*, and *LeEIL4* have been isolated from tomato, which is a model for fruit ripening. *LeEIL1*, *LeEIL2*, and *LeEIL3* have been proposed to be functionally redundant and to regulate ethylene responses throughout plant development, as it is necessary to suppress the expression of all three EILs in order to reduce ethylene sensitivity (Tieman et al. 2001). The *LeEIL1* gene, which is expressed throughout tomato fruit ripening, plays a very important role in the ethylene transduction pathway in tomato. Since an EIN3 to ERF1 to PR protein pathway has been established in *Arabidopsis* (Gu et al. 2002; Solano et al. 1998), transformation of *LeEIL1* gene into the *Arabidopsis* should test the function of LeEIL1 in the pathway.

In this research, The *LeEIL1* gene has been transformed into and expressed in the *ein2* mutant of *Arabidopsis*. The Southern blotting analysis indicated that there were

Table 1 Hypocotyl length LeEIL1 transgenic *Arabidopsis ein2*-1 plant after treatment of ethylene

($M \pm$ SD)

Length of hypocotyl (mm)	WT (Clo-0)	LeEIL1-ein2-a	LeEIL1-ein2-b	*ein2-1*
Ethylene (3 days)	2.5 ± 0.2	9.6 ± 0.8	7.0 ± 0.6	12.5 ± 0.4
Air (3 days)	8.0 ± 0.4	9.5 ± 0.5	7.2 ± 0.6	13.0 ± 0.7

Fig. 6 Phenotype of *LeEIL1* transgenic *Arabidopsis ein2-1* plants (40 days after germination) *1 Arabidopsis* (wild type) Col-0, *2* LeEIL1-ein2-a, *3* LeEIL1-ein2-b, *4 Arabidopsis ein2-1* mutant

multiple copies of *LeEIL1* in LeEIL1-ein2-a genome and a single copy in LeEIL1-ein2-b's. Northern blotting analysis with a *LeEIL1* probe suggested that the *LeEIL1* gene was over-expressed in LeEIL1-ein2-a and LeEIL1-ein2-b. The expression level of *LeEIL1* in LeEIL1-ein2-b was higher than that in LeEIL1-ein2-a. Maybe, the multi-copies of *LeEIL1* interfered in the expression of the transgenic LeEIL1-ein2-a. The ethylene insensitive phenotype of *ein2* mutant plants was partially recovered when *LeEIL1* was expressed in it. The triple response of LeEIL1-ein2-a and LeEIL1-ein2-b transgenic plants was partially restored in an *ein2* mutant background. LeEIL1-ein2-a and LeEIL1-ein2-b plants were still insensitive to ethylene. The phenotypes of LeEIL1-ein2-a and LeEIL1-ein2-b showed that flowering of LeEIL1-ein2-a and LeEIL1-ein2-b occurred earlier than the *ein2* mutant but still lagged that of the wild type of *Arabidopsis* (Col-0). Previous studies reported that both EIN3-dependent and independent pathways exist downstream of EIN2 (Binder et al. 2004; Seifert et al. 2004). In this study, the transgenic LeEIL1-ein2 seems to confer ethylene-inducible expression of the *LeEIL1*, suggesting *EIL1/EIN3* can activate the transcription of downstream genes in ethylene signaling pathway. *LeEIL1* was not highly expressed in the transgenic LeEIL1-ein2-a with the multi-copies. We suggest that these signal transductions compete with one another; the negative feedback control of ethylene signaling presumably also regulates this process.

The expression patterns of four pathology-related genes (*AtPR3*, *AtPR4*, *AtPDF1.2* and *AtGST2*), which are regulated by ethylene, have been analyzed in *LeEIL1* transgenic *ein2* mutant plants. Northern analysis of these four genes showed that the levels of expression of *AtPR3* and *AtPR4*, which were low in the *ein2* mutant, were enhanced in the two transgenic *Arabidopsis* plants. This means *LeEIL1* could up-regulate their expression. The expression levels of *AtPDF1.2* were indistinguishable among the two transgenic *Arabidopsis* plants, the *ein2* mutant and the wild-type *Arabidopsis* plants, which means the expression of *AtPDF1.2* was regulated by a branched signal pathway which crosses ethylene signal pathway at upstream of EIN3. In addition, the expression level of *AtGST2* in transgenic *Arabidopsis* plants was even lower than that in *ein2* mutant and wild-type *Arabidopsis* plants. It seemed like the expression level of *AtGST2* was negatively regulated by *LeEIL1* indirectly. Genetic and biochemical evidence has shown that EIN3/EIL1 possesses both transcriptional activator and repressor activities, depending on the target genes (Feys and Parker 2000; Roy et al. 1998). EIN3 or EIL1 may interact with other transcription factors or other regulators to repress *AtGST2* transcription. Thus, the ethylene signal transduction pathway may have cross-talk with other signaling pathways, like salicylic acid (SA) signaling pathways in which EIN3/EIL1 may act as a key transcription factor to coordinate regulation of gene expression.

Phylogenetic analysis of the EIN3 family genes showed that LeEIL1 has a high level of homology with AtEIL1 but is not identical. LeEIL1 can play a similar role to EIN3 family members but not the whole function. The ethylene insensitive phenotype of the *ein2* mutant plants has been partially recovered by expression of *LeEIL1*. This partially recovered phenotype and a delay in recovery in flowering may suggest existence of a complex signaling pathway included feedback-regulated transcriptional networks and the existence of still undefined post-transcriptional mechanism of regulating.

In Arabidopsis, constitutive expression of the Arabidopsis *EIN3*, *EIL1* and *EIL2* genes can complement the ein3-1 mutation in transgenic plants (Chao et al. 1997). Tieman has performed a similar experiment with the *LeEIL1*, *LeEIL2,* and *LeEIL3* to complement the ein3-1 mutation in transgenic Arabidopsis, respectively, indicating that all are involved in ethylene signal transduction

(Tieman et al. 2001). In tomato, reduced expression of a single EIN3 family gene such as *LeEIL1* did not exhibit ethylene response, but reduced expression of multiple tomato LeEIL genes was necessary to reduce ethylene sensitivity significantly (Tieman et al. 2001). The LeEILs are proposed functionally redundant and positive genes regulators of multiple ethylene responses throughout plant development. In our published article, a LeEIL1GFP fusion has been constitutively expressed in the non-ripening Nr tomato mutant, and the expression of LeEIL1GFP was able to restore aspects of ripening in two independently transformed plant lines indicating a role for LeEIL1 in fruit ripening (Chen et al. 2004). However, expression *LeEIL1* in Arabidopsis *ein2* mutant has restored the mutation of *ein2*, which completely insensitive to ethylene. It can confirm that LeEIL1 works downstream of EIN2 in ethylene signal transduction pathway.

In *Arabidopsis*, a significant finding is that the stability of EIN3 appears to be controlled through two MAPK phosphorylation sites, one required for stabilization of EIN3 and the other involved in its degradation in ethylene signaling (Yoo et al. 2008). In tomato, whether LeEIL1 is controlled through MAPKs like EIN3 is still worth confirming through further research. From our results, we know that ethylene-related PR genes appear with different expression patterns in LeEIL1 transgenic plants. That illustrates that LeEIL1 is not only involved in ethylene signal transduction pathway but also involved in other signal pathways such as SA pathway and JA pathway. These three stress-related signaling pathways can cross the defense function under different stresses, and they play an important role in defense-signaling pathways. Cross talk between SA-, ethylene-, and JA is thought to be essential. We also have some work for pathogen defense but no different between the *LeEIL1* transgenic *ein2* and *ein2* mutant (data has shown).

In summary, the partial restoration of the ethylene-related genes and phenotype in these experiments could indicate that LeEIL1 plays a similar role to EIN3 in *Arabidopsis.* We have demonstrated that LeEIL1 can act as a transcriptional activator to enhance ethylene-related GCC box-mediated gene transcription. Expression of *LeEIL1* in transgenic *Arabidopsis* plants confers a constitutive ethylene phenotype and induces the expression of genes containing a GCC box in a similar manner to Pti4 in *Arabidopsis* (Wu et al. 2002). In this study we present evidence that *LeEIL1* gene is involved in the regulation of a subset of ethylene-responsive genes. In future, systematic approaches including gene regulatory networks are a step forward to understand how coordinated gene expression programs in ethylene signaling pathways.

Materials and methods

Plant material

The *Arabidopsis thaliana* plants, WT(Columbia ecotype) and *ein2-1* mutant(Columbia background, NASC stock number N3071), were grown in a growth chamber (16 h of light and 8 h of darkness at 22°C) after a 3-day cold treatment. The seeds were surface-sterilized by treatment with 75% ethanol for 30 s, 10-mins incubation in 1% [v/v] sodium hypochlorite and a three-time rinse in sterile distilled water and sown on Murashige and Skoog medium.

To test the triple response of seedlings, surface-sterilized seeds were planted in Murashige and Skoog medium and cold treated at 4°C for 3 days. Seeds were then grown in the dark at 22°C for 72 h in the presence or absence of exogenous ethylene, and the hypocotyl lengths of seedlings were measured(M $\pm$ SD) (Wu et al. 2002).

Plasmid construction

The transgenic construct (pBIN-LeEIL1) was designed to constitutively over-express a functional *LeEIL1* with the Cauliflower Mosaic Virus (CaMV) 35S promoter. A 2470-bp PCR fragment of *LeEIL1*, including the full coding sequence was first digested by *Bam*HI and ligated in the sense orientation between the CaMV 35S promoter and terminator of *Bam*HI-digested pDH51 to yield pDH-LeEIL1. The sense gene was then excised from pDH-LeEIL1 by partial digestion with *Eco*RI and ligated into similarly digested pBIN19 to yield pBIN-LeEIL1. LeEIL1-Forward: 5′-AGCTGAGTTCCAGT TGAACCACAG-3′ LeEIL1-Reverse: 5′-ACAATAACAA CATTGATATCCCAAAC-3′.

Plant transformation and selection

Plasmid pBIN-LeEIL1 was identified by restriction digest analysis and by sequencing and then transformed into *Agrobacterium tumefaciens* LBA4404. The *A. tumefaciens*-mediated transformation of *Arabidopsis* was performed as described previously (Clough and Bent 1998). T1 seeds were collected and sown on sterile Murashige and Skoog media containing 50 mg l^{-1} kanamycin. To select the transformants, kanamycin-resistant seedlings were transferred to the soil. The T2 generations were segregated by kanamycin resistance and confirmed by southern blot analysis.

Southern- and northern-blot analysis

Total genomic DNA from *Arabidopsis* was extracted as described (Dellaporta et al. 1983). For Southern blots, *Arabidopsis* genomic DNA was digested with *Xba*I,

separated by Agarose gel electrophoresis, and transferred to GeneScreen Plus (NEN Life Science Products, Hounslow, UK) membranes (Sambrook et al. 1989). For northern analysis, total RNA was isolated from *Arabidopsis* tissues using the SDS-method as previously described (Chen et al. 2004). Northern blots were prepared by electrophoresis of 20 μg total RNA through Agarose gels in the presence of formaldehyde, followed by transfer to GeneScreen Plus (NEN Life Science Products, Hounslow, UK) membranes. Southern and northern blots were probed with ^{32}P-labeled probes. Probe primer: nptII-Forward: 5′-AA CTCCAGCATGAGATCC-3′; nptII-Reverse: 5′-GACAA TCGGCTGCTCTGA-3′; AtPR3-Forward: 5′-CTACACT TAC AACGCCTTTA-3′; AtPR3-Reverse: 5′-AACTCCTA TTGCTCTACCG-3′; AtPR4-Forward: 5′-AACAATGCG GTCGTCAAG-3′; AtPR4-Reverse: 5′-GGTCCACTATT CTCACAG-3′; AtGST2-Forward: 5′-TTCTCCAAACC GACTCCA-3′; AtGST2-Reverse: 5′-TGATTTCAGCC ACCCACT-3′; AtPDF1.2-Forward: 5′-GCTTCCATCATC ACCCTTATC-3′; AtPDF1.2-Reverse: 5′-TAACAACAAC GGGAAAATAAAC-3′.

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