Plant Cell Tiss Organ Cult (2013) 112:181–190
DOI 10.1007/s11240-012-0220-z

ORIGINAL PAPER

Isolation and expression analysis of three EIN3-like genes in tree peony (*Paeonia suffruticosa*)

Yanjie Wang · Chao Zhang · Peiyi Jia · Xiaoqing Wang · Weiran Wang · Li Dong

Received: 28 April 2012 / Accepted: 6 August 2012 / Published online: 19 August 2012

Abstract Ethylene is a critical signal that influences cut flower opening and senescence in tree peony (*Paeonia suffruticosa*), which is a traditional ornamental plant in China. *Arabidopsis* ETHYLENE-INSENSITIVE3 (EIN3) acts as a key transcription factor of the ethylene signaling pathway, suggesting a possible role for its homologues in regulation of cut flower postharvest development. In this study, three EIN3 homologous genes including *PsEIL1*, *PsEIL2*, and *PsEIL3* have been isolated from petals of tree peony. Deduced amino acid sequences of conserved domains of *PsEILs* share high similarities with the *Arabidopsis* EIN3 protein. Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis indicated that three *PsEILs* were differentially expressed in various tissues of tree peony, and none was flower specific. During cut flower postharvest development, *PsEIL1* transcript in petals accumulated at a relatively low level at the opening stage and reached the highest level of mRNA accumulation at senescence; *PsEIL2* and *PsEIL3* transcripts were gradually increased and peaked at full opening stage followed by a decline when petals wilted. However, these *PsEIL* genes exhibited differential responses to ethylene and 1-methycyclopropene (1-MCP) treatments. Compared with the control, the mRNA level of *PsEIL1* was not influenced by either ethylene or 1-MCP treatment, whereas both *PsEIL2* and *PsEIL3* transcripts were significantly increased by exogenous ethylene, and only *PsEIL3* was strongly inhibited by 1-MCP. These results suggest that *PsEIL* transcripts are spatiotemporally regulated, and the transcriptional regulation of *PsEIL3* may play an important role in ethylene-mediated cut flower opening and senescence in tree peony.

Keywords Tree peony (*Paeonia suffruticosa*) · EIN3 · Ethylene signaling · Cut flower · Gene expression

Abbreviations

CTR	CONSTITUTIVE TRIPLE RESPONSE
EIN	ETHYLENE-INSENSITIVE
EIL	EIN3-like
ERF	ETHYLENE RESPONSE FACTOR
1-MCP	1-methycyclopropene
RT-PCR	Reverse transcription-polymerase chain reaction
RACE	Rapid amplification of cDNA ends
ORF	Open reading frame
NCBI	National Center for Biotechnology Information
UTR	Untranslated region

Introduction

The gaseous phytohormone ethylene plays a crucial regulatory role in diverse aspects of plant growth and development (Abeles et al. 1992; Bleecker and Kende 2000; Lavee et al. 2010; Lu et al. 2011), including flower opening and senescence (Reid et al. 1989; Reid and Wu 1992; Shibuya 2012). The climacteric rise in ethylene influences flower senescence of ethylene-sensitive flowers, which is associated with changes in gene expression (Borochov and

Y. Wang · C. Zhang · P. Jia · X. Wang · W. Wang · L. Dong (✉)
Department of Landscape Architecture, National Flower Engineering Technology Research Center, Beijing Forestry University, 100083 Beijing, China
e-mail: dongleah@yahoo.com.cn

Present Address:
P. Jia
China Forestry Press, Beijing, China

Woodson 1989; Lawton et al. 1989). In this event, the ethylene perception and signal transduction is essentially required (Lawton et al. 1990; Verlinden et al. 2002).

During the past decades, progress in understanding the molecular mechanism of ethylene perception and signal transduction has been made through screening for *Arabidopsis* mutants defective in the ethylene triple response phenotype, and a framework has been established in this model plant. Ethylene is perceived by a family of membrane-associated receptors, which act as negative regulators of ethylene response, along with the downstream Raf-like serine/threonine kinase called CONSTITUTIVE TRIPLE RESPONSE1 (CTR1). In the absence of ethylene, the active receptors interact with CTR1 to repress the downstream response. While binding ethylene, the receptors are inactive, resulting in the inactivation of CTR1 and the downstream repression is relieved. Then the ethylene signal is transmitted through ETHYLENE-INSENSITIVE2 (EIN2) into the nucleus to active the EIN3/EIN3-like (EIL) transcription factors, which trigger transcription of downstream target genes, such as *ETHYLENE RESPONSE FACTOR1* (*ERF1*), ultimately inducing diverse ethylene responses (Guo and Ecker 2004; Chen et al. 2005; Lin et al. 2009).

The transcription factor EIN3, located at the most downstream position of the ethylene signaling pathway, has received more attention than any other components (Chen et al. 2005). EIN3 functions as a positive regulator of ethylene response (Chao et al. 1997) and a potential integration point for cross-talk with other signals (Yanagisawa et al. 2003; Chen et al. 2005; Zhu et al. 2011). This protein belongs to a small family, of which six members (EIN3 and EIL1 to EIL5) have been identified in *Arabidopsis*. These members share common features for nuclear-localized transcription factors whereas only EIN3 and its most closely related EIL1 have been conclusively demonstrated to function in the ethylene signaling pathway (Chao et al. 1997; Alonso et al. 2003; Guo and Ecker 2004). To date, the *EIN3/EIL* gene family has also been well documented in many other plant species including tobacco (Kosugi and Ohashi 2000; Rieu et al. 2003), tomato (Tieman et al. 2001; Yokotani et al. 2003), carnation (Waki et al. 2001; Iordachescu and Verlinden 2005), rice (Mao et al. 2006), banana (Mbeguie et al. 2008), apple (Tacken et al. 2010), and *Oncidium* (Chen et al. 2011). These *EIN3/EIL* genes are almost ubiquitously expressed throughout the plants, despite slightly different patterns and levels of their expression. For example, four *LeEILs* are expressed in all tomato tissues examined, with *LeEIL2* transcript somewhat higher than those of *LeEIL1* and *LeEIL3*, and *LeEIL4* mRNA accumulation increases during fruit ripening, whereas the three others are relatively unchanged (Tieman et al. 2001; Yokotani et al. 2003). In *Arabidopsis*, the regulation of EIN3 at the post-transcriptional level has been reported to be crucial in ethylene signaling. *EIN3* mRNA level is not affected by ethylene, however, EIN3 protein is stabilized and accumulates in response to ethylene, while it is rapidly degraded through the 26S proteasome pathway in the absence of ethylene (Guo and Ecker 2003; Yanagisawa et al. 2003). By contrast, *Arabidopsis EIL1* shows significant differences in expression upon ethylene treatment (De Paepe et al. 2004; Chen et al. 2005), and *EILs* in other species such as carnation (Waki et al. 2001; Iordachescu and Verlinden 2005), banana (Mbeguie et al. 2008), and *Oncidium* (Chen et al. 2011) have also been found to be regulated at the transcriptional level by ethylene and developmental cues.

Tree peony (*Paeonia suffruticosa*), a traditional ornamental plant in China, is popular for its attractive flowers around the world. Cut flower of tree peony exhibits short vase life, which causes a major problem in its shipping and handling. Previous studies revealed that cut flower opening and senescence of most tree peony cultivars, for example, 'Luoyang Hong', was associated with the increase of ethylene production (Jia et al. 2008), which was largely derived from the petals (Jia 2010). Moreover, exogenous ethylene was able to influence the postharvest process of the cultivar 'Luoyang Hong' as well as the endogenous ethylene production, further confirming the important role of ethylene in regulation of tree peony cut flower opening and senescence (Zhou et al. 2009). Ethylene biosynthesis, perception and signal transduction have become the molecular biology research hotspots in order to extend the vase life and improve the quality of this ornamental flower (Zhou et al. 2008, 2010; Gao et al. 2011). To gain a better understanding of ethylene signaling in regulating tree peony cut flower development at the transcriptional level, we isolated three full-length cDNAs of EIN3 homologous genes from petals of tree peony, and investigated their expression patterns in different tissues and during cut flower opening and senescence, as well as the influences of exogenous ethylene and 1-methycyclopropene (1-MCP), an ethylene action inhibitor, on the expression of these genes. The possible roles for *PsEILs* in regulation of tree peony cut flower opening and senescence are discussed.

Materials and methods

Plant materials

Tree peony (*Paeonia suffruticosa* 'Luoyang Hong') was grown in the nursery of Luoyang Tuqiao Peony and Young Plants Co., Ltd., Luoyang City, Henan Province, China. Flowers were harvested at stage 1, and different tissues including roots, stems, leaves, sepals, petals, stamens, and

pistils were collected from intact plants with fully opened flowers. Flower opening stages were described previously in Guo et al. (2004): stage 1, soft bud; stage 2, pre-opening; stage 3, initial opening; stage 4, half opening; stage 5, full opening; stage 6, wilting. After transportation to the laboratory, different tissue samples were frozen in liquid nitrogen and stored at −80 °C for further use. Meanwhile, flowers were re-cut to 25 cm in stem length with the uppermost leaves left and rehydrated for 1 h. Then those flowers each were placed in glass flasks filled with 100 mL distilled water and kept for observation in a room at 20–23 °C, 50–60 % relative humidity, and a 12-h photoperiod with an illumination of ~40 μM m^{-2} s^{-1}. Petal samples were detached from flowers at each stage during vase life, and frozen in liquid nitrogen before storage at −80 °C for later total RNA extraction.

Ethylene and 1-MCP treatments

To investigate the regulatory effect of ethylene on the expression of tree peony *EIL* genes, exogenous ethylene and 1-MCP treatments were imposed on cut flower as follows: after rehydration, flowers with their bases in distilled water were sealed in a 100 L glass chamber with 10 μL L^{-1} ethylene or 1 μL L^{-1} 1-MCP for 6 h at room temperature. Control flowers were incubated in another chamber with air. After treatment, flowers were held in glass flasks with distilled water under the same aforementioned environmental conditions. Petal samples were collected at 0, 12, and 24 h after each treatment, immediately frozen and stored until extraction of total RNA.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from different tissues of tree peony according to the method described by Chang et al. (1993), and then digested with RNase-free DNase I (Takara, Japan) to remove the residual genomic DNA. The first-stand cDNA was synthesized from 1 μg of DNA-free RNA using a PrimeScript® RT reagent Kit (Takara, Japan).

Isolation of tree peony *EIL* cDNAs and sequence analysis

PsEIL partial cDNA fragments were isolated by reverse transcription-polymerase chain reaction (RT-PCR) using total RNA extracted from senescent tree peony petals and nested degenerate primers (EIL-OF and EIL-OR as outer primers, EIL-IF and EIL-IR as inner primers). These primer sequences were designed based on highly conserved domains among plant EIN3/EIL proteins, ELERRMW and IRKLVRQS (for outer primers) or ARRKKMS and PQRRFPL (for inner primers) (Fig. 1). The PCR program was carried out with a template denaturation at 94 °C for 5 min, followed by 30 cycles of denaturing at 94 °C for 30 s, annealing at 46 °C for 30 s, and elongating at 72 °C for 1 min, then ended with the elongation at 72 °C for 7 min. The remaining 3′ and 5′ ends of each *PsEIL* cDNA were isolated by rapid amplification of cDNA ends (RACE) method as follows: the 3′ end cDNA fragment was amplified according to the manufacturer's instruction with a SMART™ RACE cDNA Amplification Kit (Clontech, Japan) by two successive 3′ RACE-PCR, using adaptors and specific primers 3′-EIL1-G1, -G2 (for *PsEIL1*), 3′-EIL2-G1, -G2 (for *PsEIL2*) or 3′-EIL3-G1, -G2 (for *PsEIL3*), which were designed based on each *PsEIL* partial cDNA sequence isolated above; the 5′ end cDNA fragment was extended using a 5′-Full RACE Kit (Takara, Japan) with nested specific primers 5′-EIL1-G1, -G2 (for *PsEIL1*), 5′-EIL2-G1, -G2 (for *PsEIL2*) or 5′-EIL3-G1, -G2 (for *PsEIL3*). Once three partial fragments of each cDNA had been aligned together, full-length cDNA for each *PsEIL* gene was generated through PCR programmed at 94 °C for 5 min, then followed by 32 cycles of 30 s at 94 °C, 30 s at 60 °C, 3 min at 72 °C, and a final elongation for 7 min at 72 °C. The details of primers used in amplifications are described in Table 1. All amplified products were cloned into the pMD18-T vector (Takara, Japan), and then putative positive clones were identified by PCR with RV-M and M13-47 sequencing primers before they were sequenced by Sunbiotech Co., Ltd. (Beijing, China).

Comparisons of the nucleotide and predicted amino acid sequences were performed using the BLAST program, while the open reading frame (ORF) was identified by ORF Finder at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). For multiple sequence alignments, the deduced full-length amino acid sequences of *PsEILs* and *Arabidopsis EIN3* were analyzed using DNAMAN (version 5.2) with default parameters, and the phylogenetic analysis was carried out based on Neighbor-Joining (NJ) model with 1000 bootstrap replications by MEGA (version 4.0).

Real-time RT-PCR analysis

For real-time RT-PCR, each primer set was designed based on the 3′ end cDNA sequence of the corresponding gene with Primer Premier 5 (Table 2 for primer sets). Gene specificity of all primer sets was tested with the following procedure. First, Primer-BLAST searches against GenBank databases were performed for primers to confirm that none of them matched with other sequences. Then primers were examined by melting peaks and dissociation curves to check that only a single product existed for each primer set. For evaluation of each primer set efficiency, a standard curve was produced using serial dilutions of a cDNA

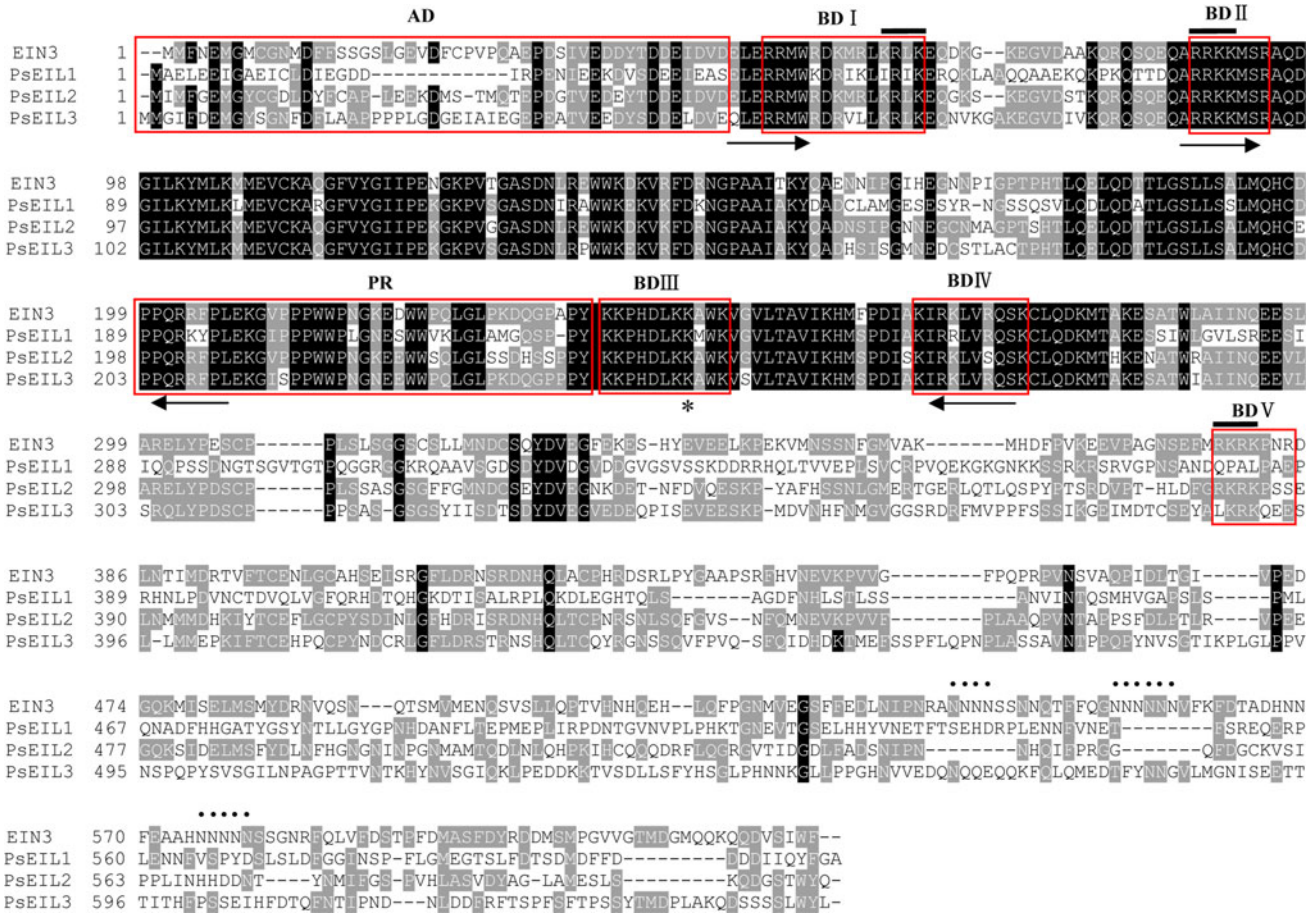


Fig. 1 Multiple amino acid sequence alignments of PsEILs and EIN3 (*Arabidopsis thaliana*, AF004216). Identities and similarities among amino acid sequences are colored in *black* and *gray*, respectively. *Box regions* designate the acidic region (*AD*), proline-rich region (*PR*), and five small clusters of basic amino acids (*BDI-V*). The putative nuclear localization signal sites are indicated by *solid lines*. *Asterisk* represents the lysine residue critical for the function of EIN3. The polyasparagine repeats present in the C-terminus of *Arabidopsis* EIN3 but not in that of PsEILs are marked by *dots*. *Arrows* refer to the primer amino acid sequences for nested RT-PCR

mixture from different tissues of tree peony, and efficiency values (ranging from 96 to 101 %) were automatically calculated by Bio-Rad CFX manager (version 2.0) (data not shown).

Real-time RT-PCR was performed on a Miniopticon Real-Time PCR instrument (Bio-Rad, USA) using a 20 μL reaction mixture comprised of 10 μL of SYBR® Premix Ex Taq™ (TaKaRa, Japan), 0.4 μL of each primer (10 μM), 2 μL of 10-fold diluted cDNA, and 7.2 μL of PCR-grade water. The PCR program was initiated at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 57 °C for 30 s, and 72 °C for 30 s, and completed with a melting curve analysis in every reaction. Real-time RT-PCR was repeated three times for each sample, with a no template reaction as a negative control, and the PCR products were analyzed on 2.0 % agarose gels stained with ethidium bromide to ensure their size in accordance with expectation. The transcript abundance of each *PsEIL* was normalized to that of the reference gene, *Paeonia suffruticosa ubiquitin* (*PsUB*), and relative transcript level was presented as mean with standard errors.

Results

Isolation and sequence analysis of *EIL* cDNAs from tree peony

With degenerate primers designed based on the conserved EIN3 domains, PCR products of about 350 bp in length were obtained from senescent petals of tree peony and subsequent sequencing revealed three distinct fragments. Based on the sequences of these three partial cDNA fragments, full-length cDNAs of the corresponding genes were isolated by the combination of RT-PCR and RACE-PCR and designated as *PsEIL1* (GenBank accession number JQ771469), *PsEIL2* (GenBank accession number JQ771470), and *PsEIL3*

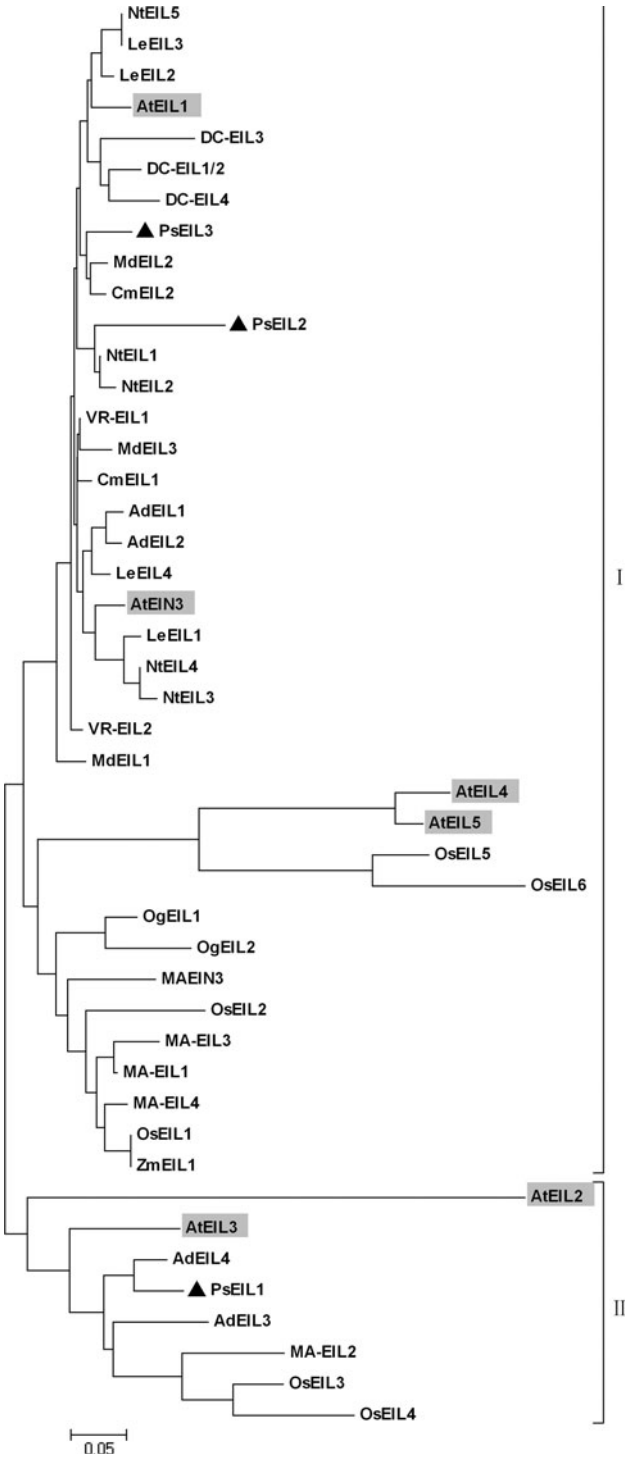
Table 1 Primers for isolation of *PsEILs*

Gene	Primer name	Primer sequence (5′–3′)	Application
PsEILs	EIL-OF (Outer forward primer)	GA[A/G/C][C/T]T[A/G/C/T]GAG[A/C][A/G][A/G/T][A/C]G[A/G/C]ATGTGG	Partial cDNAs
	EIL-OR (Outer reverse primer)	GA[C/T]TG[A/G/C/T]C[G/T][A/G/C/T]AC[A/G/C][A/T]GC[C/T]T[A/G/C/T]C[G/T][A/G/T]AT	
	EIL-IF (Inner forward primer)	GC[A/G/C/T][A/C]G[A/G/C/T][A/C]G[A/G]AA[A/G]AA[A/G]ATGTC	
	EIL-IR (Inner reverse primer)	A[A/G][A/G/C/T]GG[A/G][A/T]A[A/C/T][C/T][G/T][C/T]C[G/T][C/T]TG[A/G/C/T]GG	
PsEIL1	3′-EIL1-G1	GAGAAGGGTAAGCCCGTAAGTGGTGC	3′ end cDNA
	3′-EIL1-G2	AGATGCTACATTGGGGTCACTTTTG	
	5′-EIL1-G1	TGATGGATTCCTCTCGGCTCAAAACCC	5′ end cDNA
	5′-EIL1-G2	CTTACGGGCTTACCCTTCTCAGGAAT	
	EIL1RTF	CTCCTTCCGTTTCTCCATTACCCT	Full-length cDNA
	EIL1RTR	TGAGATATTTACATGAACCCCCTG	
PsEIL2	3′-EIL2-G1	CTCCGAGAGTGGTGGAAGGATAAAGTC	3′ end cDNA
	3′-EIL2-G2	TATGGCGGGTCCTACTTCACACACT	
	5′-EIL2-G1	AAGTGTGTGAAGTAGGACCCGCCAT	5′ end cDNA
	5′-EIL2-G2	GCCCCACCTACTGGTTTTCCCTTCTC	
	EIL2RTF	TCCTCTATCTCTTTCTTCTCCCTT	Full-length cDNA
	EIL2RTR	CCCAACACTACCTTATTAGCCTGT	
PsEIL3	3′-EIL3-G1	CTGGGATGAACGAAGACTGCTCTACT	3′ end cDNA
	3′-EIL3-G2	GCAGCATTGTGACCCACCCCAGAGGCG	
	5′-EIL3-G1	CCTGTAGGCATTTAGACTGACGAACC	5′ end cDNA
	5′-EIL3-G2	AGTAGAGCAGTCTTCGTTCATCCCAG	
	EIL3RTF	GATGATTGCTGGAGGAGACCCGAT	Full-length cDNA
	EIL3RTR	GACACCCCCAAAGTTCACAAAGTG	

Table 2 Primers for real-time RT-PCR analysis

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Size of PCR product (bp)
PsEIL1	GGACTTGGTTATCCTTCTCG	TCTGTTGGCATTCATTTGTCC	191
PsEIL2	TGTGAGTTCCTTGGTTGTCC	GAGCAGCTAGAGGGAAGACA	169
PsEIL3	CCAGCAAGAGCAACAGAAGTTTCAG	AATCGGAAGTCGTCAAGGTTGTCG	177
PsUB	GACCTATACCAAGCCGAAG	CGTTCCAGCACCACAATC	142

(GenBank accession number JQ771471). *PsEIL1* was 2,400 bp in full length consisting of a 196-bp 5′-untranslated region (UTR), a 1,833-bp ORF encoding 610 amino acids, and a 371-bp 3′-UTR. *PsEIL2* was 2,399 bp long composed of a 261-bp 5′-UTR, a 1,824-bp ORF encoding 607 amino acids, and a 314-bp 3′-UTR. The cDNA of *PsEIL3* was 2,917 bp comprising 213-bp 5′-UTR, a 1,959-bp ORF encoding 652 amino acids, and a 745-bp 3′-UTR. Deduced polypeptides encoded by *PsEIL1*, *PsEIL2*, and *PsEIL3* had predicted molecular masses of 68, 69, and 73 kDa, respectively. The overall nucleotide sequence identity between the *PsEILs* ranged from 40 to 47 %, while the ORFs were 47–58 % identical at the nucleotide level and 35–51 % at the amino acid level. In addition, *PsEIL3* shared 98 % nucleotide identity with a previously cloned cDNA fragment, *Ps-EIN3-1* (EU526839), which encodes a partial tree peony EIN3 homologue of only 180 amino acid residues (Zhou et al. 2010). Multiple sequence alignment analysis revealed that three *PsEILs* share predicted amino acid sequence similarity with the *Arabidopsis* EIN3 protein, especially in the conserved N-terminal halves (Fig. 1). All the PsEILs possess an acidic region, a proline-rich region, and five small clusters of basic amino acids in the N-terminus, and have putative sites for nuclear localization signal, as well as the lysine residue (234 for PsEIL1, 244 for PsEIL2, and 249 for PsEIL3) which is critical for the function of EIN3 (Chao et al. 1997). The



◀**Fig. 2** Phylogenetic analysis of EIN3 homologues from *Paeonia suffruticosa* (PsEIL1, JQ771469; PsEIL2, JQ771470; and PsEIL3, JQ771471), *Arabidopsis thaliana* (AtEIN3, AF004216; AtEIL1, AF004213; AtEIL2, AF004214; AtEIL3, AF004215; AtEIL4, NM_121050; and AtEIL5, NM_125909), *Nicotiana tabacum* (NtEIL1, AY248903; NtEIL2, AY248904; NtEIL3, AY248905; NtEIL4, AY248906; and NtEIL5, AY248907), *Lycopersicon esculentum* (LeEIL1, AF328784; LeEIL2, AF328785; LeEIL3, AF328786; and LeEIL4, AB108840), *Dianthus caryophyllus* (DC-EIL1/2, AY728191; DC-EIL3, AY728192; and DC-EIL4, AY728193), *Vigna radiata* (VR-EIL1, AF467784; VR-EIL2, AF467783), *Malus* x *domestica* (MdEIL1, GU732484; MdEIL2, GU732485; and MdEIL3, GU732486), *Cucumis melo* (CmEIL1, AB063191; CmEIL2, AB063192), *Actinidia deliciosa* (AdEIL1, EU170633; AdEIL2, EU887511; AdEIL3, EU887512; and AdEIL4, EU887513), *Musa acuminata* (MA-EIL1, DQ682615; MA-EIL2, AB266318; MA-EIL3, AB266319; MA-EIL4, AB266320; and MAEIN3, AB266321), *Oncidium* (OgEIL1, HQ585983; OgEIL2, HQ585984), *Oryza sativa* (OsEIL1, AB074971; OsEIL2, AB074972; OsEIL3, AK065979; OsEIL4, AP005816; OsEIL5, AP005116; and OsEIL6, AL606636), and *Zea mays* (ZmEIL1, EU973374). PsEILs and the EIN3/EILs of *Arabidopsis thaliana* are highlighted by *triangles* and *gray boxes*, respectively. The scale below means 0.05 amino acid substitutions per site

polyasparagine repeats present in the C-terminus of *Arabidopsis* EIN3 are absent in any of the PsEILs (Fig. 1).

Phylogenetic analysis of multiple EIN3 homologues with complete amino acid sequences revealed that these proteins could be classified in two clusters (Fig. 2). Cluster I containing a majority of EIN3/EIL family members is separated into two subclusters. Both PsEIL2 and PsEIL3 are grouped into the first subcluster, which consists of AtEIN3 and AtEIL1, together with other homologues from various dicotyledonous species. Within this subcluster, PsEIL2 is the closest to NtEIL1 and NtEIL2, while PsEIL3 is the closest to MdEIL2 and CmEIL2. The second subcluster mainly includes EIN3 homologues from monocotyledons. PsEIL1 belongs to the same cluster (cluster II) as AtEIL2 and AtEIL3, but it proves to be more closely related to AtEIL3 than to AtEIL2.

Tissue specificity and expression patterns of *PsEIL* genes during cut flower opening and senescence

Real-time RT-PCR was performed to investigate the tissue specificity and expression patterns of the three *PsEILs* during cut flower postharvest development. Results showed that the transcripts of *PsEILs* were found in all tree peony tissues examined, but with different accumulation patterns and levels based on the tissue and the opening stage.

None of the three *PsEIL* genes were flower specific. *PsEIL1* was constitutively expressed in all tissues except somewhat more abundance in roots. The closely related *PsEIL2* and *PsEIL3* genes exhibited different expression patterns, with *PsEIL2* transcript higher in roots and lower in flower tissues, and *PsEIL3* high mRNA accumulation in petals and low abundance in stamens and pistils compared with the vegetative tissues. Expression levels of these *PsEIL* genes were different from each other in various tissues, and the most prominent feature was that *PsEIL3* transcript accumulated considerably higher in sepals, leaves, and stems, but especially in petals, compared with the other two *PsEIL* genes (Fig. 3).

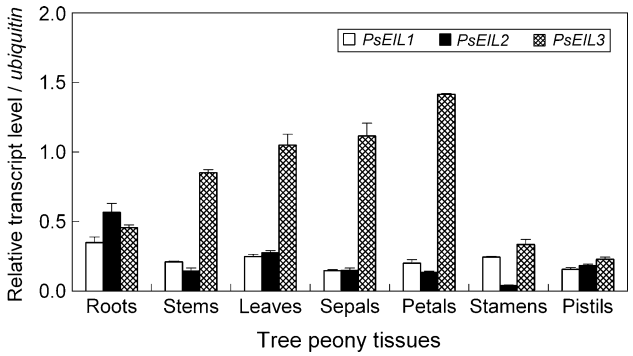


Fig. 3 Relative transcript levels of three *PsEILs* in different tissues. Real-time RT-PCR was performed to analyze the relative transcript levels of three *PsEILs* in roots, stems, leaves, sepals, petals, stamens, and pistils from intact tree peony with fully opened flowers. Data from real-time RT-PCR were normalized to *PsUB* mRNA levels, and presented as mean with standard errors (S.E.) of three replications

Throughout cut flower postharvest development, *PsEIL1* transcript in petals accumulated at a relatively low level during the early opening stages, and reached the maximum abundance when cut flower became senescent (stage 6), whereas *PsEIL2* and *PsEIL3* in petals kept increasing gradually during the opening process until reaching peak mRNA accumulation in fully opened flowers (stage 5), and subsequently started to drop with wilting. Although the expression patterns for the two genes of *PsEIL2* and *PsEIL3* were similar, *PsEIL3* exhibited approximately tenfold higher mRNA accumulation than *PsEIL2* (Fig. 4).

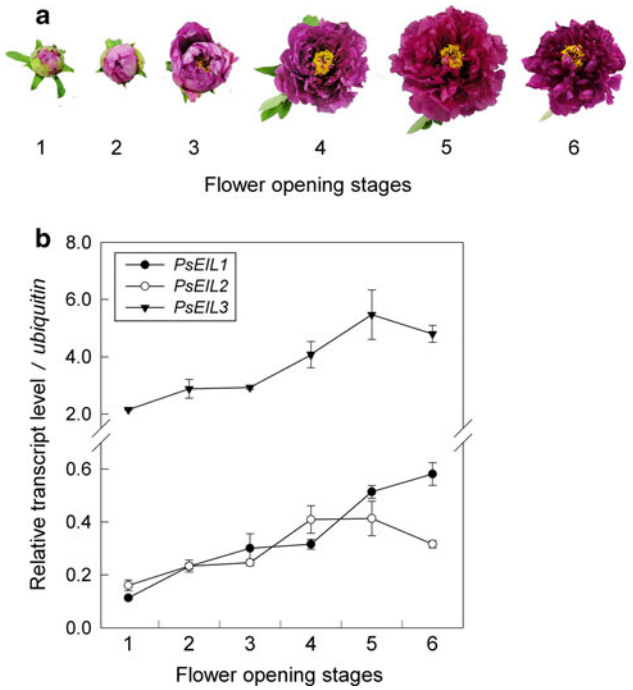


Fig. 4 **a** Phenotypes of tree peony cut flower at the following stages of postharvest development: soft bud (*stage 1*), pre-opening (*stage 2*), initial opening (*stage 3*), half opening (*stage 4*), full opening (*stage 5*), and wilting (*stage 6*). **b** Relative transcript levels of three *PsEILs* in petals throughout tree peony cut flower postharvest development. Real-time RT-PCR was performed to analyze the relative transcript levels of three *PsEILs* in petals at different flower opening stages. Data from real-time RT-PCR were normalized to *PsUB* mRNA levels, and presented as mean with standard errors (S.E.) of three replications

Effect of ethylene and 1-MCP treatments on *PsEIL* gene expression

To investigate whether the expression of the *PsEILs* was modulated by ethylene, transcript levels of these genes in

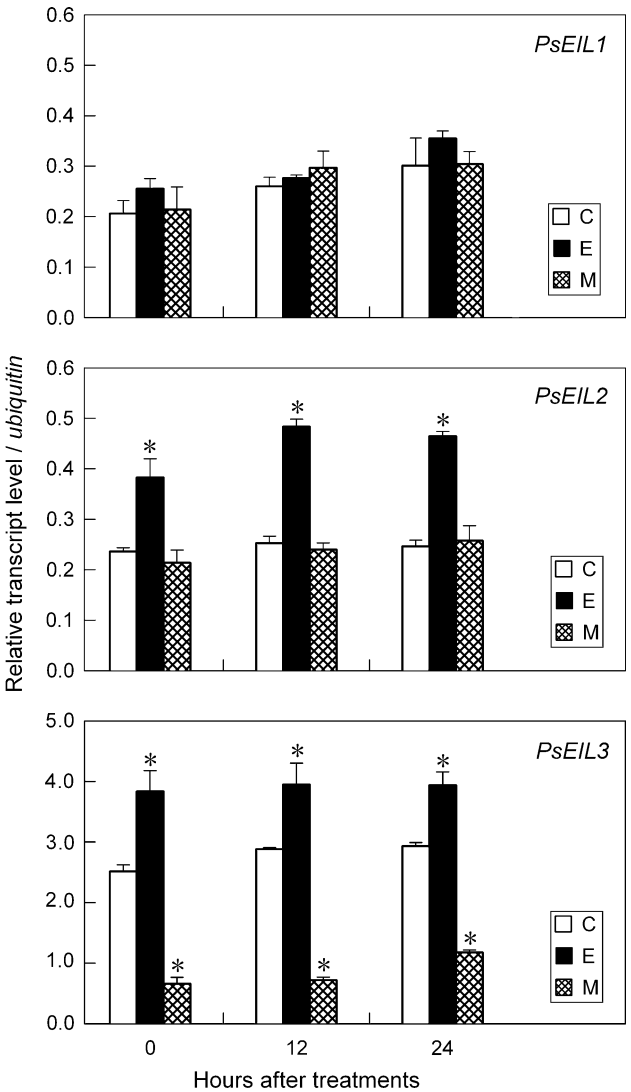


Fig. 5 Effect of exogenous ethylene and 1-MCP treatments on transcript levels of three *PsEILs* in petals. Real-time RT-PCR was performed to analyze the relative transcript levels of three *PsEILs* in petals at 0, 12, and 24 h after different treatments, including C (cut flower incubated with air), E (10 μL L^{-1} ethylene for 6 h), and M (1 μL L^{-1} 1-MCP for 6 h). Data from real-time RT-PCR were normalized to *PsUB* mRNA levels, and presented as mean with standard errors (S.E.) of three replications (*$P < 0.05$ for ethylene or 1-MCP treatment against control)

petals after exogenous ethylene and 1-MCP treatments were determined. As shown in Fig. 5, when compared with the control, the transcript level of *PsEIL1* showed no significant alteration after treatments with exogenous ethylene and 1-MCP. By contrast, as *PsEIL2* and *PsEIL3* transcripts were seemingly unchanged in control petals, they were both immediately enhanced to 1.5-fold at the end of ethylene treatment (0 h), and remained significantly induced at 12 and 24 h thereafter. The mRNA abundance of *PsEIL3* was substantially suppressed after 1-MCP treatment, with an approximately fourfold decline from 0 to 12 h, and afterwards a 2.5-fold decrease. However, *PsEIL2* transcript level was not significantly reduced by 1-MCP at any time examined.

Discussion

Ethylene plays an important role in regulating postharvest opening and senescence of cut flower, and ethylene signal transduction is a critical part in this regulation. In the present work, three full-length cDNAs for transcription factor EIN3 homologous genes, *PsEIL1*, *PsEIL2*, and *PsEIL3*, were isolated and characterized from tree peony. Deduced polypeptides of all the *PsEILs* show high sequence similarity to the *Arabidopsis* EIN3 protein, with many highly conserved domains in the N-terminus (Fig. 1). These regions are also well conserved in other EIN3/EIL proteins from different plant species (Chao et al. 1997; Lee and Kim 2003; Chen et al. 2011; Zou et al. 2011). In addition, PsEILs do not have the polyasparagine repeats near the C-terminus. Similar results have been found in predicted peptide sequences of *Arabidopsis EIL1* and tomato *LeEILs* which complement the *Arabidopsis ein3-1* mutant (Chao et al. 1997; Tieman et al. 2001). These structure conservations suggest that *PsEILs* are functional *EIL* genes (Chao et al. 1997). A previously cloned cDNA fragment from tree peony, *Ps-EIN3-1*(Zhou et al. 2010), is very similar to the corresponding region of *PsEIL3*. But the *Ps-EIN3-1* fragment encodes a short amino acid sequence, which is less than 30 % of the full-length sequence of PsEIL3 and located in the highly conserved N-terminus. Furthermore, the nucleotide sequences of *PsEIL3* and *Ps-EIN3-1* are not identical, with 7 nucleotide differences within the conserved coding region, where 8 nucleotide differences exist between *NtEIL3* and *NtEIL4* from tobacco (Rieu et al. 2003). Thus, we could not rule out the possibility that *PsEIL3* and *Ps-EIN3-1* may be different *EIL* cDNAs in tree peony.

The *PsEILs* were widely expressed in various tree peony tissues with different expression patterns and levels, such data are in accordance with those from other species (Waki et al. 2001; Rieu et al. 2003; Yokotani et al. 2003; Yin et al. 2010; Chen et al. 2011) and match multiple functions of ethylene depending on tissue types, including regulating root hair formation, stem elongation, flower senescence, and leaf abscission (Abeles et al. 1992; Bleecker and Kende 2000). Within the *PsEIL* gene family, *PsEIL1* exhibited a relatively constant expression level in all tissues and *PsEIL2* mRNA level was higher in roots and lower in flower tissues. Similar expression patterns have also been observed in kiwifruit *EIL* gene family (Yin et al. 2008; Yin et al. 2010). Compared with the above two *PsEIL* genes, *PsEIL3* was predominantly expressed in sepals, leaves, and stems, but especially in petals, where *PsEIL3* showed the highest transcript abundance. Considering the result of a previous study that the ethylene production of the intact flower is primarily derived from the petals in tree peony 'Luoyang Hong' (Jia 2010), we propose that the expression of three *PsEIL* genes, especially *PsEIL3*, could contribute to the response of tree peony to ethylene.

During cut flower opening and senescence, three *PsEIL* transcripts in petals presented clear up-regulation, although the patterns and levels of their expression were different. Since EIN3 acts as a positive regulator in the ethylene signaling pathway (Chao et al. 1997), it would be expected that the increase in mRNA levels of *PsEILs* and, in turn, proteins would enhance tissue sensitivity to ethylene, which is consistent with the fact that tree peony gradually becomes more and more ethylene sensitive with flower opening (Liu 2009). It is worth noting that the production of endogenous ethylene, which influences flower diameter and postharvest opening process, increases during cut tree peony 'Luoyang Hong' opening and reaches a peak at full opening stage, then decreases (Jia et al. 2008). These data make it possible to put forward a hypothesis that the up-regulation of *PsEILs* during vase life might be associated with the increase of endogenous ethylene. In an attempt to demonstrate the regulatory effect of ethylene on *PsEILs* at the transcriptional level, we determined the transcript abundances of *PsEILs* in petals after exogenous ethylene and 1-MCP treatments.

The *PsEIL1* transcript, which was relatively low at the early opening stages and then accumulated with the maximum at senescence, was not significantly affected by either ethylene or 1-MCP treatment. It is likely that the accumulation in the transcript level of *PsEIL1* is due to some senescence signals rather than ethylene, as in the case of melon *CmEILs* (Huang et al. 2010). The pattern of *PsEIL1* transcript unaffected by exogenous ethylene here is similar to those of *EIN3/EILs* found in *Arabidopsis* (Chao et al. 1997), tomato (Tieman et al. 2001), tobacco (Rieu et al. 2003), mung been (Lee and Kim 2003), rose (Ma et al. 2006), and kiwifruit (Yin et al. 2008), suggesting that their regulation may be controlled by ethylene at the post-

transcriptional level. Indeed, it has been reported that ethylene induces *Arabidopsis* EIN3/EIL1 protein accumulation by promoting EBF1/EBF2 proteasomal degradation (Guo and Ecker 2003; Yanagisawa et al. 2003; An et al. 2010). However, phylogenetic analysis showed that PsEIL1 is located in a cluster distant to that of the two other PsEILs but together with AtEIL3, a member of the EIN3 family that might participate in regulating sulfur acquisition and metabolism rather than ethylene response (Maruyama-Nakashita et al. 2006). Thus, further study on the regulation of PsEIL1 at the protein level would be important to confirm the involvement of PsEIL1 in the ethylene signaling pathway.

Apart from the post-transcriptional regulation, previous studies have shown that transcriptional regulation of *EIN3/EIL* genes also played an important role in ethylene-mediated plant development. In carnation, three *DC-EIL* genes were regulated at the transcriptional level, in particular, one gene (*DC-EIL3*) was induced throughout flower development and after ethylene treatment, which was considered a possible role in regulation of flower senescence (Iordachescu and Verlinden 2005). In banana, *MA-EIL2* mRNA abundance was markedly increased by ethylene and ripening, which showed a positive correlation with fruit quality development (Mbeguie et al. 2008). A recent study has reported that the increase in transcript levels of *OgEIL1* and *OgEIL2* in *Oncidium* may be attributed to ethylene (Chen et al. 2011). Similar to these findings, in the present work, *PsEIL2* and *PsEIL3* transcripts increased gradually during cut flower opening and reached peak abundance at full opening stage before decreasing, coincident with the climacteric phase of ethylene production (Jia et al. 2008). In addition, *PsEIL2* and *PsEIL3* transcripts were significantly increased following exogenous application of ethylene even at the early opening stages, but only *PsEIL3* was substantially inhibited by 1-MCP treatment. Previous studies revealed that exogenous ethylene treatment effectively enhanced the ethylene production before initial opening of the flowers, and accelerated postharvest opening and senescence of tree peony 'Luoyang Hong' cut flower, on the other hand, 1-MCP suppressed ethylene production and prolonged the vase life (Zhou et al. 2009), indicating that the pattern of *PsEIL3* mRNA accumulation was positively correlated with that of ethylene production and the postharvest process of cut flower after different treatments. Taken together, these results suggest that consistent with the high phylogenetic proximity of PsEIL3 with DC-EIL3 (Fig. 2), the transcriptional regulation of *PsEIL3* is critical in ethylene signaling and possibly involved in ethylene-medicated flower opening and senescence.

In conclusion, we have isolated and characterized three EIN3 homologous genes (*PsEIL1*, *PsEIL2*, and *PsEIL3*) from tree peony. These *PsEIL* genes were differentially expressed in various tissues and during cut flower opening and senescence, indicating they are spatiotemporally regulated. *PsEIL1* is likely associated with petal senescence, and possibly subject to post-transcriptional regulation by ethylene; *PsEIL2* and *PsEIL3* are regulated at the transcriptional level and at least *PsEIL3*, which was more abundant in petals and under positive feedback regulation by ethylene and 1-MCP, may play an essential role in ethylene-mediated postharvest development of tree peony cut flower. Further studies should let us gain insight into the accumulation of PsEIL proteins in relationship with the ethylene response and the process of cut flower opening and senescence. Our work should reveal targets for RNAi inhibition of critical *PsEILs* in ethylene signal transduction and extend the vase life of tree peony cut flower.

Acknowledgments The authors thank Assistant Professor Mengmeng Gu (Texas A&M University, USA) and the two anonymous reviewers for critically revising the manuscript. This research was supported by the National Natural Science Foundation of China (No. 30972030).

References

Abeles FB, Morgan PW, Saltveit ME Jr (1992) Ethylene in plant biology. Academic Press, San Diego

Alonso JM, Stepanova AN, Solano R, Wisman E, Ferrari S, Ausubel FM, Ecker JR (2003) Five components of the ethylene-response pathway identified in a screen for weak ethylene-insensitive mutants in *Arabidopsis*. Proc Natl Acad Sci USA 100:2992–2997

An FY, Zhao QO, Ji YS, Li WY, Jiang ZQ, Yu XC, Zhang C, Han Y, He WR, Liu YD, Zhang SQ, Ecker JR, Guo HW (2010) Ethylene-induced stabilization of ETHYLENE INSENSITIVE3 and EIN3-LIKE1 is mediated by proteasomal degradation of EIN3 binding F-Box 1 and 2 that requires EIN2 in *Arabidopsis*. Plant Cell 22:2384–2401

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

Borochov A, Woodson WR (1989) Physiology and biochemistry of flower petal senescence. Hortic Rev 11:15–43

Chang S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Mol Biol Rep 11:113–116

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

Chen YF, Etheridge N, Schaller GE (2005) Ethylene signal transduction. Ann Bot 95:901–915

Chen SY, Tsai HC, Raghu R, Do YY, Huang PL (2011) cDNA cloning and functional characterization of ETHYLENE INSENSITIVE 3 orthologs from *Oncidium* Gower Ramsey involved in flower cutting and pollinia cap dislodgement. Plant Physiol Biochem 49:1209–1219

De Paepe A, Vuylsteke M, Van Hummelen P, Zabeau M, Van Der Straeten D (2004) Transcriptional profiling by cDNA-AFLP and microarray analysis reveals novel insights into the early response to ethylene in *Arabidopsis*. Plant J 39:537–559

Gao J, Jia PY, Wang YJ, Zhang C, Wang WR, Dong L (2011) Effects of ethylene and 1-MCP treatments on the expressions of CTR genes of tree peony. Acta Bot Boreal-Occident Sin 31:19–26

Guo H, Ecker JR (2003) Plant responses to ethylene gas are mediated by SCF (EBF1/EBF2)-dependent proteolysis of EIN3 transcription factor. Cell 115:667–677

Guo H, Ecker JR (2004) The ethylene signaling pathway: new insights. Curr Opin Plant Biol 7:40–49

Guo WW, Dong L, Wang LY, Chen RX, Liu AQ (2004) The postharvest characteristics and water balance of some cultivars of tree-peony cut flowers. Scientia Silvae Sinicae 40:89–93

Huang SZ, Sawaki T, Takahashi A, Mizuno S, Takezawa K, Matsumura A, Yokotsuka M, Hirasawa Y, Sonoda M, Nakagawa H, Sato T (2010) Melon EIN3-like transcription factors (CmEIL1 and CmEIL2) are positive regulators of an ethylene- and ripening-induced 1-aminocyclopropane-1-carboxylic acid oxidase gene (*CM-ACO1*). Plant Sci 178:251–257

Iordachescu M, Verlinden S (2005) Transcriptional regulation of three EIN3-like genes of carnation (*Dianthus caryophyllus* L. cv. Improved White Sim) during flower development and upon wounding, pollination, and ethylene exposure. J Exp Bot 56:2011–2018

Jia PY (2010) Isolation and expression analysis of key genes related to ethylene signal transduction pathway in tree peony cut flowers. Dissertation, Beijing Forestry University

Jia PY, Zhou L, Guo WW, Wang LY, Dong L (2008) Postharvest behavior and endogenous ethylene pattern of *Paeonia suffruticosa* cut flowers. Acta Hortic 768:445–450

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

Lavee S, Parnes A, Avidan N (2010) Involvement of ethylene in growth induction of stationary tobacco pith tissue in vitro. Plant Cell Tissue Organ Cult 103:123–129

Lawton KA, Huang B, Goldsbrough PB, Woodson WR (1989) Molecular cloning and characterization of senescence-related genes from carnation flower petals. Plant Physiol 90:690–696

Lawton KA, Raghothama KG, Goldsbrough PB, Woodson WR (1990) Regulation of senescence-related gene expression in carnation flower petals by ethylene. Plant Physiol 93:1370–1375

Lee JH, Kim WT (2003) Molecular and biochemical characterization of *VR-EILs* encoding mung bean ETHYLENE INSENSITIVE3-LIKE proteins. Plant Physiol 132:1475–1488

Lin ZF, Zhong SL, Grierson D (2009) Recent advances in ethylene research. J Exp Bot 60:3311–3336

Liu YY (2009) The responses of 'Luoyang Hong' tree peony cut flowers at different opening stages to ethylene and 1-MCP. Dissertation, Beijing Forestry University

Lu JR, Vahala J, Pappinen A (2011) Involvement of ethylene in somatic embryogenesis in scots pine (*Pinus sylvestris* L.). Plant Cell Tissue Organ Cult 107:25–33

Ma N, Tan H, Xue JH, Li YQ, Gao JP (2006) Transcriptional regulation of ethylene receptor and *CTR* genes involved in ethylene-induced flower opening in cut rose (*Rosa hybrida*) cv. Samantha. J Exp Bot 57:2763–2773

Mao C, Wang S, Jia Q, Wu P (2006) *OsEIL1*, a rice homolog of the *Arabidopsis EIN3* regulates the ethylene response as a positive component. Plant Mol Biol 61:141–152

Maruyama-Nakashita A, Nakamura Y, Tohge T, Saito K, Takahashi H (2006) *Arabidopsis* SLIM1 is a central transcriptional regulator of plant sulfur response and metabolism. Plant Cell 18:3235–3251

Mbeguie D, Hubert O, Fils-Lycaon B, Chillet M, Baurens FC (2008) EIN3-like gene expression during fruit ripening of Cavendish banana (*Musa acuminata* cv. Grande naine). Physiol Plant 133:435–448

Reid MS, Evans RY, Dodge LL, Mor Y (1989) Ethylene and silver thiosulphate influence opening of cut rose flowers. J Am Soc Hort Sci 114:436–440

Reid MS, Wu MJ (1992) Ethylene and flower senescence. Plant Growth Regul 11:37–43

Rieu I, Mariani C, Weterings K (2003) Expression analysis of five tobacco EIN3 family members in relation to tissue-specific ethylene responses. J Exp Bot 54:2239–2244

Shibuya K (2012) Molecular mechanisms of petal senescence in ornamental plants. J Jpn Soc Hort Sci 81:140–149

Tacken E, Ireland H, Gunaseelan K, Karunairetnam S, Wang D, Schultz K, Bowen J, Atkinson RG, Johnston JW, Putterill J, Hellens RP, Schaffer RJ (2010) The role of ethylene and cold temperature in the regulation of the apple *POLYGALACTURONASE1* gene and fruit softening. Plant Physiol 153:294–305

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. Plant J 26:47–58

Verlinden S, Boatright J, Woodson WR (2002) Changes in ethylene responsiveness of senescence-related genes during carnation flower development. Physiol Plantarum 116:503–511

Waki K, Shibuya K, Yoshioka T, Hashiba T, Satoh S (2001) Cloning of a cDNA encoding EIN3-like protein (DC-EIL1) and decrease in its mRNA level during senescence in carnation flower tissues. J Exp Bot 52:377–379

Yanagisawa S, Yoo SD, Sheen J (2003) Differential regulation of EIN3 stability by glucose and ethylene signalling in plants. Nature 425:521–525

Yin XR, Chen KS, Allan AC, Wu RM, Zhang B, Lallu N, Ferguson IB (2008) Ethylene-induced modulation of genes associated with the ethylene signalling pathway in ripening kiwifruit. J Exp Bot 59:2097–2108

Yin XR, Allan AC, Chen KS, Ferguson IB (2010) Kiwifruit *EIL* and *ERF* genes involved in regulating fruit ripening. Plant Physiol 153:1280–1292

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

Zhou L, Dong L (2008) Cloning and sequence analysis of 1-aminocyclopropane-1-carboxylic acid oxidase gene cDNA from tree peony. Acta Hortic Sin 35:891–894

Zhou L, Jia PY, Liu J, Wang WR, Huo ZP, Dong L (2009) Effect of ethylene on cut flowers of tree peony 'Luoyang Hong' opening and senescence process and endogenous ethylene biosynthesis. Acta Hortic Sin 36:239–244

Zhou L, Dong L, Jia PY, Wang WR, Wang LY (2010) Expression of ethylene receptor and transcription factor genes, and ethylene response during flower opening in tree peony (*Paeonia suffruticosa*). Plant Growth Regul 62:171–179

Zhu Z, An F, Feng Y, Li P, Xue L, A M, Jiang Z, Kim JM, To TK, Li W, Zhang X, Yu Q, Dong Z, Chen WQ, Seki M, Zhou JM, Guo H (2011) Derepression of ethylene-stabilized transcription factors (EIN3/EIL1) mediates jasmonate and ethylene signaling synergy in *Arabidopsis*. Proc Natl Acad Sci USA 108:12539–12544

Zou AL, Zhang WJ, Pan QY, Zhu SM, Yin JJ, Tian RN, Gu HW, Wang XM, Qi JL, Yang YH (2011) Cloning, characterization, and expression of *LeEIL-1*, an *Arabidopsis* EIN3 homolog, in *Lithospermum erythrorhizon*. Plant Cell Tissue Organ Cult 106:71–79