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RESEARCH PAPER

Identification and expression analysis of *ERF* transcription factor genes in petunia during flower senescence and in response to hormone treatments

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Abstract

***Ethylene-responsive element-binding factor* (*ERF*) genes constitute one of the largest transcription factor gene families in plants. In *Arabidopsis* and rice, only a few *ERF* genes have been characterized so far. Flower senescence is associated with increased ethylene production in many flowers. However, the characterization of *ERF* genes in flower senescence has not been reported. In this study, 13 *ERF* cDNAs were cloned from petunia. Based on the sequence characterization, these PhERFs could be classified into four of the 12 known ERF families. Their predicted amino acid sequences exhibited similarities to ERFs from other plant species. Expression analyses of *PhERF* mRNAs were performed in corollas and gynoecia of petunia flower. The 13 *PhERF* genes displayed differential expression patterns and levels during natural flower senescence. Exogenous ethylene accelerates the transcription of the various *PhERF* genes, and silver thiosulphate (STS) decreased the transcription of several *PhERF* genes in corollas and gynoecia. *PhERF* genes of group VII showed a strong association with the rise in ethylene production in both petals and gynoecia, and might be associated particularly with flower senescence in petunia. The effect of sugar, methyl jasmonate, and the plant hormones abscisic acid, salicylic acid, and 6-benzyladenine in regulating the different *PhERF* transcripts was investigated. Functional nuclear localization signal analyses of two PhERF proteins (PhERF2 and PhERF3) were carried out using fluorescence microscopy. These results supported a role for petunia *PhERF* genes in transcriptional regulation of petunia flower senescence processes.**

Key words: ERF, ethylene, flower senescence, gene expression, petunia.

Introduction

The gaseous phytohormone ethylene is involved in many aspects of plant growth and development (Abeles *et al.*, 1992). Flower senescence is associated with increased ethylene production in many flowers. The climacteric rise of endogenous ethylene in these flowers has been shown to play a regulatory role in the events leading to the death of some of the flower organs (Iordachescu and Verlinden, 2005).

The expression of ethylene-related genes is induced through transduction of the ethylene signal from receptors to dedicated transcription factors (Giovannoni, 2004). Ethylene-responsive factors (ERFs) are uniquely present in the plant kingdom and belong to the AP2/EREBP-type transcription factors, which function as *trans*-acting factors at the last step of transduction (Ohme-Takagi and Shinshi, 1995). ERF proteins can specifically bind the GCC box *cis*-acting element (core sequence AGCCGCC) that is present in promoters of pathogenesis-related (*PR*) genes (Ohme-Takagi and Shinshi, 1995; Park *et al.*, 2001). Although *Arabidopsis* has 122 predicted *ERF* genes (Nakano *et al.*, 2006) and *Oryza sativa* (rice) has 139 predicted *ERF* genes, only a few have been characterized so

Abbreviations: ABA, abscisic acid; BA, 6-benzylaminopurine; ERF, ethylene-responsive element-binding factor; IAA, indole-3-acetic; JA, jasmonic acid; MeJA, methyl jasmonate; RACE, rapid amplification of cDNA ends; SA, salicylic acid; STS, silver thiosulphate; TF, transcription factor.

far (Sakuma *et al.*, 2002). In tomato, plum, kiwifruit, and apple, the role of *ERF* genes in fruit ripening has been reported. However, the characterization of *ERF* genes in flower senescence has not been reported.

Petunia, an important ornamental plant, often serves as a model for ethylene-sensitive flower senescence. Transgenic petunias constitutively expressing the mutant *Arabidopsis* ethylene receptor gene *etr1-1* exhibit decreased ethylene sensitivity and delayed flower senescence (Wilkinson *et al.*, 1997). The role of petunia *EIN2* was reported by Shibuya *et al.* (2004). In the present work, 13 full-length *ERF* genes of petunia were identified and transcriptional regulation of the 13 *ERF* genes was investigated during natural flower senescence in petunia and in response to different plant hormones, in order to gain further insights into their roles in petunia flower senescence. Finally, the subcellular localization of two of the PhERF proteins was examined using green fluorescent protein (GFP).

Materials and methods

Plant material

Petunia (*Petunia hybrida* 'Carpet White') plants were grown under normal greenhouse conditions (22 °C, 14 h light/10 h dark). Flowers were emasculated 1 d before flowers were fully open to prevent self-pollination. Eight to 10 petunia flowers were harvested at anthesis (corollas 90 ° reflexed) stages and then placed immediately in tap water. Within 1 h of harvest, the flowers were delivered to the laboratory; after the stems were cut to a length of 5 cm under water, the flowers were placed in distilled water for further processing. Corollas and gynoecia were collected from petunias at 0 (corollas 90 ° reflexed), 24, 36, 48, 60, 72, and 84 h after flower full opening (Fig. 3A). Stem, leaves, and roots were collected from plants at the vegetative stage when the plants were ~10 cm in height. All tissues were frozen in liquid nitrogen and stored at –80 °C until used for RNA extraction. Fresh weights were measured immediately before freezing. At least six corollas or gynoecia were pooled for each time point. All experiments were conducted at least three times with independently collected and extracted tissues, unless otherwise noted.

RNA extraction and RT-PCR

Total RNA was extracted according to previously described protocols (Woodson and Lawton, 1988; Iordachescu and Verlinden, 2005). Briefly, 2 g of frozen tissue was powdered under liquid nitrogen and homogenized in equal volumes (10 ml) of phenol:chloroform:isoamylalcohol (25:24:1, v/v/v) and an extraction buffer containing 50 mM TRIS-HCl (pH 8.0), 5% phenol (pH 8.0), 6% sodium *p*-aminosalicylate, and 1% (v/v) β-mercaptoethanol. Following phase separation, nucleic acids were precipitated by the addition of 1/20 vol. of 4 M sodium acetate (pH 6.0) and 2 vols of ethanol overnight at –20 °C. The precipitate was resuspended in diethylpyrocarbonate (DEPC)-treated water, reprecipitated by the addition of 3 vols of 4 M sodium acetate (pH 6.0), and incubated on ice for 1.5 h. The precipitated RNA was dissolved in DEPC-treated water. RNA content was determined spectrophotometrically.

A 1 μg aliquot of total RNA was reverse transcribed at 42 °C for 1 h in 20 μl final volume reaction mixture containing reaction buffer, 20 mmol l^{-1} dithiothreitol (DTT), 0.5 mmol l^{-1} dNTP, 1 μg oligo$(dT)_{15}$, and reverse transcriptase (AMV; Promega, Madison, WI, USA) according to the manufacturer's instructions.

Cloning of the petunia PhERF1–PhERF13 *genes*

The partial sequences of *PhERF1–PhERF13* were obtained by a computational identification approach (Yang *et al.*, 2010). Briefly, TBLASTN analysis against the GenBank EST database (http://www.ncbi.nlm.nih.gov) with *AtERF2* identified 13 petunia clones, FN015325, FN032926, CV301184, FN004404, FN001585, FN006171, FN014682, CV297340, FN033374, FN000871, FN005346, FN040788, and CV300585, encoding putative proteins that displayed conservation with *Arabidopsis* ERFs. The remaining 5′ and 3′ cDNA sequences of *PhERF1–PhERF13* were isolated by rapid amplification of cDNA ends (RACE). The Gene Race amplification system (Invitrogen, Carlsbad, CA, USA) and EXPAND Taq polymerase (Perkin-Elmer, Wellesley, MA, USA) were used for all RACE experiments. Once the sequence of the 5′ and 3′ ends of each cDNA had been determined, full-length cDNAs for *PhERF1–PhERF13* were isolated by RT-PCR.

Sequence analysis

Alignments were carried out on DNAMAN software, and a phylogenetic tree was generated with MEGA version 3.1 (Kumar *et al.*, 2004). An identity search for nucleotides and translated amino acids was carried out using the National Center for Biotechnology Information (NCBI) BLAST network server (http://www.ncbi.nlm.gov/BLAST).

Ethylene measurements

To measure the ethylene production, corollas and gynoecia of each individual flower were collected and placed in a 200 ml airtight container according to the method of Ma *et al.* (2006). Thus, to avoid the contamination with wound-induced ethylene, the containers were capped and incubated at 25 °C for 1 h for corollas and gynoecia. Then a 2 ml sample of head-space gas was withdrawn using a gas-tight hypodermic syringe, and injected into a gas chromatograph (GC 17A, Shimadzu, Kyoto, Japan) for ethylene concentration measurement. The gas chromatograph was equipped with a flame ionization detector and an activated alumina column. All measurements were performed with eight replicates.

Quantitative real-time PCR assays

Total RNA was extracted from the samples of corollas and gynoecia and digested with RNase-free DNase I followed by reverse transcription according to the manufacturer's instructions. PCR analysis was performed with the cDNA extracted from different samples as a template. For real-time PCR, oligonucleotide primers were designed according to each gene's untranslated region with Primer3 (version 0.4.0; http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). All primers were tested with melting peaks and dissociation curves to confirm that there was only one product for each pair of primers. In order to verify that primers were specific for amplifications of the target genes, all PCR products were purified and resequenced. The sequences of all primers used for real-time PCR analysis are described in Table 1. Petunia actin was used as the housekeeping gene to quantify cDNA abundance.

The PCR was performed in an iCycler iQ™ Real-time Detection System (Bio-Rad Laboratories, Hercules, CA, USA). For each 25 μl reaction, 1 μl of sample cDNA was mixed with 12.5 μl of IQ™ SYBR® Green Supermix (Bio-Rad Laboratories), 0.5 μl of forward primer (12 μM, final concentration 240 nM), 0.5 μl of reverse primer (12 μM, final concentration 240 nM), and 10.5 μl of sterile water. Samples were subjected to thermal cycling conditions of DNA polymerase activation at 95 °C for 4 min, 40 cycles of 45 s at 95 °C, 45 s at 52 °C or 55 °C, 45 s at 72 °C, and 45 s at 80 °C; a final elongation step of 7 min at 72 °C was performed. The melting curve was designed to increase 0.5 °C every 10 s from 62 °C. Real-time PCR analysis was performed with two different

Table 1. *Primer sequences of* PhERF1–PhERF13 *used in quantitative real-time PCR*

Gene	Forward primer (5′ →3′)	Reverse primer (5′ →3′)	PCR product size (bp)
PhERF1	ATCAAGGTGGCATACAAGAAG	ACCTTCGTGAAATTATAGAGC	145
PhERF2	GCATTTGAATCTGAGATGAAGT	ACTTAGTAGACACCTCCCATCA	167
PhERF3	TGACTGCTCTGATTTTGGTTGG	CTTTGCACTGAATTGTTCGTAC	156
PhERF4	GTATGTTGATGGCCAATCAGTG	ATAAATGAGCTGGGGACACTGG	122
PhERF5	GATTCGGTGATTCTGACCCTCT	ATGCTACTACCCAAGGGCTAAC	170
PhERF6	AGCCAAAGAAGACGGTGGATT	TCCGTCTTCGTCAGCTCTTTC	165
PhERF7	GTGGATGATCGTTGCGATGTTG	GAGACATAACGCAGTAACATGTA	135
PhERF8	GATTGCCGTAGCGACTGTGATT	AGCTCATCGGAGTCCACGTCAT	161
PhERF9	TGACTCTGCAGCTTTCAAGATG	CTCGAAATCCCAACTACGTGGAG	155
PhERF10	CTAGAGCTTATGATTGTGCTGC	TCACCATATCAATTCTCATGATCTC	139
PhERF11	CGGGACGTATGAGACATCAGAG	ATGATTTTCAAACGCCTCTTCGG	156
PhERF12	TAGATACTCCATTGACACCTTC	ATTATGAAACCAAAAGCTGAGAG	138
PhERF13	AATTCTTGGGATGGGGTTGTGA	CCTTCAGCATGTTCATCACCAA	158

cDNAs from the same time point (from two different RNAs), and each was carried out in triplicate. The amplicon was analysed by electrophoresis and sequenced once for identity confirmation. Quantification was based on analysis of the threshold cycle (Ct) value as described by Pfaffl (2001).

Ethylene treatment

Petunia flowers were treated with ethylene according to the previously described protocols (Iordachescu and Verlinden, 2005). Petunia flowers were harvested at anthesis and their stems re-cut to 5 cm, placed in flasks with distilled water, and subsequently treated with 2 μl l^{-1} ethylene for 0, 2, 4, 8, 12, and 24 h. Corollas and gynoecia from 8–10 flowers were collected at each time point, immediately frozen in liquid nitrogen, and stored at –80 °C for later RNA extraction.

Silver thiosulphate (STS), abscisic acid (ABA), indole-3-acetic acid (IAA), salicylic acid (SA), methyl jasmonate (MeJA), 6-benzyladenine (BA), and sucrose treatment

Petunia flowers harvested at anthesis (corollas 90 ° reflexed), re-cut to 5 cm in length, were placed in 1.0 l flasks containing either distilled water or STS (1.6 mM $Na_2S_2O_3{\cdot}5H_2O$, 0.2 mM $AgNO_3$), an inhibitor of ethylene action, and held in controlled environmental conditions (12/12 h, 24 °C day/night temperature, 50 μmol m^{-2} s^{-1}), or 50 μM ABA, 50 μM IAA, 0.6 mM SA, 50 μM MeJA, 50 μM BA, or 5% sucrose solution for 12 h. Then the flowers were transferred to distilled water and incubated at room temperature for 20 d. The distilled water was changed every day. After STS treatment at 0, 24, 48, and 72 h, the corollas and gynoecia were collected and frozen for later RNA extraction. After ABA, IAA, SA, MeJA, BA, or sucrose at 0, 6, and 24 h, the corollas were collected and frozen for later RNA extraction.

Subcellular localization and fluorescence microscopy

The coding sequences of *PhERF2* and *PhERF3* were cloned as a C-terminal fusion in-frame with GFP into the pGreen vector (Hellens *et al.*, 2000) and expressed under the control of the cauliflower mosaic virus (CaMV) *35S* promoter. A high fidelity PCR system was used to amplify the full-length *PhERF2* and *PhERF3* clones. The corresponding open reading frames (ORFs) were cloned using the *Bam*HI restriction site of the pGreen vector.

Particle bombardment with a Biolistic PDS-1000/He system (Bio-Rad, Hercules, CA, USA) was used to introduce GFP fusion plasmids into onion epidermal cells. Tungsten particles were coated with the plasmids 35S:GFP:PhERF2, 35S:GFP:PhERF3, and 35S:GFP, and a helium pressure of 1100 psi was employed. Tungsten particles, 500 μg, coated with 0.8 μg of DNA were used in each shot. The target distance between the stop screen and piece of onion was set at 9 cm. Onions (*Allium cepa* L.) used for particle bombardment were obtained from a local supermarket. After bombardment, onion pieces were placed on Murashige and Skoog (MS) medium and kept in darkness at 22 °C for 15 h. The onion epidermal cells were then examined under a fluorescence microscope (Nikon, Tokyo, Japan) and single optical sections were taken to visualize the resulting images for each transient expression.

Results

Identification of 13 ERF *transcription factor genes in petunia*

Thirteen *ERF* full-length cDNAs were isolated and designated as *PhERF1–PhERF13*. *PhERF1–PhERF13* (GenBank accession nos HQ259595–HQ259607) were predicted to encode proteins of 231, 378, 363, 252, 266, 220, 207, 217, 185, 190, 207, 289, and 129 amino acids, with calculated mol. wts of 25.7, 41.9, 40.1, 28.6, 29.9, 24, 22.6, 24.0, 20.9, 21.3, 23.8, 32.9, and 14.7 kDa, respectively. The relationships between the predicted amino acid sequences, as indicated by percentage similarity over the whole sequence, are presented in Table 2. The similarities of the PhERFs varied from 21.6% (PhERF1 and PhERF2) to 76.7% (PhERF2 and PhERF3). The deduced amino acid sequences of PhERFs comprise a conserved DNA-binding ERF/AP2 domain (ranging from 58 to 59 amino acids), which is characteristic of the plant *ERF* gene family (Fig. 1A).

The ERF domain of most PhERFs includes two key amino acid residues, the 14th alanine A (A) and the 19th aspartate (D), believed to contribute a functional GCC box-binding activity in many ERFs (Sakuma *et al.*, 2002), with the exception of PhERF1, in which the two amino acid residues are instead the 14th valine (V) and the 19th histidine (H) (Fig. 1A).

Phylogenetic analysis of PhERF proteins

Comparison of the amino acid sequence and phylogenetic analysis of PhERFs and 122 AtERFs revealed that PhERF

Table 2. Amino acid sequence comparison between the predicted full-length ethylene response factor (PhERF) cDNAs

	Amino acid similarity (%)											
	PhERF1	**PhERF2**	**PhERF3**	**PhERF4**	**PhERF5**	**PhERF6**	**PhERF7**	**PhERF8**	**PhERF9**	**PhERF10**	**PhERF11**	**PhERF12**
PhERF2	21.6	100										
PhERF3	21.9	76.7	100									
PhERF4	24.5	36.7	49.0	100								
PhERF5	25.4	36.5	34.2	35.5	100							
PhERF6	28.2	23.2	23.7	25.7	30.4	100						
PhERF7	27.3	27.9	24.6	27.1	29.7	32.0	100					
PhERF8	25.3	22.9	24.9	25.4	25.8	33.3	54.7	100				
PhERF9	40.2	29.3	29.5	33.6	29.2	43.0	37.6	38.5	100			
PhERF10	41.2	33.1	31.7	32.9	32.7	40.6	40.8	39.8	59.2	100		
PhERF11	35.0	26.9	27.2	29.7	30.5	37.4	41.0	34.2	35.3	36.6	100	
PhERF12	26.7	25.5	23.1	25.2	24.1	27.1	32.8	26.7	39.7	37.0	32.5	100
PhERF13	36.1	37.0	33.8	35.4	45.5	36.5	33.1	35.2	41.8	41.7	42.7	36.4

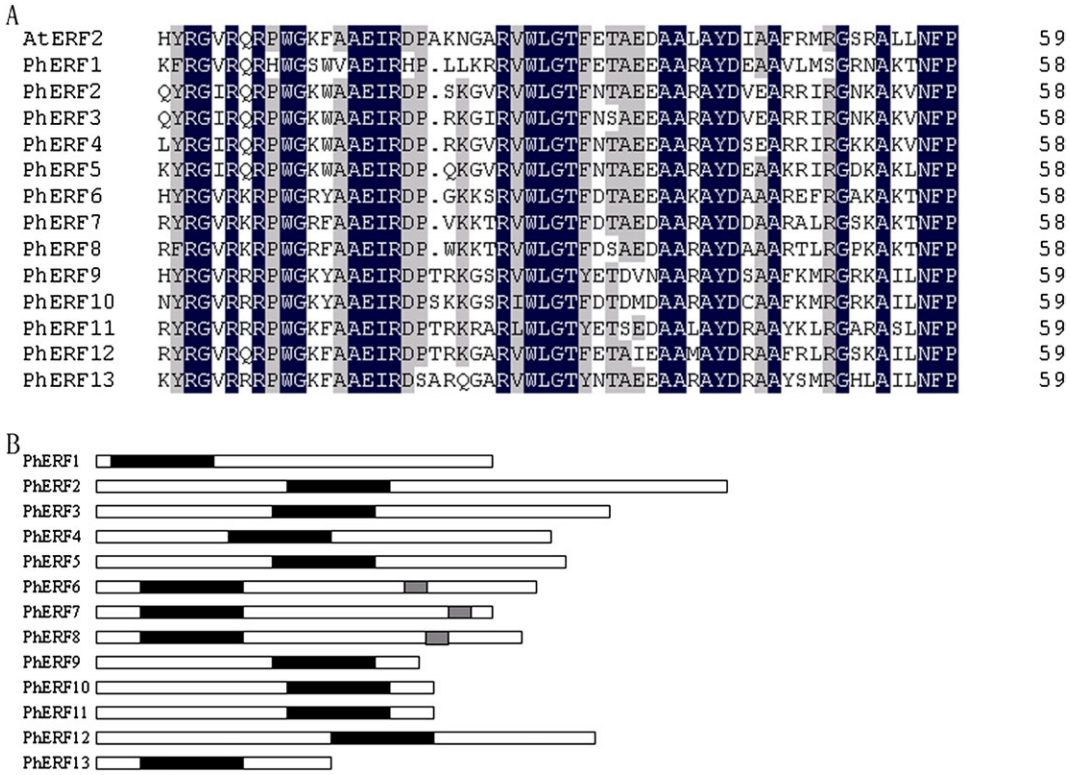

Fig. 1. Schematic analysis of PhERFs with ERF domains and EAR repressor domains. (A) Comparison of ERF domains by deduced amino acids sequences. Conserved residues are shaded in black. Grey shading indicates similar residues in 10 out of 14 of the sequences. (B) Location of ERF domains (black bars) and EAR repressor domains (grey bars).

sequences belong to four out of 12 groups of ERF proteins (Nakano *et al.*, 2006) (Fig. 2).

The predicted PhERF1 protein is classified as a members of group V ERFs (Nakano *et al.*, 2006) (Fig. 2). The PhERF1 predicted protein contains an ERF domain consisting of 58 amino acids located near the middle of the sequences (Fig. 1B). The deduced amino acid sequence of PhERF1 shares 68.1, 57.1, and 56.2% identity with *Arabidopsis* At1g15360/SHN1, At5g11190/SHN2, and At5g25390/SHN3. The four genes, belonging to subgroup Va, share two motifs, CMV-1 and CMV-2, in the C-terminal regions (see Supplementary Fig. S1a available at *JXB* online).

PhERF2–PhERF5 encode proteins that belong to group VII ERFs (Nakano *et al.*, 2006) (Fig. 2). These sequences share 33.7–50.0% similarity with their homologues in *Arabidopsis*, At1g53910/RAP2.12 and At3g14230/RAP2.2. The predicted PhERF2, PhERF3, and PhERF4 proteins share the CMVII-1 motif in the N-terminal regions with At1g53910/RAP2.12 and At3g14230/RAP2.2 (see Supplementary Fig. S1b at *JXB* online), but PhERF5 did not include this motif.

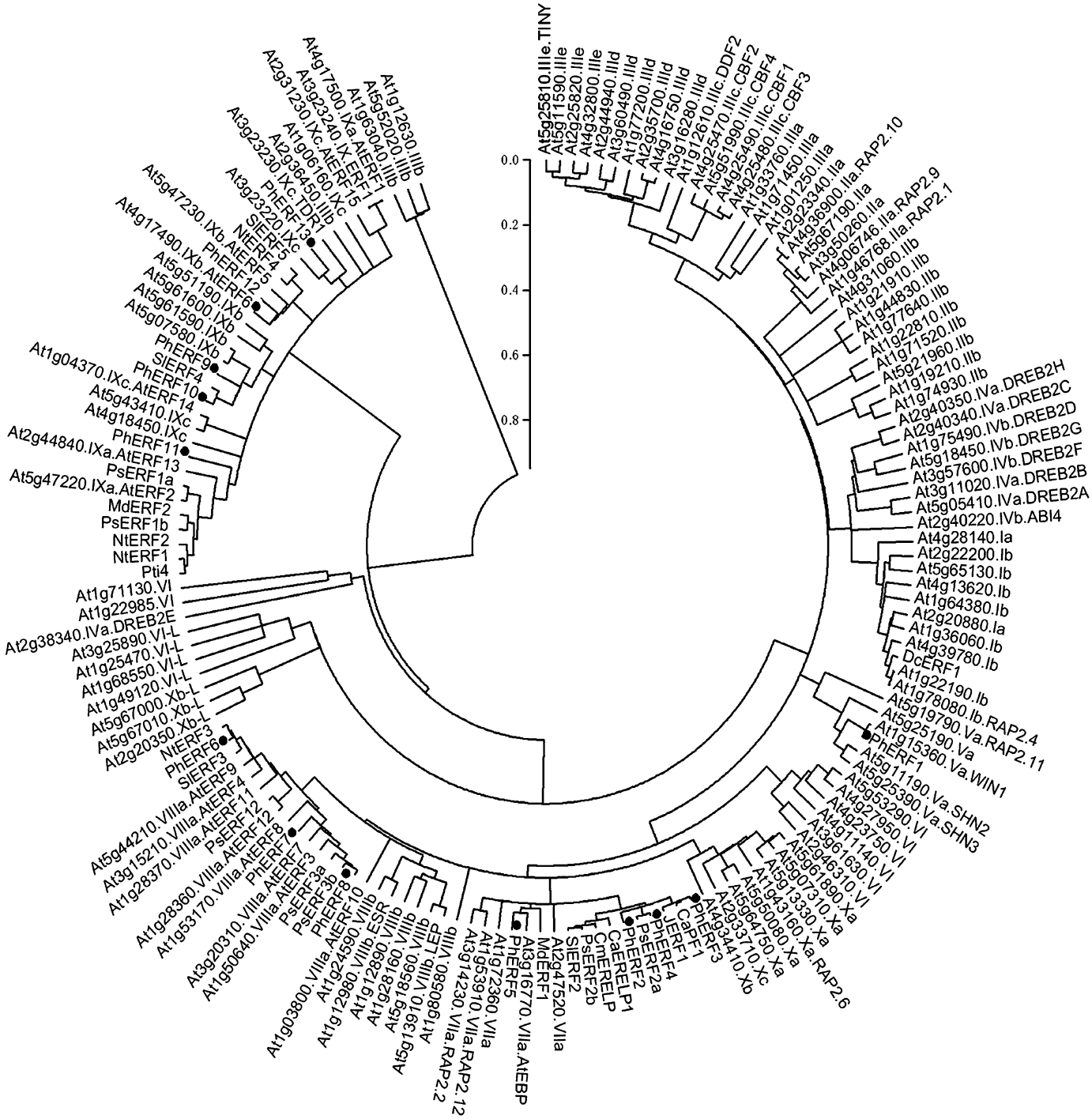

Fig. 2. Phylogenetic tree of ERFs. Thirteen petunia PhERFs (black circles) were aligned with the *Arabidopsis* ERF family, *Solanum lycopersicum* [SlERF2 (GenBank accession number AAO34704), SlERF3 (AAO34705), SlERF4 (AAO34706), SlERF5 (AY559315.1), Pti4 (AAC50047), JERF1 (AAK95687)]; *Nicotiana tabacum* [NtERF1 (UniProtKB/Swiss-Prot accession no. Q40476), NtERF2 (Q40479), NtERF3 (Q40477), NtERF4 (Q40478)]; *Capsicum annuum* [CaPF1 (GenBank accession no. AAP72289), CaERELP1 (AAS20427)], *Malusx domestica* [MdERF1 (GenBank accession no. BAF43419), MdERF2 (BAF43420)]; *Prunus salicina* [PsERF1a (GenBank accession no. FJ026009), PsERF1b (FJ026008), PsERF2a (FJ026007]), PsERF2b (FJ026006), PsERF3a (FJ026005), PsERF3b (FJ026004), PsERF12 (FJ026003)]; *Cucumis melo* [CmERELP (GenBank accession no. BAD01556)]; and *Dianthus caryophyllus* [DcERF1 (GenBank accession no. AB517647)]. The amino acid sequences of *Arabidopsis* ERFs were obtained from the Arabidopsis Information Resource or the National Center for Biotechnology Information database. The amino acid sequences were analysed with Vector NTI (version 9.0.0; Invitrogen), and the phylogenetic tree was constructed with MEGA (version 3.1) using a bootstrap test of phylogeny with minimum evolution test and default parameters.

The predicted PhERF6–PhERF8 proteins are assigned to group VIII ERFs (Nakano *et al.*, 2006) (Fig. 2). The three sequences share 36.0–63.1% similarity with their *Arabidopsis* homologues, At1g28370/AtERF11, At1g50640 /AtERF3, and At1g53170/AtERF8. The predicted PhERF6–PhERF8 proteins possess an ERF domain consisting of 57 amino acids located near the N-terminal regions (Fig. 1B) and comprise two motifs, the ERF-associated amphiphilic repression domain (EAR/CMVIII-1; Fig. 1B) and CMVIII-2, in the C-terminal region (Zhou *et al.*, 1997; Fujimoto *et al.*, 2000; Tournier *et al.*, 2003; Nakano *et al.*, 2006) (see Supplementary Fig. S1c at *JXB* online).

The predicted PhERF9–PhERF13 proteins are classified as members of group IX ERFs (Nakano *et al.*, 2006) (Fig. 2). These sequences shared 27.3–46.0% similarity with their homologues in *Arabidopsis*, At3g23240/AtERF1 and At2g31230/AtERF15.

Ethylene production and PhERF *gene expression in corollas and gynoecia of petunia during natural flower senescence*

Ethylene measurement of corollas and gynoecia of the senescence stage of petunia flower used in this study showed a typical ethylene climacteric, and ethylene production was detected after full opening (0 h) of the flowers, attaining the maximum rate in corollas and gynoecia at 72 h and 60 h, respectively (Fig. 3B, C).

Changes in the *PhERF* gene mRNA levels were monitored by using quantitative real-time PCR analysis and mRNAs isolated from corollas and gynoecia.

Figure 4 shows the expression profile of the different *PhERF* genes in corollas during natural flower senescence (0–84 h after harvest). The isolated sequences were differentially expressed throughout development and flower senescence. The levels of *PhERF2*, *PhERF3*, *PhERF4*, and *PhERF5* mRNA were low before 36 h, increased significantly at 48 h, peaked at 72 h when ethylene production reached the maximum, and then decreased. A slight increase was found in the amount of mRNAs for *PhERF8* in corollas during natural flower senescence in petunia (Fig. 4). *PhERF1*, *PhERF6*, *PhERF7*, and *PhERF9* showed a constitutive expression pattern, and *PhERF10*, *PhERF12*, and *PhERF13* expression showed a decreasing trend toward the late stage of senescence.

In gynoecia, the amount of *PhERF2*, *PhERF3*, and *PhERF4* transcripts increased significantly at 48 h and peaked at 72 h, the change being consistent with ethylene production in gynoecia during natural flower senescence in petunia (Fig. 5). As in corollas, decreasing levels of *PhERF10*, *PhERF12*, and *PhERF13* transcripts were found in gynoecia. The levels of *PhERF1*, *PhERF5*, *PhERF7*, *PhERF8*, and *PhERF9* mRNAs were almost unchanged during natural flower senescence. However, the transcripts of *PhERF11* were barely detected during natural flower senescence in both corollas and gynoecia and were not affected by exogenous ethylene (data not shown); therefore *PhERF11* was not studied further.

Expression of PhERF *in corollas and gynoecia treated by exogenous ethylene*

To determine whether *PhERF* genes are under ethylene regulation in petunia flowers, quantitative PCR was used to test their relative mRNA accumulation upon short-term exogenous ethylene treatment.

As shown in Fig. 4, ethylene treatment increased the abundance of transcripts of the various *PhERF* genes in corollas, excluding *PhERF6* that was not significantly altered by the treatment. *PhERF7*, *PhERF8*, and *PhERF12* expression was slightly induced by ethylene, and peaked at 8 h after ethylene treatment. The expression profiles of the other eight genes reached a high level at 4 h or 8 h after treatment.

Similarly, in gynoecia, the transcription of most *PhERF* genes was accelerated by ethylene treatment, excluding *PhERF10* that remained almost unchanged by the treatment (Fig. 5). *PhERF1*, *PhERF 4*, and *PhERF8* expression was

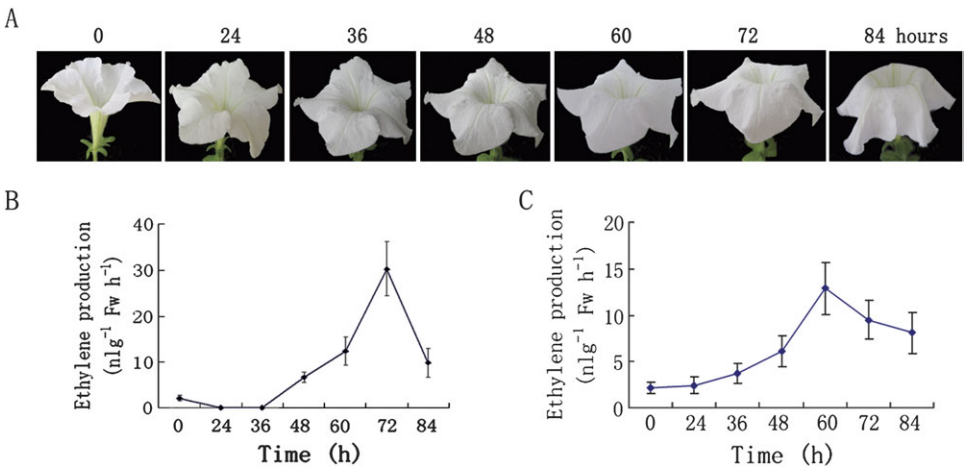

Fig. 3. Natural senescence of unpollinated wild-type (WT) *Petunia hybrida* 'Carpet White' flowers and changes in ethylene production of corollas and gynoecia. (A) Natural senescence of unpollinated WT *P. hybrida* 'Carpet White' flowers. (B) Changes in ethylene production of corollas. (C) Changes in ethylene production of gynoecia. (This figure is available in colour at *JXB* online.)

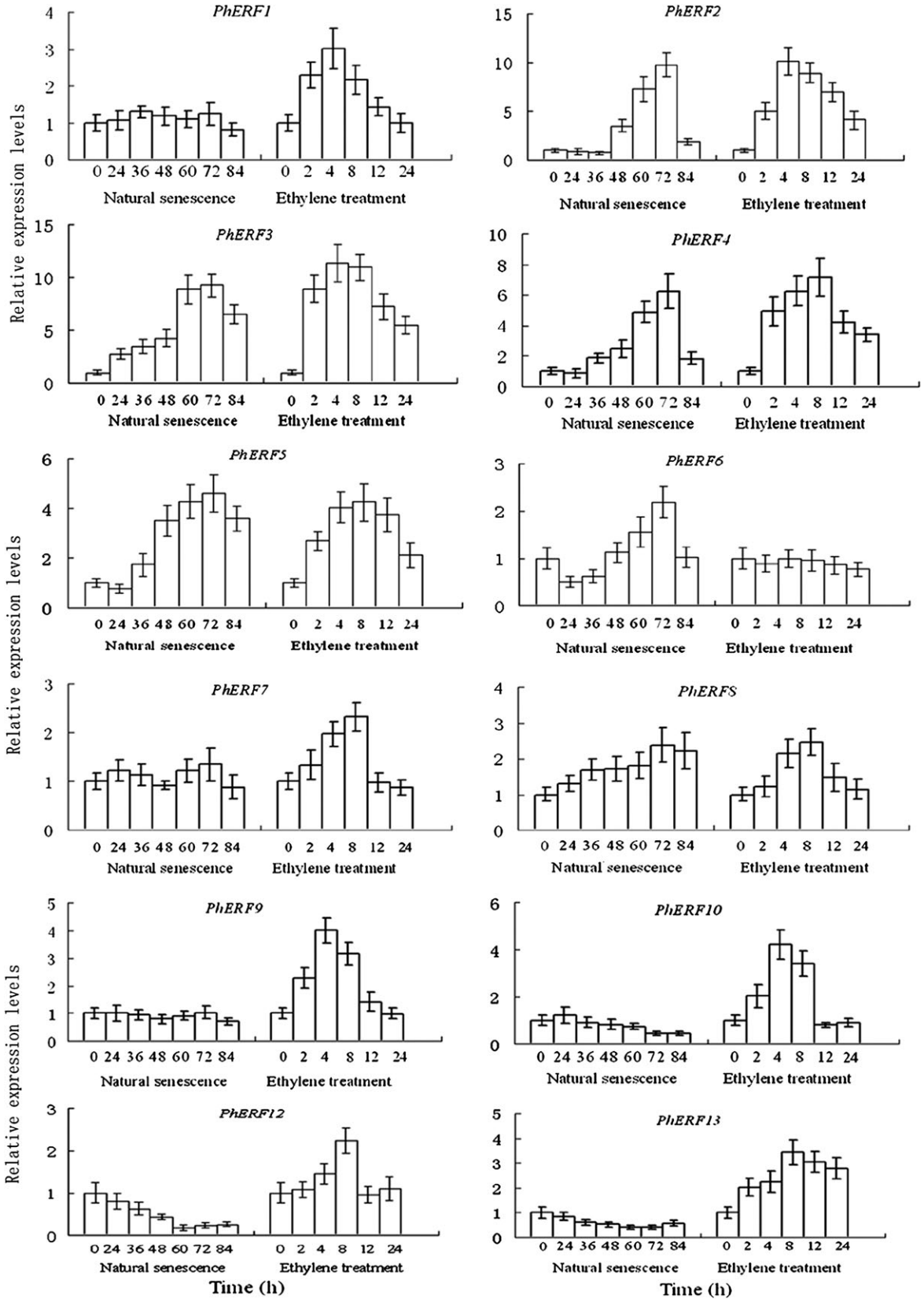

Fig. 4. Expression profile of *PhERF1-10, PhERF12*, and *PhERF13* during natural flower senescence, and effects of exogenous ethylene on the expression of these genes by quantitative PCR in petunia corollas. The mRNA level in corollas was measured by quantitative PCR. Relative expression levels are shown as fold change values (1=time 0). Data are means ±SD (n–3).

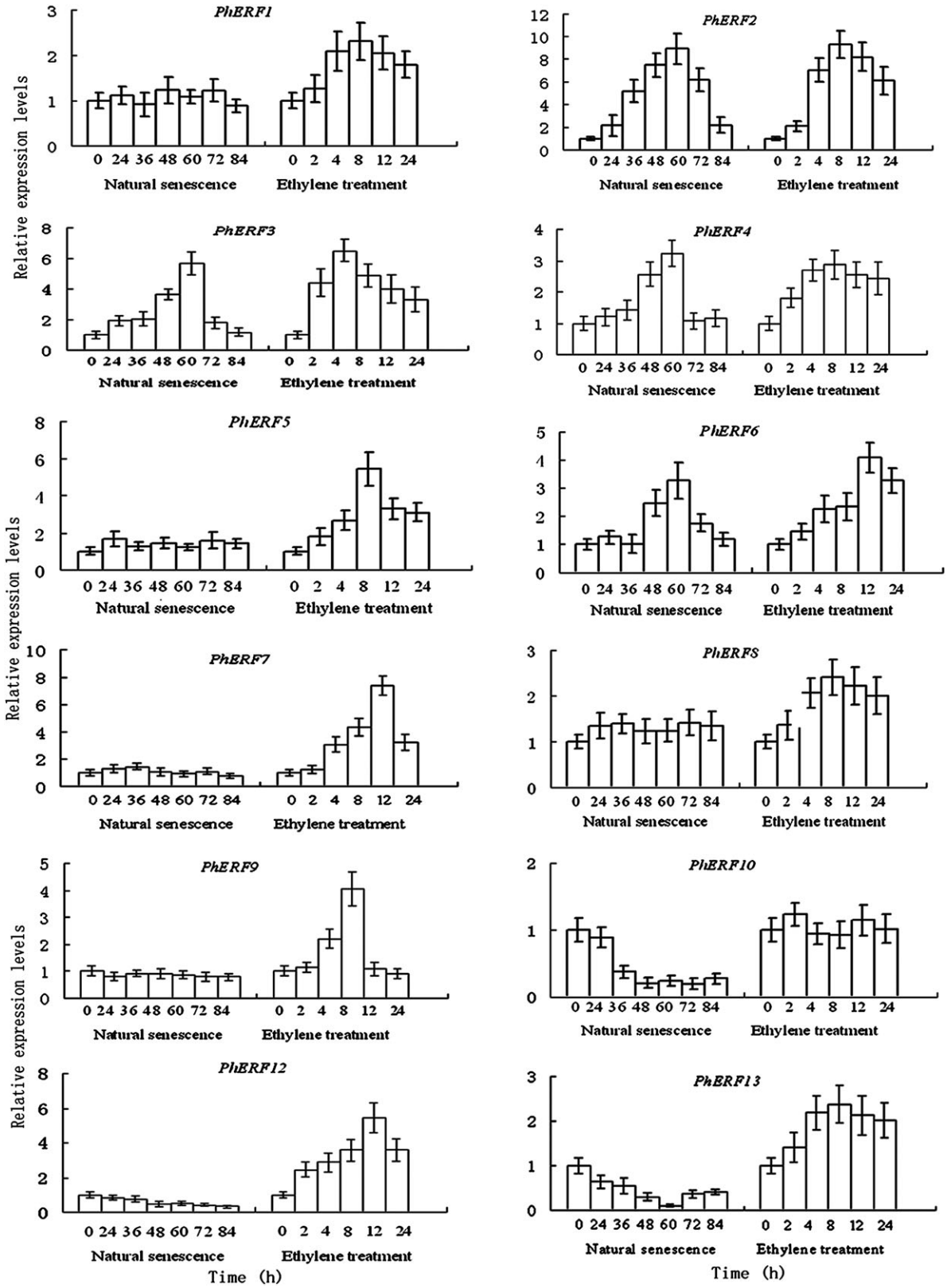

Fig. 5. Expression profile of *PhERF1-10, PhERF12,* and *PhERF13* during natural flower senescence and effects of exogenous ethylene on the expression of these genes by quantitative PCR in petunia gynoecia. The mRNA level in gynoecia was measured by quantitative PCR. Relative expression levels are shown as fold change values (1=time 0). Data are means ±SD (n=3).

slightly increased by ethylene, and peaked at 8 h after ethylene treatment. The expression profiles of the other eight genes reached a high level at 4 h or 8 h after treatment (Fig. 5).

Expression of PhERF *in corollas and gynoecia treated by STS*

STS inhibits the climacteric increase of ethylene by blocking the ethylene-binding site of the ethylene receptor and activating the ethylene receptor (Veen, 1983; Woltering and Van Doorn, 1988). Petunia flowers treated with 0.2 mM STS delayed corolla senescence by 5 d, and no ethylene production was detected during the incubation period (data not shown). The effect of STS on the levels of mRNA for *PhERF* genes was investigated in corollas and gynoecia (Fig. 6).

After STS treatment, the levels of *PhERF1*, *PhERF2*, *PhERF3*, *PhERF4*, *PhERF5*, *PhERF8*, and *PhERF9* mRNA remained low and did not increase compared with the control; STS did not affect the levels of *PhERF6* and *PhERF7* mRNA. *PhERF10*, *PhERF 12*, and *PhERF13* transcripts decreased slightly from 24 h to 72 h after STS treatment. It should be noted that in the non-treated flowers, *PhERF10*, *PhERF12*, and *PhERF13* transcripts decreased significantly from 24 h to 72 h; therefore, after STS treatment, the decrease in their transcripts was delayed in corollas compared with the control (Fig. 6A).

Similar results were observed in gynoecia, with the exception of *PhERF8*, which did not change significantly compared with the control, and *PhERF6*, whose transcripts were decreased when compared with the control (Fig. 6B).

Effect of other hormones and sugar treatments on the expression of the PhERF *genes in corollas*

To investigate the control mechanisms of other hormones underlying *PhERF* gene expression, petunia flowers were treated with ABA, IAA, SA, and MeJA, and the changes in transcript abundance of these genes were analysed using quantitative PCR.

At 6 h after ABA treatment, *PhERF1–PhERF6* and *PhERF8* expression increased significantly, and *PhERF7* expression did not increase significantly, while *PhERF9, PhERF10*, *PhERF12*, and *PhERF13* expression decreased significantly (Fig. 7). The expression of *PhERF1–PhERF8* kept increasing significantly, that of *PhERF10*, *PhERF12*, and *PhERF13* did not change significantly, and that of *PhERF9* kept decreasing from 6 h to 24 h after ABA treatment (Fig. 7).

After IAA treatment, most *PhERF* mRNAs showed accumulation at 6 h and 24 h, with the exception of *PhERF1*, which showed a constitutive expression pattern (Fig. 7).

As shown in Fig. 7, SA treatment did not affect the levels of *PhERF3*, *PhERF4*, *PhERF6*, *PhERF7*, *PhERF12*, and *PhERF13*, it up-regulated the expression of PhERF*2*, *PhERF5*, *PhERF6*, *PhERF8*, *PhERF9*, and *PhERF10*, and it down-regulated that of *PhERF1* in corollas.

JA treatment increased the levels of *PhERF1–PhERF8* mRNA in corollas and did not affect that of *PhERF9*, *PhERF10*, *PhERF12*, and *PhERF13* mRNAs (Fig. 7).

None of the *PhERF* genes responded significantly to BA treatment at least under the conditions used in this study (Fig. 7).

Following treatment of flowers with 5% sucrose, the amount of *PhERF1*, *PhERF5*, *PhERF6*, *PhERF8*, *PhERF12*, and *PhERF13* transcripts in corollas was not changed, and that of *PhERF2*, *PhERF3*, and *PhERF7* increased, while that of *PhERF4*, *PhERF9*, and *PhERF10* decreased at 6 and 24 h (Fig. 7).

Nuclear localization of two PhERF proteins

To confirm that the PhERFs identified are indeed targeted to the nucleus, two *PhERF* genes of group VII were selected for analysis *in vivo*, based on their potential functions associated with senescence (Tournier *et al.*, 2003; Wang *et al.*, 2007; Yin *et al.*, 2010). The coding regions of *PhERF2* and *PhERF3* were fused to the N-terminus of GFP and expressed in onion epidermal cells under the control of the constitutive CaMV *35S* promoter. In two cases, green fluorescent signals were observed only in the nucleus (Fig. 8A, B). With the control vector alone, GFP signals were distributed in both the cytoplasm and nucleus (Fig. 8C). These results indicated that *PhERF2* and *PhERF3* are indeed nuclear-localized proteins, which is consistent with their predicted function as transcription factors.

Discussion

Flower senescence is associated with increased ethylene production in many flowers (Borochov and Woodson, 1989). In this study, 13 members of the AP2/ERF superfamily were identified from petunia. In *Arabidopsis*, 122 of the 147 genes identified as possibly encoding AP2/ERF domain(s) have been assigned to the ERF family, and the *Arabidopsis* ERF family was then classified into 12 groups, groups I–X, VI-L, and Xb-L (Nakano *et al.*, 2006). The 13 PhERFs fall into four out of the12 different groups of the previously characterized ERF proteins (Fujimoto *et al.*, 2000; Tournier *et al.*, 2003; Nakano *et al.*, 2006) (Fig. 2).

PhERF1 belongs to group V ERFs, which share two motifs, CMV-1 and CMV-2, in the C-terminal regions. PhERF2–PhERF5 belong to group VII ERFs. PhERF2–PhERF4 proteins shared the CMVII-1 motif while PhERF5 did not include this motif. PhERF6–PhERF8 belong to group VIII ERFs. The three predicted proteins comprise two motifs, EAR and CMVIII-2. The ERFs of tobacco, *Arabidopsis*, and rice, which contain the CMVIII-1 and CMVIII-2 motifs, have been shown to repress GCC box-mediated transcription via a transient assay (Fujimoto *et al.*, 2000; Ohta *et al.*, 2000, 2001). In addition, *AtERF4* was shown to be a negative regulator in the expression of

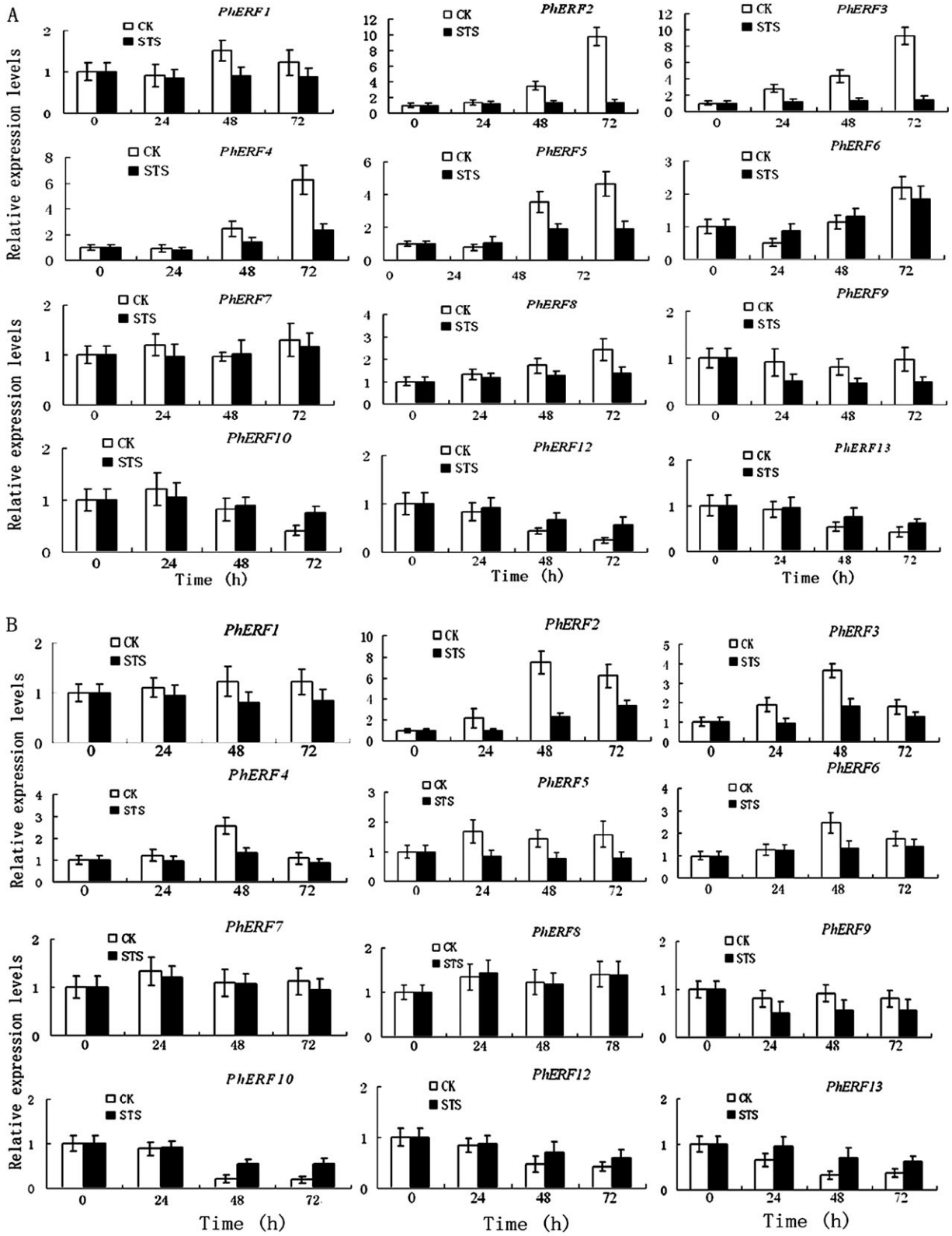

Fig. 6. Effects of STS on the expression of *PhERF1-10*, *PhERF12*, and *PhERF13* by quantitative PCR in corollas and gynoecia. (A) Effects of STS on the expression of *PhERF1-10*, *PhERF12*, and *PhERF13* in corollas. (B) Effects of STS on the expression of *PhERF1-10*, *PhERF 12*, and *PhERF 13* in gynoecia. The mRNA level was measured by quantitative PCR. Relative expression levels are shown as fold change values (1=time 0). Data are means ±SD (n=3).

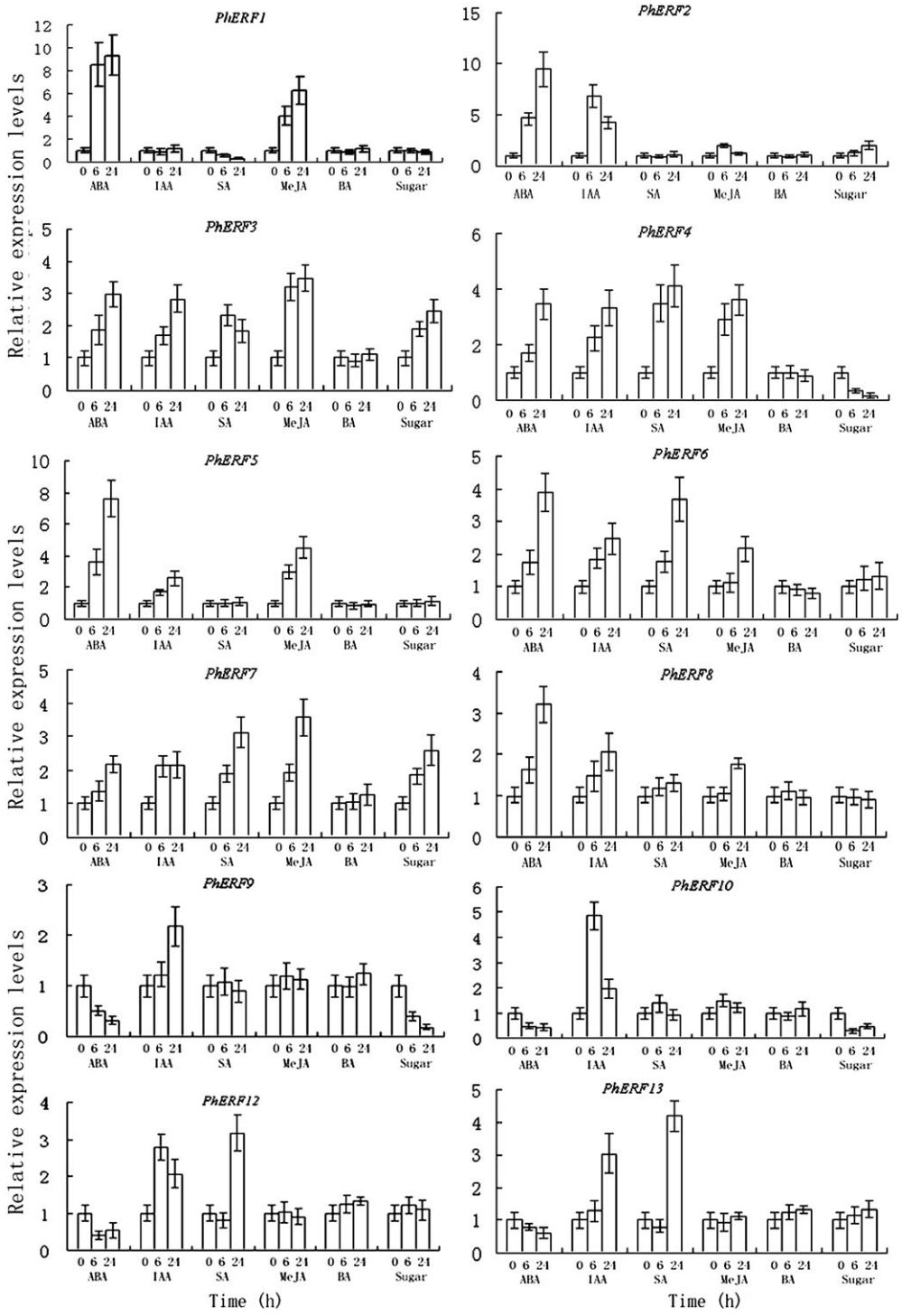

Fig. 7. Effects of ABA, IAA, SA, MeJA, BA, and sugar on the expression of *PhERF1-10*, *PhERF12*, and *PhERF13* by quantitative PCR in corollas of petunia. The mRNA level in corollas was measured by quantitative PCR. Relative expression levels are shown as fold change values (1=time 0). Data are means ±SD (n=3).

ethylene-, jasmonate-, and ABA-responsive genes (McGrath *et al.*, 2005; Yang *et al.*, 2005), and *AtERF7* was shown to play an important role in ABA response in plants (Song *et al.*, 2005). PhERF9–PhERF13 belong to group IX ERFs that have been shown to function as activators of transcription (Solano *et al.*, 1998; Fujimoto *et al.*, 2000; Tournier *et al.*, 2003; Wang *et al.*, 2007).

In addition, the ERF family is also grouped into two major subfamilies, ERF conserving the 14th alanine (A14) and the 19th aspartic acid (D19), and CBF/DREB in which valine (V14) and glutamic acid (E19) are present at the respective positions (Yang *et al.*, 2002; Sakuma *et al.*, 2002; Tournier *et al.*, 2003; Nakano *et al.*, 2006). The predicted amino acid sequences of PhERF2–PhERF13 contain A14

and D19, indicating that they belong to the ERF subfamily, and that of PhERF1 has V14 and H19, indicating that it belongs to the DRE subfamily.

In the present study, *PhERF* mRNAs, with the exception of *PhERF11*, which was below the detection limits in corollas and gynoecia, were present at different levels in different tissues and stages of petunia flower, and showed different responses to exogenous ethylene, indicating that their expression is spatially regulated. In particular, *PhERF6* in corollas did not respond to exogenous ethylene but in gynoecia it increased gradually after ethylene, the expression of *PhERF8* increased slightly from 24 h to 72 h but in gynoecia showed a constitutive expression, and that of *PhERF10* in corollas increased gradually after ethylene but in gynoecia it did not respond to exogenous ethylene (Figs 4, 5).

In the *ERF* gene family, subfamilies III and IV have been associated with the stress response (Novillo *et al.*, 2004; Sakuma *et al.*, 2006; Qin *et al.*, 2008), while subfamilies VII, VIII, and IX contain members that are ethylene responsive (Gu *et al.*, 2000; Tournier *et al.*, 2003; Yang *et al.*, 2005; Onate-Sanchez *et al.*, 2007; Champion *et al.*, 2009; Yin *et al.*, 2010). Subfamily VII genes have been particularly associated with fruit ripening and senescence; for example, the tomato *LeERF2* (Tournier *et al.*, 2003) and apple *MdERF1* (Wang *et al.*, 2007) genes accumulate during fruit ripening, plum *PsERF2a* and *PsERF2b* (El-Sharkawy *et al.*, 2009) genes accumulate in flowers after fertilization, and kiwifruit *AdERF4* and *AdERF6* were markedly stimulated at the later fruit senescence stage (Yin *et al.*, 2010). In this study, most *PhERF* genes, with the exception of *PhERF11*, showed increases in corollas or gynoecia after exogenous ethylene treatment (Figs 4, 5), which supported that subfamilies VII, VIII, and IX contain members that are ethylene responsive. In addition, *PhERF2–PhERF5* which are group VII members, and in particular *PhERF2* and *PhERF3*, showed a strong association with the rise in ethylene production in both petals and gynoecia, which was confirmed by a reduction in expression with STS treatment and the positive response to exogenous ethylene (Figs 4–6), a pattern that has been observed for some senescence-related genes, such as SR8 and SR12 in carnation (Lawton *et al.*, 1989; Jones *et al.*, 1995; Verlinden *et al.*, 2002). This pattern of mRNA accumulation would also be expected of a gene regulating ethylene responsiveness and therefore timing of petal senescence. Therefore, *PhERF2* and *PhERF3* might be associated particularly with flower senescence in petunia. Future studies demonstrating functionality through *in vitro* and *in vivo* experiments including transgenic plants should confirm this.

It should be pointed out that several *PhERF* genes, for example *PhERF12* and *PhERF13*, decreased during natural flower senescence, when ethylene production increased gradually, but increased with exogenous ethylene treatment (Figs 4, 5). This suggested that these *PhERF* genes were regulated not only by ethylene, but also by other factors. Similarly, kiwifruit *AdERF1*, *AdERF7*, *AdERF11*, and *AdERF12* transcripts were slightly higher directly after harvest in control fruit and then declined during ripening (Yin *et al.*, 2010).

Exogenous application of ABA accelerates flower senescence in ethylene-sensitive plants such as petunia (Vardi and Mayak 1989) and in ethylene-insensitive plants such as daylily (Panavas *et al.*, 2000). The endogenous content of ABA increases during senescence in several flowers (Serrano *et al.*, 1999; Hunter *et al.*, 2004). ABA treatment increased ethylene production of the *Rosa* flower (Müller *et al.*, 1999). In this study, *PhERF1–PhERF5*, *PhERF7*, and *PhERF8* transcripts were increased by both ABA and ethylene (Fig. 7) and it is possible that these genes respond to ABA in an ethylene-dependent manner. The increase in transcripts of *PhERF2–PhERF5* of group VII due to ABA treatment was consistent with the accelerating flower senescence caused by ABA treatment, which strengthens the idea that *PhERF2–PhERF5* of group VII are actively involved in flower senescence.

In *Arabidopsis*, the expression of *AtERF4* and *AtERF7*, members of group VIII, can be induced by ABA and were shown to play an important role in ABA response in plants (McGrath *et al.*, 2005; Song *et al.*, 2005; Yang *et al.*, 2005). The expression of *PhERF6*, also a member of group VIII, which was not affected by ethylene, was increased by ABA (Fig. 7), showing that it might not respond to ABA in an ethylene-dependent manner. It is worth mentioning that the expression of three members of group IX, *PhERF10*, *PhERF12*, and *PhERF13*, which increased by ethylene treatment but decreased during natural flower senescence, was decreased by ABA treatment (Fig. 7), showing that the decrease in the transcripts of these gene during natural flower senescence might be associated with the increase in endogenous ABA of corellas in petunia (Vardi and Mayak, 1989). In addition, in the three members of group IX of soybean, *GmERF069* was subjected to negative regulation by ABA, while *GmERF061* and *GmERF079* increased after ABA treatment (Zhang *et al.*, 2008).

Treatment with auxin is useful to reduce drop of flower bracts in *Bougainvillea* (Chang and Chen, 2001). 1-Naphthaleneacetic acid is effective in improving post-harvest life of cut *Eustoma* flowers (Shimizu-Yumoto and Ichimura, 2010). In plum fruits, the high levels of accumulation of *PsERF2a*, *PsERF2b*, *PsERF3a*, and *PsERF12* might be enhanced in an auxin-dependent manner (El-Sharkawy *et al.*, 2009). In this study, treatment with the auxin IAA increased expression of most of the *PhERF* genes, with the exception of *PhERF13* (Fig. 7), showing the direct or indirect response of *PhERF* genes of group VII, VIII, and IX to IAA.

SA can interfere with the biosynthesis and action of ethylene in plants (Raskin, 1992) and delay the ripening of some fruits, probably through inhibition of ethylene biosynthesis or action (Srivastava and Dwivedi, 2000; Zhang *et al.*, 2003). However, in *Arabidopsis* the *AtACS7* promoter was responsive to SA; treatment with SA increased the expression of *AtACS7* (Tang *et al.*, 2008). In this study, the expression of *PhERF2*, *PhERF3*, *PhERF8*, *PhERF9*, and *PhERF10*, as with ethylene treatment, increased after SA

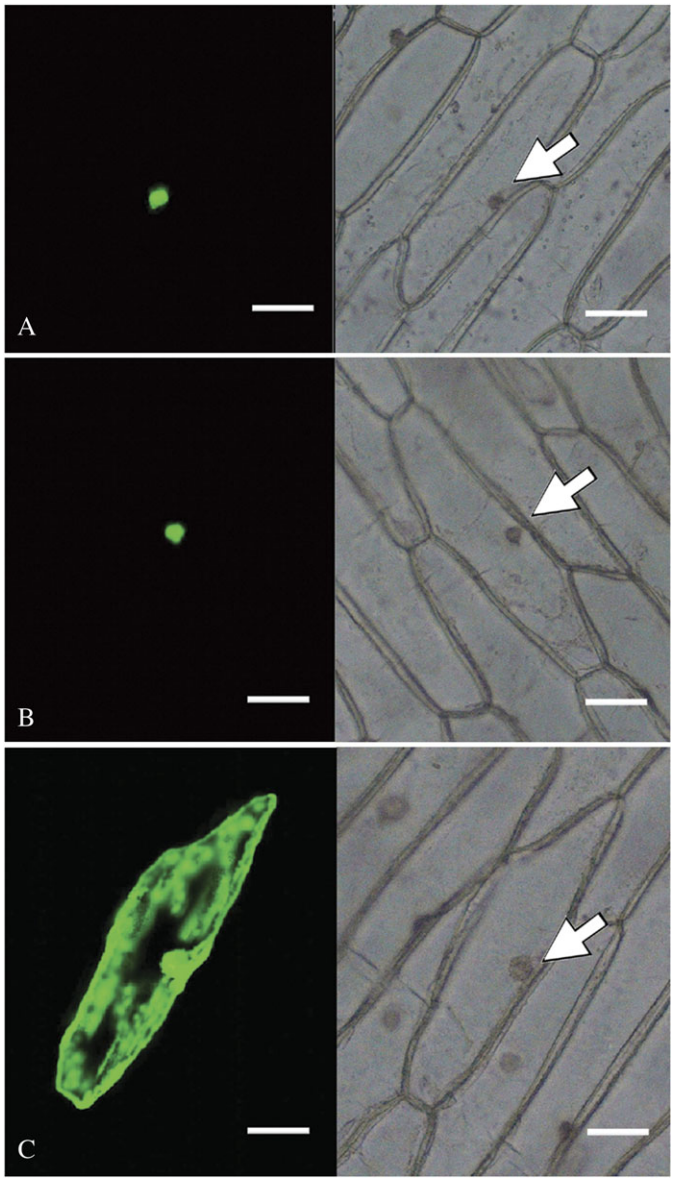

Fig. 8. Subcellular localization of PhERF2 and PhERF3 proteins. (A) Nuclear localization of GFP–PhERF2 fusion protein in onion epidermal cells. (B) Nuclear localization of GFP–PhERF3 fusion protein in onion epidermal cells. (C) Onion epidermal cells transformed with a translational construct of GFP as a positive control showed localization throughout the cell, with the strongest signals in the cytoplasm and nucleus. GFP fluorescence (left panel) and differential contrast imagery (right panel) were compared. The positions of nuclei are indicated by arrows. The scale bars indicate 100 μm.

treatment (Fig. 7), which supported a positive interaction between SA and ethylene in petunia flower. Similarly, in soybean leaf, the expression levels of nine *GmERF* genes, belonging to groups V, VI VII, VIII, IX, and X, were all induced and increased after SA treatment (Zhang *et al.*, 2008).

Jasmonates have been shown to be powerful promoters of plant senescence and there is some evidence that jasmonic acid (JA) or MeJA may promote senescence via ethylene (Sembdner and Parthier, 1993; Emery *et al.*, 1996; Hung, 2006). In *Arabidopsis*, 10 members of the AP2/ERF family, primarily from the VIII and IX subclusters, were induced by both a pathogen and MeJA (McGrath *et al.*, 2005). *AtERF4* of group VIII acts as a negative regulator of JA-responsive defence gene expression and antagonizes JA inhibition of root elongation (McGrath *et al.*, 2005). In contrast, *AtERF2* of group IXa is a positive regulator of JA-responsive defence genes and enhances JA inhibition of root elongation (McGrath *et al.*, 2005). In this study, MeJA treatment increased the levels of mRNA of all the four group VII *PhERF* genes, *PhERF2–PhERF5*, three group VIII *PhERF* genes, *PhERF6–PhERF8*, and *PhERF1* in corollas (Fig. 7). The transcript increases of *PhERF2–PhERF5* of group VII by MeJA were consistent with the accelerating flower senescence caused by MeJA treatment, which supported that *PhERF2–PhERF5* of group VII are actively involved in flower senescence. It is not clear whether the response of *PhERF1*, *PhERF6*, *PhERF7*, and *PhERF8* to JA depended on ethylene production. Also, for JA treatment, the expression of nine *GmERF* genes in soybean leaf all increased (Zhang *et al.*, 2008).

Exogenous treatments with cytokinin may delay senescence of cut flowers (Cook *et al.*, 1985; Van Staden *et al.*, 1990; Lukaszewska *et al.*, 1994). Overproduction of cytokinins in petunia flowers transformed with P_{SAG12}-IPT delays corolla senescence and decreases sensitivity to ethylene (Chang *et al.*, 2003). In plum, *PsERF1a*, *PsERF1b*, *PsERF3a*, and *PsERF12* transcription levels were accelerated in response to cytokinin application, *PsERF2a* and *PsERF3b* were negatively regulated by the treatment, and *PsERF2b* did not respond significantly (El-Sharkawy *et al.*, 2009). However, in this study, when treated with BA, the transcripts of *PhERF1–PhERF13* all accumulated in a constitutive manner and did not respond to BA (Fig. 7). The difference between the responses of *ERF* genes of the two plants to BA need to be researched in the future.

Sucrose is a global regulator of growth and metabolism that is evolutionarily conserved in plants (Smeekens, 2000; Rolland *et al.*, 2001). Sucrose has also been shown to decrease ethylene responsiveness in carnation petals, and sugars are known to extend the vase-life of flowers (Verlinden and Garcia, 2004). Sucrose prevented up-regulation of senescence-associated genes in carnation petals (Hoeberichts *et al.*, 2007). In this study, after 5% sugar treatment, the amount of *PhERF2*, *PhERF3*, and *PhERF7* transcripts increased slightly, and that of *PhERF4*, *PhERF9*, and *PhERF10* decreased at 6 h and 24 h (Fig. 7), which showed that some *PhERF* genes might respond to sugar treatment and play a role in flower senescence.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Amino acid sequence alignment of group V ERFs (a) PhERF1, At1g15360/SHN1, At5g11190/SHN2, and At5g25390/SHN3; (b) PhERF2, PhERF3, PhERF4,

At1g53910/RAP2.12, and At3g14230/RAP2.2; and (c) PhERF6, PhERF7, PhERF8, At1g28370/AtERF11, At1g50640/AtERF3, and At1g53170/AtERF8 using the DNAMAN program.

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