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RESEARCH PAPER

Molecular characterization of seven genes encoding ethylene-responsive transcriptional factors during plum fruit development and ripening

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Abstract

Seven ERF cDNAs were cloned from two Japanese plum (*Prunus salicina* L.) cultivars, 'Early Golden' (EG) and 'Shiro' (SH). Based on the sequence characterization, these Ps-ERFs could be classified into three of the four known ERF families. Their predicted amino acid sequences exhibited similarities to ERFs from other plant species. Functional nuclear localization signal analyses of two Ps-ERF proteins (Ps-ERF1a and -1b) were carried out using confocal microscopy. Expression analyses of Ps-ERF mRNAs were studied in the two plum cultivars in order to determine the role of this gene family in fruit development and ripening. The seven Ps-ERFs displayed differential expression pattern and levels throughout the various stages of flower and fruit development. The diversity in Ps-ERFs accumulation was largely due to the differences in their responses to the levels of ethylene production. However, other plant hormones such as cytokinin and auxin, which accumulate strongly throughout the various developmental stages, also influence the Ps-ERFs expression. The effect of the plant hormones, gibberellin, cytokinin, auxin, and ethylene in regulating the different Ps-ERF transcripts was investigated. A model was proposed in which the role played by the plant hormone auxin is as important as that of ethylene in initiating and determining the date and rate of ripening in Japanese plums.

Key words: Double sigmoid curve growth pattern, ethylene-responsive factor (ERF), plant hormones, plum fruit development and ripening.

Introduction

Ethylene, a gaseous phytohormone, mediates diverse developmental and physiological processes throughout the entire life cycle of plants (Abeles *et al.*, 1992). The involvement of ethylene in the ripening of climacteric fruit is a symbolic incident of ethylene regulation. However, the developmental processes and the signal transduction mechanisms involved in such a phenomenon are less understood (Giovannoni, 2001). The expression of ethylene-related genes is induced through transduction of the ethylene signal from receptors to dedicated transcription factors (Giovan-

noni, 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). The ERF transcription factors contain a highly conserved DNA-binding domain (Ohme-Takagi and Shinshi, 1995) that interacts monomerically with the target DNA (Allen *et al.*, 1998). Many ERF proteins have been shown specifically to bind the so-called GCC box with a strictly conserved GCCGCC core domain

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Abbreviations: AD, acidic activator domain; aa, amino acids; BA, benzyladenine; DAB, days after bloom; EAR, ERF-associated amphiphilic repression; 'EG', 'Early Golden'; ERF, ethylene-responsive factor; GFP, green fluorescent protein; IAA, indole acetic acid; NLS, nuclear localization signal; 1-MCP, 1-methylcyclopropene; 'SH', 'Shiro'; S1, S2, S3, S4, the four stages of plum fruit development.

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to modulate transcription of a wide variety of ethylene-responsive genes, indicating that a transcriptional cascade is involved in ethylene signalling (Ohme-Takagi and Shinshi, 1995; Büttner and Singh, 1997; Zhou *et al.*, 1997; Solano *et al.*, 1998; Gu *et al.*, 2002). Some ERFs function as activators of GCC box-dependent transcription and others act as active repressors that down-regulate, not only basal transcription levels of a reporter gene, but also the trans-activation of other transcription factors (Fujimoto *et al.*, 2000). Although *Arabidopsis* has ~122 predicted ERF genes (Nakano *et al.*, 2006), only a few have been characterized so far (Sakuma *et al.*, 2002). In fact, only in tomato and apple, the role of ERFs in fruit ripening has been reported (Tournier *et al.*, 2003; Wang *et al.*, 2007).

The molecular mechanisms involved in fruit ripening have been studied to gain a broader insight into the factors that contribute to the differences in the date and rate of ripening among *Prunus* spp., including plum cultivars (Abdi *et al.*, 1997; Tonutti *et al.*, 1997; El-Sharkawy *et al.*, 2007, 2008; Trainotti *et al.*, 2007; Ziliotto *et al.*, 2008). The difference in ethylene production rate observed among several plum cultivars (Abdi *et al.*, 1997) could partially be due to the differences in the accumulation levels and/or pattern of various ethylene biosynthesis, perception, and signal transduction elements, which could be further related to ‘allelo-type’ or ‘genotype’ variance (El-Sharkawy *et al.*, 2007, 2008). However, allelic form alone still does not explain all the significant phenotypic disparity in fruit ripening. Furthermore, significant accumulation of certain ripening-related genes in fruits treated with the ethylene antagonist 1-methylcyclopropene (1-MCP) suggests that ethylene signalling is not the only essential signal that contributes to fruit ripening and indicates the possibility of more signalling pathways that are as crucial as that of ethylene (El-Sharkawy *et al.*, 2007, 2008; Ziliotto *et al.*, 2008).

The objective of this study was to understand the role of *ERF* gene family members in fruit development and ripening in Japanese plums. The ethylene evolution and the accumulation profile of seven *Ps-ERF* mRNAs were studied during fruit development and ripening in early ‘EG’ and late ‘SH’ cultivars. The aim was to determine whether there are dissimilarities that could account for the diversity in ripening behaviour. Finally, the localization of two of *Ps-ERF* proteins and the function of their putative NLS sites were visualized by confocal microscopy.

Materials and methods

Plant material and post-harvest treatments

Fruits from two Japanese plum (*Prunus salicina* L.) cultivars ‘Early Golden’ (‘EG’) and ‘Shiro’ (‘SH’) were harvested and treated as described previously (El-Sharkawy *et al.*, 2007). Other tissues such as flowers and early developmental stages were collected from ‘EG’ cultivar. All plant material was frozen in liquid nitrogen and stored at -80°C .

Hormones and hydrogen peroxide treatments

‘EG’ leaves were transferred to 250 ml flasks containing 100 ml of appropriate treatment. The flasks were incubated on a rotary shaker (100 rpm) for 2 h at room temperature. The treatments include gibberellin- GA_3 , cytokinin-BA, and auxin-IAA (all 0.4, 4, and 40 μM), ethylene as ethephon (1, 2, and 4 mM), and hydrogen peroxide- H_2O_2 (0.5, 1, and 2 mM). At the end, all the treated-leaves were removed, briefly blotted dry, immediately frozen in liquid nitrogen, and stored at -80°C for RNA analysis. Leaves without any treatment were used as control.

RNA isolation

Total RNA from fruit samples was extracted using the methods described by Boss *et al.* (1996). For vegetative tissues and flowers, total RNA was extracted using the Plant Total RNA Purification kit (Norgen, Thorold, ON, Canada), as per the manufacturer’s instructions. All RNA extracts were treated with DNase I (Promega, Madison, WI, USA) then cleaned up with the RNeasy mini kit (Qiagen, Mississauga, ON, Canada).

Isolation and in silico analysis of plum cDNA sequences

For the isolation of plum ethylene-responsive factors homologues (*Ps-ERFs*), first strand cDNA synthesis was carried out using 20 μg of total DNase-treated RNA in a 50 μl aliquot. One μl of cDNA was used in a PCR with the appropriate degenerate primers. Several sets of degenerate primers (Tournier *et al.*, 2003) were used to isolate *Ps-ERF* clones that shared the structural characteristics associated with functional *ERF* transcription factors. The isolated fragments were cloned in pGEM-T easy vector (Promega), sequenced, and compared with database sequences using the BLAST program (Altschul *et al.*, 1997). Extension of the partial cDNA clones was carried out using the 3’- and 5’- RACE kit (Invitrogen, Burlington, ON, Canada). Full-length amplification of cDNA sequences, designated *Ps-ERF1a*, *Ps-ERF1b*, *Ps-ERF2a*, *Ps-ERF2b*, *Ps-ERF3a*, *Ps-ERF3b*, and *Ps-ERF12*, was carried out using the Platinum *Taq* DNA Polymerase High Fidelity following the instructions provided by the manufacturer (Invitrogen). Alignments of the predicted protein sequences were performed with ClustalX (Jeanmougin *et al.*, 1998) and GeneDoc (Nicholas and Nicholas, 1997). The Neighbor-Joining tree was constructed with PAUP* 4.0b3. Bootstrap values from 1000 replicates were obtained. The tree was visualized with the TreeView program (Page, 1996).

Real-time quantitative RT-PCR

DNase-treated RNA (5 μg) was reverse transcribed in a total volume of 50 μl using SuperScript III Reverse Transcriptase (Invitrogen). Real-time quantitative PCR (QPCR) was performed using 20 ng of total RNA in a 20 μl reaction volume using Sybr Green PCR MasterMix (Qiagen) on a M \times 4000[®] multiplex Quantitative PCR system (Stratagene,

La Jolla, CA, USA). M×4000[®] v 4.20 software (Stratagene, La Jolla, CA, USA) was used to design gene-specific primers (see Supplementary Table S1 at *JXB* online). Other details are well described in the supplemental online materials and methods part.

Protoplast isolation and transient expression of Ps-ERF::GFP fusion proteins

The coding sequences of Ps-ERF1a and -1b were cloned as a C-terminal fusion in-frame with the GFP into the pGreen vector (Hellens *et al.*, 2000) and expressed under the control of the 35S promoter. A high fidelity PCR system was used to amplify the full-length Ps-ERF1a and -1b clones. The corresponding ORFs were cloned using the *Bam*HI restriction site of the pGreen vector. Protoplasts used for transfection were obtained from suspension-cultured tobacco (*Nicotiana tabacum*) BY-2 cells according to the method described by Leclercq *et al.* (2005). Protoplasts were transfected by a modified polyethylene glycol method as described by Abel and Theologis (1994). Typically, 0.2 ml of protoplast suspension (0.5×10^6) was transfected with 50 µg of shared salmon sperm carrier DNA and 30 µg of either 35S::GFP (control), or 35S/Ps-ERF1a::GFP, or 35S/Ps-ERF1b::GFP plasmid DNA. Transfected protoplasts were incubated 16 h at 25 °C and analysed for GFP fluorescence by confocal microscopy (Leclercq *et al.*, 2005). All transient expression assays were repeated at least three times.

The resulting plasmids, Ps-ERF1a::GFP and Ps-ERF1b::GFP, were engineered further by replacing amino acid residues, as well as by removing either one or the two clusters of the basic charged residues of the putative NLS from the full-length Ps-ERF1a and -1b sequences using the QuikChange II XL Site-Directed Mutagenesis kit (Stratagene, San Diego, CA, USA). The protoplast suspension was transfected with the different mutant versions as described previously.

Results

Molecular characteristics and organization of Ps-ERFs

Seven novel putative *ERF* clones were isolated from plum using an RT-PCR approach in order to investigate their involvement in fruit development and ripening. Ps-ERF1a,

-1b, -2a, -2b, -3a, -3b, and -12 predicted to encode proteins of 282, 261, 381, 327, 231, 235, and 172 amino acid residues (Table 1), with calculated molecular weights of 30.2, 29.4, 42.1, 36, 25.1, 25.5, and 18.3 kDa, respectively. The relationships between the predicted amino acid sequences, as indicated by percentage similarity over the whole sequence, are presented in Table 1. Although the various Ps-ERF cDNAs exhibited low sequence homology between them (13% identity, 20% similarity), several signature elements were detected. Multiple alignments of full-length predicted Ps-ERF proteins with other reported ERF sequences (see Supplementary Fig. S1 at *JXB* online) highlighted a number of conserved motifs and structural similarities that are commonly associated with the ERF/AP2 family of plant transcription factors (Fujimoto *et al.*, 2000; Sakuma *et al.*, 2002; Tournier *et al.*, 2003). The deduced amino acid sequences of Ps-ERFs comprise a conserved DNA-binding ERF/AP2 domain (ranging from 58 to 59 aa), which is characteristic of the plant ERF gene family (see Supplementary Fig. S1 at *JXB* online). The ERF domain of Ps-ERF proteins and other plant ERFs showed high sequence homology (67–94% identity and 81–100% similarity). By contrast, full-length Ps-ERFs exhibited a considerable divergence, not only to other characterized ERFs (15–66% similarity), including *Arabidopsis*, tomato, and other plant species, but also among themselves (Table 1). Such low overall sequence similarity is largely due to the low level of homology outside the ERF domain. Furthermore, the ERF domain in all plum sequences includes two key amino acid residues, 14th A (Ala) and the 19th D (Asp), believed to contribute a functional GCC box-binding activity in many ERFs (Sakuma *et al.*, 2002). In addition, all the seven cDNAs possess a basic region in their predicted proteins that might function as a nuclear localization signal (NLS) (Raikhel, 1992), and all, except Ps-ERF12, hold an acidic domain that might act as an activation domain (AD) for transcription (Yang *et al.*, 2002).

Comparison of the amino acid sequence and phylogenetic analysis of 34 characterized ERFs (Fig. 1), from various plant species, revealed that Ps-ERF sequences belong to three out of the four classes of ERF proteins (Fujimoto *et al.*, 2000; Tournier *et al.*, 2003). Predicted Ps-ERF1a and -1b proteins are classified as members of ‘B-3’/‘Class I’ ERFs (Sakuma *et al.*, 2002; Tournier *et al.*, 2003) (Fig. 1).

Table 1. Amino acid sequence comparison between the predicted full length ethylene responses factors (Ps-ERF) cDNAs

	Protein size (No. of aa)	Amino acid similarity percentage						
		Ps-ERF1a	Ps-ERF1b	Ps-ERF2a	Ps-ERF2b	Ps-ERF3a	Ps-ERF3b	Ps-ERF12
Ps-ERF1a	282	100						
Ps-ERF1b	261	45	100					
Ps-ERF2a	381	21	25	100				
Ps-ERF2b	327	22	26	45	100			
Ps-ERF3a	231	23	20	20	24	100		
Ps-ERF3b	235	23	20	22	23	60	100	
Ps-ERF12	172	24	23	21	23	35	36	100

Both sequences shared 39–63% similarity to their homologues in *Arabidopsis*, tomato, tobacco, and apple (see Supplementary Fig. S1a at *JXB* online). Like all ‘Class I’ gene members, Ps-ERF1a and -1b predicted proteins possess a putative NLS motif near the C-terminal region, hold an ERF domain consisting of 59 aa located near the middle of the sequences and comprise an acidic activator domain placed in the N-terminal region (see Supplementary Fig. S1a at *JXB* online; Fig. 2) (Zhou *et al.*, 1997; Fujimoto *et al.*, 2000; Tournier *et al.*, 2003). Despite the presence of all these common features, phylogenetic analysis pointed out the presence of at least two clearly separated branches among ‘Class I’ ERFs (Fig. 1). The first branch, Group A, includes Ps-ERF1a, At-ERF1, and At-ERF2 that exhibited a relatively high sequence similarity (59–64%) and characterized by the presence a short (25 aa) acidic domain (see Supplementary Fig. S1a at *JXB* online). The second branch gene members, Group B, are structurally similar in their long acidic domain, but displayed considerable sequence divergence (41–51% similarity). However, the acidic domain in Ps-ERF1b and MdERF2 sequences are much longer (70–130 aa) when compared to other group B members (53–55 aa) and occupy almost all of the N-terminal region (see Supplementary Fig. S1c at *JXB* online; Fig. 2). The previous observations suggest that Ps-ERF1b and MdERF2 sequences could represent a new clade under ‘Class I’ ERFs.

Ps-ERF2a and -2b encode for proteins that belong to the recently identified ‘B-2’/‘Class IV’ ERFs (Fig. 1) (Tournier *et al.*, 2003). Ps-ERF2a, -2b and their putative homologues from other species in this class are well conserved even outside the ERF domain (76–86% similarity) (see Supplementary Fig. S1b at *JXB* online). Consistent with other ‘Class IV’ ERFs, Ps-ERF2a and -2b have a conserved ERF domain (58 aa) near the N-terminus, a putative NLS [KRKRK/106–110 aa and KRARK/71–75 aa for Ps-ERF2a and -2b, respectively] located just before the ERF domain (see Supplementary Fig. S1b at *JXB* online; Fig. 2). In addition, a highly conserved N-terminal signature sequence of unknown function [MCGGAIL/L] (Tournier *et al.*, 2003) was also identified in both clones (see Supplementary Fig. S1b at *JXB* online). However, sequence analysis of different ERFs from ‘Class IV’ revealed that gene members of this class could be further divided into two groups (Fig. 1) based on the position of the acidic domain (Fig. 2). Group A includes Ps-ERF2a and its closest homologues (Fig. 1), which exhibit a long amino acid sequence (369–381 aa; see Supplementary Fig. S1b at *JXB* online) with an acidic domain (22–24 aa) near the N-terminus (Fig. 2) (Yi *et al.*, 2004; Zhang *et al.*, 2004). Conversely, Ps-ERF2b and its closest homologues of group B (Fig. 1), exhibits a shorter amino acid sequence (260–327 aa; see Supplementary Fig. S1b at *JXB* online) with a slightly longer acidic domain (26–32 aa) near the C-terminal end (Fig. 2) (Tournier *et al.*, 2003; Lee *et al.*, 2004; Mizuno *et al.*, 2006).

Ps-ERF3a, -3b, and -12 predicted proteins assign to ‘B-1’/‘Class II’ (Sakuma *et al.*, 2002; Tournier *et al.*, 2003) (Fig. 1) that includes all ERFs possess the conserved EAR repressor motif (ERF-associated amphiphilic repression) in

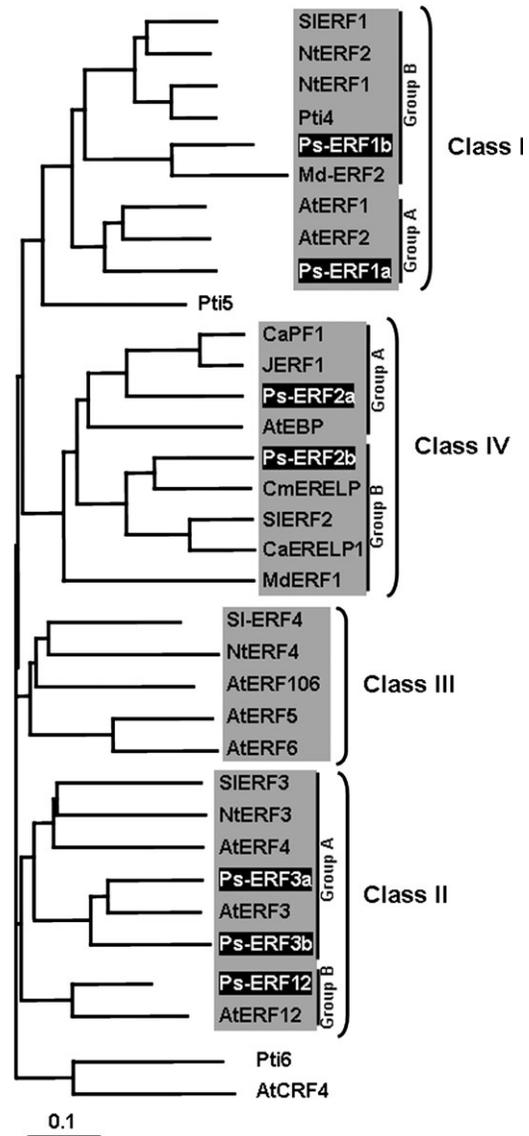


Fig. 1. Phylogenetic relationships between *Prunus salicina* [Ps-ERF1a (FJ026009), Ps-ERF1b (FJ026008), Ps-ERF2a (FJ026007), Ps-ERF2b (FJ026006), Ps-ERF3a (FJ026005), Ps-ERF3b (FJ026004), Ps-ERF12 (FJ026003)]; *Arabidopsis thaliana* [AtERF1 (BAA32418), AtERF2 (BAA32419), AtERF3 (BAA32420), AtERF4 (BAA32421), AtERF5 (BAA32422), AtERF6 (NP_567529), AtERF12 (Q94ID6), AtEBP (CAA05084), AtERF106 (Q9LY05), AtCRF4 (NP_194524)]; *Solanum lycopersicum* [SIERF1 (AAO34703), SIERF2 (AAO34704), SIERF3 (AAO34705), SIERF4 (AAO34706), Pti4 (AAC50047), Pti5 (AAC49740), Pti6 (AAC49741), JERF1 (AAK95687)]; *Nicotiana tabacum* [NtERF1 (Q40476), NtERF2 (Q40479), NtERF3 (Q40477), NtERF4 (Q40478)]; *Capsicum annuum* [CaPF1 (AAP72289), CaERELP1 (AAS20427)], *Malus×domestica* [MdERF1 (BAF43419), MdERF2 (BAF43420)] and *Cucumis melo* [CmERELP (BAD01556)] based on amino acid sequence. ‘Class I’, ‘Class II’, ‘Class III’, and ‘Class IV’ represent the different classes of the ERF/AP2 family of plant transcription factors. Groups A and B signify the two separated branches under each ERF class.

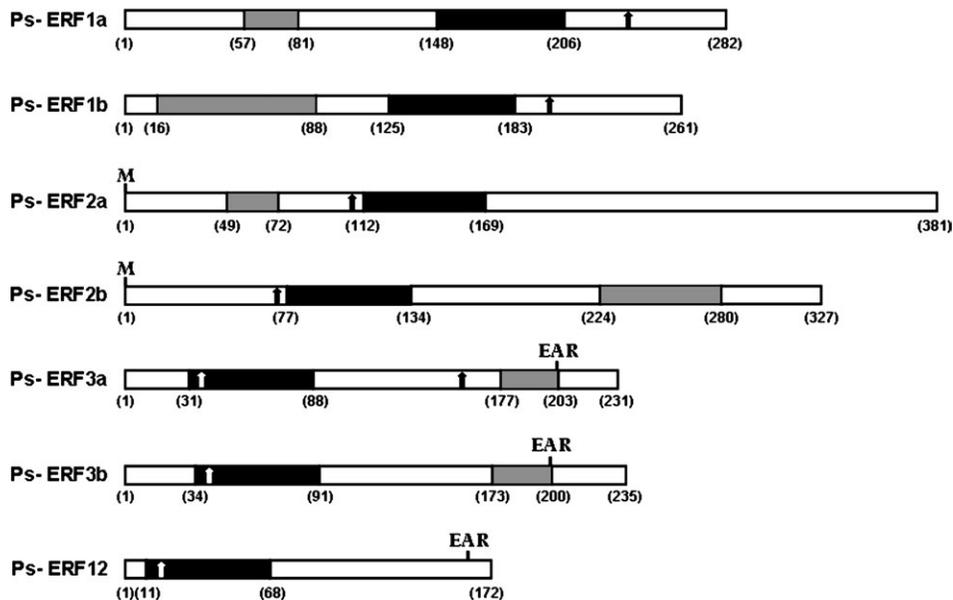


Fig. 2. Structure of Ps-ERF proteins (Ps-ERF1a, -1b, -2a, -2b, -3a, -3b, and -12). Black and hatched boxes represent the ERF and acidic domains, respectively. The putative nuclear localization signals (NLS) are marked with filled or open arrows within the boxes. 'M' indicates the (MCGGAIL/L) N-terminal motif and 'EAR' corresponds to the C-terminal ERF-associated amphiphilic repression motif. Numbers below the boxes indicate the positions of amino acid residues.

the C-terminal end (Ohta *et al.*, 2001) (see Supplementary Fig. S1c at *JXB* online; Fig. 2). The ERF domain for 'Class II' gene members is located close to the N-terminal region and consists of 58 aa (Fig. 2). A putative NLS motif was identified within the ERF domain region (see Supplementary Fig. S1c at *JXB* online) (Tournier *et al.*, 2003; Long-Guo and Jin-Yuan, 2008). Interestingly, a second putative NLS motif near C-terminal region has been identified in Ps-ERF3a (see Supplementary Fig. S1c at *JXB* online). However, sequence analysis of 'Class II' ERFs revealed that this class could also be divided into two groups (Fig. 1). Group A members include Ps-ERF3a, Ps-ERF3b, AtERF3, AtERF4, SIERF3, and NtERF3, which are characterized by a long amino acid sequence (221–235 aa) (see Supplementary Fig. S1c at *JXB* online). Moreover, Ps-ERF3a and -3b hold an acidic domain of 27 aa and 28 aa, respectively, just before the EAR motif. On the other hand, Ps-ERF12 orthologues was only found in *Arabidopsis*, AtERF12. Plum and *Arabidopsis* ERF12, which are so far the only members of the 'Class II'/group B (Fig. 1), are characterized by having a short N-terminal end and an overall short amino acid sequence length (172–189 aa) (see Supplementary Fig. S1c at *JXB* online). Both plum and *Arabidopsis* ERF12 predicted proteins lack the acidic domain.

Expression of Ps-ERFs during fruit development and ripening

To understand the possible role of the various ethylene responsive factors in fruit physiology, the expression of the seven isolated *Ps-ERF* mRNAs was quantified by real-time

PCR to determine their accumulation patterns throughout fruit development and ripening.

Figure 3 shows the expression profile of the different *Ps-ERFs* during flowering and early fruit development (0–15 DAB). The isolated sequences were differentially expressed throughout this stage of development. Out of the seven studied cDNAs only *Ps-ERF1a* and -12 were greatly expressed in flowers. Although *Ps-ERF1b* and -3a were expressed in lower levels, but in the same time their accumulation throughout this stage represented the highest levels among the whole experiment. The accumulation of the different *Ps-ERF* mRNAs were considerably stimulated at bloom (~4 DAB for *Ps-ERF1a*, -1b, and -3b) or after fertilization (~7 DAB for *Ps-ERF2a*, -2b, -3a, and -12), and decreased gradually afterwards in initiated fruit (10–15 DAB).

Stone fruit development period (22–77 DAB) could be divided into three different stages (S1–S3) (Tonutti *et al.*, 1997; El-Sharkawy *et al.*, 2007). The first stage (S1, 22–37 DAB) is illustrated by intense cell division and differentiation, and rapid growth. Low levels of ethylene production, ranging from 0.8–2 nl g⁻¹ h⁻¹ ± 0.2, have been detected during S1 of fruit development (data not shown). Throughout S1 (Fig. 4; Stage 1), six out of the seven *Ps-ERF* transcripts steadily increased to a peak early, ~27 DAB (for *Ps-ERF1a*, -1b, -2a, and -2b) or later, ~32 DAB (for *Ps-ERF3b* and -12) and declined thereafter. The drop pattern in the case of early peaked transcripts, ~27 DAB, was much sharper than in later accumulated ones, ~32 DAB. *Ps-ERF3a* was expressed at high constant levels during the same period.

In the second stage (S2, 42–52 DAB), there is hardly any increase in fruit size but the endocarp hardens to form

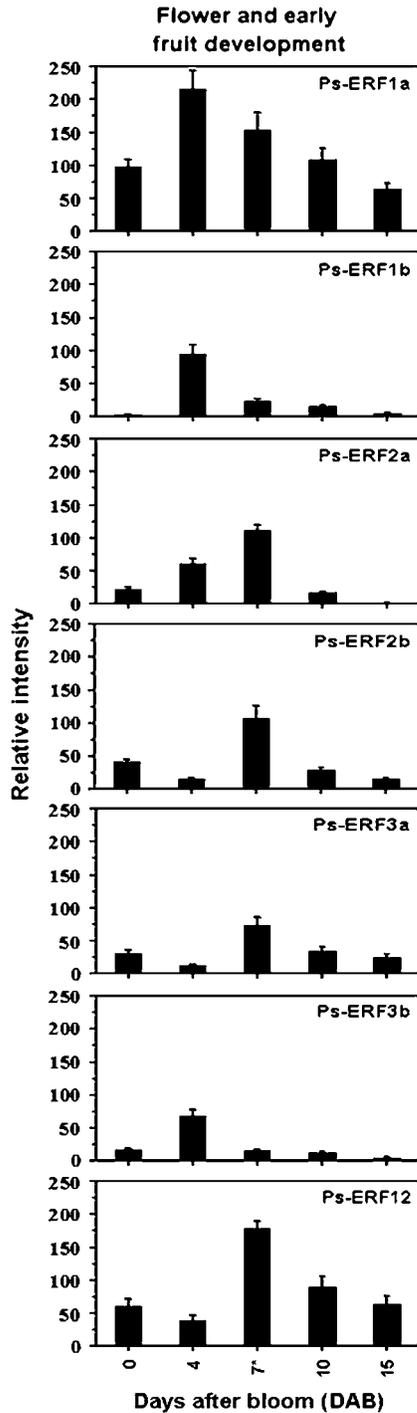


Fig. 3. Steady-state transcript levels of *Ps-ERFs* mRNA assessed by real-time quantitative PCR during flowering and early fruit development of the ‘EG’ cultivar. The fertilized flowers stage is marked with an asterisk. The experiments were carried out in triplicate. The x-axis represents the developmental stages indicated by the number of days after bloom (DAB). Relative intensity in the y-axis of each figure refers to the fold difference in gene expression relative to the sample that exhibited the minimum level of transcripts in the whole experiment (as indicated in the supplementary online materials and methods part).

a solid stone (pit hardening). *Ps-ERF1a*, *-2a*, and *-2b* mRNAs steadily decreased with the progress of fruit age to reach their basal levels at the end of this stage, ~52 DAB, while those of *Ps-ERF3b* were weakly accumulated (Fig. 4; Stage 2). By contrast, *Ps-ERF1b*, *-3a*, and *-12* transcript signals were strongly detected, but their accumulation patterns were different. *Ps-ERF1b* and *-3a* reached their relative maximal levels ~47 DAB, while those of *Ps-ERF12* reached it earlier, ~42 DAB. The three transcripts subsequently dropped to their normal low levels (*Ps-ERF3a* and *-12*) or were almost undetectable (*Ps-ERF1b*) at the end of this stage, ~52 DAB.

The third stage (S3, 57–77 DAB) is accompanied by rapid cell division resulting in a significant increase in fruit size. During the S3 stage when the pulp (mesocarp) separates from the seed (endocarp+embryo), *Ps-ERFs* expression profile in the pulp was, generally, parallel to those in the seed (Fig. 4; Stage 3). However, markedly higher accumulation of *Ps-ERF1b*, *-2a*, *-2b*, and *-3a* mRNAs (2–11-fold) were detected in the seed compared with the pulp. *Ps-ERF3b* and *-12* transcripts were uniformly accumulated in fruit pulp and seed. *Ps-ERF1a* accumulation was slightly higher in the pulp than in the seed. In the pulp, transcripts of *Ps-ERF2b*, *-3a*, *-3b*, and *-12* seem to be constitutively expressed throughout S3, although those of *Ps-ERF12* were present at much higher levels. A slight stimulation in *Ps-ERF1a*, *-1b*, and *-2a* transcription levels were detected (~62 DAB) and steadily declined thereafter. In the seed, *Ps-ERF1a*, *-1b*, *-2a*, *-2b*, and *-3a* clones displayed very similar expression patterns. Their mRNAs were strongly accumulated ~62 DAB and decreased afterwards, reaching a basal level at the end of this stage, ~77 DAB. However, those of *Ps-ERF3b* and *-12* were induced at low and high constant levels, respectively.

The last stage of fruit development (S4) denotes the fruit ripening or climacteric stage. Throughout this stage the fruit starts to enhance ripening in an ethylene-dependent manner (El-Sharkawy *et al.*, 2007, 2008). ‘EG’ fruit displayed an early, rapid ripening, and short and rapid (maximal 5 d) ethylene production profile (see Supplementary Fig. S2 at *JXB* online). A dramatic increase in *Ps-ERFs* transcription levels has been accelerated throughout ‘EG’ fruit ripening, 78–83 DAB (Fig. 5). Their transcripts were barely detectable at the non-climacteric stage, ~78 DAB. As ripening progressed and higher levels of autocatalytic ethylene produced, their expression levels increased in abundance. However, their patterns of transcript accumulation were different. In pulp, *Ps-ERF1a*, *-1b*, *-3b*, and *-12* increased to a peak ~82 DAB, and declined afterward at the post-climacteric stage, ~83 DAB (Fig. 5). Their accumulation pattern correlated well with the evolution of ethylene production and their maximal transcript levels coincided with the climacteric ethylene peak (see Supplementary Fig. S2 at *JXB* online; Fig. 5), whereas, those of *Ps-ERF2b* and *-3a* transcripts were gradually augmented along with the progression of fruit ripening and continued to increase past the climacteric peak, reaching their maximal levels in post-climacteric fruit, ~83 DAB. *Ps-ERF2a* peaked early at the pre-climacteric stage, ~80 DAB, and declined thereafter.

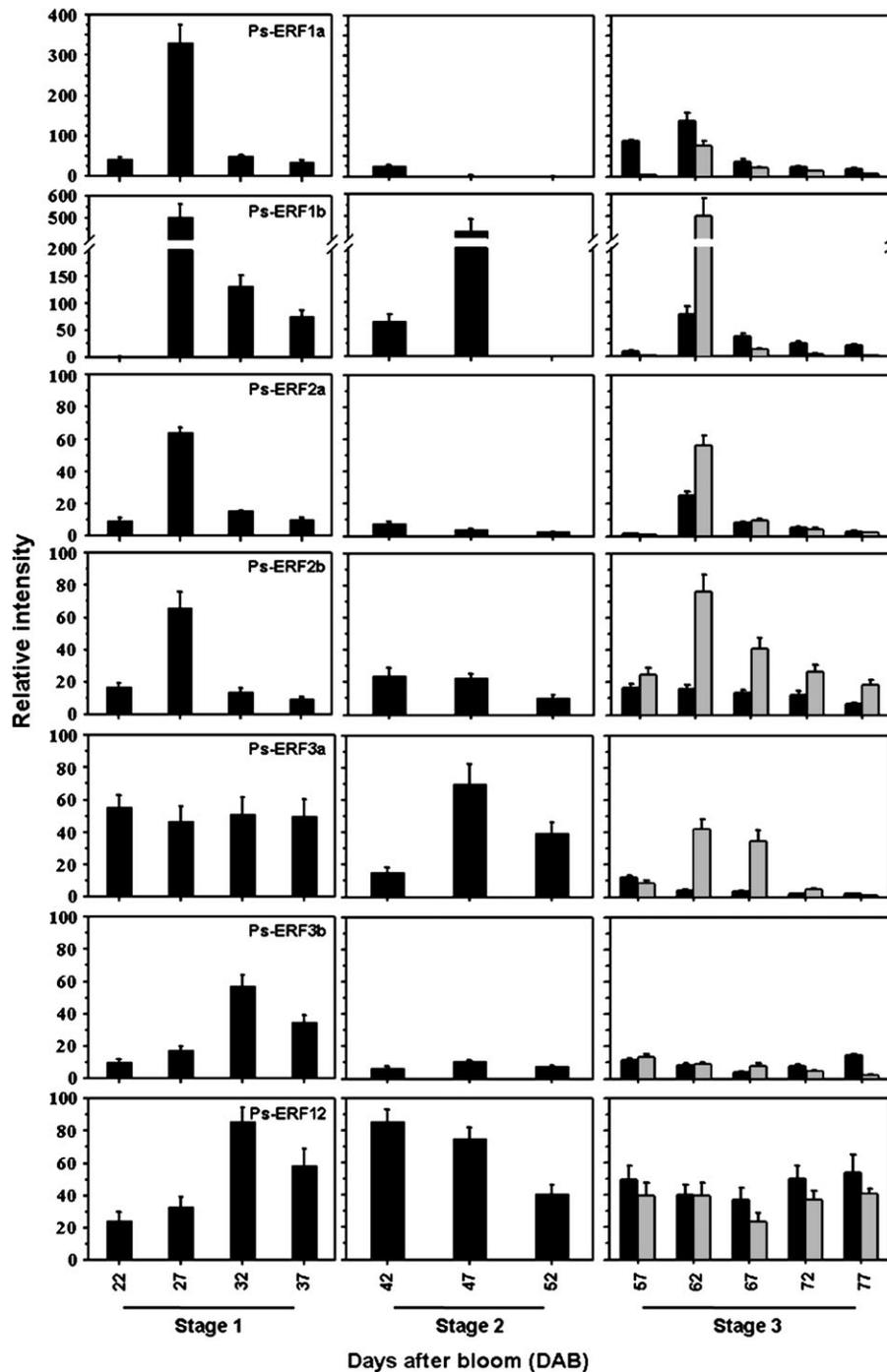


Fig. 4. Steady-state transcript levels of *Ps-ERFs* mRNA assessed by real-time quantitative PCR during S1 and S2 of fruit development using the whole 'EG' fruit. Throughout S3 of fruit development, expression was studied in the pulp (black filled bars) and in seeds (grey filled bars). The experiments were carried out in triplicate. The x-axis represents the developmental stage indicated by the number of days after bloom (DAB) and by the name of the stage. Relative intensity in the y-axis of each figure refers to the fold difference in gene expression. Other details are as described in Fig. 3.

All *Ps-ERFs*, except for the *Ps-ERF2b*, expression pattern in seed had a similar trend as in pulp, but at much lower levels. The *Ps-ERF2b* accumulation profile, in 'EG' seed, mimics that of *Ps-ERF2a*, and they reached their highest levels in the pre-climacteric stage, ~80 DAB (Fig. 5).

The association of *Ps-ERF* transcripts induction with fruit ripening was further investigated using 1-MCP, which

completely abolished the ethylene burst and ripening in the pretreated fruits (data not shown). Surprisingly, the increases of *Ps-ERF1b* and *-2a* mRNAs during 'EG' fruit ripening were not found to be ethylene-dependent, but seemed to be ripening-related, since their transcripts were still present in considerable amounts in MCP-treated fruits (Fig. 5). MCP completely inhibited the ethylene-associated

accumulation of *Ps-ERF2b*, *-3a*, and *-12* in the whole fruit. However, *Ps-ERF1a* and *-3b* mRNAs accumulation were fully inhibited in the pulp and did not significantly respond to 1-MCP treatment in the seed.

The differences in ripening behaviour between various plum cultivars might be due to the differences in their capacity to produce and respond to ethylene (El-Sharkawy *et al.*, 2007, 2008). In order to determine the involvement of the different *Ps-ERFs* in ethylene sensitivity and, sub-

sequently, in the capacity of the fruit to ripen, their expression was investigated during ‘SH’ fruit ripening. ‘SH’ fruit exhibited a suppressed climacteric pattern and ripened later than ‘EG’ (see Supplementary Fig. S2 at *JXB* online).

Throughout ripening of ‘SH’ fruit (90–105 DAB), the expression pattern of *Ps-ERF1a*, *-1b*, *-2a*, and *-2b* in the pulp was stimulated in parallel with the evolution of autocatalytic ethylene production (see Supplementary Fig. S2 at *JXB* online; Fig. 6), however, their accumulation

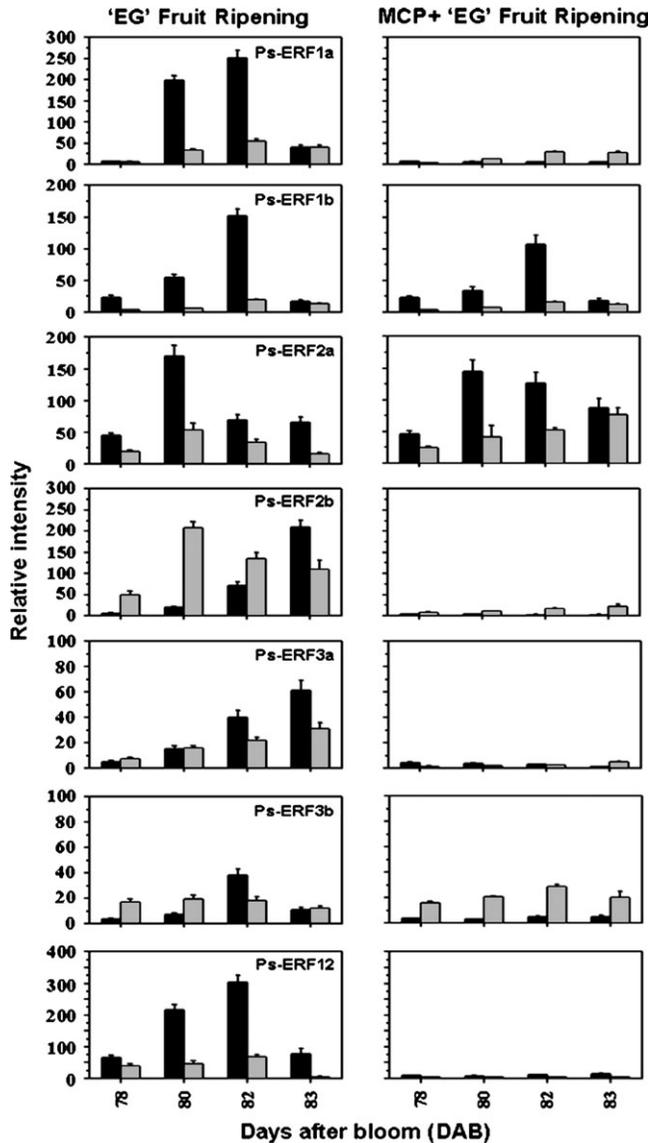


Fig. 5. Steady-state *Ps-ERFs* transcript levels assessed by real-time quantitative PCR throughout ‘EG’ fruit ripening, non-treated (left panel) and treated (right panel) with 1-MCP. The expression was studied in pulp (black filled bars) and in seeds (grey filled bars). For MCP treatment, fruits were exposed overnight immediately at harvest (76 DAB) to 1-MCP ($1 \mu\text{l l}^{-1}$) before the onset of endogenous ethylene. The experiments were carried out in triplicate. The x-axis represents the developmental stage indicated by the number of days after bloom (DAB). Relative intensity in the y-axis of each figure refers to the fold difference in gene expression. Other details are as described in Fig. 3.

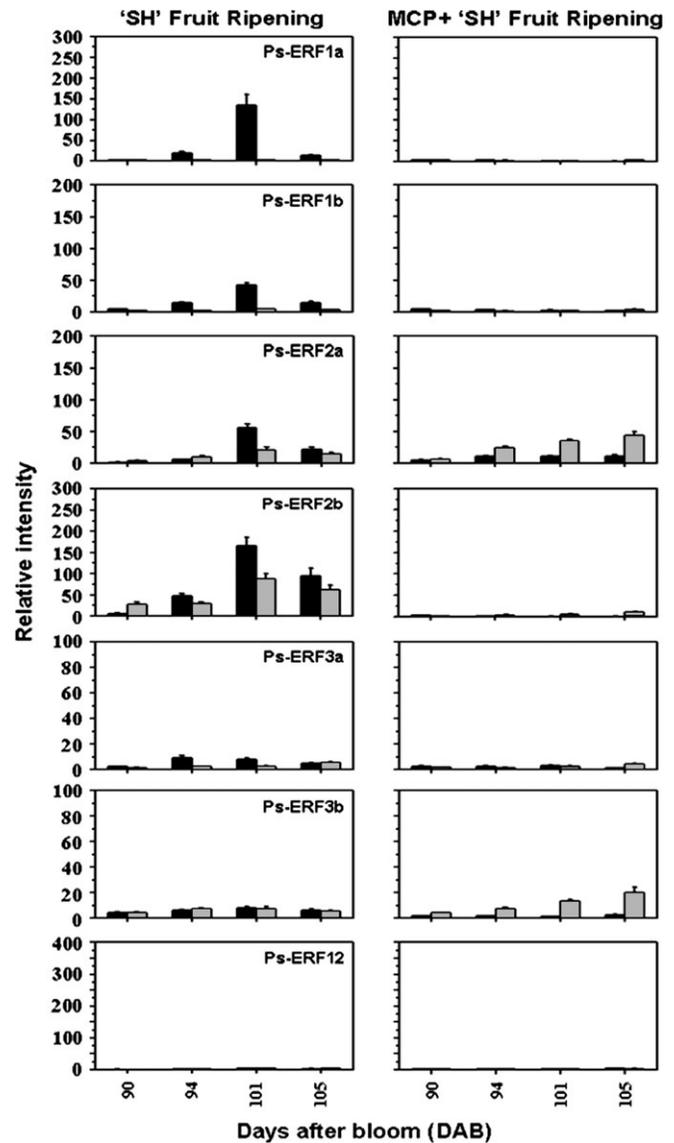


Fig. 6. Steady-state *Ps-ERFs* transcript levels assessed by real-time quantitative PCR throughout ‘SH’ fruit ripening, non-treated (left panel) and treated (right panel) with 1-MCP. The expression was studied in pulp (black filled bars) and in seeds (grey filled bars). For MCP treatment, fruits were exposed overnight immediately at harvest (88 DAB) to 1-MCP ($1 \mu\text{l l}^{-1}$) before the onset of endogenous ethylene. The experiments were carried out in triplicate. The x-axis represents the developmental stage indicated by the number of days after bloom (DAB). Relative intensity in the y-axis of each figure refers to the fold difference in gene expression. Other details are as described in Fig. 3.

levels were obviously lower than in case of 'EG' fruit (compare Figs 5 and 6). The accumulation of *Ps-ERF3a* and *-3b* transcripts remained temporally constant and low; however, those of *Ps-ERF12* were totally absent. Only *Ps-ERF2a* and *-2b* transcripts were detected in the seed. *Ps-ERF2a* and *-2b* accumulated in 'SH' seeds in the same manner as in pulp but at slight lower levels (Fig. 6).

MCP treatment completely eliminated the ethylene burst and ripening in the treated 'SH' fruits (data not shown). MCP-'SH' fruit strongly repressed the ethylene-related induction of *Ps-ERF1a* and *-3a*, and, contrary to the situation in the 'EG' cultivar, also for *Ps-ERF1b* and *-2b*. Although, *Ps-ERF2a* and *-3b* transcripts accumulation were totally inhibited in the pulp, their expression was significantly enhanced in the seed. Similar to the situation during 'SH' fruit ripening, *Ps-ERF12* transcripts were totally missing in MCP-'SH' treated fruit (Fig. 6).

Effect of different hormones and hydrogen peroxide treatments on the expression of the Ps-ERFs

In view of the inducible nature of *Ps-ERFs* during fruit development and ripening, the question was raised whether any of the *Ps-ERFs* are regulated by one or more of the various hormones and/or enzymes that are associated with fruit development in plum. In particular, that significant *Ps-ERFs* transcript levels were accumulated in non-ethylene-related tissues such as young fruit, seed, and MCP-treated fruits. To address this hypothesis, the expression profile of the seven transcripts was assessed in leaves exposed to different treatments (gibberellin, cytokinin, auxin, ethylene, and hydrogen peroxide).

None of the *Ps-ERFs* significantly respond to gibberellin treatment at least under the conditions used in this study (data not shown).

Only *Ps-ERF1a*, *-1b*, *-3a*, and *-12* transcription levels were accelerated in response to cytokinin application (Fig. 7). *Ps-ERF1a*, *-1b*, and *-3a* mRNAs were steadily augmented with increasing cytokinin concentrations, while *Ps-ERF12* decreased. The effect of cytokinin treatment on the induction of the four *Ps-ERF* transcripts was significantly higher for *Ps-ERF1b* and *-3a* (~26-fold) than those of *Ps-ERF1a* and *-12* (~8-fold). However, *Ps-ERF2a* and *-3b* were negatively regulated by the treatment and *Ps-ERF2b* did not significantly respond.

Leaves treated with auxin exhibited a remarkable increase of all the studied *Ps-ERFs*. *Ps-ERF1a* and *-1b* transcripts were strongly accumulated but only with low auxin concentration (0.4 μ M) and gradually decreased with higher concentrations. The other five *Ps-ERF* mRNAs were largely and selectively detected in leaves treated with 4 μ M auxin concentration (Fig. 7).

Ethylene treatment accelerates the transcription of the various *Ps-ERFs*, excluding *Ps-ERF2a* that was not significantly altered by the treatment (Fig. 7). *Ps-ERF1a*, *-1b*, and *-12* expressions were slightly induced by low ethylene concentration (1 mM) and strongly released thereafter to reach a maximal level with higher concentrations. Contrary,

Ps-ERF2b signal was greatly detected with low ethylene concentration and declined afterward, however, those of *Ps-ERF3a* and *-3b* increased to a peak with 2 mM ethylene concentration.

Accumulation of all *Ps-ERFs* mRNAs, excluding *Ps-ERF1a*, was significantly enhanced in H₂O₂-treated leaves (Fig. 7). Expression of *Ps-ERF1b* and *-2b* was strongly detected in leaves treated with a low H₂O₂ concentration (0.5 mM) and declined gradually with higher concentrations. *Ps-ERF2a*, *-3a*, *-3b*, and *-12* transcripts clearly showed the same manner of accumulation when they were selectively peaked with 1 mM H₂O₂ concentration.

Ps-ERF1a and -1b encoded proteins are targeted to the nucleus

Inspection of the amino acid sequences of all 'Class I' ERFs characterized so far revealed that each ERF member belonging to this class holds typical nuclear localization sequences (NLSs) of the bipartite class with two clusters of basic residues separated by 12–18 amino acids (see Supplementary Fig. S1a at *JXB* online). The two NLS clusters in 'Class I'/Group A ERFs (Fig. 1) consist of the basic amino acids (KR) and (K^{K/R}R^{R/K}K); however, there are (KR^{R/K}) and (KRRRK) in Group B (see Supplementary Fig. S1a at *JXB* online). Although *Ps-ERF1b* and *MdERF2* belong to Group B, they appear to exhibit a particular putative NLS sequence. The amino acid residues 'Ser-Ala' and 'Thr-Ala-Ala-Thr' located in the middle of the second NLS cluster of *Ps-ERF1b* and *MdERF2*, respectively, (see Supplementary Fig. S1a at *JXB* online), are unique to these clones while all other 'Class I' ERFs, including *Ps-ERF1a*, have the basic amino acids 'Lys' and/or 'Arg'. It was checked that this modification in the *Ps-ERF1b* NLS sequence was not caused by a cloning or sequencing error by sequencing several clones from three independent PCRs and using a high fidelity DNA polymerase. Due to the strong difference between these amino acids in terms of charge, polarity, and hydrophobicity, it was decided to examine the subcellular localization of *Ps-ERF1b* and compare it with its closest homologue in plum *Ps-ERF1a*. Their coding regions were fused to the GFP tag and were transiently expressed in tobacco protoplasts. Fluorescence microscopy analysis demonstrated that control cells transformed with the GFP gene alone displayed fluorescence spread throughout the cytoplasm and nucleus, in accordance with the expected cytosolic localization of the GFP proteins (Fig. 8A). By contrast, *Ps-ERF1a* and *Ps-ERF1b::GFP* fusions were localized exclusively in the nucleus, indicating that both *Ps-ERF1s* were fully able to redirect the GFP from the cytosol to the nucleus.

To investigate the structural role of the two amino acid residues 'Ser' and 'Ala' located at the *Ps-ERF1b* NLS site, the two amino acids 'Arg' found in the putative NLS site of *Ps-ERF1a* were changed to 'Ser' and 'Ala' by site-directed mutagenesis, *MuERF1a/SA*. Similarly, the two amino acids 'Ser' and 'Ala' in *Ps-ERF1b* were modified into 'Arg', *MuERF1b/RR*. Surprisingly, protoplasts transfected with a *MuERF1a/SA::GFP* fusion showed green fluorescence

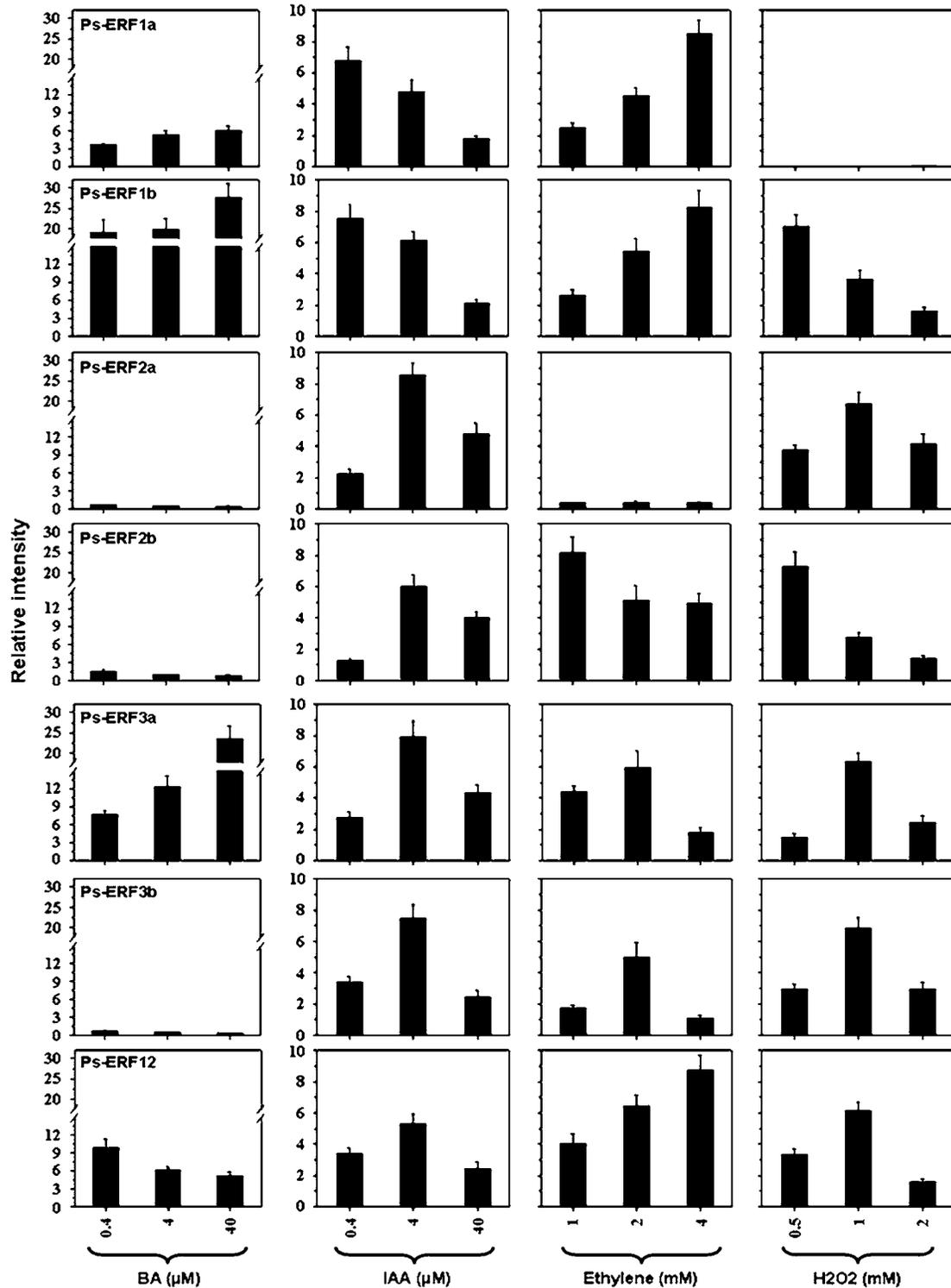


Fig. 7. Steady-state *Ps-ERFs* transcript levels assessed by real-time quantitative PCR in ‘EG’ leaves treated with cytokinin (BA), auxin (IAA), ethylene (ethephon), and hydrogen peroxide (H₂O₂) using the indicated concentrations. The experiments were carried out in triplicate. The x-axis represents the type of the treatment and the respective concentration used. Relative intensity in the y-axis of each figure refers to the fold difference in gene expression relative to the control.

through the entire cytoplasm and nucleus. By contrast, *MuERF1b/RR::GFP* fusion still localized exclusively to the nucleus (Fig. 8B). Taken together, these data strongly suggest that the second NLS cluster of Ps-ERF1a is essential for proper nuclear localization.

To examine this domain further, the two clusters of the basic charged residues of the NLS from the full-length Ps-ERF1a (Δ ERF1a-KR/KRRKK) and -1b (Δ ERF1b-KRK/KSAKR) sequences were deleted, and the resulting mutants were transfected with tobacco protoplasts. This deletion

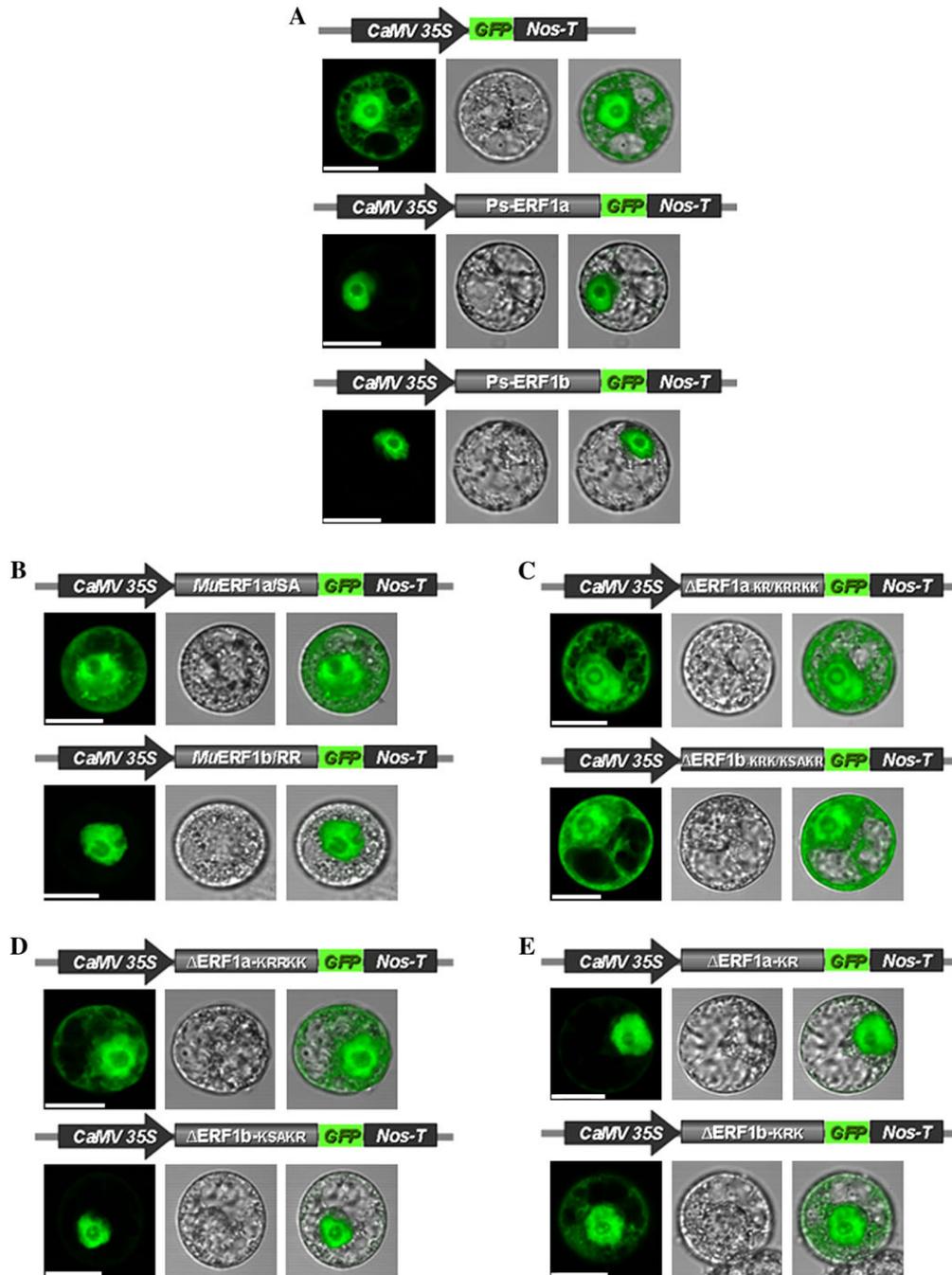


Fig. 8. Ps-ERF1a, and -1b proteins and their modified versions were fused to the GFP tag. *N. tabacum* protoplasts were transfected with the following constructs: (A) the control 35S::GFP, Ps-ERF1a::GFP and Ps-ERF1b::GFP; (B) the site-directed mutagenesis versions of Ps-ERF1s; designated *MuERF1a/SA* and *MuERF1b/RR*; (C) Ps-ERF1s versions with deletion of the basic amino acid residues that form the two NLS clusters; designated Δ ERF1a_{-KR/KRRKK} and Δ ERF1b_{-KRK/KSAKR}; (D) Ps-ERF1s versions with deletion of the basic amino acids that form the second NLS cluster; designated Δ ERF1a_{-KRRKK} and Δ ERF1b_{-KSAKR}; (E) Ps-ERF1s versions with deletion of the basic residues that form the first NLS cluster; designated Δ ERF1a_{-KR} and Δ ERF1b_{-KRK}. GFP fluorescence was visualized using confocal laser scanning microscopy. Light micrographs (centre panels) and fluorescence (left panels) images are merged (right panels) to illustrate the different locations of the seven proteins. The length of the bar corresponds to 10 μ m.

caused the loss of nuclear localization of both Ps-ERF1s, and the green fluorescence was uniformly distributed throughout the cytoplasm and nucleus (Fig. 8C). Therefore, this bipartite domain region is the sole NLS necessary to target such proteins into the nucleus.

To characterize the bipartite NLS domain of both Ps-ERF1s further, the amino acid residues KRRKK and KSAKR were removed from the full-length Ps-ERF1a (Δ ERF1a_{-KRRKK}) and -1b (Δ ERF1b_{-KSAKR}), respectively. As shown in Fig. 8D, this elimination dramatically altered

the normal targeting of the protein via the nucleus, but only for Ps-ERF1a. The Ps-ERF1b localization was not significantly affected. These results confirm again the importance of the 'KRRKK' sequence in the correct Ps-ERF1a nuclear localization and that the 'KSAKR' domain in Ps-ERF1b plays only a minor role in protein localization.

After that, the amino acid residues KR and KRK were deleted from the full-length Ps-ERF1a (Δ ERF1a_{KR}) and -1b (Δ ERF1b_{KRK}), respectively, in order to discover their role in protein nuclear localization. Δ ERF1a_{KR} was exclusively localized to the nucleus, while the Δ ERF1b_{KRK} protein was visualized in the nucleus as well as in the cytoplasm (Fig. 8E). Therefore, it seems that 'KRK' domain in Ps-ERF1b play the major role to target the protein via the nucleus.

Discussion

ERF proteins were first identified as transcription factors, which possess GCC box binding activity (Ohme-Takagi and Shinshi, 1995; Büttner and Singh, 1997). Although sequence identity can be as low as 13% among the different ERFs, all have highly conserved signature elements associated with the ERF/AP2 transcription factors (Fujimoto *et al.*, 2000; Ohta *et al.*, 2001; Sakuma *et al.*, 2002; Tournier *et al.*, 2003; Cao *et al.*, 2006), including the presence of a typical ERF domain that is responsible for the DNA binding activity, the acidic activator domain (AD), and a basic amino acid reach region putatively functions as a nuclear localization signal (NLS). However, variations within the ERFs are reasonable enough to divide them into four main classes based on sequence structure and similarities (Fujimoto *et al.*, 2000; Sakuma *et al.*, 2002; Tournier *et al.*, 2003). These variant characteristics include the length and position of the ERF domain. Moreover, the presence of the highly conserved C-terminal EAR repressor and N-terminal (MCGGAIL/L) motifs in all ERF proteins belong to Class 'II' and 'IV', respectively (Ohta *et al.*, 2001; Tournier *et al.*, 2003). Further, the NLS motif is located near the C-terminal end for 'Class I', just before the ERF domain for 'Class II', and within the ERF domain for 'Class IV' ERFs. Some other minor features that might distinguish between ERF proteins within the same class have been identified, such as the length, the position, and the presence of the acidic domain for Class 'I', 'II', and 'IV' ERFs, respectively. Despite the extensive structural differences between them, all are functional ERF transcription factors, as defined by their DNA binding ability and their function as transcriptional activators or repressors (Fujimoto *et al.*, 2000; Tournier *et al.*, 2003).

In this study, seven Ps-ERFs were isolated. Based on their structural organizations and dendrogram analysis, a well-defined branch, 'Class III', has *Arabidopsis*, tomato, and other plant species sequences but lack plum sequences, suggesting that there are likely to be as yet unidentified ERF genes within the plum genome. In species where multiple ERFs have been characterized, each protein

appears to have a unique mode of function (Fujimoto *et al.*, 2000). Accordingly, the Ps-ERFs fall into three out of the four different classes of the previously characterized ERF proteins (Fujimoto *et al.*, 2000; Tournier *et al.*, 2003). Ps-ERF1a and -1b belong to 'Class I' ERFs that have been shown to function as activators of transcription (Zhou *et al.*, 1997; Solano *et al.*, 1998; Fujimoto *et al.*, 2000; Ohta *et al.*, 2000; Tournier *et al.*, 2003; Wang *et al.*, 2007). Ps-ERF3a, -3b, and -12 represent members of 'Class II' ERFs. Several members of this class have been demonstrated to be active repressors of transcription (Fujimoto *et al.*, 2000; Ohta *et al.*, 2000, 2001). Ps-ERF2a and -2b are categorized as 'Class IV' ERFs based on the location of the putative NLS site and the presence of the conserved N-terminal motif (Tournier *et al.*, 2003). The function of this motif (MCGGAIL/L) is unknown yet, but it is unlikely to be required for nuclear localization or for binding to the GCC box (Tournier *et al.*, 2003). Moreover, the function of the 'Class IV' ERFs is also unclear.

As expected for transcription factors, Ps-ERF1a and -1b were localized in the nucleus of tobacco cells. All 'Class I' ERF gene members contain typical bipartite NLSs with two clusters of basic residues. However, functionality of either of these NLSs (Ps-ERF1b and MdERF2 or Ps-ERF1a, At-ERF1, and At-ERF2) is often sufficient to target the protein via the nucleus, although in some instances (Sl-ERF1, Pti4, Nt-ERF1, and Nt-ERF2) both are active (Gu *et al.*, 2002).

Presuming that the sequences isolated in this study encode functional ethylene-responsive factors, their expression profile was studied in different plant tissues and under various conditions in order to determine their role in fruit development in terms of a double sigmoid growth pattern, their involvement in ethylene sensitivity, and the capacity of the fruit to ripen, and their hormone-dependent accumulation.

The difference in the *Ps-ERFs* accumulation profile throughout flower development suggested the contribution of different plant hormones, strongly occurring during this stage, in the regulation of the various *Ps-ERFs*. Based on the times of transcripts induction in flowers and the expression of *Ps-ERFs* post-application of the different hormones, it appears that *Ps-ERF1a*, *-1b*, and *-3b* mRNAs are induced in opened flowers, before fertilization (~4 DAB), in an ethylene-dependent manner. Interestingly, the expression pattern of these three transcripts was entirely similar to the previously characterized ethylene biosynthesis elements (*ACC* synthesis, *Ps-ACS*), which supports the possible role of ethylene in their accumulation (El-Sharkawy *et al.*, 2008). After fertilization, large amounts of auxin and cytokinin might be accelerated in response (Miller *et al.*, 1987; Hartmann *et al.*, 2002), which leads to the up-regulation of different transcripts associated with the presence of these two hormones. The large accumulation of *Ps-ERF3a* and *-12* post-fertilization (~7 DAB) might be enhanced in auxin- and/or cytokinin-dependent manners. However, those of *Ps-ERF2a* and *-2b* accumulate in an auxin-dependent manner since cytokinin has no effect in

their expression. Importantly, the up-regulation of *Ps-ERF2a*, *-2b*, *-3a*, and *-12*, post-fertilization, coincided with the complete inhibition of the different *Ps-ACS* transcripts, which eliminate any feasible role played by ethylene in their accumulation (El-Sharkawy *et al.*, 2008).

The increase of the various *Ps-ERF* transcripts during the S1 stage corresponds to a strong accumulation of *Ps-ACS* mRNAs, low levels of ethylene production, high ACC and MACC content, and massive levels of IAA and cytokinin content, all of which are important for cell division during early embryogenesis (Miller *et al.*, 1987; Hartmann *et al.*, 2002; DeDios *et al.*, 2006; El-Sharkawy *et al.*, 2008). Mantiri *et al.* (2008) clearly showed that *Medicago ERF* (*MtSERF1*) is essential for somatic embryogenesis and is induced by auxin and cytokinin that are both accelerated during embryo development. Thus, *Ps-ERFs* accumulation may be enhanced in an ethylene-, auxin-, and/or a cytokinin-dependent manner throughout this stage.

Ps-ERF1b, *-2b*, *-3a*, and *-12* transcripts were detected in considerably high concentrations during the S2 stage. To determine the role of these four transcripts, it was essential to understand clearly the physiological aspects that characterize the S2 stage. As mentioned previously, during this stage, there is hardly any increase in fruit size which coincided with a significant reduction in auxin content (Miller *et al.*, 1987; Trainotti *et al.*, 2007), indicating a minor role for auxin. Furthermore, the evaluation of different ethylene biosynthesis and perception elements in plum fruits (i.e. the same developmental stages studied in the present work) revealed the absence of all these ethylene elements throughout this stage, and also suggesting an insignificant role for ethylene (El-Sharkawy *et al.*, 2007, 2008). The only fruit development process accelerated during this stage was the lignification of the endocarp in a H₂O₂-dependent manner to form a solid stone (seed). The role of H₂O₂ in the lignification of the cell wall was clearly determined by Ros Barceló (2005), and Kärkönen and Fry (2006). These results led to the belief that the transcription of these four selected *Ps-ERFs* could be altered due to the high levels of endogenous H₂O₂. Interestingly, application of H₂O₂ activated the accumulation not only of these four transcripts but also of *Ps-ERF2a* and *-3b*.

Although there is hardly any ethylene emission detected throughout S3 of fruit development, *Ps-ERF* transcripts were generally abundant in young fruit at this stage. On the other hand, S3 seed exhibited much greater *Ps-ERFs* transcription contents than those found in the pulp. The seed in this stage is highly lignified and dry, and ethylene synthesis is usually inhibited (Rodríguez-Gacio and Matilla, 2001), which eliminate any possible ethylene regulation.

During S3, the fruit recovered its activity by strongly accelerated cell division and expansion, which results in a significant increase in fruit size in an auxin-dependent manner (Abel and Theologis, 1996; Christian *et al.*, 2006; Trainotti *et al.*, 2007). Furthermore, seed from this stage exhibited the relatively high auxin contents necessary for embryogenesis (Miller *et al.*, 1987). Finally, our data showed that application of auxin significantly enhanced the

transcription levels of all *Ps-ERFs*. Consequently, it seems that auxin is the logic hormone, which controls the transcription of *Ps-ERFs* throughout the S3 stage.

It is almost certain that the series of modifications that transform a mature green fruit into a ripe fruit occur during S3 (El-Sharkawy *et al.*, 2007, 2008; Trainotti *et al.*, 2007) and involve many different metabolic pathways. Therefore, these ethylene-independent factors that control the transition of a fruit from the end of growth to the onset of ripening are of primary importance. Previous studies showed that auxin content steadily increased in peach fruits during the S3 stage and throughout fruit ripening (Miller *et al.*, 1987; Ohmyia, 2000; Trainotti *et al.*, 2007), which strengthens the idea that auxin is actively involved in the fruit ripening process.

Expression analysis during 'EG' fruit ripening revealed that all *Ps-ERFs* accumulated greatly in the whole fruit (pulp and seed). Such high levels of accumulation could be cytokinin-, ethylene-, and/or auxin-dependent. Cytokinin can increase ethylene biosynthesis via a post-transcriptional mechanism that increases the ACS protein stability (Chae and Kieber, 2005). Similarly, auxin can affect ethylene levels through an increase in *ACS* transcription levels (Abel *et al.*, 1995; Trainotti *et al.*, 2007; El-Sharkawy *et al.*, 2008). Ethylene also can affect its own biosynthesis, either by increasing (autocatalysis) or decreasing (autoinhibition) its rate of production (Nakatsuka *et al.*, 1998). Furthermore, the three hormones could accelerate the transcription of either all (as in case of auxin) or some (as in the case of cytokinin and ethylene) plum *ERFs* characterized in this study.

Despite the important role played by cytokinin in enhancing ethylene production, as well as its role in the induction of several *Ps-ERF* transcripts, this hormone could not be present in such adult tissue and, consequently, could not participate in any fruit ripening process (Hartmann *et al.*, 2002). However, *Ps-ERFs* and, accordingly, the ripening process might be enhanced during fruit ripening in both ethylene- and auxin-dependent manners.

The differences in the accumulation levels and/or pattern of the various ethylene elements throughout ripening of early and late fruits (El-Sharkawy *et al.*, 2007, 2008; this paper) might be largely due to the variation in the levels of auxin content and ethylene produced among the two plum cultivars. Such variations affect the capacity of the fruit to produce and respond to ethylene, which results in the differentiation in ripening behaviour thereafter (Miller *et al.*, 1987; Ohmyia, 2000). If this is the case, auxin might be accumulated rapidly and in much higher levels in early cultivars during S3 (Miller *et al.*, 1987), which leads to the up-regulation of different transcripts and proteins associated with auxin, including different ethylene synthesis, perception, and signal transduction elements (El-Sharkawy *et al.*, 2007, 2008; this paper). When such high levels of ethylene-related proteins accumulate during S3, the mature green fruit transit into the S4 ripening stage early (~78 DAB). The ripening process in fruit during the non-climacteric stage (before the onset of autocatalytic ethylene) progresses only in

an auxin-dependent manner (Miller *et al.*, 1987; Ohmyia, 2000; Trainotti *et al.*, 2007). Once the fruit initiates autocatalytic ethylene (pre-climacteric), the ripening process will be enhanced in both auxin- and ethylene-dependent manners, since transcripts of some ethylene elements still present in considerable levels in non-ethylene associated tissues such as seed and MCP-treated fruits (Miller *et al.*, 1987; Trainotti *et al.*, 2007; El-Sharkawy *et al.*, 2008; Ziliotto *et al.*, 2008; this paper). Therefore, the possibility of regulating many ethylene-related genes by auxin is the best alternative that explains the significant accumulation of such transcripts during fruit ripening in an ethylene-independent manner.

Late cultivars, including 'SH', seem to produce insufficient quantities of auxin to co-ordinate the transition into ripening stage. The low level of auxin throughout the S3 stage results in minimal accumulation of ethylene-related proteins and, consequently, the fruit reaches the S4 stage late (~90 DAB) accompanied by a slow ripening process thereafter. Ethylene production in 'EG' fruit reached a maximum ($33.3 \text{ nl g}^{-1} \text{ h}^{-1} \pm 1.0$) after approximately 5 d post-harvest, however, those of 'SH' fruit reached its maximum ($5.7 \text{ nl g}^{-1} \text{ h}^{-1} \pm 0.7$) after approximately 11 d post-harvest. On the other hand, treatment of such late varieties with exogenous auxin or ethylene restored the typical climacteric pattern (Abdi *et al.*, 1997; Ohmyia, 2000). Taken together, it seems that auxin is at least one of the most important factors, if not the only factor, which commands the transition of a fruit from the S3 growth stage to the S4 ripening stage. The role of auxin continues thereafter in association with ethylene in enhancing fruit ripening.

In this investigation, as well as, in our previous reports (El-Sharkawy *et al.*, 2007, 2008) many ethylene related genes were characterized. These ethylene-related proteins can accumulate either in an ethylene-dependent, or auxin-dependent, or both ethylene- and auxin-dependent manners. However, the role played by auxin is more significant in controlling the ripening behaviour. The importance of auxin comes from its being essential in initiating the ripening phenomenon itself, and after that, in concert with ethylene, in accelerating the ripening process. Any genetic and/or environmental factors that negatively affect the accumulation of auxin in the early developmental stages (before ripening) resulted in the significant delay in fruit ripening. Characterization of many auxin-related elements will be our next step in order to establish the role of auxin in fruit ripening and to investigate the existence of any cross-talk between ethylene and auxin through controlling the regulation of auxin-related genes by ethylene.

Supplementary data

The supplementary data available at *JXB* online consists of a detailed description of the real-time PCR condition and the method used for expression levels calculation.

Supplementary Table S1. The real-time PCR primers used in this study.

Supplementary Fig. S1. The amino acid sequence alignment of 'Class I' (a), 'Class IV' (b), and 'Class II' (c) ERFs.

Supplementary Fig. S2. The ethylene production ($\text{nl g}^{-1} \text{ h}^{-1}$) of five fruits during ripening of early 'EG' and late 'SH' plum cultivars.

References

- Abdi N, Holford P, McGlasson WB, Mizrahi Y.** 1997. Ripening behavior and responses to propylene in four cultivars of Japanese type plums. *Postharvest Biology and Technology* **12**, 21–34.
- Abel S, Theologis A.** 1994. Transient transformation of *Arabidopsis* leaf protoplasts: a versatile experimental system to study gene expression. *The Plant Journal* **5**, 421–427.
- Abel S, Theologis A.** 1996. Early genes and auxin action. *Plant Physiology* **111**, 9–17.
- Abel S, Nguyen MD, Chow W, Theologis A.** 1995. ACS4, a primary indoleacetic acid-responsive gene encoding 1-aminocyclopropane-1-carboxylate synthase in *Arabidopsis thaliana*. Structural characterization, expression in *Escherichia coli*, and expression characteristics in response to auxin. *Journal of Biological Chemistry* **270**, 19093–19099.
- Abeles FB, Morgan PW, Saltveit ME.** 1992. *Ethylene in plant biology*, 2nd edn. San Diego: Academic Press.
- Allen MD, Yamasaki K, Ohme-Takagi M, Tateno M, Suzuki M.** 1998. A novel mode of DNA recognition by a beta sheet revealed by the solution structure of the GCC-box binding domain in complex with DNA. *EMBO Journal* **17**, 5485–5496.
- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389–3402.
- Boss PK, Davies C, Robinson SP.** 1996. Analysis of the expression of anthocyanin pathway genes in developing *Vitis vinifera* cv. Shiraz grape berries and the implications for pathway regulation. *Plant Physiology* **111**, 1059–1066.
- Büttner M, Singh KB.** 1997. *Arabidopsis thaliana* ethylene responsive element binding protein (ATEBP), an ethylene-inducible, GCC box DNA-binding protein interacts with an ocs element binding protein. *Proceedings of the National Academy of Sciences, USA* **94**, 5961–5966.
- Cao Y, Song F, Goodman RM, Zheng Z.** 2006. Molecular characterization of four rice genes encoding ethylene-responsive transcriptional factors and their expressions in response to biotic and abiotic stress. *Journal of Plant Physiology* **163**, 1167–1178.
- Chae HS, Kieber JJ.** 2005. *Eto Brute?* Role of ACS turnover in regulating ethylene biosynthesis. *Trends in Plant Science* **10**, 291–296.
- Christian M, Steffens B, Schenck D, Burmester S, Böttger M, Lüthen H.** 2006. How does auxin enhance cell elongation? Roles of auxin-binding proteins and potassium channels in growth control. *Plant Biology (Stuttgart)* **8**, 346–352.
- DeDios P, Matilla AJ, Gallardo M.** 2006. Flower fertilization and fruit development prompt changes in free polyamines and ethylene in

- damson plum (*Prunus insititia* L.). *Journal of Plant Physiology* **163**, 86–97.
- EI-Sharkawy I, Kim WS, El-Kereamy A, Jayasankar S, Svircev AM, Brown DCW.** 2007. Isolation and characterization of four ethylene signal transduction elements in plums (*Prunus salicina* L.). *Journal of Experimental Botany* **58**, 3631–3643.
- EI-Sharkawy I, Kim WS, Jayasankar S, Svircev AM, Brown DCW.** 2008. Differential regulation of four members of ACC synthase gene family in plum. *Journal of Experimental Botany* **59**, 2009–3027.
- Fujimoto SY, Ohta M, Usui A, Shinshi H, Ohme-Takagi M.** 2000. *Arabidopsis* ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. *The Plant Cell* **12**, 393–404.
- Giovannoni J.** 2001. Molecular biology of fruit maturation and ripening. *Annual Review of Plant Physiology and Plant Molecular Biology* **52**, 725–749.
- Giovannoni JJ.** 2004. Genetic regulation of fruit development and ripening. *The Plant Cell* **16**, 170–180.
- Gu YQ, Wildermuth MC, Chakravarthy S, Loh YT, Yang C, He X, Han Y, Martin GB.** 2002. Tomato transcription factors Pti4, Pti5, and Pti6 activate defense responses when expressed in *Arabidopsis*. *The Plant Cell* **14**, 817–831.
- Hartmann HT, Kester DE, Davies FT, Geneve RL.** 2002. *Plant propagation*, 7th edn. New Jersey, USA: Pearson Education.
- Hellens RP, Edwards AE, Leyland NR, Bean S, Mullineaux P.** 2000. pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. *Plant Molecular Biology* **42**, 819–832.
- Jeanmougin F, Thompson JD, Gouy M, Higgins DG, Gibson TJ.** 1998. Multiple sequence alignment with Clustal X. *Trends in Biochemical Science* **23**, 403–405.
- Kärkönen A, Fry SC.** 2006. Effect of ascorbate and its oxidation products on H₂O₂ production in cell-suspension cultures of *Picea abies* and in the absence of cells. *Journal of Experimental Botany* **57**, 1633–1644.
- Leclercq J, Ranty B, Sanchez-Ballesta MT, Li Z, Jones B, Jauneau A, Pech JC, Latche A, Ranjeva R, Bouzayen M.** 2005. Molecular and biochemical characterization of LeCRK1, a ripening-associated tomato CDPK-related kinase. *Journal of Experimental Botany* **56**, 25–35.
- Lee JH, Hong JP, Oh SK, Lee S, Choi D, Kim WT.** 2004. The ethylene-responsive factor like protein 1 (CaERFLP1) of hot pepper (*Capsicum annuum* L.) interacts *in vitro* with both GCC and DRE/CRT sequences with different binding affinities: possible biological roles of CaERFLP1 in response to pathogen infection and high salinity conditions in transgenic tobacco plants. *Plant Molecular Biology* **55**, 61–81.
- Long-Guo J, Jin-Yuan L.** 2008. Molecular cloning, expression profile and promoter analysis of a novel ethylene responsive transcription factor gene *GhERF4* from cotton (*Gossypium hirsutum*). *Plant Physiology and Biochemistry* **46**, 46–53.
- Mantiri FR, Kurdyukov S, Lohar DP, Sharopova N, Saeed NA, Wang XD, VandenBosch KA, Rose RJ.** 2008. The transcription factor MtSERF1 of the ERF subfamily identified by transcriptional profiling is required for somatic embryogenesis induced by auxin plus cytokinin in *Medicago truncatula*. *Plant Physiology* **146**, 1622–1636.
- Miller AN, Walsh CS, Cohen JD.** 1987. Measurement of indole-3-acetic acid in peach fruits (*Prunus persica* L. Batsch cv. Redhaven) during development. *Plant Physiology* **84**, 491–494.
- Mizuno S, Hirasawa Y, Sonoda M, Nakagawa H, Sato T.** 2006. Isolation and characterization of three DREB/ERF-type transcription factors from melon (*Cucumis melo*). *Plant Science* **170**, 1156–1163.
- Nakano T, Suzuki K, Fujimura T, Shinshi H.** 2006. Genome wide analysis of the ERF gene family in *Arabidopsis* and rice. *Plant Physiology* **140**, 411–432.
- Nakatsuka A, Murachi S, Okunishi H, Shiomi S, Nakano R, Kubo Y, Inaba A.** 1998. Differential expression and internal feedback regulation of 1-aminocyclopropane-1-carboxylate synthase, 1-aminocyclopropane-1-carboxylate oxidase, and ethylene receptor genes in tomato fruit during development and ripening. *Plant Physiology* **118**, 1295–1305.
- Nicholas KB, Nicholas HBJ.** 1997. *GeneDoc: a tool for editing and annotating multiple sequence alignments* (distributed by the authors).
- Ohme-Takagi M, Shinshi H.** 1995. Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. *The Plant Cell* **7**, 173–182.
- Ohmyia A.** 2000. Effects of auxin on growth and ripening of mesocarp discs of peach fruit. *Scientia Horticulturae* **84**, 309–319.
- Ohta M, Ohme-Takagi M, Shinshi H.** 2000. Three ethylene responsive transcription factors in tobacco with distinct transactivation functions. *The Plant Journal* **22**, 29–38.
- Ohta M, Matsui K, Hiratsu K, Shinshi H, Ohme-Takagi M.** 2001. Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. *The Plant Cell* **13**, 1959–1968.
- Page RDM.** 1996. TREEVIEW: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* **12**, 357–358.
- Raikhel N.** 1992. Nuclear targeting in plants. *Plant Physiology* **100**, 1627–1632.
- Rodríguez-Gacio MC, Matilla AJ.** 2001. The last step of the ethylene-biosynthesis pathway in turnip tops (*Brassica rapa*) seeds: alterations related to development and germination and its inhibition during desiccation. *Physiologia Plantarum* **111**, 273–279.
- Ros Barceló A.** 2005. Xylem parenchyma cells deliver the H₂O₂ necessary for lignification in differentiating xylem vessels. *Planta* **220**, 747–756.
- Sakuma Y, Liu Q, Dubouzet JG, Abe H, Shinozaki K, Yamaguchi-Shinozaki K.** 2002. DNA-binding specificity of the ERF/AP2 domain of *Arabidopsis* DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. *Biochemical and Biophysical Research Communications* **290**, 998–1000.
- Solano R, Stepanova A, Chao Q, Ecker JR.** 1998. Nuclear events in ethylene signalling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE RESPONSE FACTOR1. *Genes and Development* **12**, 3703–3714.
- Tonutti P, Bonghi C, Ruperti B, Tornielli GB, Ramina A.** 1997. Ethylene evolution and 1-aminocyclopropane-1-carboxylate oxidase gene expression during early development and ripening of peach fruit.

Journal of the American Society of Horticultural Science **122**, 642–647.

Tournier B, Sanchez-Ballesta MT, Jones B, Pesquet E, Regad F, Latché A, Pech JC, Bouzayen M. 2003. New members of the tomato ERF family show specific expression pattern and diverse DNA-binding capacity to the GCC box element. *FEBS Letters* **550**, 149–154.

Trainotti L, Tadiello A, Casadoro G. 2007. The involvement of auxin in the ripening of climacteric fruits comes of age: the hormone plays a role of its own and has an intense interplay with ethylene in ripening peaches. *Journal of Experimental Botany* **58**, 3299–3308.

Wang A, Tan D, Takahashi A, Li TZ, Harada T. 2007. MdERFs, two ethylene-response factors involved in apple fruit ripening. *Journal of Experimental Botany* **58**, 3743–3748.

Yang HJ, Shen H, Chen L, Xing YY, Wang ZY, Zhang JL, Hong MM. 2002. The OsEBP-89 gene of rice encodes a putative EREBP transcription factor and is temporally expressed in developing

endosperm and intercalary meristem. *Plant Molecular Biology* **50**, 379–791.

Yi SY, Kim JH, Joung YH, Lee S, Kim WT, Yu SH, Choi D. 2004. The pepper transcription factor CaPF1 confers pathogen and freezing tolerance in *Arabidopsis*. *Plant Physiology* **136**, 2862–2874.

Zhang H, Huang Z, Xie B, Chen Q, Tian X, Zhang X, Zhang H, Lu X, Huang D, Huang R. 2004. The ethylene-, jasmonate-, abscisic acid- and NaCl-responsive tomato transcription factor JERF1 modulates expression of GCC box-containing genes and salt tolerance in tobacco. *Planta* **220**, 262–270.

Zhou J, Tang X, Martin GB. 1997. The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a *cis*-element of pathogenesis-related genes. *EMBO Journal* **16**, 3207–3218.

Ziliotto F, Begheldo M, Rasori A, Bonghi C, Tonutti B. 2008. Transcriptome profiling of ripening nectarine (*Prunus persica* L. Batsch) fruit treated with 1-MCP. *Journal of Experimental Botany* **59**, 2781–2791.