Mol. Cells, Vol. 10, No. 5, pp. 533–539

Molecules and Cells

Molecular Cloning and Expression Analysis of 3-Ketoacyl-ACP Synthases in the Immature Seeds of *Perilla frutescens*

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(Received on June 1, 2000)

We report the isolation and expression analysis of two cDNAs encoding 3-ketoacyl-acyl carrier protein synthases (KAS) that are involved in the *de novo* synthesis of fatty acids in plastids of perilla (*Perilla frutescens* L.). The cDNAs, designated *PfFAB1* and *PfFAB24*, encoded polypeptides with high sequence identities to those of KAS I and KAS II/IV, respectively, of various plants. Genomic Southern blots revealed that there was a single *PfFAB1* gene but two *PfFAB24* genes in the perilla genome. Of interest is that the expression of both genes was developmentally regulated in seeds. Their mRNA expression patterns in seeds were also discussed in comparison with the profile of fatty acid accumulation.

Keywords: Fatty Acid Biosynthesis; KAS; *Perilla frutescens*.

Introduction

In plastids of higher plants, *de novo* fatty acid biosynthesis occurs through a primary metabolic pathway known as a dissociated (or type II) fatty acid synthase system that differs from the multifunctional (or type I) fatty acid synthases found in fungi and mammals (for a review see Ohlrogge and Browse, 1995; Slabas and Fawcett, 1992; Topfer *et al*., 1995). A series of discrete enzymes concomitantly catalyze the condensation of a two-carbon unit (C2) derived from acetyl-CoA with the growing acyl chains. The initial step of fatty acid synthesis is driven by 3-ketoacyl-ACP synthase III (KAS III) to condense acetyl-CoA with malonyl-ACP. KAS I catalyzes the condensation of acetyl-ACP with malonyl-ACP to yield acyl-ACPs up to myristoyl-ACP (C14:0) or palmitoyl-ACP (C16:0), whereas KAS II is involved in the final elongation to stearoyl-ACP (C18:0). In addition, the existence of a fourth condensing enzyme KAS IV, which has a different acyl-chain specificity from other three KAS species, has been reported from *Cuphea* sp. (Dehesh *et al*., 1998) that produces a high amount of medium-chain fatty acids. Subsequent to the condensation reactions of acyl-chains by KAS enzymes, each cycle of elongation is completed by the sequential actions of NADH-dependent 3-ketoacyl-ACP reductase, 3-hydroxyacyl-ACP dehydratase, and finally enoyl-ACP reductase. Recently, the X-ray crystal structures of KAS I (Olsen *et al*., 1999), KAS II (Huang *et al*., 1998; Moche *et al*., 1999), and KAS III (Qiu *et al*., 1999) from *Escherichia coli* were determined. These reports may confer valuable information for further studies on structure-to-function relationship of the enzymes.

Perilla (*Perilla frutescens* L.) is known to accumulate a high amount of α-linolenic acid (C18:3) in seed oils, ranging from 60% to 70% depending upon the variety (Shin and Kim, 1994). Thus this species can be considered as a useful model plant for the study of the biosynthesis of polyunsaturated fatty acids. As an initial step toward understanding the expression profiles of genes involved in fatty acid synthesis in perilla, we have previously cloned and characterized two cDNAs encoding KAS III from this plant (Hwang and Hwang, 2000). For further study, we also isolated and characterized two cDNAs homologous to KAS I and KAS II/IV in the present study. The expression levels of both KAS genes were examined in various perilla tissues. Furthermore, in order to understand the relationship between gene expression and fatty acid accumulation, we compared the transcript levels of both genes with the

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Abbreviations: ACP, acyl carrier protein; CoA, coenzyme A; FAME, fatty acid methyl ester; KAS, 3-ketoacyl-ACP synthase; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; WPF, week post flowering.

fatty acid profiles of storage lipids in seeds harvested at different developmental stages.

Materials and Methods

Perilla cultivar Okdong was used in this study for DNA and RNA isolation. Genomic DNA was isolated from frozen leaf tissues using polyvinylpyrrolidone (PVP) and cetyltriethylammonium bromide (CTAB) according to the method of Kim *et al.* (1997). Total RNA was extracted from different plant tissues according to the method of Pawlowski *et al.* (1994). Restriction digestions, cloning, and DNA- and RNA-blottings were performed following standard protocols (Sambrook *et al.*, 1989). DNA probes were radioactively labeled with [α-^{32}P]dCTP using the random labeling kit (Boehringer-Mannheim). DNA- and RNA-blot hybridizations were performed in a solution containing 0.5 M sodium phosphate, pH 7.2, 7% (w/v) SDS, and 1% (w/v) bovine serum albumin (Church and Gilbert, 1984). Membranes were washed either under low-stringency conditions (at 60°C in 2 × SSC/0.2% SDS) or under high-stringency conditions (at 65°C in 2 × SSC/0.2% SDS followed by in 0.1 × SSC/0.1% SDS).

A lambda ZAP II cDNA library prepared from the poly(A) mRNA of perilla immature seeds (15 d after flowering) was generously provided by S.-K. Lee, NIAST, RDA (Korea). In order to prepare gene-specific probes for library screening an aliquot (5×10^5 plaque-forming units) of the cDNA library was directly used as a template for PCR amplification. The specific probes for the KAS I gene consisted of a 1.1-kb PCR-amplified fragment produced using the degenerate primers FabB-F, 5′-GG(ACGT)TA(CT)AT(ACT)GA(CT)GG(ACGT)AA(AG)AA-3′ [GYIDGKN], and FabB-R, 5′-TT(AG)AA-(ACGT)GC(ACGT)(CG)(AT)(AG)AA(ACGT)GC(ACGT)-AC(ACGT)AC-3′ [VVAFSAFK]. The KAS II-specific probe consisted of a 0.6-kb PCR-amplified fragment obtained using the primers FabF-F, 5′-AA(AG)(AC)G(ACGT)ATGGA-(CT)AA(AG)TT(CT)ATG-3′ [KRMDKFM], and FabF-R, 5′-GC(AG)TC(ACGT)(GC)(AT)(ACGT)GT(AG)AA(ACGT)(CG)(AT)(ACGT)CC(ACGT)CC-3′ [GGSFTSDA]. The PCR condition was a cycle for 2 min at 95°C, followed by 35 cycles of 30 s at 95°C, 1 min at 55°C, and 1 min at 72°C with final extension for 10 min at 72°C. Each PCR product amplified from the cDNA library was sequenced with the DNA cycle sequencing kit (Promega) and databases were searched for sequence similarities using the BLAST program of the NCBI (Altschul *et al.*, 1997). The two PCR fragments were radioactively labeled and used to isolate full-length clones from the cDNA library. The duplicate membranes were hybridized overnight at 60°C with each of the probes. The membranes were washed twice under low-stringency conditions for 20 min, then visualized by autoradiography at −80°C. Double-positive phage clones were picked and purified by two further rounds of screening. The restriction digests of *in vivo*-excised plasmids were subjected to Southern blot hybridization under the same conditions as the library screening. Plasmids containing the longest insert were selected and their 5′-ends were sequenced to identify full-length clones.

Fatty acid analysis of seed samples was performed according to the method of Metcalfe *et al.* (1966) with slight modifications. Perilla seeds at different developmental stages were pulverized in a mortar and pestle in the presence of liquid nitrogen. One hundred milligrams of each sample was supplemented sequentially with 5 ml of $CHCl_3$-CH_3OH (1:1, v/v), 4 ml of 0.58% NaCl and 1 ml (0.1 mg) of pentadecanoic acid as an internal standard. The mixture was then homogenized and partitioned into two layers. The organic layer was collected and evaporated to near dryness by the flow of nitrogen gas. Then, 0.5 ml of toluene and 2 ml of 0.5 N NaOH in methanol were added, and the sealed vial was placed in boiling water for 5 min and cooled to room temperature; 2.5 ml of BF_3 in methanol was added and each vial was placed again in boiling water for 3 min. After the addition of 10 ml of petroleum ether and 10 ml of water to each sample, the sample was sonicated for 10 min. The upper organic phase was collected and evaporated with nitrogen gas to near dryness after removing water with Na_2SO_4. The FAMEs were resuspended in petroleum ether and separated by gas liquid chromatography (GLC) with a HP 5890 Series II system using a Carbowax capillary column (HP-20M, Hewlett-Packard). The oven temperature was isothermal at 180°C and that for the injector and detector was 200°C. The FAMEs were detected by flame ionization, all peaks were integrated, and quantities were calculated with reference to the internal standard.

Results and Discussion

We identified two full-length cDNA clones homologous to known plant KAS I and KAS II/IV sequences that were hereafter referred to as *PfFAB1* cDNA (1,841-bp) and *PfFAB24* cDNA (2,072-bp), respectively. The *PfFAB1* cDNA without the poly(A) tail was 1,818-bp long and contained an open reading frame (ORF) that encoded a protein of 474 amino acids with a calculated molecular mass of 50,023 Da. The deduced amino acid sequence of *PfFAB1* cDNA showed 85.3%, 84.2%, 82.4%, 66.5%, and 31.5% identities with those of castor bean (*Ricinus communis*), soybean (*Glycine max*), Arabidopsis (*Arabidopsis thaliana*) (Millar and Kunst, 1995), *Capsicum chinense* (Aluru *et al.*, 1998) and *Escherichia coli* (Kauppinen *et al.*, 1988), respectively. The *PfFAB24* cDNA without the poly(A) tail was 2,053-bp long and contained an ORF encoding a protein of 530 amino acids with a calculated molecular mass of 57,017 Da. Comparison of the deduced protein sequence of *PfFAB24* showed 74.0%, 74.0%, 72.7%, 72.6%, 72.6%, 72.0%, 71.8%, and 34.9% identities with those of castor bean, *Cuphea wrightii* (KAS II_2 and KAS II_1) (Slabaugh *et al.*, 1998), soybean (KAS II), *Cuphea hookeriana* (KAS IV) (Dehesh *et al.*, 1998), *Arabidopsis* (KAS II), *Cuphea pulcherrima* (KAS IV), and *E. coli* (KAS II) (Magnuson *et al.*, 1995), respectively. It is noteworthy that lower but still significant sequence identity (44.6%) and long stretches of identical residues were found between perilla KAS I (referred to as PfFAB1) and KAS II/IV (referred to as PfFAB24) (Fig. 1). Both PfFAB1 and PfFAB24 contained the

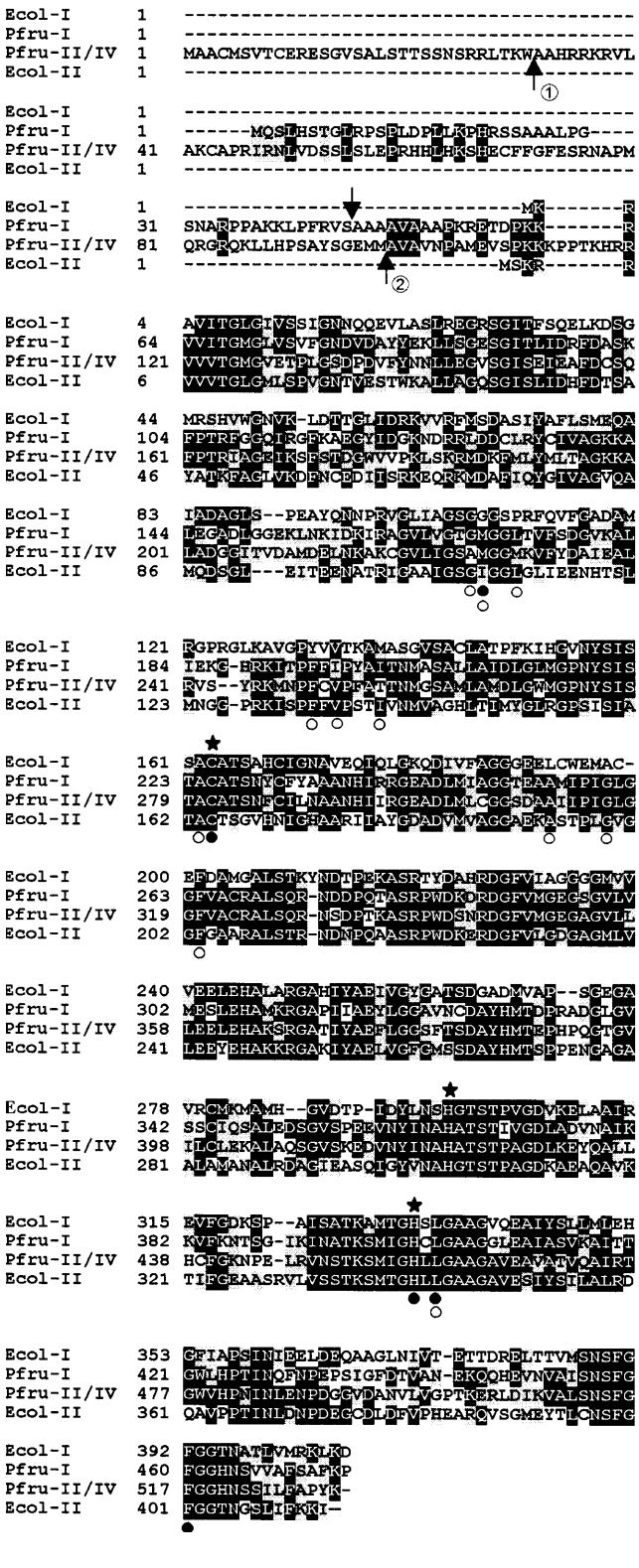

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Fig. 1. Comparison of the predicted perilla KAS polypeptides with *E. coli* KAS I and KAS II. The putative cleavage sites, indicated by arrows, for chloroplast transit peptides were predicted using the ChloroP method (Emanuelsson *et al*., 1999) at the web site (http://www.cbs.dtu.dk/services/Chloropl). Three amino acid residues, including cysteine as a centerpiece, of the active site are indicated by asterisks above the sequences. The residues whose conformation are changed to provide access for cerulenin to the active site are indicated by closed circles and the binding sites for the hydrophobic part of the inhibitor are indicated by open circles. Residues that are identical in at least two of the sequences are highlighted. Biologically similar residues are shown by gray boxes. Dashes indicate gaps introduced for optimization of the alignment. The nomenclature for each sequence is shown in Fig. 2.

putative transit peptide sequences for targeting into plastids at their N-termini, indicating that the proteins seem to represent plastidial isoforms. On the basis of statistical analysis using the ChloroP program (Emanuelsson *et al*., 1999), a single cleavage site was predicted for PfFAB1, whereas two sites were predicted for PfFAB24. Multiple alignment of the protein sequences of PfFAB1 and PfFAB24 with those of the corresponding *E. coli* KASs revealed many conserved sequence motifs throughout the overall sequences except for the putative transit peptide sequences. Several structural features of both proteins were predicted by comparison with the known crystal structures of *E. coli* KAS I (Olsen *et al*., 1999) and KAS II (Huang *et al*., 1998; Moche *et al*., 1999) (Fig. 1). Three amino acid residues, including cysteine as a centerpiece, of the active site were well conserved both in PfFAB1 and PfFAB24. The other residues whose conformations are changed to build a cavity for the binding of cerulenin, a potent KAS inhibitor to the active site, and those interacting with the hydrophobic part of the inhibitor were also well conserved in both proteins, although several residue divergences were found. These divergences between the FabB and FabF proteins may explain different substrate specificities as suggested by Olsen *et al*. (1999).

The deduced protein sequences of the two cDNA clones and the published sequences of KAS homologs from various plant sources were used to generate the phylogenetic tree shown in Fig. 2. The tree consisted of two distinct groups, KAS I and KAS II/IV. It is apparent that PfFAB1 (Pfru-I) was well clustered with other known KAS I sequences. However, PfFAB24 (Pfru-II/IV) was placed on the same branch with both the KAS II and KAS IV sequences. The closest neighbors of PfFAB1 and PfFAB24 were those of caster bean.

The copy numbers of the *PfFAB1* and *PfFAB24* genes were assessed by Southern-blot hybridization under high-stringency conditions (Fig. 3). Genomic DNA of perilla was digested with three restriction enzymes: *Eco*RI, *Hin*dIII and *Bam*HI. Since relatively high sequence similarity was present between *PfFAB1* and *PfFAB24*, we prepared gene-specific probes that did not cross-hybridize with each other under high-stringency conditions (data not shown). As shown in

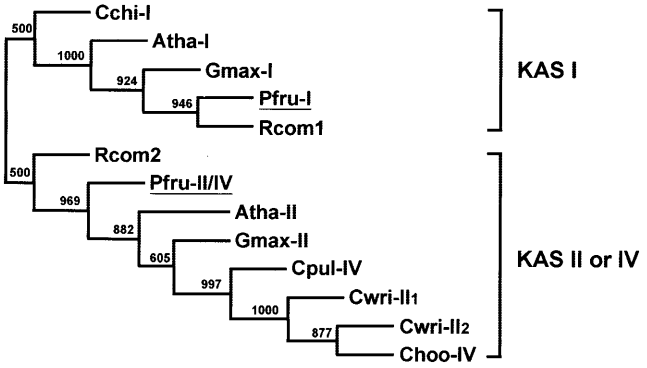

Fig. 2. Phylogenetic tree based on the deduced KAS protein sequences. The amino acid sequences representative of plant, fungal, and bacterial KASs were aligned using the multiple alignment program Clustal X (Jeanmougin *et al*., 1998) with a PAM250 residue weight table. The tree was constructed by programs of the PHYLIP package using the neighbor-joining method with Kimura's formula as described by Hwang and Kim (1998). Bootstrap values from 1,000 replicates are given above each branch. The identities of the proteins are: Pfru-I (*P. frutescens* KAS I, AF026148); Rcom1 (*R. communis* KAS, L13242); Atha-I (*A. thaliana* KAS I, U24177); Gmax-I (*G. max* KAS I, AF243182); Cchi-I (*C. chinense* KAS I, AF085148); Pfru-II/IV (*P. frutescens* KAS II/IV, AF026149); Gmax-II (*G. max* KAS II, AF244518); Rcom2 (*R. communis* KAS, L13241); Cwri-II_1 (*C. wrightii* KAS II, U67317); Cwri-II_2 (*C. wrightii* KAS II, U67316); Cpul-IV (*C. pulcherrima* KAS IV, AF060518); Choo-IV (*C. hookeriana* KAS IV, AF060519); Atha-II (*A. thaliana* KAS II, AC013258).

Fig. 3A, the *PfFAB1*-specific probe strongly hybridized to a single fragment in each digest. Since there were no restriction enzyme sites for *Eco*RI, *Hin*dIII, and *Bam*HI in the probe of the *PfFAB1* cDNA, we conclude that a *Hin*dIII site is probably present within an intron and that the *PfFAB1* gene is a single copy in the perilla genome. These results are similar to the previous results of genomic analyses for barley (Kauppinen, 1992) and Arabidopsis (Millar and Kunst, 1995). For *PfFAB24* two predominant hybridizing bands were detected in all digests (Fig. 3B). Although the three restriction enzymes, *Eco*RI, *Hin*dIII and *Bam*HI, do not cut within the DNA probe of the *PfFAB24* cDNA, we could not rule out the possible presence of the restriction sites in the intron sequence of the *PfFAB24* gene. Thus, further assessment of the copy numbers of the *PfFAB24* gene was performed using the perilla genomic fragment that was obtained by PCR amplification (data not shown). The restriction results and sequence analysis revealed that the intron-containing the 1.4-kb PCR products of the *PfFAB24* gene had no restriction sites for the enzymes. Together with the results of Southern-blot analysis (Fig. 3B), these observations strongly imply that two *PfFAB24* genes encoding closely related isoforms exist at one or two loci or that they make up a small gene family.

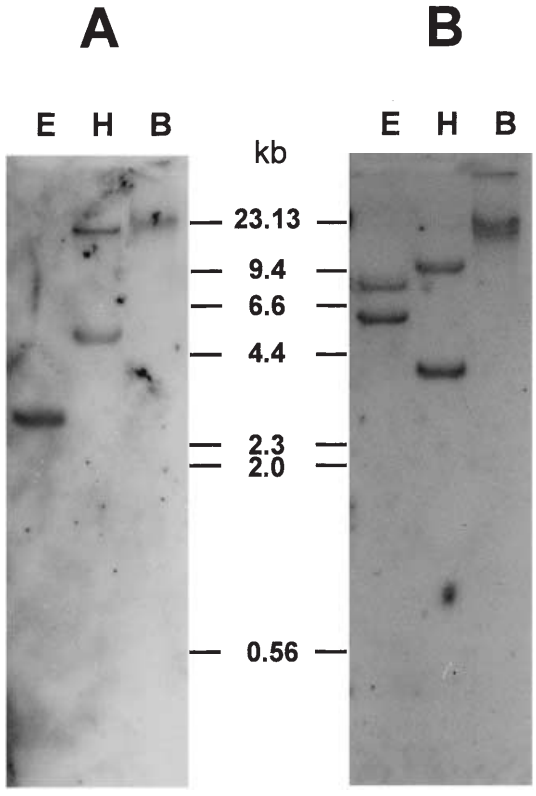

Fig. 3. Genomic DNA hybridization of *PfFAB1* (**A**) and *PfFAB24* (**B**). The genomic DNA (10 μg for each lane) was digested with *Eco*RI (E), *Hin*dIII (H) and *Bam*HI (B) as specified by the manufacturer (Boehringer-Mannhein), separated on 0.8% (w/v) agarose gels, and alkali transferred onto a Hybond-N^+ membrane (Amersham) (Sambrook *et al*., 1989). The blots were hybridized with each of the KAS gene-specific probes: a 0.5-kb *Eco*RI/*Xho*I fragment of the *PfFAB1* cDNA and a 0.6-kb *Eco*RI/*Bst*XI fragment of the *PfFAB24* cDNA. The blots were washed under high-stringency conditions. The positions of the DNA size markers (in kb) are indicated between the blots.

To understand the mode of expression of the two KAS genes in perilla, Northern-blot analysis was performed using the same gene-specific probes as for the genomic DNA analyses (Fig. 3). Total RNAs were prepared from leaves, roots, and stems under light conditions. As shown in Fig. 4A, the results of RNA-blot hybridization revealed that mRNA expression of both genes, *PfFAB1* (1.9-kb) and *PfFAB24* (2.1-kb), was detected equally at high levels in leaves, whereas slightly lower levels of both transcripts were detected in stems and only very weakly in roots. In addition, to investigate the expression patterns of both genes during seed maturation, we also performed Northern-blot analyses using total RNAs prepared from seeds and sepals at different developmental stages. An increase of *PfFAB1* mRNA levels was detectable at 1-week post flowering (WPF), reaching the highest levels of accumulation at 2-WPF when the seeds were turning into

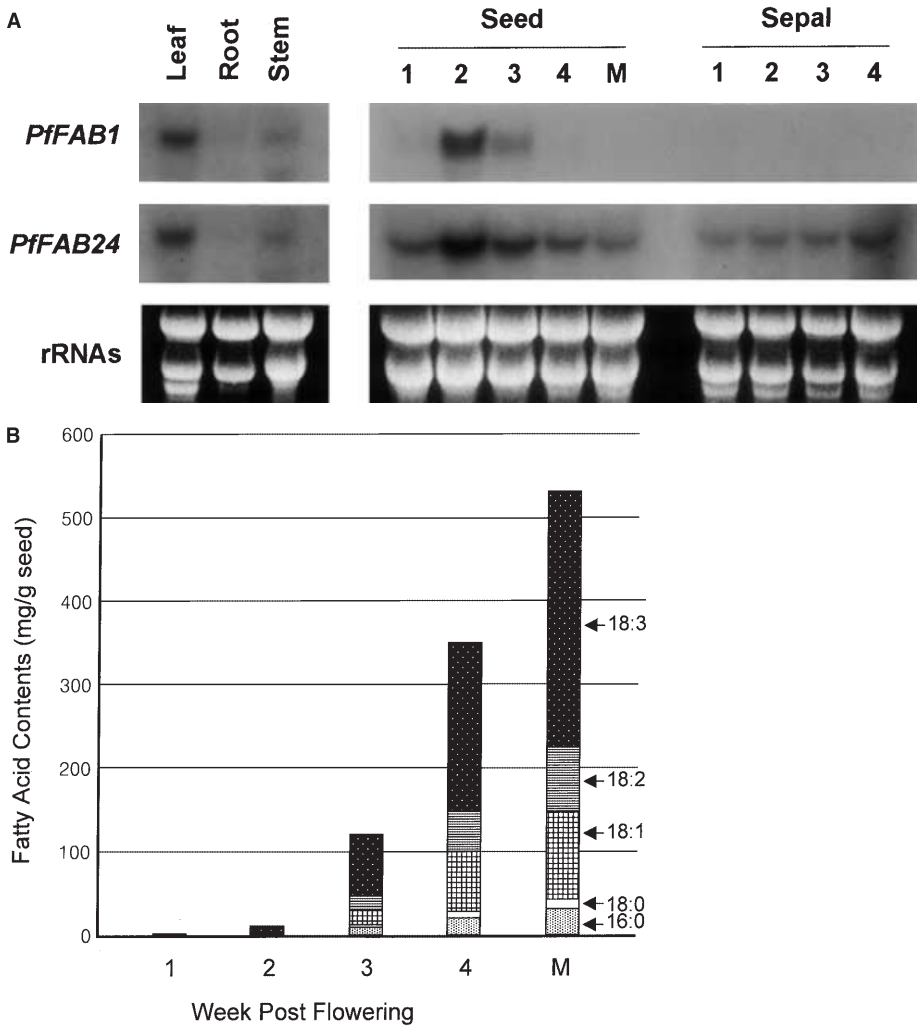

Fig. 4. Northern blot analyses of the *PfFAB1* and *PfFAB24* genes (**A**) and the fatty acid profile (**B**) in developing seeds. **A**. Equal amounts (20 μg) of total RNA were separated by electrophoresis on a 1.2% (w/v) formaldehyde-agarose gel and blotted onto a Hybond-N nylon membrane (Amersham) as described by Sambrook *et al.* (1989). The blot was then hybridized with a probe specific to either the *PfFAB1* or the *PfFAB24* gene as for genomic Southern hybridization (Fig. 3). The numbers above the lanes indicate the ages of the seeds in weeks. An ethidium bromide-stained RNA gel was used as a control for a visual evaluation of RNA quality and equal loading. **B**. Perilla seeds collected at different developmental stages were subjected to fatty acid analysis (see **Materials and Methods**). The fatty acid contents are presented as average values from three independent experiments.

a light brown color. However, this accumulation was transient, since transcript levels decreased thereafter. A very low, barely detectable accumulation of the *PfFAB1* transcript was detected in seeds at subsequent stages and in sepals at all stages examined.

A similar expression profile was observed for *PfFAB24* mRNA in that *PfFAB24* showed the highest expression level in seeds at 2-WPF and decreased thereafter. However, the relatively unchanged accumulation was detectable at basal levels both in seeds and sepals throughout all developmental stages. We assume that the basal levels of the transcripts at all stages were derived from the other *PfFAB24* isoform that was suggested from the results of Southern-blot analysis (Fig. 3). Taken together, these results indicate that expression of the *PfFAB1* and *PfFAB24* genes are at their maximum, but at transient levels at 2-WPF, indicating that fatty acid biosynthesis may occur actively at early stages of seed development.

In a previous report (Kauppinen, 1992), the barley KAS I gene was highly expressed in leaves, but barely in developing kernels, germinating embryos and roots. Aluru *et al.* (1998) also reported that the *C. chinense* KAS I gene was abundantly expressed and developmentally regulated in the placenta of immature fruit, whereas the transcripts were not detected in other tissues of the plant, including the leaf, stem, root, flower, seed and the fruit wall. This result indicates that expression of the gene is placenta-specific in *C. chinense*. The KAS IV gene of *C. hookeriana* was

detected only in seed and embryo tissues (Dehesh *et al*., 1998). In addition, Slabaugh *et al*. (1998) observed that expression of the KAS I protein was detected in all tissues of *C. wrightii*, whereas KAS A1 (KAS II) was confined to seed tissues. However, in our case, since high expression of *PfFAB1* and *PfFAB24* was detected in both leaves and immature seeds (2-WPF), it appears that the expression is not tissue-specific but developmentally regulated in seeds. In our previous report (Hwang and Hwang, 2000), similar profiles of gene expression have been observed for the perilla KAS III genes. Thus we conclude that genes encoding at least the condensing enzymes for fatty acid biosynthesis are expressed at their maximal levels at early stages of seed maturation.

Because the expression of *PfFAB1* and *PfFAB24* was developmentally regulated in seeds, we further analyzed fatty acid profiles of storage lipids at different developmental stages to examine whether gene expression is coincident with fatty acid accumulation. After extraction of lipids and the preparation of methyl esters, we determined the contents and composition of fatty acids in seeds. As shown in Fig. 4B, accumulation of fatty acids drastically increased to about 12 fold at 3-WPF and their increase continued thereafter, suggesting that fatty acid accumulation is not in accord with mRNA accumulation (Fig. 4A) since there was an eclipsed period (1-week) between the two results. Furthermore, the transcript levels of both genes decreased significantly after 3-WPF while the accumulation of lipids increased continually. Our plausible explanation for this difference is that although significantly short-lived changes in both transcripts took place immediately after 2-WPF, the proteins encoded by the transcripts might be stable and may maintain their activities during the developmental process, whereby a continuous increase in the contents of fatty acids was able to occur. This assumption is supported in part by the previous observation (Slabaugh *et al*., 1998) that KAS I protein was expressed at an early stage of development in *C. wrightii* seeds and was maintained thereafter without significant changes in expression level. However, further work is needed to determine if this is the case for the *PfFAB1* and *PfFAB24* proteins in perilla, and, if so, whether the proteins maintain their activities during seed development. The existence of other fatty acid-condensing enzymes including the *PfFAB24* isoform cannot be ignored, which may explain the discrepancy between the levels of gene expression versus the profile of fatty acid accumulation.

To our knowledge, although up to now several plant genes encoding KAS I, KAS II or KAS IV have been cloned from diverse species, this is the first study to investigate the relationship between gene expression and fatty acid accumulation in terms of seed development.

Acknowledgements We thank Dr. Jeong-Bong Kim for valuable technical assistance. We also thank Drs. Donghern Kim and Varindra for critical reading of the manuscript. This work was supported in part by a research grant from the Rural Development Administration (RDA) and in part from the Korean Organization of Science and Engineering Foundation (KOSEF). S. K. Hwang is the recipient of a KOSEF post-doctoral fellowship (1997).

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