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3-Ketoacyl-Acyl Carrier Protein Synthase III from Spinach (*Spinacia oleracea*) Is Not Similar to Other Condensing Enzymes of Fatty Acid Synthase[1]

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A cDNA clone encoding spinach (*Spinacia oleracea*) 3-ketoacyl-acyl carrier protein synthase III (KAS III), which catalyzes the initial condensing reaction in fatty acid biosynthesis, was isolated. Based on the amino acid sequence of tryptic digests of purified spinach KAS III, degenerate polymerase chain reaction (PCR) primers were designed and used to amplify a 612-bp fragment from first-strand cDNA of spinach leaf RNA. A root cDNA library was probed with the PCR fragment, and a 1920-bp clone was isolated. Its deduced amino acid sequence matched the sequences of the tryptic digests obtained from the purified KAS III. Northern analysis confirmed that it was expressed in both leaf and root. The clone contained a 1218-bp open reading frame coding for 405 amino acids. The identity of the clone was confirmed by expression in *Escherichia coli* BL 21 as a glutathione *S*-transferase fusion protein. The deduced amino acid sequence was 48 and 45% identical with the putative KAS III of *Porphyra umbilicalis* and KAS III of *E. coli*, respectively. It also had a strong local homology to the plant chalcone synthases but had little homology with other KAS isoforms from plants, bacteria, or animals.

Fatty acid biosynthesis in higher plants is a plastid-localized pathway catalyzed by a set of nuclear-encoded enzymes. During the past several years, our understanding of how this universal pathway functions in plants and bacteria has undergone a fundamental change as a result of the discovery of an additional condensing enzyme, KAS III (Jackowski and Rock, 1987b; Jackowski et al., 1989; Jaworski et al., 1989; Clough et al., 1992). Fatty acids are synthesized by the sequential addition of C_2 units, resulting in principally C_{16} to C_{18} fatty acids. Each cycle of C_2 addition is initiated by a reaction catalyzed by a KAS and involves the condensation of a malonyl-ACP with an acyl acceptor. The initial studies of fatty acid synthase revealed that there were two isoforms of KAS, KAS I and KAS II (Shimakata and Stumpf, 1982). KAS I was thought to catalyze the early condensation reactions of malonyl-ACP with an acyl-ACP, leading to the synthesis of C_{14} to C_{16} fatty acids, whereas KAS II catalyzed only the final condensation reactions. The discovery in *Escherichia coli* and spinach (*Spinacia oleracea*) of a third KAS isoform, KAS III, significantly altered our understanding of the role of KAS I.

The in vitro (Jackowski and Rock, 1987b; Jackowski et al., 1989; Jaworski et al., 1989; Clough et al., 1992) and subsequent in vivo (Jaworski et al., 1993) evidence supported the proposal that KAS III catalyzes the initial condensation reaction in place of KAS I. Furthermore, KAS III uses acetyl-CoA as the acyl acceptor and thus relegates acetyl-ACP and acetyl-CoA:ACP transacylase to minor roles in fatty acid synthesis. To further understand the role of KAS III in plant fatty acid metabolism, Clough et al. (1992) purified this enzyme from spinach. Sufficient amino acid sequence analysis was carried out to allow the cloning of the spinach KAS III using a PCR approach. We report here the first cloning and analysis of a cDNA for KAS III from a higher plant.

MATERIALS AND METHODS

DNA and RNA Isolation

Spinach leaves used for the isolation of genomic DNA and RNA were frozen in liquid nitrogen and stored at −80°C until use. Genomic DNA was extracted in cetyltrimethylammonium bromide buffer at 60°C (Murray and Thompson, 1980). Total RNA was isolated using guanidine hydrochloride buffer as described by Logeman et al. (1987). Poly(A)$^+$ RNA was prepared from total RNA by poly(dT)-cellulose (Sigma) chromatography (Jacobson, 1987). Root poly(A)$^+$ RNA was a gift from Katherine Schmid, Butler University. The methods used for DNA isolation from bacteriophage and plasmids were adapted from Sambrook et al. (1989).

Synthesis of KAS III PCR Fragment

Partial amino acid sequences of the spinach KAS III were obtained from a tryptic digest of the purified enzyme blotted to nitrocellulose. Sequences of four peptides that were homologous to the *Escherichia coli* KAS III (Tsay et al., 1992) were used to prepare degenerate PCR primers. The PCR fragment was obtained by amplification of the first-strand cDNA of spinach leaf total RNA. Reverse transcription and amplification were performed according to the Perkin-Elmer Cetus GeneAmp protocols using oligo(dT) as a reverse transcription primer. Typical temperature conditions for the PCR were: denature 2 min at 95°C, 1 cycle; denature 1 min at

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Abbreviations: ACP, acyl carrier protein; GST, glutathione *S*-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; KAS, 3-ketoacyl-acyl carrier protein synthase; ORF, open reading frame; PCR, polymerase chain reaction.

95°C, anneal 1 min at 45°C, and extend 2 min at 72°C, 45 cycles; and finally extend for 10 min at 72°C. When necessary, a second round of amplification of 30 cycles was performed using 1 μL of PCR product directly from the first-round PCR product. A 612-bp fragment generated from the RNA-PCR was subcloned into dT-tailed (Marchuk et al., 1991) pBluescript SK(−) (Stratagene) to produce pHTP1.

Isolation and Characterization of KAS III Clone

The 612-bp fragment was cut from pHTP1 with *Bam*HI, separated electrophoretically on a low-melting-temperature agarose gel, and purified with Magicprep (Promega). The purified fragment was labeled with [α-^{32}P]dATP by the random priming reaction and used as a probe to screen a spinach root cDNA library (Schmid and Ohlrogge, 1990) in λZAP, a gift from Katherine Schmid. Approximately 20,000 plaques were screened in duplicate. The filters were hybridized at 68°C in a solution of 6× SSC, 0.25% nonfat dried milk, and the final wash was at 68°C in 1× SSC, 0.1% SDS. After a second round of screening, positive clones were isolated and excised in vivo into a plasmid according to the protocol of Stratagene. The identity and orientation of the insert were confirmed by PCR using a nondegenerate primer co-linear to a part of the probe sequence and a sequencing primer for the vector. A set of nested deletions was generated using exonuclease III/S1 nuclease (Promega) to sequence the clone to completion (Fig. 1). Single-stranded DNA was prepared and sequenced by the dideoxy method using Sequenase (United States Biochemical). The opposite strand was prepared by reversing the orientation of the clone and sequenced with custom sequencing primers. The GCG (Genetics Computer Group, University of Wisconsin) software and BLAST (Basic Local Alignment Search Tool) network service by National Center for Biotechnology Information were used to analyze the sequence.

Southern and Northern Analyses

Spinach genomic DNA was further purified by passing through Centricon-100 filters (Amicon). DNA (15 μg) was digested with restriction enzymes and separated on a 0.7% (w/v) agarose gel. The DNA was transferred to a nylon membrane (Tropilon, Tropix) and baked for 30 min in an 80°C vacuum oven. Electrophoresis of RNA was performed on a 1% (w/v) agarose gel containing formaldehyde (Sambrook et al., 1989), and the RNA was transferred to a nylon membrane.

Both Southern and northern blots were prehybridized in a solution of 6× SSC, 0.5% SDS, and 100 μg mL^{-1} of salmon sperm DNA at 68°C. The probe was the 612-bp PCR fragment labeled with [α-^{32}P]dATP by random priming. Hybridization was performed in the same solution at 68°C overnight. The final washing condition was 68°C in 2× SSC, 0.5% SDS. The filters were rinsed in 0.2× SSC at room temperature and exposed to x-ray film.

Expression of Spinach KAS III in *E. coli*

A PCR fragment corresponding in size to the coding region of *E. coli* KAS III was generated from spinach KAS III cDNA. The fragment was ligated into the *Nco*I site of the expression vector pGEX-KG, which codes for a GST fusion protein (Guan and Dixon, 1991), and transformed into *E. coli* strain BL 21 containing pLysS (Studier et al., 1990). Cultures of the transformed cells were grown at 22°C with or without induction by 0.1 mM IPTG. The IPTG was added at the beginning of logarithmic growth (A_{600} 0.2), and the cultures were allowed to grow 6 h more to reach late logarithmic phase. Cells were collected by centrifugation and lysed by freezing-thawing (Studier et al., 1990), and cell debris was removed by centrifugation in a microfuge for 5 min at 4°C. The KAS III-GST fusion protein was purified by affinity chromatography using GSH-agarose and subsequently cleaved with thrombin (Guan and Dixon, 1991). KAS III activity was measured as previously described (Clough et al., 1992).

RESULTS

Generation of a KAS III PCR Fragment

Partial amino acid sequences of purified spinach KAS III (Clough et al., 1992) were obtained from a tryptic digest of the blotted enzyme. The sequence information was used to design PCR primers to obtain a probe to screen a spinach cDNA library. Four of 10 tryptic digest peptides obtained from the purified KAS III had sequences with homology to *E. coli* KAS III (Tsay et al., 1992). Two nested pairs of degenerate primers were prepared from a six-amino acid stretch of each peptide. Two of the primers, corresponding to peptides QMANVN (upstream) and HQANQQ (downstream) (Fig. 1), generated a 612-bp fragment after two rounds of RNA-PCR, as described in "Materials and Methods." Although other combinations of the degenerate primers failed to produce a visible band on an agarose gel after the second round of the PCR, Southern analysis of the products showed bands of the expected size range when the 612-bp fragment was used as a probe (data not shown). The PCR fragment was subcloned into pBluescript SK(−) (pHTP1) and partially sequenced. The deduced amino acid sequence matched exactly with 11 internal amino acid residues of the available peptide sequence in addition to five of the six amino acids used to design the primer.

Isolation of cDNA Clone

Screening of a spinach root cDNA library in λZAP with the PCR probe resulted in four positive clones. The two longest clones were both 1.9 kb (λHT3 and λHT4), and two others were 1.4 kb (λHT2) and 1.2 kb(λHT1). Nondegenerate primers to the 612-bp PCR probe fragment were used in conjunction with a sequencing primer for pBluescript, present in λZAP cloning site, to confirm the identity and orientation of the clones. Clones λHT3 and λHT4 (1.9 kb) were in the same orientation, and clone λHT2 (1.4 kb) clone was in the opposite orientation. The shortest clone (λHT1) failed to produce a PCR band, and partial sequencing revealed that its sequence was unrelated to other clones. The λHT3 was excised in vivo with a helper phage Ex-Assist (Stratagene) to a pBluescript clone (pBHT3) and sequenced in both orientations. The nucleotide and deduced amino acid sequence of the clone and the sequencing scheme are presented in Figure 1.

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1                                                    CTCCCTGTCTGTAGTTCACCCATCCTCCTCATAATTTCTCCCAATTGAATCCATTCTGTGTCAAAT   66

67   GGGCTCAAAATACTCTCAAAGTTTCATCTTTCACAATCTCTCCAGCTAAAGATCTCTTCTTCTAAATCAACTTTTACTGTTCGTCTTGTTCTTTTGATCA  166

167  ATGGCGACTTCATATGGCTTCTTCTCACCTTCTGTACCTTCTAGTCTCAACAACAAAATTTCGCCATCTTTGGGCATAAATGGATCTGGGTTTTGCTCC  265
1     M  A  T  S  Y  G  F  F  S  P  S  V  P  S  S  L  N  N  K  I  S  P  S  L  G  I  N  G  S  G  F  C  S    33

266  CATTTGGGTATCAGTAAAAGGGTTTTCTGTTCGTCCATTGAAGCTTCTGAGAAACATGCTGCAGCTGGGGTTTCTTCCAGTGAATCGAGAGTTTCTAGG  364
34    H  L  G  I  S  K  R  V  F  C  S  S  I  E  A  S  E  K  H  A  A  A  G  V  S  S  S  E  S  R  V  S  R    66

365  CTAGTAAATAGGGGTTGCAAGCTAGTTGGTTGTGGTTCTGCTGTTCCAAAGCTGCAGATCTCCAATGATGACCTTTCTAAATTTGTTGAAACTTCGGAT  463
67    L  V  N  R  G  C  K  L  V  G  C  G  S  A  V  P  K  L  Q  I  S  N  D  D  L  S  K  F  V  E  T  S  D    99
                                                                                                (1)

464  GAATGGATAGCTACTCGAACTGGGATCCGCCAACGACATGTTCTTTCAGGTAAGGATAGCTTGGTAGACTTGGCTGCCGAAGCAGCAAGGAATGCTCTT  562
100   E  W  I  A  T  R  T  G  I  R  Q  R  H  V  L  S  G  K  D  S  L  V  D  L  A  A  E  A  A  R  N  A  L   132

563  CAGATGGCCAACGTTAATCCTGATGATATTGACCTTATCTTGATGTGTACTTCTACACCAGAGGACCTCTTTGGCAGTGCTCCTCAGGTACAAAGAGCA  661
133   Q  M  A  N  V  N  P  D  D  I  D  L  I  L  M  C  T  S  T  P  E  D  L  F  G  S  A  P  Q  V  Q  R  A   165
                  (2)

662  TTGGGATGCAGCCGAACTCCGTTGTCTTATGATATTACTGCAGCCTGCAGTGGATTTATGCTGGGTTTGGTGTCAGCTGCTTGTCACGTAAGGGGTGGT  760
166   L  G  C  S  R  T  P  L  S  Y  D  I  T  A  A  C  S  G  F  M  L  G  L  V  S  A  A  C  H  V  R  G  G   198
                                    ↑

761  GGATTTAAGAACGTGCTAGTTATAGGTGCCGATGCTCTTTCTCGTTTCGTTGACTGGACTGATAGAGGTACTTGCATACTTTTTGGTGATGCTGCTGGA  859
199   G  F  K  N  V  L  V  I  G  A  D  A  L  S  R  F  V  D  W  T  D  R  G  T  C  I  L  F  G  D  A  A  G   231

860  GCTGTAGTTGTGCAGGCTTGTGACAGTGAAGAAGACGGCATGTTTGCTTTTGACCTGCATAGTGATGGCGGGGGCGGAAGGCATTTGAATGCATCCCTG  958
232   A  V  V  V  Q  A  C  D  S  E  E  D  G  M  F  A  F  D  L  H  S  D  G  G  G  G  R  H  L  N  A  S  L  264

959  TTAAACGATGAGACTGATGCAGCAATAGGCAACAATGGTGCGGTGACAGGTTTCCCCCCGAAGCGGCCATCTTATTCATGTATTAATATGAATGGGAAA 1057
265   L  N  D  E  T  D  A  A  I  G  N  N  G  A  V  T  G  F  P  P  K  R  P  S  Y  S  C  I  N  M  N  G  K   297

1058 GAGGTCTTTCGCTTTGCTGTTAGATGTGTGCCCCAGTCTATTGAGGCTGCACTTCAAAAGGCTGGTCTAACTAGTTCCAACATTGATTGGTTATTATTG 1156
298   E  V  F  R  F  A  V  R  C  V  P  Q  S  I  E  A  A  L  Q  K  A  G  L  T  S  S  N  I  D  W  L  L  L   330
                                                                                        (3)

1157 CATCAGGCAAACCAGAGGATTATTGATGCGGTTGCAACACGTTTGGAAGTTCCTTCAGAAAGGGTATTGTCTAATTTGGCAAATTATGGTAACACCAGT 1255
331   H  Q  A  N  Q  R  I  I  D  A  V  A  T  R  L  E  V  P  S  E  R  V  L  S  N  L  A  N  Y  G  N  T  S   363
                                                                                    (4)

1256 GCTGCATCAATCCCCTTGGCATTGGATGAAGCCGTTCGGAGTGGAAAAGTTAAGCCAGGAAATATAATTGCGACATCCGGATTTGGTGCTGGTCTTACA 1354
364   A  A  S  I  P  L  A  L  D  E  A  V  R  S  G  K  V  K  P  G  N  I  I  A  T  S  G  F  G  A  G  L  T   396

1355 TGGGGTTCGTCCATTATTAGATGGGGATAAACAAAGAAAGACAGATTTATGGACATCATTACAGTGTTGAAGATGAATGGGAAAGGAGGAAGTGTGTTTG 1454
397   W  G  S  S  I  I  R  W  G                                                                          405

1455 TTTAACACTTGCAATCCATTCCATACTCAAGAAGAATTTCACTCCCTTTTGGTTAAGATTAAGATGTTACAAGAGCTGCAACTTCTCGTAAATTCGTACT 1554

1555 GCACAGAAGCTCAATCAGAGAGGCCACTAGTGTTATACCTTTTCCCCTATATCTGCTAAATAAGTCTATTTGTGGTCACCTAGGAAAATAAAAATTTCCC 1654

1655 TTCAATTCCCTGAGTTGTAATTCATTGTTTTTCGCAGTCAGTCAGTTAGCAGAACTAGGAATTTTTTCAGGCAGAGCGATTCAAATAGCTTCTTCAGGCT 1754

1755 TGCTCTTCCTATCTGTTAGACCTTGTATCAGTTATTCAGTTGTATGTTGTACTCTTTCGTTATGCACTTTTCATCTTCATTCAATCATTAATCAGAGTTA 1854

1855 CTTGCTGATTTACTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 1920
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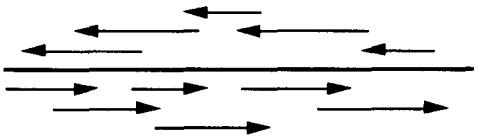

Figure 1. Nucleotide sequence of the spinach KAS III clone. The complete DNA sequence of the cDNA insert in pBHT3 and the deduced amino acid sequence of the ORF are shown. The initiating 5′ ATG is located at nucleotide position 167, and the stop codon is marked at position 1382 (•). Possible polyadenylation signal sequences are marked with an overline in the 3′ untranslated region at nucleotides 1612, 1640, and 1843. Underlined peptide sequences indicate where tryptic digest sequences obtained from purified KAS III match. Four of these [(1)–(4)] had homology to *E. coli* KAS III and were used to design PCR primers. At residue 181, the location of the proposed active-site Cys of the KAS III is also marked by an arrow. A scaled schematic of the size and direction of the fragments of pBHT3 that were sequenced is shown at the bottom.

Characterization of KAS III cDNA Clone

The 1920-bp cDNA clone pBHT3 contained a 1218-bp ORF (nucleotides 167–1384). The deduced amino acid sequence matched the sequences obtained from the 10 tryptic peptides of the purified KAS III (Fig. 1).

The size of the message, estimated from the northern blot (Fig. 2), was in the range of 1.9 to 2.0 kb, suggesting that the clone was full length. The message was expressed in both leaf and root, from which the PCR probe and the clone were obtained, respectively. The large difference in the intensity of the bands from leaf and root suggested that KAS III may be more highly expressed in leaf than in root, but a more detailed analysis is required to confirm this. Southern blot analysis (Fig. 2) produced one major band and one minor band when spinach genomic DNA was hybridized with the 612-bp PCR probe, which was used to screen the cDNA library. Within the region corresponding to the probe there were no restriction sites for the enzymes used for the analysis. Thus, the simplest interpretation is that there is a single copy of KAS III, represented by the major band, and a sequence closely related to that of KAS III, shown as a minor band. Generally, two or more copies of the genes of fatty acid synthase have been found in other plants. For example, there are at least two copies each of the genes for enoyl-ACP reductase (Kater et al., 1991) and 3-ketoacyl-ACP reductase (Klein et al., 1992) and multiple copies of the gene for ACP (Schmid and Ohlrogge, 1990; Hlousek-Radojcic et al., 1992).

The 5′ ATG start codon at position 167 was assigned by the following criteria. The sequence surrounding the ATG at this position conformed to the known consensus sequence (AACAATGGC) for the initiating Met in plants (Lutcke et al., 1987), with the exception of a single T/A substitution (underlined). Furthermore, this ORF would code for a protein of approximately the anticipated size. On the other hand, other potential 5′ ATGs (nucleotides 165 and 566) would have initiated ORFs that code for peptides that are much smaller than the known size of KAS III, and thus they were eliminated from consideration. The deduced amino acid sequence of the 1218-bp ORP corresponds to a protein of 405 amino acids with calculated molecular mass of 42,534 D. In higher plants, all known fatty acid synthesis enzymes are nuclear encoded but found in the plastid. KAS III, therefore, would require a transit peptide. Because the N-terminal sequence of the KAS III was not available, the cleavage site for the transit peptide was not established. However, several lines of evidence suggest that the transit peptide is 43 to 48 residues long. Hydropathy analysis (Kyte and Doolittle, 1982) of the peptide indicated that the hydrophobic character of this region of the protein was lost near residue 43. Acidic residues, rarely found in transit peptides, did not appear until residue 47. Finally, at residue 41 and at 46 were found sequences that approximated a proposed consensus motif for the cleavage site of chloroplast transit peptides, i.e. (I or V)-X-(A or C) ↓ A (Gavel and von Heijne, 1990). The calculated molecular mass of the deduced KAS III sequence after the cleavage of the transit peptide was in the range of 37,568 to 38,056 D. This compared reasonably with the size of the purified enzyme that was estimated to be 40.5 kD on SDS-PAGE (Clough et al., 1992).

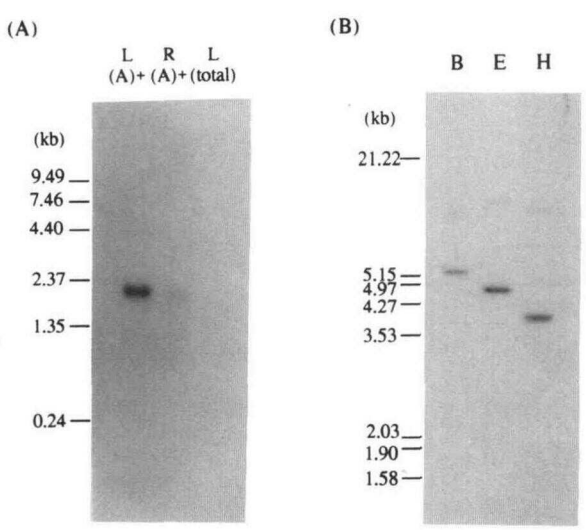

Figure 2. Northern and Southern blot analyses of spinach KAS III. A, For northern blot, 2.6 μg of poly(A)$^+$ RNA of both leaf (L) and root (R) was loaded onto each lane. Also, an equal mass of leaf total RNA (L total) was loaded. Indicated kb corresponds to RNA kb marker (Bethesda Research Laboratories). B, Spinach genomic DNA (15 μg) was digested with *Bam*HI (lane B), *Eco*RI (lane E), and *Hin*dIII (lane H) and separated on 0.7% agarose gel. Indicated kb corresponds to DNA kb markers (λ*Hin*dIII/*Eco*RI digest).

A probable polyadenylation signal was also identified for the KAS III clone (Fig. 1). A consensus polyadenylation signal ([A or U]AAUAA) had been identified in some plants, but it is not well conserved and is sometimes even absent, in contrast to its high conservation in animals (Heidecker and Messing, 1986). Although the consensus sequence of polyadenylation was observed in two regions (nucleotides 1,612 and 1,640) of the 3′ untranslated region of the KAS III clone, their positions relative to the poly(A)$^+$ tail appeared to be too remote. Polyadenylation signals typically precede the end of message by 15 to 30 nucleotides (Heidecker and Messing, 1986; Grierson and Covey, 1988). However, at nucleotide 1843, 27 bases upstream from the start of the poly(A)$^+$ tail, a sequence (UAAUCA) close to the proposed consensus sequence was observed, which is the probable polyadenylation signal.

The identity of the spinach KAS III clone was confirmed by inserting a PCR fragment generated from the clone, corresponding in size to the coding region of *E. coli* KAS III, into the expression vector pGEX-KG. This plasmid was then expressed in *E. coli* BL 21, producing a KAS III-GST fusion protein. Addition of IPTG resulted in a nearly 16-fold increase in KAS III activity in cell-free extracts when compared to extracts from uninduced cells (Table I). The KAS III activity was purified to near homogeneity from the cell extract by GSH-agarose affinity matrix. The induced activity remained high in the purified fraction both before and after thrombin cleavage of KAS III from the fusion protein (Table I). Moreover, no KAS III activity from the uninduced cells bound to the GSH-agarose affinity matrix.

Sequence Comparison

The amino acid sequence of spinach KAS III was compared to all known sequences available in data bases. A search of

Table I. *Expression of spinach KAS III in E. coli*

Induction and purification of the fusion protein are described in "Materials and Methods." The KAS III activity assay was performed as described by Clough et al. (1992) using cell homogenate of cell number equivalent to 10^4 cells. ND, Not detectable.

	KAS III Activity	
	0.1 mM IPTG	Minus IPTG
	pmol min^{-1}	
Cell-free homogenate	41.5	2.6
Unadsorbed to GSH-agarose	7.8	0.4
GSH-agarose purified	8.4	ND
GSH-agarose purified and cleaved with thrombin	10.4	ND

nonredundant peptide sequence data bases using BLAST revealed significant similarities to the putative KAS III of *Porphyra umbilicalis* (red alga) (Reith, 1993) and KAS III of *E. coli*, encoded by *fabH* (Tsay et al., 1992). The extent of homology and alignment of the spinach KAS III sequence to these other KAS III sequences was carried out using the GAP program of the Genetics Computer Group. This program uses the algorithm of Needleman and Wunsch (1970) to align two complete sequences with maximum matches and minimum gaps. This analysis indicated that the spinach KAS III had 69% similarity and 48% identity with KAS III of *P. umbilicalis* and 64% similarity and 45% identity with *E. coli* KAS III. Alignment of these sequences is shown in Figure 3. Other peptides with a high degree of homology included an ORF located upstream of *Rhodobacter capsulatus himA* and a partial ORF located upstream of *phnA* from *Pseudomonas aeruginosa*, which may actually be KAS III sequences as suggested earlier (Reith, 1993). The BLAST search did not produce a match of KAS III with any sequences of other KAS isoforms, such as KAS I from barley or *E. coli*.

It is interesting that numerous sequences detected in the search were chalcone synthases, plant-specific condensing enzymes that catalyze the synthesis of naringenin chalcone from 4-coumaroyl-CoA and malonyl-CoA. GAP analysis indicated that the percentages of similarity and identity of spinach KAS III with barley KAS I (48 and 21%, respectively) and chalcone synthases (46 and 20%, respectively) were essentially the same. However, there were local homologies between KAS IIIs and chalcone synthases that were more significant than with barley KAS I and resulted in the matches to chalcone synthase in the BLAST search. For example, residues 297 to 350 had a high degree of similarity (51%), and residues 356 to 379 had nearly 50% identity with all chalcone synthases (Fig. 3), whereas no similar regions were observed between KAS IIIs and KAS Is.

All previously studied condensing enzymes have an active-site Cys residue, to which the growing fatty acid chain is bound as a thioester during fatty acid synthesis. Only one (at position 181) of the 12 Cys's in spinach KAS III and its surrounding region shows high similarity to the proposed active sites of KAS III (Tsay et al., 1992) and other condensing enzymes (Fig. 4) (Revill and Leadlay, 1991). There is a nine-residue region that is highly conserved for the KAS III but is only partially conserved among other KASs. The active-site Cys's of fatty acid synthase-condensing enzymes are surrounded predominantly by Ser/Thr and small neutral amino acids (Gly or Ala) (Lanz et al., 1991), such as those found near Cys[181] of the spinach KAS III sequence (Fig. 4). A notable

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            1                                                  51                                                 100
Spinach     MATSYGFFSP SVPSSLNNKI SPSLGINGSG FCSHLGISKR VFCSSIEASE KHAAAGVSSS ESRVSRLVNR GCKLVGCGSA VPKLQISNDD LSKFVETSDE
P. umbil                                                                                 M .VHILST..S ..NFSVE.QQ FEDII....H
E. coli                                                                                  M YT.II.T..Y L.EQVRT.A. .E.M.D....

            101                                                150                                                199
Spinach     WIATRTGIRQ RHVLSGKDSL VDLAAEAARN ALQMANVNPD DIDLILMCTS TPEDLFGSAP -QVQRALGCS RTPLSYDITA ACSGFMLGLV SAACHVRGGG
P. umbil    ..S.....KK SILPLLLP.. TK......ND ..SK.SI.AE .....ILA.. ..D......S -.L.AEI.AT SST-AF.... .....IIA.. T.SQFIQA.S
E. coli     ..V......E ..IAAPNETV STMGF...TR .IE..GIEK. Q.G..VVA.T SATHAFP..A C.I.SM..IK GC.-AF.VA. ..A..TYA.S V.DQYVKS.A

            200                                                250                                                299
Spinach     FKNVLVIGAD ALSRFVDWTD RGTCILFGDA AGAVVVQACD SEEDGMFAFD LHSDGGGGRH LNASLLNDET DAAIGNNGAV TGFPPKRPSY SCINMNGKEV
P. umbil    YNK...V... TM..WI..S. .SS......G ....LI--GE .SINSILG.K .CT..RLNS. .Q--.M.SPS .---SQQFGL .TV.KG.--. DS.R......
E. coli     V.YA..V.S. V.A.TC.P.. ...I.I...G ...A.LA.-- ...P.IISTH ..A..SY.EL .T---.PNAD RVNPE.SIHL .--------- ----.A.N..
CHS                                                                                                                .D.

            300                                                350                                                399
Spinach     FRFAVRCVPQ SIEAALQKAG LTSSNIDWLL LHQANQRIID AVATRLEVPS ERVLSNLANY GNTSAASIPL ALDEAVRSGK VKPGNIIATS GFGAGLTWGS
P. umbil    YK...FQ..I V.KNC.NDVN ISIDEV..FI .....I..LE .I....SI.L SKMIT..E.. .......... .....IKEK. IQ..QVVVLA .........A
E. coli     .KV..TELAH IVDET.AANN .DR.QL...V P....L...S .T.KK.GMSM DN.VVT.DRH .......V.C .......D.R I...QLVLLE A..G.F....
CHS         PGLISKNIEK .LDE.FKPL. ISDW.SLFWI A.PGGPA.L. D.EKK.GLKA .KM   .SE. ..M...CVLF I...MR.KS.

            400
Spinach     SIIRWG*
P. umbil    IVLK.Q*
E. coli     ALV.F-
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Figure 3. Alignment of the deduced amino acid sequences of spinach KAS III clone (Spinach), *P. umbilicalis* putative KAS III (*P. umbil*) (Reith, 1993), and *E. coli* KAS III (*E. coli*) (Tsay et al., 1992). A portion of the chalcone synthase 3 sequence from *Sinapsis alba* (CHS) (Lanz et al., 1991) was also aligned where high homology was found. Identical residues are indicated by dots and gaps are shown as dashes.

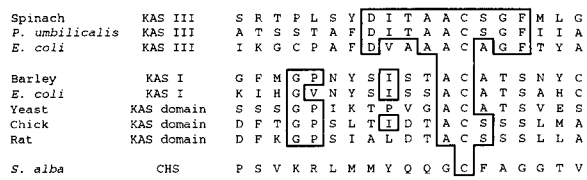

Figure 4. Sequence comparison of proposed active sites of KAS IIIs with KAS Is and other related enzymes. Residues conserved with spinach KAS III near the active-site Cys's are boxed. In addition, a consensus sequence at eight and nine residues upstream of the active Cys observed in KASs other than KAS III is boxed.

difference between the active-site region of KAS IIIs and those of other KASs is a Gly-Pro domain eight residues upstream from the Cys that is highly conserved in other KASs (Revill and Leadlay, 1991) but missing from the KAS IIIs.

A biochemical property distinguishing KAS III from other KAS enzymes is the complete absence of inhibition by the antibiotic cerulenin. It has been established that the mechanism of cerulenin inhibition of other KAS enzymes involves alkylation of the active-site Cys (Kauppinen et al., 1988). Comparison of these active-site sequences might reveal residues, e.g. the Gly-Pro sequence mentioned above, that are related to cerulenin sensitivity. It should be noted, however, that chalcone synthase is also inhibited by cerulenin, presumably also by alkylation of the active-site Cys (Lanz et al., 1991). The active-site Cys of chalcone synthase has been identified by site-directed mutagenesis (Lanz et al., 1991), and the sequence surrounding the active site has no similarity to the active sites of the condensing enzymes in fatty acid biosynthesis (Fig. 4). Thus, if the lack of homology at the active-site region of chalcone synthase with all other fatty acid synthase-condensing enzymes is considered, it is clear that cerulenin sensitivity is not conferred as a result of the primary sequence in this region.

DISCUSSION

We have cloned and sequenced the first KAS III from a higher plant. Its sequence shares regions of conservation with that of the KAS IIIs from such diverse organisms as the bacterium *E. coli* (Tsay et al., 1992) and the red alga *P. umbilicalis*, in which KAS III is chloroplast encoded (Reith, 1993). In contrast, KAS III sequences are unlike those of any of the other fatty acid synthesis-condensing enzymes from bacteria, plants, or animals. This observation is somewhat surprising, because the reactions of fatty acid synthesis are essentially the same in all organisms. Moreover, there is clear homology between the KAS regions of the multifunctional fatty acid synthases found in animals and yeast and the KAS I of fatty acid synthases found in plants and bacteria (Revill and Leadlay, 1991). Thus, it appears likely that all of these fatty acid synthases evolved from a common origin. However, the KAS III reaction is different from those catalyzed by the other condensing enzymes of bacteria and plants, i.e. it is specific for acetyl-CoA rather than for acyl-ACP (Jackowski and Rock, 1987a; Clough et al., 1992). Furthermore, it has been suggested that the acetyl transacylase reaction may actually be catalyzed in vivo by KAS III and that there may not be a separate acetyl transacylase enzyme (Jackowski and Rock, 1987a; Clough et al., 1992). We attempted to align the KAS III sequence to the acetyl transacylase domain of the β subunit of the yeast fatty acid synthase (Chirala et al., 1987) and found that there was essentially no homology. Thus, these observations may suggest that KAS III is an additional enzyme among the dissociated fatty acid synthases, having no analogy in the multifunctional fatty acid synthases.

The mechanism of regulation of fatty acid biosynthesis in plants has not been established. Because KAS III catalyzes the initial condensation reaction, which, along with the reaction catalyzed by acetyl-CoA carboxylase is the initial reaction of fatty acid synthesis, it is a reasonable candidate to serve a regulatory function. Analysis of the in vivo levels of acyl-ACPs in plants and bacteria suggest that, in addition to acetyl-CoA carboxylase, KAS III may play a regulatory role (Jackowski and Rock, 1987a; Post-Beittenmiller et al., 1991, 1993; Clough et al., 1992). A question that can be addressed further with the availability of a KAS III clone from higher plants is its metabolic role. Altering the levels of expression of this enzyme will help to define more clearly the influence of KAS III on regulation of fatty acid biosynthesis in plants.

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LITERATURE CITED

Chirala SS, Kuziora MA, Spector DM, Wakil SJ (1987) Complementation of mutations and nucleotide sequence of *FAS1* gene encoding β subunit of yeast fatty acid synthase. J Biol Chem **262:** 4231–4240

Clough RC, Matthis AL, Barnum SR, Jaworski JG (1992) Purification and characterization of 3-ketoacyl-acyl carrier protein synthase III from spinach. J Biol Chem **267:** 20992–20998

Gavel Y, von Heijne G (1990) A conserved cleavage-site motif in chloroplast transit peptides. FEBS Lett **261:** 455–458

Grierson D, Covey SN (1988) Structure and expression of nuclear gene. *In* D Grierson, SN Covey, eds, Plant Molecular Biology. Blackie, New York, pp 22–46

Guan K, Dixon, JE (1991) Eukaryotic proteins expressed in *Escherichia coli:* an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. Anal Biochem **192:** 262–267

Heidecker G, Messing J (1986) Structural analysis of plant genes. *In* WR Briggs, RL Jones, eds, Annual Review of Plant Physiology, Vol 37. Annual Reviews Inc, Palo Alto, CA, pp 439–466

Hlousek-Radojcic A, Post-Beittenmiller D, Ohlrogge JB (1992) Expression of constitutive and tissue-specific acyl carrier protein isoforms in *Arabidopsis.* Plant Physiol **98:** 206–214

Jackowski S, Murphy CM, Cronan JE, Rock CO (1989) Acetoacetyl-acyl carrier protein synthase. A target for the antibiotic thiolactomycin. J Biol Chem **264:** 7624–7629

Jackowski S, Rock CO (1987a) Acetoacetyl-acyl carrier protein synthase, a potential regulator of fatty acid biosynthesis in bacteria. J Biol Chem **262:** 7927–7931

Jackowski S, Rock CO (1987b) Altered molecular form of acyl carrier protein associated with β-ketoacyl-acyl carrier protein synthase II (*fabF*) mutants. J Bacteriol **169:** 1469–1473

Jacobson A (1987) Purification and fractionation of poly(A)+ RNA. Methods Enzymol **152:** 254–261

Jaworski JG, Clough RC, Barnum SR (1989) A cerulenin insensitive short chain 3-ketoacyl-acyl carrier protein synthase in *Spinacia oleracea* leaves. Plant Physiol **90:** 41–44

Jaworski JG, Post-Beittenmiller D, Ohlrogge JB (1993) Acetyl-acyl carrier protein is not a major intermediate in fatty acid biosynthesis in spinach. Eur J Biochem **213:** 981–987

Kater MM, Konigstein GM, Nijkamp HJ, Stuitje AR (1991) cDNA cloning and expression of *Brassica napus* enoyl-acyl carrier protein reductase in *Escherichia coli*. Plant Mol Biol **17:** 895–909

Kauppinen S, Siggard-Anderson M, von Wettstein-Knowles P (1988) β-Ketoacyl-ACP synthase I of *Escherichia coli*: nucleotide sequence of the *fabB* gene and identification of the cerulenin binding residue. Carlsberg Res Commun **53:** 353–370

Klein B, Pawlowski K, Höricke-Grandpierre C, Schell J, Töpfer R (1992) Isolation and characterization of a cDNA from *Cuphea lanceolata* encoding a β-ketoacyl-ACP reductase. Mol Gen Genet **233:** 122–128

Kyte J, Doolittle RF (1982) A method for displaying the hydropathic character of a protein. J Mol Biol **157:** 105–132

Lanz T, Tropf S, Marner F-J, Schroder J, Schroder G (1991) The role of cysteines in polyketide synthase. J Biol Chem **266:** 9971–9976

Logemann J, Schell J, Willmitzer L (1987) Improved method for the isolation of RNA from plant tissues. Anal Biochem **163:** 16–20

Lutcke HA, Chow KC, Mickel FS, Moss KA, Kern HF, Scheele GA (1987) Selection of AUG initiation codons differs in plants and animals. EMBO J **6:** 43–48

Marchuk D, Drumm M, Saulino A, Collins FS (1991) Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. Nucleic Acids Res **19:** 1154

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res **8:** 4321–4325

Needleman SB, Wunsch CD (1970) A general method applicable to the search for similarities in the amino acid sequence of two proteins. J Mol Biol **48:** 443–453

Post-Beittenmiller D, Jaworski JG, Ohlrogge JB (1991) *In vivo* pools of free and acylated acyl carrier proteins in spinach—evidence for sites of regulation of fatty acid biosynthesis. J Biol Chem **266:** 1858–1865

Post-Beittenmiller D, Jaworski JG, Ohlrogge JB (1993) Regulation of lipid synthesis in castor seeds: analysis of the in vivo acyl-ACP pools. *In* SL MacKenzie, DC Taylor, eds, Seed Oils for the Future. American Oil Chemist Society, Champaign, IL, pp 44–51

Reith M (1993) A β-ketoacyl-acyl carrier protein synthase III gene (*fabH*) is encoded on the chloroplast genome of the red alga *Porphyra umbilicalis*. Plant Mol Biol **21:** 185–189

Revill WP, Leadlay PF (1991) Cloning, characterization, and high-level expression in *Escherichia coli* of the *Saccharopolyspora erythraea* gene encoding an acyl carrier protein potentially involved in fatty acid biosynthesis. J Bacteriol **173:** 4379–4385

Sambrook J, Fritsh EF, Maniatis T (1989) Molecular Cloning, A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Schmid KM, Ohlrogge JB (1990) A root acyl carrier protein-II from spinach is also expressed in leaves and seeds. Plant Mol Biol **15:** 765–778

Shimakata T, Stumpf PK (1982) Isolation and function of spinach leaf β-ketoacyl-[acyl-carrier-protein] synthases. Proc Nat Acad Sci USA **79:** 5808–5812

Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW (1990) Use of T7 RNA polymerase to direct expression of cloned gene. Methods Enzymol **185:** 60–89

Tsay J-T, Oh W, Larson TJ, Jackowski S, Rock CO (1992) Isolation and characterization of the β-ketoacyl-acyl carrier protein synthase III gene (*fabH*) from *Escherichia coli* K-12. J Biol Chem **267:** 6807–6814