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Modification of *Brassica napus* seed oil by expression of the *Escherichia coli fabH* gene, encoding 3-ketoacyl-acyl carrier protein synthase III

Ira I. G. S. Verwoert¹, Karin H. van der Linden¹, Michael C. Walsh², H. John J. Nijkamp¹ and Antoine R. Stuitje^{1,*}

¹Department of Genetics, Institute for Molecular Biological Sciences, BioCentrum Amsterdam, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands (* author for correspondence);

²E. C. Slater Institute, BioCentrum Amsterdam, University of Amsterdam, 1018 TV Amsterdam, The Netherlands

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Abstract

The *Escherichia coli fabH* gene encoding 3-ketoacyl-acyl carrier protein synthase III (KAS III) was isolated and the effect of overproduction of bacterial KAS III was compared in both *E. coli* and *Brassica napus*. The change in fatty acid profile of *E. coli* was essentially the same as that reported by Tsay *et al.* (J Biol Chem 267 (1992) 6807–6814), namely higher C14:0 and lower C18:1 levels. In our study, however, an arrest of cell growth was also observed. This and other evidence suggests that in *E. coli* the accumulation of C14:0 may not be a direct effect of the KAS III overexpression, but a general metabolic consequence of the arrest of cell division. Bacterial KAS III was expressed in a seed- and developmentally specific manner in *B. napus* in either cytoplasm or plastid. Significant increases in KAS III activities were observed in both these transformation groups, up to 3.7 times the endogenous KAS III activity in mature seeds. Only the expression of the plastid-targeted KAS III gene, however, affected the fatty acid profile of the storage lipids, such that decreased amounts of C18:1 and increased amounts of C18:2 and C18:3 were observed as compared to control plants. Such changes in fatty acid composition reflect changes in the regulation and control of fatty acid biosynthesis. We propose that fatty acid biosynthesis is not controlled by one rate-limiting enzyme, such as acetyl-CoA carboxylase, but rather is shared by a number of component enzymes of the fatty acid biosynthetic machinery.

Introduction

De novo synthesis of fatty acids occurs through the combined action of the acetyl-CoA carboxylase and the fatty acid synthetase. The fatty acid synthetases (FAS) of bacteria and plants, are classified as type II synthases, in which each individual reaction is carried out by a discrete and dissociable enzyme. This is in contrast to the

type I synthases found in yeasts and vertebrates in which the components are combined within one or two high-molecular-weight multi-functional polypeptides [1]. 3-ketoacyl-ACP synthase (KAS) is the enzyme that catalyses the initial reaction of each cycle of fatty acid synthesis, the condensation between malonyl-ACP and the growing acyl-ACP chain. Shimakata and Stumpf [26] were the first to resolve two KAS isoforms

in spinach leaf extracts. KAS I catalyses the condensations, using acyl-ACPs ranging from C2 to C14 as substrate, whereas KAS II is specific for long-chain acyl-ACPs (C14 and C16). The first KAS enzyme to be purified to homogeneity from a plant source was KAS I from *Brassica napus* [17]. cDNAs of KAS I have been isolated from barley leaf and castor seed [29, 6].

For a long time, the primer of fatty acid synthesis was thought to be acetyl-ACP. Acetyl-ACP is produced by the transfer of an acetyl moiety from CoA to ACP. This reaction is catalysed by acetyl-CoA:ACP transacylase (ACAT). *In vitro* data, of the specific activities of a number of enzymes involved in plant lipid biosynthesis, suggested that this reaction was potentially a rate-limiting step in fatty acid synthesis [30]. Recently, however, a third condensing enzyme (KAS III) was identified in *E. coli* [9], which is specific for short-chain ACPs (C2-C4 substrates), and prefers acetyl-CoA instead of acetyl-ACP as the primer unit. The presence of this short-chain condensing activity has now been demonstrated in a wide variety of plant species and tissues [11, 34, 23]. This condensation reaction is five-fold faster than the ACAT reaction in spinach leaf extracts [11]. A more recent study [12] showed that acetyl-ACP is actually the least effective primer of fatty acid synthesis in spinach extracts when compared to acetyl-CoA, butyryl-ACP or hexanoyl-ACP. In addition to the condensing activity, KAS III also possesses, at least *in vitro*, ACAT activity [4, 31]. This could indicate that ACAT is merely a partial reaction of KAS III. These data suggest that KAS III is the enzyme that initiates the synthesis of acyl chains and that it may play an important role in controlling the rate of fatty acid synthesis in plants and bacteria. With the discovery of KAS III there does not seem to be an absolute requirement for the existence of ACAT. However, direct evidence for the presence of acetyl-ACP in spinach leaf has recently been obtained [20], and a separate enzyme with ACAT activity has been purified from *E. coli* [16]. A recent paper by Gulliver and Slabas [7], however, suggests that the enzyme purified from *E. coli* by Lowe and Rhodes [16] was actually a

purification of the *E. coli* KAS III. The paper of Gulliver and Slabas [7] also demonstrated the presence of a separable KAS III and ACAT activity in avocado mesocarp tissue. Additional experiments will be required to determine the role of ACAT in fatty acid synthesis.

A synthase III mutant was used to isolate the KAS III gene from *E. coli* [31]. Overproduction of KAS III in *E. coli* resulted in a decrease in the amount of *cis*-vaccenate, and an accumulation of shorter-chain fatty acids (mainly myristate) in the membrane lipids [31]. These results are consistent with the proposed role of KAS III in the initiation of fatty acid synthesis.

Recently, we constructed a seed-specific promoter-terminator cassette, consisting of a rapeseed napin gene, and the 3' terminator region of the petunia chalcone synthase gene (*chs*) to study the effects of overproduction of a bacterial FAS component in the seeds of transgenic plants [33]. It was shown that the *E. coli fabD* gene, encoding malonyl-CoA:ACP transacylase (MCAT), can be expressed to a high level (up to 55 times the maximum endogenous MCAT activity) in a seed- and developmental-specific manner. Plastid targeting of the bacterial FAS component was successfully accomplished by the use of the *Brassica napus* enoyl-ACP reductase leader-sequence. In the present study, the same promoter-terminator cassette and leader sequence were used for the transgenic expression of the *E. coli* KAS III gene (*fabH*) in rapeseed. The potentials of this 'heterologous gene approach' are discussed not only in terms of modification of fatty acid biosynthesis, but also in terms of identification of the regulatory and controlling elements of fatty acid biosynthesis.

Materials and methods

Materials

Brassica napus cv. Westar was greenhouse-grown. Acyl carrier protein (ACP) was prepared from *E. coli* by the procedure of Rock and Cronan [24]. Restriction enzymes, isopropyl- β -thiogalactopyranoside (IPTG), NADH and NADPH

were purchased from Boehringer Mannheim. [^{14}C]-acetyl CoA (specific activity 56 mCi/mmol) was obtained from Amersham International. Malonyl-CoA was from Sigma. Nitroblue tetrazolium (NBT) substrate, 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) substrate and mouse anti-IgG alkaline phosphatase conjugate for immunodetection were purchased from Promega. Cerulenin was from Serva.

Bacterial strains, plasmids and growth media

The bacterial strain JM101 and cloning vectors pUC19 and pBS-KS (Stratagene) were used for standard molecular cloning techniques. The binary vector pMOG402 was provided by MOGEN International [33]. Routine growth conditions were as described in Verwoert *et al.* [32].

DNA manipulations

Plasmid DNA isolation, standard molecular cloning, transformation and electrophoresis procedures were performed as described by Sambrook *et al.* [25].

Cloning of the E. coli fabH gene

In order to clone the *E. coli fabH* gene encoding 3-ketoacyl-ACP synthase III (KAS III), a PCR was performed on chromosomal DNA of *E. coli* strain JM101 to introduce the appropriate restriction sites. The following primers were used: (1) 5'-GAATTGCCGCTCGTCTAGAATCTG-3' which introduces a *Xba* I site 130 bp upstream of the starting ATG of the coding region [31]; (2) 5'-GCGGATCCGAGAACCCTGTCCAGGG-3', which introduces a *Bam* HI site at the 3' end, beyond the stop codon, 40 bp into the coding region of the *E. coli fabD* gene [32]. The following thermal cycle was used: denaturation at 94 °C for 30 s; annealing at 52 °C for 60 s; and extension at 72 °C for 90 s, repeated 30 times. The PCR

product was cloned in vector pBS-KS as a *Xba* I-*Bam* HI fragment, resulting in plasmid pIV90.

E. coli expression studies and production of antibodies

E. coli vector pUC90, containing the *E. coli fabH*-coding region, was derived from pUC50, in which the *E. coli fabD* gene was cloned as a *Xba* I-*Eco* RI PCR fragment in pUC19, as described in Verwoert *et al.* [32] by using the primers 5'-CGTCTAGAATAAGGATTAACCATG-GCGC-3' and 5'-CGGCGAATTCCG-AAGCTTGC-3' respectively. Vector pUC50 thus provides an expression cassette for any ORF with a *Nco* I compatible restriction site at the initiation codon and appropriate restriction sites downstream of the ORF. In pUC90 the *fabD* gene of pUC50 was thus replaced by a 1 kb *Afl* III-*Eco* RI fragment of pIV90, containing the *fabH*-coding region. In pUC90, the expression of the *fabH* gene is thus placed under transcriptional control of the *lacZ* promoter of the cloning vector pUC19, the translation initiation, however, is controlled by the intergenic region present between the *fabH* and *fabD* genes. *E. coli* strain JM101 was transformed with both the ampicillin-resistant vectors pUC19 and pUC90. The plasmid-encoded proteins were specifically expressed as described earlier [32]. The KAS III protein was purified from *E. coli* JM101 harbouring pUC90, by a 55–80% ammonium sulfate precipitation, desalting over a PD-10 Sephadex G-25M column (Pharmacia), and Blue Sepharose CL-6B chromatography, essentially as described by Tsay *et al.* [31]. The enzyme-containing fractions were desalted and concentrated using a Centricon-10 microconcentrator (Amicon), run on a 12% SDS-polyacrylamide gel and electrotransferred from the gel onto PVDF membrane (Millipore). The part of the blot containing the 33 kDa polypeptide was directly used for N-terminal amino acid sequence analysis, and immunization of a Swiss female mouse. SDS-PAGE immunoblotting, N-terminal amino acid sequence analysis

and the production of anti-*E. coli* KAS III antibodies were performed as described in Verwoert *et al.* [32].

Quantitative laser densitometric scanning

Laser densitometric scanning of Coomassie-stained polyacrylamide gels and western blots stained with the alkaline phosphatase method was performed using the Cybertech Imaging System (Cybertech GmbH).

Enzyme and protein assays

Crude *E. coli* protein extracts were prepared as described in Verwoert *et al.* [32], plant protein extracts as in Verwoert *et al.* [33]. Protein was routinely assayed as described by Bradford [3]. The standard assay mixture (50 μ l) for acetoacetyl-ACP synthase III (KAS III) activity contained 100 mM sodium phosphate (pH 7.0), 1 mM dithiotreitol (DTT), 1 mM malonyl-CoA, 65 μ M ACP, 1.5 mM acetyl-CoA (of which 150 μ M [$1\text{-}^{14}\text{C}$] acetyl-CoA), 100 μ M cerulenin and 10 μ l crude protein extract. Cerulenin was maintained as a 1 mM stock solution in methanol and stored at -20°C . The sodium phosphate, DTT and ACP were incubated for 30 min at 37°C prior to the addition of the other components of the assay mixture. The protein extract and cerulenin were preincubated at room temperature for 10 min, and added last to the assay mixture to initiate the reaction. Termination of the reaction, after a 5 min incubation at 25°C , and liquid scintillation counting were performed as described earlier [32]. Acetyl-CoA:ACP transacylase activity, which accounted for less than 1.5% of the KAS III activity, was not subtracted.

Data analysis

Kinetic parameters were determined by using Eadie-Hofstee plots, and all data were analysed

by computer-assisted non-linear regression by using Enzfitter software. Standard errors were estimated by Enzfitter.

Construction of the chimaeric napin-KAS III genes

The *Afl* III-*Bam* HI fragment of pIV90 containing the *E. coli fabH* gene was inserted into the seed-specific promoter-terminator cassette pAR4 [33], resulting in plasmid pAR90. The promoter-terminator cassette is obtained from a rapeseed napin gene and the 3' terminator region of the petunia chalcone synthase gene [33]. In order to allow for plastid targeting of the *E. coli fabH* gene product in transgenic plants, plasmid pAR93 was constructed. To create pAR93, the 230 bp leader sequence of the *Brassica napus* enoyl-ACP reductase [13] was inserted between the napin promoter and the *fabH*-coding region, essentially as described for the chimaeric napin MCAT constructs by Verwoert *et al.* [33]. The chimaeric gene constructs present in plasmids pAR90 and pAR93 were digested with *Xba* I and *Sal* I, and ligated to the binary vector pMOG402 digested with *Xba* I and *Xho* I resulting in plasmids pBAR90 and pBAR93.

Transformation and growth of plants

The binary vectors, pBAR90 and pBAR93, were introduced into *Agrobacterium tumefaciens* strain C58C1Rif (pMP90), and the *Agrobacterium* strain was subsequently used to transform 7-day old *Brassica napus* cotyledons [33]. Primary transformants were grown in the greenhouse, self-fertilized and seeds of the different developmental stages were collected, as described in Verwoert *et al.* [33].

Northern blotting, fatty acid analysis and total lipid extraction

Northern blotting, fatty acid analysis and total lipid extraction were performed as described earlier [33].

Results

Expression studies in Escherichia coli

Recently, a conditional *E. coli* mutant (*fabD*) with a temperature-sensitive malonyl-CoA:ACP transacylase (MCAT), was used to isolate the wild-type *E. coli* MCAT gene by complementation cloning [32]. Sequence analysis followed by a protein data base search suggested that *fabD* is part of an operon encoding several other components of the *E. coli* FAS II system, including a ketoacyl-ACP reductase and a ketoacyl-ACP synthase [32]. Tsay *et al.* [31] demonstrated that this ketoacyl-ACP synthase gene (*fabH*), immediately upstream of *fabD*, is the ketoacyl-ACP synthase III (KAS III). As described in detail in Material and methods, a PCR was performed on chromosomal DNA of *E. coli* strain JM101 introducing *Xba* I and *Bam* HI restriction sites, and the PCR product was cloned in vector pBSKS. An *Afl* III-*Eco* RI fragment, containing the *E. coli* KAS III coding region, was subsequently cloned in vector pUC50, resulting in plasmid pUC90, to accomplish overexpression of KAS III (see Materials and methods). JM101 cells harbouring pUC90 over-express KAS III after IPTG induction (Fig. 1A, lane 1 and 2). The specific activity of KAS III was measured in cell extracts of strains JM101, and JM101 harbouring pUC90. The latter possessed significantly higher KAS III activity (5 $\mu\text{mol}/\text{min}$ per mg protein) compared to JM101 (9.2 nmol/min per mg protein), demonstrating that pUC90 expressed the *fabH* gene. By measuring the optical density of the Coomassie-stained protein bands (Fig. 1A, lane 2) with a densitometer (see Materials and methods), it was estimated that cells expressing the *E. coli fabH* gene accumulate KAS III to ca. 0.86% of their total soluble protein. From these data it can be calculated that, as a comparison, the endogenous KAS III comprises ca. 0.002% of the total soluble protein fraction of *E. coli*.

Overexpression of the *E. coli* KAS III had a significant impact on cell growth and overall membrane fatty acid composition. On solid medium in the presence of IPTG, growth of JM101

Fig. 1. A. Expression and purification of the *fabH* gene product. Growth conditions, methods for the purification of KAS III, and extract preparation are described in Materials and methods. SDS-PAGE of ca. 10 μg of crude protein extract of *E. coli* JM101 harbouring pUC90 without (lane 1) and after IPTG induction (lane 2), and ca. 0.15 μg purified KAS III protein eluted from Blue Sepharose (lane 3). B. Quantitative western blot of purified *E. coli* KAS III protein. Quantified amounts (10, 5, 2.5 and 1 ng) of purified KAS III protein were run on a 12% SDS-PAGE, electrotransferred to nitrocellulose, and detected using mouse antibodies raised against *E. coli* KAS III and goat anti-mouse IgG-conjugated alkaline phosphatase as described in Materials and methods.

harbouring pUC90 was severely inhibited, resulting in a decreased colony size, and altered morphology, compared to control JM101. After IPTG induction of *fabH* expression in exponentially growing cells, in liquid medium, growth stopped after approximately one generation. Elevated levels of KAS III resulted in an increase in amount of C14:0 (from 6 to 14% of the total fatty acids), whereas the C18:1 content decreased markedly (from 20 to 5%) (data not shown). A similar result has been reported by Tsay *et al.* [31].

E. coli KAS III antibodies

The *E. coli* KAS III protein was partially purified from strain JM101/pUC90, essentially as described by Tsay *et al.* [31] (see Materials and methods for details). The KSCN eluate from a Blue Sepharose column contained predominantly

a single protein with a molecular mass around 32 kDa (Fig. 1A, lane 3). Using quantitative laser densitometry, it was estimated that the predominant protein made up for 96% of the total soluble protein in the purified fraction. N-terminal sequencing of this protein revealed the sequence MYTKIIG, confirming that it represented KAS III [31].

Subsequently antibodies were raised against *E. coli* KAS III, as described in Materials and methods. Quantified amounts of the purified *E. coli* KAS III were subjected to SDS-PAGE, and transferred to nitrocellulose. Immunodetection was performed with the mouse anti-*E. coli* KAS III antibodies. Figure 1B shows, that the *E. coli* KAS III antibodies can detect amounts as small as 1 ng of bacterial protein.

Transformation of B. napus with the chimaeric fabH constructs

As overexpression of the *E. coli* KAS III gene had a significant effect on the fatty acid profile of bacterial membrane lipids [31], the consequences of expressing this bacterial FAS component in transgenic rape seeds were investigated. A 1 kb *Afl* III-*Bam* HI fragment, derived from pIV90, containing the *E. coli* KAS III-coding region was inserted into the seed-specific promoter-terminator cassette pAR4 [33], consisting of a napin promoter and the petunia chalcone synthase 3' terminator region. For plastid targeting, the *B. napus* enoyl-ACP reductase transit peptide [13] was linked to the bacterial gene (see Materials and methods for details).

The chimaeric KAS III genes, with or without the transit peptide (pAR93 and pAR90, respectively), were inserted into the binary vector pMOG402. *Agrobacterium*-mediated transformation of *B. napus* cv. Westar cotyledons [18] resulted in 15 transgenic BN90 plants, derived from the construct encoding the non plastid-targeted protein, and 23 transgenic BN93 plants, derived from the construct encoding the plastid-targeted protein. Transformation frequencies, based on the percentage of explant material producing kana-

mycin-resistant shoots, were ca. 10% for both transformation groups. PCR and Southern blot analysis of total leaf DNA demonstrated the presence of the chimaeric napin-KAS III gene in 87% of the kanamycin-resistant transgenic plants.

Expression of the E. coli KAS III transgene during seed development

Transgenic seeds were harvested at various developmental stages and analysed for expression of the bacterial KAS III gene using western immuno-blotting. Crude rape seed protein extracts, equivalent to 0.2 seed per developmental stage, were size-fractionated on a 12% SDS-PAGE and transferred to nitrocellulose membranes. The mouse anti-*E. coli* KAS III antibodies showed, however, no hybridization with the transgenic rape seed extracts. Mixing small amounts of bacterial KAS III enzyme, 1–10 ng, with excess of plant protein, did not result in any detectable quenching of the signal under the experimental conditions used. This implies that if the bacterial gene is expressed in the transformants, the amount of *E. coli* KAS III protein present, is below the detection level of the antibody (see Fig. 1B).

The primary rape transformants (15 of each, BN90 and BN93) were then analysed for KAS III enzyme activity. The K_m of acetyl-CoA for *B. napus* KAS III activity in crude seed protein extracts was determined. When assayed in the presence of saturating levels of malonyl-CoA and ACP, KAS III exhibited normal Michaelis-Menten kinetics with respect to acetyl-CoA. Analysis of the data using Eadie-Hofstee plots (data not shown), revealed a K_m for acetyl-CoA of $45 \pm 3 \mu\text{M}$. The V_{max} value was $3.9 \pm 0.1 \text{ nmol/min per mg protein}$. Since the affinity of the acetyl-CoA substrate for the plant and bacterial KAS III enzymes are different (K_m of $45 \mu\text{M}$ (this study) and $150 \mu\text{M}$ [10], respectively), the assays were performed under optimal conditions for measuring *E. coli* KAS III, which had the lower affinity of the two enzymes for acetyl-CoA. En-

ogenous KAS III levels of control plants peaked around 5 to 6 weeks after flowering, and decreased markedly in mature seeds (Fig. 2). Transgenic plants expressing the bacterial gene essentially followed this expression pattern, but the level of KAS III activity was higher, and remained higher in mature seeds (Fig. 2). In Table 1, KAS III activity levels in mature seeds of the highest expressors are compared to the endogenous KAS III level in mature seeds of untransformed control plants. The level of KAS III activity of the highest expressor (BN93.15) was 3.7 times the endogenous KAS III activity in mature seeds. The highest level of KAS III activity that can be attributed to the bacterial transgene (225 pmol/min per seed) was also found in the mature seeds of transformant BN93.15, yet this was still not detected by immunoblotting. Using these data and the quantitative densitometry analyses it can be estimated that bacterial KAS III comprises ca. 0.0005% of the total soluble protein of seeds of BN93.15. Since 15 μ g of soluble seed protein was routinely loaded on SDS-PAGE for immunodetection, the maximum amount of bacterial KAS III therein was below the detection limit of the antibody (Fig. 1B).

Fig. 2. Comparison of relative KAS III activity levels during seed development of transformant BN93.15, and control rape. For each developmental stage, crude seed protein extracts, the equivalent of 0.2 seed, were assayed for KAS III. The value of KAS III activity, measured in control plants at 5 weeks after anthesis, is normalized at 1 (1 = 0.48 nmol/min per seed). KAS III activity of the transformant is quantified to this value. The numerals on the x-axis indicate the stages of seed development in weeks after anthesis, M, mature seeds.

Table 1. 3-ketoacyl-ATP synthase III relative activities^a in transgenic rapeseed, compared to untransformed control plants.

Source of extract	Specific activity (nmol/min per seed) ^a
Control plants	1.0 $\pm$ 0.1
BN90 (– transit peptide)	
BN90.1	1.4 $\pm$ 0.1
BN90.8	3.2 $\pm$ 0.2
BN90.9	3.0 $\pm$ 0.2
BN90.17	1.5 $\pm$ 0.2
BN93 (+ transit peptide)	
BN93.15	3.7 $\pm$ 0.3
BN93.22	2.6 $\pm$ 0.2
BN93.27	2.3 $\pm$ 0.2
BN93.35	2.1 $\pm$ 0.3
BN93.60	3.3 $\pm$ 0.2

^a KAS III activity is measured in protein extracts of mature seeds. The average value of KAS III activity ($\pm$ SD, $n = 5$), measured in untransformed control plants, is normalized at 1 (1 = 0.08 nmol/min per seed). The KAS III activities found in the primary transformants (average value of three independent measurements, $\pm$ SD) are quantified to this value.

To determine whether the significant increase in KAS III activity found in these transformants could be attributed to the expression of the bacterial KAS III gene, mRNA steady-state levels of the transgene encoding the plastid-targeted protein were analysed using northern blot analysis. Transcripts of the expected length (1.2 kb) were detected in the five highest expressors. The transcript of transformant BN93.15 was most abundant, followed by those in transformants BN93.60, 22, 35 and 27 (data not shown). To study the expression of the chimaeric KAS III gene in more detail, seeds of these five transformants, and a control plant, were grown in the greenhouse and self-pollinated. Northern blots were prepared containing 10 μ g of total seed RNA isolated from different developmental stages of the S2 progeny of these plants. The blots were hybridized with a radiolabelled *E. coli fabH* probe. Figure 3 shows the steady state mRNA levels present at different stages of seed development in the S2 progeny of the primary transformant BN93.15. The transgene mRNA steady-

Fig. 3. Northern blot analyses of *E. coli* KAS III mRNA steady-state levels in transgenic *B. napus* seeds. Total seed mRNA (10 µg) of different developmental stages of the S2 progeny of transformant BN93.15 were electrophoresed, blotted and hybridized with a ³²P-labelled *E. coli* KAS III probe as described in Material and methods. The numerals above the panels indicate the stages of seed development in weeks after anthesis. M, mature seeds; C, control seed 5 weeks after anthesis.

state level reached its maximum at 6 weeks after flowering, and was hardly detectable in mature seeds.

Fatty acid analysis and total lipid extraction of transgenic seeds

To determine the effect of KAS III overexpression on plant lipid synthesis, total lipid content and total fatty acid composition of transgenic *B. napus* seeds were determined (see Materials and methods). Significant differences in total lipid

contents could not be detected due to variation in both control and transgenic data (data not shown). However, BN93 transformants (plastid-targeted protein) with a KAS III activity level in mature seeds of 2.1 to 3.7 times that of control KAS III levels, exhibited a significant difference in the fatty acid profile compared to control plants (Table 2). The relative amount of C18:1 in the storage lipid fraction of these transformants decreased from approximately 64 to 54% of the total fatty acids. This decrease was for the most part compensated for by an increase in the C18:2 and C18:3 content. Enhanced levels of medium-chain fatty acids, as found by overexpression of the bacterial KAS III in *E. coli* [31] were not observed. The fatty acid profiles found in the storage lipid fraction of BN90 transformants (non plastid-targeted protein) were not significantly different from those of untransformed control plants.

Discussion

In this study, we have examined the effects of increasing the initial condensation reaction of fatty acid biosynthesis by overexpression of the bacterial *fabH* gene, encoding 3-ketoacyl-ACP synthase III (KAS III), in both *E. coli* and *B.*

Table 2. Fatty acid composition of *E. coli* KAS III transformed rape seeds.

Plants	Fatty acid							
	14:0	16:0	16:1	18:0	18:1	18:2	18:3	> 20:0
Controls ^a	0.4 ± 0.3	4.9 ± 0.4	0.9 ± 0.3	1.7 ± 0.4	64.1 ± 4.9	18.0 ± 4.7	6.0 ± 1.8	2.4 ± 0.2
BN90 (– transit peptide) ^a	0.3 ± 0.2	4.6 ± 0.3	0.5 ± 0.1	1.9 ± 0.5	66.1 ± 4.5	17.0 ± 4.2	6.4 ± 2.0	2.2 ± 0.3
BN93 (+ transit peptide) ^b								
BN93.13	0.4 ± 0.2	6.0 ± 0.3	1.1 ± 0.2	1.5 ± 0.4	56.0 ± 1.5	23.7 ± 2.8	7.3 ± 1.8	2.2 ± 0.2
BN93.14	0.4 ± 0.2	5.3 ± 0.3	1.3 ± 0.3	1.8 ± 0.3	53.5 ± 2.4	26.5 ± 2.5	6.6 ± 1.2	2.2 ± 0.2
BN93.15	0.4 ± 0.3	5.1 ± 0.4	1.1 ± 0.2	1.3 ± 0.3	54.1 ± 2.3	26.5 ± 2.4	8.0 ± 1.5	2.4 ± 0.2
BN93.23	0.3 ± 0.2	6.0 ± 0.2	1.1 ± 0.2	1.8 ± 0.3	52.5 ± 2.0	24.7 ± 3.0	11.0 ± 1.5	2.1 ± 0.3
BN93.35	0.4 ± 0.3	5.1 ± 0.3	0.8 ± 0.3	1.5 ± 0.4	56.4 ± 1.6	23.6 ± 3.2	9.3 ± 2.0	2.3 ± 0.2
BN93.50	0.3 ± 0.3	4.6 ± 0.4	0.7 ± 0.3	1.2 ± 0.4	53.8 ± 1.8	26.0 ± 3.1	10.8 ± 1.6	2.0 ± 0.3
BN93.60	0.4 ± 0.3	5.0 ± 0.2	0.8 ± 0.1	1.2 ± 0.3	52.5 ± 2.1	27.5 ± 2.3	10.0 ± 1.7	2.4 ± 0.3

^a The values are % (mol/mol) ± SD (*n* = 15 for BN90 plants; *n* = 10 for controls).

^b The values are the average (in % mol/mol ± SD) of three independent analyses per plant.

napus seeds. For seed-specific expression in plants, the bacterial KAS III gene was linked to the plastid transit peptide sequence of the *B. napus* enoyl-ACP reductase gene, and a napin promoter. These sequences were successfully used in an earlier study to express the *E. coli fabD* gene, encoding malonyl-CoA:ACP transacylase (MCAT), in transgenic plants, and to direct its gene product to the plastids [33].

The presence of the chimaeric KAS III gene in transgenic rape was confirmed by PCR, Southern and northern blot analysis (Fig. 3). Furthermore, levels of KAS III enzyme activity in transformants were significantly higher than the level in control plants. Western immunoblot analysis, however, could not detect the bacterial KAS III gene product in developing rape seeds. This was despite the fact that the antibody raised against the *E. coli* KAS III can detect the bacterial protein as low as 1 ng (Fig. 1B), and that plant protein extracts mixed with low amounts of bacterial enzyme did not quench the signal. The explanation for this apparent contradiction was that even the KAS III level of the highest expressor (BN93.15, see Table 1) remained, under the experimental conditions used, below the detection limit of the antibody. Enzyme assays showed, that the expression pattern of the chimaeric KAS III gene was seed and developmentally specific. Endogenous rape KAS III levels peaked around 5–6 weeks after anthesis, and had decreased markedly in mature seeds (Fig. 2). In transgenic plants, essentially the same expression pattern was found, although KAS III levels were higher, and remained higher in mature seeds (Fig. 2). The steady-state mRNA of the chimaeric KAS III gene was hardly detectable in mature seeds (Fig. 3), suggesting that the bacterial protein is quite stable in the plant cell environment. A similar temporal expression pattern of steady-state mRNA levels was found in an earlier study in which the bacterial MCAT gene was expressed in transgenic plants [33].

The level of KAS III enzyme activity of the highest expressor was 3.7 times the endogenous KAS III activity in mature seeds. This activity level is very low in comparison to the level of

overexpression of the *E. coli* MCAT gene in transgenic plants, which reached up to 55 times the maximum endogenous MCAT activity [33]. Overexpression of the bacterial MCAT in transgenic plants did not, however, have an effect on the fatty acid composition of their storage lipids, presumably because MCAT does not catalyse a controlling step in plant and bacterial fatty acid biosynthesis [33]. If the first condensation reaction, catalysed by KAS III, is one of the controlling steps, high levels of this enzyme might not be tolerated. Overproduction of KAS III in transgenic seeds could hypothetically lead to either, an increase in the rate of lipid synthesis and thereby a higher seed oil content [15], or an increase in shorter-chain fatty acids and a concomitant decrease in longer-chain fatty acids [5, 27]. Consistent with the latter possibility, in *E. coli*, elevated levels of KAS III result indeed in an accumulation of myristate (C14:0), and a large decrease of *cis*-vacenate (C18:1) in the membrane lipid fraction [31; and this study]. It has not been demonstrated, however, whether this change in the fatty acid profile in *E. coli* is a direct result of the overexpression of this FAS component. Overproduction of KAS III in *E. coli* not only has a severe impact on the fatty acid composition of the membrane lipids, but also affects the growth behaviour of this organism. Upon induction of *fabH* expression, growth stops after approximately one generation, and the amount of myristic acid (C14:0) increases. This change in fatty acid composition might well be a general metabolic phenomenon, a result of the cell responding to stress. The synthesis of 3-hydroxymyristic acid is at a branchpoint of the core fatty acid biosynthesis pathway. At this point, 3-hydroxymyristoyl-ACP can be a substrate for outer membrane lipopolysaccharide biosynthesis, where the 3-hydroxymyristoyl moiety is attached to lipid A; alternatively, it can be elongated to palmitic acid and hence to phospholipids [22]. This increase in C14:0 has been observed in a variety of *E. coli* strains and mutants when placed under stress. For example, a mutant with a temperature-sensitive MCAT, when grown at the restrictive temperature of 39 °C accumulates myristate [8].

A recombinant *E. coli* strain expressing a temperature-sensitive rapeseed enoyl-ACP reductase, also accumulates myristate when grown at 42 °C [14]. Similarly, in a phosphotransferase deletion mutant of *E. coli* [2], accumulation of C14:0 was detected after cells growing on gluconate were transferred to a phosphotransferase substrate such as mannitol (Verwoert and Walsh, unpublished data), and wild-type *E. coli* cells in late stationary growth phase also accumulate myristic acid [21]. These data strongly suggest that accumulation of C14:0 in *E. coli* is a general metabolic effect due to the arrest in cell division.

The effect of the chimaeric KAS III overproduction on plant fatty acid biosynthesis is even more complex. Although significant increases in KAS III activities were found in plants expressing both the plastid-targeted or the non-targeted gene construct, only the expression of the plastid-targeted KAS III gene affected the fatty acid profile of the storage lipids. If, as suggested by others [20] the flux through fatty acid biosynthesis is controlled primarily by the acetyl-CoA carboxylase (ACCase), then increasing KAS III activity should have little effect on flux. In this case, however, overexpression of KAS III does result in a change in the fatty acid composition of transformed rape seeds. In a previous study [33], overexpression of the bacterial MCAT had no effect on the fatty acid profiles of transformed rape seeds. This is not surprising since, considering its high activity and high equilibrium constant, MCAT is unlikely to exert a high degree of control on fatty acid biosynthesis. The KAS III and ACCase may be functionally related as they share a common substrate pool, namely acetyl-CoA, and the malonyl-CoA product of the ACCase reaction provides the elongation unit for the condensation reaction, namely malonyl-ACP. Since no significant change could be confirmed in the total lipid biosynthetic flux in the plants overexpressing KAS III, it would appear that KAS III does not exert a high degree of control, however, changes have been detected in the fatty acid composition, indicating that some, perhaps more subtle, changes in regulation and control of fatty acid biosynthesis have taken place. We suggest,

therefore, that control of fatty acid biosynthesis may be shared, for example, by both KAS III and ACCase and perhaps other components, this could be via a common intermediate or by a product later in fatty acid biosynthesis. In this case it is interesting to note that one of the main changes in fatty acid composition is in the amount of C18:1, and recently C18:1 has been implicated to cause feedback inhibition on fatty acid biosynthesis [28]. Such an idea of enzymes sharing control on biosynthetic flux is not new, and indeed such a mechanism has been demonstrated for tryptophan biosynthesis in yeast where the idea of one rate-limiting step has been superseded by a model of various enzymes in the pathway sharing control over biosynthetic flux [19]. Changes in fatty acid composition may reflect only subtle changes in the control of biosynthesis, perhaps as in this case by the change in the stoichiometric ratios of some of the FAS components. The test of such a model will be the concomitant elevation of several fatty acid biosynthetic components.

From the data presented in this paper it seems evident that bacterial FAS proteins can interact with the plant FAS system, and that the expression of heterologous FAS components in transgenic plants can contribute to the understanding of the regulation and control of plant lipid biosynthesis.

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