octadecatetraenoyl)-6""-*O*-acetyl-chitopentaose [abbreviated NodRlv-V(Ac,C18:4)] (*7*). Both molecules are characterized by the presence of a highly unsaturated lipid moiety and an *O*-acetyl substituent. Besides these two nodulation signals, other Nod metabolites were reported (*7*) that differ in the acyl substituent or that lack the *O*-acetyl group. When the acyl substituent is *cis*-vaccenic acid, the corresponding Nod factors are designated NodRlv-IV(Ac,C18:1) and NodRlv-V(Ac,C18:1). When the *O*-acetyl substituent is absent, the corresponding Nod factors are designated NodRlv-IV(C18:4) and NodRlv-V(C18:4). A Tn*5* mutation in the rhizobial nodulation gene *nodE* causes the absence of the NodRlv-IV(Ac,C18:4) and NodRlv-V(Ac,C18:4) Nod factors, and instead the corresponding Nod factors NodRlv-IV(Ac,C18:1) and NodRlv-V(Ac,C18:1) are formed (*7*). A *nodL*::Tn*5* mutation results in the absence of the *O*-acetyl group in all Nod factors produced, such as NodRlv-IV(C18:4) and NodRlv-V(C18:4) (*7*).

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20. Methods used for Fig. 1, A, B, and D: Induction of nodulation phenotypes by *R. leguminosarum* bv. viciae bacteria, crude Nod factors, or purified Nod factors was tested with *Vicia sativa* ssp. *nigra* plants in glass containers with the roots shielded from light (*7*). *Rhizobium leguminosarum* bv. viciae strain RBL5601 (Nod$^+$Fix$^+$) was grown for 3 days on solidified yeast mannitol broth medium [P. J. J. Hooykaas, P. M. Klapwijk, M. P. Nuti, R. A. Schilperoort, A. Rörsch, *J. Gen. Microbiol.* **98**, 477 (1979)], suspended in sterile water, and added to the *Vicia* plant growth medium at 10^5 to 10^6 cells per milliliter. Crude Nod factors were extracted with the use of butanol (*7*) from *Rhizobium* cultures of the strains RBL5601 (containing the Sym plasmid pRL1JI) for wild-type crude Nod factors, RBL5602 (pRILJI*nodE*::Tn*5*) for crude Ac,C18:1 Nod factors, and RBL5793 (pRL1JI*nodL*589::Tn*5phoA*) for crude C18:4 Nod factors [A. A. N. van Brussel *et al.*, *J. Bacteriol.* **172**, 5394 (1990)]. The butanol phase was evaporated, and the residue was redissolved in an acetonitrile and water mixture (1:1, v/v; 25 ml per liter of the original culture medium). Nod factors produced by *R. leguminosarum* bv. viciae strain RBL5601 were purified as described (*7*). Purified and crude Nod factors were tested as follows. Samples (100 μl) of solutions in acetonitrile-water (1:1, v/v) were evaporated under sterile conditions at 80°C. Sterile medium (20 ml) was added, yielding final factor concentrations of 10^{-7} M for the purified Nod factor and a final concentration of the crude Nod factor corresponding to one-fifth of the concentration in the *Rhizobium* culture from which the factors were extracted. Crude Nod factor samples containing the lipooligosaccharides NodRlv-IV(Ac,C18:1), NodRlv-V(Ac,C18:1) or NodRlv-IV(C18:4), and NodRlv-V(C18:4) required solubilization in sterile 1% CHAPS (Sigma) (*7*). Seedlings were mounted in glass containers, and after 4 or 5 days of incubation with *Rhizobium* or with Nod factors, the roots were processed for thin sectioning as described (*12*), with the exception that the first and second fixation periods were reduced to 1 hour and 45 min, respectively. Transverse serial sections of 8 μm thickness were made of the developing nodule meristems and investigated by light microscopy. For Fig. 1C, induction of nodulation phenotypes was tested with pea seedlings grown and inoculated with *Rhizobium leguminosarum* bv. viciae strain 248 (Nod$^+$Fix$^+$) as described [C. L. Diaz *et al.*, *Planta* **168**, 350 (1986)]. The roots were processed for electron microscopy (*12*), and serial thin sections were examined with a Philips 300 electron microscope operating at 60 kV.
21. We thank A. Wijfjes for technical assistance, G. P. G. Hock for drawing Fig. 2, and O. Geiger for valuable discussions. Supported in part by the Royal Netherlands Academy of Arts and Sciences (H.P.S.). The first three authors have made equal contributions to this work.

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Fatty Acid Biosynthesis Redirected to Medium Chains in Transgenic Oilseed Plants

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Medium-chain fatty acids (FAs), found in storage lipids of certain plants, are an important renewable resource. Seeds of undomesticated California bay accumulate laurate (12:0), and a 12:0–acyl-carrier protein thioesterase (BTE) has been purified from this tissue. Sequencing of BTE enabled the cloning of a complementary DNA coding for a plastid-targeted preprotein. Expression of the complementary DNA in the seeds of *Arabidopsis thaliana* resulted in BTE activity, and medium chains accumulated at the expense of long-chain (≥16) FAs. Laurate became the most abundant FA species and was deposited in the storage triacylglycerols. These results demonstrate a mechanism for medium-chain FA synthesis in plants.

In plants, FAs are assembled in the plastids where the fatty acid synthase (FAS) sequentially condenses two-carbon units onto the growing fatty acyl chain. Whereas the end products are usually 16- or 18-carbon FAs (*1*), members of several plant families synthesize large amounts of predominantly 8- to 14-carbon (medium-chain) FAs. Some are harvested for dietary or industrial purposes—for example laurate, which is currently extracted from the seeds of tropical trees at a rate approaching one million tons annually (*2*). We investigated the feasibility of producing laurate in an annual, temperate crop by genetic engineering.

In the developing oilseeds of California bay (*Umbellularia californica*) that accumulate caprate (10:0) and laurate (12:0), a medium-chain acyl acyl-carrier protein (acyl-ACP) BTE has been identified (*3*). This BTE, by prematurely hydrolyzing the growing acyl thioesters, is thought to play a critical role in medium-chain production (*3*). We report here on the transfer of this activity into seeds of *A. thaliana*.

Peptide sequences of BTE were obtained (*4*, *5*), enabling the synthesis of primers for polymerase chain reaction (PCR) amplification of reverse-transcribed BTE mRNA (*6*). The PCR-derived BTE DNA sequence fragment was used to obtain a full-length cDNA (*7*). The ATG nearest the 5′ end of the single, large open reading frame is surrounded by a sequence matching the motif for initiation of translation in plants (*8*). A 382–amino acid polypeptide (molecular weight 33782) containing most of the BTE peptide sequences (*5*) was predicted (Fig. 1).

Mature BTE, when isolated from developing seeds, appears to be a processed form of the predicted polypeptide, having its NH_2-terminus at amino acid 84 (Fig. 1) (*5*). A search of all currently available gene banks with the derived amino acid sequence yielded no significant matches. Using ribonuclease protection, we detected no BTE transcripts in bay leaves. In developing cot-

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MATTSLASAFCSMKAVMLARDGRGMKPRSSDLQLRAGNAP  40
TSLKMINGTKFSYTESLKRLPDWSMLFAVITTIFSAAEKQ  80
WTNLEWKPKPKLPQLLDDHFGLHGLVFRRTFAIRSYEVGP 120
DRSTSILAVMNHMQEATLNHAKSVGILGDGFGTTLEMSKR 160
DLMWVVRRTHVAVERYPTWGDTVEVECWIGASGNNGMRRD 200
FLVRDCKTGEILTRCTSLSVLMNTRTRRLSTIPDEVRGEI 240
GPAFIDNVAVKDDEIKKLQKLNDSTADYIQGGLTPRWNDL 280
DVNQHVNNLKYVAWVFETVPDSIFESHHISSFTLEYRREC 320
TRDSVLRSLTTVSGGSSEAGLVCDHLLQLEGGSEVLRART 360
EWRPKLTDSFRGISVIPAEPRV 382
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Fig. 1. Amino acid sequence of bay 12:0-ACP TE derived from the cDNA of pCGN3822 (*7*). The NH_2-terminus of the BTE, as obtained from the purified protein (*5*), is indicated by a dot below the respective amino acid. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. GenBank accession number M94159.

Calgene, Inc., 1920 Fifth Street, Davis, CA 95616.

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Table 1. Laurate accumulation in individual transformants. All plants were independently transformed with pCGN3828 (*14*). The 12:0-ACP TE activities (TE Act.) of pools of 40 mid-maturation seeds were measured (1 U = 1 nmol of substrate hydrolyzed in 30 min at 30°C) (*3*). Extracts from control plants had 7 mU of 12:0-ACP hydrolytic activity per seed. For FA analysis (*19*), 100 mature seeds were pooled; no laurate was detected in the control.

Plant	TE Act./ seed (mU)	12:0/seed (nmol)	12:0 (mole % of total FA)
21	324	4.4	10.6
16	260	4.6	12.1
19	903	6.7	16.4
13	1080	6.4	23.5

yledons, BTE mRNA was found only during the time of medium-chain FA production (*9*).

The NH_2-terminal, 83–amino acid portion of the derived polypeptide contains conserved features of plastid transit peptides. This was expected because the plant FAS resides in that organelle (*1*, *10*). However, hydropathy analysis reveals an alanine-rich hydrophobic domain at the COOH-terminal end of the transit sequence, typical of thylakoid targeting (*10*, *11*). The location of BTE within the plastids of bay cotyledons is not known, although its substrate is likely to be in the stroma (*12*). Long-chain thioesterases (TEs) of safflower seeds have stroma-targeting transit sequences (*13*). But it is possible that in bay, the synthesis of storage lipid occurs in its own intracellular compartment.

The function of the cloned BTE was analyzed in a heterologous plant system: transformed *A. thaliana*. The BTE cDNA was fused with a *Brassica rapa* seed storage protein (napin) promoter (*14*). Transformation of *A. thaliana* with this construct did not significantly affect the number, size, or viability of the seeds.

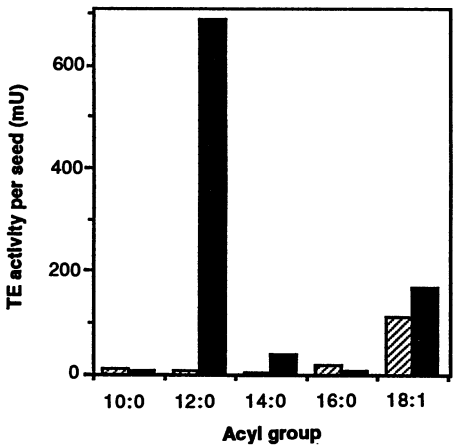

Fig. 2. Acyl-ACP hydrolysis profiles of *A. thaliana* seeds. Forty immature seeds were harvested, homogenized in BTE assay buffer, and their acyl-ACP hydrolysis activity was measured (*3*). Analysis of control (hatched bars) and the BTE transformant 3828-13 (*14*) (solid bars) is shown.

Acyl-ACP hydrolytic activities were measured in extracts of the developing seeds. Control plants showed predominantly 18:1-ACP TE activity and only a trace of 12:0-ACP TE activity. In plants transformed with BTE, the 12:0-ACP TE activity was elevated up to 70-fold, surpassing even that of the endogenous 18:1-ACP TE (Fig. 2). Also, the low 14:0-ACP hydrolytic activity was increased up to sevenfold in the transformants. The ratio of the introduced 12:0-ACP and 14:0-ACP TE activities was the same as that observed with the purified bay enzyme (*4*). These results confirm that the BTE cDNA encodes a medium-chain TE activity.

To examine the effects of BTE on the heterologous plant FAS in vivo, we determined the lipid composition of mature seeds from the transgenic plants. Laurate, absent from untransformed *A. thaliana*, accumulated in all transformants that showed BTE activity (Table 1). The proportion of laurate to the total FAs varied between the individual transformed plants but was approximately correlated with the BTE enzyme activities of seed extracts. This production of laurate in the seeds of transformed *A. thaliana* showed that the bay TE was able to access acyl-ACPs in vivo, thereby intercepting the FAS.

Seeds of control plants showed the near-exclusive accumulation of long-chain (≥ 16) FAs (*15*). In one transgenic plant, laurate was the predominant FA. Myristate (14:0) was also present (Fig. 3). In all BTE-expressing plants, the amount of myristate that accumulated was about 16% that of accumulated laurate, regardless of the absolute amount of medium-chain FAs produced (*9*). In vitro, the hydrolytic activity of BTE on 14:0-ACP was 5% of that on 12:0-ACP (Fig. 2) (*4*). Therefore, the BTE's action in vivo was quite similar to its specificity as determined in vitro with substrates prepared with *Escherichia coli* ACP. This suggests that in vivo the introduced enzyme accessed approximately equal-sized pools of 12:0-ACP and 14:0-ACP [the nearly total absence of myristate in bay seeds (*3*) suggests that BTE intercepts the FAS at 12:0 very efficiently].

Medium-chain FA accumulation caused reductions in the contents of most of the other FAs, but the total seed FA contents remained unchanged. This would be expected if BTE was removing metabolites from the FAS pathway to produce 12:0 and 14:0. The exception was linolenate (18:3), which was unaffected by BTE action (Fig. 3). Linolenate amounts were also sustained

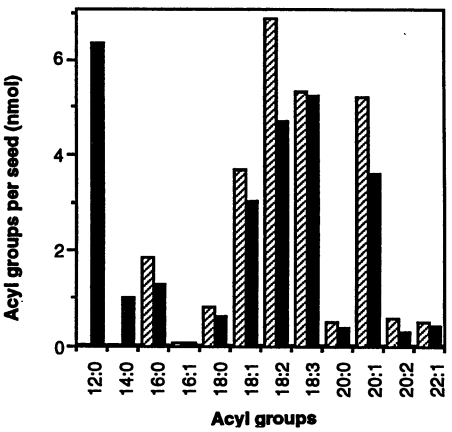

Fig. 3. Engineering of medium-chain FA production. One hundred mature seeds of each plant were pooled for fatty acyl determination. We quantitated FAs as methyl esters by gas-liquid chromatography (*18*). Control, hatched bars; BTE transformant 3828-13, solid bars.

in an *A. thaliana* mutant that had a large amount of palmitate (*16*) and in a transgenic rapeseed that had reduced seed-specific stearoyl-ACP desaturase activity (*17*).

In plants, the FAs destined for storage leave the plastid after their synthesis and are subsequently esterified to glycerol in the endoplasmic reticulum to form triacylglycerols (TAGs). In the transgenic plants, laurate also entered the TAG fraction and was therefore accepted for storage lipid formation (*9*).

These results illustrate the probable role of BTE in California bay (*3*) and demonstrate a mechanism for medium-chain FA synthesis in plants (*1*). Transformation of the annual oil crop, rapeseed, with the napin-BTE gene resulted in the accumulation of laurate (*9*), which shows that genetic engineering may be used to alter the FA chain lengths in crops of commercial importance.

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5. The pooled active fractions from the ACP affinity column (*4*) were concentrated by ultrafiltration. Proteins were separated by SDS–polyacrylamide electrophoresis and electroblotted to polyvinylidene difluoride (NH_2-terminal sequence) or nitrocellulose (for peptide production). Peptides obtained by trypsin or Asp-N protease digestion [R. Aebersold, *A Practical Guide to Protein and Peptide Purification for Microsequencing*, P. Matsudaira, Ed. (Academic Press, San Diego, 1989), pp. 71–88] were separated by a C18 reverse-phase column (Vydac) on an Applied Biosystems model 130A liquid chromatograph {7 to 52.5% v/v gradient of acetonitrile in 0.1 mM sodium phosphate (pH 2.2) [M. R. Rosner and P. W. Robbins,

J. Cell. Biochem. **18**, 37 (1982), modified]} and sequenced on an Applied Biosystems 477A Sequencer. We obtained a sequence from 27 peptides from both 34-kD bands of the purified BTE, and 24 matched the derived cDNA sequence. No preferential matches with peptides from either member of the protein doublet were apparent. We obtained the eight NH_2-terminal residues of the 34-kD polypeptides.

6. We followed in principle the approach of C. C. Lee *et al.* [*Science* **239**, 1288 (1988)]. Degenerate oligonucleotides were synthesized after reverse-translation of the respective peptide sequences. The sequence of the antisense oligonucleotide 696-2a was: GCCTCGAGCC(IC) CC(TC)TGIAT(AG)TA(AG)TC(IC)GC (I = desoxyinosine, degenerate residues in brackets. A 5′ Xho I site was added). The sequence of the sense oligonucleotide 701-2 was: CTGGATCCGA(CT) AT(ACT)(CT)T(IC)GC(IC)GT(IC)ATGAA (A 5′ Bam HI site was added). RNA from bay cotyledons at the stage of rapid accumulation of bay TE was extracted according to T. H. Turpen and O. M. Griffith [*BioTechniques* **4**, 11 (1986)]. Polyadenylate [poly(A)] RNA, obtained by oligo(dT) cellulose chromatography, was reverse-transcribed, and the single-strand cDNA (1 to 10 ng per 100 μl of reaction) was used for PCR. A Perkin-Elmer thermal cycler was used with primers at 2 μM, 30 cycles of 1 min at 94°C, 1 s at 65°C, 2 min-ramp to 50°C, 1 s at 50°C, and 2 min at 72°C.
7. A cDNA library of the plasmid vector pCGN1703 [D. C. Alexander, *Methods Enzymol.* **154**, 41 (1987)] was made with the use of poly(A) RNA from developing bay cotyledons. The PCR generated a 0.8-kb BTE cDNA fragment (*6*) that was used as a probe. The clone with the longest insert was named pCGN3822. DNA sequencing was according to M. Hattori and Y. Sakaki [*Anal. Biochem.* **152**, 232 (1986)].
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14. For plant expression, we engineered a Bam HI site 6 bp upstream of the proposed translational start. The BTE cDNA from this engineered Bam HI site to a Pst I site at bp 1361 was inserted into pCGN3223. This plasmid contains 1750 bp of a napin promoter fragment, followed by a napin 3′ untranslated sequence isolated from *Brassica rapa* [J. C. Kridl *et al.*, *Seed Sci. Res.* **1**, 209 (1991)]. The chimeric gene was inserted into a binary vector, pCGN1578 [K. E. McBride and K. R. Summerfelt, *Plant Mol. Biol.* **14**, 269 (1990)], resulting in pCGN3828. *Arabidopsis thaliana*, ecotype No-0 [M. C. Whalen, R. W. Innes, A. F. Bent, B. J. Staskawicz, *Plant Cell* **3**, 49 (1991)], was transformed by *Agrobacterium tumefaciens* cocultivation, and plants were regenerated [D. Valvekens, M. Van Montagu, M. Van Lijsebettens, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5536 (1988), modified].
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Evidence That Eukaryotes and Eocyte Prokaryotes Are Immediate Relatives

Maria C. Rivera and James A. Lake

The phylogenetic origin of eukaryotes has been unclear because eukaryotic nuclear genes have diverged substantially from prokaryotic ones. The genes coding for elongation factor EF-1α were compared among various organisms. The EF-1α sequences of eukaryotes contained an 11–amino acid segment that was also found in eocytes (extremely thermophilic, sulfur-metabolizing bacteria) but that was absent in all other bacteria. The related (paralogous) genes encoding elongation factor EF-2 and initiation factor IF-2 also lacked the 11–amino acid insert. These data imply that the eocytes are the closest surviving relatives (sister taxon) of the eukaryotes.

The evolutionary antecedents of eukaryotes have been difficult to identify because eukaryotic nuclear gene sequences have diverged extensively from their prokaryotic homologs. Their frequent nucleotide and amino acid changes confound tree reconstruction (*1*) and multiple sequence alignment (*2*) algorithms and cause incorrect trees to be artifactually selected (*3*). The probability of these artifacts could be reduced, however, if slowly changing and well-aligned features, such as inserts that consist of many nucleotides, could be observed and analyzed. We report here the identification, sequencing, and analysis of an 11–amino acid segment within the gene coding for protein synthesis factor EF-1α that occurs in all eukaryotes and eocytes (*4*) but not in other prokaryotes.

Elongation factor EF-1α (termed EF-Tu in eubacteria) is an ubiquitous protein that transports aminoacyl-tRNAs to the ribosome and participates in their selection by the ribosome. The structure of the guanosine diphosphate (GDP)–binding domain of EF-Tu from *Escherichia coli* has been determined by x-ray diffraction (*5*). Within this domain (Fig. 1), sequence $DCPGH_{84}$ (*6*) ends a β strand, forms part of the GDP binding site, and initiates a short α helix. The sequence $KNMITG_{94}$, which is conserved in EF-1α and EF-Tu sequences, terminates this helix. The β strand that follows is terminated by $GPMP_{113}$ at the GDP binding site; $QTREH_{118}$ then starts a 3_{10} helix. The amino acid motifs of the eukaryotic EF-1α are similar, except that the four–amino acid sequence $GPMP_{113}$ is replaced by the 11–amino acid sequence GEFEAGISKDG and its variants.

Unlike that in other prokaryotes, the EF-1α sequence from the eocyte *Sulfolobus acidocaldarius* (*7*) contained an 11–amino acid segment similar to the eukaryotic one. In order to investigate this segment in all three taxonomic subdivisions of the eocytes [the Pyrodictiales, the Thermoproteales, and the Sulfolobales (*8*)], we amplified genomic DNA (*9*) from the eocytes *Pyrodictium occultum*, *Desulfurococcus mucosus*, and *Acidianus infernus* with the polymerase chain reaction (PCR) and primers to the $KNMITG_{94}$ and $QTREH_{118}$ regions (Table 1).

The amino acid sequences, translated from DNA, shared the eukaryotic motif rather than that found in methanogens, halobacteria, and eubacteria (Table 2). The longer, 11–amino acid segment, present in eocytes and eukaryotes, shares little obvious similarity with the shorter, four–amino acid segment found in other prokaryotes. In order to ascertain which form of the segment existed first, we compared sequences of the related (paralogous) proteins EF-2 (termed EF-G in eubacteria) and IF-2

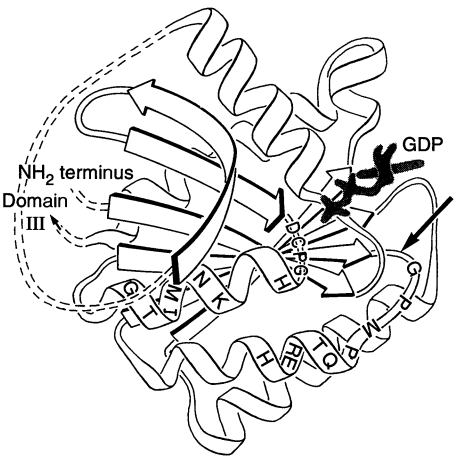

Fig. 1. Structure of the GDP binding domain of trypsin-treated elongation factor EF-Tu from *E. coli* (*5*). Open arrows represent β strands, spirals represent helices, and GDP (labeled) is represented by shading. The dashed line represents a sequence removed by trypsin treatment. The connection to structural Domain III is shown by a small, solid arrow. The large, solid arrow indicates the NH_2-terminus of the amino acid segment ($GPMP_{113}$) analyzed here. The $DCPGH_{84}$, $KNMITG_{94}$, and $QTREH_{118}$ regions are indicated (*6*).

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