# Identification of a Long-Chain Polyunsaturated Fatty Acid Acyl-Coenzyme A Synthetase from the Diatom *Thalassiosira pseudonana*[1]

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The draft genome of the diatom *Thalassiosira pseudonana* was searched for DNA sequences showing homology with long-chain acyl-coenzyme A synthetases (LACSs), since the corresponding enzyme may play a key role in the accumulation of health-beneficial polyunsaturated fatty acids (PUFAs) in triacylglycerol. Among the candidate genes identified, an open reading frame named *TplacsA* was found to be full length and constitutively expressed during cell cultivation. The predicted amino acid sequence of the corresponding protein, TpLACSA, exhibited typical features of acyl-coenzyme A (acyl-CoA) synthetases involved in the activation of long-chain fatty acids. Feeding experiments carried out in yeast (*Saccharomyces cerevisiae*) transformed with the algal gene showed that TpLACSA was able to activate a number of PUFAs, including eicosapentaenoic acid and docosahexaenoic acid (DHA). Determination of acyl-CoA synthetase activities by direct measurement of acyl-CoAs produced in the presence of different PUFA substrates showed that TpLACSA was most active toward DHA. Heterologous expression also revealed that *TplacsA* transformants were able to incorporate more DHA in triacylglycerols than the control yeast.

Long-chain acyl-CoA synthetases (LACSs) play a critical role in the biosynthetic pathways of nearly all fatty acid (FA)-derived molecules. These enzymes esterify free FAs to CoA to form acyl-CoAs, a key activation step necessary for the utilization of FAs by most lipid metabolic enzymes (Groot et al., 1976). The enzymatic mechanism is a two-step reaction that proceeds via the formation of an acyl adenylate (acyl-AMP) intermediate (Kornberg and Pricer, 1953). Acyl-CoAs serve as important intermediates in many metabolic pathways, such as elongation and $\beta$-oxidation of FAs, enzyme activation, cell signaling, and transcriptional regulation (Watkins, 1997). Consistent with the diverse roles of acyl-CoA synthetases (ACSs) in cell metabolism, many eukaryotic organisms encode several different ACSs that specifically activate short- (C6–C8), medium- (C10–C12), long- (C14–C20), or very long- (>C22) chain FAs (Watkins, 1997). Moreover, some organisms possess multiple enzymes for each set of acyl chain lengths. In plants, LACS activity has been localized to several subcellular compartments (Schnurr et al., 2002, 2004; Shockey et al., 2002; Fulda et al., 2004), enabling acyl chains produced by de novo FA synthesis to be activated to their CoA esters and subsequently used for metabolic pathways such as those involved in the synthesis of membrane glycerolipids and storage lipids (triacylglycerols [TAGs]) in developing seeds (Ohlrogge et al., 1991). In addition, LACS enzymes play an important role in intracellular FA homeostasis (Choi and Martin, 1999) and in FA transport. This process has been studied in detail in bacteria (Black et al., 1992), yeast (*Saccharomyces cerevisiae*; Faergeman et al., 2001), and mammalian cells (Mashek et al., 2004). Recently, Fulda et al. (2004) demonstrated the involvement of two Arabidopsis (*Arabidopsis thaliana*) peroxisomal ACSs, LACS6 and LACS7, in the delivery of acyl-CoA substrates into peroxisomes for breakdown via the $\beta$-oxidation pathway in an as yet undefined process that also involves an ATP-binding cassette transporter.

Very long-chain polyunsaturated fatty acids (VLCPUFAs) are important components of infant and adult human nutrition because they play key roles in various biological functions (Lauritzen et al., 2001). They can be classified into the omega-3 ($\omega$3) and omega-6 ($\omega$6) families and are derived mainly from the diet or from the metabolism of dietary linoleic acid (18:2n6) and $\alpha$-linolenic acid (18:3n3). Among the $\omega$3 FAs, eicosapentaenoic acid (EPA; 20:5n3) and docosahexaenoic acid (DHA; 22:6n3) have been linked to a variety of health benefits, such as decreased risk of cardiovascular diseases (Harper and Jacobson, 2003), protection against cancers (Leitzmann et al., 2004), and amelioration of metabolic syndrome (Graham et al.,

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**Table I.** *FA and acyl-CoA composition in T. pseudonana cells*

Microalgal cells were harvested during the late exponential phase of growth. Values, expressed as mol % of total FAs and mol % of total acyl-CoAs, represent the mean ± SD for three replicates within a representative culture. nd, Not detected.

| Fatty Acids | | Acyl-CoAs | |
|---|---|---|---|
| | *mol%* | | *mol%* |
| 14:0 | 6.8 ± 0.1 | 14:0 | 8.8 ± 0.4 |
| 14:1n5 | 0.2 ± 0.0 | 14:1n5 | 0.3 ± 0.0 |
| 16:0 | 27.9 ± 0.5 | 16:0 | 16.1 ± 0.5 |
| 16:1n7 | 19.4 ± 0.2 | 16:1n7 | 19.2 ± 0.7 |
| 16:1n5 | 0.2 ± 0.1 | 18:0 | 1.4 ± 0.2 |
| 16:2n4 | 2.1 ± 0.0 | 18:1n9 | 6.1 ± 0.7 |
| 16:3n4 | 4.7 ± 0.2 | 20:4n6, 22:6n3 | 4.4 ± 0.2 |
| 18:0 | 1.3 ± 0.1 | 20:5n3 | 29.2 ± 2.3 |
| 18:1n9 | 1.8 ± 0.1 | 18:4n3 | 7.7 ± 0.8 |
| 18:2n6 | 1.5 ± 0.0 | 20:0 | 0.4 ± 0.1 |
| 18:3n6 | 0.4 ± 0.0 | 20:3n6 | nd |
| 18:3n3 | 0.7 ± 0.0 | 20:3n3 | nd |
| 18:4n3 | 6.4 ± 0.1 | 22:1n9 | 3.5 ± 0.2 |
| 20:0 | 0.3 ± 0.0 | | |
| 20:3n6 | nd | | |
| 20:4n6 (ARA) | 0.7 ± 0.0 | | |
| 20:5n3 (EPA) | 18.7 ± 0.5 | | |
| 22:1n9 | 0.1 ± 0.0 | | |
| 22:6n3 (DHA) | 4.1 ± 0.0 | | |

2004). Changes in the dietary habits of western societies have seen a decrease in EPA and DHA intake. The principal dietary source of these very long-chain ω3 FAs is fish. However, wild fish are a declining resource, and fish farming does not represent a reliable alternative to sustain the growing demand for EPA and DHA because of environmental issues (Pauly et al., 2003; Hites et al., 2004). Some marine microalgae produce and store high levels of EPA and DHA (for examples, see Tonon et al., 2002). Therefore, oilseed crops engineered with algal genes involved in VLCPUFA metabolism could provide a sustainable supply of these valuable FAs. This would require the introduction of multiple enzymatic reactions, including FA desaturation, elongation, and activation to form substrates suitable for incorporation into TAGs. Recently, successful engineering of EPA biosynthesis has been demonstrated in oil-synthesizing seeds of higher plants by heterologous expression of three genes encoding a Δ6-desaturase, a Δ6-elongase, and a Δ5-desaturase (Abbadi et al., 2004). EPA production has also been achieved in the model plant Arabidopsis (*Arabidopsis thaliana*) by constitutive expression of the alternate Δ8-desaturation pathway (Qi et al., 2004). In addition, Meyer et al. (2004) and Pereira et al. (2004) have described the production of DHA in transgenic yeast transformed with newly characterized algal Δ5-elongases and Δ4-desaturases. These results indicate that all the activities necessary for the reconstitution of DHA synthesis in plants are now available. The next step will be to achieve a commercially viable level of this VLCPUFA in plant oil. To fulfill this goal, coexpression in plants of activities such as acyltransferases and ACSs is likely to be required to improve the flux of intermediates through the C22 polyunsaturated fatty acid (PUFA) biosynthetic pathway and to ensure the accumulation of DHA in plant storage lipids (TAGs).

Here, we report the characterization of the *TplascA* gene of *Thalassiosira pseudonana*, encoding one of the eight putative LACS genes identified after analysis of the algal draft genome. This enzyme exhibits high activity toward the health-beneficial VLCPUFAs EPA and DHA and has been shown to increase the quantity of DHA stored in yeast TAGs.

## RESULTS

### FA and Acyl-CoA Composition of *T. pseudonana*

FA profiling of Thalassiosira cells showed that palmitic acid (16:0), palmitoleic acid (16:1n7), and EPA were the most abundant FAs in algal cells (Table I). Only a low percentage of ω6 C20 PUFAs were measured in contrast with the significant amounts of ω3 stearidonic acid (18:4n3) and DHA, indicating that the ω3 pathway is the most active in these diatom cells. The acyl-CoA profile followed that of FAs in that palmitic, palmitoleic, and EPA-CoA were the most abundant, with the latter representing almost 30% of the acyl-CoA pool (Table I; Fig. 1). This high level of EPA-CoA could potentially act as an intermediate in the synthesis of DHA through elongation to 22:5n3 and desaturation to 22:6n3.

### Identification of Putative LACS Genes in *T. pseudonana*

Sequence analysis of the *T. pseudonana* draft genome revealed eight putative genes encoding putative LACS proteins with previously characterized plant and

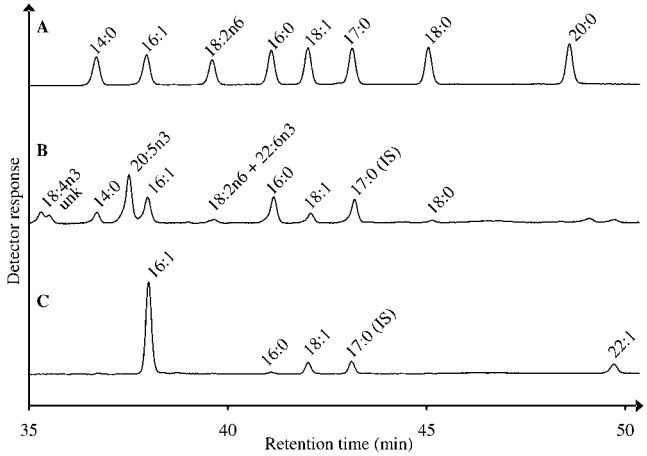


**Figure 1.** Representative acyl-CoA profiles from yeast and algal samples. HPLC profiles are shown for standard acyl-CoAs (A), *T. pseudonana* cells harvested in midexponential phase (B), and *faa4Δ* cells containing the plasmid pYES2 (C). None of the acyl-CoAs produced in the PUFA feeding experiments on *faa4Δ* transformants coeluted with the endogenous yeast acyl-CoAs. IS, Internal standard; unk, unknown.

A

| Culture time | t1 | t2 | t3 | t4 |
|---|---|---|---|---|
| Incubation (h) | 95 | 173 | 237 | 311 |
| % nitrate degraded | 5 | 30 | 60 | 100 |

B

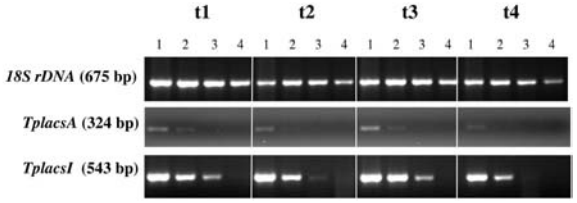


**Figure 2.** RT-PCR expression analysis of *TplacsA* and *TplacsI* genes. Thalassiosira cells were harvested at different stages of growth for total RNA extraction and cDNA synthesis. PCR was then performed on undiluted (lane 1) and 5-fold serial dilutions (lanes 2–4) of each cDNA using *TplacsA*- and *TplacsI*-specific primer pairs. The *18S rRNA* gene was used as a control for cDNA synthesis. Size of the diagnostic fragment for each locus is given in parentheses.

mammalian LACS. Only two of them, designated *TplacsA* and *TplacsI*, respectively, were found to be full length in current sequence data. *TplacsI* was intronless, while *TplacsA* was predicted to contain two introns. To monitor the transcription of these two loci in Thalassiosira cells, temporal expression analysis was carried out by reverse transcription (RT)-PCR. Figure 2 shows that both genes were expressed throughout cell cultivation, the *TplacsI* transcript being the more abundant of the two. Amplification and sequencing of the *TplacsA* open reading frame (ORF) from algal cDNA (accession no. AY730618) shows that it was 2,025-bp long and encodes a protein of 674 amino acids. Alignment of this ORF with the corresponding genomic DNA sequence confirmed the presence of 2 introns of 96 and 88 bp, respectively, in the second half of the sequence. Comparison of TpLACSA amino acid sequence with functionally characterized LACS showed that the algal enzyme exhibits 35% to 40% identity with both plant and mammalian LACS, with high homology in the region containing a putative AMP-binding domain. Our further studies focused on the functional characterization of *TplacsA*.

### Evaluation of FA Activation Deletion Mutants of Yeast

To identify an optimal yeast strain for the functional characterization of *TplacsA*, several FA activation (FAA) deletion mutants from the Euroscarf collection were tested. Proteins encoded by the genes *FAA1* and *FAA4* have been shown to be the primary enzymes involved in activation of endogenous and imported C12 to C18 saturated and monounsaturated FAs, while FAA3 was found to be most active toward saturated FAs longer than C18 (Knoll et al., 1994; Faergeman et al., 2001). Wild-type strain BY4741 and deletion strains *Y06477* (*faa1Δ*), *Y01401* (*faa3Δ*), and *Y00833* (*faa4Δ*) were transformed with the empty vector control, pYES2, and incubated simultaneously in the presence of three ω6 (18:2n6, 18:3n6, 20:3n6) or three ω3 (18:4n3, 20:5n3, 22:6n3) PUFAs. Table II shows the acyl-CoA composition after 1-h incubation at 25°C in these different strains. Surprisingly, neither C20 nor C22 PUFA-CoAs could be detected in wild type or FAA mutants, suggesting that the cells were not able to produce the corresponding acyl-CoAs during this short time of incubation. However, the FAs used as substrates were incorporated by the four strains, since FA profiling showed they were present in washed yeast cells (data not shown). No 14:0-, 16:0-, or 18:0-CoAs could be detected in *faa1Δ* cells, suggesting that the FAA1 gene product is involved in the activation of the corresponding saturated FAs. Similar percentages of 18:3n6- and 18:4n3-CoAs were measured in wild-type cells, but their amounts were lower than the values determined for 18:2n6. In all the different lines, a higher 18:2n6-CoA percentage suggested that this FA is the most efficiently incorporated and/or activated in yeast cells. Compared with the wild type, *faa4Δ* cells exhibited the lowest content of acyl-CoAs synthesized from exogenously fed unsaturated C18 FA. This suggests that the FAA4 gene product plays a major role in

**Table II.** *Acyl-CoA composition of wild-type and FAA yeast mutants transformed with the control plasmid pYES2 before ($t_0$) and 1 h after ($t_1$) incubation in the presence of ω6 and ω3 PUFAs*

Values expressed as mol% of total acyl-CoAs represent the average ± SD for four replicates. nd, Not detected.

| | Wild Type | | | *faa1Δ* | | | *faa3Δ* | | | *faa4Δ* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $t_1$ | | | $t_1$ | | | $t_1$ | | | $t_1$ | |
| Acyl-CoAs | $t_0$ | ω6 | ω3 | $t_0$ | ω6 | ω3 | $t_0$ | ω6 | ω3 | $t_0$ | ω6 | ω3 |
| 14:0 | 1.3 ± 0.1 | 1.0 ± 0.2 | 1.2 ± 0.1 | nd | nd | nd | 1.2 ± 0.4 | 1.3 ± 0.3 | 1.3 ± 0.4 | 1.3 ± 0.4 | 1.4 ± 0.2 | 1.0 ± 0.3 |
| 16:0 | 1.0 ± 0.5 | 1.8 ± 0.2 | 1.5 ± 0.1 | nd | nd | nd | 1.3 ± 0.3 | 2.6 ± 0.3 | 1.6 ± 0.7 | 1.6 ± 0.2 | 2.1 ± 0.5 | 1.7 ± 0.2 |
| 16:1 | 80.7 ± 0.3 | 66.6 ± 0.7 | 75.0 ± 1.2 | 88.6 ± 0.1 | 60.8 ± 4.2 | 76.2 ± 0.9 | 80.4 ± 2.0 | 71.2 ± 3.2 | 79.2 ± 1.4 | 78.4 ± 1.1 | 73.2 ± 1.7 | 79.7 ± 0.3 |
| 18:0 | 0.7 ± 0.1 | 0.5 ± 0.1 | 0.6 ± 0.1 | nd | nd | nd | 0.7 ± 0.1 | 0.6 ± 0.1 | 0.6 ± 0.1 | 0.8 ± 0.1 | 0.8 ± 0.1 | 0.6 ± 0.1 |
| 18:1 | 7.7 ± 0.8 | 6.7 ± 0.5 | 7.2 ± 0.7 | 4.8 ± 0.5 | 3.2 ± 0.5 | 4.6 ± 0.1 | 7.8 ± 0.7 | 6.8 ± 0.7 | 7.8 ± 0.6 | 8.2 ± 0.3 | 8.4 ± 0.8 | 9.6 ± 0.4 |
| 22:1 | 9.2 ± 1.1 | 7.1 ± 0.3 | 8.6 ± 1.0 | 6.7 ± 0.5 | 4.1 ± 1.2 | 4.9 ± 0.1 | 10.1 ± 1.7 | 8.5 ± 1.5 | 6.7 ± 1.1 | 8.6 ± 0.4 | 8.8 ± 1.5 | 6.8 ± 0.3 |
| 18:2n6 | | 10.2 ± 0.2 | | | 18.5 ± 1.4 | | | 6.2 ± 0.3 | | | 3.0 ± 0.2 | |
| 18:3n6 | | 6.1 ± 0.1 | | | 12.7 ± 0.5 | | | 4.4 ± 0.7 | | | 0.7 ± 0.1 | |
| 20:3n6 | | nd | | | nd | | | nd | | | nd | |
| 18:4n3 | | | 6.1 ± 0.8 | | | 12.6 ± 1.2 | | | 4.0 ± 0.3 | | | 0.8 ± 0.2 |
| 20:5n3 (EPA) | | | nd | | | nd | | | nd | | | nd |
| 22:6n3 (DHA) | | | nd | | | nd | | | nd | | | nd |

**Table III.** *Acyl-CoA composition of pYES2 and pYLACSA faa4Δ transformants before ($t_0$) and after different times of incubation in the presence of 18:3n6 and 20:4n6 FAs*

Values, given as mol% of total acyl-CoAs, represent the average ± SD of four replicates. nd, Not detected.

| | | Acyl-CoA | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Time after Feeding | Plasmid | 14:0 | 16:0 | 16:1n7 | 18:0 | 18:1n9 | 18:3n6 | 20:4n6 (ARA) | 22:1n9 |
| $t_0$ | pYES2 | 2.9 ± 0.2 | 2.1 ± 0.0 | 74.4 ± 0.1 | 0.7 ± 0.1 | 17.3 ± 0.5 | nd | nd | 3.2 ± 0.2 |
| | pYLACSA | 5.4 ± 1.1 | 1.9 ± 0.2 | 68.2 ± 2.0 | 0.71 ± 0.1 | 19.7 ± 1.3 | nd | nd | 3.0 ± 0.3 |
| 5 min | pYES2 | 3.4 ± 0.4 | 1.8 ± 0.3 | 69.1 ± 1.9 | 0.8 ± 0.1 | 19.3 ± 1.4 | 1.9 ± 0.3 | nd | 2.9 ± 0.2 |
| | pYLACSA | 6.7 ± 0.8 | 1.8 ± 0.2 | 53.3 ± 1.5 | 0.7 ± 0.2 | 17.1 ± 1.2 | 2.7 ± 0.4 | 15.1 ± 2.4 | 2.9 ± 0.3 |
| 60 min | pYES2 | 4.3 ± 0.3 | 2.8 ± 0.3 | 67.6 ± 0.5 | 1.1 ± 0.1 | 17.3 ± 0.4 | 2.3 ± 0.3 | nd | 3.7 ± 0.4 |
| | pYLACSA | 9.2 ± 0.9 | 3.8 ± 0.3 | 54.1 ± 3.3 | 0.8 ± 0.1 | 12.7 ± 1.1 | 3.4 ± 0.6 | 14.8 ± 1.5 | 3.5 ± 0.2 |
| 24 h | pYES2 | 2.5 ± 0.4 | 8.6 ± 0.8 | 72.0 ± 2.8 | 1.9 ± 0.3 | 7.3 ± 0.5 | 1.5 ± 0.2 | 0.1 ± 0.0 | 5.9 ± 0.7 |
| | pYLACSA | 8.4 ± 0.3 | 16.7 ± 1.9 | 53.3 ± 4.1 | 5.5 ± 0.8 | 4.3 ± 0.2 | 4.0 ± 0.8 | 8.2 ± 1.5 | 2.6 ± 0.5 |

the activation of exogenously fed PUFAs. The corresponding deletion strain was therefore selected as a useful line for heterologous expression studies aimed at identification of genes encoding PUFA synthetase activity on the basis that it has much lower background ACS activity with PUFAs, and zero activity with 20:5n3 and 22:6n3.

### Heterologous Expression of *TplacsA* in Yeast FAA Deletion Strain *faa4Δ*

To establish the function of the TpLACSA protein, the full-length *TplacsA* cDNA was cloned behind the Gal-inducible GAL1 promoter of pYES2 to generate the plasmid pYLACSA. The results of incubation experiments conducted separately in the presence of the ω6 18:3n6 and 20:4n6 and the ω3 18:4n3 and 20:5n3 FAs are presented in Tables III and IV, respectively. After 5 min of incubation, C18 PUFA-CoAs were found in both empty vector control pYES2 and pYLACSA *faa4Δ* transformants, with a higher percentage in the latter. No C20 PUFA-CoAs were detected in the empty vector control *faa4Δ* transformants, in contrast with *faa4Δ* cells containing the *TplacsA* gene. Arachidonic acid (ARA; 20:4n6)-CoA was the most abundant of the PUFA-CoAs measured in pYLACSA transformants, peaking in concentration after a 5-min incubation and then falling to approximately one-half this initial concentration over the following 24 h. The four exogenously fed FAs accumulated in the cells and did not follow the temporal variation exhibited by the corresponding acyl-CoAs (data not shown). C20 PUFA-CoAs were not detected in the empty vector controls after 60 min but were detected 24 h after feeding. C18 ω3 and ω6 FAs followed a similar pattern of accumulation as ARA-CoA in pYLACSA transformants, with values increasing during the first hour of incubation and then decreasing after 24 h. In contrast, mol% of EPA-CoA increased throughout the duration of the experiment. TpLACSA also led to a 2-fold increase in the endogenous saturated 14:0-, 16:0-, and 18:0-CoAs, while 16:1n7- and 18:1n9-CoAs decreased and 22:1n9-CoA was only slightly changed.

### Measurement of ACS Activities by in Vitro Assay

To determine the substrate specificity of TpLACSA directly, several FAs were tested using an assay adapted to measure the enzymatic production of acyl-CoA in the presence of free FAs, ATP, and free-CoA. A commercially available ACS from *Pseudomonas* sp. that utilizes a broad range of FA substrates was included as a positive control. Results shown in Figure 3 confirm the broad specificity of this enzyme. Com-

**Table IV.** *Acyl-CoA composition of pYES2 and pYLACSA faa4Δ transformants before ($t_0$) and after different times of incubation in the presence of 18:4n3 and 20:5n3 FAs*

Values, given as mol% of total acyl-CoAs, represent the average ± SD of four replicates. nd, Not detected.

| | | Acyl-CoA | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Time after Feeding | Construct | 14:0 | 16:0 | 16:1n7 | 18:0 | 18:1n9 | 18:4n3 | 20:5n3 (EPA) | 22:1n9 |
| $t_0$ | pYES2 | 1.4 ± 0.2 | 2.6 ± 0.3 | 76.1 ± 3.1 | 0.7 ± 0.2 | 17.9 ± 2.6 | nd | nd | 4.7 ± 0.3 |
| | pYLACSA | 2.2 ± 0.1 | 1.5 ± 0.2 | 76.1 ± 1.3 | 0.6 ± 0.1 | 16.3 ± 2.0 | nd | nd | 5.6 ± 0.3 |
| 5 min | pYES2 | 1.5 ± 0.1 | 1.5 ± 0.1 | 76.1 ± 2.0 | 0.4 ± 0.1 | 17.9 ± 2.0 | 0.3 ± 0.1 | nd | 4.3 ± 0.3 |
| | pYLACSA | 2.3 ± 0.2 | 1.2 ± 0.0 | 77.3 ± 1.4 | 0.4 ± 0.0 | 13.0 ± 0.5 | 1.3 ± 0.2 | 1.6 ± 0.5 | 6.2 ± 0.3 |
| 60 min | pYES2 | 1.4 ± 0.1 | 2.4 ± 0.6 | 77.1 ± 3.3 | 0.6 ± 0.2 | 17.2 ± 2.7 | 0.5 ± 0.1 | nd | 4.6 ± 0.2 |
| | pYLACSA | 3.3 ± 0.6 | 2.1 ± 0.1 | 78.9 ± 1.7 | 0.5 ± 0.1 | 8.9 ± 0.8 | 2.3 ± 0.7 | 1.4 ± 0.4 | 7.1 ± 0.9 |
| 24 h | pYES2 | 1.2 ± 0.2 | 4.9 ± 0.3 | 85.1 ± 0.7 | 1.1 ± 0.1 | 7.4 ± 0.3 | 0.5 ± 0.1 | 0.2 ± 0.0 | 7.8 ± 0.5 |
| | pYLACSA | 2.8 ± 0.5 | 9.7 ± 0.8 | 69.4 ± 3.9 | 4.1 ± 0.7 | 6.8 ± 0.2 | 1.3 ± 0.2 | 3.6 ± 1.0 | 8.3 ± 1.0 |

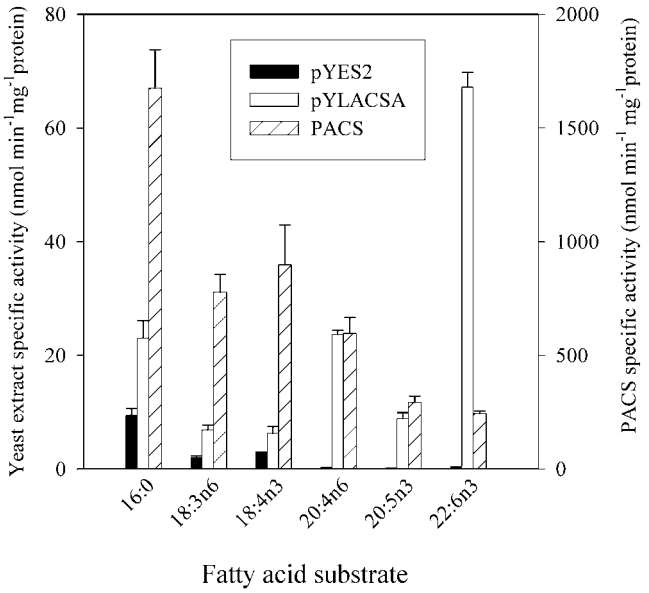


**Figure 3.** LACS enzyme-specific activity measurement from cell-free lysates of overexpressing *faa4Δ* transformants and from PACS (Sigma). Cell-free extracts from yeast containing the plasmid pYES2 (control) and pYLACSA were used as enzyme sources for in vitro LACS assay in parallel with the commercially available PACS. Values represent the average ± SD of acyl-CoA samples from three separate experiments.

parison of specific activities determined in the extract obtained from the pYES2 and the pYLACSA *faa4Δ* transformants showed that TpLACSA is very active on C20 and C22 PUFAs. Effectively, activities were 75- to 198-fold higher for 20:4n6, 20:5n3, and 22:6n3 FAs in the TpLACSA extract compared to the empty vector control, while values in the assays conducted in the presence of palmitic acid or C18 PUFAs only increased by a factor of 2 to 4. Production of acyl-CoAs in the presence of ARA, EPA, and DHA free FAs was barely detectable in the pYES2 yeast extract.

### DHA Storage in Yeast Expressing *TplacsA*

To establish whether the expression of the *TplacsA* gene might result in an increased quantity of 22:6n3 (DHA) stored in yeast storage lipids, total and TAG FAs were extracted from pYES2 and pYLACSA *faa4Δ* transformants after a 4-d incubation at 30°C in the presence of DHA. Table V shows that *faa4Δ* cells containing the *TplacsA* gene showed approximately 6 times the amount of DHA and an associated doubling of total FAs in TAGs on a dry-weight basis compared to the empty vector control. Only a slight increase was observed for endogenous saturated and monounsaturated FAs (data not shown).

## DISCUSSION

Examination of *T. pseudonana* FA and acyl-CoA profiles revealed that ω3 FAs and acyl-CoAs were the most abundant in these diatom cells. These profiles demonstrate that *T. pseudonana* contains the necessary FA synthesis, desaturation, elongation, and acylation mechanisms to accumulate PUFAs in TAGs. However, despite recent advances in understanding the elongation and desaturation mechanisms important for PUFA production in several organisms (Domergue et al., 2003; Meyer et al., 2004; Pereira et al., 2004), little is known about how the production of acyl-CoA substrates required for these reactions may limit or control the ultimate accumulation of FAs in TAGs. In this regard, ACSs are likely to play an important role in regulating the size and composition of the cytoplasmic acyl-CoA pool used as substrates for FA desaturation, and particularly elongation, prior to their incorporation into TAG. Therefore, we were interested in isolating and characterizing the activity of ACS enzymes, particularly LACS enzymes, which would be responsible for providing PUFA-CoA substrates.

From the analysis of the publicly available genome of *T. pseudonana*, eight candidate genes encoding amino acid sequences with high homology to LACS sequences isolated from plants and mammals were assembled. Only two assemblies, *TplacsA* and *TplacsI*, appeared to contain a full-length LACS coding sequence. RT-PCR analysis of their expression showed that both were constitutively expressed during cell cultivation, with the *TplacsI* gene being more abundantly transcribed than *TplacsA*. We focused on *TplacsA* because it was the first putative LACS gene annotated through our in-house analysis. Before attempting the expression of this gene in yeast, we screened several FAA mutants for their ability to activate different PUFAs to their CoA esters. This analysis revealed that Euroscarf strain *faa4Δ* was the most compromised in PUFA activation. In addition, no 14:0-, 16:0-, or 18:0-CoA was produced from endogenous substrates in *faa1Δ* cells, confirming that FAA1 is mainly involved in the activation of these FAs (Knoll et al., 1994; Faergeman et al., 2001).

After expression of *TplacsA* in *faa4Δ* cells, we observed that the encoded protein was involved in the activation of 18:3n6, 20:4n6, 18:4n3, and 20:5n3 FAs. The production of the corresponding acyl-CoAs was rapid, demonstrating the availability of these molecules as intermediates for PUFA elongation during VLCPUFA biosynthesis (Domergue et al., 2003).

**Table V.** *Partitioning of FAs into TAGs in pYES2 and pYLACSA faa4Δ transformants after incubation in the presence of DHA*

Values given are the average ± SD of four replicates.

| Parameters Considered | pYES2 | pYLACSA |
|---|---|---|
| Percent of FA incorporated in TAG | 50 ± 1 | 63 ± 4 |
| Nanomoles of FA incorporated in TAG per milligram dry weight | 140 ± 13 | 272 ± 28 |
| Percent of DHA incorporated in TAG | 54 ± 2 | 81 ± 5 |
| Nanomoles of DHA incorporated in TAG per milligram dry weight | 13 ± 1 | 86 ± 10 |

Presence of the *TplacsA* gene not only affected the production of PUFA-CoAs, but also significantly increased the percentage amount of endogenous saturated acyl-CoAs and decreased that of monounsaturated acyl-CoAs after 1 d of incubation.

The ability of TpLACSA to accommodate a wide range of acyl chain lengths and degrees of desaturation was measured directly in the presence of different types of FAs. The method used to determine the specific activities in the presence of the six substrates tested showed that ARA, EPA, and DHA were the FAs most efficiently activated. Very low levels of activity in yeast extracts from the empty vector controls were observed, as expected from the results of the cofeeding experiments. To our knowledge, this is the first time that a LACS enzyme active on VLCPUFAs has been reported outside mammals. At least nine LACS enzymes have been recently characterized from Arabidopsis (Schnurr et al., 2002; Shockey et al., 2002), but their specificity against VLCPUFAs has not been reported. Bearing in mind that *T. pseudonana* produces high quantities of EPA and DHA, our results suggest that TpLACSA might be involved in the activation of the EPA that is elongated to 22:5n3 by a Δ5-elongase and then desaturated to 22:6n3. After release from phospholipids, this FA can be activated by TpLACSA and incorporated in storage lipids, as suggested by the results of partitioning of fed DHA to TAG in *faa4Δ* transformants. We previously reported that DHA is efficiently partitioned into TAGs in Thalassiosira cells (Tonon et al., 2002).

Isolation of such an enzyme highly active on DHA has potential biotechnological applications. Increasing the quantity of DHA stored in oilseed TAGs will be an important target after the metabolic pathway for DHA synthesis is reconstructed in plants. This goal is now becoming more realistic with the recent reconstitution of EPA synthesis in plants (Abbadi et al., 2004; Qi et al., 2004) and the production of DHA in transgenic yeast (Meyer et al., 2004; Pereira et al., 2004).

## MATERIALS AND METHODS

### Identification of a Set of Genomic DNA Sequences Putatively Encoding LACS

The draft genome of the diatom *Thalassiosira pseudonana* has been sequenced to approximately 9 times coverage by the whole-genome shotgun method. The raw sequence data were downloaded onto a local server from the U.S. Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/). Batch tBLASTn searches were carried out using protein sequences of the following 12 known LACSs as query, including 3 mammalian proteins, mouse MmLACS4 (BC016416), rat RnLACS4 (D85189), and human HsLACS4 (BC034959), and 9 Arabidopsis (*Arabidopsis thaliana*) sequences, AtLACS1 (AF503751), AtLACS2 (AF503752), AtLACS3 (AF503753), AtLACS4 (AF503754), AtLACS5 AF503755), AtLACS6 (AF503756), AtLACS7 (AF503757), AtLACS8 (AF503758), and AtLACS9 (AF503759). All nonredundant sequences with an E-value less than 0.001 were retrieved and assembled into contigs using the CAP3 sequence assembly program (Huang and Madan, 1999). The contigs were translated into amino acid sequences in three frames in the orientation indicated by the tBLASTn result. Eight putative LACS gene models were constructed manually based on sequence homology, and in-frame GT-AG intron boundaries were identified.

### Cultivation of *T. pseudonana*, RNA Extraction, and RT-PCR Analysis

*T. pseudonana* was cultivated as previously described (Tonon et al., 2004). Cell density was monitored by counting cells with a hemocytometer. Nitrate concentration was determined periodically during the culture time by measuring the change of the medium $A_{220}$ (Collos et al., 1999).

Total RNA was extracted from cells harvested at different stages of growth with an RNeasy plant mini kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized from 3 $\mu$g DNAse-treated RNA using a Prostar first-strand RT-PCR kit (Stratagene, La Jolla, CA). PCRs with primer pairs specific of putative Thalassiosira LACS genes *TplacsA* and *TplacsI* were performed using undiluted and 5-fold dilutions of cDNAs as follows: the reactions were heated to 95°C for 5 min followed by 35 cycles at 95°C for 30 s and 30 s at 55°C (*TplacsA*, *18S rRNA*) or 60°C (*TplacsI*), according to the primer pair used, and 72°C for 2 min, then a single step at 72°C for 10 min. The *18S rRNA* gene was used to ensure that the same quantity of cDNA was used for PCR on the different RNA samples. Aliquots of PCR reaction were electrophoresed through a 1% agarose gel.

### Heterologous Expression of *TplacsA* in Yeast

*T. pseudonana* cDNA was synthesized using the SuperScript III Rnase H$^-$ Reverse Transcriptase (Invitrogen, Carlsbad, CA) and used to amplify the entire *TplacsA* coding region with primers TpLACSANH 5′-CCCAAGCTT-ACC**ATG***GCT*ACGAACAAATGGT-3′ (ORF start codon is indicated by bold type; underlined sequence is a *Hin*dIII site; italic sequence is an added Ala codon not present in the original sequence of *TplacsA*) and TpLACSACE 5′-GCGAATTC**TTA**CAACTTGCTCTGTGGAGA-3′ (ORF stop codon is indicated in bold type; underlined sequence is an *Eco*RI site). The Expand Long Template PCR System (Roche, Mannheim, Germany) was employed to minimize potential PCR errors. The amplified product was first cloned using the TOPO TA cloning kit (Invitrogen) and fidelity of the cloned PCR product was checked by sequencing. The recombinant vector was then restricted with *Hin*dIII and *Eco*RI and cloned in the corresponding sites behind the Gal-inducible GAL1 promoter of pYES2 (Invitrogen) to yield the plasmid pYLACSA. The control vectors pYES2 and pYLACSA were then transformed into yeast (*Saccharomyces cerevisiae*) by a lithium acetate method, and transformants were selected on minimal medium plates lacking uracil. Host yeast strains were obtained from the Euroscarf yeast deletion strain collection (Frankfurt): wild-type BY4741 (MATa; his3Δ1; leu2Δ0, met15Δ0; ura3Δ0) and deletion strains Y06477 (YOR317w::kanMX4, *faa1Δ*), Y01401 (YIL009w::kanMX4, *faa3Δ*), and Y00833 (YMR246w::kanMX4, *faa4Δ*). These three mutated strains are congenic to BY4741.

For the feeding and cofeeding experiments, cultures were grown at 25°C or 30°C in the presence of 2% (w/v) raffinose and 1% (w/v) Tergitol NP-40 (Sigma, St. Louis). Expression of the transgene was induced at $OD_{600\ nm}$ 0.2 to 0.3 by supplementing Gal to 2% (w/v). At that time, the appropriate FAs were added to a final concentration of 50 $\mu$M. For acyl-CoA analysis, samples of 3 mL of cells were harvested after 5 min, 1 h, and 24 h of incubation at 25°C. For total content and TAG FA analysis, cells (1.5 mL by sample) were harvested after a 4-d incubation at 30°C.

### Enzyme Overproduction in Yeast and ACS Assays

Cells were grown overnight in minimum medium lacking uracil containing 2% raffinose and 2% Gal. Following growth, cells were harvested by centrifugation and resuspended in 100 mM MOPS, pH 7.5, 0.4 mM EDTA, 5 mM 2-mercaptoethanol, 10% glycerol, 0.01% Triton X-100, and protease inhibitor mix (Sigma). This suspension was then transferred in 2-mL Eppendorf tubes containing 500 $\mu$L acid-washed glass beads (425–600 $\mu$m; Sigma) and cells lysed by bead milling for 1 min, 5 times. Samples were clarified by centrifugation at 18,000*g* and supernatants used to assess acyl-CoA activities. Protein concentration in these enzyme extracts was determined using the Bradford assay and bovine serum albumin as a standard (Bradford, 1976).

ACS activities were determined in yeast cell-free lysates following a protocol adapted from a method based on the use of the *Pseudomonas* sp. acyl-CoA synthetase (PACS; Sigma) to enzymatically synthesize acyl-CoAs from free FAs, ATP, and free-CoA (Taylor et al., 1990). Twenty nanomoles of total free FAs were dried down in a 1.5-mL Eppendorf tube. The assay mixture (final volume 20 $\mu$L) containing 100 mM MOPS, pH 7.5, 10 mM $MgCl_2$, 10 mM ATP, 1 mM dithiothreitol, 0.1% Triton X-100, and 5 mM of CoA was added to the

tubes and sonicated for 5 min. The reaction was initiated by adding 2 $\mu$L of *Pseudomonas* sp. enzyme (Sigma) or the same volume of yeast protein extract in tubes placed in a sonicating bath, and incubation was carried out at 25°C for 25 min. Tubes were sonicated for 5 and 10 min after starting the assay. The reaction was stopped by addition of 100 $\mu$L of 9:2 methanol:chloroform (v/v), 2 $\mu$L of saturated $(NH_4)_2SO_4$, 10 $\mu$L of internal standard (17:0-CoA, stock solution at 0.12 mM), and vortexing. After spinning down 5 min at 18,000*g* to precipitate proteins, 5 $\mu$L of supernatant were transferred to a tapered vial, dried, and 1 mL of chloroacetaldehyde derivitizing buffer was added. Samples were then heated in an oven at 85°C for 20 min and 20 $\mu$L were used for acyl-CoA determination as described below.

## FA and Acyl-CoA Analyses

Yeast and algal cells were harvested by centrifugation. FA and acyl-CoA extraction and measurement were carried out from the same pellet as reported previously (Larson and Graham, 2001; Larson et al., 2002).

For TAG analysis, yeast cells were harvested by centrifugation in preweighed tubes, washed with distilled water, and centrifuged overnight in a speedy vacuum blotter to determine the dry weight. The next day, the pellet was rehydrated with 10 $\mu$L of water, then 10 $\mu$L of tripentadecanoin (5 mg/mL), and 700 $\mu$L of 2:1 chloroform:methanol (v/v) were added. Cells were transferred to a 1.5-mL Eppendorf tube containing 300 $\mu$L of acid-washed glass beads (425–600 $\mu$m; Sigma) and lysed by bead milling twice for 3 min. Extraction and measurement of total FAs and TAG FAs were conducted as described previously (Tonon et al., 2002).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AY730618.

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