New Insight into *Phaeodactylum tricornutum* Fatty Acid Metabolism. Cloning and Functional Characterization of Plastidial and Microsomal Δ12-Fatty Acid Desaturases[^1][^w]

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In contrast to 16:3 plants like rapeseed (*Brassica napus*), which contain α-linolenic acid (18:3[^9,12,15]) and hexadecatrienoic acid (16:3[^7,10,13]) as major polyunsaturated fatty acids in leaves, the silica-less diatom *Phaeodactylum tricornutum* contains eicosapentaenoic acid (EPA; 20:5[^5,8,11,14,17]) and a different isomer of hexadecatrienoic acid (16:3[^6,9,12]). In this report, we describe the characterization of two cDNAs having sequence homology to Δ12-fatty acid desaturases from higher plants. These cDNAs were shown to code for a microsomal and a plastidial desaturase (*PtFAD2* and *PtFAD6*, respectively) and heterologous expression in yeast (*Saccharomyces cerevisiae*) and *Synechococcus* respectively. Using these systems in the presence of exogenously supplied fatty acids, the substrate specificities of the two desaturases were determined and compared with those of the corresponding rapeseed enzymes (*BnFAD2* and *BnFAD6*). The microsomal desaturases were similarly specific for oleic acid (18:1[^9]), suggesting that *PtFAD2* is involved in the biosynthesis of EPA. In contrast, the plastidial desaturase from the higher plant and the diatom clearly differed. Although the rapeseed plastidial desaturase showed high activity toward the ω9-fatty acids 18:1[^9] and 16:1[^7], in line with the fatty acid composition of rapeseed leaves, the enzyme of *P. tricornutum* was highly specific for 16:1[^9]. Our results indicate that in contrast to EPA, which is synthesized in the microsomes, the hexadecatrienoic acid isomer found in *P. tricornutum* (16:3[^6,9,12]) is of plastidial origin.

Diatoms (Bacillariophyceae) represent a significant group of eukaryotic microalgae found in marine and freshwater habitats and in terrestrial environments. In ocean ecosystems, they are thought to be responsible for as much as 25% of the global primary productivity (Scala and Bowler, 2001). Furthermore, they play a key role in the biogeochemical cycling of silica because most of them are surrounded by a highly structured silica cell wall (Tréguer et al., 1995). The plastids of diatoms contain xanthophylls like fucoxanthin as the major accessory pigments for photosynthesis, which give these organisms their brownish color and their denomination as chromophytes (Bhya and Grossman, 1991). Phylogenetically, diatoms are thought to have originated from the engulfment of a photoautotrophic eukaryotic cell, most probably an ancestor of the modern red algae, by a heterotrophic heterokont flagellate (McFadden, 2001). Because of this secondary endocytobiosis, the chromophytic plastids of diatoms are surrounded by four membranes and referred to as “complex plastids.” The two inner membranes of the plastid are thought to represent the original envelope of the plastid, whereas the two outer ones most probably evolved from the plasma membrane of the first endosymbiont and the phagotrophic membrane of the host cell (Kroth and Strotmann, 1999). The transport of nuclear-encoded proteins into such plastids was shown to depend on multisignal presequences and to be a two-step process (Lang et al., 1998).

The object of this study, *Phaeodactylum tricornutum*, is a silica-less diatom mainly known as a potential source for the industrial production of eicosapentaenoic acid (EPA; 20:5[^5,8,11,14,17]; Molina Grima et al., 1996). In the fatty acid profile of *P. tricornutum* shown in Figure 1, EPA (30%), palmitoleic acid (16:1[^9]; 26%), palmitic acid (16:0; 17%), hexadecatrienoic acid (16:3[^6,9,12]; 10%), and myristic acid (14:0; 5%) are the major fatty acids. The first data concerning the fatty

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acid metabolism of *P. tricornutum* were obtained from labeling experiments. Incubation of the diatom with $^{14}$C-acetate suggested that palmitic and oleic acid are the main products of the de novo synthesis (Moreno et al., 1979). Incubation with C18 or C20- $^{14}$C-fatty acids showed that EPA was synthesized by desaturation and elongation of oleic acid (Arao and Yamada, 1994). In Figure 1, oleic acid and all the intermediates of the EPA biosynthetic pathway (18:2 $^{9}$H9004 9,12, 18:3 $^{9}$H9004 6,9,12, 20:3 $^{9}$H251 8,11,14,17) are only present in trace amounts. This may indicate that this organism has developed highly efficient mechanisms to accumulate specifically EPA. Recently, we reported the cloning and characterization of the 5- and the 6-desaturases involved in EPA biosynthesis in *P. tricornutum* (Domergue et al., 2002). Both desaturases are microsomal enzymes, which indicates that several steps involved in EPA biosynthesis are taking place in the endoplasmic reticulum (ER). On the other hand, most of the EPA found in *P. tricornutum* is present in the plastidial glycolipids (Arao et al., 1987; Yongmanitchai and Ward, 1993), suggesting an import of EPA into the plastid after its synthesis in the ER. Although the subcellular origin of EPA appears to be microsomal, that of the hexadecatrienoic acid isomer found in *P. tricornutum*, 16:3 $^{9}$H9004 7,10,13, remains unclear.

In contrast to the fatty acid metabolism of diatoms, that of higher plants is well documented. In higher plants, the de novo fatty acid synthesis is catalyzed in the plastid by a type II (dissociable) fatty acid synthase, leading primarily to the synthesis of 16:0-ACP. Most of this 16:0-ACP is then elongated to 18:0-ACP and desaturated to 18:1 $^{9}$-ACP by a soluble $^{9}$-acyl-ACP desaturase. Genetic and biochemical analyses of mutants of the model plant Arabidopsis have shown that two inter-connected pathways are then responsible for the synthesis of 16:3 $^{7}$,10,13 and 18:3 $^{9}$,12,15, the two major polyunsaturated fatty acids (PUFAs) found in the leaves of 16:3 plants like Arabidopsis and rapeseed (*Brassica napus*; Browse and Somerville, 1991; Wallis and Browse, 2002). The so-called “eukaryotic” pathway is located in the ER and involved in the synthesis of 18:2 $^{9}$,12 and $\omega$-linolenic acid, 18:3 $^{9}$,12,15. In the chloroplast, the “prokaryotic” pathway catalyzes similar reactions, but is also responsible for the entire synthesis of the hexadecatrienoic acid isomer, 16:3 $^{9}$,10,13. Each pathway possesses its own set of $\Delta$12- and $\Delta$15-fatty acid desaturases (FADs), but they differ in both lipid substrates and electron donors. Although the plastidial desaturases of the prokaryotic pathway use primarily glycolipids as acyl-carriers and ferredoxin/ferredoxin oxidoreductase as electron donors, the microsomal desaturases involved in the eukaryotic pathway use phospholipids and cytochrome b5/cytochrome b5 oxidoreductase (Los and Murata, 1998). Such parallel sets of FADs may also exist in the plastidial and ER compartments of diatoms, and the cloning and functional characterization of these activities should help in understanding the origin of the predominating fatty acids found in *P. tricornutum*.

In the present paper, we report the cloning of two $\Delta$12-FADs from *P. tricornutum* and their functional
characterization as microsomal and plastidial desaturase by expression in yeast (*Saccharomyces cerevisiae*) and a cyanobacterium, respectively. The substrate specificity of each desaturase was determined in these heterologous expression systems and compared with those of the corresponding homologs from rapeseed. The microsomal desaturase of *P. tricornutum* was shown to be most active with oleic acid, whereas the plastidial desaturase was highly specific for 16:1\[^{9}\]. It could be concluded from these experiments that the microsomal desaturase is most probably involved in the biosynthesis of EPA, whereas the plastidial enzyme contributes to the synthesis of the hexadecatrienoic acid isomer characteristic to *P. tricornutum*, 16:3\[^{6,9,12}\], which in contrast to EPA, is most probably of prokaryotic origin.

**RESULTS**

**Isolation of Two Δ12-Desaturase cDNA Clones**

Two full-length clones coding for putative Δ12-desaturases were isolated from a *P. tricornutum* cDNA library by mass sequencing. The first clone was 1,651 bp long and contained an open reading frame (ORF) of 1,488 bp, which encoded a polypeptide of 495 amino acids. The second clone was 1,526 bp long, with an ORF of 1,311 bp coding for a polypeptide of 436 amino acids. The two proteins encoded by these ORFs were 28% identical (42% similar), and both showed high sequence similarities to Δ12-desaturases from various organisms, including the rapeseed 6-desaturase (BnFAD2; GenBank accession no. AF243045). This latter was 25% identical (38% similar) to the first *P. tricornutum* clone and 35% identical (49% similar) to the second one. When the N-terminal amino acid sequences were analyzed for the presence of targeting signals (Emanuelsson et al., 2000), the protein encoded by the first clone was predicted to be of plastidial origin. With respect to the nomenclature developed for such enzymes in Arabidopsis (Falcone et al., 1994; Okuley et al., 1994), the first and second ORFs identified in *P. tricornutum* were annotated as *PtFAD6* and *PtFAD2*, respectively.

Figure 2 shows the amino acid sequences of the proteins encoded by *PtFAD2* and *PtFAD6* together with the plastidial and microsomal homologs from rapeseed (BnFAD6 and BnFAD2, respectively). All these proteins contain the three conserved His clusters most likely involved in the coordination of the diiron center of the active site (Shanklin and Cahoon, 1998) and the four potential transmembrane helices of the topological model developed for membrane-bound desaturases (Shanklin et al., 1994). Both FAD6 proteins contain an N-terminal extension, the one of *PtFAD6* being longer, as expected for the bipartite structure of the presequence encountered in diatoms (Lang et al., 1998).
Presequence Analysis and Expression of Enhanced Green Florescent Protein (EGFP) Fusion Proteins

The presequence of PtFAD6 contains the two domains that are typical for the import of nuclear-encoded proteins into the complex plastids of diatoms. The N-terminal domain of PtFAD6 is basic with an Arg in the third position and contains a hydrophobic portion (Fig. 2, broken line) similar to a classical ER-targeting signal. This sequence is followed by a domain rich in hydroxylated amino acids (Ser and Thr; Fig. 2, dots), which is characteristic for transit peptides involved in the transport into the plastid.

To confirm the cellular localization of PtFAD6, the DNA sequence encoding the first 113 amino acids (Fig. 2, arrow) was fused with the EGFP gene and cloned in pPha-T1. The resulting vector was used to transform *P. tricornutum* (see “Materials and Methods”). As shown in Figure 3, the EGFP fluorescence was colocalized with the red fluorescence of chlorophyll in the plastid. Although the EGFP fluorescence was not evenly distributed within the plastid, but most probably accumulated within the pyrenoid, these results strongly support that PtFAD6 is a plastidial desaturase. In contrast, when the first 55 amino acids of PtFAD2 were fused to the N terminus of EGFP, the fluorescence appeared to be cytoplasmic (data not shown).

Functional Expression of the Desaturases in Yeast

The ORF of the putative microsomal desaturase PtFAD2 was cloned in the yeast expression vector pYES2 (Invitrogen, Leek, Netherlands) and expressed in yeast to confirm its enzymatic activity. Using the empty vector pYES2 as control and pYES2-BnFAD2 for comparison, the different constructs were transformed into the yeast strain C13ABYS86 (Bröker et al., 1991) and expressed for 48 h at 20°C. The yeast cells transformed with the empty vector pYES2 showed the typical yeast fatty acids (16:0, 16:1, 18:0, and 18:1) and traces of 17:0 and 17:1 (Fig. 4A, top). The expression of PtFAD2 and BnFAD2 resulted in two additional peaks corresponding to 16:2 and 18:2 (Fig. 4A, middle and bottom, respectively). The high proportions of 18:2 were correlated with a significant decrease of 18:1 as expected for an educt-product relation.

**Figure 3.** Expression of the PtFAD6-EGFP fusion protein in *P. tricornutum*. The DNA sequence encoding the first 113 amino acids of PtFAD6 was fused to the 5’ end of the EGFP gene and cloned in pPha-T1. The resulting vector was used to transform *P. tricornutum* UTEX 646 as indicated in “Materials and Methods.” Top, Light microscopical image of *P. tricornutum* with the plastids visible in brown. Bottom, Red (chlorophyll) and green (EGFP) fluorescence. Scale bar = 10 μm.
ship. PtFAD2 desaturated as much as 51% of oleic acid and about 14% of palmitoleic acid, whereas BnFAD2 was slightly less active, converting 40% and 8% of oleic and palmitoleic acid, respectively.

The yeast expression system was then used to determine the substrate specificity of PtFAD2 in more detail. The most efficiently desaturated substrate was oleic acid (50% conversion), but 16:1\(^{\Delta 9}\) and 17:1\(^{\Delta 9}\) were also accepted as substrates (15% and 22% conversion to 16:2\(^{\Delta 9,12}\) and 17:2\(^{\Delta 9,12}\), respectively; Table 1). No activity was measured with 18:1\(^{\Delta 11}\) or 22:1\(^{\Delta 13}\) but about 4% of 20:1\(^{\Delta 11}\) was converted to 20:2\(^{\Delta 11,14}\).

Similar results were obtained with BnFAD2 (data not shown), indicating that PtFAD2 has the substrate specificity typical for FAD2 enzymes. Although such enzymes can be denominated as \(\omega 6\)- or \(\Delta 12\)-

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**Figure 4.** Fatty acid profiles of transgenic yeast expressing microsomal (A) and plastidial (B) desaturases. The C13ABYS86 yeast strain was transformed with the indicated constructs. The transformants were grown for 48 h at 20°C, and FAMEs from whole cells were prepared and analyzed by gas-liquid chromatography (GLC) as indicated in "Materials and Methods." The traces in B were magnified to show the small peaks representing the products of the plastidial desaturases, with the consequence that the peaks corresponding to 16:1\(^{\Delta 9}\) and 18:1\(^{\Delta 9}\) go off scale.
desaturases, it should be noted that they convert 16:1\(^{9}\) to 16:2\(^{9,12}\), 17:1\(^{9}\) to 17:2\(^{9,12}\), and 20:1\(^{11,14}\) to 20:2\(^{11,14}\) (Table I), inserting the new double bond in the \(\omega-4\), \(\omega-5\), and \(\omega-6/\omega-14\)-position, respectively. Therefore, a correct assignment of the regioselectivity for FAD2 enzymes would be \(\mu + 3\) because the position of the incipient double bond is determined relative to a preexisting double bond by placing it three carbons closer to the methyl end (Meesapyodsuk et al., 2000).

Interestingly, when the FAD6 desaturases from \textit{P. tricornutum} and rapeseed were expressed in yeast, small but significant proportions of 16:2\(^{9,12}\), 17:1\(^{9}\) to 17:2\(^{9,12}\), and 20:1\(^{11,14}\) to 20:2\(^{11,14}\) (Table I), inserting the new double bond in the \(\omega-4\), \(\omega-5\), and \(\omega-6/\omega-14\)-position, respectively. Therefore, a correct assignment of the regioselectivity for FAD2 enzymes would be \(\mu + 3\) because the position of the incipient double bond is determined relative to a preexisting double bond by placing it three carbons closer to the methyl end (Meesapyodsuk et al., 2000).

### Table 1. Substrate specificity of PtFAD2 expressed in Saccharomyces cerevisiae

<table>
<thead>
<tr>
<th>Substrate Total Fatty Acids (Educt)</th>
<th>Product Total Fatty Acids</th>
<th>Desaturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{16}:1^{9}) 38.4 ± 1.6</td>
<td>16:2(^{9,12}) 6.6 ± 0.5</td>
<td>14.7</td>
</tr>
<tr>
<td>(^{17}:1^{9}) 2.9 ± 0.2</td>
<td>17:2(^{9,12}) 0.8 ± 0.1</td>
<td>22.3</td>
</tr>
<tr>
<td>(^{18}:1^{9}) 15.9 ± 1.1</td>
<td>18:2(^{9,12}) 16.1 ± 0.9</td>
<td>50.3</td>
</tr>
<tr>
<td>(^{18}:1^{11}) 36.6 ± 2.2</td>
<td>18:2(^{11,14}) 0</td>
<td>0</td>
</tr>
<tr>
<td>(^{20}:1^{11,14}) 9.5 ± 0.9</td>
<td>20:2(^{11,14}) 0.4 ± 0.1</td>
<td>4.1</td>
</tr>
<tr>
<td>(^{22}:1^{11,14}) 2.0 ± 0.3</td>
<td>22:2(^{11,16}) 0</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^{a}\) In the absence of exogenously fed fatty acid. \(^{b}\) In the presence of 2 mM exogenously fed \(17:0\). \(^{c}\) In the presence of 2 mM exogenously fed fatty acid.

The data presented in Figure 5 suggest that the favorite substrates of PtFAD6 and BnFAD6 differed and were 16:1\(^{9}\) and 18:1\(^{9}\), respectively, in agreement with the results obtained in yeast (Fig. 4B). Nevertheless, the origin of 18:2\(^{11,14}\) remained unclear. To clarify the origin of 18:2\(^{11,14}\) and to determine the substrate specificity of each desaturase in more detail, transgenic \textit{Synechococcus} cultures were supplemented with 16:1\(^{9}\), 18:1\(^{9}\), or 18:1\(^{11}\), and grown for 1 week before fatty acid analysis (Fig. 6). In \textit{Synechococcus} that had been transformed with pFP1 as control (Fig. 6, top), exogenously supplied 16:1\(^{9}\) increased only slightly the 16:1\(^{9}\) and 18:1\(^{11}\) content, confirming that 18:1\(^{11}\) results from the elongation of 16:1\(^{9}\). The fact that supplying 16:1\(^{9}\) had only a slight impact on the fatty acid composition of \textit{Synechococcus} wild type was probably due to the high proportion of the endogenous 16:1\(^{9}\) (about 35%) present in the wild-type strain. In contrast, the proportion of 18:1\(^{9}\), which represents only about 4% of the total fatty acids in the wild-type strain, was increased more than 7 times.

### Functional Expression of the Desaturases in Synechococcus PCC 7942

For the functional characterization of the putative plastidial FADs, the \textit{Synechococcus} R2-PIM8 strain was used as a heterologous expression system (Fig. 5). \textit{Synechococcus} transformed with the empty vector pFP1 presented a rather simple fatty acid profile with 16:0, 16:1\(^{9}\), 18:1\(^{9}\), and 18:1\(^{11}\) as the major fatty acids, whereas 18:0 was present in trace amounts (Fig. 5, top). When \textit{Synechococcus} had been transformed with pFP1-PtFAD2, the fatty acid profile did not change, indicating that the microsomal desaturase was not active in the cyanobacterium. In contrast, transformation with pFP1-BnFAD6 resulted in the expression of an active desaturase as indicated by the presence of three new fatty acids (Fig. 5, middle) corresponding to 16:2\(^{9,12}\), 18:2\(^{9,12}\), and 18:2\(^{11,14}\) as characterized by GLC-MS analysis of the 4,4-dimethylloxazoline derivatives. The expression of BnFAD6 resulted in the conversion of nearly all of the 18:1\(^{9}\) to 18:2\(^{9,12}\), whereas only a small proportion of 16:1\(^{9}\) was desaturated to 16:2\(^{9,12}\). The presence of 18:2\(^{11,14}\) could result either from the desaturation of 18:1\(^{11}\) or from the elongation of 16:2\(^{9,12}\), similar to the formation of 18:1\(^{11}\) from 16:1\(^{9}\). When PtFAD6 was expressed in \textit{Synechococcus}, the same new fatty acids were detected but in different proportions (Fig. 5, bottom). 18:2\(^{9,12}\) was present in a very low percentage, whereas 16:2\(^{9,12}\) and 18:2\(^{11,14}\) were highly accumulated at the expense of 16:1\(^{9}\) and 18:1\(^{11}\).

### Substrate Specificity of FAD6 Desaturases

The data presented in Figure 5 suggest that the favorite substrates of PtFAD6 and BnFAD6 differed and were 16:1\(^{9}\) and 18:1\(^{9}\), respectively, in agreement with the results obtained in yeast (Fig. 4B). Nevertheless, the origin of 18:2\(^{11,14}\) remained unclear. To clarify the origin of 18:2\(^{11,14}\) and to determine the substrate specificity of each desaturase in more detail, transgenic \textit{Synechococcus} cultures were supplemented with 16:1\(^{9}\), 18:1\(^{9}\), or 18:1\(^{11}\), and grown for 1 week before fatty acid analysis (Fig. 6). In \textit{Synechococcus} that had been transformed with pFP1 as control (Fig. 6, top), exogenously supplied 16:1\(^{9}\) increased only slightly the 16:1\(^{9}\) and 18:1\(^{11}\) content, confirming that 18:1\(^{11}\) results from the elongation of 16:1\(^{9}\). The fact that supplying 16:1\(^{9}\) had only a slight impact on the fatty acid composition of \textit{Synechococcus} wild type was probably due to the high proportion of the endogenous 16:1\(^{9}\) (about 35%) present in the wild-type strain. In contrast, the proportion of 18:1\(^{9}\), which represents only about 4% of the total fatty acids in the wild-type strain, was increased more than 7 times.
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Figure 5. Fatty acid profiles of transgenic Synechococcus expressing plastidial desaturases. The Synechococcus R2-PIM8 strain was transformed with the indicated constructs. The transformants were grown at 23 °C, and FAMES from whole cells were prepared and analyzed by GLC as indicated in "Materials and Methods."

upon exogenous supply of 18:1Δ9. Finally, supplementing 18:1Δ11 doubled its proportion, reaching about 40% of the total fatty acids. In Synechococcus expressing BnFAD6 (Fig. 6, middle), exogenously supplied 16:1Δ9 barely changed the fatty acid composition, whereas supply of 18:1Δ9 resulted in a dramatic increase of 18:2Δ11,14, indicating that BnFAD6 had desaturated more than 87% of 18:1Δ9. In the presence of 18:1Δ11, the proportion of 18:1Δ11 was increased about 3 times but 18:2Δ11,14 remained nearly unchanged. When Synechococcus had been transformed with pFP1-PtFAD6 (Fig. 6, bottom), supplementing 16:1Δ9 doubled the proportions of 18:1Δ11 but did not change that of 16:1Δ9, 16:2Δ9,12, or 18:2Δ11,14. In the presence of 18:1Δ9, although the proportion of 18:2Δ9,12 was increased, 18:1Δ9 accumulated to high levels, indicating that PtFAD6 had desaturated only 20% of the 18:1Δ9. When 18:1Δ11 was supplemented, the 18:2Δ11,14 percentage was lowered by about 50%, although the 18:1Δ11 proportion had been increased by more than 4 times, suggesting that 18:2Δ11,14 was produced by elongation of 16:2Δ9,12 rather than by desaturation of 18:1Δ11. This was further supported by supplying exogenous 16:2Δ9,12 to Synechococcus transformed with pFP1. Although 16:2Δ9,12 was very poorly incorporated, 30% to 40% of it was converted to 18:2Δ11,14 in the absence of any Δ12-desaturase, confirming the presence in Synechococcus of an endogenous elongation activity converting 16:2Δ9,12 to 18:2Δ11,14. If we consider that none of the 18:2Δ11,14 was due to the action of PtFAD6 on 18:1Δ11, then more than 70% of 16:1Δ9 had been desaturated by PtFAD6.

The specificity of both desaturases was further evaluated by feeding other potential substrates for ω6-desaturation to the transgenic Synechococcus cultures (Table II). Both desaturases did not accept 22:1Δ13 as substrate, in contrast to previous data obtained in vitro for the plastidial desaturase from spinach (Spinacia oleracea; Schmidt and Heinz, 1993). Nevertheless, because the authors used an ω6-desaturase solubilized by Triton X-100 from chloroplast envelopes, the high activity with 22:1Δ13 obtained in this study may be artifactual and due to the presence of detergent in the assays. Similar to the FAD2 desaturases (Table I), BnFAD6 converted about 19% of 20:1Δ11 to 20:2Δ11,14, but PtFAD6 did not display activity toward that fatty acid. Because the plastidial desaturases of the 16:3 higher plants like rapeseed are involved in the synthesis of 16:3Δ7,10,13, 16:1Δ7 was chemically synthesized and tested as substrate. In Synechococcus transformed with pFP1, 16:1Δ7 accumulated to about 10% of the total fatty acids and was most probably not elongated to 18:1Δ9 because the content of oleic acid was not increased (data not shown). In the transgenic cyanobacteria expressing PtFAD6, only about 5% of 16:1Δ7 was converted to 16:2Δ7,10, whereas upon expression of BnFAD6, more than 80% of 16:1Δ7 was desaturated to 16:2Δ7,10 (Table II). The results presented in Table II clearly show that PtFAD6 is highly specific for 16:1Δ9, whereas BnFAD6 is as active with 16:1Δ9 as with 18:1Δ9, in line with its involvement in the synthesis of the two majors PUFAs of rapeseed leaves, 16:1Δ7,10,13 and 18:3Δ9,12,15. Similar to the microsomal desaturases, both plastidial enzymes display ω6 as well as ω4 activity, as already suggested by Hitz et al. (1994) for BnFAD6. It can be added that 16:1Δ7 was not accepted as substrate by the microsomal desaturases (PtFAD2 and BnFAD2) expressed in yeast (data not shown).

DISCUSSION

In this paper, we report the cloning and functional characterization of the microsomal and the plastidial Δ12-desaturase from the diatom P. tricornutum, PtFAD2 and PtFAD6, respectively. Similar to FADs from other species, both enzymes contain the three His clusters most likely coordinating the diiron center of the active site as well as long hydrophobic
stretches involved in membrane binding (Fig. 2; Shanklin et al., 1994). Although PtFAD2 presents moderate sequence homology to microsomal desaturases from higher plants (about 35% identity/50% homology), PtFAD6 has no homology to the corresponding plastidial desaturases (less than 14% identity/27% homology). As shown in the phylogenetic tree of desaturases presented in Figure 7, PtFAD2 and PtFAD6 form a separate branch between the microsomal desaturases from fungi (Mucor rouxii and Mortierella alpina) and another group containing the ω3- and ω6-desaturases of the nematode Caenorhabditis elegans (FAT1 and FAT2, respectively). The cyanobacterial Δ12-desaturase (DesA) groups together with the plastidial Δ12-desaturases from higher plants (Fig. 7) in line with the phylogenetic origin of

Figure 6. Fatty acid composition of transgenic Synechococcus expressing plastidial desaturases in the presence or absence of different exogenous fatty acids. The Synechococcus R2-PIM8 strain was transformed with the indicated constructs. The transformants were grown for a week at 23°C in the presence or absence of 75 μM 16:1Δ9, 18:1Δ9, or 18:1Δ11, and FAMEs from whole cells were prepared and analyzed by GLC as indicated in “Materials and Methods.” Each value is the mean ± SD from three to five independent experiments.
results are in agreement with the recent analysis of about 1,000 expressed sequence tags (ESTs) from \textit{P. tricornutum}, which has shown that many sequences were more similar to animals than to plant counterparts, reflecting the different phylogenetic histories of diatoms and higher plants (Scala et al., 2002).

Because of their origin via secondary endocytobiosis, the plastids of the chromophytic diatoms are surrounded by four membranes. The transport of nuclear-encoded proteins into such complex plastids is a two-step process that relies on the bipartite structure of the targeting signal (Lang et al., 1998). The N-terminal sequence of the plastidial desaturase of \textit{P. tricornutum} PtFAD6 (Fig. 2) contains a typical signal peptide for cotranslational transport through the ER membranes and a transit peptide for posttranslational protein targeting into the plastid. This bipartite presequence is sufficient to target EGFP into the plastid (Fig. 3). In contrast, fusing the N-terminal sequence of the microsomal desaturase PtFAD2 to EGFP led to cytoplasmic fluorescence (data not shown). In another study (Apt et al., 2002), the fusion of the N-terminal sequence of a luminal protein to EGFP led to fluorescence within a network of membranes most probably representing the ER. In accordance, the N-terminal extremity of PtFAD2 preceding the first transmembrane domain (Fig. 2) seemed to contain no information for a localization within the microsomes. It should be added that the three microsomal desaturases from \textit{P. tricornutum} characterized so far, PtFAD2 (this study), PtD5, and PtD6 (Domergue et al., 2002), do not contain the putative ER retention signal for diatoms, DDEL, at their C terminus. Immunocytological studies have shown that the FAD2 desaturases from higher plants are localized in the ER and face the cytosol (Dyer and Mullen, 2001). If PtFAD2 is similarly located in the ER, the information for its localization is most likely present within the transmembrane domains of the protein sequence.

The functional characterization of PtFAD2 and PtFAD6 in yeast and \textit{Synechococcus}, respectively, confirmed that the former enzyme is a microsomal desaturation that uses cytochrome b5 and cytochrome b5 oxidoreductase as electron donors, whereas the latter desaturase is a plastidial protein requiring ferredoxin and ferredoxin oxidoreductase for electron donation. In addition, these heterologous expression systems enabled a detailed study of substrate specificities (Table I and II, respectively), which in turn led to a better understanding of the fatty acid metabolism in \textit{P. tricornutum}. The clear preference of PtFAD2 for 18:1\textsuperscript{9} (50% conversion to 18:2\textsuperscript{9,12}; Table I) fits with previous labeling experiments that had shown that EPA (20:5\textsuperscript{6,8,11,14,17}) was synthesized by desaturation and elongation of oleic acid (Arao and Yamada, 1994). Together with the recently cloned Δ5- and Δ6-desaturases (Domergue et al., 2002), PtFAD2 is the third microsomal enzyme involved in the biosynthesis of EPA to be characterized. It should be added

### Table II. Substrate specificity of the plastidial desaturases BnFAD6 and PtFAD6 expressed in \textit{Synechococcus}

\textit{Synechococcus} was transformed with pFP1-BnFAD6 or pFP1-PtFAD6 and grown for 7 d at 23°C in the presence of different fatty acid substrates (75 μM) in separate experiments. FAMES from the whole cells were prepared and analyzed by GLC as indicated in “Materials and Methods.” Desaturation (percentage) was calculated as ([product(s)] \times 100)/[educt + product(s)] using values corresponding to percent of total fatty acids. Each value is the mean ± sd from three to five independent experiments.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product(s)</th>
<th>BnFAD6</th>
<th>PtFAD6</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:1\textsuperscript{Δ7}</td>
<td>16:2\textsuperscript{Δ7,10}</td>
<td>79.6 ± 5.8</td>
<td>5.2 ± 0.8</td>
</tr>
<tr>
<td>16:1\textsuperscript{Δ9}</td>
<td>16:2\textsuperscript{Δ9,12}, 18:2\textsuperscript{Δ11,14α}</td>
<td>15.5 ± 1.9</td>
<td>70.3 ± 3.1</td>
</tr>
<tr>
<td>18:1\textsuperscript{Δ9}</td>
<td>18:2\textsuperscript{Δ9,12}</td>
<td>87.3 ± 4.9</td>
<td>20.6 ± 2.5</td>
</tr>
<tr>
<td>20:1\textsuperscript{Δ11}</td>
<td>20:2\textsuperscript{Δ11,14}</td>
<td>19.4 ± 2.2</td>
<td>0</td>
</tr>
<tr>
<td>22:1\textsuperscript{Δ13}</td>
<td>22:2\textsuperscript{Δ13,15}</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Due to the endogenous elongation of the desaturase product 16:2\textsuperscript{Δ9,12}.

The large separation between DesA and PtFAD6 does not suggest that the latter one is derived from a cyanobacterial symbiont. On the other hand, it is accepted that a single primary endosymbiosis event represents the origin for the evolution of both the green (and the higher plants) and the red algae (Moreira et al., 2000). Because the evolution of both the green (and the higher plants) and the red algae (more than 1,000 expressed sequence tags (ESTs) from \textit{P. tricornutum}) may have a more complicated history. Because PtFAD6 is more similar to ER-localized desaturases, it could have evolved from the microsomal Δ12-desaturase of the host of either the first or the second endosymbiosis. In the latter case, the gene of the microsomal desaturase of the heterotrophic flagellate that engulfed the photoautotrophic eukaryote could have been duplicated and the protein encoded by one copy redirected to the newly acquired plastid. A similar gene duplication may have given rise to the \textit{a3-} and \textit{a6-} desaturase of \textit{C. elegans} (FAT1 and FAT2, respectively; Fig. 7) and is supported by the presence of conserved intron-exon junctions in both genes (Napier and Michaelson, 2001). Although the phylogenetic relationship of PtFAD2 and PtFAD6 looks similar to that of CeFAT1 and CeFAT2 in Figure 7, the two Δ12-desaturases from \textit{P. tricornutum} are much more similar and the absence of introns in the genomic sequences (data not shown) does not provide additional support for a similarly late gene duplication. It could also have happened that the gene of the microsomal Δ12-desaturase from the ancestral red algae, the host of the first endosymbiont, has been transferred to the nuclear genome of the second host and that, after appropriate modifications, the product of its expression was targeted back to the first host, which during evolution became the complex plastid of \textit{P. tricornutum}. Whatever the true origin of PtFAD6, these results are in agreement with the recent analysis of about 1,000 expressed sequence tags (ESTs) from \textit{P. tricornutum}, which has shown that many sequences were more similar to animals than to plant counterparts, reflecting the different phylogenetic histories of diatoms and higher plants (Scala et al., 2002).
that the different intermediates of the EPA biosynthetic pathway were only present in trace amounts in the fatty acid profile obtained from *P. tricornutum* cells (Fig. 1) but found in much more abundant proportions in phospholipids, particularly in phosphatidylcholine (Arao et al., 1987). In contrast, the proportion of EPA was about the same in phosphatidylcholine, phosphatidyglycerol, and the total fatty acids (30%; Arao et al., 1987). From this, it may be concluded that in addition to the Δ12-, Δ6-, and Δ5-desaturation steps required for EPA biosynthesis, the Δ6-elongation and the ω3-desaturation steps are taking place in the microsomal fraction (Fig. 8). In contrast, the high specificity of PtFAD6 for 16:1Δ9 (Table II) suggests that 16:2Δ9,12 and most probably 16:3Δ6,9,12, the second major PUFA found in *P. tricornutum*, are synthesized within the plastid, similar to the hexadecatrienoic acid isomer found in 16:3 plants. Although 16:2Δ9,12 could leave the plastid, be converted to 16:3Δ6,9,12 by a microsomal Δ6-desaturase, and then re-incorporated into the plastid, this scenario seems unlikely. Because the microsomal Δ6-desaturase PtD6 is highly active with 18:2Δ9,12 but poorly active with both 16:1Δ9 (Domergue et al., 2002) and 16:2Δ9,12 (data not shown), we rather speculate that *P. tricornutum* contains a plastidial Δ6-desaturase that is involved in the synthesis of 16:3Δ6,9,12 (Fig. 8). It should be added that among the 3,860 ESTs that have been identified, no ESTs corresponding to an acylACP desaturase, to a plastidial Δ6 desaturase, or to an ω3-desaturase were detected, and only one sequence having similarities to mammalian stearoyl-CoA desaturase was detected. Nevertheless, this clone was not full length and functionally characterized; therefore, its role in the fatty acid metabolism of *P. tricornutum* remains to be studied. In accordance, the number of Δ9-desaturases present in *P. tricornutum* remains unclear: Is there a microsomal Δ9-desaturase specific for 18:0 and a plastidial Δ9-desaturase specific for 16:0, or is a single desaturase in the plastid responsible for the synthesis of both 16:1Δ9 and 18:1Δ9 as in cyanobacteria? Alternatively, a soluble Δ9-acyl-ACP desaturase and a membrane-bound Δ9-acyl-lipid desaturase, responsible for the synthesis of 18:1Δ9 and 16:1Δ9, respectively, could co-exist in the plastid of diatoms, similar to the situation found in higher plants.

Although the expression of a rapeseed FAD6 cDNA in *Synechococcus* has already been achieved (Hitz et al., 1994), this is the first time, to our knowledge, that the substrate specificity of a plastidial desaturase is characterized in detail. The use of the *Synechococcus* R2-PIM8 strain as a heterologous expression system was instrumental in this, as evidenced by the high desaturation activities reported in Figures 5 and 6 and in Table II. These results confirm the strong activity of the *nptII* promoter in *Synechococcus* and demonstrate the usefulness of the R2-PIM8 strain for the functional characterization of plastidial enzymes involved in fatty acid metabolism. The substrate specificity of BnFAD6 reported in Table II confirms that the plastidial Δ12-desaturases of 16:3 plants are involved in the synthesis of the two trienoic fatty acids found in the leaves of such plants, 16:3Δ7,10,13 and 18:3Δ9,12,15. In addition, the high activity of the microsomal desaturase BnFAD2 with 18:1Δ9 (Fig. 4A) is in agreement with the involvement of this enzyme in the synthesis of 18:2Δ9,12 in the ER. In 16:3 plants like rapeseed, two glycerolipid biosynthetic pathways co-exist: The prokaryotic pathway in the plastid leads to the synthesis of glycolipids with 18:3Δ9,12,15 and 16:3Δ7,10,13 at the sn-1 and sn-2 position, respectively, whereas the eukaryotic pathway in
the ER is contributing to the synthesis of glycolipids with 18:3\(^{19,12,15}\) at both positions (Browse and Somerville, 1991; Wallis and Browse, 2002). In *P. tricornutum*, the most abundant glycolipids contain EPA and unsaturated 16 carbon fatty acids at the sn-1 and sn-2 position, respectively (Fig. 8; Arao et al., 1987; Yongmanitchai and Ward, 1993), which is considered to be a typically prokaryotic diacylglycerol backbone. Such a denomination is nevertheless ambiguous in the case of *P. tricornutum* because the results presented in this study demonstrate that the 16 carbon fatty acids at the sn-2 position are most probably synthesized exclusively by a plastidial prokaryotic pathway, whereas EPA at the sn-1 position is of eukaryotic origin. Whether EPA is imported into the chromoplast of *P. tricornutum* as a free fatty acid in reversal of the fatty acid export from plastids, or whether it is linked to a glycerol backbone similar to the situation prevailing in higher plants, deserves further investigation. The lipid exchange between plastids and other compartments may differ to some extent regarding nature of intermediates and direction of transport when looking at organisms such as algae, which differ in fatty acid diversity from the monotonous situation encountered in higher plants.

**MATERIALS AND METHODS**

**Materials**

Restriction enzymes, polymerases, and DNA-modifying enzymes were obtained from New England BioLabs (Frankfurt) unless indicated otherwise. All other chemicals were from Sigma (St. Louis).

**Culture of Phaeodactylum tricornutum**

*P. tricornutum* UTEX 646 was grown in brackish water medium (Schloesser, 1993) at 23°C with moderate shaking under long-day light conditions (35 h at 30 \( \mu \)E m\(^{-2} \) s\(^{-1} \)). Cells were harvested by centrifugation, washed with water, and used for fatty acid analysis.

**Isolation of *P. tricornutum* cDNA Clones**

A *P. tricornutum* cDNA library was constructed and subjected to random sequencing as previously described (Domergue et al., 2002). Among the 3,860 nonredundant clones obtained, three sequences presented high homologies to various \( \Delta \)2-desaturases and were fully sequenced. Two clones originating from a single gene overlapped, but P80107//2031 and P80107//2030 differed and each contained a full-length ORF.

**Isolation of Rapeseed Brassica napus cDNA Clones**

Using an excised rapeseed cv Askari cDNA library as template and primers designed according to the sequences available in databases, clones corresponding to the rapeseed plastidial and microsomal desaturases...
(BnFAD6 and BnFAD2, respectively) were amplified by PCR and sequenced. The nucleotide sequences of the clones BnFAD6 and BnFAD2 presented in this study were 95% and 96% identical to that of the rapeseed plastidial ω6-desaturase (accession no. L29214) and that of the rapeseed Δ12-olote desaturase (accession no. AF243045), respectively. The deduced proteins differed slightly from those already published (accession nos. AAF78778 and AAA50157). BnFAD2 presented three amino acid changes (T20N, A246V, and L266F) whereas BnFAD6 had eight substitutions at the N terminus (Q17H, C18S, P32Q, L53F, F66S, N76S, D79H, and E83D), respectively.

**Functional Characterization in Yeast (Saccharomyces cerevisiae)**

For functional characterization, the four desaturase sequences were cloned in the yeast expression vector pYES2 (Invitrogen). The ORFs of BnFAD6, PtFAD2, and PtFAD6 were modified by PCR to create BamHI and Xhol restriction sites adjacent to the start and stop codons, respectively, and to insert the yeast consensus sequence for enhanced translation (Donahue and Cigan, 1990) in front of the start codon. The ORF of BnFAD6 was similarly amplified but with KpnI and Xhol sites. All these PCR products were cloned into the pGEM-T vector (Promega, Madison, WI), and the ORFs were released by BamHI/Xhol (or KpnI/Xhol) digestion. Cloning of the PtFAD2, PtFAD6, BnFAD6, and BnFAD6 ORFs in pYES2 using the same sites yielded pYES2-PtFAD2, pYES2-PtFAD6, pYES2-BnFAD6, and pYES2-BnFAD6, respectively.

**Functional characterization in Synechococcus**

The Synechococcus PCC7972 strain R2-PIM8, which contains an integrative vector pFP1. The PFP1-PtFAD2, PtFAD6, and BnFAD6 ORFs were cut out of pYES2-PtFAD2, pYES2-PtFAD6, and pYES2-BnFAD6, respectively, by BamHI/Xhol digestion, blunt ended with Klenow enzyme, and phosphorylated with T4 polynucleotide kinase. Each ORF was then inserted in the correct orientation in pFP1-1 opened with Smal, yielding pFP1-PtFAD2, pFP1-PtFAD6, and pFP1-BnFAD6, respectively.

The Synechococcus R2-PIM8 strain was cultured in BG11 medium (Rippka et al., 1979) supplemented with 30 μg mL⁻¹ Met and 10 μg mL⁻¹ streptomycin at 23°C with moderate shaking and under long-day light conditions (15 h at 30 μmol m⁻² s⁻¹). Transformation was conducted according to Windholz et al. (1994), and transformants were selected on BG11 plates (1.5% [w/v] Bacto-agar) containing 30 μg mL⁻¹ Met, 1 μg mL⁻¹ ampicillin, and 10 μg mL⁻¹ kanamycin. When fatty acids were exogenously supplied, growing cultures were centrifuged and resuspended in 30 mL of BG11 containing Met, ampicillin, and kanamycin, and 75 μM fatty acid. The cultures were further grown for a week at 23°C, harvested by centrifugation, and the pellet was washed once with 0.1 M NaHCO₃ before being used for fatty acid analysis.

**Fatty Acid Analysis**

Cell sediments were directly transesterified with 1 N sulfuric acid in methanol containing 2% [v/v] dimethyloxazoline (1 h at 80°C) to prepare FAMEs. FAMEs were extracted in petroleum ether and analyzed by GLC as already described (Domergue et al., 2002). Fatty acids were identified by comparison with appropriate reference substances or by GLC-MS of 4,4-dimethyloxazoline derivatives as described earlier (Sperling et al., 2000).

**Δ12-Fatty Acid Desaturases from Phaeodactylum tricornutum**

BnFAD6 presented three amino acid changes (T20N, A246V, and L266F) and of the rapeseed Δ12-olote desaturase (accession no. AF243045), respectively. The deduced proteins differed slightly from those already published (accession nos. AAF78778 and AAA50157). BnFAD2 presented three amino acid changes (T20N, A246V, and L266F) whereas BnFAD6 had eight substitutions at the N terminus (Q17H, C18S, P32Q, L53F, F66S, N76S, D79H, and E83D), respectively.

**GFP Expression in P. tricornutum**

The N-terminal part of the plastidial or the microsomal desaturase from P. tricornutum was fused to the N-terminus of EGFP. For this purpose, the first 165 and 339 bp of the ORF of PtFAD2 and PtFAD6, respectively, were cloned in frame at the 5' end of the nucleotide sequence coding for EGFP. The resulting EGFP fusions were inserted into the P. tricornutum transformation vector pPha-T1, and transformation of the diatom was achieved by microparticle bombardment (Zaslavskiak et al., 2000). Selection and culture of the transformants and light and fluorescence microscopy were carried out according to Apt et al. (2002).

**Chemical Synthesis of 16:1Δ7 and 16:2Δ9,12**

The synthesis of (7Z)-hexadeca-7-enoic acid (16:1Δ7) was achieved by a cis-selective Wittig reaction (Bestmann et al., 1976) of 7-phosphoranylpentanoic acid methyl ester and nonanol in 40% (w/v) yield. (9Z,12Z)-hexadeca-9,12-dienoic acid (16:2Δ9,12) was synthesized along a protocol described for the synthesis of homoconjugated trienoic acids (Pohnert and Boland, 2000; Zank et al., 2002). Successive treatment of the symmetrical bisphosphonate with butan-1-ol and methyl 9-oxononanoate, using a carefully optimized regime of temperatures, yielded the methyl ester of 16:2Δ9:12 in a single operation and 42% (w/v) yield. The free fatty acids were obtained by saponification of the respective methyl ester with LiOH in THF:water (3:1 v/v); Nicolaou et al., 1986).

Details of the syntheses and spectroscopic data are available as supplemental material (see www.plantphysiol.org for supplemental material).

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