Production of ricinoleic acid-containing monoestolide triacylglycerides in an oleaginous diatom, *Chaetoceros gracilis*

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Ricinoleic acid (RA), a hydroxyl fatty acid, is suitable for medical and industrial uses and is produced in high-oil-accumulating organisms such as castor bean and the ergot fungus *Claviceps*. We report here the efficient production of RA in a transgenic diatom *Chaetoceros gracilis* expressing the fatty acid hydroxylase gene (*CpFAH*) from *Claviceps purpurea*. In transgenic *C. gracilis*, RA content increased at low temperatures, reaching 2.2 pg/cell when cultured for 7 d at 15 °C, without affecting cell growth, and was enhanced (3.3 pg/cell) by the co-expression of a palmitic acid-specific elongase gene. Most of the accumulated RA was linked with monoestolide triacylglycerol (ME TAG), in which one RA molecule was esterified to the α position of the glycerol backbone and was further esterified at its hydroxy group with a fatty acid or second RA moiety, or 1-OH TAG, in which RA was esterified to the glycerol backbone. Overall, 80% of RA was accumulated as ME TAGs. Furthermore, exogenous RA-methyl ester suppressed the growth of wild-type diatoms in a dose-dependent manner and was rapidly converted to MET AG. These results suggest that *C. gracilis* masks the hydroxyl group and accumulates RA as the less-toxic ME TAG.

Ricinoleic acid (RA) is an unsaturated fatty acid with a double bond and hydroxyl group at positions of C9 and C12 from the carboxy end, respectively. RA is synthesised from oleic acid by fatty acid hydroxylase (FAH), which hydroxylates the carbon at position 12 in the oleic acid molecule. FAH genes have been isolated from castor bean *Ricinus communis*1 (*RcFAH*) as well as the fungus *Claviceps purpurea*2 (*CpFAH*). The *RcFAH* protein is localised in the endoplasmic reticulum of *R. communis*, where it preferentially hydroxylates oleic acid moieties linked at sn-2 positions in phosphatidylcholine3,4. Furthermore, the cellular localisation and substrate specificity of *CpFAH* are predicted to be similar to those of *RcFAH*5; however, *CpFAH* exhibits a higher sequence similarity to *C. purpurea*Δ12-desaturase than to *RcFAH*2.

RA serves as a precursor for the production of many bioproducts, including polyesters, biodiesel, and lubricants6, and castor bean seed oil is currently the only commercial source. However, castor bean is not considered agronomically suitable, because its seeds contain the harmful protein ricin, as well as allergenic 2S albumins7. In addition, because the ergot fungus *C. purpurea* that also produces RA is a pathogenic fungus of crop plants and grows slowly, it has been deemed unsuitable for commercial RA production. Therefore, attempts have been made to produce RA heterologously by expressing the hydroxylase gene in other oil-producing organisms. To date, heterologous RA production has been achieved by introducing the *RcFAH* gene into tobacco1, *Arabidopsis thaliana*8–10, and *Camelina sativa*11, as well as by introducing the *CpFAH* gene into *A. thaliana*2. To express FAH genes in the oilseed of *A. thaliana*2 and *C. sativa*11, seed-specific promoters were used, and when *CpFAH* expression was driven by a seed-specific promoter in the *fad2/FAE1* mutant of *A. thaliana*, which contains elevated levels of oleic acid (a preferred substrate of FAH), the RA level accounted for up to 18% of the total seed oil content2.

Attempts have also been made to produce RA in microorganisms. For example, *RcFAH* has been expressed in baker’s yeast, *Saccharomyces cerevisiae*2, and in later studies, *CpFAH* was introduced into the fission yeast *Schizosaccharomyces pombe*2, *S. cerevisiae*13, the methylo trophic yeast *Pichia pastoris*13, and the oleaginous yeast *Yarrowia lipolytica*14. In addition, *CpFAH*-expressing *Y. lipolytica* lines have been shown to accumulate higher

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levels of RA than \( RcfAH \)-expressing lines\(^1\); however, the expression of \( CfAH \) and the resulting RA production was also shown to markedly suppress cell growth in fission yeast\(^2\). This cellular toxicity was probably caused by the incorporation of RA into phospholipid fractions, which could affect membrane properties\(^2\).

At low culture temperatures, Piolic et al\(^{13} \) improved RA production and prevented its cellular toxicity, and Yazawa et al\(^{4} \) reported that the co-expression of \( CfAH \) and a phospholipase gene suppressed RA toxicity as well. Furthermore, these authors also demonstrated that phospholipase-expressing fission yeast lines secreted RA into the culture medium\(^{15,16} \). However, the microorganisms utilised in these studies were heterotrophs, which require exogenously added organic carbon sources to produce RA. To achieve carbon-neutral RA production based on photosynthesis without the supply of organic carbon, microalgae could be a good biological material.

In the present study, we used the oleaginous diatom \( Chaetoceros gracilis \)\(^{17,18} \) as a platform for RA production, because \( C. gracilis \) is used commercially as food for larval and post-larval shrimp\(^{19} \) and a transformation system for this species was established recently\(^{20} \). Here, we report that a \( CfAH \)-expressing transgenic \( C. gracilis \) produced RA in photoautotrophic conditions, without any negative effects on cell growth, and that increased RA levels were achieved by co-expressing a palmitic acid (16:0)-specific fatty acid elongase, \( Mortierella alpina \) long chain fatty acid elongase 1 (MALCE1). Notably, most of the synthesised RA accumulated as monoestolide triacylglycerols (ME TAGs), in which the RA hydroxyl group was masked by other fatty acids, which might explain its reduced cellular toxicity.

### Results

#### Isolation of transgenic \( CfAH \)-expressing \( C. gracilis \) cell lines.

In order to produce RA in transgenic \( C. gracilis \) cell lines via \( FAH \) expression, a \( CfAH \) cDNA fragment was obtained from cDNA pools of \( C. purpurea \) NBCR 6263. In an open reading frame (ORF) of \( CfAH \) cloned from the NBRC 6263 strain, 13 nucleotides were found to differ from a previously reported \( CfAH \) sequence (NCBI/EMBL/DDBJ accession number; EU661785; Supplementary Fig. S1), and one of these polymorphisms caused an amino acid substitution A327T (Supplementary Fig. S2). Therefore, the enzymatic activity of the encoded protein was determined by heterologous expression in \( S. cerevisiae \) cells. The transgenic \( S. cerevisiae \) cell line harbouring \( CfAH \) accumulated significant amounts of RA (Supplementary Fig. S3). Two \( \Delta^{12} \)-desaturated fatty acids: 9,12-hexadecadienoic acid (16:2(9,12)) and linoleic acid (LA, 18:2\( \Delta^9,12 \)) were also detected in the \( CfAH \)-expressing line (Supplementary Fig. S3). These three fatty acids did not present in the vector control line.

After confirming the enzymatic activity of \( CfAH \), the \( CfAH \) ORF was cloned into an expression plasmid under the control of the promoter of the fucoxanthin chlorophyll a/c-binding protein 5 (\( Lhc5 \)) gene from \( C. gracilis \) with a clonNAT-resistant gene expression cassette, which was used as a selection marker. The resulting expression plasmid (pLhc5p-CfAH; Fig. 1a) was used to transform \( C. gracilis \) cells by electroporation\(^{20} \), and four independent transgenic lines (Cp1, Cp3, Cp4, and Cp6) that contained the \( CfAH \)-expression cassette were selected from the 11 clonNAT-resistant transformants using genomic PCR (Supplementary Fig. S4).

Both quantitative reverse transcription PCR (qRT-PCR) and gas chromatography–mass spectrometry (GC-MS) analyses confirmed that all four transgenic lines expressed \( CfAH \) (Fig. 1b) and produced RA (Fig. 1c, Supplementary Fig. S5). In addition, the hydroxyl fatty acid (12OH-16:1) via \( CfAH \)-catalysed hydroxylation, was also detected at 8.6 min (Supplementary Fig. S5), and the MS profiles of their trimethylsilyl derivatives including three diagnostic fragments at \( m/z \) 159, \( m/z \) 270, and \( m/z \) 299 were identical to those reported previously\(^{2} \) (Supplementary Fig. S5). Of the four lines, Cp4 exhibited the highest expression of \( CfAH \) after 3 d, and the largest accumulation of RA (1.2 pg/cell) after 7 d in aerated culture at 20°C (Fig. 1b,c). Thus, line Cp4 was used for further analyses.

#### Low-temperature-dependent ricinoleic acid production in Cp4.

Optimal temperature conditions for RA accumulation were determined using Cp4 cells cultured at seven different temperatures: 10.0°C, 12.5°C, 15.0°C, 17.5°C, 20.0°C, 22.5°C, and 25°C. At 10.0°C and 12.5°C, the cells grew poorly, and the cell density (2.2 pg/cell; 8.8% of TLs) observed after 7 d at 15°C (Fig. 2a, Supplementary Fig. S7). In contrast, RA was not detected in the extracts of three spots (Nos 1, 2, and 3 in Fig. 3a), which contained 61% (1.3 pg/cell), 9% (0.2 pg/cell), and 17% (0.4 pg/cell) of the total cellular RA (2.2 pg/cell), respectively (Fig. 3b). In contrast, RA was not detected in the extracts from the origin, the other lipid spots, or any other parts of the TLC. In addition, the RF values of spots Nos 1 and 2 were identical to those of normal TAG, in which three endogenous fatty acids were linked with a glycerol backbone, and free fatty acids (FFAs), respectively, and the signal intensities of the TAG and FFA spots from Cp4 cells were notably higher than those from WT cells, whereas spot No. 3 was only detected in Cp4 cells (Fig. 3a).

Characterisation of estolide triacylglycerol structures containing ricinoleic acid. To identify the lipid compounds containing RA, TLs were extracted from WT and Cp4 cells cultured for 7 d at 15°C and separated by thin-layer chromatography (TLC). Each of the resulting lipid spots, a spot of origin, and the other portions without spots was then extracted and analysed by GC-MS (Supplementary Fig. S8). Consequently, RA was detected only in the extracts of three spots (Nos 1, 2, and 3 in Fig. 3a), which contained 61% (1.3 pg/cell), 9% (0.2 pg/cell), and 17% (0.4 pg/cell) of the total cellular RA (2.2 pg/cell), respectively (Fig. 3b). In contrast, RA was not detected in the extracts from the origin, the other lipid spots, or any other parts of the TLC. In addition, the RF values of spots Nos 1 and 2 were identical to those of normal TAG, in which three endogenous fatty acids were linked with a glycerol backbone, and free fatty acids (FFAs), respectively, and the signal intensities of the TAG and FFA spots from Cp4 cells were notably higher than those from WT cells, whereas spot No. 3 was only detected in Cp4 cells (Fig. 3a).
The structures of the RA-containing lipid molecules from each spot were determined using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) (Table 1, Fig. 4, Supplementary Figs S9 and S10). Consequently, the RA-containing lipids from spots designated as Nos 1 and 2 in Fig. 3a were identified as ME TAG, which was reported previously from ergot oil22. In these molecules, one RA molecule was linked to the glyceride backbone of TAG, and an additional fatty acid was esterified with the hydroxy group of the intracellular RA moiety (Fig. 3c).

In the LC-MS/MS analysis, total six species of ME TAG were identified from the spot No. 1 extract (Table 1). Among these, an endogenous 16:0 or 16:1 fatty acid was esterified with the hydroxy group of the RA moiety (Table 1), whereas in ME TAG detected from spot No. 2, the second RA was esterified with the hydroxyl group of the RA moiety (Fig. 3c, Supplementary Fig. S9). Therefore, we denoted ME TAG from spot No. 2 as 1-OH ME TAG. In addition, we also observed that ME TAG and 1-OH ME TAG co-migrated with TAG and FFAs, respectively. The RA-containing lipid molecules from spot No. 3 were identified as 1-OH TAG, in which an RA moiety was linked with the glyceride backbone of TAG (Fig. 3c, Supplementary Fig. S10).

To determine the RA-linked position on the ME TAG glycerol backbone, a lipid extract from spot No. 1 was hydrolysed using a site-specific lipase from *Rhizopus arrhizus* (Supplementary Fig. S11), which hydrolyses acyl chains at the α (sn-1 and sn-3) positions of lipids. Following lipase treatment, we observed that the signal intensity of the TAG spot decreased; however, the intensities of the monoacylglycerol (MAG) and FFAs increased (lane 3 in Supplementary Fig. S11). In addition, RA and 12OH-16:1Δ9 were detected from the FFAs spot but not from the MAG spot in the lipase-treated sample (Supplementary Fig. S11).

**Effect of exogenous hydroxyl fatty acids on cell growth of *C. gracilis*.** Previously, RA production was shown to severely suppress the cell growth of *CpFAH*-expressing fission yeast12. However, in the present study, the growth of Cp4 and WT *C. gracilis* cells were similar (Supplementary Fig. S6). In order to evaluate the toxicity of RA in *C. gracilis* cells, various concentrations of exogenous RA-methyl ester (RAME) was added to WT cultures at 15°C, and oleic acid-methyl ester (OAME), which has the same structure as RAME, except for the lack of the hydroxy group, was used as a control. In addition, we used the methyl ester of each fatty acid, because the carboxy groups in FFAs are generally toxic to cells23,24, and acyl-CoA esters are unstable in the neutral
aqueous solution, that is, the diatom's medium conditions. Consequently, RAME inhibited cell growth in a dose-dependent manner (Fig. 5a), and addition of 4.0 μg/ml RAME arrested cell growth completely, whereas OAME had no effect on cell growth (Fig. 5a).

To find out the destination of exogenously fed RAME in different lipid classes inside the WT cell, cellular lipid extracts from WT cells that had been cultured with 1.2 μg/ml RAME were separated using TLC, and the content of RA in each spot was quantified (Fig. 5b). RA was not detected from cells harvested immediately after the addition of RAME to the medium. At d 0.5, RA was detected in four lipid spots, which corresponded to the expected migration of ME TAG, 1-OH ME TAG, 1-OH TAG co-migrating with RAME, and polar lipids. In particular, 38% and 20% of the exogenous RA was incorporated into 1-OH TAG (0.46 μg/ml culture) and 1-OH ME TAG (0.24 μg/ml culture), respectively. Their content decreased time-dependently and became undetectable at d 3 and d 7 (Fig. 5b). Furthermore, only the level of ME TAG increased during the first 3 d, whereas the other RA-containing lipids decreased in a time-dependent manner (Fig. 5b). Consequently, 83% of the exogenously fed RAME had been incorporated into the ME TAG (1.01 μg/ml culture) at d 3, and this RA level in the ME TAG was still maintained at d 7 (0.89 μg/ml culture). At d 7, the cell density of the RAME-treated culture reached 0.9, which was the same as that observed in the control and OAME-treated cultures.

Figure 2. Temperature-dependent accumulation of ricinoleic acid in Cp4 cells. (a,b) Time-dependent changes in ricinoleic acid (RA) content per cell (a) or as percent of total lipids (b) in Cp4 line cultured at 15 °C, 17.5 °C, 20 °C, 22.5 °C, and 25 °C. CpFAH (c) and Lhcr5 (d) gene expression in Cp4 cells cultured at 15 °C and 25 °C. Amounts of fatty acids (FAs) derived from triacylglycerol (TAG) (e) and total lipids (f) in wild-type (WT) and Cp4 cells at 15 °C.

Co-expression of CpFAH and MALCE1 encoding C16-fatty acid-specific elongase. Oleic acid (18:1 Δ⁹) only made a minor contribution to the fatty acid composition of transgenic C. gracilis cells (2.6% of total fatty acids in Cp4; Supplementary Fig. S7) but, nonetheless, it might limit the cellular content of RA. Similarly, stearic acid (18:0) was also identified as a minor fatty acid (4.2%) in Cp4 cells; whereas 16:0 was the most
abundant (52.4%; Supplementary Fig. S4). Because oleic acid is synthesised from 16:0 by sequential fatty acid elongation and Δ⁹-desaturation, to increase the level of intra-cellularly accumulated RA, we attempted to increase the levels of C18 fatty acids, by providing additional 18:0 and 18:1 Δ⁹ as fatty acid substrates for CpFAH-catalysed hydroxylation.

To achieve this goal, a fungal gene encoding C16-FA-specific elongase, MALCE1²⁴, was introduced into the transgenic Cp4 cells using an additional expression plasmid construct with a Zeocin-resistance gene, Streptomyces coelicolor (Shble), as a second selection marker, and the promoter region of an endogenous nitrate reductase gene²⁰ (CgNR promoter; Fig. 6a). In addition, a partial DNA fragment of CgpsbO (132 bp), which encodes a chloroplast transit peptide, was fused to MALCE1 (CgpsbO-tp-MALCE1; Fig. 6a) for expression of the MALCE1 protein in the chloroplast, in which 16:0 is synthesised by de novo biosynthesis. From screening 62 Zeocin-resistant transformants with Cp4 backgrounds using genomic PCR, seven lines (Cp4-ML17, 18, 20, 25, 43, 45, and 47) that contained the CgpsbO-tp-MALCE1 expression cassette were

Figure 3. Three types of triacylglycerol containing ricinoleic acid were accumulated in Cp4 cells growing at 15 °C. (a) Thin-layer chromatography (TLC) analysis of lipid extracts from wild-type (WT) and Cp4 lines. Three Cp4-specific spots (No. 1–3), in which ricinoleic acid (RA) was detected, are indicted by arrows. Spot positions of lipid standards (1,2-DAG, 1,2-diacylglycerol; 1,3-DAG, 1,3-diacylglycerol; FFA, free fatty acids; MAG, monoacylglycerol; PLs, polar lipids; SE, sterol ester; TAG, triacylglycerol; UK, unknown) are shown on the left side. (b) Distribution pattern of RA in each spot (left) and total content of RA (right) in Cp4 cells on d 7. (c) Structures of three types of TAG molecules containing RA extracted from each spot in (a). 1. mono-esterified (ME) TAG, 2. 1-OH ME TAG, and 3. 1-OH TAG. These structures were identified by liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) analysis shown in Fig. 4 and Supplemental Figs S9–S10.

N, acyl chain from endogenous normal fatty acids (mainly 14:0, 16:0, and 16:1); R, acyl chain from ricinoleic acid, WT, wild-type.
CpFAH transcript increased temporally during the first 3 d of culture at 15 °C and that it might promote RA (Supplemental Fig. S14). The contents of 18:0 (1.5 pg/cell) and 18:1 observed to increase in cells cultured at either 15 °C or 25 °C. These results suggested that the stability of the TAG. APCI, atmospheric pressure chemical ionization; DAG, diacylglycerol; MAG, monoacylglycerol; PPG, polypropylene glycol; TAG, triacylglycerol.

Table 1. Major molecular species from Spot 1 in TLC analysis of Cp4 line shown in Fig. 3 identified by MS/MS analysis. Individual structures of these lipid molecular species were estimated by the MS/MS analysis. *MS/MS profiles of peaks at 15.1 and 15.8 min are shown in Fig. 4 as representatives of monoestolide (ME) TAG. APCI, atmospheric pressure chemical ionization; DAG, diacylglycerol; MAG, monoacylglycerol; PPG, polypropylene glycol; TAG, triacylglycerol.

| Peak No. | Retention time (min) | Molecular weight | APCI (+) [M+H]+ | APCI (-) [M−H]− | fatty acid ion(s) | Estimated molecular species |
|----------|----------------------|------------------|-----------------|-----------------|-----------------|----------------------------|
| 1        | 1.4                  | 330              | 330.3           | 255.3           | MAG             | 16:0                       |
| 2        | 1.6                  | 358              | 359.3           | 283.3           | MAG             | 18:0                       |
| 3        | 3.4                  | 1176             | 1177.7          | 1175.7          | PPG             |                            |
| 4        | 3.9                  | 662              | 663.4           | 255.3           | PPG             |                            |
| 5        | 4.8                  | 568              | 551.5           | 255.3           | DAG             | 16:0-16:0                  |
| 6        | 5.9                  | 596              | 597.5           | 255.3, 283.3    | DAG             | 16:0-18:0                  |
| 7        | 11.7                 | 802              | 803.7           | 253.3, 255.3    | TAG             | 16:1-16:1-16:0             |
| 8        | 12.7                 | 804              | 805.7           | 253.3, 255.3    | TAG             | 16:1-16:0-16:0             |
| 9        | 13.6                 | 806              | 807.6           | 255.3           | TAG             | 16:0-16:0-16:0             |
| 10       | 14.2                 | 1028             | 1029.7          | 1027.8          | TAG             | 14:0-16:1-16:0             |
| 11       | 14.2                 | 1054             | 1055.8          | 227.5, 253.3    | TAG             | 14:0-16:1-16:0             |
| 12       | 14.4                 | 1080             | 1081.9          | 1079.8          | TAG             | 16:1-16:1-16:0             |
| 13       | 15.0                 | 1056             | 1057.8          | 1055.8          | TAG             | 14:0-16:1-16:0             |
| 14       | 15.1*                | 1082             | 1083.9          | 1082.0          | TAG             | 16:1-16:1-16:0             |
| 15       | 15.8*                | 1084             | 1085.9          | 253.3, 255.3, 297 | TAG | 16:1-16:0-16:0             |

Discussion

RA is suitable for medical and industrial uses. In this study, carbon-neutral production of RA (2.2 pg/cell, 8.8% of TLs) was achieved by expression of the CpFAH gene in a transgenic oleaginous diatom C. gracilis in phototrophic conditions, without any apparent negative effects on cell growth. Furthermore, co-expression of 16:0-specific fatty acid elongase MALCE1 with CpFAH increased RA levels to 3.3 pg/cell (11.5% of TLs). Sixty-one percent of the synthesised RA was accumulated as ME TAGs, in which the RA hydroxyl group was masked by other fatty acids, which might explain its reduced cellular toxicity.

The cellular level of RA in CpFAH-expressing C. gracilis increased in a low-temperature-dependent manner (Fig. 2). Accordingly, maximum RA accumulation (1.7 pg/cell) was observed after 7 d at 15 °C, which was 8-fold greater than that observed when cultured at 25 °C (0.2 pg/cell), which is the optimal growth temperature for C. gracilis cells. In addition, expression analysis indicated that the abundance of CpFAH mRNA increased during the first 3 d of culture at 15 °C (optimal temperature for RA accumulation), after which it decreased. However, such temporal increases in CpFAH expression were not observed in cells cultured at 25 °C, and the expression pattern of the endogenous Lhcr5 gene70, the promoter of which was used to drive CpFAH expression, was not observed to increase in cells cultured at either 15 °C or 25 °C. These results suggested that the stability of the CpFAH transcript increased temporally during the first 3 d of culture at 15 °C and that it might promote RA
accumulation. An increase in the proportion of RA in the TLs occurred concomitantly with this increase in CpFAH mRNA stability during first 3 d (Fig. 2). Although the mRNA stability was not maintained after 3 d, the cellular content of RA was increased even after 3 d, and the proportion of RA in TLs (8.8–11%) was also maintained in this period, suggesting the protein stability of CpFAH and its enzymatic activity might be maintained at a high level at 15 °C even after the mRNA stability of CpFAH had declined.

When WT C. gracilis was cultivated with RAME, cell growth was inhibited in a dose-dependent manner (Fig. 5a). In contrast, C. gracilis cells cultivated with OAME exhibited the same growth patterns as cells cultivated with ethanol, which indicates that the RA hydroxyl group was toxic to C. gracilis. Similar to the present results, RAME has been shown to inhibit cell proliferation and viability in yeast via intracellular hydroxyl groups. In addition, when a lower concentration of RAME was added to cultures (1.2 μg/ml), cell growth was restored at the late growth stage (3 d to 7 d). At 3 d, 83% of exogenously fed RA was incorporated into ME TAG, and the same levels of ME TAG were maintained still at 7 d without metabolic breakdown. On the other hand, at the early stage (0.5 d), 38% and 20% of exogenous RA was incorporated into 1-OH TAG and 1-OH ME TAG, respectively. Their content decreased time-dependently and became undetectable at d 3 and d 7 (Fig. 5b). These results suggest that half of the exogenous RAME was taken up at the early stage into TAG with free hydroxyl groups, and at least a large part of these lipids might be converted to ME TAG by an endogenous metabolic process. The accumulation of ME TAG might contribute to decrease the cellular toxicity of RA and restore cell growth. Feeding experiments...
using $^{13}$C-labelled RAME as a tracer will be necessary to identify the metabolic process of exogenous RAME.

Estolides in the biological samples can be extracted efficiently without change of its property27 using a Bligh and Dyer liquid-liquid extraction method28, which was adopted in this study. However, in the industrial-scale extraction and purification of the diatom oil containing ME TAG, the effects of estolides structure on the yield at each process should be validated.

Estolide TAG has been detected in the oils of $C$. purpurea, from which $CpFAH$ was isolated22, and also in the seed oils of Physaria spp29, Cardamine impatiens L.30, and Trewia nudiflora L.31. However, the pathway of estolide TAG synthesis remains unknown, and the related biosynthetic enzyme(s) have yet to be characterised. In $CpFAH$-expressing transgenic diatom $C$. gracilis, 61% of total RA was recovered from the TAG and ME TAG-co-eluting spots, whereas the remaining 9% and 17% were recovered from the 1-OH ME TAG and 1-OH TAG spots, respectively (Figs 3 and 4, Supplementary Figs S8–S10). This suggested that $C$. gracilis possesses an acyltransferase that catalyses the esterification of RA hydroxyl groups with the carboxy end of common fatty acids or other RA molecules. Furthermore, because RA moieties were only observed to occur at the $\alpha$-position of the TAGs in $CpFAH$-expressing $C$. gracilis, ME TAG may be produced in one of two ways. First, esterification of RA hydroxy groups with the carboxy end of common fatty acids may occur on phospholipid-linked RA moieties or on RA-CoA in the ER, after which the resulting estolide is integrated at the $\alpha$-position of diacylglycerol (DAG), or alternatively, the RA is initially integrated into DAG to produce 1-OH TAG by diacylglycerol acyltransferase or phospholipid acyltransferase, after which common fatty acids are esterified with the hydroxyl groups of 1-OH TAG RA moieties. Because no hydroxyl fatty acids (HFAs), such as RA or estolide TAG, were detected in WT $C$. gracilis cells in the first place, it is unclear why the diatom possesses the biosynthetic activity of estolide TAG. However, we suggest that either (1) HFAs themselves, or diatom-infectious bacteria that produce HFA, are present in the habitat of $C$. gracilis, and so $C$. gracilis has a specific defence system for detoxifying exogenous HFAs; or (2) the production of estolide TAG could be a side-effect of producing acyltransferases involved in endogenous lipid metabolism. Nevertheless, the isolation and functional characterisation of the genes and enzymes involved in estolide synthesis are required to provide definitive answers to these hypotheses.

The amounts of RA and 16:0 accumulated by cells of the MALCE1-$CpFAH$-coexpression line Cp4-ML47 in inductive medium were 1.4-fold greater and 0.6-fold lower than that of the same line cultured in non-inductive medium, respectively. Consistently, the proportions of RA and 16:0 in TLs of the Cp4-ML47 line in inductive medium were 1.8-fold greater and 0.8-fold lower, respectively, than that of the same line cultured in non-inductive medium. Furthermore, the proportions of 18:0 and 18:1$\Delta^9$ in TLs of the Cp4-ML47 line cultured in inductive medium were significantly higher than those in non-inductive medium. These results suggested that the expression of MALCE1 enhanced the conversion of 16:0 to 18:0 and promoted RA production. The condensing enzyme MALCE1 is normally located on ER and uses acyl-CoA as the substrate for acyl chain elongation by working with three other ER enzymes: ketoacyl-CoA reductase, acyl-CoA dehydratase, and enoyl reductase in the fatty acid elongase complex32. In this study, MALCE1 was fused to a plastid-transit peptide at the N-terminus, suggesting that this enzyme can also use acyl-ACP as a substrate in cooperation with three endogenous plastidic enzymes.

**Figure 5.** Dose-dependent effect of supplied exogenous ricinoleic acid on growth of diatom cells. (a) Growth curve of the wild-type line supplemented with ricinoleic acid (RA) methyl ester (RAME) or oleic acid methyl ester (OAME) in the culture medium, respectively; (b) Change in RA amount integrated into glycerolipids in WT cells supplemented with 1.2$\mu$g/ml RAME. Mock, addition of solvent (ethanol) as control. MAG, monoacylglycerol; PLs, polar lipids; ME TAG, monoestolide triacylglycerol.
ketoacyl-ACP reductase, acyl-ACP dehydratase, and enoyl reductase. The accumulated levels of 18:0 and 18:1 Δ9 in the Cp4-ML47 line did not show any difference between cells cultured in inductive and non-inductive conditions, suggesting that the Δ9-desaturation from 18:0 to 18:1 Δ9 is not a rate-limiting step in the production of RA. Furthermore, the RA content of the Cp4-ML47 line in non-inductive conditions was slightly greater than that of the Cp4 line expressing the \( \text{CpFAH} \) gene. This might be caused by the leaky expression of \( \text{MALCE1} \) regulated by the \( \text{CgNR} \) promoter, triggered by the depletion of ammonium ions in the medium during the late growth phase. Supply of exogenous OAME in the Cp4 culture increased the TAG and RA contents in cells (Supplemental Fig. S15), suggesting that \( \text{C. gracilis} \) cells could take up exogenous OAME from the medium and utilize it for endogenous lipid metabolic processes. Unexpectedly, the fatty acid composition of TAG fatty acids was not changed between the Cp4 cells cultured with OAME and without OAME. This indicated that all fatty acid moieties in TAG increased with keeping each ratio. At first, we predicted that the exogenously fed OAME enhanced only the downstream lipid metabolism, that is, production of RA by \( \text{CpFAH} \) and production of the linoleic acid and other C18- to C22-polyunsaturated fatty acids, such as EPA and DHA, by endogenous fatty acid desaturation and elongation processes. We suggest that either (1) OAME was incorporated into cellular lipids or converted to OA-CoA to synthesize downstream lipid, and endogenous supply of OA from 16:0 fatty acid was suppressed to maintain the balance of the overall fatty acid ratio; or (2) when exogenous OAME was taken up into \( \text{C. gracilis} \) cells, it was digested through catabolic pathways and used for de novo fatty acid synthesis. Actually, in WT \( \text{C. gracilis} \) cells cultured with the exogenous OAME, TAG contents also increased to maintain the fatty acid ratio (Supplemental Fig. S15). This suggested that \( \text{C. gracilis} \) maintained metabolic homeostasis of fatty acids by adapting to the supply of exogenous fatty acids.

To enhance the conversion of acyl chains, including RA, from phospholipids to TAG in \( \text{A. thaliana} \), a castor phospholipid:diacylglycerol acyltransferase (PDAT) was co-expressed with the \( \text{RcFAH} \) gene, which resulted in an RA content of 19% in the seed oil. However, in \( \text{CpFAH} \)-expressing \( \text{C. gracilis} \), TAG accounted for 87% of the TLs, and RA was only found in TAG molecules. Therefore, the co-expression of PDAT and \( \text{CpFAH} \) might not be necessary in \( \text{C. gracilis} \) considering that the TAG content of WT \( \text{C. gracilis} \) reaches 84% of TLs. Alternatively, use

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**Figure 6.** Expression and Lipid analyses of \( \text{CpFAH}- \) and \( \text{MALCE1} \)-co-expression lines. (a) Structure of \( \text{MALCE1} \)-expression plasmid. \( \text{MALCE1} \) gene was fused with a chloroplast transit signal from the \( \text{CgpsbO} \) gene and cloned under the control of the \( \text{NO}_3^- \)-inductive \( \text{CgNR} \) promoter. A \( \text{HindIII} \) site for linearisation and primer sites (arrow heads) using genomic PCR are shown. (b) Expression level of exogenic \( \text{MALCE1} \) and endogenic \( \text{CgNR} \) normalised by expression of the endogenous α-tubulin gene in a transgenic line, \( \text{Cp4-ML47} \) isolated from the second transformation of the \( \text{MALCE1} \)-expression plasmid to \( \text{Cp4} \) line. The cells were cultured in normal \( \text{Daigo’s IMK} \) medium containing \( \text{NO}_3^- \) (filled circles) or modified \( \text{Daigo’s IMK} \) medium containing \( \text{NH}_4^+ \) (open circles). (c) Change in ricinoleic acid (RA) amount in the \( \text{Cp4-ML47} \) and parental \( \text{Cp4} \) lines. \( \text{P}_{\text{CgpsbO}} \), promoter of \( \text{Chaetoceros gracilis} \) nitrate reductase (\( \text{CgNR} \)) gene; \( \text{P}_{\text{CgACAT}} \), promoter of \( \text{C. gracilis} \) acetyl-CoA acyltransferase gene; \( \text{CgpsbO}-\text{tp} \), chloroplast transit signal of \( \text{CgpsbO} \) gene; \( \text{Sh ble} \), Zeocin-resistance gene.
of more powerful promoters than \( \text{Lhcr5} \) and \( \text{NR} \) in this study for the expression of \( \text{CpFAH} \) and \( \text{MALCE1} \) genes, and knockout or knockdown of any endogenous \( \Delta_{12} \)-desaturase gene in RA-producing lines may be effective methods for further enhancing the production of RA in \( \text{C. gracilis} \) cells. Furthermore, TAG and total fatty acid contents in \( \text{CpFAH} \)-expressing \( \text{C. gracilis} \) cultured at 15 °C were 1.8-fold that in WT cells (Fig. 2), which has not been reported in other heterologous RA-producing organisms. Their production may have been promoted to compensate for the heterologous production of RA, as well as for the production of ME TAG molecules. These findings suggest that \( \text{CpFAH} \)-expressing \( \text{C. gracilis} \) can be used as a resource of biofuel production. Notably, \( \text{CpFAH} \)-expressing \( \text{C. gracilis} \) accumulates modest level of RA in estolide TAG without growth inhibition, potentially because estolide reduces the cellular toxicity of RA. Both estolide TAG\(^{34,35}\) and estolides\(^{36}\) have several other valuable chemical properties as well, which could be utilised for industrial and medical uses in the future. Therefore, we conclude that \( \text{C. gracilis} \) is an attractive producer of RA and estolide TAG.

**Methods**

**Strains and culture conditions of \( \text{C. gracilis} \).** A wild-type \( \text{Chaetoceros gracilis} \) strain (UTEX LB2658) was used for transformation. It was cultured in Daigo's IMK medium (Nihon Pharmaceutical, Osaka, Japan) supplemented with sea salts (Sigma-Aldrich, St. Louis, MO, USA) and 0.2 mM \( \text{Na}_2\text{SiO}_3 \). The cells were grown at 20 °C in continuous light conditions at 50 \( \mu \)mol photons/m\(^2\)/s. At the start of culture, the cell density at 730 nm was adjusted to 0.07. For feeding experiments, the stated concentration of RAME (Sigma-Aldrich) and OAME (Sigma-Aldrich) was added at the start of culture.

**cDNA isolation from \( \text{C. purpurea} \).** To obtain a cDNA fragment encoding the \( \text{CpFAH} \) gene, cDNA pool of a WT \( \text{C. purpurea} \) NBRC 6263 was constructed by reverse-transcription (RT). \( \text{C. purpurea} \) NBRC 6263 was provided by the National Institute of Technology and Evaluation (NITE). Total RNA for the RT reaction was extracted using a RNasy mini Kit (Qiagen, Hilden, Germany) from \( \text{C. purpurea} \) cells cultured in C medium\(^{37} \) for 1 month at 28 °C. The resultant total RNA (0.81 \( \mu \)g) was subjected to an RT reaction using a PrimeScript High Fidelity RT-PCR kit (TaKaRa Bio, Shiga, Japan) in accordance with the manufacturer's instructions. The ORF of the \( \text{CpFAH} \) gene was amplified for the construction of a yeast expression plasmid with gene-specific primers shown in Supplementary Table S1, and sequenced.

**Transformation of yeast cells.** To express the \( \text{CpFAH} \) gene in yeast cells, the \( \text{CpFAH} \) ORF derived from \( \text{C. purpurea} \) NBRC 6263 was cloned under the control of a galactose-inducible \( \text{GAL1} \) promoter in a yeast expression vector pYES2 (Thermo Fisher Scientific, Waltham, MA, USA). \( \text{S. cerevisiae} \) INVSc1 (Thermo Fisher Scientific) was transformed using a lithium acetate-mediated transformation procedure\(^{38} \). The resultant clone was pre-cultured for 2 d at 28 °C in SC minimal medium and then cultured for 2 d at 20 °C in the SC minimal medium containing 2% (w/v) galactose substituted for glucose.

**Vectors construction and transformation of \( \text{C. gracilis} \) cells.** To obtain transgenic \( \text{C. gracilis} \) lines expressing the \( \text{CpFAH} \) gene, the \( \text{CpFAH} \) ORF derived from \( \text{C. purpurea} \) NBRC 6263 was amplified by PCR using primers \( \text{CpFAH-BglII-fw} \) and \( \text{CpFAH-NsiI-rev} \) and cloned into the \( \text{BamHI-PsrI} \) site of a pCgLhcr5p plasmid (accession number: AB981621) downstream of the \( \text{CgNR} \) promoter for a light-harvesting fucoxanthin chlorophyll protein (\( \text{fcp} \)) gene\(^{20} \). The resultant plasmid was linearised by digestion using \( \text{HindIII} \) for transformation. Transformation was performed by a multi-pulse electroporation method using NEPA21 apparatus (Nepagene, Shiga, Japan) with \( \text{C. gracilis} \) cells. Furthermore, TAG and total fatty acid contents in \( \text{C. gracilis} \) cultured at 15 °C were 1.8-fold that in WT cells (Fig. 2), which has not been reported in other heterologous RA-producing organisms. Their production may have been promoted to compensate for the heterologous production of RA, as well as for the production of ME TAG molecules. These findings suggest that \( \text{C. gracilis} \)-expressing \( \text{C. gracilis} \) can be used as a resource of biofuel production. Notably, \( \text{C. gracilis} \)-expressing \( \text{C. gracilis} \) accumulates modest level of RA in estolide TAG without growth inhibition, potentially because estolide reduces the cellular toxicity of RA. Both estolide TAG\(^{34,35}\) and estolides\(^{36}\) have several other valuable chemical properties as well, which could be utilised for industrial and medical uses in the future. Therefore, we conclude that \( \text{C. gracilis} \) is an attractive producer of RA and estolide TAG.

**Quantitative real-time PCR.** Quantitative real-time PCR was performed using SYBR Premix Ex Taq GC (Takara Bio) and a LightCycler 480 Instrument (Roche, Basel, Switzerland), as described previously\(^{46} \). The \( \text{Cg} \alpha_{-}\text{tubulin} \) gene was used as an internal control. The primers used for qRT-PCR are described in Supplementary Table S1.
Lipid analysis. TLs were extracted from the yeast and diatom cells in a chloroform–methanol–water system. For transesterification of the TLs, the extracted TLs were incubated in 2.5% hydrogen chloride/methanol at 85 °C for 2.5h, and fatty acid methyl esters (FAMEs) were extracted by 4 ml of petroleum ether with 0.4 ml of 5M NaCl, and dried under N2 gas. 20 μl of 1 mM heptadecanoic acid (17:0) was added to the each lipid sample before the transesterification, and used as an internal standard. The resultant FAMEs dissolved in 30 μl of acetonitrile, were trimethylsilylated by adding 30 μl of N,O-bis(TMS)-acetamide/pyridine (1:1), and heating at 90 °C for 30 min. Then, 1 μl of total FAMEs and TMS derivatives were analyzed using a Shimadzu gas chromatography mass spectrometer, GCMS-QP2010 system (Shimadzu, Kyoto, Japan) equipped with a DB-23 column (30 μm × 0.25 mm) with 0.25-μm film thickness (Agilent Technologies, CA, Santa Clara). The column temperature was maintained at 160 °C for 1 min, and then increased to 210 °C at a rate of 4 °C/min. For MS, the mass selective detector under electron impact conditions (70 eV) was scanning an effective m/z range of 40–500 at 1,666 amu/sec. Neutral lipids were separated into sterol esters, MAG, diacylglycerol, and triacylglycerol (TAG) by TLC using silica plates (TLC silica gel 60, 20 × 20 cm; Merck Millipore, Darmstadt, Germany) developed with n-hexane/diethyl ether/acetic acid (70:30:1 v/v). After drying, the plate was sprayed with 80% aqueous aceton containing 0.01% primuline. TAG spots were scraped for transesterification as above. Then, 20 μl of 1 mM 17:0 added to the sample before transesterification was used as the internal standard for quantification. The amount of TAG was quantified by gas–liquid chromatography (GC) using a Shimadzu (Kyoto, Japan) GC-1700 gas chromatograph equipped with a flame ionization detector and a split-less injection system, fitted with a capillary column (TC-70, 60 μm × 0.25 mm I.D., GL Sciences, Tokyo, Japan) after derivatisation to FAMEs. The initial column temperature was maintained at 60 °C for 2.5 min, increased to 180 °C at range of 40 °C/min, maintained at 180 °C for 10 min, increased to 260 °C at range of 5 °C/min, and then maintained for 8.5 min. The injector and detector were operated at 250 °C. The fatty acid peaks were identified by comparing the retention times to known standards.

Positional analysis of TAG. Positional analysis of TAG was performed using R. arrhizus lipase (Sigma-Aldrich) as reported previously. Lipids were extracted from the C. gracilis cells and resolved by TLC as described above. TAG was extracted from silica gel using the chloroform–methanol–water system as above. Approximately 10 mg was dried under a N2 gas stream and resuspended in 350 μl of 0.1 M PBS buffer (pH 7.4) containing 4.28 mM Triton X-100. The sample was dispersed by sonication (Sonicator UR-21P, Tomy) for 6 × 10s (output control: 8) on ice. Then, 20 μl of R. arrhizus lipase dissolved in the 100 μl of PBS buffer was added to the emulsified TAG preparation, and incubated at 22 °C for 3 h. The lipase-treated lipids were extracted from the reaction mixtures, and resolved by TLC then developed with n-hexane/diethyl ether/acetic acid (70:140:3 v/v) for the separation of lipids containing HFAs, as described previously. FFAs and MAG spots were scraped for transesterification as above.

Liquid chromatography coupled with tandem mass spectrometry analysis. LC-MS/MS analysis was performed using a LCMS-8040 tandem quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). LabSolution LCMS software Ver. 5.65 (Shimadzu) was used to control the instrument and to process the data. The system used in the analysis consisted of a system controller CBM-20A, two pumps LC-30AD, and autosampler SIL-30AC, a column heater CTO-30AC, and a degasser DGU-20AC.

Lipids were extracted from the silica gel derived from the each TLC spot by the Bligh and Dyer method and dried by evaporation. Then, the extracts were resolved with 1,000 μl of 2-propanol and subjected to LC-MS/MS analysis. The liquid chromatography conditions were optimised as follows: solvent A was water, and solvent B was 2-propanol. The gradient profile was as follows: 100% B (1–25.0 min); 80% B (25.1–30 min). The flow rate was set to 0.2 ml/min, and the column temperature was 40 °C. Chromatographic separation was carried out on a Shim-pack XR-ODSII (75 × 2.0 mm, 2.2 μm, Shimadzu GLC, Tokyo, Japan). The injection volume was 5 μl. An LCMS-8040 tandem quadrupole mass spectrometer was operated in both positive and negative mode with APCI source in the range of m/z 200–1200. The operating parameters were optimised as follows: nebulizer gas flow, 4.0 l/min; drying gas flow, 5.0 l/min; desolation line (DL) temperature, 200 °C; heat block temperature, 200 °C.

Accession number of the CpFAH gene used in this study. The CpFAH sequence in C. purpurea NBRC 6263 used in this study was deposited in the NCBI/EMBL/DDJB database as accession number LC149858.

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