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Selection of a DGLA-producing mutant of the microalga *Parietochloris incisa*: I. Identification of mutation site and expression of VLC-PUFA biosynthesis genes

Umidjon Iskandarov · Inna Khozin-Goldberg · Zvi Cohen

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Abstract Chemical mutagenesis of the phototrophic green microalga *Parietochloris incisa*, producing high amounts of arachidonic acid (ARA), resulted in selection of a mutant deficient in ARA and rich in dihomo-γ-linolenic acid (DGLA) and thus Δ5 desaturase defective. The mutagenesis produced a nonsense mutation in the Δ5 desaturase gene, resulting in alteration of the 62nd codon TGG into a stop codon. Thus, the polypeptide encoded by the mutant Δ5 desaturase gene is severely truncated and biochemically inactive, as was confirmed by heterologous expression in *Saccharomyces cerevisiae*. The mutation did not affect the oleogenic nature of the strain, and the total fatty acid content in the mutant biomass reached 39%, in comparison to 34% in the wild type, after 14 days of nitrogen starvation; biomass yields amounted to 5.1 and 3.6 g/l, respectively. While in the wild type, DGLA and ARA comprised about 1% and 58% of total fatty acids, respectively; the mutation annulled ARA almost totally but increased the DGLA proportion to 32% only with a corresponding increase in the proportion of oleic acid. Consequently, DGLA comprised 12.3% of dry weight, in comparison to 19.4% ARA in the wild type. The expression profiles of the genes coding enzymes involved in VLC-PUFA biosynthesis, Δ12, Δ6, Δ5 desaturases and Δ6 PUFA elongase, during nitrogen starvation, were compared. The transcript levels of all four genes, which were coordinately up-regulated in the wild type, appeared to be drastically reduced in the mutant, indicating their co-regulated transcription.

Keywords LC-PUFA · DGLA · ARA · Arachidonic acid · Δ5 Desaturase · Dihomo-γ-linolenic acid · Microalgae · MNNG mutagenesis · *Parietochloris incisa* · VLC-PUFA

Introduction

The freshwater microalga *Parietochloris incisa* (Trebouxiophyceae, Chlorophyta) is the richest plant source of the ω-6 very-long-chain polyunsaturated fatty acid (VLC-PUFA) arachidonic acid (ARA, 20:4 ω-6; Bigogno et al. 2002a; Cohen and Khozin-Goldberg 2005). Under nitrogen starvation, the total fatty acid (TFA) content of *P. incisa* can constitute more than 35% of dry weight which contains about 60% of TFA as ARA (Khozin-Goldberg et al. 2002). Oleaginous microalgae accumulate substantial amounts of storage triacylglycerols (TAG) as a carbon and energy reserve in response to nitrogen depletion, and their TAG is mainly composed of saturated and monounsaturated fatty acids (Cohen and Khozin-Goldberg 2005). As a unique case, *P. incisa* accumulates ARA-rich TAG, including a rare

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U. Iskandarov · I. Khozin-Goldberg (✉) · Z. Cohen (✉)
French Associates Institute of Agriculture and Biotechnology of Drylands, J. Blaustein Institutes for Desert Research, Ben-Gurion University of the Negev, Midreshet Ben-Gurion 84990, Israel
e-mail: khozin@bgu.ac.il

Z. Cohen
e-mail: cohen@bgu.ac.il

Present Address:
U. Iskandarov
University of Nebraska-Lincoln, Lincoln, NE 68588-0660, USA

triarachidonylglycerol (Bigogno et al. 2002a, b; Khozin-Goldberg et al. 2002). The high proportion of ARA suggested that *P. incisa* could also be used as a platform for obtaining mutants with altered ω-6 VLC-PUFA composition. We were particularly interested in obtaining an oleaginous, Δ5 desaturase-deficient algal strain that would be rich in dihomo-γ-linolenic acid (DGLA, 20:3 ω-6). DGLA is the Δ6 elongation product of γ-linolenic acid (GLA, 18:3 ω-6) and the immediate precursor of ARA. Normally, it is found only in trace levels in a few algae and fungi. The only known source of DGLA is Δ5-deficient mutants of the fungus *Mortierella alpina* (Jareonkitmongkol et al. 1993; Abe et al. 2005) featuring different ratios of DGLA to ARA.

Studies in mammals have shown that DGLA competes with ARA in binding to cyclooxygenase, and thus causes a reduction in the levels of the pro-inflammatory dienoic eicosanoids, prostaglandin E2 and leukotriene LP4, derived from ARA, and an increase in prostaglandin E1 (Chavali and Forse 1999). The latter, which is derived from DGLA, has been shown to have a positive effect in a variety of diseases, e.g., atopic eczema, psoriasis, asthma, and arthritis (Fan and Chapkin 1998), due to its anti-inflammatory properties and modulation of vascular reactivity.

P. incisa deposits ARA in cytoplasmic oil globules in the form of TAG (Bigogno et al. 2002b; Merzlyak et al. 2007). We have shown that the alga can utilize stored ARA as a reservoir of VLC-PUFA for membrane modifications under sudden changes in environmental conditions, e.g., a sudden low-temperature shift or recovery from nitrogen starvation (Bigogno et al. 2002b; Khozin-Goldberg et al. 2005). We have further hypothesized that a mutant defective in its ability to produce ARA would have an impaired growth at reduced temperature. In this work, we report the selection of a Δ5 desaturase-deficient mutant of *P. incisa*, the identification of the mutation site and the time-course of VLC-PUFA biosynthesis gene expression.

Materials and methods

Strains

The wild-type (WT) strain of *P. incisa* Watanabe comb. nov., basionym *Myrmecia incisa* Reisigl, (Trebouxiophyceae, Chlorophyta) was identified and reclassified by Watanabe et al. (1996). Recent molecular phylogenic studies (Karsten et al. 2005) suggested considering *M. incisa* as a member of *Lobosphaera* Reisigl, and the new combination, *Lobosphaera incisa* (Reisigl) comb. nov., was proposed (the accession number is SAG 2007 in the SAG Culture Collection of microalgae, University of Göttingen). The accession number of a mutant strain is PTA-8497 in the American Type Culture Collection (ATCC) depository.

Mutagenesis

During cell division, *P. incisa* produces 16–32 daughter cells that later form aggregates. To separate cells, aliquots of daily diluted semi-continuous culture were sonicated in a water bath and observed by a light microscope (Carl Zeiss, Germany). Ten milliliters of suspension, containing mostly single cells, were exposed to the mutagen, N-methyl-N′-nitro-N-nitrosoguanidine (MNNG, Sigma-Aldrich, St. Louis, MO) at a final concentration of 100 μg/ml for 1 h in an incubator shaker. The stock solution of MNNG (5 mg/ml) was prepared in dimethyl sulfoxide to ease the penetration of the mutagen across the tough cell wall of the alga. The cells were pelleted and washed several times with BG-11 medium (Stanier et al. 1971). Finally, the cultures were sonicated in 10 ml of fresh medium, and cell numbers of untreated and treated cultures were counted. The cultures were sequentially diluted to 10^3 cell/ml and plated on BG-11 agar plates. Plates were maintained under fluorescent light at room (25 °C) and low (15 °C) temperature. Colonies, which showed decreased performance (as estimated by decreased pigmentation, poor growth) at low temperature, were selected and grown in liquid medium.

Growth conditions

Cultures were cultivated phototrophically on BG-11 nutrient medium (Stanier et al. 1971) in 1,000-ml glass columns under controlled temperature and light conditions. The columns were placed in a temperature regulated water bath at 25 and 15 °C and illuminated by cool white fluorescent lamps from one side at a light intensity of 170 μmol photon/m^2 s, as previously described (Bigogno et al. 2002a). Light intensity was measured at the middle and the center of the empty column with a quantum meter (Lamda L1-185, LiCOR, USA). The cultures were provided with a continuous bubbling of air and CO_2 mixture (98.5:1.5, *v/v*) from the bottom of the column. To induce nitrogen-starvation conditions the complete BG-11 medium, containing 1.5 g/l $NaNO_3$ and 6 mg/l ferric ammonium citrate was replaced by nitrogen-free BG-11 medium where $NaNO_3$ was omitted and ferric ammonium citrate was replaced by the similar amount of ferric citrate (Solovchenko et al. 2010).

Growth parameters

Growth of the cultures was estimated on the basis of chlorophyll volumetric content and dry weight measurements (Solovchenko et al. 2010). The biomass concentration was estimated by dry weight determination on pre-weighed glass fiber paper filters (Schleicher & Schuell Co, Germany).

Fatty acid analysis

Fatty acid profile and content in the samples were determined as their methyl esters by capillary GC (Cohen et al. 1992). Transmethylation of fatty acids were carried out by incubation of the freeze-dried cells, total lipid extracts, or individual lipids, in dry methanol containing 2% H_2SO_4 (*v/v*) at 70 °C for 1.5 h under argon atmosphere and continuous mixing. Heptadecanoic acid (Sigma-Aldrich, St. Louis, MO) was added as an internal standard.

Gas chromatographic analysis of fatty acid methyl esters (FAME) was performed on a ZB-WAX+(Phenomenex, USA) fused silica capillary column (30×0.32 mm) on Trace GC ultra Gas Chromatograph (Thermo, Italy) equipped with a flame ionization detector (FID) and a programmed temperature vaporizing (PTV) injector. The FID temperature was fixed at 280 °C; and a PTV injector was programmed to increase the temperature from 40 °C at time of injection to 300 °C at time of sample transfer. The oven temperature was programmed as follows: initial temperature of 130 °C was maintained for 1 min, then raised to 200 °C at a rate of 10 °C/min and held for 6 min then raised to 230 °C at a rate of 15 °C/min for 2 min. Helium was used as a carrier gas. FAME were identified by co-chromatography with authentic standards (Sigma-Aldrich) and by gas chromatography mass spectrometry (GC-MS) on HP 5890 (Hewlett Packard, Aglient, USA) equipped with a mass selective detector HP 5971A as their pyrrolidine derivatives utilizing HP-5 capillary column (Aglient, USA) with a liner temperature gradient from 120 to 300 °C. Pyrrolidide derivatives were prepared by reacting FAME with pyrrolidine, in the presence of acetic acid (Christie 2003).

The data shown represent mean values with a range of less than 5% for major peaks (over 10% of fatty acids) and 10% of minor peaks, of at least two independent samples, each analyzed in duplicate.

DNA and RNA manipulations

RNA was isolated from cells harvested from log phase cultures (time 0) and cells were cultured on nitrogen-free medium for 1.5, 3, 7, and 14 days according to Iskandarov et al. (2009). Genomic DNA of *P. incisa* was isolated as described by Doyle and Doyle (1987).

An ORF of the mutant Δ5 desaturase was PCR-amplified from cDNA with a proof-reading *PfuUltra* II fusion HS DNA polymerase (Stratagene, La Jolla, CA), using the forward (CCAAAGCTT**AAA**ATGATGGCTGTAACAGA) and reverse (GCTCTAGA**CTA**TCCCACGGTGGCCA) primers, containing the *Hin*dIII and *Xba*I restriction sites (underlined), respectively (Iskandarov et al. 2010). The forward primer contained a yeast translation consensus (boldfaced), followed by ATG; the reverse primer contained a restriction site (underlined) and a stop codon (boldfaced). The PCR product of the expected size was excised, purified from the gel (NucleoSpin Extract II purification kit, Machery-Nagel, Duren, Germany), cloned to *Escherichia coli* through pGEM T-Easy vector (Promega, Madison, WI) and sequenced (ABI PRISM 3100 Genetic Analyzer). A fragment of the Δ5 desaturase gene corresponding to the mutation site in genomic DNA was amplified by PCR with *PfuUltra* II fusion HS DNA polymerase using the gene specific primers (Des5For 5′-CCAAAGCTTAAAATGATGGCTGTAACAGA-3′ and Des5Rev 5′-TGTACGCCAAGTCGCTGACCATCC-3′), on genomic DNA isolated from mutant *P. incisa* cells.

Functional validation of the *MutPiDes5* gene

The ORFs encoding for the WT and mutant Δ5 desaturases were cloned to the yeast expression vector pYES2 (Invitrogen, Carlsbad, CA, USA), yielding the pY*PiDes5* and pY*MutPiDes5* constructs. The constructs were used to transform *S. cerevisiae* strain W303 by the PEG/lithium acetate method (Gietz et al. 1995), as previously described (Iskandarov et al. 2010). The *S. cerevisiae* cells harboring the empty pYES2 vector were used as control. Transformants were selected by uracil prototrophy on yeast synthetic medium lacking uracil. For functional expression, a minimal selection medium containing 2% (*w/v*) raffinose was inoculated with the pY*PiDes5*, pY*MutPiDes5*, or pYES2 transformants and grown at 27 °C for 24 h in a water bath shaker. Five milliliters of sterile YSM, containing 1% (*w/v*) Tergitol-40 and 250 μM of DGLA (20:3 ω-6), were inoculated with raffinose-grown cultures to obtain an OD of 0.2 at 600 nm. Expression was induced by adding galactose to a final concentration of 2% (*w/v*), and cultures were further grown at 27 °C for 48 h. Cells were harvested by centrifugation, washed twice with 0.1% $NaHCO_3$, freeze-dried and used for fatty acid analysis.

Semi-quantitative PCR

The cDNA samples for semi-quantitative PCR were synthesized using 1 μg of Dnase treated total RNA in a total volume of 20-μL, using random hexamers (Verso ™ cDNA Kit, ABgene, UK). Each 20-μL cDNA reaction mixture was then seven- and tenfold diluted with PCR grade water to amplify the fragments of actin and LC-PUFA biosynthesis genes, respectively. This was done due to the substantially higher expression of desaturases in the WT. PCR products were visualized in 2% agarose gel.

Results

Cultures of *P. incisa* were chemically mutagenized with N-methyl-N′-nitro-N-nitrosoguanidine (MNNG). Several colonies with reduced growth at low temperature (15 °C) were isolated and analyzed for fatty acid (FA) composition. Following growth on liquid medium, one of the colonies (P127), proved to be deficient in ARA; instead, its precursor, DGLA was accumulated. The chemical structure of DGLA was confirmed by GC-MS analysis of the pyrollidine derivatives of the FA (Fig. 1). We further compared the FA composition and content of the WT and P127 strains after 2 days of growth on nitrogen-replete BG-11 medium (+N) and after 14 days of nitrogen starvation (−N) condition, triggering TAG accumulation in *P. incisa*. ARA was detected in the mutant at very low levels (less than 0.2% TFA) in comparison to over 20% and 58% in the wild type, on nitrogen-replete and nitrogen-deplete medium, respectively (Table 1).

The proportion of DGLA, the immediate precursor of ARA, increased from about 1% in the wild type to over 30% in P127 under nitrogen starvation. We thus assumed that the mutant was defective in its Δ5 desaturase gene. Interestingly, following 2 days of growth on N-replete medium (a linear phase of growth), the proportion of DGLA was only slightly lower than that of ARA in the WT. However, under N-starvation, the proportion of DGLA amounted to only one half of that of ARA in the WT. Correspondingly, the proportion of oleic acid almost tripled, suggesting global changes in the VLC-PUFA pathway. Instead of low levels of eicosapentaenoic acid (EPA, 20:5 ω-3) in the WT, the mutant produced eicosatetraenoic acid (20:4 ω-3), indicating that the ω-3 (Δ17) desaturation of C20 PUFA was not affected. Also, the capacity of P127 to accumulate TAG was not impaired as indicated by the appearance of large oil bodies (Supplementary Fig. 1) and high TFA biomass content under starvation conditions (Table 1).

The nucleotide sequence of the WT gene (GenBank accession number GU390533), encoding for an ORF of 482 residues, was compared with that of the mutant using the CLUSTAL W2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The alignment showed that the 186th nucleotide, downstream the start codon in the WT gene, A (boldfaced), was replaced by the nucleotide G (boldfaced) in the *MutPiDes5* gene (Fig. 2). A PCR amplification of the fragment of the mutant Δ5 desaturase from the genomic DNA resulted in a 570-bp nucleotide sequence starting from the start codon ATG and containing the mutation site and a 192-bp intron within the amplified DNA region (Fig. 2). The mutation is stable as it did not revert during 3 years of sub-culturing.

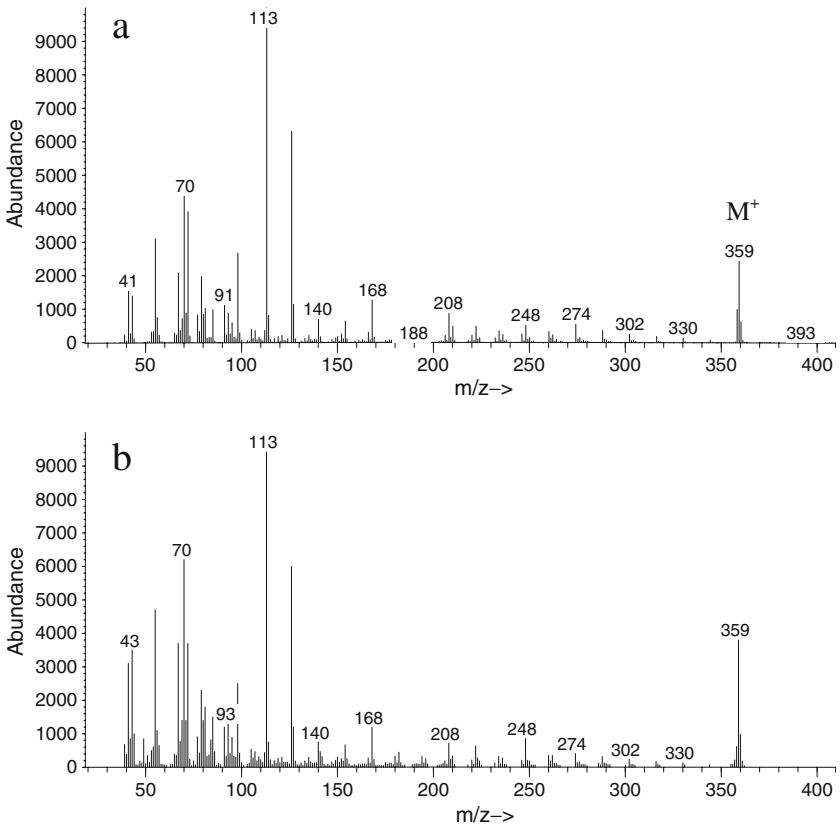

Fig. 1 GS-MS spectrum of the peak corresponding to, DGLA in the mutant *P. incisa* (**a**) as compared to the GS-MS spectrum of the DGLA pyrrolidine derivative from the reference database (**b**). A diagnostic molecular ion (M^+) of 359 m/z and gaps of 12 amu between *m/z*= 182 and 194, 222 and 234, and 262 and 274 (visible after magnification) are important for the identification of the double bonds in positions 8, 11, and 14, respectively (Cristie 2003)

Table 1 Fatty acid composition (relative percentage, *w/w*) and content of the wild type and the Δ5 desaturase mutant (P127) after 2-days cultivation on nitrogen replete (+N) and 14 days on nitrogen-deplete (−N) media

Strain	CultCon.[a]	Fatty acid composition (% of total)														TFA	DW
		16:0	16:1	16:1	16:2ω-6	16:3ω-3	18:0	18:1ω-9	18:1ω-7	18:2ω-6	18:3ω-6	18:3ω-3	20:3ω-6 DGLA	20:4ω-6 ARA	20:4ω-3	20:5ω-3 EPA	% DW	g/l
WT	+N	17.0	2.8	1.1	3.7	3.4	4.1	9.7	1.9	19.8	2.4	7.9	0.7	22.5	–	1.1	10.0	1.9
P127		16.6	2.4	1.1	2.9	4.6	2.8	14.3	2.3	19.2	2.8	11.8	16.7	tr	1.0	–	9.5	2.0
WT	−N	9.3	0.3	0.2	0.2	0.3	3.1	10.8	4.0	9.0	0.9	0.7	1.1	57.7	–	0.8	33.6	5.1
P127		7.7	0.2	0.2	0.3	0.2	2.0	36.4	3.0	13.9	1.1	1.1	31.5	tr	0.6	–	38.9	3.6

tr, traces

[a] Culture conditions

To assure the absence of activity, *S. cerevisiae* pY*MutPiDes5* transformants were fed with DGLA, the ω6 substrate of the Δ5 desaturase, in the presence of tergitol (1%) and galactose (2%). DGLA was not desaturated by either the pY*MutPiDes5* transformant or the empty pYES2-harboring negative control cells (Fig.3).

The expression profiles of VLC-PUFA biosynthesis genes coding for Δ12 (PiDes12, GenBank accession number GU390531), Δ6 (PiDes6, GenBank accession number GU390532), Δ5 (PiDes5, GenBank accession number GU390533) desaturases and Δ6 PUFA elongase (PiElo1, GenBank accession number FJ548973) in the WT and mutant *P. incisa* were compared by semi-quantative RT-PCR using the actin gene, a constitutively expressed gene, as a control (Fig. 4). Results have shown that the transcript levels of all four genes appeared to be drastically reduced in the mutant. At the same time, the expression patterns of the Δ6 and Δ5 desaturase genes (*PiDes6*, *PiDes5*) as well as the Δ6 PUFA elongase gene (*PiElo1*) were not affected in the mutant (Fig. 4), with the maximal transcript level being observed on the third day of N-starvation (Iskandarov et al. 2009, 2010), however, at a substantially lower level in comparison to that of the wild type.

Discussion

One of the proposed roles of ARA in *P. incisa* is to provide a high level of unsaturation for membranes at low temperatures (Bigogno et al. 2002b). We thus expected that mutants impaired in ARA production could be selected using their diminished ability to grow under low temperature. Indeed, one of the slow-growing mutants at 15 °C (designated P127) appeared to be incapable of desaturating DGLA to ARA and was shown to be Δ5 desaturase defective. Comparison of the genomic and cDNA sequences of the Δ5 desaturase gene isolated from the WT (Fig. 2) revealed a single-point mutation in a tryptophan (W) encoding codon, upstream of the HPGG quartet, that is highly conserved within a fused cytochrome *b5* domain in all cloned Δ5 and Δ6 desaturases regardless of their origin (Napier et al. 1999, 2003). The cytochrome *b5* domain functions as an intermediate electron donor in NADH-dependent acyl-group desaturations. The mutation resulted in the appearance of a stop codon, leading to premature termination of translation (Fig. 2). Thus, the deduced polypeptide of the mutant Δ5 desaturase, carrying the nonsense mutation, is severely truncated, rendering it biochemically inactive (Fig. 3). Several Δ5 desaturase mutants were obtained by treating spores of *M. alpina* (the wild-type strain IS-4 or its ω3-defficient mutant) with MNNG (Abe et al. 2005). These mutants were found to be ARA deficient due to mutations causing frame-shift, or

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WtPiDes5     atgatggctgtaacagagggcgctgggggtgtaacggccgaggttggtttgcacaaacgc 60
MutPiDes5    atgatggctgtaacagagggcgctgggggtgtaacggccgaggttggtttgcacaaacgc 60
MutPiDes5a   atgatggctgtaacagagggcgctgggggtgtaacggccgaggttggtttgcacaaacgc 60
              M  M  A  V  T  E  G  A  G  G  V  T  A  E  V  G  L  H  K  R

WtPiDes5     agttctcagccgcgtcccgcagctccccgcagcaagctgttcacgttggatgaggttgca 120
MutPiDes5    agttctcagccgcgtcccgcagctccccgcagcaagctgttcacgttggatgaggttgca 120
MutPiDes5a   agttctcagccgcgtcccgcagctccccgcagcaagctgttcacgttggatgaggttgca 120
              S  S  Q  P  R  P  A  A  P  R  S  K  L  F  T  L  D  E  V  A

WtPiDes5     aagcacgacagcccgactgactgctgggtggtcattcggcggagggtttacgacgtgacg 180
MutPiDes5    aagcacgacagcccgactgactgctgggtggtcattcggcggagggtttacgacgtgacg 180
MutPiDes5a   aagcacgacagcccgactgactgctgggtggtcattcggcggagggtttacgacgtgacg 180
              K  H  D  S  P  T  D  C  W  V  V  I  R  R  R  V  Y  D  V  T
                       W
WtPiDes5     gcgtggggtgccgcagcatcctggcggaaacctgatctttgtgaaagctggccgcgactgt 240
MutPiDes5    gcgtgagtgccgcagcatcctggcggaaacctgatctttgtgaaagctggccgcgactgt 240
MutPiDes5a   gcgtgagtgccgcagcatcctggcggaaacctgatctttgtgaaagctggccgcgactgt 240
              A  -  V  P  Q  H  P  G  G  N  L  I  F  V  K  A  G  R  D  C

WtPiDes5     acccagctgttcgattcctaccacccctaagtgccagg---------------------- 279
MutPiDes5    acccagctgttcgattcctaccacccctaagtgccagg---------------------- 279
MutPiDes5a   acccagctgttcgattcctaccacccctaagtgccaggtgggcgatgaagctctttcag 300
              T  Q  L  F  D  S  Y  H  P  L  S  A  R

MutPiDes5    ------------------------------------------------------------
MutPiDes5    ------------------------------------------------------------
MutPiDes5a   ggaccttcgccccgcctgccttgttgcttgacccatggcccagctgtgtgcctcgagtca 360
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Fig. 2 Comparison of partial cDNAs of WT (Wt*PiDes5*) and mutant (Mut*PiDes5*) *P. incisa* Δ5 desaturase genes, using CLUSTAL 2.0.10 multiple sequence alignment. The mutation site is *highlighted*. The intron in the fragment of the *P. incisa* Δ5 desaturase mutant genomic sequence (Mut*PiDes5*[a]) is *underlined*. The deduced amino acid sequence (*uppercase*) was generated using the Translate tool program (Expasy Proteomics, http://www.expasy.ch/tools/dna.html)

amino acid substitution in the Δ5 desaturase gene (Abe et al. 2005). However, in order to obtain maximal DGLA production, a further mutation of the ω-3 desaturase was required, to avoid the conversion of DGLA to an ω-3 PUFA. In contrast, *P. incisa* has a very limited capacity for extrachloroplastic ω-3 activity.

We suggest that in *P. incisa*, like in some other organisms from which Δ5 desaturase genes were cloned (e.g., Knutzon et al. 1998; Michaelson et al. 1998; Kajikawa et al. 2004), a single copy of the Δ5 desaturase gene is present. This feature is advantageous when biotechnological applications with Δ5 desaturase mutants are considered. Distinctly, the slime mold *Dictyostelium discoideum*, has two copies of functional Δ5 desaturase genes in its genome (Saito and Ochiai 1999; Saito et al. 2000).

Under N starvation, the proportion of DGLA was almost 50% of that of ARA in the WT (Table 1) while the proportion of 18:1 increased threefold in the mutant. The comparative study of the expression levels of Δ12, Δ6, Δ5 desaturases and Δ6 elongase genes can offer a possible explanation for the increased accumulation of oleic acid in the mutant. The transcriptional up-regulation of all genes involved in VLC-PUFA biosynthesis was severely decreased indicating that the impaired transcription of the Δ5 desaturase gene seems to affect the transcription of the preceding sequential genes, resulting, apparently, in reduced translation. Consequently, the excess of 18:1 was deposited in TAG most likely via the Kennedy pathway enzymes (Kennedy 1961). Despite the impaired Δ5 desaturation and the altered fatty

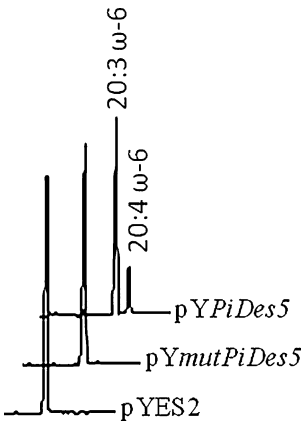

Fig. 3 A fragment of GC chromatogram of FAME of recombinant *S. cerevisiae*, harboring either *pYES2* (empty vector control), *pYPiDes5*, or *pYMutPiDes5*, and fed with 20:3 ω-6

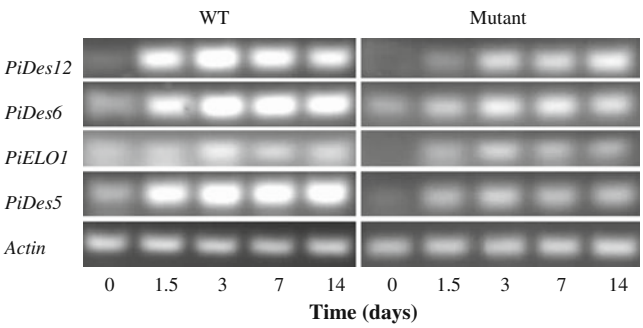

Fig. 4 Time-course of VLC-PUFA biosynthesis gene expression in the WT and the mutant *P. incisa* under N-starvation (time 0—nitrogen-sufficient conditions)

acid composition, TAG accumulation was not affected in the mutant. The data on semi-quantative estimation of mRNA levels are in line with our previous findings on a concerted transcriptional upregulation of the ARA biosynthesis genes in the WT of *P. incisa* obtained by means of quantative real-time PCR (Iskandarov et al. 2009, 2010). It should be noted, however, since steady-state RNA levels were examined, reduced mRNA accumulation in the mutant might be due to reduced RNA stability.

We suggest that a general expression control may exist over the ARA biosynthetic pathway in *P. incisa*. Global transcriptional signals were shown to control gene expression of sequential stages in the biosynthesis pathways, e.g., fatty acid and lipid biosynthesis (Chirala 1992; Baud et al. 2007; Wang et al. 2007). Recent findings demonstrated that mRNAs that encode functionally related proteins are coordinately regulated during cell growth and differentiation as post-transcriptional RNA operons or regulons, through a ribonucleoprotein-driven mechanism (Keene 2007). Likewise, it was shown that genes in the protozoon *Perkinsus marinus*, encoding the sequential activities responsible for ARA acid biosynthesis, are genomically clustered and positioned in the same order as their encoded biochemical reactions and co-transcribed as an operon (Venegas-Calerón et al. 2007). We tried to identify a possible gene clustering by PCR using 5′ and 3′ primers of *PiDes12* and *PiDes5*, as well *PiDes6* and *PiDes5*, respectively, but did not obtain any amplification (not shown).

DGLA is a precursor of anti-inflammatory prostaglandin E_1 and have a beneficial impact on the cardiovascular system and appearance of associated health risk factors, and is, therefore, of potential pharmacological significance. However, the lack of sources for large scale production has prevented its clinical research and, consequently, its nutraceutical or pharmaceutical use. Whereas higher plants or fungi and algae accumulate other PUFA, DGLA normally occurs only as an intermediate in the biosynthesis of ARA; it is not appreciably accumulated in any organism. Instead, GLA-rich oils from several plant species are utilized as a DGLA precursor (Johnson et al. 1997). However, the conversion of GLA to DGLA in the body is, under certain conditions, e.g., low calcium, significantly diminished, and in such cases, GLA cannot replace DGLA (Horrobin and Huang 1987). Moreover, the administration of DGLA is expected to be more effective than the administration of GLA (Umeda-Sawada et al. 2006). Thus, the *P. incisa* Δ5 desaturase mutant may represent a promising green source of DGLA for further up-scaling and optimization of the biotechnology of its cultivation.

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