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Accumulation of arachidonic acid-rich triacylglycerols in the microalga *Parietochloris incisa* (Trebuxiophyceae, Chlorophyta)

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Abstract

The freshwater green microalga *Parietochloris incisa* is the richest known plant source of the polyunsaturated fatty acid (PUFA), arachidonic acid (20:4ω6, AA). While many microalgae accumulate triacylglycerols (TAG) in the stationary phase or under certain stress conditions, these TAG are generally made of saturated and monounsaturated fatty acids. In contrast, most cellular AA of *P. incisa* resides in TAG. Using various inhibitors, we have attempted to find out if the induction of the biosynthesis of AA and the accumulation of TAG are codependent. Salicylhydroxamic acid (SHAM) affected a growth reduction that was accompanied with an increase in the content of TAG from 3.0 to 6.2% of dry weight. The proportion of 18:1 increased sharply in all lipids while that of 18:2 and its down stream products, 18:3ω6, 20:3ω6 and AA, decreased, indicating an inhibition of the Δ12 desaturation of 18:1. Treatment with the herbicide SAN 9785 significantly reduced the proportion of TAG. However, the proportion of AA in TAG, as well as in the polar lipids, increased. These findings indicate that while there is a preference for AA as a building block of TAG, the latter can be produced using other fatty acids, when the production of AA is inhibited. On the other hand, inhibiting TAG construction did not affect the production of AA. In order to elucidate the possible role of AA in TAG we have labeled exponential cultures of *P. incisa* kept at 25 °C with [1-^{14}C]arachidonic acid and cultivated the cultures for another 12 h at 25, 12 or 4 °C. At the lower temperatures, labeled AA was transferred from TAG to polar lipids, indicating that TAG of *P. incisa* may have a role as a depot of AA that can be incorporated into the membranes, enabling the organism to quickly respond to low temperature-induced stress. © 2002 Published by Elsevier Science Ltd.

Keywords: *Parietochloris incisa*; Trebuxiophyceae; Arachidonic acid; LC-PUFA; Salicylhydroxamic acid; SAN 9785; Triacylglycerols

1. Introduction

Arachidonic acid (AA, 20:4ω6) is a long chain polyunsaturated fatty acid (LC-PUFA) that is important as a constituent of biological membranes and a precursor of numerous eicosanoids. AA as well as docosahexaenoic acid (DHA, 22:6ω3) are major components of brain membrane phospholipids (Hansen et al., 1997). Newborns construct these membranes from AA and DHA supplied via human milk feeding. Formulae-fed infants, especially preterm and under weight, require an external supply of these PUFAs (Clandinin et al., 1992).

AA can be obtained from marine fish oil, animal tissues and fungi (Gill and Valivety, 1997). The interest in AA and other LC-PUFAs inspired the search for new sources of these PUFAs. Several microalgae were shown

Abbreviations: AA, arachidonic acid (20:4ω6); C_n fatty acid, fatty acid with *n* carbon atoms; DGDG, digalactosyldiacylglycerol; DGTS, diacylglyceryltrimethylhomoserine; DHA, docosahexaenoic acid (22:6ω3); DMSO, dimethyl sulfoxide; EPA, eicosapentaenoic acid (20:5ω3); MGDG, monogalactosyldiacylglycerol; *P. incisa*, *Parietochloris incisa*; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PUFA, polyunsaturated fatty acid; SHAM, salicylhydroxamic acid; SQDG, sulfoquinovosyldiacylglycerol; TAG, triacylglycerols. X:Y, a fatty acyl group containing X carbon atoms and Y double bonds (*cis*). Pairs of numbers representing the fatty acids when separated by a slash designate the components in the *sn*-1 and *sn*-2 positions respectively of the molecular species.

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to contain high proportions of LC-PUFAs among their fatty acids. For example *Nannochloropsis* (Seto et al., 1984), *Porphyridium cruentum* (Cohen et al., 1988), *Phaeodactylum tricornutum* (Yongmanitchai and Ward, 1992) and *Monodus subterraneus* (Cohen, 1994) are some of the best algal sources of eicosapentaenoic acid (EPA, 20:5ω3), whereas *Crypthecodinium cohnii* (Jiang et al., 1999) and *Chroomonas salina* (Henderson and MacKinlay, 1992) are rich in DHA. When present, LC-PUFAs are generally constituents of membrane lipids while triacylglycerols (TAG) are rather poor in PUFAs and are mostly made of saturated and monounsaturated fatty acids (Ben Amotz et al., 1985). TAG of only a few algae contain significant amounts of LC-PUFAs, e.g. *P. cruentum* (EPA and AA, Cohen, 1990) and *Ectocarpus fasciculatus* (EPA, Makewicz et al., 1997). It was thus extremely interesting to find a green freshwater microalga, *Parietochloris incisa* comb. nov. (Trebuxiophyceae, Chlorophyta, Watanabe et al., 1996), capable of accumulation of large quantities of TAG that were rich in AA (Bigogno et al., 1998).

While the biosynthesis of C_{16} and C_{18} PUFAs in plants was elucidated by Browse and Somerville (1991), very little is known about that of LC-PUFAs, which occur in lower plants, fungi and bacteria. AA can be produced via several different biosynthetic pathways. In *P. cruentum,* 18:1 is stepwise desaturated to 18:2 and 18:3ω6 before it is elongated to 20:3ω6 and Δ5 desaturated to AA (Fig. 1, bottom) (Khozin et al., 1997), whereas in *Euglena gracilis* the elongation of 18:2ω6 to 20:2ω6 was claimed to precede any further desaturations (Fig. 1, top) (Nichols and Appleby, 1969).

The study of the biosynthesis of LC-PUFAs in microalgae was aided by the use of various inhibitors. The substituted pyridazinone, 4-chloro-5(dimethylamino)-2-phenyl-3(2H) pyridazinone (SAN 9785, BASF 13–338) was shown to be a selective inhibitor of the ω3 chloroplastic desaturase (Norman and St. John, 1987). Its use indicated the existence of two different pathways leading to the biosynthesis of EPA in *P. cruentum* and in *M. subterraneus* (Khozin and Cohen, 1996). In *Pavlova lutheri* however, SAN 9785 inhibited the assembly of TAG (Hanggi and Eichenberger, 1998). Salicylhydroxamic acid (SHAM) was shown to affect the Δ12 and Δ15 microsomal desaturases in wheat root seedlings and linseed cotyledons (Banas et al., 1997). Khozin-Goldberg et al. (1999) have recently shown that in *P. cruentum*, SHAM primarily affects the Δ6 desaturation of linoleic acid, resulting in a decrease in the proportion of downstream products of the ω6 pathway and in an increase in that of the ω3 products.

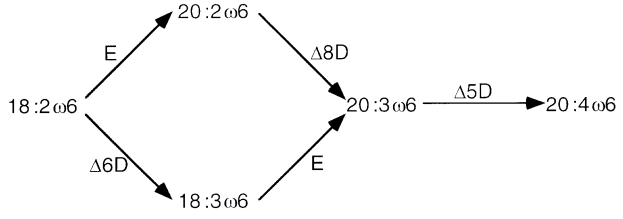

Fig. 1. Suggested pathways for the biosynthesis of arachidonic acid in various microalgae.

Treating cultures of *P. incisa* with SHAM or SAN 9785 has shown that it is possible to inhibit either AA production or TAG accumulation, suggesting that the enhanced accumulation of TAG and the induction of synthesis of AA in this alga can be carried out independently. Labeling studies indicated that AA accumulated in TAG could be exported to polar lipids at low temperatures.

2. Results

2.1. Salicylhydroxamic acid

Incubation of exponential cultures of *P. incisa* for 3 days in the presence of SHAM resulted in an inhibition of growth (as reflected in chlorophyll concentration) that was proportional to the concentration of the inhibitor (Fig. 2). The inhibitor also affected a rise in the fatty acid content. At an inhibitor concentration of 0.3 mM the fatty acid content was enhanced from 5.5 to 8.5% (of dry weight, Table 1), predominantly due to the accumulation of TAG, which increased from 54 to 72.5% (of total fatty acids, Table 2). The proportion of 18:1 sharply rose with increasing concentration of the inhibitor (Fig. 3) from 15.6 to 55.3% at the expense of almost all other fatty acids (Table 1). Among cytoplasmic lipids, 18:1 increased most dramatically, in diacylglyceryltrimethylhomoserine (DGTS) from 5.9 to 53.9%, in phosphatidylcholine (PC) from 13.8 to 56.9 and in TAG from 27.9 to 66.4% (Table 2), indicating an inhibition of the Δ12 desaturation. Subsequently, decreases were observed in the proportion of 18:2 and most of its apparent down stream products, 18:3ω3,

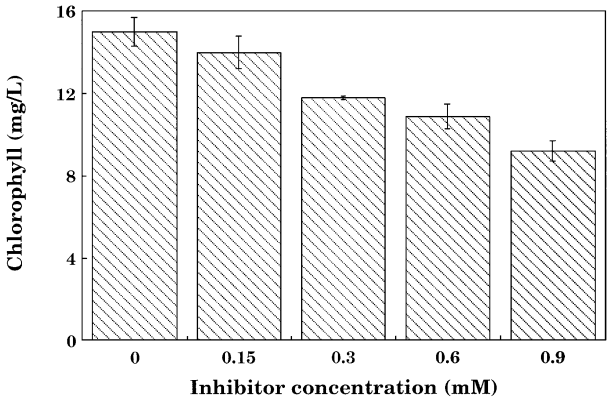

Fig. 2. Growth inhibition of *Purietochloris incisa* following a 3 day treatment with different concentrations of salicylhydroxamic acid (SHAM). Error bars show standard deviation, $n = 3$.

18:3ω6, 20:3ω6, 20:4ω6 and 20:5ω3. For example, the proportion of AA in TAG decreased from 39.8 to 12.0%. In phosphatidylethanolamine (PE), 18:1 increased from 25.3 to 43.4% but 18:2 was not significantly affected. Decreases in C_{16} fatty acids were also observed, predominantly in PC and DGTS. This could possibly be the result of a shift of the fatty acid flux towards the production of C_{18} fatty acids, to overcome the inhibition.

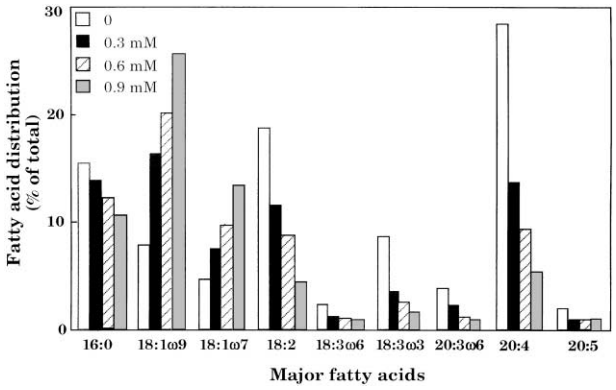

Fig. 3. Effect of SHAM on the fatty acid composition of *Parietochloris incisa*.

2.2. SAN 9785

Treatment of cultures of *P. incisa* with the herbicide SAN 9785 also resulted in an inhibition of growth (Fig. 4). With increasing concentration of the inhibitor (Fig. 5), the proportion of 18:2 (% of total fatty acids) in the lipid extract increased, while that of ω3 PUFAs decreased (Table 1). This effect was particularly evident in the chloroplastic lipids (Table 2). For example, in monogalactosyldiacylglycerol (MGDG), the proportion of 18:3ω3 and 16:3ω3, decreased from 32.6 and 23.4 to 24.4 and 17.0%, respectively. Subsequently, increases were noted in the proportions of their respective precursors, 18:2 and 16:2, from 15.3 and 9.6% to 21.9 and 15.0%, respectively. Digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG) contain much less C_{16} PUFAs and therefore the major effect on these lipids was manifested in the increase of 18:2 at the expense of 18:3ω3. We interpret these findings as an indication to an inhibition of the chloroplastic ω3 desaturation.

Significant changes were also noted in the relative distribution of the lipid classes. At an inhibitor concentration of 0.2 mM, the proportion of TAG was sharply reduced from 54.0 to 24.8% (of total fatty

Table 1
Effect of salicylhydroxamic acid (SHAM, 0.3 mM) and SAN 9785 (SAN, 0.2 mM) on the fatty acid composition of *Parietochloris incisa*

Inhibitor	Fatty acid content[a]	Fatty acid composition (% of total fatty acids)												
		16:0	16:1[b]	16:2 ω6	16:3 ω3	18:0	18:1[c]	18:2 ω6	18:3 ω6	18:3 ω3	20:2 ω6	20:3 ω6	20:4 ω6	20:5 ω3
CON	5.5	14.6	2.7	1.4	4.3	2.0	15.6	15.5	1.9	10.1	0.3	1.0	28.0	1.5
SHAM	8.5	12.8	1.8	0.6	1.6	1.5	55.3	6.7	0.4	4.0	0.4	0.2	13.1	0.7
SAN	4.5	16.6	4.5	3.3	3.3	1.4	7.9	19.7	1.0	9.0	0.2	0.5	29.4	1.7

The fatty acids 20:0 and 20:1 were present at less than 1%.
[a] % of dry weight.
[b] Total of several isomers.
[c] Total of 18:1ω9 and 18:1ω7.

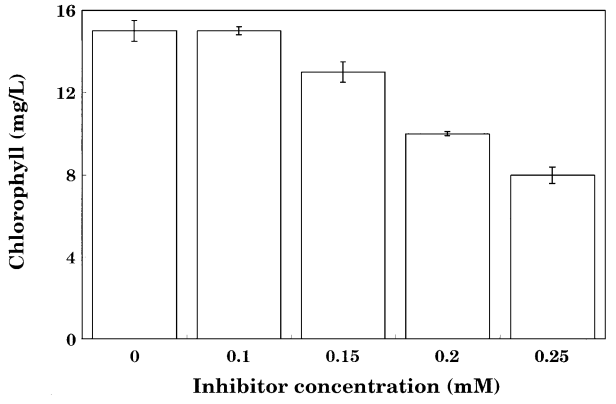

Fig. 4. Growth inhibition of *Parietochloris incisa* following 3 day treatment with various concentrations of SAN 9785. Error bars show standard deviation, $n = 3$.

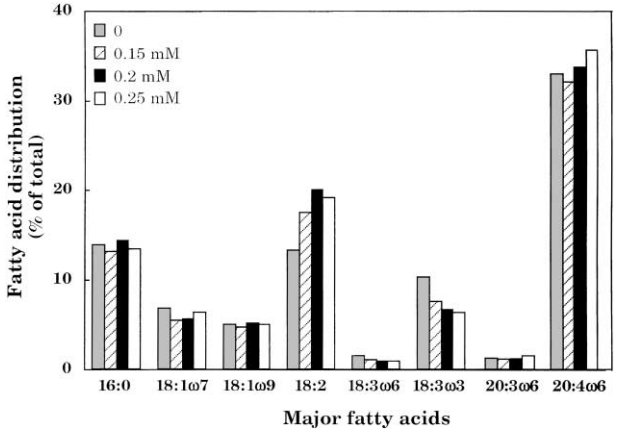

Fig. 5. Effect of SAN 9785 on the fatty acid composition of *Parietochloris incisa*.

Table 2
Effect of SHAM (0.3 mM) and SAN 9785 (SAN, 0.2 mM) on the fatty acid composition of the major lipids of *Parietochloris incisa*

Lipid	Inhibitor	Lipid distribution (% TFA[a])	Lipid content (% dw[b])	Fatty acid composition (% of total fatty acids)												
				16:0	16:1[c]	16:2 ω6	16:3 ω3	18:0	18:1[d]	18:2 ω6	18:3 ω6	18:3 ω3	20:2 ω6	20:3 ω6	20:4 ω6	20:5 ω3
MGDG	CON	7.1	0.39	2.1	1.2	9.6	23.4	0.3	3.9	15.3	0.7	32.6	0.1	0.1	8.7	0.7
	SHAM	4.7	0.40	1.9	2.7	8.3	29.3	0.3	6.9	10.4	0.2	35.5	0.1	0.3	3.9	0.3
	SAN	18.3	0.82	1.5	0.6	15.0	17.0	0.5	1.5	21.9	0.7	24.4	0.1	0.1	15.2	1.5
DGDG	CON	9.6	0.53	19.2	1.3	2.7	3.5	1.4	7.7	22.2	1.2	26.1	0.1	0.4	12.3	1.2
	SHAM	5.8	0.49	15.5	2.0	–	4.6	0.4	12.4	21.7	1.1	23.7	0.2	0.4	12.3	1.2
	SAN	21.3	0.96	12.1	0.8	4.2	2.2	2.3	2.8	33.2	1.3	15.1	0.7	0.6	22.6	1.9
SQDG	CON	7.7	0.42	49.6	0.8	0.3	0.2	1.2	12.4	16.1	0.7	14.2	0.3	–	3.8	–
	SHAM	3.5	0.30	58.3	0.9	–	–	0.8	18.1	12.0	–	9.4	–	–	0.5	–
	SAN	12.3	0.55	54.8	0.7	–	0.1	0.9	6.8	24.7	0.7	9.3	0.1	–	1.2	–
PG[e]	CON	2.4	0.13	36.8	7.2	0.8	0.3	1.4	12.5	22.9	4.8	4.5	0.4	–	3.9	–
	SHAM	1.3	0.11	40.0	6.9	0.8	0.6	0.8	37.6	7.6	0.3	1.3	–	–	2.5	–
	SAN	6.8	0.31	29.1	9.3	1.5	0.1	1.4	4.9	22.8	0.6	2.9	0.3	–	0.3	–
PC	CON	7.6	0.42	29.5	2.7	0.2	0.5	4.2	13.8	16.0	4.5	2.3	0.3	1.9	21.0	1.8
	SHAM	3.7	0.31	14.9	1.5	–	0.1	1.5	56.9	8.0	1.2	1.3	0.6	0.1	12.9	0.4
	SAN	7.3	0.33	20.2	0.3	0.3	0.5	3.1	10.3	12.8	2.2	3.1	0.5	1.1	42.5	1.5
DGTS	CON	4.8	0.26	35.8	2.0	1.1	2.3	3.4	5.9	17.3	6.3	4.6	–	0.6	17.3	1.6
	SHAM	3.8	0.32	27.9	2.3	0.2	0.3	1.7	53.9	8.3	0.8	1.2	–	–	3.5	–
	SAN	5.0	0.23	37.3	1.7	0.6	0.4	4.9	6.2	14.9	2.3	3.0	0.4	0.4	25.8	0.9
PE	CON	2.4	0.13	10.5	1.7	–	1.2	4.9	25.3	5.8	1.8	1.4	0.3	6.3	36.9	1.5
	SHAM	2.4	0.20	7.8	0.8	0.2	0.8	3.3	43.4	4.9	1.9	1.4	0.4	1.1	29.4	1.3
	SAN	3.3	0.15	13.6	1.6	–	0.4	4.9	28.7	4.6	0.7	2.1	0.2	2.3	34.4	0.8
TAG	CON	54.0	3.0	11.3	0.2	0.2	0.4	1.6	27.9	11.8	1.3	1.4	0.1	0.4	39.8	2.2
	SHAM	72.5	6.2	10.5	0.8	0.1	0.2	1.5	66.4	4.8	0.2	0.8	0.4	0.2	12.0	0.7
	SAN	24.8	1.1	9.4	0.4	0.2	0.5	2.3	12.0	8.9	0.7	1.6	0.3	0.8	57.9	3.9

The fatty acids, 20:0 and 20:1, were present at less than 1%. CON, control.
[a] TFA—total fatty acids.
[b] % Of dry weight.
[c] Total of several isomers.
[d] Total of 18:1ω9 and 18:1ω7.
[e] In PG, 16:1tΔ3 accounted for 4.6% (CON), 1.5% (SHAM) and 26.5% (SAN), respectively.

acids) while that of the chloroplastic lipids increased (Table 2). For example, MGDG increased from 7.1 to 18.3% and DGDG from 9.6 to 21.3%. However, the total fatty acid content was only slightly reduced (Table 1), indicating that the de novo synthesis of fatty acids was not severely inhibited.

The proportion of AA in TAG increased from 39.8 to 57.9%. Similar increases were observed in AA-rich polar lipids except for PE. In phosphatidylglycerol (PG) however, we have observed a sharp increase in the proportion of 16:1tΔ3 from 4.6 to 26.5% at the expense of 16:0, 18:3ω6 and AA. We cannot offer an explanation for the latter increase.

2.3. Role of TAG

In an attempt to elucidate the role of AA, we radiolabeled cultures of *P. incisa* and followed the turnover of radioactivity in the various lipids following changes in growth conditions. We thus radiolabeled with [1-^{14}C]arachidonic acid a stationary culture of *P. incisa* that was maintained at 25 °C and resuspended it in fresh medium to allow exponential growth. As expected, total radioactivity decreased, almost entirely at the expense of TAG (data not shown). This finding suggested that at the optimal growth temperature, TAG are predominantly utilized as a source of energy. In an attempt

to assess the effect of a sudden temperature drop on the mobility of acyl groups from TAG, we have similarly labeled an exponentially growing culture. After 12 h at 25 °C, the culture was transferred to either 12 or 4 °C and was compared with a control culture that was kept at 25 °C. Total radioactivity did not decline significantly and AA was still the only labeled fatty acid at the end of the experiment, indicating that no breakdown and reassembly of fatty acids have occurred. At 25 °C, the level of radioactivity remained relatively stable in both TAG and polar lipids, suggesting that any transfer of acyl groups, between polar lipids and TAG, was already in equilibrium (Fig. 6). However, at 12 °C and even more so at 4 °C, TAG rapidly and continuously turned over its label to polar lipids.

In order to find out if AA accumulation is correlated with cold acclimation, we have reduced the growth temperature of exponentially growing cultures from 25 to 12 °C. After 7 h, we could not observe any significant differences in the total fatty acid composition. However, the proportion of polar lipids increased from 50.7 to 56.7% at the expense of TAG (Table 3).

3. Discussion

SHAM has been shown previously to affect the Δ12 and Δ15 desaturations in roots of wheat seedlings and linseed cotyledons (Banas et al., 1997). Recently, we have shown that SHAM inhibits extrachloroplastic desaturations in the red alga *P. cruentum* (Khozin-Goldberg et al., 1999). At a relatively low concentration, the Δ6 desaturation was affected and at higher concentrations, also the Δ12 desaturation. Our data indicate that in *P. incisa*, SHAM inhibits the extrachloroplastic Δ12 and possibly also the Δ6 desaturation. The decrease in 18:2 was particularly evident in PC, phosphatidylglycerol (PG) and DGTS, but not in PE, indicating the former lipids as likely substrates for the Δ12 desaturation. The effect on PG, which was much more intense than on any of the chloroplastic lipids, suggests that PG of *P. incisa* is partly extrachloroplastic.

The effect of SAN 9785 on chloroplastic lipids suggests an inhibition of the chloroplastic ω3 desaturation. Our findings clearly indicate an inhibition of the chloroplastic ω3 desaturation of the eukaryotic molecular species 18:2/18:2 to 18:3ω3/18:3ω3 and in MGDG, also on the desaturation of the prokaryotic molecular species 18:2ω6/16:2ω6 to 18:3ω3/16:3ω3. However, 18:3ω3 in

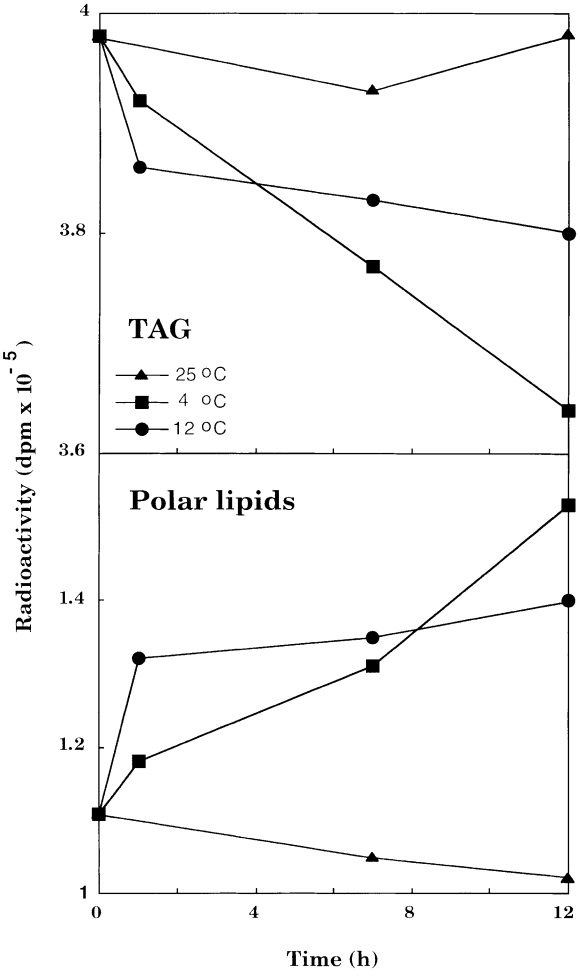

Fig. 6. Effect of temperature on the lipid composition of *P. incisa*. Exponential cultures were pulse labeled with [1-^{14}C]arachidonic acid for 12 h at 25 °C and cultivated for another 12 h at 25, 12 or 4 °C.

Table 3
Fatty acid composition of TAG and polar lipids (PL) of cultures of *Parietochloris incisa* following a cool-down from 25 to 12 °C for 7 h

Lipid	Temp. (°C)	Lipid content[a]	Fatty acid composition (% of total fatty acids)												
			16:0	16:1[b]	16:2 ω6	16:3 ω3	18:0	18:1 ω9	18:1 ω7	18:2 ω6	18:3 ω6	18:3 ω3	20:3 ω6	20:4 ω6	20:5 ω3
TAG	25	49.3	7.4	0.1	–	–	3.2	16.8	3.9	10.8	0.9	0.6	3.1	50.1	1.7
	12	43.3	7.9	0.3	–	–	3.9	17.2	4.2	11.0	1.0	0.6	1.8	48.3	1.7
PL	25	50.7	14.7	8.7	5.6	3.1	1.4	6.7	3.7	25.8	2.9	7.9	1.0	16.0	1.0
	12	56.7	14.6	8.1	5.1	3.1	1.6	6.6	3.9	25.3	2.8	8.6	0.9	16.4	1.2

[a] % of total fatty acids.
[b] Total of several isomers.
The fatty acids 20:0, 20:1 and 20:2ω6 were present at less than 0.5%.

cytoplasmatic lipids was not significantly affected. This finding is in keeping with previous works demonstrating a similar effect on *Arabidopsis thaliana* (Norman and St. John, 1987) and *P. cruentum* (Cohen et al., 1993). Our data supports the existence of two different ω3 desaturases acting respectively on 18:2 that is linked to extraplastidial lipids and on 18:2 and 16:2 of chloroplastic lipids, previously demonstrated in higher plants (Browse and Somerville, 1991). Similar findings were also reported for *Chlorella* and other green algae (Heinz, 1993).

We interpret the SAN 9785-affected decrease in TAG as an indication to an inhibition of the assembly of TAG. Similarly, SAN 9785 affected a decrease in the content of TAG and a corresponding increase in that of MGDG in the alga *Pavlova lutheri* (Hanggi and Eichenberger, 1998). We have recently found that treatment of the eustigmatophyte *M. subterraneus* with SAN 9785 resulted in a decrease of the TAG content from 36.3% (of total fatty acids) to 7.6% (Cohen et al., unpublished results). Our findings show that while the accumulation of TAG is reduced, the production of AA is not. A similar amount of AA is deposited in half the normal content of TAG, resulting in an enhancement of the proportion AA in TAG. The sharp increase in the proportion of AA in PC and DGTS indicate these lipids as possible sources of AA to TAG.

Many microalgae are capable of accumulating high contents of TAG, especially in the stationary phase or under nitrogen starvation (Ben Amotz et al., 1985). However, even in algae that are relatively rich in PUFAs, the fatty acids that are accumulated in TAG are mostly saturated and monounsaturated. Relatively high proportions of PUFAs are found only in TAG of a few algae. For example, the TAG of *P. cruentum* are rich in AA and EPA (Cohen, 1990) and those of *E. fasciculatus*, in EPA (Makewicz et al., 1997). We have recently shown that *P. incisa* (code name T12) is unique in its ability to accumulate TAG that are rich in AA (Cohen et al., 2000). Under nitrogen starvation conditions, TAG accounted for over 90% of total acyl lipids and AA constituted up to 60% of the fatty acids of TAG (Bigogno, 2000). It was thus interesting to find out if the accumulation of TAG and the production of AA can be carried out independently. In the presence of SHAM, the content of TAG doubled from 3.0 to 6.2% (of dry weight) while that of AA decreased in TAG from 39.8 to 12.0% (of fatty acids) (Table 2). Consequently, the amount of AA occurring in TAG decreased from 1.2 to 0.7% (of dry wt.), whereas that of 18:1 increased from 0.8 to 4.1%. It follows that inhibiting the biosynthesis of AA does not interfere with the accumulation of enhanced levels of TAG. On the other hand, in the presence of SAN 9785, the inhibition of TAG assembly did not significantly affect the production of AA. While the dry weight content of TAG decreased from 3.0 to 1.1%, the proportion of AA in TAG increased sharply. These findings show that although there is a preference for AA as a building block of TAG, when the biosynthesis of AA is inhibited TAG can be produced using other fatty acids. However, we cannot yet say whether the production of 18:1 rich and AA-rich TAG proceeds via the same pathway.

TAG are generally considered as storage materials that have no role in the biosynthesis of polar lipids. However, the low temperature-induced transfer of label from TAG indicates that under conditions that require enhanced desaturation or production of new AA-containing chloroplastic membranes, cells of *P. incisa* can utilize TAG as a reservoir of AA. Further support for this is found in the SAN 9785 induced shift of AA from TAG to galactolipids (Table 3). Similarly, Adlerstein et al. (1997) and Khozin-Goldberg et al. (2000) have recently shown that in the microalga *P. cruentum*, AA and EPA of TAG can be exported to the chloroplast for the build up of the 20:4/20:4 and 20:5/20:5 molecular species of MGDG. Likewise, Garces et al. (1994) and Sarmiento et al. (1998) have shown that following a temperature drop in sunflowers, 18:1 of TAG was transferred to PC where it was desaturated to 18:2. Adaptation of higher plants and algae to low temperature is associated with an increase in fatty acid unsaturation or a reduction in the chain length of membranal fatty acids (Sato and Murata, 1980; Tatsuzawa and Takizawa, 1995) and has been interpreted as an attempt to maintain membrane fluidity (Wada et al., 1990; Vigh et al., 1993). Other effects on fatty acids include exposure to high light that results in damage to the photosynthetic machinery, particularly at low temperatures. The main site of the damage is the D1 protein of photosystem II (PSII). Fatty acid desaturation was shown to be important for the tolerance of strong light, especially at low temperatures by accelerating the synthesis of the D1 protein (Gombos et al., 1997).

The optimal growth temperature of *P. incisa* is 25 °C, however, the alga was isolated from the slopes of a snow mountain in Japan (Watanabe et al., 1996). Recently, measuring oxygen evolution and variable fluorescence we have shown that *P. incisa* is more resistant to low temperatures than *Chlorella*, its resistance being almost as high as that of cryophyte *Chlamydomonas nivalis* (Bigogno, 2000). It is thus possible that the low temperature-induced transfer of AA from TAG to membrane lipids could be a utilized as a mechanism for a quick response of the cells to conditions which require a higher level of AA in the membranes on the one hand, and prevent, or slow down, the *de novo* synthesis of PUFAs on the other hand. We hypothesize, therefore, that one of the roles of TAG in *P. incisa* is to serve as a reservoir of AA that enables the organism to quickly respond to low temperature-induced stress.

4. Experimental

4.1. Growth conditions

Cultures of *P. incisa* in the logarithmic phase were cultivated on BG11 medium (Stanier et al., 1971) in 150 ml Erlenmeyer flasks under an air:CO_2 (99:1) atmosphere. The flasks were placed in an incubator shaker at 25 °C and illuminated from above at a light intensity of 115 μmol quanta m^{-2} s^{-1}. For lipid analysis, cultures were grown in tubes under an air:CO_2 (99:1) atmosphere at 25 °C. The tubes were placed in a water bath and illuminated with a light intensity of 150 μmol quanta m^{-2} s^{-1}. Cultures were daily diluted for at least 4 d prior to the onset of the experiment. The specific growth rate was estimated by measurement of the chlorophyll concentration.

4.2. Inhibitor studies

Stock solutions of 10 mM SHAM or SAN 9785 in DMSO were added to exponentially growing cultures to achieve a final range of concentrations of 0.15–1 mM and 0.1–0.25 mM, respectively.

4.3. Lipid extraction

Freeze-dried samples of *P. incisa* biomass were extracted with methanol containing 10% DMSO, by warming to 40 °C for 5 min and stirring at 4 °C for another hour. The mixture was centrifuged, the supernatant removed and the pellet was re-extracted with a mixture of diethyl ether and hexane (1:1, v/v). Diethyl ether, hexane and water were added to the supernatant to form a ratio of 1:1:1:1 (v/v/v/v). The mixture was shaken and then centrifuged for 5 min at 3500 rpm and the upper phase was collected. The water phase was re-extracted twice with the ether–hexane mixture. The organic phases were combined and evaporated to dryness. The diethyl ether utilized in the extractions and the lipid extractions was peroxide-free and contained 0.01% butylatedhydroxytoluene (BHT).

4.4. Lipid analysis

Polar lipids were separated by two-dimensional TLC using a solvent system of chloroform:methanol:water (65:25:4, v/v/v) for the first direction and of chloroform: methanol:1-ethylpropylamine:conc. ammonia (65:35:0.5:5, v/v/v/v) for the second direction. Neutral lipids were resolved with petroleum ether:diethyl ether:acetic acid (80:20:1, v/v/v).

Freeze-dried cells, lipid extracts and individual lipids were transmethylated with 2% H_2SO_4 in methanol (Khozin et al., 1997). Heptadecanoic acid was added as an internal standard. Gas chromatographic analysis was performed with a Supelcowax 10 fused silica capillary column (30 m × 0.32 mm) at 200 °C (injector and flame ionization detector temperatures 280 °C, split ratio 1:100). Fatty acid methyl esters were identified by co-chromatography with authentic standards (Sigma Chemical Co., St. Louis). The data shown represent mean values with a range of less then 5% for major peaks (over 10% of fatty acids) and 10% for minor peaks, of at least two independent samples, each analyzed in duplicate.

4.5. Incorporation of [1-^{14}C]arachidonic acid and redistribution at low temperatures

Cells in the logarithmic phase of growth were reconstituted in one half of the original volume and pulse-labeled with 10 μCi of the ammonium salt of [1-^{14}C]arachidonic acid (55 mCi/mmol, Amersham) for 4 h. The ammonium salt was obtained by neutralization of labeled AA with an equimolar amount of 2 M ammonium hydroxide. After the pulse, cells were centrifuged, washed repeatedly with fresh medium and resuspended in fresh medium to the original volume. Cultures were grown as described above for 12 h at 25 °C and for another 12 h at either 12 °C or 4 °C. Lipids were extracted and separated as described above and the distribution of radioactivity was detected by autoradiography with X-ray films (X-OMAT AR, Kodak) exposed to the TLC plates for 24 h. Lipid spots were scraped directly into scintillation vials containing 1 ml of methanol and 1 ml of scintillation cocktail and radioactivity was measured in a liquid scintillation counter (Rackbeta LKB, model 1217, LKB, Wallac Oy, Finland) as previously described (Khozin et al., 1997).

4.6. Incorporation of [1-^{14}C]arachidonic acid into stationary cells

Cells of *P. incisa* were cultivated in batch for 12 days to the stationary phase (chlorophyll conc., 53 μg/ml). The cells were pulse-labeled with 10 μCi of the ammonium salt of [1-^{14}C]arachidonic acid for 1 h. After the pulse, the cells were centrifuged, washed repeatedly and diluted with label-free medium to a chlorophyll concentration of 6 μg/ml, and cultivated as mentioned above. The distribution of radioactivity in the lipid classes was determined as previously described (Khozin et al., 1997).

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