Expression of the Isochrysis C18-Δ^9 Polyunsaturated Fatty Acid Specific Elongase Component Alters Arabidopsis Glycerolipid Profiles[1]

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A cDNA isolated from the prymnesiophyte micro-alga *Isochrysis galbana*, designated *IgASE1*, encodes a fatty acid elongating component that is specific for linoleic acid (C18:2n-6) and α-linolenic acid (C18:3n-3). Constitutive expression of *IgASE1* in Arabidopsis resulted in the accumulation of eicosadienoic acid (EDA; C20:2n-6) and eicosatrienoic acid (ETrA; C20:3n-3) in all tissues examined, with no visible effects on plant morphology. Positional analysis of the various lipid classes indicated that these novel fatty acids were largely excluded from the *sn*-2 position of chloroplast galactolipids and seed triacylglycerol, whereas they were enriched in the same position in phosphatidylcholine. EDA and ETrA are precursors of arachidonic acid (C20:4n-6), eicosapentaenoic acid (C20:5n-3), and docosahexaenoic acid (C22:6n-3) synthesized via the so-called $\omega 6$ $\Delta 8$ desaturase and $\omega 3$ $\Delta 8$ desaturase biosynthetic pathways, respectively. The synthesis of significant quantities of EDA and ETrA in a higher plant is therefore a key step in the production of very long chain polyunsaturated fatty acid in oil-seed species. The results are further discussed in terms of prokaryotic and eukaryotic pathways of lipid synthesis in plants.

The very long chain polyunsaturated fatty acids (VLCPUFAs), arachidonic acid (AA; C20:4n-6), docosahexaenoic acid (DHA; C22:6n-3), and eicosapentaenoic acid (EPA; C20:5n-3) are considered to have profound effects on cell function and development. The reduction in nonfatal and fatal cardiovascular events that is associated with the consumption of EPA and DHA may be due to the stabilization of atherosclerotic plaques (Thies et al., 2003). AA and DHA are considered to be important in pre- and postnatal development (Crawford, 2000; for review, see Lauritzen et al., 2001). Such VLCPUFAs are also precursors of the physiologically active prostaglandins, thromboxanes, and leukotrienes. No higher plants contain these fatty acids and hence it is considered desirable to genetically engineer the capacity to synthesize them in oilseed species and so provide an important source for the nutraceutical/pharmaceutical industries.

Isochrysis galbana is a marine microalga that is rich in DHA and EPA (Lopez et al., 1994). Recently we isolated and characterized a cDNA from *I. galbana*, which encodes a novel fatty acid elongase component designated IgASE1 (Qi et al., 2002, 2003). Transgenic yeast-expressing *IgASE1* converted linoleic acid (LA; C18:2n-6) and α-linolenic acid (ALA; C18:3n-3) to eicosadienoic acid (EDA; C20:2n-6) and eicosatrienoic acid (ETrA; C20:3n-3), respectively (Qi et al., 2002). The substrate specificity of IgASE1 is consistent with it catalyzing the condensation reaction of fatty acid elongation (Qi et al., 2003). We have further suggested (Qi et al., 2002, 2003) that the IgASE1 elongating activity is the first committed step in VLCPUFA synthesis and hence AA, EPA, and DHA formation via the so-called $\omega 3$ $\Delta 8$ and $\omega 6$ $\Delta 8$ pathways, respectively (Fig. 1; Nichols and Appleby, 1969).

Here we report the constitutive expression of *IgASEI* in Arabidopsis in order to assess (1) glycerolipid synthesis with VLCPUFAs and its effect on plant morphology; and (2) whether the introduction of such novel fatty acids can help in the further understanding of so-called prokaryotic/eukaryotic lipid partitioning in higher plants (for reviews, see Somerville and Browse, 1991; Maréchal et al., 1997; Wallis and Browse, 2002).

RESULTS

Expression of the *IgASE1* Elongase Gene in Arabidopsis

The entire coding region of the *I. galbana* elongase component *IgASE1* was expressed under the control of the cauliflower mosaic virus (CaMV 35S) promoter in transgenic Arabidopsis. Primary Liberty herbicide-resistant transformants (T1) that yielded herbicide-resistant and herbicide-sensitive T2 progeny in a ratio

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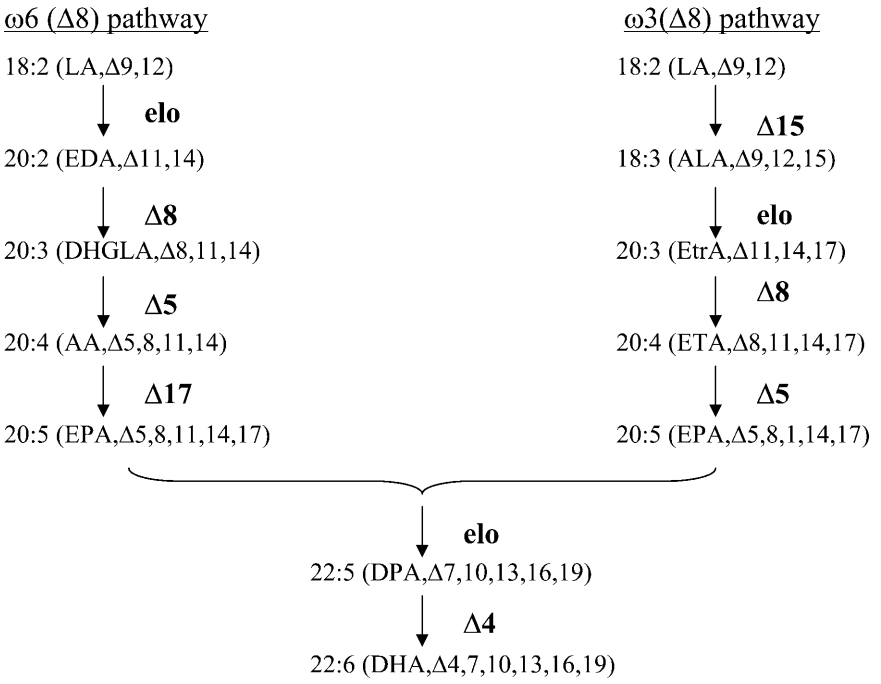

Figure 1. Proposed modes of ω3 and ω6 (Δ8) VLCPUFA biosynthesis in *I. galbana*. The first committed step in VLCPUFA biosynthesis in the ω3 (Δ8) and ω6 (Δ8) pathways is the elongation of LA and ALA by the IgASE1 elongase component resulting in EDA and ETrA formation. A Δ8 desaturase then inserts a double bond at the Δ8 position of EDA and ETrA yielding dihomo-γ-linolenic acid (DHGLA) and ω3-eicosatetraenoic acid (ETA) respectively. DHGLA and ETA are further desaturated by a Δ5 desaturase forming AA and ETA. EPA is produced from the AA and ETA by Δ5 and Δ17 desaturase activities, respectively. A proportion of the EPA is elongated to docosapentaenoic acid (DPA). In the final step DHA is produced from the DPA precursor by a Δ4 desaturase activity.

of 3:1 were considered to carry a single transgene. Single-copy T2 plants that yielded all herbicide-resistant progeny (T3) were further identified as being homozygous for the transgene. Leaf fatty acid methyl esters (FAMEs) of homozygous, single-copy T3 plants were analyzed for total fatty acid to determine which plants had the highest C20 content. Line CA1-9 was identified as having the highest product formation. It therefore had the greatest potential for accurate quantification of novel C20 content and was selected for further study. Gas chromatography analysis of FAMEs from all the different tissues examined showed that the transgenic plants contained two additional fatty acids compared to the wild type (Fig. 2; Table I). These were identified by comigration and spiking with known standards as EDA and ETrA (Fig. 2) and by comparison with the fatty acid products observed previously in transformed yeast (Qi et al., 2002). They were considered, therefore, to be the elongation products of the IgASE1 elongase component. A complete fatty acid analysis of all plant organs was carried out. The results (Table I) show that, with the exception of leaf tissue, EDA was present in relatively greater amounts than ETrA in all parts of the plant, even though ALA was the predominant fatty acid precursor in stem, flower, and leaf tissues. In roots, where LA was present at particularly high levels, EDA was produced in higher amounts than in other tissues. The morphology of line CA1-9 plants was characterized by the following traits: rosette geometry and maximum diameter; infloresence stalk height; leaf dimensions; petiole length; root-to-shoot wet weight ratio; and flower and silique development. Transformants were morphologically indistinguishable from wild-type plants by the above criteria.

Leaf Glycerolipids and Fatty Acid Composition

In order to assess the effect of *IgASE1* expression and EDA/ETrA formation on lipid metabolism, the glycerolipid and fatty acid content of leaves of transformants were determined (Table II). The results show that *IgASE1* expression had little effect on the proportions of lipid classes. Fatty acid profiles, however, showed that all the glycerolipids examined in the CA1-9 plants contained the fatty acid elongation products, EDA and ETrA. The chloroplast lipids monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and phosphatidylglycerol (PG) always had more ETrA than EDA, with DGDG having substantially greater levels of ETrA. The relative amounts (mol%) of the elongation products in these lipids varied in the order DGDG > MDGD > PG. In the extraplastidially-synthesized lipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE), EDA was always in excess of ETrA and this was also true of phosphatidate (PA) and phosphatidylinositol (PI).

Positional Analysis of the Fatty Acids in the Leaf Glycerolipids

The previous results indicated that the fatty acid elongation products were present in both the (so-called) prokaryotic and eukaryotic glycerolipids. To investigate this further, positional analysis of the fatty acids in the leaf lipids was performed (Tables III and IV). The results show that EDA and ETrA were largely present at the *sn*-1 position of the chloroplast lipids MGDG and DGDG (Table III). PC, on the other hand, had substantially more EDA and ETrA at the *sn*-2 position than the *sn*-1 (Table IV). While both PC and

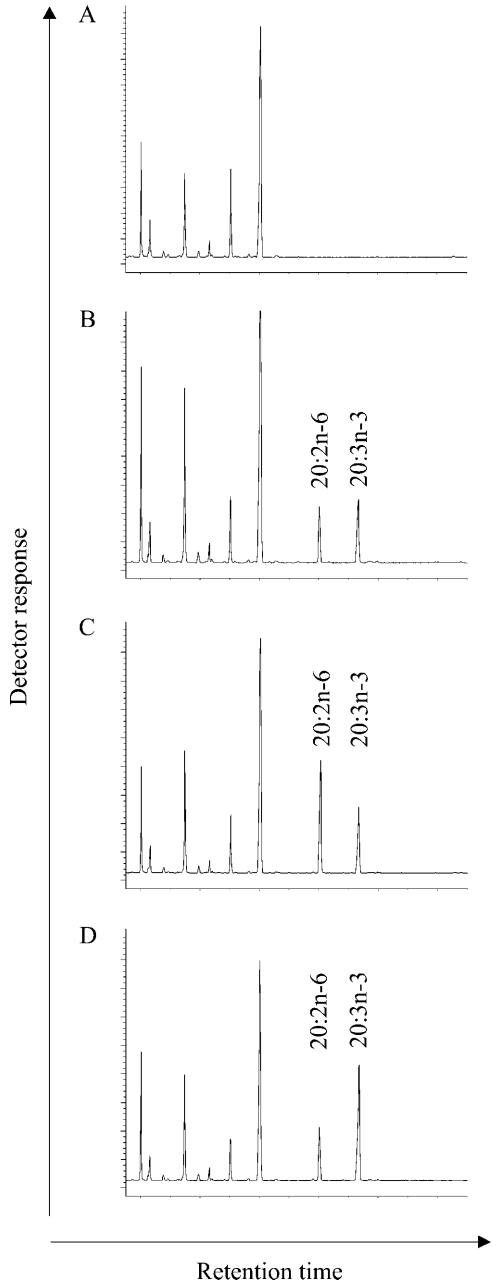

Figure 2. Gas chromatograms of leaf FAMEs from wild-type and *IgASE1* transgenic plants. A, Wild-type; B, line CA1-9; C, transformant plus EDA (C20:2n-6); D, transformant plus ETrA (C20:3n-3).

PA contained relatively large quantities of these fatty acids, the positional distribution of acyl groups in PC and PA was different, with PA having more elongation products at the *sn*-1 position.

Positional Fatty Acid Distribution in Seed Triacylglycerol

Stereo-specific analysis of fatty acid composition was carried out on Arabidopsis seed triacylglycerol (TAG). The results (Table V) show that TAG in the CA1-9 transformants contained EDA and ETrA and that they were confined to the *sn*-1 + 3 positions with little, if any, at the *sn*-2 position. The TAG also contained the seed-specific fatty acids C20:0, C20:1, and C22:1. However, these fatty acids were present in similar amounts in both wild-type and transgenic plants. Wild-type plants did not contain any ETrA and had only traces of EDA.

DISCUSSION

A component of a polyunsaturated fatty acid (PUFA) elongase from *I. galbana* has been expressed successfully in Arabidopsis. Expression of *IgASE1* in yeast had previously demonstrated its substrate specificity to be restricted to LA and ALA (Qi et al., 2002). Consistent with these findings we have now observed the accumulation of EDA and ETrA in all transgenic plant tissues/organs examined, with the novel C20 fatty acid content reaching 15 mol% of total leaf fatty acid. Despite these high levels of VLCPUFAs we could not discern any effect on plant morphology.

A fatty acid elongation gene (*FAE1*) has been isolated from Arabidopsis (James et al., 1995). Expression of *FAE1* is seed specific, and the enzyme is involved in the synthesis of seed TAG with C20 and C22 fatty acids (Kunst et al., 1992). Ectopic expression of *FAE1* resulted in the formation of saturated, mono-, and polyunsaturated C20 and C22 fatty acids in the leaf, with some plants containing >30% very long chain fatty acid (VLCFA; Millar et al., 1998). Morphological changes were reported in plants with 10% or more VLCFAs. In this study, IgASE1 was completely specific for LA and ALA as previously described (Qi et al., 2002). The absence of ectopic saturated VLCFAs in this study may account for the normal phenotype of plants with >10% VLCPUFA content.

VLCFA synthesis involves the addition of C2 moieties to preexisting fatty acids, with each cycle of elongation requiring the four enzymatic reactions of condensation, reduction, dehydration, and further reduction (Fehling and Mukherjee, 1991). The condensation reaction catalyzed by a substrate-specific condensing enzyme is considered to be rate limiting; hence, *FAE1* most probably encodes the condensing component (Kunst et al., 1992; Millar and Kunst, 1997).

The chloroplast lipids PG, MGDG, and DGDG contained C20 PUFAs largely confined to the *sn*-1 position, whereas they were at both positions in PC but predominated at *sn*-2. It is considered that while some 60% of leaf PC is associated with the chloroplast, it is all derived from the eukaryotic synthetic pathway in the cytoplasm (Browse et al., 1986). Glycerolipids synthesized by the prokaryotic and eukaryotic pathways are characterized by having C16 and C18 fatty acids at the *sn*-2 position, respectively (Heinz and Roughan, 1983). Table III therefore shows that >70% of MGDG and 80% of PG are prokaryotic in Arabidopsis,

Table I. *Fatty acid composition of different tissues from wild-type and IgASE1 transgenic plants*

The results are averages from three independent measurements ±SD. Dashes indicate not detected.

Tissue	Line	Fatty Acid										
		16:0	16:1	16:3	18:0	18:1	18:2[a]	18:3[b]	20:0	20:2[c]	20:3[d]	22:0
		mol % of total										
Leaf	WT[e]	17.5 ± 0.8	4.8 ± 0.7	12.3 ± 0.7	0.9 ± 0.3	2.9 ± 0.7	13.7 ± 1.0	48 ± 1.0	–	–	–	–
	CA1-9	17.4 ± 0.1	3.2 ± 0.6	13.3 ± 0.1	1.2 ± 0.2	1.4 ± 0.2	7.0 ± 0.2	41.6 ± 0.3	–	6.1 ± 0.4	8.9 ± 0.1	–
Stem	WT	15.6 ± 1.0	2.9 ± 0.4	12.9 ± 0.7	0.8 ± 0.2	0.9 ± 0.2	15.1 ± 2.2	52 ± 1.7	–	–	–	–
	CA1-9	16.7 ± 2.8	2.2 ± 0.1	11.8 ± 1.7	1.2 ± 0.4	1.7 ± 0.2	14.2 ± 2.3	37.5 ± 4.2	–	8.4 ± 1.4	6.4 ± 0.4	–
Flower	WT	21.7 ± 0.9	1.6 ± 0.2	3.0 ± 0.4	1.3 ± 0.6	3.7 ± 0.7	29.0 ± 4.2	39.6 ± 4.2	–	–	–	–
	CA1-9	20.5 ± 0.7	2.0 ± 0.4	3.7 ± 0.4	1.9 ± 0.4	4.1 ± 0.3	25.6 ± 1.9	28.7 ± 4.4	–	7.6 ± 0.8	5.8 ± 1.1	–
Root	WT	16.2 ± 1.7	1.1 ± 0.2	1.0 ± 0.2	1.8 ± 0.1	4.6 ± 0.5	38.3 ± 3.4	21.9 ± 1.4	4.1 ± 0.4	–	–	11.0 ± 2.5
	CA1-9	14.0 ± 2.2	2.1 ± 0.2	0.8 ± 0.2	1.4 ± 0.3	6.0 ± 0.8	29.8 ± 2.2	15.7 ± 2.1	5.5 ± 0.6	11.3 ± 1.1	3.6 ± 0.6	9.8 ± 0.4
Silique	WT	17.1 ± 2.0	2.2 ± 0.2	3.9 ± 0.5	2.1 ± 0.4	10.1 ± 1.4	32.2 ± 3.6	32.3 ± 5.1	–	–	–	–
	CA1-9	19.0 ± 2.5	1.9 ± 0.3	2.3 ± 0.2	2.5 ± 0.5	10.0 ± 1.0	30.7 ± 5.1	25.0 ± 2.9	–	6.3 ± 1.1	2.5 ± 0.5	–

[a]LA. [b]ALA. [c]EDA. [d]ETrA. [e]Wild type.

whereas >80% of DGDG is derived from the eukaryotic pathway. These results confirm previous observations (Browse et al., 1986), and this study also shows that the C20 PUFA content largely mirrors the eukaryotic component of the various lipid classes. The difference in intramolecular distribution of the fatty acids in PC and DGDG requires consideration, especially with respect to the large component of elongation products at the *sn*-2 position of PC. It has been suggested that MGDG and DGDG derived from the eukaryotic pathway contain diacylglycerol (DAG) moiety from PC that typically does not contain palmitate at the *sn*-1 (Browse et al., 1986). Assuming that much of the PC with palmitate at the *sn*-1 position would have VLCPUFAs at *sn*-2, their selective retention in PC would ultimately decrease the C20 PUFAs at *sn*-1 of PC and increase them in eukaryotically derived MGDG and DGDG. It is interesting, however, that the positional distribution of PA was more similar to that observed in the chloroplast lipids than PC, and we have previously suggested that DAG moiety returned to the chloroplast may emanate from PA (Griffiths et al., 1986; Fig. 3).

Although DGDG is produced from MGDG, both the eukaryotic component and relative amount of VLCPUFAs are very different between these two lipids. The data are therefore consistent with the existence of two discrete largely pro- and eukaryotic pools of MGDG, with the smaller, more eukaryotic, pool taking part in DGDG production. In Arabidopsis MGDG is synthesized by at least two classes of MGDG synthetases, designated type A and type B (Miège et al., 1999). The type A synthetase, which is responsible for the bulk of MGDG synthesis, utilizes both prokaryotic and eukaryotic DAG and is probably located at the inner chloroplast membrane, whereas

Table II. *Fatty acid composition of major leaf glycerolipid classes from wild-type and IgASE1 transgenic plants*

Lipid Class	Line	Percentage of Total Lipid	Fatty Acid								
			16:0	16:1	16:3	18:0	18:1	18:2[a]	18:3[b]	20:2[c]	20:3[d]
			mol % of total								
MGDG	WT[e]	42	2.5 ± 0.4	0.9 ± 0.1	37.9 ± 3.0	tr[f]	0.9 ± 0.2	3.1 ± 0.6	54.9 ± 4.1	–	–
	CA1-9	40	2.8 ± 0.5	1.9 ± 0.6	37.2 ± 3.6	tr	2.0 ± 0.8	2.2 ± 0.4	51.0 ± 3.3	0.9 ± 0.4	2.0 ± 0.6
DGDG	WT	19	17.2 ± 2.9	–	1.9 ± 0.4	1.0 ± 0.3	0.8 ± 0.6	2.7 ± 0.2	76.3 ± 2.6	–	–
	CA1-9	18	13.0 ± 1.2	–	2.3 ± 0.6	0.9 ± 0.7	2.7 ± 0.7	3.6 ± 0.8	59.4 ± 1.6	1.3 ± 0.3	16.8 ± 0.8
PG	WT	11	32.1 ± 6.5	33.7 ± 5.1	–	1.1 ± 0.6	3.8 ± 1.4	6.5 ± 1.8	22.9 ± 5.8	–	–
	CA1-9	13	30.6 ± 4.4	33.4 ± 5.4	–	2.8 ± 0.7	5.1 ± 2.2	6.8 ± 2.0	18.1 ± 3.1	1.0 ± 0.2	2.1 ± 0.4
PC	WT	12	28.9 ± 4.8	0.8 ± 0.6	–	4.5 ± 1.1	6.3 ± 1.0	24.1 ± 1.3	35.4 ± 2.4	–	–
	CA1-9	11	28.0 ± 3.0	0.7 ± 0.3	–	3.8 ± 0.4	5.3 ± 0.4	12.3 ± 1.8	25.3 ± 3.0	14.8 ± 0.7	9.7 ± 1.7
PA	WT	6.9	22.9 ± 2.9	3.3 ± 0.5	–	3.0 ± 0.4	3.6 ± 0.3	28.6 ± 1.6	38.6 ± 2.4	–	–
	CA1-9	6.4	22.1 ± 3.0	3.6 ± 0.6	–	2.3 ± 0.4	3.9 ± 0.8	16.6 ± 2.5	27.9 ± 2.1	15.7 ± 1.5	7.9 ± 0.2
PE	WT	5.5	39.5 ± 4.0	1.5 ± 0.9	–	1.6 ± 0.1	1.6 ± 0.6	31.0 ± 4.4	24.8 ± 3.5	–	–
	CA1-9	5.1	34.8 ± 3.7	0.6 ± 0.2	–	1.2 ± 0.6	0.9 ± 0.3	22.4 ± 3.9	25.4 ± 2.1	11.1 ± 1.5	3.7 ± 0.6
PI	WT	4.2	50.2 ± 4.6	–	–	1.2 ± 0.2	1.1 ± 0.3	19.9 ± 3.3	27.5 ± 1.6	–	–
	CA1-9	4.3	49.8 ± 3.6	–	–	2.0 ± 0.6	2.3 ± 0.9	14.5 ± 2.4	20.7 ± 1.2	10.3 ± 1.2	0.6 ± 0.3

The results are averages from three independent measurements ±SD. Dashes indicate not detected. [a]LA. [b]ALA. [c]EDA. [d]ETrA. [e]Wild type. [f]Trace.

Table III. *Positional analysis of leaf galactolipids from wild-type and IgASE1 transgenic plants*

Lipid	Line	Fatty Acid									
		16:0	16:1	16:3	18:0	18:1	18:2[a]	18:3[b]	20:2[c]	20:3[d]	Total *sn*-2 C16[e]
		mol% of total									
MGDG											
sn-1	WT[f]	3.9 ± 0.4	–	6.4 ± 0.5	0.6 ± 0.2	1.5 ± 0.4	2.7 ± 1.1	84.9 ± 1.5	–	–	
	CA1-9	4.2 ± 1.3	–	6.0 ± 1.8	0.9 ± 0.4	3.8 ± 0.2	2.2 ± 0.3	78.2 ± 2.8	0.5 ± 0.2	4.2 ± 0.7	
sn-2	WT	2.1 ± 0.6	0.6 ± 0.4	70.0 ± 2.0	0.4 ± 0.1	0.6 ± 0.5	2.2 ± 0.3	24.4 ± 2.0	–	–	72.7
	CA1-9	2.6 ± 0.6	2.4 ± 0.8	68.5 ± 1.7	0.6 ± 0.3	2.5 ± 0.7	1.9 ± 0.62	21.3 ± 3.2	–	–	73.5
DGDG											
sn-1	WT	18.2 ± 0.6	–	–	2.5 ± 0.4	2.6 ± 0.6	3.5 ± 1.1	73.1 ± 0.6	–	–	
	CA1-9	14.4 ± 1.9	–	–	2.8 ± 0.5	4.6 ± 0.9	3.4 ± 0.8	40.6 ± 1.0	5.5 ± 1.3	28.5 ± 2.3	
sn-2	WT	15.9 ± 2.0	–	3.6 ± 0.6	–	–	2.2 ± 0.2	78.3 ± 1.5	–	–	19.5
	CA1-9	11.0 ± 2.7	–	4.9 ± 1.2	–	–	4.1 ± 0.8	76.6 ± 3.4	–	3.4 ± 0.8	15.9

The results are averages from three independent measurements ±SD. Dashes indicate not detected. [a]LA. [b]ALA. [c]EDA. [d]ETrA. [e]Total *sn*-2 C16 fatty acid (mol%). [f]Wild type.

type B synthetases are probably located at the outer membrane and have greater substrate specificity for eukaryotic DAG (Miège et al., 1999). DGDG synthesis also appears to be controlled by a multigene family, with DGDG1 and DGDG2 playing the major and minor roles, respectively, in DGDG biosynthesis in Arabidopsis (Klaus et al., 2002). While both isoforms are involved in eukaryotic DGDG synthesis, DGDG2 appears to be exclusively eukaryotic and is located at the outer chloroplast membrane (Klaus et al., 2002). The results from those studies and the data reported in this paper are consistent with discrete prokaryotic and eukaryotic pools of MGDG and DGDG synthesized at the inner and outer chloroplast membranes of Arabidopsis, respectively (Fig. 3).

The apparent correlation between the eukaryotic component determined by *sn*-2 C16 fatty acids and VLCPUFA content suggests that the various isoforms of MGDG and DGDG synthetases do not discriminate against VLCPUFAs.

CA1-9 transformants had substantially more C20:2 than C20:3 in PC and PE, whereas the major chloroplast lipids MGDG and DGDG had more C20:3 than C20:2 (see Table II). This suggests that the chloroplast ω3 desaturases have a higher activity with C20:2 fatty acid precursor than their endoplasmic reticulum (ER) counterpart(s). Similar results were reported using transgenic expression of *FAE1* from the CaMV 35S promoter (Millar et al., 1998). In wild-type plant leaf lipids the C18:3 to C18:2 ratio was also higher in the chloroplast galactolipids than PC and PE, consistent with combined chloroplast ω3 desaturase activity being greater than equivalent ER desaturase activity with C18:2 or C20:2 precursors.

The VLCPUFA content (mol%) of transgenic Arabidopsis seeds was much higher than that of the wild

Table IV. *Positional analysis of the leaf phospholipids from wild-type and IgASE1 transgenic plants*

Lipid	Line	Fatty Acid								
		16:0	16:1	18:0	18:1	18:2[a]	18:3[b]	20:2[c]	20:3[d]	Total *sn*-2 C16[e]
		mol% of total								
PG										
sn-1	WT	26.1 ± 4.3	11.9 ± 1.6	2.0 ± 0.5	8.8 ± 0.8	11.7 ± 2.1	39.4 ± 4.0	–	–	
	CA1-9	24.4 ± 1.9	9.2 ± 0.8	5.5 ± 0.8	15.5 ± 1.4	8.8 ± 1.2	31.5 ± 2.5	1.9 ± 0.4	3.1 ± 0.4	
sn-2	WT	35.6 ± 3.4	47.4 ± 2.8	–	0.6 ± 0.3	7.6 ± 1.6	8.7 ± 0.9	–	–	83.0
	CA1-9	32.5 ± 1.8	50.9 ± 3.2	–	0.3 ± 0.2	7.7 ± 0.7	8.5 ± 1.5	–	–	83.4
PC										
sn-1	WT	50.4 ± 5.2	1.3 ± 0.6	6.0 ± 0.6	7.2 ± 1.4	8.8 ± 2.4	26.3 ± 2.1	–	–	
	CA1-9	52.4 ± 2.2	0.6 ± 2.2	6.2 ± 1.4	5.2 ± 0.5	5.0 ± 0.4	15.7 ± 1.2	7.4 ± 0.6	7.5 ± 0.4	
sn-2	WT	2.5 ± 0.4	0.7 ± 0.2	3.1 ± 1.0	6.6 ± 1.3	40.3 ± 2.0	46.6 ± 3.9	–	–	3.2
	CA1-9	2.4 ± 1.0	0.6 ± 0.3	2.4 ± 0.6	6.5 ± 0.7	24.3 ± 2.6	32.3 ± 2.9	22.7 ± 3.2	8.5 ± 1.5	3.0
PA										
sn-1	WT	40.0 ± 1.9	4.2 ± 0.3	4.6 ± 0.7	4.0 ± 0.7	15.6 ± 2.9	31.5 ± 1.8	–	–	
	CA1-9	39.4 ± 1.8	3.2 ± 0.7	4.1 ± 0.3	5.0 ± 0.6	7.5 ± 0.7	10.4 ± 0.6	17.8 ± 1.1	12.5 ± 1.0	
sn-2	WT	6.0 ± 0.9	2.8 ± 0.7	–	3.7 ± 0.3	40.9 ± 0.9	46.6 ± 2.2	–	–	8.8
	CA1-9	5.6 ± 1.1	5.1 ± 1.8	–	4.3 ± 0.2	25.7 ± 1.8	45.0 ± 3.9	9.8 ± 1.6	4.4 ± 0.4	10.7

The results are averages from three independent measurements. Dashes indicate not detected. [a]LA. [b]ALA. [c]EDA. [d]ETrA. [e]Total *sn*-2 C16 fatty acid (mol%).

Table V. *Positional analysis of seed TAG from wild-type and IgASE1 transgenic plants*

Position	Line	Fatty Acid										
		16:0	Other C16[a]	18:0	18:1	18:2[b]	18:3[c]	20:0	20:1	20:2[d]	20:3[e]	22:1
		mol% of total										
sn-1+3	WT[f]	16	–	5	17	22	16	2	19	2	–	2
	CA1-9	16	–	5	16	19	13	2	19	7	1	2
sn-2	WT	–	–	–	17	52	31	–	–	–	–	–
	CA1-9	–	–	–	16	51	33	–	–	–	–	–
Total	WT	10	–	4	16	32	21	2	12	1	–	1
	CA1-9	11	–	3	15	31	20	1	13	5	–	1

The results are averages from two independent measurements. Dashes indicate not detected. [a]16:1 plus 16:3. [b]LA. [c]ALA. [d]EDA. [e]ETrA. [f]Wild type.

type. Expression of the transgene was therefore surprisingly successful in light of the comparatively weak activity reported for the CaMV 35S promoter in seeds (Eccleston and Ohlrogge, 1998). Positional analysis of TAG showed that VLCPUFAs were largely excluded from the *sn*-2 position in both wild-type and transgenic plants, similar to the absence of VLCFAs from the *sn*-2 position of TAG in rapeseed oil (*Brassica napus*; Mattson and Volpenhein, 1961; Norton and Harris, 1983). In vitro assays using microsomal membranes from developing rapeseed embryos also showed that lysophosphate acid acyltransferase did not utilize erucoyl-CoA (Sun et al., 1988; Bernerth and Frentzen, 1990; Cao et al., 1990). While all seed TAG is probably derived from the eukaryotic pathway (no C16 *sn*-2 fatty acids), there was a clear difference between the acyl composition at the *sn*-2 position of TAG and the leaf eukaryotic lipid PC, and PA, which has a similar

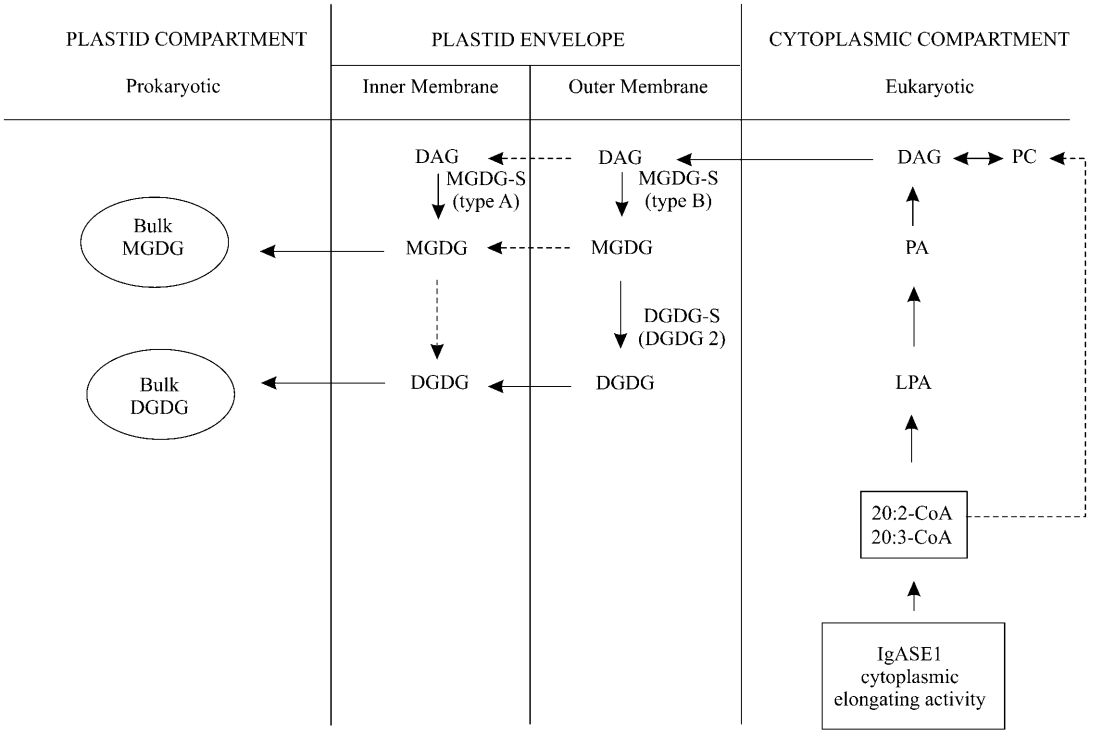

Figure 3. Partitioning of VLCPUFAs in leaves of transgenic Arabidopsis. VLCPUFA (EDA and ETrA) production occurs by the elongation of LA and ALA CoAs utilizing IgASE1 and the other elongating components in the cytoplasm. The VLCPUFAs are largely esterified to the *sn*-1 position of G3P to yield eukaryotic DAG. The DAG can be further metabolized to PC that, in conjunction with lysophosphatidylcholine acyl-CoA acyltransferase activity, yields pool(s) of PC with VLCPUFAs at the *sn*-1 and *sn*-2 positions. Enzymes in the outer membrane of the chloroplast convert the DAG to eukaryotic DGDG (via MGDG) and this pool of DGDG forms the bulk pool of DGDG in the chloroplast. Major routes for VLCPUFA utilization are given in solid lines with possible minor contributions in broken lines.

C20:2 to C20:3 ratio to eukaryotic lipids and an *sn*-2 C16 content consistent with it being of some 90% eukaryotic origin. Both these leaf lipids contained appreciable quantities of VLCPUFAs at the *sn*-2 position; indeed in PC they were more prevalent at this position than at *sn*-1. Selective channeling of PC containing *sn*-1 VLCPUFAs back to the chloroplast in the leaf but not in the seed may account for some of the apparent enrichment of VLCPUFAs at the *sn*-2 position of PC compared to the *sn*-1. However, it is inconsistent with their presence at the *sn*-2 position of PA but their absence from this position in seed TAG. This, therefore, provides biochemical evidence for different extraplastidic lysophosphate acid acyltransferase isozymes expressed in the seed and leaf, analogous to those observed in *Limnanthes douglasii* (Brown et al., 1995).

While in vivo kinetics of acetate incorporation into chloroplast lipids are consistent with PC providing the DAG moiety for incorporation into plastid glycerolipids (Roughan and Slack, 1982; Heinz and Roughan, 1983; Browse et al., 1986), the current data suggest that PA may also play a role in the transfer of fatty acids from the ER to the chloroplast envelope. Certainly the use of novel/foreign fatty acid synthesis in transgenic Arabidopsis and other species should provide a better understanding of the trafficking of acyl components between the chloroplast and the cytoplasm.

CONCLUSIONS

The expression of the elongase component *IgASE1* resulted in the production of substantial quantities of C20 VLCPUFAs accumulating in all glycerolipids and without adverse morphological effects. The formation of foreign fatty acids in the model plant Arabidopsis and other species offers a novel approach in understanding the trafficking of acyl components between the chloroplast and the cytoplasm for prokaryotic and eukaryotic lipid synthesis.

MATERIALS AND METHODS

Vector Construction and Plant Transformation

The cloning of the *IgASE1* cDNA is described in Qi et al. (2002). The cDNA was released from plasmid vector pCR2.1-TOPO by digestion with *Kpn*I and ligated into the *Kpn*I site of the intermediate vector pBlueBac 4.5 (Invitrogen, Carlsbad, CA). Recombinant plasmids were screened for insert orientation with *Eco*RI. The insert was released from a selected plasmid by digestion with *Bam*HI plus *Spe*I and ligated into the corresponding sites of pCB302-3 (Xiang et al., 1999, in which the map of pCB302-3 is incorrect. The CaMV 35S promoter [plus omega sequence] and nos terminator regions are reversed with respect to MCS2). The recombinant binary vector was transferred to *Agrobacterium tumefaciens* strain GV3101 by electroporation, and transformed colonies were selected on medium containing 50 μg mL^{-1} kanamycin. A selected colony was grown to stationary phase at 28°C, then the cells were concentrated by centrifugation and resuspended in a dipping solution containing 5% Suc, 0.03% Silwet-177, and 10 mM $MgCl_2$.

Seeds of Arabidopsis ecotype Columbia 4 were germinated on one-half-strength Murashige and Skoog medium, and seedlings were transferred to compost in 15-cm flowerpots. Plants were grown to flowering stage in a growth cabinet at 21°C, with a 23-/1-h photoperiod at 150 μmol photons m^{-2} s^{-1}. Plant transformation was carried out by the floral dipping method of Clough and Bent (1998), essentially as follows:

Two pots containing 16 plants each were inverted in the dipping solutions containing transformed *A. tumefaciens* (described above). The plants were then covered with a plastic bag and left overnight at room temperature in the dark. The bag was then removed and the plants transferred to the growth cabinet. Dipping (with fresh *A. tumefaciens* solution) was repeated after 5 d and the plants were allowed to set seed. Bulked seeds from dipped plants (=T1 seed) were collected, approximately 10,000 seeds sprinkled onto compost in a seed tray and after stratification at 4°C for 2 d cultivated in the growth cabinet. When seedlings had reached the 2 to 4 true-leaf stage they were sprayed with 0.5% Liberty herbicide (Aventis, Essex, UK; 0.5 g glufosinate-ammonium L^{-1}), and spraying was repeated 1 week later. Twelve herbicide-resistant plants were selected and potted and allowed to self-fertilize. Samples of T2 seed collected from these plants were germinated on one-half-strength Murashige and Skoog medium containing Liberty (5 mg glufosinate-ammonium L^{-1}). T3 seed collected from individual surviving plants were then again germinated on Liberty plates to screen for homozygous lines that had ceased segregating for herbicide resistance. Total fatty acids extracted from leaves of such lines were analyzed and line CA1-9 was identified as having the greatest C20 content and characterized further.

Lipid Extraction

Leaves were homogenized manually using a custom-made glass homogenizer with 12 mL of chloroform to methanol to formic acid (10:10:1, v/v) and stored overnight at −20°C. The extract was washed with 6 mL of 0.2 M H_3PO_4 and 1 M KCl, and the lipids recovered in the chloroform phase were dried and taken up in 0.5 mL chloroform.

Separation of Lipids

Individual lipids were purified from the extracts by one-dimensional thin layer chromatography (TLC) on precoated silica gel plates. PA, PI, MGDG, PG, and PE were separated using chloroform:methanol:7 M NH_4OH (65:30:4, v/v). DGDG and PC were separated with chloroform:methanol:25% w/v ammonia (60:30:7.5, v/v). Lipids were visualized by light iodine staining, identified by comparison with reference standards, and scraped from the plate. They were either transmethylated in situ by the methods of Lightner et al. (1997) for analysis of purified lipid classes or eluted using chloroform-methanol and treated with lipase for positional analysis of individual lipid classes as described below.

Positional Analysis of Phospholipids

Positional analysis of phospholipids was carried out using phospholipase A_2. *Ophiophagus hannah* venom (25 units) was dissolved in 0.5 mL of 2 mM calcium chloride and 50 mM borate buffer pH 7.5. Phospholipid dissolved in 1 mL of diethyl ether was added to the phospholipase A_2 solution and shaken vigorously for 1 h at room temperature. The mixture was washed into a conical flask with 10 mL of methanol followed by 20 mL of chloroform. The solution was dried with anhydrous sodium sulfate. The liquid phase was decanted and evaporated under N_2. The products were taken up in a small volume of chloroform:methanol (2:1) and applied to a silica gel-coated TLC plate and double developed in the same direction first in chloroform:methanol:acetic acid:water (85:15:10:3.2) to about 60% of the height of the plate and then redeveloped to the top of the plate in hexane:diethyl ether:acetic acid (70:30:1).

Positional Analysis of Galactolipids and Triacylglycerol

Rhizopus lipase (25 units) was dissolved in 2 mL of 2 mM calcium chloride, 50 mM borate buffer, pH 7.5. Lipids dissolved in 0.5 mL of diethyl ether were added to the lipase solution and shaken vigorously for 1 h at room temperature in a small sealed glass tube. Subsequent procedures were the same as described above for phospholipids, apart from the TLC of TAG lipase hydrolysis products, where the first development was omitted.

Fatty Acid Analysis

Fatty acids from leaves, roots, stems, flowers, siliques, and the various TLC-purified individual lipids, their lyso-derivatives and free fatty acids were

extracted and methylated according to Lightner et al. (1997). The FAMEs were analyzed by gas chromatography on a 30 m × 0.25 mm fused silica DB-23 column (J&W Scientific, Folsom, CA) using heptadecanoic acid (C17:0) as internal standard and quantified by flame ionization detection. The chromatograph was programmed for an initial temperature of 140°C for 5 min, followed by a 20°C/min temperature ramp to 185°C and a secondary ramp of 1.5°C/min to 220°C. The final temperature was maintained for 2 min. Injector and detector temperatures were maintained at 230°C and 250°C, respectively. The head pressure of the carrier gas (He) was 80 kPa; a split injection was used.

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

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