lipid metabolism. We identified a transfer-DNA (T-DNA)-tagged *Arabidopsis* mutant with an interrupted *ACS* gene which we believe to encode a plastidial isoform. This paper describes the partial characterization of this ACS isoform. A better understanding of the biochemistry and biological function of the plastidial ACS isoform will be critical to the overall knowledge of lipid synthesis in leaf and seed tissues.

Results

Analysis of RNA expression profiles

The RNA expression profiles of several isoforms of *A. thaliana* ACS were examined. The pattern of transcript accumulation of ACS2 suggests that it might be localized in the plastid. The ACS2 transcript is most abundant in young leaves, developing seeds, roots and flowers. Northern analysis indicates that the ACS2 transcript abundance decreases with age in developing siliques (results not shown).

Chloroplast import assay with ACS2

Chloroplast import assays [9] were performed in order to determine if the ACS2 protein was targeted to the plastid. After import, intact chloroplasts were isolated and treated with lysis buffer (25 mM Hepes + 5 mM $MgCl_2$), 3 M NaCl or 100 mM Na_2CO_3. The negative control, luciferase, is not imported. Pea Rubisco small subunit is targeted to the soluble fraction. A tomato hydroperoxide lyase (LeHPL, an outer-envelope-targetted protein; J. Froehlich and G. Howe, unpublished work) and ACS2 are localized in the chloroplast membranes. Harsh chaotropic conditions (Na_2CO_3) dissociate ACS2 from the chloroplasts.

Analysis of a T-DNA-tagged ACS2-knockout mutant

The T-DNA-tagged populations of *A. thaliana* were screened for the presence of an individual with a T-DNA insertion in the ACS2 coding region. A plant homozygous for such an insertion in the ACS2 coding region would display stunted, wrinkled leaves, a shortened floral bolt and relatively low seed yield. Analysis of the fatty acid composition was done on the ACS2-knockout mutant to determine if the absence of ACS2 has a pronounced effect on the fatty acid profile. Fatty acids were extracted and analysed by GC. There was no significant difference between the fatty acid profile of the wild-type and mutant plants.

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Enhancement of seed oil content by expression of glycerol-3-phosphate acyltransferase genes

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Abstract

Arabidopsis thaliana was transformed with a plastidial safflower glycerol-3-phosphate acyltransferase (GPAT) and an *Escherichia coli* GPAT. The genes were used directly and in modified forms with, as applicable, the plastidial targeting sequence removed, and with an endoplasmic reticulum targeting sequence added. Seeds of plants transformed using only the vector were indistinguishable in oil content from wild-type control plants. All other gene constructs increased seed oil content. The unmodified safflower gene (*spgpat*) produced oil increases ranging from 10 to 21%.

Key words: *Arabidopsis*, safflower, triacylglycerol.

Abbreviations used: GPAT, glycerol-3-phosphate acyltransferase; tp, transit peptide; ERrs, endoplasmic reticulum retention sequence.

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On average, the greatest increase (+22%) was observed in seeds of transformants carrying the *spgpat* with the targeting peptide removed. The *E. coli plsB* gene increased seed oil content by an average of 15%.

Introduction

Glycerol-3-phosphate acyltransferase (GPAT) catalyses the first reaction in triacylglycerol synthesis via the Kennedy pathway. We hypothesized that enhancing GPAT production might result in an increase in carbon flux through the Kennedy pathway, resulting in higher seed oil content.

We expressed, in *Arabidopsis thaliana*, a plastidial (P)-GPAT gene (*ctpgpat*) from safflower [1] and the GPAT gene (*plsB*) from *Escherichia coli*. The genes were used directly and in modified forms with, as applicable, the plastidial targeting sequence (tp) removed, and with an endoplasmic reticulum retention sequence (ERrs) added [2]. Here we demonstrate a role for GPAT in regulating the amount of oil in *A. thaliana* seeds.

Experimental

Preparation of chimaeric genes and expression vectors

An open reading frame ($\approx$ 1.1 kb) without tp was PCR-amplified from the ctpgpat cDNA. Another chimaeric gene containing an ERrs at its 3′ end was also PCR-amplified. The open reading frame of the *E. coli plsB* gene ($\approx$ 2.5 kb) was PCR-amplified from bacterial DNA without modification or with an ERrs at its 3′ end. The blunt-end PCR fragments generated using *Pfu* DNA polymerase were cloned into the pSKII (Stratagene) cloning vector and were sequenced to confirm the nucleotide sequence and the incorporation of restriction sites and ERrs sequences into the chimaeric genes. The genes were labelled *ctpgpat-tp*, *ctpgpat-tp+ERrs*, *plsB* and *plsB+ERrs*. The intact *ctpgpat* cDNA was also used.

For yeast expression studies, the *ctpgpat* or *plsB* chimaeric genes were cloned into the yeast expression vector, pYES2 (Invitrogen), under the transcriptional control of a galactose-inducible promoter. For plant expression studies, chimaeric genes were cloned into the plant transformation vector, pHS737 (G. Selvaraj and R. Hirji, unpublished work), under the control of a tandem 35 S cauliflower mosaic virus promoter with avian myeloblastosis virus translational enhancer and 35 S poly(A)$^+$ for constitutive expression. The recombinants were transferred into *Agrobacterium tumefaciens* GV 3101 for transformation of *A. thaliana* L. Heynh. ecotype Columbia.

Transformation of yeast and *A. thaliana*

The INVSc 1 strain (Invitrogen) of yeast was transformed by the heat-shock method [3] to assess the functionality of the genes and the derived proteins. *Arabidopsis* plants were transformed by the floral dip method [4]. Seeds (T_1) from these plants were collected and selected on a growth medium containing kanamycin. Transgenic plants were grown to maturity and seeds (T_2) from 10 individual plants were collected and used for fatty acid analysis. Wild-type plants and plants transformed with vector only were grown as controls.

Enzyme assay

Yeast cells containing chimaeric *GPAT* genes were grown in SC-Ura (Bio 101) containing glucose. *GPAT* gene expression was induced by transferring cells to growth medium containing galactose. Protein was extracted from the control and transformed cells and GPAT activity was assayed [5]. The products of the reaction were identified by TLC [6].

Results and discussion

All the *GPAT* genes used produced functionally active protein when expressed with a galactose-inducible promoter in yeast cells. GPAT activity was greater in the transformants than in the control cells. Increased GPAT activity also led to enhanced lipid production *in vitro*.

The oil compositions and seed sizes of selected lines representing each construct are shown in Table 1. Seeds of plants transformed using only the pHS737 vector were indistinguishable in oil content from wild-type control plants. All other gene constructs produced higher seed oil contents. The unmodified *ctpgpat* produced oil increases ranging from 10 to 21%. On average, the greatest increase in oil was observed in seeds of transformants carrying the *ctpgpat-tp* gene (average +22%). Addition of an ERrs had no apparent effect on seed oil content.

The *plsB* gene increased seed oil content by an average of 15%. The addition of an ERrs resulted in an average seed oil increase of 18% but the difference was not significant. It is somewhat

Table 1

Oil contents of wild-type and transformed *A. thaliana* seeds

Oil percentages for transformed plants represent the means from three analyses. S.D. values were in the range 0.48–1.52%. Values for the wild-type and vector-only (pHS737) plants represent means±S.D., $n = 7$.

Sample	Transformant	Oil content		Weight/100 seeds (mg)
		Wt%	% Increase	
Wild type	—	26.9±0.8	—	1.42±0.29
pHS737	—	26.8±1.09	—	1.41±0.26
ctpgpat	315-2	32.4	20.9	2.47
	315-3	29.4	9.7	1.92
	315-4	30.1	12.3	1.90
	315-7	29.6	10.4	1.85
ctpgpat-tp	301-2	34.0	26.9	2.03
	301-3	34.6	29.1	1.90
	301-5	29.3	9.3	1.79
ctpgpat-tp+ERrs	302-2	29.4	9.7	1.44
	302-6	32.7	22.0	2.45
plsB	303-2	29.8	11.2	1.28
	303-3	30.8	14.9	1.98
	303-4	29.1	8.6	1.89
	303-7	33.2	23.9	1.53
plsB+ERrs	304-1	30.9	15.2	1.51
	304-2	32.5	21.3	1.60
	304-3	30.3	13.1	1.38
	304-15	32.5	21.3	2.20

unexpected that the ctpGPAT protein, which ordinarily acts on an acyl-acyl carrier protein (ACP) substrate and has a much reduced activity with acyl-CoA substrates, effected a greater seed oil increase (on average) than the plsB protein.

Seeds of plants transformed with the vector only did not differ significantly in average weight from wild-type plants (Table 1). Seeds of individual plants from each construct were significantly heavier than the wild type and the pHS737 control; e.g. transformants 315-2, 301-2, 302-6, 303-3 and 304-15. However, increased seed oil content was not always positively correlated with increased seed weight; e.g. 303-7 and 304-1.

Ohlrogge and Jaworski [7] have proposed a fatty acid supply (source) and demand (sink) model for determining the regulation of oil synthesis. An increase of 5% was obtained in rapeseed by expressing a cytosolic form of acetyl-CoA carboxylase in chloroplasts [8]. Larger increases, 8–48%, were obtained in members of the Brassicaceae by expressing a mutant form of yeast *lyso*-phosphatidic acid acyltransferase (LPAAT) [9]. In our study, the oil content of *Arabidopsis* seeds was increased from 8 to 29% in selected transgenic lines by expressing a plant plastidial and a bacterial *GPAT* gene. Our results are consistent with the 'creation of a sink' theory. It should be noted that no increases were obtained by expressing wild-type plant *LPAAT* genes [10]. However, the use of either mutant or heterologous genes, such as those used in this study and by others [9], has proven to be useful in elevating oil content. Transgenic plants producing triacylglycerols with altered fatty acid chain lengths and saturation have also been produced [7].

In conclusion, we have demonstrated that expression of both a bacterial and a plant *GPAT* gene increase plant seed oil content and seed weight.

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Mutagenesis of a plastidial lysophosphatidic acid acyltransferase

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Abstract

A combination of site-directed and random mutagenesis generated sequence variants of a plastidial lysophosphatidic acid acyltransferase. Alanine substitutions of residues present within two conserved motifs including the putative catalytic histidine resulted in a loss of acyltransferase activity assessed as complementation competance. Substitutions at five sites within the central core resulted in reduced or loss of activity. Truncation mutants reveal that sequences in the C-terminal moiety are essential for function.

Introduction

As a consequence of a strong variation in substrate preference, isozymes of lysophosphatidic acid acyltransferase (LPAAT, EC 2.3.1.51) located in organellar and cytoplasmic compartments play an important role in determining the acyl composition of phosphatidic acid, a key intermediate in the biosynthesis of membrane and storage lipids [1]. In oilseeds that accumulate unusual fatty acids additional isoforms of the microsomal LPAAT exhibit variation in substrate preference and selectivity and thus play a crucial role in determining triacylglycerol composition. Phylogenetic sequence alignments reveal the existence of two classes of plant LPAATs; class A, containing ubiquitously expressed eukaryotic enzymes, and class B, containing the prokaryotic types and the plastidial enzyme, together with microsomal isoforms expressed only in the seeds of plants that accumulate unusual fatty acids [2]. We have isolated genes encoding microsomal and plastidial LPAATs (Bourgis et al. [3]) and we are attempting to determine the structural domains that confer acyltransferase activity and substrate specificities. We describe here the use of a combination of site-directed and random mutagenesis to identify residues essential for function of the plastidial LPAAT.

Key words: protein engineering.

Abbreviation used: LPAAT, lysophosphatidic acid acyltransferase.

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Materials and methods

Site-directed mutagenesis

This was performed via PCR with a plasmid DNA containing the *Brassica napus* cDNA BAT2 encoding a plastidial LPAAT (accession number A111161) as template using the following oligonucleotides (5′ → 3′ direction): BAT215, TACGTTTCGAACGCCCAAAGCAGCTTTCTGG; BAT213, CCAGAAAGCTTTGGGCGTTCGAAACGTA; BAT225, CACCAAAGCTTTCTGGCTATATACACACTTCTC; BAT223, GAGAAGTGTGTATATAGCCAGAAAGCTTTGGTG; BAT235, GTCTTTTTCTTCCCAGCGGGAACGAGGAGTAAGG; BAT233, CCTTACTCCTCGTTCCCGCTGGGAAGAAAAAGAC.

The PCR followed by digestion of the parental template with *Dpn*I was performed according to the supplier's (Quik-change, Stratagene) conditions. The modified plasmid was transformed into JC201 [4], a temperature-sensitive *Escherichia coli* JC201 LPAAT-deficient strain (supplied by J. Coleman, Lousianna State University School of Medicine, New Orleans, LA, U.S.A.), and plasmid DNA was sequenced to verify the presence of the desired mutations.