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Full Length Research Paper

Ectopic expression of *Crambe abyssinica* lysophosphatidic acid acyltransferase in transgenic rapeseed increases its oil content

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Lysophosphatidic acid acyltransferase (LPAAT) is a pivotal enzyme controlling the metabolic flow of lysophosphatidic acid into different phosphatidic acids in diverse organisms. Here we identified and cloned LPAAT from *Crambe abyssinica* Hochst. ex R. E. Fries (Ca*LPAAT,* GenBank accession number EF208088). To study its function *in vivo*, Ca*LPAAT* was introduced into *Brassica napus* by *Agrobacterium*. The expression profile of several genes in the glycerolipids synthesis pathway was determined by quantitative RT-PCR. Interestingly, higher expression of *CaLPAAT* led to elevated expression of these genes. Further analysis of the fatty acyl compositions in the self-cross seeds of the T_1 generation demonstrated that they were at the similar level in both transgenic plants and their non-transgenic counterparts. However, the total oil content of transgenic seeds was increased. We conclude that Ca*LPAAT* can be ectopically expressed in *B. napus* and increase its total oil content.

Key words: *Brassica napus*, *Crambe abyssinica* Hochst. ex R. E. Fries, lysophosphatidic acid acyltransferase, oil content.

INTRODUCTION

Glycerolipids are the most abundant lipids in seed plants (Somerville et al., 2000; Voelker and Kinney, 2001). In plants, they are synthesized in plastids (prokaryotic system) and the endoplasmic reticulum (eukaryotic system) through a cascade of enzymatic reactions. These two systems are similar in the first step of this cascade, in which glycerol-3-phosphate (GP) is acylated to lysophosphatidic acid (LPA) by GP acyltransferase (GPAT, EC 2.3.1.15). Subsequently, LPA is converted to phosphatidic acid (PA) by LPA acyltransferase (LPAAT, EC 2.3.1.51) in different cellular compartments. This conversion precludes the fate of PA in different parts of the organism. Thus, the regulation of PA synthesis is pivotal in controlling of glycerolipid synthesis (Kim and Huang, 2004). Synthesis of PA from LPA is catalyzed by distinct LPAATs in different cellular compartments (Kim et al., 2005). In maturing seeds, the PA, converted to triacylglycerols (TGs) for storage, is synthesized by cytoplasmic LPAATs. Although many of the biochemical steps in TG biosynthesis are known, its regulation remains unclear.

Achieving the goal of increasing seed oil content will be of importance not only for feeding a growing world population, but also for meeting the need of the growing biofuel industry. There are many ongoing studies that are trying to increase seed oil content and cytoplasmic LPAATs are believed to play a key role in determining the overall lipid acyl composition and oil content in seeds (Brown et al., 1995; Cao et al., 1990; Frentzen, 1998; Icihara et al., 1987; Laurant and Huang, 1992; Maisonneuve et al., 2009; Oo and Huang, 1989; Zou et al., 1997).

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Abbreviations: GP, Glycerol-3-phosphate; **GPAT,** glycerol-3-phosphate acyltransferase; **LPA,** lysophosphatidic acid; **LPAAT,** lysophosphatidic acid acyltransferase; **PA,** phosphatidic acid; **CaLPAAT,** cloned LPAAT from Crambe abyssinica Hochst. ex R. E. Fries; **TGs,** triacylglycerols; **6-BA**, 6-benzylaminopurine; PCR, polymerase chain reaction; **RT-PCR,** reverse transcription-PCR.

Although many LPAAT genes have been identified from different species (Coleman, 1990; Knutzon et al., 1995; Nagiec et al., 1993) and LPAATs of different species have been found to share two conserved motifs (NHX_4D and EGT), which have been shown to be the catalytic site and GP-binding site, respectively (Heath and Rock, 1998; Lewin et al., 1999), yet there is little information available for *Crambe abyssinica* Hochst. ex R. E. Fries LPAAT (Brassicaceae = Cruciferae). *C. abyssinica* is an important industrial crop in many countries and is one of the richest known sources of erucic acid. Here, we identified a *LPAAT* gene (Ca*LPAAT*) in *C. abyssinica*, ectopically expressed in *Brassica napus* and determined the fatty acyl compositions and total oil content of the transgenic plants.

MATERIALS AND METHODS

General molecular biology techniques

Unless stated, all molecular biological techniques (PCR, DNA, RNA and plasmid preparations, restriction digestion etc.) were performed by methods generally prescribed by Sambrook and Russell (2001).

Cloning of LPAAT gene from *C. abyssinica* Hochst. ex R. E. Fries

One pair of degenerate primers was designed from both ends of *Arabidopsis thaliana* LPAAT2 cDNA (At*LPAAT2*, GenBank accession No.: NM115625) and *B. napus* LPAAT2 cDNA (Bn*LPAAT2*, GenBank accession No: Z95637). The forward and reverse primers are C1 (5'-ATGGYGAT KGCWGCAGCWG-3') and C2 (5'-TTACTT CTSCTTCTCCRYTTCTGT-3'), respectively. RT-PCR was carried out to obtain the targeted sequence from leaves. Purified PCR products were subcloned into pMD18-T vector (Takara, Japan) and sequenced from both orientations.

On the basis of the cDNA sequence, one pair of specific primers was designed to clone the genomic DNA (C3: 5'-ATGGCGATGGC TGCAGCTGTAAT-3' and C4: 5'-TTACTTCTGCTTCTCCGCTTC TGT-3'). The purified PCR products were also subcloned into pMD 18-T vector and sequenced.

Sequence analysis

The nucleotide and putative amino acid sequence were aligned in the NCBI (National Center for Biotechnology Information) database using BLAST analysis sever. A homology tree of putative amino acid sequences of proteins derived from Ca*LPAAT* and related putative LPAAT proteins of other plant species in GenBank was created by using the observed divergency method of DNAMAN software (Lynnon Biosoft).

Construction of expression vectors

Ca*LPAAT* cDNA was directed cloned between *Bam*HI and *Sac*I sites of pBI121 instead of GUS gene to construct the CaLPAAT expression vector 35S: LPAATF. For cloning, one pair of specific primers was designed (F-BF: 5'-CGCGGATCCATGGCGATGGCTG CAGCTGTAAT-3' and F-SR: 5'-CGCGAGCTCTTACTTCTGCTT CTCCGCTTCTGT-3'), which contains *Bam*HI and *Sac*I sites, respectively. Anti-sense CaLPAAT vector 35S: LPAATR was also constructed through reverse-directed cloning the key conservative sequence of Ca*LPAAT* between *Bam*HI and *Sac*I sites of pBI121. The primers for reverse-directed cloning are R-BF (5'-CGCG GATCCGATCTCTGCACCACTGTG CAAT-3') and R-SR (5'-CGCGAGCTCTGGATCGTTGACTGGTGG GCT-3').

Plant transformation

Transformation was performed on *B. napus* cv. "84100-18" with low content of erucic acid. Hypocotyl explants were prepared as described by Mukhopadhyay (1991). *Agrobacterium* strain EHA105 containing 35S:LPAATF (or 35S:LPAATR) was grown overnight (220 rpm, 28°C in dark) in 100 ml of liquid LB containing 15 mg/L rifampicin, 50mg/L kanamycin and 25 mg/L streptomycin. Agrobacteria were pelleted (2100 g) and suspended in liquid MS medium (Murashige and Skoog, 1962) with 100 mg/L acetosyringone (As). The OD of the bacterial suspension was adjusted to around A_{600} = 0.6. Explants plated on the medium were removed and incubated in the bacterial suspension for 1 min. Excess fluid was removed by placing explants on a filter paper. Subsequently, explants were replated on co-cultivation media (MS with 0.2mg/L 6-benzylaminopurine (6-BA), 100 mg/L As and 5.0 mg/L silver nitrate, pH = 5.4) in dark. After 2 days, explants were transferred to callus induction medium (MS with 1.0 mg/L 2, 4-dichlorophenoxyacetic acid, 500mg/L carbenicillin and 5.0 mg/L $AgNO_3$, pH = 5.8). 7 days later, explants were transferred to MS medium supplemented with 3.0 mg/L 6 BA, 0.01 mg/L gibberellin, 500mg/L carbenicillin, 5.0mg/L $AgNO_3$, 0.1mg/L α-naphthaleneacetic acid and 15 mg/L kanamycin (pH = 5.8). Green plantlets that differentiated on selection media were rapid propagated, rooted and transferred to soil and grown to maturity (Mukhopadhyay et al., 1991).

Confirmation of positive transformation

PCR was carried out to confirm the transgenic nature of the regenerated plants. The forward primer 35S (5'-ATTTCATTT GGAGAGAACACGG-3') was designed according to the upstream 35S promoter sequence to avoid false positive results. F-SR (for sense) and R-SR (for anti-sense) were used as reverse primer, respectively.

RT-PCR analysis

Total RNA was extracted from different parts of PCR positive transgenic and non-transformed plants (flowers, stems, leaves and mature seeds) for quantitative and semi-quantitative RT-PCR analysis. Primers for *B. napus ACTIN* gene (as internal standard, GenBank accession No.AF111812) are A1 (5'-TCTTCCTCACG CTATCCTCCG-3') and A2 (5'-CGATGTTTCCATACAGATCCTT CC-3'). Primers for *LPAAT* are R-BF and R-SR. Amplification was also performed for several genes of the glycerolipids synthesis pathway [fatty acid desaturase-2 (Bn*FAD2*, AY577313), fatty acid desaturase-3 (Bn*FAD3*, L22962), fatty acid desaturase-7 (Bn*FAD7*, L22963), 3-ketoacyl-CoA reductase (Bn*KCR*, AY196197), At*GPAT* (AY093169) and diacylgycerol acyltransferase (Bn*DGAT*, AF251794)]. Primers are (forward: -up, reverse: -dn) FAD2up (5'-AACATG GGTGCAGGTGG-3') and FAD2dn (5'-CTTCACCATCATCCTCA TAACTT-3'), FAD3up (5'-CGGGATCCATGGTTGTCGCTATGGAC-3') and FAD3dn (5'-CGAGCTCTTAGTTGATTTTGGATTTGT-3'), FAD7up (5'-ATGGC GAACTTGGTCTTAT-3') and FAD7dn (5'-CTTCATTTCAATCTGC TGTTAC-3'), KCRup (5'-ATGGAGATCTG CACTTACTTC-3') and KCRdn (5'-TTACTCCTTTTTACTACTG GAGTC-3'), GPATup (5'-CGGTTTATCCACTCG CTTCGTC-3') and GPATdn (5'-ACGATT CCAGACCGCCAGTTGA-3'), as well as DGATup (5'-CTGGCGG AGATGCCGAAAC-3') and DGATdn (5'-ACGAGCCACCCAA CCCTTC-3'). PCR conditions: annealing for

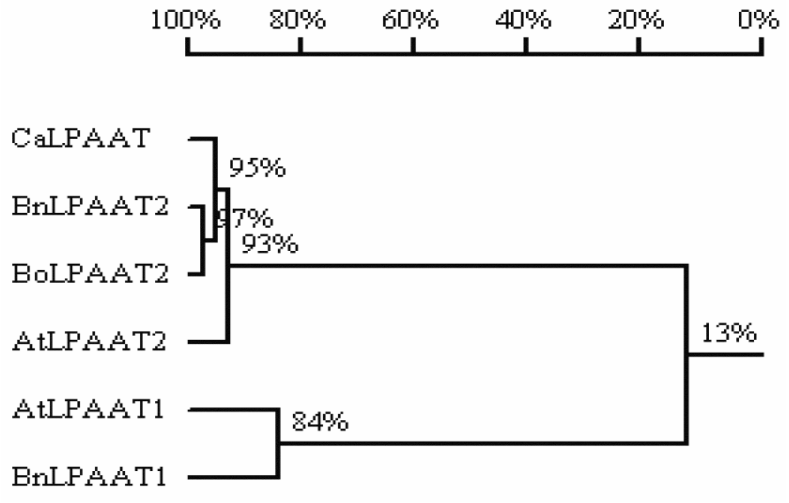

Figure 1. A homology tree of putative LPAAT of *C. abyssinica* and related LPAATs of other plant species constructed on the basis of their predicted amino acid sequences. Genes were obtained after a BLAST search of the databases of NCBI with use of the deduced amino acid sequences of *Crambe LPAAT* (Ca*LPAAT*, EF208088) and *B. napus LPAAT1* (Bn*LPAAT1*, AF111161) as queries. They were of *A. thaliana* (At*LPAAT1*, AK118028; At*LPAAT2*, NM115625), *B. napus* (Bn*LPAAT2*, Z95637) and *B. oleracea* (Bo*LPAAT2*, AY616009).

30 s at 57°C, extension for 30 s at 72°C, in 1.5 mM $MgCl_2$. Semi-quantitative RT-PCR was performed for 27 cycles. Quantitative RT-PCR was performed for 40 cycles using Lightcycler DNA Master SYBRgreen I (Roche) in 384 well plates using Lightcycler 480 (Roche). For each sample, the ratio of the fluorescence intensity of the target gene band to that of the internal standard band was used to overcome the variability between samples caused by RNA quality, RNA quantification errors and random tube-to-tube variation in PCR and reverse transcription reactions. Results presented are mean values of at least three independent lines and three repeats per line to exclude the result of insertional mutagenesis.

Lipid analysis

The fatty acyl compositions and total oil contents of mature seeds were determined by normative method (ISO 5509-1978, ISO 5508-1990, ISO 659-1988) (http://www.iso.org) in Testing Center of Sichuan Academy of Agricultural Sciences (Chengdu, China). The results are also mean values of at least three independent lines and three repeats per line.

RESULTS AND DISCUSSION

Identification of *LPAAT* in *C. abyssinica*

The cDNA obtained from *C. abyssinica* leaves contained an open reading frame encoding 390 amino acids (CaLPAAT). The predicted pI and molecular weight of the deduced protein was 9.18 and 43.8 kDa, respectively. The genomic DNA of Ca*LPAAT* was 2941 bp long including 11 exons. The cDNA (Accession No.: EF208088) and genomic DNA (Accession No.: EF208089) of Ca*LPAAT* were submitted to the GenBank.

Homology tree was created with six known as well as putative LPAAT proteins in GenBank (Figure 1). The homology tree demonstrated that CaLPAAT is closer to cytoplasmic LPAAT2 of *A. thaliana and B. napus* than plastid LPAAT1. The multiple sequence alignments also indicated that CaLPAAT shares two similar conserved motifs: NHX_4D and EGT with *A. thaliana*, *B. napus* and *Brassica oleracea*. CaLPAAT is 93.1 and 94.1% identical with cytoplasmic LPAAT2 of *A. thaliana* and *B. napus*, respectively. In addition, amino acid residues essential for the catalysis, including His, Asp, Glu and Gly, are all conserved (Lewin et al., 1999) (Figure 2).

Generation of transgenic *B. napus* expressing *CaLPAAT*

To study the function of CaLPAAT *in vivo,* transgenic *B. napus* expressing Ca*LPAAT* (35S: LPAATF) and a cDNA containing reverse Ca*LPAAT* sequence (35S: LPAATR) were generated by agrobacterium-mediated transformation, respectively. Transcripts from reverse Ca*LPAAT* can function as antisense RNA to decrease the level of endogenous *LPAAT* in *B. napus* (Figure 4B). Potential transgenic plants were genotyped by PCR to identify ones that contain 35S: LPAATF or 35S: LPAATFR.

Out of 41 35S: LPAATF lines characterized, 21 contained *CaLPAAT* (Figure 3A) and 8 out of 15 potential 35S: LPAATR lines contained reversed Ca*LPAAT* (Figure 3B). Consistent with the report by Zou et al. (1997), there were no discernible phenotypic effects on vegetative growth nor on development although Ca*LPAAT* was

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AtLPAAT2 (1)…(70) VFADNETFNRMGKEHALVVQNHRSDIDWLVGWILAQRSGCLGSALAVMKKSSKFLPVIGWSMWFSEYLFL
BnLPAAT2 (1)…(70) VFADDETFNRMGKEHALVVQNHRSDIDWLVGWILAQRSGCLGSALAVMKKSSKFLPVIGWSMWFSEYLFL
BoLPAAT2 (1)…(71) VFADDETFNRMGKEHALVVQNHRSDIDWLVGWILAQRSGCLGSALAVMKKSSKFLPVIGWSMWFSEYLFL
 CaLPAAT (1)…(70) VFADNETFNRMGKEHALVVQNHRSDIDWLVGWILAQRSGCLGSALAVMKKSSKFLPVIGWSMWFSEYLFL

AtLPAAT2 (140) ERNWAKDESTLKSGLQRLSDFPRPFWLALFVEGTRFTEAKLKAAQEYAASSELPIPRNVLIPRTKGFVSA…(390)
BnLPAAT2 (140) ERNWAKDESTLQSGLQRLNDFPRPFWLALFVEGTRFTEAKLKAAQEYAASSELPVPRNVLIPRTKGFVSA…(391)
BoLPAAT2 (141) ERNWAKDESTLKSGLQRLNDFPRPFWLALFVEGTRFTEAKLKAAQEYAATSQLPVPRNVLIPRTKGFVSA…(392)
 CaLPAAT (140) ERNWAKDESTLQSGLQRLNDFPRPFWLALFVEGTRFTEAKLKAAQEYAASSELPVPRNVLIPRTKGFVSA…(391)
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Figure 2. Multiple alignments of LPAATs. Two motifs (NHX_4D and EGT) are shaded.

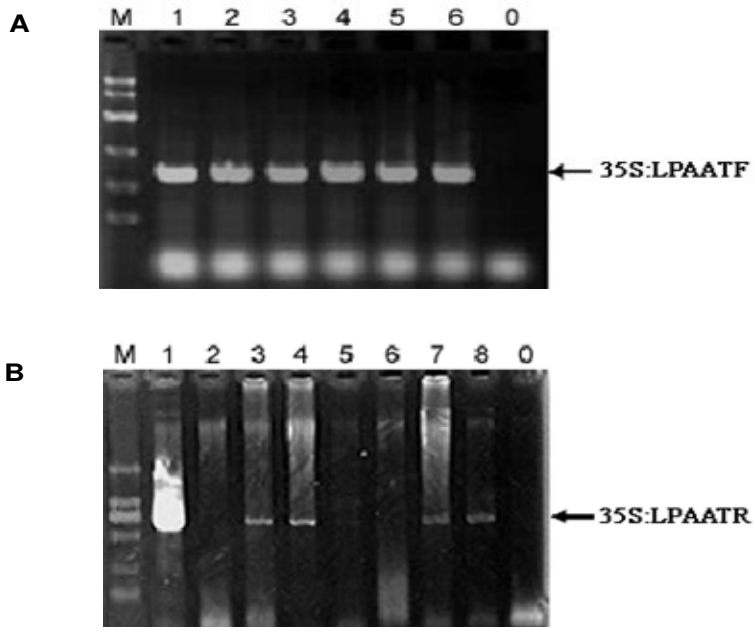

Figure 3. PCR amplification of Ca*LPAAT* in putative transgenic lines of 35S:LPAATF (A) and 35S:LPAATR (B). A: M – MarkerVI, lane 0: negative control (non-transgenic 84100-18), lane 1: positive control (35S:LPAATF plasmid), lanes 2 – 6: putative transgenic plants of 35S:LPAATF, arrow indicates the 1221 bp fragment of 35S:LPAATF. B: M – DL2000, lane 0: negative control (84100-18), lane 1: positive control (35S:LPAATR plasmid), lanes 2 – 8: putative transgenic plants of 35S:LPAATR, arrow indicates the 694 bp fragment of 35S:LPAATR.

expressed under the control of a constitutive (35s) promoter.

Expression profiles of genes in the glycerolipids synthesis pathway

To analyze the expression level of Ca*LPAAT* and endogenous Bn*LPAAT2*, semi-quantitative and quantitative RT-PCR were performed in transgenic lines. In flowers (F), leaves (L), stems (St) and mature seeds (S), an elevated level of *LPAAT* was observed in 35S: LPAATF, compared to the non-transgenic counter-parts (84100-18) (Figure 4A). As our primers can amplify both endogenous Bn*LPAAT2* and exogenous Ca*LPAAT*, it is possible that

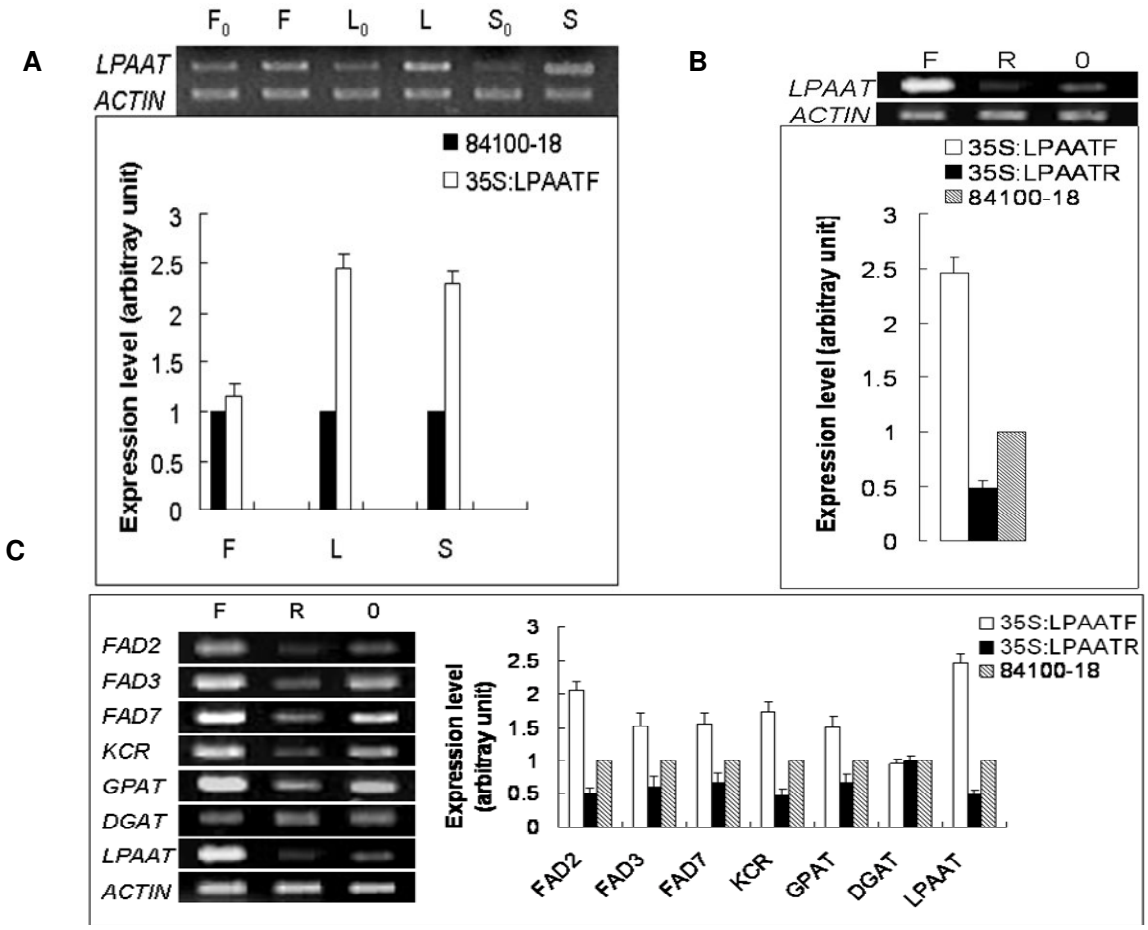

Figure 4. Analyses of *LPAAT* and several genes of glycerolipids synthesis pathway by RT-PCR. Each mean represents at least three replications from independent lines and three repeats per line. Vertical bars represent the standard error. Actin level was used as a normalization standard. Results from semi-quantitative PCR were resolved by agarose DNA gel and stained by ethidium bromide. Results from quantitative RT-PCR are shown as bar graph in each panel. A: Different expression levels of *LPAAT* in flowers (F), leaves (L), stems (St) and mature seeds (S). F_0, L_0, St_0 and S_0 are samples of non-transgenic 84100-18. F, L, St and S are samples of 35S:LPAATF transgenic plants. B: Different expression levels of *LPAAT* in mature seeds of 35S:LPAATF transgenic plants (F), 35S:LPAATR transgenic plants (R) and non-transgenic 84100-18 (0). C: Different expression levels of several genes involved in glycerollipids synthesis in mature seeds of 35S:LPAATF (F), 35S:LPAATR (R) and 84100-18 (0).

for unknown reason, the transgenic protocol stimulated the expression of endogenous *LPAAT*. However, this is unlikely because in our negative control (35S:LPAATR, which express antisense RNA to Bn*LPAAT2*), the level of total LPAAT did not increase. In contrast, a decreased level of LPAAT was observed in mature seeds of 35S: LPAATR (Figure 4B). Therefore, we consider the observed increase is mostly likely caused by the exogenous LPAAT. Interestingly, the expression of several genes (*FAD2*, *FAD3*, *FAD7*, *KCR*, *GPAT* and *DGAT*) of the glycerolipids synthesis pathway in mature seeds, were also increased in 35S: LPAATF, but decreased in 35S: LPAATR, compared to the non-transgenic counterparts (Figure 4C), indicating that there was a positive feedback (*FAD2*, *FAD3*, *FAD7*, *KCR* and *GPAT*) and feed forward (*DGAT*). Similar results were observed in leaves except the expression of *DGAT*, which expression was similar in leaves of all lines (data not shown). This could be due to the fact that *DGAT* mainly functions in maturing seeds (Jako et al., 2001) and has low activity in other tissues of the plant examined in this study.

Analysis of the lipid composition and total oil content in seeds

Because LPAAT is a key enzyme in the glycerolipids synthesis pathway, we analyzed the lipid composition as well as the total oil content in self-cross seeds of the T_1 generation. The composition as well as their relative percentage of fatty acids generated by LPAAT was similar in transgenic plants and their non-transgenic counterparts (Table 1). This is not surprising because CaLPAAT and BnLPAAT2 proteins shares 94.1%

Table 1. The relative percentage of fatty acid (mol %) and the total oil content (dry weight %) in T_1 generation seeds.

Sample	16:0 mol %	16:1 mol %	18:0 mol %	18:1 mol %	18:2 mol %	18:3 mol %	20:4 mol %	22:1 mol %	total oil content dry weight%
35S:LPAATF	4.31 (0.10)	0.22 (0.06)	2.45 (0.08)	64.33 (1.10)	18.80 (0.36)	8.76 (0.24)	0.79 (0.11)	0.34 (0.17)	42.33 (1.04)
35S:LPAATR	4.37 (0.17)	0.23 (0.07)	2.63 (0.18)	62.13 (2.03)	20.30 (0.23)	9.07 (0.14)	0.87 (0.19)	0.40 (0.25)	27.13 (1.10)
84100-18	4.51 (0.06)	0.24 (0.07)	2.53 (0.02)	62.43 (0.24)	19.80 (0.06)	9.11 (0.11)	1.01 (0.06)	0.37 (0.18)	35.97 (0.86)

Values within parentheses represent the standard error of three determinations from each line, and three repeats per line.

sequence identity (data not shown), indicating they have similar substrate specificities.

Previous studies demonstrated that there are two classes of cytoplasmic LPAAT genes (Maisonneuve et al., 2009; Frentzen, 1998). The class A LPAATs possesses substrate preferences for C18:1CoA typical of enzymes involved in membrane lipid synthesis and are ubiquitously expressed in the plant. In contrast, individual members of the class B LPAATs display preferences for distinct, unusual saturated or unsaturated acyl groups and are normally expressed in storage organs. In this study, we cloned class A CaLPAAT from *C. abyssinica* Hochst. ex R. E. Fries. In future study, it would be of interest to clone class B LPAAT of *C. abyssinica* to compare to other class B LPAATs.

In sharp contrast, the total oil content of mature seeds (on a dry weight basis) was increased in transgenic lines expressing Ca*LPAAT* (42.33%), compared to the non-transgenic counterpart (35.97%) (Table 1). The difference was statistically significant (P value < 0.05). This demonstrates that overexpression of Ca*LPAAT* can promote the production of total oil content in *B. napus*, possibly due to the upregulation of other genes in the glycerolipids synthesis pathway, consistent with previous reports (Maisonneuve et al., 2009; Sharma et al., 2008; Zou et al., 1997). Consistent with this notion, in plants expressing reverse Ca*LPAAT* sequence (35S: LPAATR), which represses the expression of endogenous *LPAAT*, the total oil content was decreased to 27.13% (Table 1).

As there seems no obvious effect in the vegetative portions of the transgenic plants and in addition, the industrial value of rapeseed is mostly in the seeds, lipids of other tissues are not determined in the current experiments. In other studies, analyses of leaf lipids in transgenic *B. napus* and *A. thaliana* showed no significant changes in fatty acid content and composition, although exogenous LPAAT gene was expressed under the control of a constitutive (tandem 35S) promoter (Zou et al., 1997), just like here.

Thus, we conclude that Ca*LPAAT* can be ectopically expressed in *B. napus*, and increase its total oil content in the context of transgenic plants.

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