Plant Biotechnology Journal

Plant Biotechnology Journal (2009) **7**, pp. 602–610 doi: 10.1111/j.1467-7652.2009.00427.x

A 10-kDa acyl-CoA-binding protein (ACBP) from *Brassica napus* enhances acyl exchange between acyl-CoA and phosphatidylcholine

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Received 1 March 2009;
revised 5 April 2009;
accepted 6 April 2009.
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Keywords: acyl-CoA-binding protein, *Arabidopsis*, lysophosphatidylcholine acyltransferase, phosphatidylcholine, polyunsaturated fatty acid, triacylglycerol.

Summary

The gene encoding a 10-kDa acyl-CoA-binding protein (ACBP) from *Brassica napus* was over-expressed in developing seeds of *Arabidopsis thaliana*. Biochemical analysis of T_2 and T_3 *A. thaliana* seeds revealed a significant increase in polyunsaturated fatty acids (FAs) (18:2$^{cis\Delta 9,12}$ and 18:3$^{cis\Delta 9,12,15}$) at the expense of very long monounsaturated FA (20:1$^{cis\Delta 11}$) and saturated FAs. *In vitro* assays demonstrated that recombinant *B. napus* ACBP (rBnACBP) strongly increases the formation of phosphatidylcholine (PC) in the absence of added lysophosphatidylcholine in microsomes from ΔYOR175c yeast expressing *A. thaliana* lysophosphatidylcholine acyltransferase (*AthLPCAT*) cDNA or in microsomes from microspore-derived cell suspension cultures of *B. napus* L. cv. Jet Neuf. rBnACBP or bovine serum albumin (BSA) were also shown to be crucial for AthLPCAT to catalyse the transfer of acyl group from PC into acyl-CoA *in vitro*. These data suggest that the cytosolic 10-kDa ACBP has an effect on the equilibrium between metabolically active acyl pools (acyl-CoA and phospholipid pools) involved in FA modifications and triacylglycerol bioassembly in plants. Over-expression of *ACBP* during seed development may represent a useful biotechnological approach for altering the FA composition of seed oil.

Introduction

Seed oil is one of the most important sources of dietary fats in human nutrition and is being extensively investigated for both nutraceutical and industrial applications (Cahoon *et al*., 2007; Davies, 2007). Fatty acid (FA) composition largely determines the nutritional and functional properties of various seed oils and their suitability for particular applications. Despite the broad variety of FAs found in plants, seed oil of the most agriculturally important oilseed crops is composed of a limited number of FAs, ranging from 16 to 18 carbons in length, with up to three double bonds, present in different proportions depending on the species and variety. Over the past decade, genetic engineering has been used to modify the FA composition of seed oil with varying degrees of success (Cahoon *et al*., 2007; Graham *et al*., 2007; Napier, 2007).

In developing seeds of oleaginous plants, FA synthesized in the plastids is released into the cytosol in the form of acyl-CoA, which is a major intermediate in seed oil biosynthesis (Rawsthorne, 2002). The FA moieties of acyl-CoA can undergo further modifications, such as elongation and desaturation, in the endoplasmic reticulum (ER). FA elongation is performed on the acyl-CoA substrate, whereas the introduction of second and third double bonds requires the acyl group to be esterified to phosphatidylcholine (PC) (Jaworski, 1987). It has been shown that the majority of FAs synthesized in plastids enter PC through acyl editing of PC rather than *de novo* synth-

esis (Williams *et al*., 2000; Bates *et al*., 2007). Desaturated FA can be either returned to the acyl-CoA pool, where it can be used in the Kennedy pathway, or can be channelled to TAG biosynthesis via acyl-CoA-independent mechanisms (Weselake, 2005).

Most of the cytosolic acyl-CoAs are expected to be associated with small, soluble, acyl-CoA-binding proteins (ACBPs) (Rasmussen *et al*., 1993). These proteins are believed to fulfill housekeeping functions in acyl-CoA pool maintenance and protection, as well as to participate in the intracellular transport of acyl-CoAs (Færgeman and Knudsen, 2002; Burton *et al*., 2005). In *Arabidopsis thaliana*, six ACBP family members have been identified that differ in structure, cellular location and acyl-CoA binding properties (Engeseth *et al*., 1996; Chye *et al*., 1999, 2000; Leung *et al*., 2004, 2006). The only *B. napus* ACBP (BnACBP) identified so far represents a small cytosolic protein of 92 amino acids and displays 84% amino acid sequence identity to the *A. thaliana* 10-kDa ACBP (Hills *et al*., 1994). BnACBP is highly expressed in flowers and developing embryos, with the peak concentration of BnACBP in developing embryos coinciding with the peak of TAG accumulation (Engeseth *et al*., 1996). *In vitro* experiments have shown that recombinant *B. napus* ACBP (rBnACBP) stimulates *sn*-glycerol-3-phosphate acyltransferase (GPAT) activity in a manner dependent on the ACBP to acyl-CoA ratio in the reaction mixture (Brown *et al*., 1998). These findings suggest that ACBP may have an important role in TAG accumulation in developing seeds. Binding studies with a limited number of substrates have revealed different affinities of ACBP towards the most common acyl-CoAs found in plants, suggesting that binding/transport of some acyl-CoA species by the protein may be preferred over the others, or that different ACBP isoforms specialize in the accommodation of a particular acyl-CoA class (Brown *et al*., 1998).

Over-expression of ACBP in yeast and in animal systems has been shown to increase the acyl-CoA pool size (as a result of an increase in certain acyl-CoA species) and rates of glycerolipid synthesis (Mandrup *et al*., 1993; Huang *et al*., 2005). Thus, there is reason to expect that the manipulation of ACBP expression in plants can affect the size and composition of the cellular acyl-CoA pool. One of the few published results on ACBP over-expression in plants has demonstrated that the expression of the *A. thaliana* 10-kDa ACBP in low- and high-erucic acid rapeseed varieties results in modifications of the FA composition of seed oil in a manner dependent on the vector structure and genetic background of the transformed plants (Enikeev and Mishutina, 2005). The over-expression of the *A. thaliana* 10-kDa ACBP using a 35S promoter enhanced freezing tolerance and was accompanied by an increase in phosphatidic acid and a decrease in PC content in leaf tissue (Chen *et al*., 2008).

Our primary interest in ACBP is to study its physiological role in seed oil biosynthesis and to investigate its potential as a molecular tool for the modification of FA composition and/or enhancement of seed oil content. In this study, we transformed *A. thaliana* with *B. napus* cytosolic *ACBP* cDNA to facilitate the seed-specific expression of the protein. *In vitro* assays using yeast and plant cell-free systems were conducted to study the effect of rBnACBP on lysophosphatidylcholine acyltransferase (LPCAT), an enzyme postulated to be involved in acyl exchange between acyl-CoA and PC metabolic pools (Stymne and Stobart, 1984).

Results

Seed-specific expression of the gene encoding the 10-kDa cytosolic BnACBP affects the FA composition of seed oil in *A. thaliana*

cDNA of the cytosolic BnACBP (GenBank accession number X77134) was expressed in *A. thaliana* under the control of the phaseolin promoter. Null vector control lines were also produced. The expression of rBnACBP in developing *A. thaliana* seeds was confirmed by Western blot analysis (Figure 1).

The analysis of seed oil from T_2 lines revealed a significant increase in 18:2$^{cis\Delta 9,12}$ (18:2) and 18:3$^{cis\Delta 9,12,15}$ (α-18:3) in lines transformed with *ACBP* (Table 1). The increase in polyunsaturated fatty acid (PUFA) content in the seed oil appeared

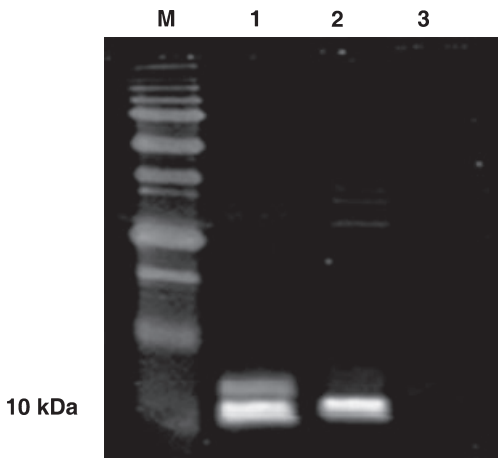

Figure 1 Western blot analysis of the total seed protein from *Arabidopsis thaliana* T_3 seeds. The blot was incubated with polyclonal antibodies against *Brassica napus* acyl-CoA-binding protein (BnACBP). Lane M, protein marker (Precision Plus Protein™ Standards, Bio-Rad); lane 1, protein extract from transgenic mature seeds; lane 2, protein extract from transgenic developing seeds [16 days after flowering (DAF)]; lane 3, protein extract from wild-type developing seeds (16 DAF). Amount of protein loaded per lane, 2.5 μg.

Table 1 Fatty acid (FA) composition of the seed oil from T_2 seeds. Mean weight percentage ± SD ($n = 4$, biological replicates). ▲/▼, values significantly greater/smaller than the wild-type (WT) at $\alpha = 0.05$

Construct	16:0	18:0	18:1	18:2	18:3	20:1
WT	7.38 ± 0.05	3.66 ± 0.04	15.51 ± 0.06	26.79 ± 0.08	17.03 ± 0.14	21.06 ± 0.04
T_2 null vector	7.73 ± 0.19	3.30 ± 0.71	16.78 ± 0.27▲	26.84 ± 1.76	15.87 ± 0.70	21.17 ± 0.79
T_2 acyl-CoA-binding protein	6.64 ± 0.36▼	2.57 ± 0.25▼	16.28 ± 0.36▲	29.78 ± 0.72▲	19.04 ± 1.32▲	17.71 ± 0.55▼

Table 2 Fatty acid (FA) composition of seed oil of T_3 seeds. Mean weight percentage ± SD ($n = 4$, biological replicates). ▲/▼, values significantly greater/smaller than the wild-type (WT) at $\alpha = 0.05$

Construct	16:0	18:0	18:1	18:2	18:3	20:1
WT	7.32 ± 0.03	3.58 ± 0.04	15.40 ± 0.04	26.92 ± 0.08	17.35 ± 0.10	21.02 ± 0.06
T_3 null segregate	7.51 ± 0.03	3.25 ± 0.08	15.69 ± 0.71	27.62 ± 0.31	16.92 ± 0.57	20.67 ± 0.31
T_3 acyl-CoA-binding protein	6.93 ± 0.11▼	2.59 ± 0.21▼	16.86 ± 0.65 ▲	28.89 ± 0.73 ▲	18.36 ± 0.72▲	18.18 ± 0.75▼

to occur at the expense of monounsaturated fatty acids (MUFAs), particularly 20:1$^{cis\Delta 11}$ (20:1), and saturated fatty acids (SFAs). Levels of 18:1$^{cis\Delta 9}$ (18:1) were also altered in transgenic lines, but this change appears to be nonspecific, as the null vector control also displayed an increase in the proportion of 18:1 at the expense of α-18:3. It has been observed previously that the *Agrobacterium*-mediated transformation of *A. thaliana* with genes not directly involved in lipid metabolism may cause significant changes in seed oil composition, particularly in 18:1 and α-18:3 levels (J. Metz, pers. commun., Market BioSciences, Boulder, CO, USA). Four lines showing the largest changes in FA composition of seed oil were selected to produce the next generation (T_3) of seeds. Four T_3 lines that segregated back to the wild-type phenotype were also selected and included in the analysis as a null segregate control. The analysis of T_3 seeds expressing *BnACBP* confirmed our previous findings (Table 2) of this heritable trait. The magnitude of the FA composition changes, however, were more subtle in T_3 seeds relative to the changes observed in the T_2 data. The results obtained from both data sets demonstrate the major effects of the *ACBP* transgene on the FA composition of seed oil: an increase in the proportions of 18:2 and α-18:3 and a decrease in the proportion of 20:1.

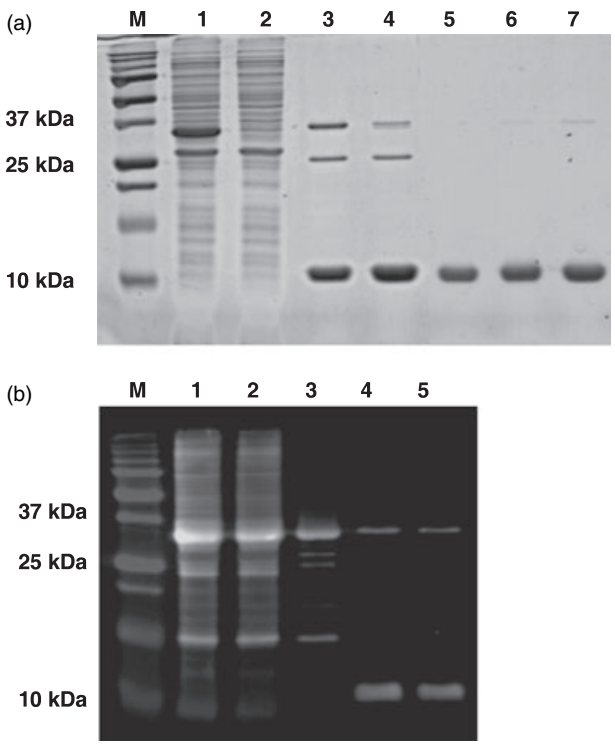

Figure 2 Analysis of protein and recombinant *Brassica napus* acyl-CoA-binding protein (rBnACBP) following gel electrophoresis of *Escherichia coli* sonicate and fractions obtained during purification. (a) Protein staining following 15% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Lane M, protein markers (Precision Plus Protein™ Standards, Bio-Rad); lane 1, *E. coli* sonicate (~15 μg); lane 2, eluate of the sonicate from the Glutathione Sepharose 4B column; lanes 3 and 4, eluate of rBnACBP cleaved from glutathione *S*-transferase (GST) with thrombin protease (~2.5 μg); lanes 5–7, rBnACBP re-eluted through the column (~1.5 μg). (b) Western blot of rBnACBP on 15% SDS-PAGE incubated with polyclonal antibodies against BnACBP. Lane M, protein markers (Precision Plus Protein™ Standards, Bio-Rad); lane 1, *E. coli* sonicate; lane 2, *E. coli* total soluble protein; lane 3, GST-rBnACBP fusion protein; lanes 4 and 5, eluate of rBnACBP cleaved from GST with thrombin protease.

rBnACBP binds to acyl-CoAs *in vitro*

We expressed and purified recombinant *B. napus* cytosolic ACBP in *Escherichia coli* as a glutathione *S*-transferase (GST)-fusion protein (Figure 2a). The GST tag was cleaved off with thrombin protease, resulting in 95%–99% pure rBnACBP. The only impurities that remained in the rBnACBP sample were the GST-rBnACBP fusion protein and cleaved GST. The

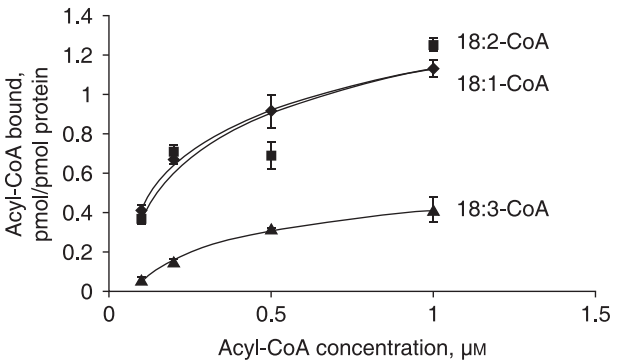

Figure 3 Lipidex-1000 binding assay of recombinant *Brassica napus* acyl-CoA-binding protein (rBnACBP) with [1-^{14}C]18:1-CoA, [1-^{14}C]18:2-CoA or [1-^{14}C]18:3-CoA.

identity of the recombinant protein was confirmed by Western blot analysis with polyclonal antibodies raised against rBnACBP (Figure 2b). The 36-kDa band in lanes 4 and 5 represents the uncut GST-rBnACBP fusion protein. The ability of rBnACBP to bind acyl-CoA was confirmed using the Lipidex-1000 binding assay (Figure 3). Substantial binding of 18:1- and 18:2-CoA occurred at low concentrations (< 1 μM) of total acyl-CoA, whereas the binding of α-18:3-CoA was considerably weaker. The interaction of 18:1-CoA with rBnACBP was similar to that observed by Brown *et al*. (1998).

rBnACBP stimulates *A. thaliana* LPCAT (AthLPCAT) activity *in vitro*

We hypothesized that the changes in FA composition of seed oil from developing seeds over-expressing *BnACBP* might be a result of an increase in cytosolic ACBP affecting the partitioning of 18:1-CoA between desaturation and elongation pathways. As desaturation of 18:1 to 18:2 and, subsequently, to α-18:3 takes place on PC, we set out to determine the effect of soluble ACBP on LPCAT activity, which, in the forward reaction, catalyses the acyl-CoA-dependent acylation of the *sn*-2 position of lysophosphatidylcholine (LPC) to form PC.

The yeast LPCAT knock-out mutant ΔYOR175c was transformed with the recently cloned cDNAs encoding AthLPCAT isoforms (Ståhl *et al*., 2008). The microsomal fraction from transformed yeast was incubated with [1-^{14}C]18:1-CoA and 18:1-LPC substrates. rBnACBP or bovine serum albumin (BSA) was added to the reaction mixture in various molar ratios to [1-^{14}C]18:1-CoA (0.33–1.66 binding protein to acyl-CoA ratio). BSA is known to bind medium- to long-chain acyl-CoA esters (Richards *et al*., 1990), and was included in the experiment as a control. rBnACBP increased AthLPCAT activity by 40% *in vitro* when the ACBP to acyl-CoA ratio reached unity, and

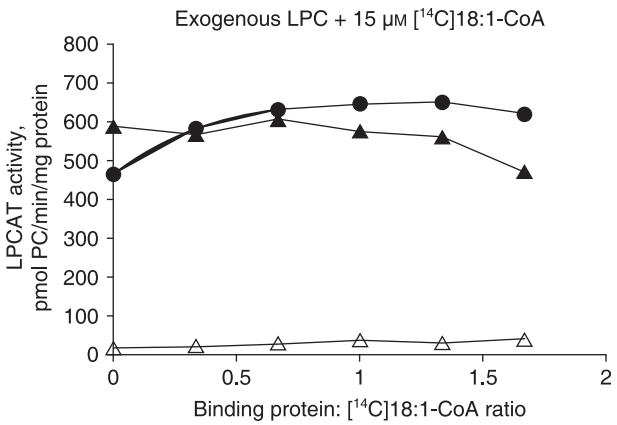

Figure 4 Effect of recombinant *Brassica napus* acyl-CoA-binding protein (rBnACBP) (circles) and bovine serum albumin (BSA) (triangles) on lysophosphatidylcholine acyltransferase (LPCAT) activity in the presence of added lysophosphatidylcholine (LPC). [1-^{14}C]18:1-CoA (15 μM) and LPC (75 μM) were incubated with microsomes (~50 μg) from ΔYOR175c yeast transformed with *AthLPCAT-1* (filled symbols) or lacZ (open symbols) (n = 3, mean ± SE).

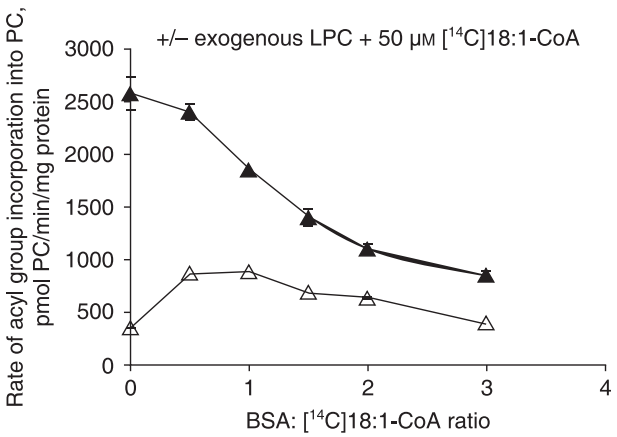

Figure 5 Effect of bovine serum albumin (BSA) on [1-^{14}C]18:1 incorporation from acyl-CoA into phosphatidylcholine (PC) when exogenous lysophosphatidylcholine (LPC) was included (filled triangles) or omitted (open triangles) from the assay. [1-^{14}C]18:1-CoA (50 μM) and CoA (50 μM) were incubated with microsomes (~30 μg) from ΔYOR175c yeast transformed with *Arabidopsis thaliana* lysophosphatidylcholine acyltransferase-1 (*AthLPCAT-1*) (n = 3, mean ± SE).

thereafter the AthLPCAT activity was not affected significantly by further addition of binding protein (Figure 4). BSA appeared to have no effect on the amount of [1-^{14}C]PC produced at low concentrations of BSA but, at BSA to acyl-CoA ratios of 0.66–1.66, AthLPCAT activity decreased by 23%. The inhibitory effect of BSA on AthLPCAT in the forward reaction was even more pronounced when higher concentrations of acyl-CoA were used in the reaction mixture (Figure 5).

The incorporation of [1-^{14}C]18:1 from the acyl-CoA pool into PC was studied in microsomes from yeast or in micro-

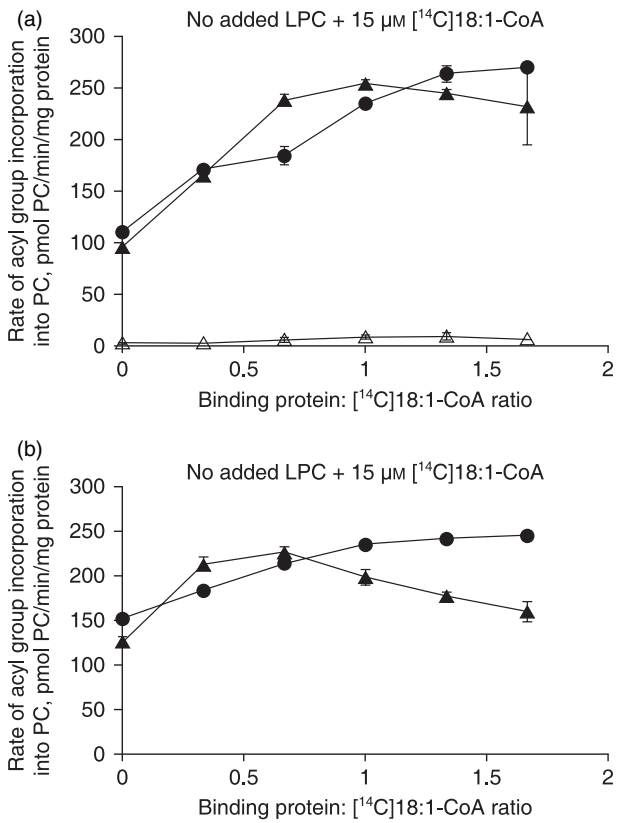

Figure 6 Effect of recombinant *Brassica napus* acyl-CoA-binding protein (rBnACBP) (circles) and bovine serum albumin (BSA) (triangles) on [1-^{14}C]18:1 incorporation from acyl-CoA into phosphatidylcholine (PC) in the absence of added lysophosphatidylcholine (LPC). [1-^{14}C]18:1-CoA (15 μM) and CoA (50 μM) were incubated with microsomes (~50 μg) from (a) ΔYOR175c yeast transformed with *Arabidopsis thaliana lysophosphatidylcholine acyltransferase-2 (AthLPCAT-2)* (filled markers) or lacZ (open markers), or (b) microspore-derived cell suspension cultures of *B. napus* L. cv. Jet Neuf ($n = 3$, mean ± SE).

spore-derived (MD) cell suspension cultures of *B. napus* L. cv. Jet Neuf under conditions in which no exogenous LPC was provided. These MD cell suspension cultures have previously been used for studies of TAG biosynthesis and acyltransferase action (Weselake and Taylor, 1999; Furukawa-Stoffer *et al.*, 2003). In the microsomal fraction of yeast expressing *AthLPCAT*, BSA or rBnACBP stimulated the synthesis of [1-^{14}C]PC. The amount of the radiolabel incorporated into PC from [1-^{14}C]18:1-CoA increased with increasing ACBP concentration up to the highest ratio tested (2.75 ratio of ACBP to acyl-CoA), where the incorporation was 2.5 times higher than in the absence of ACBP (Figure 6a). BSA also stimulated incorporation up to a BSA to acyl-CoA ratio of unity, after which incorporation decreased (Figures 5 and 6a). Similar results were observed when microsomes from *B. napus* MD cell suspension cultures were used (Figure 6b). The synthesis of radiolabelled PC when no exogenous LPC was provided can partially be explained by the acylation of the endogenous LPC. Another mechanism that could contribute to the formation of radiolabelled PC is acyl exchange between acyl-CoA and endogenous PC, catalysed by the reverse and forward reactions of LPCAT.

The effect of rBnACBP on the ability of AthLPCAT to catalyse the transfer of acyl groups from PC to acyl-CoA was also studied. Exogenous PC with [1-^{14}C]18:2 at the *sn*-2 position was introduced to the lyophilized membranes from yeast expressing AthLPCATs, and the appearance of the radiolabel in the acyl-CoA fraction was monitored. No radiolabelled acyl-CoA was formed when BSA or rBnACBP was omitted from the reaction medium, nor in assays with membranes from the yeast expressing *lacZ* (Figure 7). The addition of BSA to the assay resulted in a linear increase in the production of radiolabelled acyl-CoA up to a BSA to acyl-CoA ratio of unity. rBnACBP also stimulated the transfer of [1-^{14}C]18:2 to acyl-CoA even beyond an ACBP to acyl-CoA ratio of unity.

Discussion

Small cytosolic ACBPs, found in a number of higher plants, are believed to perform a housekeeping function in all cells by maintaining the acyl-CoA pool and facilitating the intracellular transport of acyl-CoAs. ACBP can also acquire more specialized functions in tissues with high rates of metabolic processes involving acyl-CoAs (Walz *et al.*, 2004; Suzui *et al.*, 2006). In *A. thaliana*, the 10-kDa ACBP has been demonstrated to play a role in mediating the freezing stress responses associated with phospholipid metabolism (Chen *et al.*, 2008). ACBP levels were found to be highest in developing seeds of *A. thaliana* and *B. napus*, indicating that this protein may have a specialized role in seed development and maturation (Engeseth *et al.*, 1996).

In this study, the expression of *BnACBP* cDNA in *A. thaliana* developing seeds resulted in changes in the FA composition of seed oil. Elevated amounts of PUFAs in seed oil were observed primarily as a result of the increase in 18:2 and α-18:3 at the expense of 20:1 and, to a lesser extent, SFAs. On the basis of these observations, we proposed that an increase in the concentration of acyl-CoA-binding sites in the cytosol would affect the partitioning of 18:1-CoA between elongation and incorporation into PC and subsequent desaturation.

The results of *in vitro* LPCAT assays suggested that ACBP may enhance the incorporation of 18:1 into PC by promoting acyl exchange between PC and acyl-CoA catalysed by LPCAT. An increased rate of 18:1 esterification to PC creates more

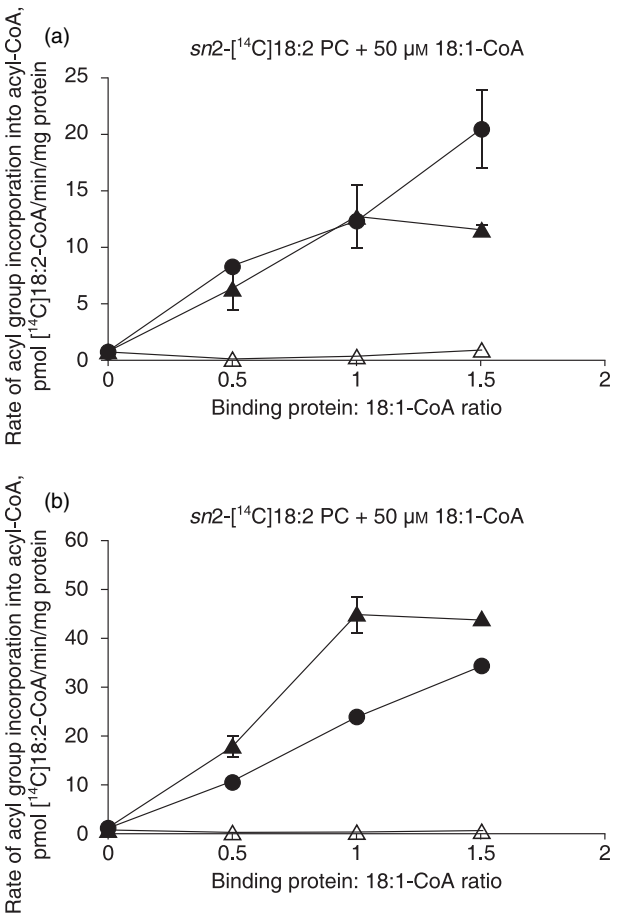

Figure 7 Effect of recombinant *Brassica napus* acyl-CoA-binding protein (rBnACBP) (circles) and bovine serum albumin (BSA) (triangles) on the transfer of acyl groups from phosphatidycholine (PC) to acyl-CoA by lysophosphatidylcholine acyltransferase (LPCAT) (reverse reaction). *sn*-2[1-^{14}C]18:2 PC (50 μM), 18:1-CoA (50 μM) and CoA (0.2 mM) were incubated with microsomes (~15 μg) from ΔYOR175c yeast transformed with (a) *AthLPCAT-1* or (b) *AthLPCAT-2* (filled symbols) or *lacZ* (open symbols) ($n = 2$, mean ± SE).

substrate for FAD2 desaturase, which may result in a greater production of 18:2, which may be desaturated further to α-18:3 by FAD3. FAD2 and FAD3 desaturases are known to catalyse the formation of 18:2 and α-18:3 in PC, respectively (Ohlrogge and Browse, 1995). Whether PUFAs produced on PC return to the cytosolic acyl-CoA pool via acyl editing, or are channelled to seed oil though acyl-CoA-independent pathways, remains unknown. On the basis of the results of our *in vitro* assays, we propose that the over-expression of the gene encoding the 10-kDa ACBP enhances the removal of PUFA from PC, thus enriching the pool of endogenous LPC that can be re-acylated with *de novo* synthesized 18:1.

Our results agree with those previously published by Stymne and Stobart (1984), who demonstrated a stimulatory effect of BSA on the reverse reaction of LPCAT in microsomal preparations from safflower cotyledons. The authors' rationale was that BSA binding of acyl-CoAs affected the free acyl-CoA to CoA ratio in the cytosol, which, in turn, shifted the LPCAT reaction towards the removal of acyl groups from PC. BSA, however, is a mammalian protein. ACBP is the likely candidate to play a role in LPCAT dynamics in plants. A possible explanation for the changes in FA composition of seed oil obtained from *Arabidopsis* over-expressing *BnACBP* is that increased cytosolic ACBP lowers the concentration of free cytosolic acyl-CoA. This shifts the equilibrium of acyl exchange between the acyl-CoA pool and PC, promoting the release of the polyunsaturated fatty acyl moieties into the cytosolic pool. Recently, it has been demonstrated that recombinant *A. thaliana* cytosolic 10-kDa ACBP can bind PC *in vitro* (Chen *et al*., 2008). Whether ACBP binds PC *in vivo*, and whether it has a role in the ACBP effect on LPCAT activity, remains to be determined.

Changes in the FA composition of seed oil in *B. napus* expressing *A. thaliana* cytosolic *ACBP* were also observed by Enikeev and Mishutina (2005). In a high-erucic-acid cultivar transformed with the sense *ACBP* construct, decreased levels of long-chain MUFAs (20:1$^{cis\Delta 11}$ and 22:1$^{cis\Delta 13}$) were documented, whereas lines expressing antisense *ACBP* constructs showed 1.5–2-fold increases in 22:1 content in seed oil. These data support our findings and hypothesis that ACBP levels in developing seeds may play an important role in the partitioning of acyl-CoA between FA elongation and desaturation.

Conclusion

This study has shown that the expression of the 10-kDa cytosolic BnACBP during seed maturation in *Arabidopsis* results in an increase in PUFAs at the expense of FA elongation. Assays of the forward and combined forward/reverse reactions of LPCAT suggest that BnACBP stimulates acyl exchange between PC and the acyl-CoA pool. Over-expression of ACBP during seed development may be useful in genetic engineering strategies aimed at modifying the FA composition of seed oils.

Experimental procedures

Preparation of genetic constructs for *ACBP* expression in developing seeds

cDNA encoding *B. napus* cytosolic ACBP was obtained from Picoscript (Houston, TX, USA) based on a cDNA sequence available from the GenBank database (accession number X77134). Restriction endonuclease sites were added to BnACBP cDNA (*Nco*I and *Xho*I at the 5′ end,

and *HindIII* at the 3′ end) for molecular cloning of cDNA into the pSBS4006 binary vector containing the phaseolin promoter/terminator expression system. The pSBS4004 binary vector lacking the phaseolin promoter and terminator was used as a null vector. Molecular constructs were introduced into *Agrobacterium tumefaciens* EHA101 by electroporation using a standard protocol (Mattanovich *et al*., 1989). The presence of the vector conferring the T-DNA of interest in *Agrobacterium* was confirmed by polymerase chain reaction (PCR) with vector-specific primers.

Growth conditions and transformation *of A. thaliana*

Arabidopsis thaliana plants (C-24) were grown in Sunshine LA4 soil mix (SunGro, Vancouver, BC, Canada) in a growth chamber with a 16h light period at a light intensity of 350 $\mu E/m^{2/s}$ and a constant temperature of 20 °C. *Agrobacterium tumefaciens*-mediated transformation of *A. thaliana* was performed using a floral dip method (Clough and Bent, 1998). The dipped plants were placed under a dome for 16–24 h to maintain high humidity, and grown normally until the seeds became mature. T_1 seeds were germinated on selective medium containing D,L-phosphinothricin at a concentration of 50 μM. The herbicide-resistant T_1 plants were transferred to soil 7 days after germination, and grown individually in a growth chamber to produce mature T_2 seeds for seed oil analysis.

Analysis of seed oil

Seed oil was extracted from 10 mg of mature T_2 seeds using a hexane–isopropanol method (Hara and Radin, 1978). Methanolic HCl was used as a methylation reagent for the conversion of FAs to FA methyl esters (FAMEs) for subsequent separation by gas chromatography (GC). FAMEs were analysed on an Agilent 6890N Gas Chromatograph (Wilmington, DE, USA) with a 5975 inert XL Mass Selective Detector equipped with an autosampler. FAMEs were separated using a DB-23 (30 m) capillary column (0.25 mm × 0.25 μm × 30 m) with a constant Helium flow of 1.2 mL/min and the following temperature programme: 90–180 °C at 10 °C/min, hold at 180 °C for 5 min, 180–230 °C at 5 °C/min. Integration events were detected and identified between 9 and 20 min, and compared against a NuChek 463 gas–liquid chromatography standard (Elysian, MN, USA).

Statistical analysis

The FA composition of T_2 seeds was compared with that of wild-type and null vector lines using analysis of variance (ANOVA) with Dunnett's test. Four technical replicates of the insertion event for T_2 lines and four biological replicates for T_3 lines were used in the analysis. Four T_2 or T_3 lines were used as biological replicates of the construct. SAS 9.1 software (SAS Institute Inc., Cary, NC, USA) was used to perform all statistical procedures.

Expression of rBnACBP in *E. coli*

The GST Gene Fusion System (GE Healthcare, Baie d'Urfé, QC, Canada) was used for the expression and purification of rBnACBP in *E. coli*. *BnACBP* cDNA (X77134) was cloned into the pGEX-4T1 expression vector using *Bam*HI and *Xho*I restriction endonuclease sites. The GST-BnACBP fusion protein was purified from the *E. coli* culture sonicate using a Glutathione Sepharose 4B column. The GST tag was removed from GST-rBnACBP by treatment with thrombin protease. The concentration of the recombinant protein was determined using the Bio-Rad (Mississauga, ON, Canada) protein microassay based on the Bradford (1976) procedure, with BSA as a standard. The purity of rBnACBP was analysed by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using standard protocols (Sambrook *et al*., 1989) and stained with Coomassie Brilliant Blue R 250 or blotted for Western analysis. Blotted samples were probed with polyclonal antibody directed against BnACBP (Brown *et al*., 1998). ACBP was detected using secondary donkey anti-rabbit IRDye® 800 CW (LiCor Biosciences, Lincoln, NE, USA) and analysed using an Odyssey Infrared Imaging System (LiCor Biosciences).

Lipidex-1000 binding assay

The binding assay for the examination of the interaction of radiolabelled acyl-CoAs with rBnACBP was performed using Lipidex-1000 (Packard BioScience, Groningen, the Netherlands), as described in Zeng *et al*. (2006) with minor modifications. Briefly, 40 pmol of rBnACBP were incubated with one radiolabelled acyl-CoA (0.1–1 μM) at a time in 200 μL of binding buffer (10 mM potassium phosphate, pH 7.4) at 30 °C for 30 min. The mixture was then placed on ice for 10 min, and 400 μL of ice-cold 50% Lipidex-1000 slurry in binding buffer (v/v) were added. Samples were incubated on ice with occasional mixing for 20 min, and then centrifuged at 12 000 ***g*** for 5 min at 4 °C. Two hundred microlitres of the supernatant from each sample were combined with 5 mL of Ecolite™(+) scintillant (MP Biochemicals, Irvine, CA, USA) and analysed for radioactivity in a scintillation counter (Beckman Coulter, LS-6500, Mississauga, ON, Canada). All assays were performed in three replicates, and control samples with no protein added were used to correct for the amount of acyl-CoA that was not absorbed by Lipidex-1000 beads.

Yeast strain and growth conditions

Yeast BY4742 strain *LPCAT* knock-out ΔYOR175c was transformed with expression vectors harbouring *AthLPCAT-1* and *AthLPCAT-2* cDNA, pYES2/NT C-At1g12640 and pYES2/NT C-At1g63050. Yeast cultures were inoculated in 2% liquid glucose synthetic medium lacking uracil, and grown on a rotary shaker at 30 °C to early stationary phase ($A_{600} = 8–10$). Cells were harvested by centrifugation at 2500 ***g*** for 7 min at 4 °C and washed once with the induction buffer (SC-U, 2% galactose). Cells were resuspended in 50 mL of induction medium to obtain $A_{600} = 0.4$ and grown on a rotary shaker for 20–22 h before harvesting for the microsome preparation.

Preparation of microsomes

Yeast cultures were centrifuged at 2500 ***g*** for 7 min at 4 °C, washed once with distilled water and resuspended in ice-cold 50 mM Tris-HCl, pH 8.0 buffer, containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT) and 10% glycerol. To each sample, approximately the same volume of 0.5-mm Zirconia/Silica beads (BioSpec Products Inc., Bartlesville, OK, USA) was added, and the cells were disrupted in a bead beater (BioSpec Products Inc.) by shaking

for 3 × 1 min with intervening periods of 1–2 min of cooling on ice. The extracts were centrifuged at 2500 ***g*** for 5 min at 4 °C and the supernatant was subjected to ultracentrifugation at 100 000 ***g*** for 1 h at 4 °C. The pellet was resuspended in 10 mM Hepes-NaOH, pH 7.4 buffer, frozen in liquid N_2 and stored at –80 °C.

The MD cell suspension culture of *B. napus* L. cv. Jet Neuf was maintained according to Orr *et al*. (1986). Harvested cells were washed on a 60-μm nylon mesh, blotted dry, frozen in liquid N_2 and stored at –80 °C. Frozen tissue was thawed on ice and ground in 4 vol of grinding buffer (0.2 M Hepes-NaOH, pH 7.4, 0.5 M sucrose) with a PowerGen700 grinder (Fisher Scientific, Whitby, ON, Canada). The homogenate was centrifuged at 10 000 ***g*** for 30 min at 4 °C and the resulting pellet was discarded. The supernatant was filtered through cheesecloth and centrifuged at 100 000 ***g*** for 1 h at 4 °C. The resulting pellet was resuspended in 10 mM Hepes-NaOH, pH 7.4 buffer, frozen in liquid N_2 and stored at –80 °C. One millilitre of resuspension buffer was used for every 10 g of original tissue weight. The protein content of the microsomal extracts was determined using the same method as employed for rBnACBP.

LPCAT assays

LPCAT (forward reaction) assay conditions were modified from Furukawa-Stoffer *et al*. (2003). The microsomal fraction from ΔYOR175c yeast expressing *AthLPCAT-1* (~25–50 μg) was incubated in 80 mM Tris-HCl, pH 7.5 buffer, containing 0.21 M sorbitol, 0.13 mM EDTA, 15 μM [1-^{14}C]18:1-CoA (5.7 mCi/mmol, GE Healthcare) and 75 μM 18:1-LPC (Avanti Polar Lipids Inc., Alabaster, AL, USA), and varying amounts of rBnACBP or BSA (5–25 μM) were incubated in the total volume of 50 μL for 10 min at 30 °C. The reaction was terminated by the addition of 2 mL of chloroform–methanol (2 : 1, v/v). Phase separation was induced by the addition of 1 mL of 0.9% (w/v) KCl. Following brief centrifugation, the lower organic phase was transferred to a clean glass tube. Lipid residues in the aqueous phase were re-extracted with 1 mL of chloroform, and combined with the first extract of the organic phase. The solvent was dried under a gentle stream of N_2 gas at 37 °C. Samples were resuspended in 70 μL of chloroform and applied in 2.2-cm lanes to a 20 × 20-cm pre-coated thin layer chromatography (TLC) plate SIL G-25. A control lane with 5 μL of 100 μM *sn*-1–16:0, *sn*-2-[1-^{14}C]18:1-PC (56 mCi/mmol, GE Healthcare) was used to confirm the identity of the radioactive product. The TLC plate was developed in a chloroform–methanol–acetic acid–water (85 : 15 : 10 : 3.5, v/v) solvent system. The PC product was visualized by a phosphorimaging system (Typhoon Trio Variable Mode Imager, GE Healthcare, Baie d'Urfe, QC, Canada). TLC spots corresponding to the position of the PC product were scraped from the plate, combined with 5 mL of Ecolite™(+) scintillant and analysed for radioactivity.

Acylation of PC in the absence of added LPC in microsomes from ΔYOR175c yeast expressing *AthLPCAT-2* or in *B. napus* MD cell suspension cultures was assayed using the same protocol as described above, but with the omission of 18:1-LPC.

The protocol for assaying the LPCAT reverse reaction was modified from that of Stymne and Glad (1981). Microsomes from ΔYOR175c yeast expressing *AthLPCAT-1* or *AthLPCAT-2* (~15 μg) were lyophilized and washed with benzene containing 5 nmol *sn*-1-16:0, *sn*-2-[1-^{14}C]18:2-PC (5.17 mCi/mmol, GE Healthcare). Benzene was immediately evaporated under a gentle stream of N_2 gas, and 100 μL of the assay medium were added. The reaction medium consisted of 50 μM 18:1-CoA (Sigma, Oakville, ON, Canada), 2 mM CoA (Sigma) and varying amounts of rBnACBP or BSA (25–75 μM) in 0.1 mM phosphate buffer, pH 7.2. The non-radioactive 18:1-CoA was added in order to trap [1-^{14}C]18:2-CoA formed by the reverse reaction of LPCAT, which otherwise would be directly re-acylated to the simultaneously formed LPC. The reaction was allowed to proceed for 30 min at 30 °C and was terminated with 375 μL of chloroform–methanol (1 : 2, v/v). Extraction of the chloroform-soluble lipids from the reaction mixture was performed according to a standard procedure (Bligh and Dyer, 1959). The volume of the aqueous fraction was reduced to approximately 50 μL under vacuum (SpeedVac with Vapor Net AES2000, Savant), and then applied to a 20 × 20-cm pre-coated TLC plate SIL G-25. The TLC plate was developed in a methyl acetate–*n*-propanol–chloroform–methanol–0.25% KCl in water (25 : 25 : 25: 10 : 9, v/v) solvent system. Acyl-CoAs were visualized using phosphorimaging, removed from the TLC plate and combined with 5 mL of Ecolite™(+) scintillant for analysis using a liquid scintillation counter.

Acknowledgements

The authors acknowledge the Alberta Crop Industry Development Fund Student Development Program, and support provided to R.J.W. via a Discovery Grant from the Natural Sciences and Engineering Research Council of Canada and the Canada Research Chairs Program. The authors also acknowledge Dr Matthew Hills (John Innes Centre, Norwich, UK) for the primary antibodies against BnACBP and Crystal Snyder (University of Alberta, AB, Canada) for technical support in the biochemical analysis of seed oil and *in vitro* enzyme assays.

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