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Identification of *Brassica napus* lysophosphatidylcholine acyltransferase genes through yeast functional screening

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Abstract:

Acyl-CoA: lysophosphatidylcholine acyltransferase (LPCAT), which acylates lysophosphatidylcholine (LPC) to produce phosphatidylcholine (PC), is a key enzyme in the Lands cycle. The combined reverse and forward reactions of LPCAT have been proposed to mediate acyl exchange between the acyl-CoA pool and the sn-2 position of PC in plants. There is also evidence that acyl exchange involving LPCAT is a prevailing metabolic process in acyl flux during triacylglycerol (TAG) synthesis in seeds. In this study, by complementing the yeast *lca1*Δ mutant deficient in LPCAT activity with an *Arabidopsis* seedling cDNA library, we found that the previously reported lysophospholipid acyltransferases, At1g12640 and At1g63050, were the only two acyltransferase genes that restored hyposensitivity of the *lca1*Δ mutant to lyso-platelet-activating factor (lyso-PAF). A developing seed cDNA library from *Brassica napus* L. cv Hero was constructed to further explore the heterologous yeast complementation approach. Six *B. napus* LPCAT cDNAs encoding five LPCAT isoforms were identified. The five protein isoforms could be divided into two subgroups, BnLPCAT1 and BnLPCAT2, that are homologous to *Arabidopsis* AtLPCAT1 (At1g12640) and AtLPCAT2 (At1g63050), respectively. Two proteins (BnLPCAT1-1 and BnLPCAT2) were chosen for further study. Enzymatic assays demonstrated that both proteins exhibited a substrate preference for LPCs and unsaturated fatty acyl-CoAs. In addition to the enzymatic properties of plant lysophosphatidylcholine acyltransferases uncovered in this study, our report describes a useful technique that facilitates subsequent analyses into the role of LPCATs in PC turnover and seed oil biosynthesis.

Keywords:

Brassica napus

Yeast complementation cloning

Phosphatidylcholine turnover

Acyl exchange

Lysophosphatidylcholine acyltransferase (LPCAT)

Abbreviations: PC, phosphatidylcholine; LPC, lysophosphatidylcholine; LPCAT, acyl-CoA: lysophosphatidylcholine acyltransferase ; PLA2, phospholipase A2; lyso-PAF, lyso-platelet-activating factor; DAG, diacylglycerol; TAG, triacylglycerol; MBOAT, membrane-bound O-acyltransferase; AGPAT, 1-acylglycerol-3-phosphate O-acyltransferase; LPLAT, lysophospholipid acyltransferase; LPA, lysophosphatidic acid; LPE, lysophosphatidylethanolamine

1. Introduction

Phospholipids are the principal components of biological membranes and are involved in a range of cellular processes. Phosphatidylcholine (PC) is a major class of phospholipids that accounts for >50% of membrane lipid in most eukaryotic organisms (van Meer et al., 2008). PC is not only a fundamental component of cell membranes but is also a significant fatty acyl donor for neutral lipid biosynthesis, and in plant cells, is an indispensable substrate for modification of fatty acids including desaturation and hydroxylation (van Meer et al., 2008; Stymne and Appelqvist, 1978; Moreau and Stumpf, 1981). In most eukaryotic cells, PC is predominantly synthesized from diacylglycerol (DAG) and CDP-choline through a *de novo* pathway called the Kennedy pathway (Kennedy and Weiss, 1956). After *de novo* synthesis, the sn-2 acyl moiety of PC undergoes rapid turnover, which is generally known as the Lands cycle (Lands, 1960). In the Lands cycle, PC is first deacylated to lysophosphatidylcholine (LPC), and the LPC is then reacylated to PC by acyl-CoA-dependent lysophosphatidylcholine acyltransferase (LPCAT; EC2.3.1.23). Hence, LPCAT is a key enzyme that can influence the fatty acid composition and properties of PC through the modulation of acyl moieties at the sn-2 position of PC.

In plant systems such as safflower (*Carthamus tinctorius* L.), the forward reaction of LPCAT provides a mechanism to transfer monounsaturated fatty acids (oleate) to position 2 of PC where they are further desaturated into polyunsaturated fatty acids. The newly synthesized linoleate in PC is then returned to the acyl-CoA pool by the reversal of LPCAT, which sustains seed oil synthesis (Stymne and Stobart, 1984). In *Brassica napus* developing seed, where very long chain fatty acids accumulate, LPCAT is also implicated in influencing the fatty acid composition of seed oil. At least one-half of the 18:1 exported from plastids enters an intermediate pool before elongation (Bao et al, 1998), and the shuffling of 18:1 is expected to impact the size of the acyl-CoA pool necessary for malonyl-CoA-dependent elongation in the cytosol (Bao et al., 1998). A recent study in developing soybean embryos provided evidence that the acyl editing of PC is a prevailing metabolic process in the acyl flux of triacylglycerol (TAG) synthesis

(Bates et al., 2009). It has been suggested that the acyl editing mechanisms and the substrate preferences of enzymes may affect the incorporation of unusual fatty acids into TAG (reviews by Napier, 2007; Snyder et al., 2009). Thus, investigating the role of LPCAT in acyl editing is essential to engineering unusual fatty acid accumulation in transgenic plants.

Although the Lands cycle was discovered 50 years ago, cloning of LPCATs was only accomplished recently, beginning with several independent reports of YOR175c (LCA1), a lysophosphatidylcholine acyltransferase in the yeast *Saccharomyces cerevisiae* (Benghezal et al., 2007; Chen et al., 2007; Jain et al., 2007; Riekhof et al., 2007; Tamaki et al., 2007). LCA1 belongs to the membrane-bound O-acyltransferase (MBOAT) family (Hofmann, 2000), and possesses an acyltransferase activity against not only LPC but also lyso-platelet-activating factor (lyso-PAF; 1-O-alkyl-LPC). The discovery of yeast LPCAT paved the road for the identification of LPCAT3 and LPCAT4, which are the MBOAT homologs in mammals that possess LPC acylation activity (Hishikawa et al., 2008; Kazachkov et al., 2008; Zhao et al., 2008; Jain et al., 2009). LPCAT3 prefers polyunsaturated fatty acyl-CoAs including 20:4-CoA and 18:2-CoA as substrates instead of saturated fatty acyl-CoAs, while LPCAT4 has a strong preference for 18:1-CoA. Previously, two mammalian enzymes, LPCAT1 and LPCAT2, were characterized as exhibiting LPCAT activities and proposed to function in respiratory physiology and the inflammatory response, respectively (Nakanishi et al., 2006; Chen et al., 2006; Shindou et al., 2007). However, they are members of the AGPAT (1-acylglycerol-3-phosphate O-acyltransferase) family, not MBOAT proteins. A report of two members of the MBOAT family from *Arabidopsis*, AtLPLAT1 (Atlg12640) and AtLPLAT2 (At1g63050), that share high sequence identity to yeast LCA1 soon followed (Ståhl et al., 2008). Heterologous expression of AtLPLAT1 and AtLPLAT2 in the yeast *lca1*Δ mutant resulted in increased acylation activity of several classes of lysophospholipids, but LPC was the most prominent and preferred acyl acceptor. Therefore, we herein propose to rename these enzymes AtLPCAT1 and AtLPCAT2, respectively.

To date, molecular identification of LPCATs from various species has been chiefly pursued through sequence homology searches to the yeast LPCAT gene. However, even in species such as *Arabidopsis* with a relatively small genome, a large number of MBOAT proteins homologous to the yeast LPCAT are present. In this study, building on our previous discovery that the yeast *lca1*Δ mutant is hypersensitive to lyso-PAF, an ester-linked analogue of LPC, we devised a biochemical screening approach to identify LPCAT cDNAs through functional complementation. In the screening process, lyso-PAF was used as a selective agent due to its toxicity to the yeast *lca1*Δ mutant (Chen et al., 2007). Five Brassica napus lysophosphatidylcholine acyltransferase (BnLPCAT) isoforms were isolated. Two of these isoforms,

BnLPCAT1-1 and BnLPCAT2, were chosen for subsequent enzymatic assays and expression profile analysis. This research contributes a useful tool for both identifying plant LPCATs and understanding triacylglycerol synthesis in different oil crop species.

2. Results

2.1. Isolation of *Arabidopsis* LPCATs by complementation of the yeast *lca1*Δ mutant

The *Saccharomyces cerevisiae* mutant strain Y02431, previously designated as *lca1*Δ, is deficient in LPCAT activity and hypersensitive to lyso-PAF (Chen et al., 2007). The sensitivity of the *lca1*Δ mutant towards lyso-PAF likely reflects the fact that the yeast LPCAT is the only enzyme capable of acylating lyso-PAF, which, if left unacylated, will exert growth inhibition and toxicity through strong detergent effects. Hence, complementation of LPCAT activity by heterologous expression of LPCAT genes from other species could suppress the lyso-PAF sensitivity of the *lca1*Δ mutant, thereby serving as a LPCAT functional screening technique. Two *Arabidopsis* LPCATs have been reported and characterized (Ståhl et al., 2008). Therefore, we first tested the feasibility of the screening method by attempting complementation of the *lca1*Δ mutant with sequences from an *Arabidopsis* seedling cDNA library (Minet et al., 1992). We employed 10 μg/ml of lyso-PAF for yeast selection. Although a higher level of lyso-PAF (20 μg/ml) suppressed the growth of *lca1*Δ more efficiently, obvious toxic effects on the wild-type strain were also observed (data not shown). After transforming the *Arabidopsis* cDNA library into the *lca1*Δ mutant, approximately 2×10^6 cells were spread as evenly as possible on 150×25 mm petri dishes containing SC-Ura medium supplemented with 10 μg/ml lyso-PAF. Based on the calculated transformation efficiency (1 transformant/ 150 cells), about 1×10^4 transformants per plate would appear if lyso-PAF was not added. A higher plating density would have seriously reduced the selective ability of lyso-PAF.

After screening about 8×10^5 transformants, 29 clones that exhibited lyso-PAF resistance were obtained and sequenced. Using the cDNA sequences of these clones as queries, BLAST searches indicated that 9 of these clones had high similarities to AtLPCAT genes (Table 1). Among these 9 clones, 6 were identified as AtLPCAT1 (At1g12640), while the other 3 were AtLPCAT2 (At1g63050). All 9 clones containing LPCAT genes contained the complete gene coding region. The other 20 clones showed no similarity to AtLPCAT

genes or other MBOAT family genes, and they were not repeatedly recovered in our complementation assay. Therefore, these clones may have been the result of random reverting events of the mutant (Table 1). Despite the presence of many other MBOAT genes and several other confirmed sn-2 lyso-phospholipid acyltransferases in the *Arabidopsis* genome (Stålberg et al., 2009), our screen only recovered the two previously reported AtLPCATs. Thus, our results suggest that AtLPCAT1 and AtLPCAT2 are the only two *Arabidopsis* genes that complement the deficiency of *lca1*Δ in the presence of lyso-PAF. In addition, these results strongly suggest that the complementation selection method was stringent and efficient, and should be applicable for isolating LPCAT genes from other species.

2.2. Construction of a *Brassica napus* seed cDNA library and identification of *B. napus* LPCAT cDNAs

Brassica napus cv Hero is a high erucic acid producing rapeseed cultivar (Scarth et al, 1991). Erucic acid is synthesized through elongation of oleic acid (18:1) in the cytosolic compartment. Current evidence suggests that erucic synthesis draws a significant portion of its 18:1-CoA substrate from an intermediate 18:1-CoA pool that originates from PC editing (Bao et al., 1998). As a part of our ongoing research on the functional significance of LPCAT in acyl-CoA channeling for very long chain fatty acid production, we constructed a cDNA library in a yeast expression vector with RNA isolated from *B. napus* cv Hero seeds at different developmental stages (see Experimental 5.3 and 5.4). Following yeast transformation and complementation screening for putative *B. napus* LPCAT cDNAs, seventy-two positive colonies were obtained from approximately 1×10^6 transformants. Sequence analyses of the plasmids recovered from yeast indicated that 44 of the 72 clones exhibited high similarity to the *Arabidopsis* LPCATs. Among the 44 plasmids, five cDNAs were highly homologous to AtLPCAT2, which we termed BnLPCAT2. The other 39 plasmids displayed high sequence similarity to AtLPCAT1 and could be furthered classified into five different subgroups, BnLPCAT1-1 (25 clones), BnLPCAT1-2 (4 clones), BnLPCAT1-3 (3 clones), BnLPCAT1-4 (5 clones) and BnLPCAT1-5(2 clones), based on minor sequence differences (Supp. Fig. 1). The remaining 28 plasmids did not have any similarities to LPCAT genes. Twenty-one of the plasmids contained independent and seemingly random inserts; three were empty vectors and four were homologous to At2g35860. At2g35860 is annotated as a fasciclin-like arabinogalactan protein 16 precursor (FLA16) in The *Arabidopsis* Information Resource (TAIR) and is involved in cell adhesion. This gene was not further characterized in this study.

The deduced amino acid sequences of BnLPCATs were aligned with AtLPCAT1 and AtLPCAT2 (Fig. 1A). All of these sequences possessed the three conserved MBOAT motifs (motifs A–C) that are essential for lysophospholipid acyltransferase (LPLAT) activities (Shindou et al., 2009a). In motif B, we found a highly conserved histine residue, which is predicted to be a putative active-site residue within a long hydrophobic region in the MBOAT family (Hofmann, 2000). Because their single nucleotide difference is located in the degenerate site of a serine codon, BnLPCAT1-3 and BnLPCAT1-5 shared the same amino acid sequence (Supplementary Fig. 1). Thus, we successfully identified 5 LPCAT proteins that belong to the MOBAT family in *B. napus* via cDNA library complementation screening.

To examine the evolutionary relationship between the BnLPCATs and other known LPLATs exhibiting LPCAT activities from different species, a phylogenetic tree was generated based on amino acid sequence identities (Fig. 1B). The results revealed that the lysophospholipid acyltransferases bifurcated into two groups. The first group contained the AGPAT family members, including LPCAT1 and LPCAT2 in mouse and human, while the second group was composed of the MBOAT members, such as LPCATs from *B. napus*, *Arabidopsis*, yeast and the mammalian LPCAT3 and LPCAT4. Four BnLPCAT1 proteins (BnLPCAT1-1, BnLPCAT1-2, BnLPCAT1-3 and BnLPCAT1-4) were in the same branch as AtLPCAT1, while BnLPCAT2 and AtLPCAT2 diverged to form a different subgroup, which suggested that plant LPCAT1 and LPCAT2 diverged during evolutionary history. Moreover, BnLPCATs and AtLPCATs exhibited higher similarities to mammalian LPCAT4 than to yeast LPCATs.

2.3. Substrate specificity assays for BnLPCATs

The yeast *lca1*Δ strain has a very low LPCAT background activity (Chen et al., 2007), thereby providing an ideal heterologous expression system for assessing LPCAT enzymatic properties. We amplified BnLPCAT cDNAs from their original plasmids and cloned them into the pYES2.1 vector. To determine whether these constructs properly expressed LPCAT after being transformed into the *lca1*Δ strain, a lyso-PAF sensitivity test was conducted (Fig. 2). Each *lca1*Δ mutant expressing a BnLPCAT gene grew well on plates containing 10 μg/ml lyso-PAF. By contrast, the *lca1*Δ strain harboring the empty vector (VO) exhibited severe growth defects on lyso-PAF plates. When plated with 20 μg/ml lyso-PAF, we observed no growth for the VO negative control. However, the *lca1*Δ strain expressing BnLPCATs were capable of

growing, except when plated at a very low cell density. BnLPCAT1-1, the most abundant LPCAT1 in *B. napus*, and BnLPCAT2 were selected for further enzymatic property analyses.

We first assessed the acyltransferase activity of BnLPCAT1-1 and BnLPCAT2 using different lysophospholipids as acyl acceptors (Fig. 3). In the presence of all tested lysophospholipids, the activity of BnLPCAT2 was consistently higher when compared with BnLPCAT1-1. Both showed higher activities with LPC substrates compared to lysophosphatidic acid (LPA) and lysophosphatidylethanolamine (LPE). These results thus confirmed that the *B. napus* BnLPCAT1-1 and BnLPCAT2 are lysophosphatidylcholine acyltransferases. Further analyses revealed that the two BnLPCATs have an approximately equal preference for 16:0 LPC, 18:0 LPC and 18:1 LPC. However, their activities were dramatically decreased when very-long-chain acyl-CoA (20:0 LPC) was used as the acyl acceptor. Moreover, the two BnLPCATs utilized lyso-PAF efficiently, although lyso-PAF is not known to be a metabolite of significance in plants or yeasts.

We also tested the preference of BnLPCAT1-1 and BnLPCAT2 towards various acyl-CoA donors (Fig. 4). Similar to the assays for lysophosphalipid preference, BnLPCAT2 exhibited a much higher activity compared to BnLPCAT1-1 with all of the tested acyl-CoAs. Both preferred unsaturated fatty acyl-CoAs, such as 16:1, 18:1, 18:2 and 18:3, to saturated fatty acyl-CoAs including 16:0 and 18:0. The activity order for BnLPCAT1-1 with the selected acyl-CoAs was 16:1 ≈ 18:2 > 18:3 > 18:1, while the acyl-CoA preference of BnLPCAT2 was 16:1 > 18:1 ≈ 18:3 > 18:2. Both enzymes showed limited activities with 22:1-CoA, which suggested that this very-long-chain unsaturated fatty acyl-CoA is a very poor substrate for the two BnLPCATs.

2.4. Tissue-specific expression of the BnLPCAT genes in *B. napus*

To investigate the expression patterns of BnLPCAT1-1 and BnLPCAT2 in different tissues of *B. napus* plants, quantitative real-time PCR (qRT-PCR) was performed using RNA prepared from 1-week-old seedlings, roots, stems, leaves, buds, flowers, 10 DAF seeds and 25 DAF seeds (Fig. 5). Gene-specific primers were used to detect the expression of the two genes in all of the tested tissues. BnLPCAT1-1 was present at much higher levels than BnLPCAT2 in all of the tested tissues. The highest expression level of BnLPCAT1-1 was detected in 1-week-old seedlings, followed by leaf, bud and flower, whereas BnLPCAT2 showed its highest expression level in the bud, compared to leaf and flower. In our assays, the expression of the two genes did not vary in 10 and 25 DAF developing seeds.

3. Discussion

In recent years, many acyl-CoA: lysophospholipid acyltransferases (LPLATs) that function in the Lands cycle have been identified (reviewed by Shindou and Shimizu, 2009; Shindou et al., 2009b). The discovery of these enzymes has furthered our understanding of the diversity and asymmetry of membrane and acyl editing. To our knowledge, most lysophosphatidylcholine acyltransferases have been identified based on homology searches (Hishikawa et al., 2008; Zhao et al., 2008; Kazachkov et al., 2008; Jain et al., 2009; Nakanishi et al., 2006; Chen et al., 2006; Shindou et al., 2007). The heterologous complementation approach described in this study allows LPCAT gene cloning without the need for access to sequence data. By complementation of the *lca1*Δ mutant with an *Arabidopsis* seedling cDNA library, we found that AtLPCAT1 and AtLPCAT2 were the only two acyltransferase genes that were capable of rescuing the hypersensitivity of the *lca1*Δ mutant to lyso-PAF.

Further, we have presented the cloning of lysophosphatidylcholine acyltransferases cDNAs from *B. napus*. Five cDNAs, which encode two paralogs of LPCATs homologous to AtLPCAT1 and AtLPCAT2, were identified in our study. All BnLPCATs are members of the MBOAT family. Sequence alignments and phylogenetic analyses showed that four of the BnLPCAT1 isoforms, BnLPCAT1-1, BnLPCAT1-2, BnLPCAT1-3, BnLPCAT1-4, belong to the same subgroup as AtLPCAT1, whereas BnLPCAT2 is an apparent homolog of AtLPCAT2 (Hofmann, 2000). In mammals, some members of the AGPAT family have been shown to exhibit an LPCAT activity (Nakanishi et al., 2006; Chen et al., 2006; Shindou et al., 2007; Cao et al., 2008). In *Arabidopsis* lysophosphatidylethanoliamine acyltransferases of the AGPAT family have also been reported (Stålberg 2009). However, we did not recover any LPCATs that were not members of the MBOAT family in both the *Arabidopsis* and *B. napus* cDNA libraries. Although not specifically determined in this study, it is likely that the method described will be useful for screening LPCATs and other lyso-PAF metabolizing enzymes in animal systems as well.

We chose BnLPCAT1-1 and BnLPCAT2 for further study because they represented the two sub-groups of BnLPCATs. Of the 44 positive complementation clones, clones that expressed BnLPCAT1-1 were the most prevalent at 25. The mRNA levels of BnLPCAT1-2, BnLPCAT1-3, BnLPCAT1-4 could not be determined individually using quantitative real-time RT-PCR due to their high sequence similarity. Our results indicated that the mRNAs of both BnLPCAT1-1 and BnLPCAT2 were detected ubiquitously. Interestingly, the level of BnLPCAT1-1 mRNA was much higher than BnLPCAT2 in all of the tested tissues;

this correlates with the recovery of more clones of BnLPCAT1-1 in the complementation assay. However, the *in vitro* enzyme activity assay indicated that the BnLPCAT2 protein showed a remarkably higher activity when compared with BnLPCAT1-1 (Figs. 3, 4). In addition, the mRNA levels of BnLPCAT1-1 and BnLPCAT2 did not significantly change in 10 and 25 DAF developing seeds.

After testing the enzymatic activities of BnLPCAT1-1 and BnLPCAT2 in the presence of various lysophospholipids or acyl-CoAs, we found that the two enzymes preferred LPC as a substrate and showed higher activities toward 16:0 LPC, 18:0 LPC and 18:1 LPC compared to 20:0 LPC and lyso-PAF. Neither showed significant activities towards LPE and LPA. The assessment of the acyl-CoA substrate preference indicated that the two *B. napus* LPCATs efficiently utilized unsaturated acyl-CoAs, including 16:1, 18:1, 18:2 and 18:3, while they showed low activity towards saturated acyl-CoAs such as 16:0 and 18:0. Our results are consistent with previous findings on LPCATs from microspore-derived cell suspension cultures of *B. napus* L. cv Jer Neuf, which indicated an LPCAT was more active with 18:1-CoA compared to 18:0-CoA or 16:0-CoA at acyl-CoA concentrations higher than 20 μM (Furukawa-Stoffer et al., 2003). Thus, similar to microsomal lysophosphatidic acid acyltransferase (LPAAT) (Hares and Frentzen, 1987; 1991), LPCATs participate in excluding saturated fatty acid from the sn-2 position of phospholipids. Bernerth and Frentzen (1990) have shown that *B. napus* LPCAT strongly discriminates against 22:1-CoA based on assays using subcellular fractions of developing seeds. Similarly, we found that the two types of BnLPCATs exhibited very low activities towards 22:1-CoA. Lastly, the two BnLPCATs exhibited significantly higher activities towards 16:1-CoA; this is surprising since we detected very low levels of 16:1 in seed oil from *B. napus* cv. Hero (data not shown).

4. Conclusions

LPCAT is a key enzyme in PC remodeling. We identified LPCATs from *Arabidopsis* and *B. napus* through functional complementation of yeast. Following the identification of plant LPCAT genes, our study now provides molecular tools for investigating the functional significance of acyl exchange during the accumulation of erucic acid in seed oil biosynthesis in transgenic plants. The methodology described in our study will be useful for identifying LPCATs from other species that accumulate unusual fatty acids.

5. Experimental

5.1. Plant materials

Brassica napus L. cv Hero was grown in a growth cabinet with a 16-h light/ 8-h dark regime with a photosynthetic photon flux density of 200 µmol photons m^{-2} s^{-1}. The day/night temperature was controlled at 22/17°C. Siliques of early, middle and late developmental stages were collected 6 to 15, 16 to 25 and 26 to 40 days after flowering (DAF), respectively. Seeds were dissected out of the silique, immediately placed on dry ice, and frozen at -80°C until further use. Seeds at the same developmental stage were pooled for RNA extraction.

5.2. Yeast strain and reagents

The yeast strain: Y02431 (*lca1*Δ, MATa *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 YOR175c::KanMX4) was purchased from the European *Saccharomyces cerevisiae* Archive for Function Analysis (EUROSCARF). Various lysophospholipids and acyl-CoAs were obtained from Avanti Polar Lipids and Sigma. [^{14}C]oleoyl-CoA and [^{14}C]oleoyl-LPC were purchased from American Radiolabeled Chemicals Inc.

5.3. cDNA library construction

A yeast expression library from *B. napus* seeds was constructed with the Creator SMART cDNA Library Construction kit (Clontech). Because the kit does not provide a yeast expression vector, we modified the pFL61 vector (Minet et al., 1992) according to previously described techniques (Tan et al., 2008). Oligomer1 (5'-CGGCCATTACGGCCTGCAGGATCCGGATCCGGCCGCCTCGGCCCCACA-3') and its complementary strand Oligomer2 (5'-

GGGCCGAGGCGGCCGGATCCGGATCCTGCAGGCCGTAATGGCCGCACA-3'), which contain *Sfil*A and *Sfil*B restriction sites and *Bst*XI (5'-CACA-3') sticky-ends at their 3' ends, were mixed at an equal molar value and ligated with *Bst*XI digested pFL61. The correctly-oriented plasmid was verified by DNA sequencing and termed pFL61-Sfil.

Total RNA was extracted from seeds of Hero plants grown to different developmental stages as previously described (Oñate-Sánchez and Vicente-Carbajosa, 2008) and further purified using an RNeasy

Midi Kit (Qiagen). Equivalent amounts of total RNA from different developmental stages were pooled together for isolating Poly A+ RNA using an Oligotex mRNA Mini Kit (Qiagen). poly A+ RNA (0.5 μg) was used to construct the cDNA library using a SMART cDNA Library Construction kit (Clontech). After synthesis of the second strand, the cDNAs were digested with *Sfi*I and then inserted into a *Sfi*I digested pFL61-SfiI vector to create the cDNA library. The library had approximately 4.8×10^6 plaque-forming units before amplification. Based on 15 randomly selected clones, the average insert size was estimated to be 800 bp.

5.4. Complementation of the *lca1*Δ mutant with the cDNA libraries

Yeast transformation was performed as previously described (Pan et al., 2004). *Arabidopsis* or *B. napus* cDNA (1.5 μg) was introduced into each aliquot of *lca1*Δ competent cells. About 2×10^6 cells were spread on one 150×25 mm petri dish containing SC-Ura medium supplemented with 10 μg/ml lyso-PAF and incubated at 28°C for 4 to 7days for selection. The average transformation efficiency was 1 transformant/150 cells. Colonies from selective plates were transferred onto fresh SC-Ura plates without lyso-PAF to determine that they grew to similar degrees. To decrease false positive clones, each colony was cultured in liquid SC-Ura medium overnight and diluted 20 times (about OD_{600}=0.2). Each dilution (5 μl) was inoculated on SC-Ura plates containing 10 μg/ml lyso-PAF and cultured for another 2 days. Clones without growth deficiencies were chosen for sequencing. The plasmids were isolated from individual colonies using a QIAprep Spin Miniprep kit (Qiagen) and amplified by subcloning into *E. coli*. The inserts were sequenced from the 5' end using primer FL61F (5'-GTAGAACCTCGTGAAACTTAC-3').

5.5. Sequence alignment and phylogenetic analysis

Five different BnLPCATs were obtained from the library screening, which included four BnLPCAT1 isoforms (BnLPCAT1-1, BnLPCAT1-2, BnLPCAT1-3, and BnLPCAT1-4) and one BnLPCAT2. The alignment was performed using ClustalW2 multiple sequence alignment with 5 BnLPCATs and 2 AtLPCATs. Lysophospholipid acyltransferases from various species, previously shown to display LPCAT activity, were obtained from the UniProt database. The phylogenetic analysis was performed using the Neighbor-Joining method from MEGA version 5.0 software (Tamura et al., 2007; MEGA version 4.0).

5.6. Gene expression vector construction

For the enzymatic assay, the coding sequences of *B. napus* LPCATs were PCR-amplified from their original library plasmids and placed under the control of a yeast inducible promoter in vector pYES2.1 using the pYES2.1 TOPO TA Expression kit (Invitrogen). Correctly-oriented plasmids were identified by DNA sequencing and subsequently introduced into the yeast strain Y02431.

5.7. Lyso-PAF sensitivity test

Yeast strains were grown in 10 ml of SC-Ura medium supplemented with 2% glucose overnight at 28°C with shaking. To induce protein expression, the culture was then diluted to an OD_{600} of 0.4 in 10 ml of SC-Ura medium supplemented with 2% galactose and 1% raffinose and incubated for another 12 h. Cultures were serially diluted 1:10 starting from OD_{600} = 2.0 to $OD_{600} = 2 \times 10^{-3}$. Five microliters from each dilution was spotted on SC-Ura plates containing 0, 5, 10, 20 μg/ml lyso-PAF, respectively. The plates were incubated at 28°C for 2 days.

5.8. Enzyme activity assays

Preparations of yeast cultures and microsomal proteins were performed as previously described (Kazachkov et al., 2008). The substrate specificities of the BnLPCATs were determined by measuring the incorporation of [^{14}C]oleoyl-CoA or [^{14}C]oleoyl-LPC into phosphotidylcholine. Microsomal proteins (0.5 μg) from *lca1*Δ harboring an empty vector (VO) or expressing BnLPCAT1-1, and 0.1 μg protein from *lca1*Δ expressing BnLPCAT2 were added to each reaction. To analyze lysophospholipid preference, assays were conducted in 50 μl of 0.1 M potassium phosphate (pH 7.2) containing 60 μM [^{14}C] oleoyl-CoA (5 nCi/nnol), 200 μM lysophospholipids and the indicated amount of microsomal proteins. To assess acyl-CoA preference, assays were performed in 50 μl of 0.1 M potassium phosphate (pH 7.2) containing 200 μM [^{14}C] oleoyl-LPC (1.5 nCi/nnol), 60 μM acyl-CoA species and the indicated amount of microsomal protein. The reactions were initiated by adding the microsomal proteins into the pre-warmed mixture at 30°C. Reactions were performed at 30°C for 4 min with shaking at 700 rpm, stopped by the addition of 170 μl of 5% acetic acid and 500 μl of chloroform: methanol (2:1, v/v) and vortexed immediately. After

centrifugation, the lower phase was transferred to a new glass tube and evaporated under a nitrogen stream. The lipid powder was dissolved in 40 µl of chloroform and separated on silica G60 TLC plates (Merck) using a solvent system of chloroform: methanol: acetic acid: water (85:15:10:2.5, v/v/v/v). Phospholipid species were identified by comparison to known standards and scraped off the TLC plate. The incorporation of ^{14}C into the phospholipid products was quantified by a scintillation counter (Beckman Coulter LS6500).

5.9. Quantitative real-time RT-PCR analysis

Total RNA was extracted from various tissues other than seeds using an RNeasy Mini Kit (Qiagen). Seed RNA was prepared as previously described (Oñate-Sánchez and Vicente-Carbajosa, 2008) and further purified using an RNeasy Mini Kit (Qiagen). For real-time quantitative RT-PCR (real-time qRT-PCR), 1 µg of total RNA was used for the cDNA synthesis with a QuantiTect Reverse Transcription kit (Qiagen, Ontario, Canada).

Following multiple sequence alignment of *BnLPCAT* gene sequences, regions of low similarity within each homolog were usued for PCR primer design. Specific primers (Tm, 57°C – 63°C) were designed to generate PCR products between 75 and 130 bp. The specificity of the two primers was tested using BLASTn searches against *B. napus* EST databases. *BnActin 7* (AF111812) was used as an endogenous control for standardization (Fujisawa et al., 2009). The primers used for qRT-PCR are listed in Supplementary Table 1. Real-time qRT-PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems), and amplification was monitored with an ABI StepOne Realtime PCR System (Applied Biosystems). A standard thermal profile was used for PCR: 50°C for 2 min; 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 57°C for 30 s, and 72°C for 30 s. Data acquisition and analyses were performed using StepOne software 2.0 (Applied Biosystems). Results from three biological replicates are shown.

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Appendix A. Supplementary data

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Figures and Legends

Fig. 1. Sequence analysis of predicted BnLPCAT proteins. (A) Deduced amino acid sequence alignment of BnLPCATs and AtLPCATs. Five BnLPCATs were obtained from the complementation assay, including four BnLPCAT1 isoforms (BnLPCAT1-1, BnLPCAT1-2, BnLPCAT1-3, BnLPCAT1-4) and one BnLPCAT2. The alignment was done using ClustalW multiple sequence alignment. Conserved amino acids are marked with asterisks (*). The three MBOAT motifs (Motifs A, B and C) are boxed. A highly conserved histine residue in motif B is highlighted in black. (B) Phylogenetic tree of lysophospholipid acyltransferases exhibiting LPCAT activity. The phylogenetic tree was drawn using MEGA5. The bar represents an evolutionary distance of 0.2. The UniProt accession numbers are as follow: *Arabidopsis thaliana* AtLPCAT1, Q8RWH4; *Arabidopsis thaliana* AtLPCAT2, Q9CAN8; *Homo sapiens* HsLPCAT4, Q6ZWT7; *Mus musculus* MmLPCAT4, Q8R3I2; *Schizosaccharomyces pombe* SpLPCAT, O42916; *Saccharomyces cerevisiae* ScLPCAT, A9EDP4; *Homo sapiens* HsLPCAT3, Q6P1A2; *Bos taurus* BtLPCAT3, Q3SZL3; *Mus musculus* MmLPCAT3, Q91V01; *Rattus norvegicus* RnLPCAT3, Q5FVN0; *Homo sapiens* HsLPEAT2, Q643R3; *Mus musculus* MmLPEAT2, Q6NVG1; *Xenopus laevis* XlLPEAT2, Q6DCK1; *Xenopus tropicalis* XtLPEAT2, Q28C60; *Mus musculus* MmLPCAT1, Q3TFD2; *Rattus norvegicus* RnLPCAT1, Q1HAQ0; *Homo sapiens* HsLPCAT1, Q8NF37; *Danio rerio* DrLPCAT1, Q1LWG4; *Danio rerio* DrLPCAT2, Q502J0; *Mus musculus* MmLPCAT2B, Q9D5U0; *Rattus norvegicus* RnLPCAT2B, Q4V8A1; *Homo sapiens* HsLPCAT2, Q7L5N7; *Mus musculus* MmLPCAT2, Q8BYI6; *Rattus norvegicus* RnLPCAT2, P0C1Q3.

Fig. 2. Lyso-PAF sensitivity test of *lca1Δ* harboring empty vector (VO) or expressing BnLPCATs. Yeast cells were grown in SC-Ura + 2% glucose medium overnight at 28 °C with shaking then transferred to induction medium for 12 h to induce protein expression. Cultures were serial diluted 1:10 from OD_{600} = 2.0. A measure of 5 µl from each dilution was spotted on SC-Ura plates containing 0, 5, 10, 20 µg/ml lyso-PAF respectively. The plates were incubated at 28 °C for 2 days.

Fig. 3. Lysophospholipid preferences of BnLPCATs. Assays were performed with 60 µM [^{14}C]18:1-CoA (5nCi/nnol), 200 µM lysophospholipids in a total volume of 50 µl. The amount of microsomal protein added in the reactions were 0.5 µg for *lca1Δ* harboring an empty vector (VO; white bar) or expressing BnLPCAT1-1 (gray bar), and 0.1 µg for *lca1Δ* expressing BnLPCAT2 (black bar). Reactions were performed at 30 °C for 4 min with shaking at 700 rpm. The data were shown as a mean of three assays.

Fig. 4. Acyl-CoA substrate preference of BnLPCATs. Assays were performed with 200 μM [^{14}C]18:1-LPC (1.5nCi/nnol), 60 μM acyl-CoA species in a total volume of 50 μl. The amount of microsomal protein added in the assays were 0.5 μg for *lca1Δ* harboring an empty vector (VO; white bar) or expressing BnLPCAT1-1(gray bar), and 0.1 μg for *lca1Δ* expressing BnLPCAT2 (black bar). Reactions were performed at 30 °C for 4 min with shaking at 700 rpm. The data were shown as a mean of three assays.

Fig. 5. Tissue expression analysis of *BnLPCAT*s using Real-time quantitative RT-PCR. The relative expression levels of *BnLPCAT1-1*(black bar) and *BnLPCAT2* (white bar) from different tissues as revealed by real-time qRT analysis. For comparison, the expression level of *BnLPCAT1-1* from seedling was normalized to 1 using StepOne software 2.0 (Applied Biosystems). The values represent the average of three independent biological replicates. Each biological repeat contains four technical replicates

Table1 Genes isolated from complementation of *lca1*Δ with *Arabidopsis* seedling cDNA library.

Clone	Inserts	Annotation of Encoded Protein
A1	AT1G63050.	MBOAT; AtLPCAT2
A2	AT1G02780	Structural constituent of ribosome
A5	AT3G20050	A putative cytoplasmic chaperonin that is similar to mouse Tcp-1
A12	AT5G51280	DEAD-box protein abstrakt, putative
A13	AT1G12640	MBOAT; AtLPCAT1
A16	AT1G12640	MBOAT; AtLPCAT1
A19	AT1G28290	An atypical arabinogalactan protein
A21	AT1G12640	MBOAT; AtLPCAT1
A27	AT1G63050	MBOAT; AtLPCAT2
A43	AT1G64470	Ubiquitin family protein
A44	AT1G12640	MBOAT; AtLPCAT1
A46	AT3G57520	ATSIP2 (ARABIDOPSIS THALIANA SEED IMBIBITION 2)
A47	AT1G12640	MBOAT; AtLPCAT1
A53	AT1G12640	MBOAT; AtLPCAT1
A56	AT1G53140	Dynamin family protein
A57	AT1G25530	Lysine and histidine specific transporter
A59	AT1G07670	Calcium-transporting ATPase
A60	AT3G53020	STV1 (SHORT VALVE1)
A61	AT2G43150	Proline-rich extensin-like family protein
A69	AT1G12640	MBOAT; AtLPCAT1
A73	AT2G26980	CIPK3 (CBL-INTERACTING PROTEIN KINASE 3)
A76	AT2G22870	EMB2001 (EMBRYO DEFECTIVE 2001)
A80	AT5G20900	JAZ12/TIFY3B (JASMONATE-ZIM-DOMAIN PROTEIN 12)
A83	AT5G10450	GRF6 (G-BOX REGULATING FACTOR 6)
A84	AT3G07630	ADT2 (AROGENATE DEHYDRATASE 2)
A85	AT2G47000	ABCB4 (ATP BINDING CASSETTE SUBFAMILY B4)
A87	AT3G47070	Unknown protein
A96	AT1G63050	MBOAT; AtLPCAT2
A99	AT3G19170	ATPREP1 (PRESEQUENCE PROTEASE 1)

Figure 1A

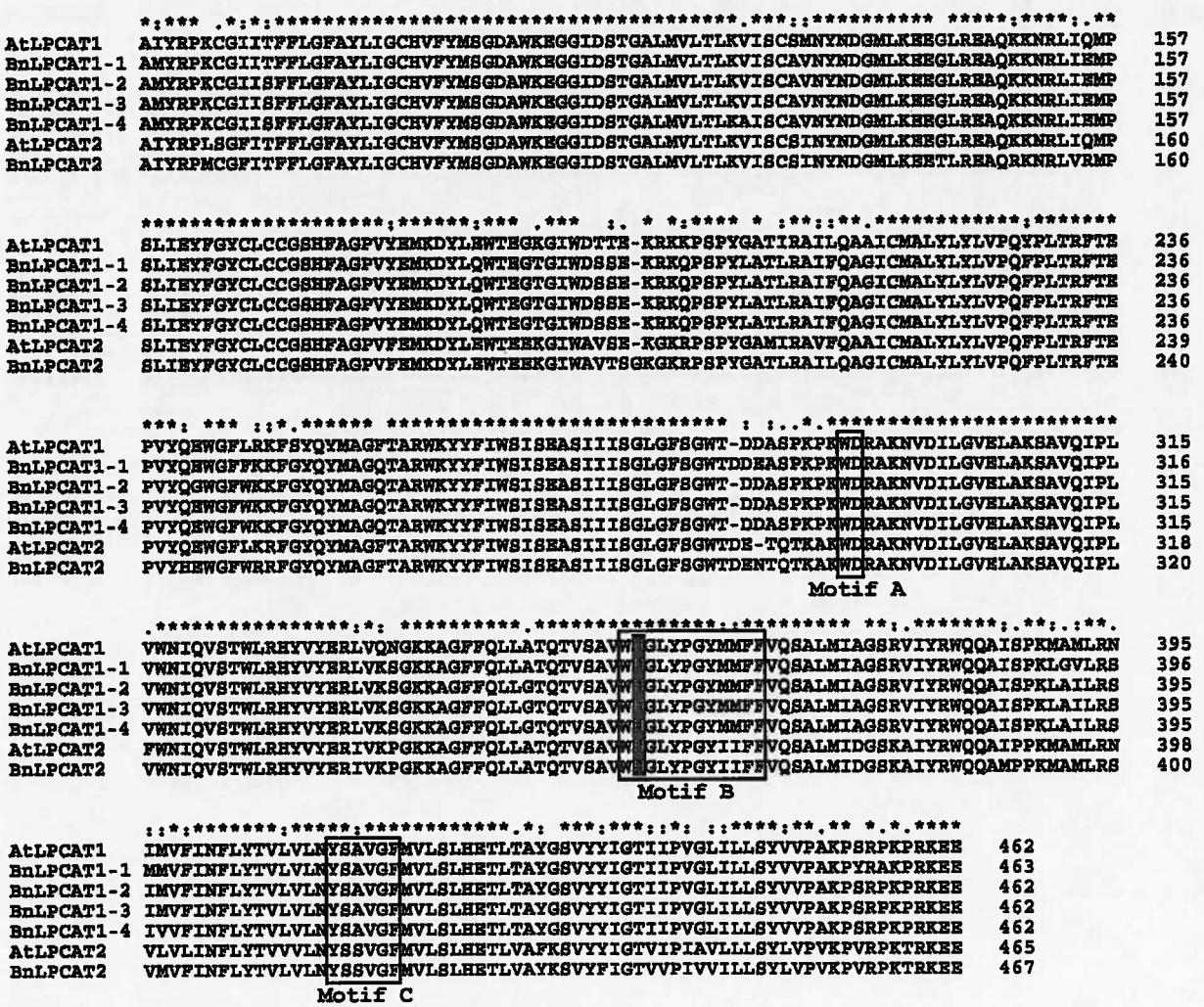
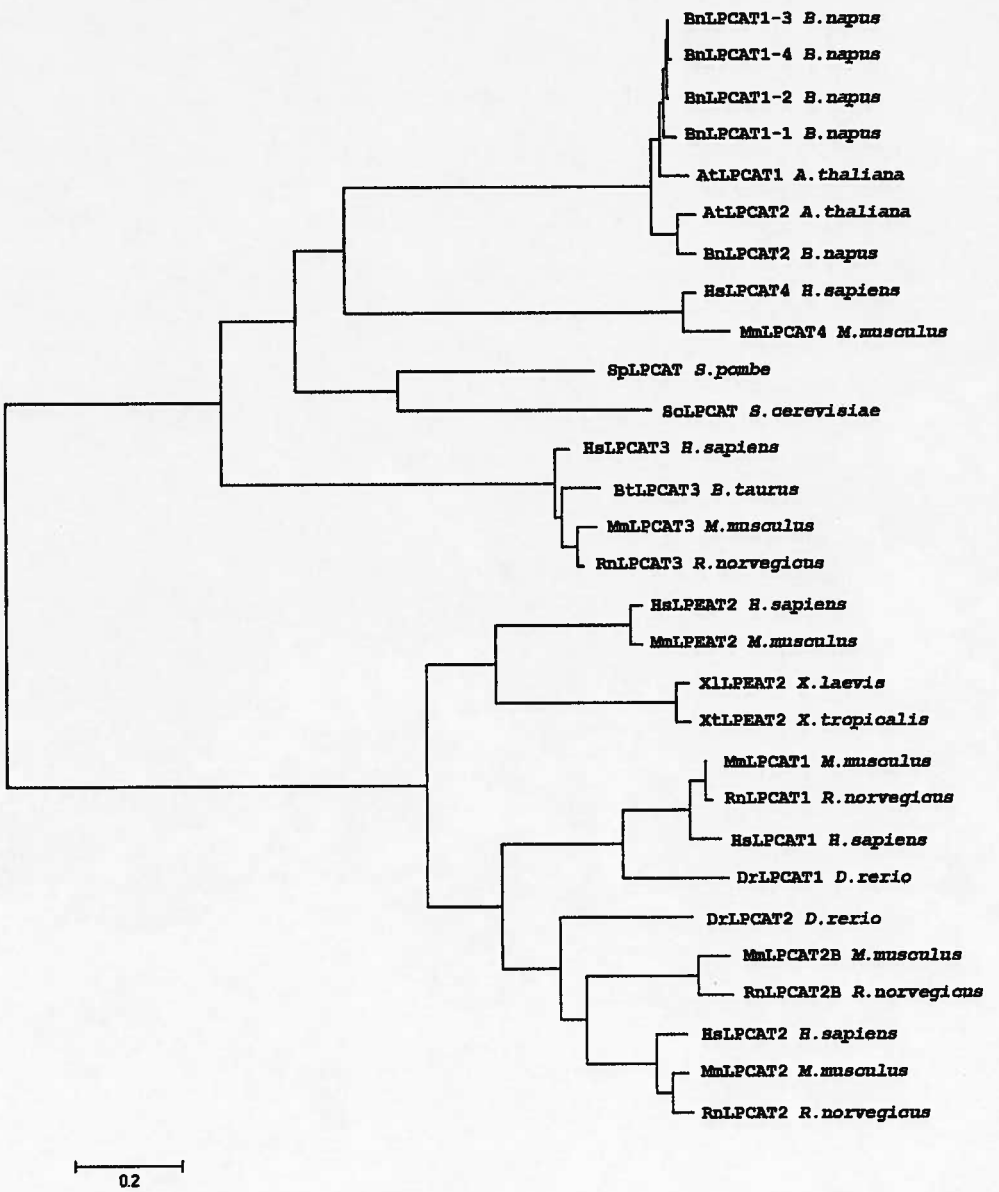

AtLPCAT1
BnLPCAT1-1
BnLPCAT1-2
BnLPCAT1-3
BnLPCAT1-4
AtLPCAT2
BnLPCAT2
Motif A
Motif B
Motif C

Figure 1B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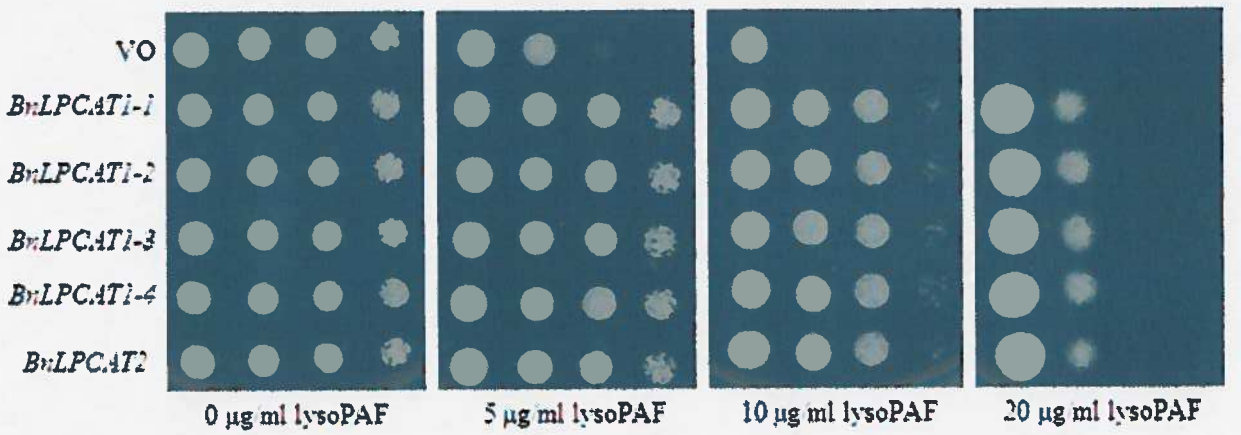
BnLPCAT1-4 B.napus
BnLPCAT1-2 B.napus
BnLPCAT1-1 B.napus
AtLPCAT1 A.thaliana
AtLPCAT2 A.thaliana
BnLPCAT2 B.napus
HsLPCAT4 H.sapiens
MmLPCAT4 M.musculus
SpLPCAT S.pombe
ScLPCAT S.cerevisiae
HsLPCAT3 H.sapiens
BtLPCAT3 B.taurus
MmLPCAT3 M.musculus
RnLPCAT3 R.norvegicus
HsLPEAT2 H.sapiens
MmLPEAT2 M.musculus
XlLPEAT2 X.laevis
XtLPEAT2 X.tropicalis
MmLPCAT1 M.musculus
RnLPCAT1 R.norvegicus
HsLPCAT1 H.sapiens
DrLPCAT1 D.rerio
DrLPCAT2 D.rerio
MmLPCAT2B M.musculus
RnLPCAT2B R.norvegicus
HsLPCAT2 H.sapiens
MmLPCAT2 M.musculus
RnLPCAT2 R.norvegicus
0.2

Figure 2


VO
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BnLPCAT1-2
BnLPCAT1-3
BnLPCAT1-4
BnLPCAT2
0 μg ml lysoPAF
5 μg ml lysoPAF
10 μg ml lysoPAF
20 μg ml lysoPAF

Figure 3

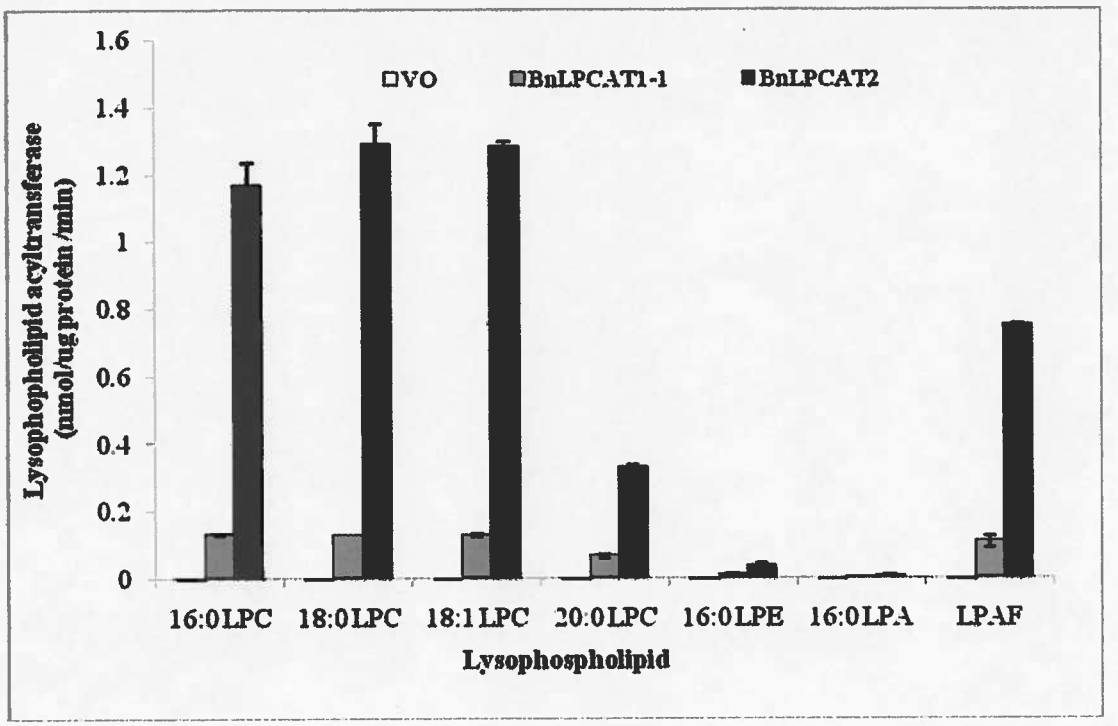
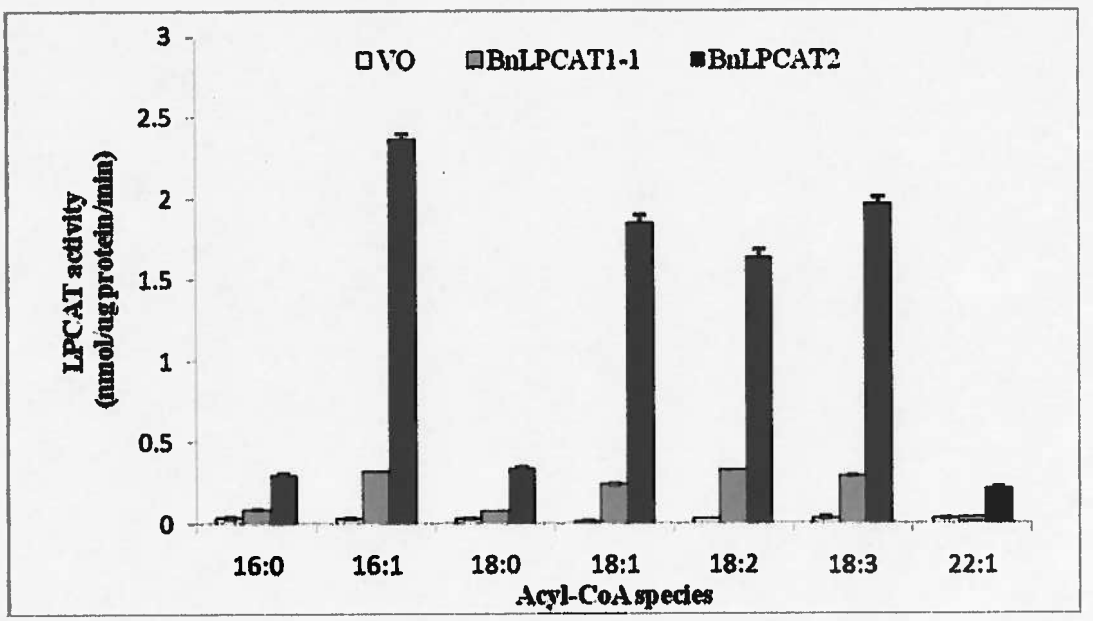

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
VO
BnLPCAT1-1
BnLPCAT2
Lysophopholipid acyltransferase (nmol/ug protein /min)
16:0LPC
18:0LPC
18:1LPC
20:0LPC
16:0LPE
16:0LPA
LPAF
Lysophospholipid

Figure 4


VO
BnLPCAT1-1
BnLPCAT2
LPCAT activity (nmol/ug protein/min)
3
2.5
2
1.5
1
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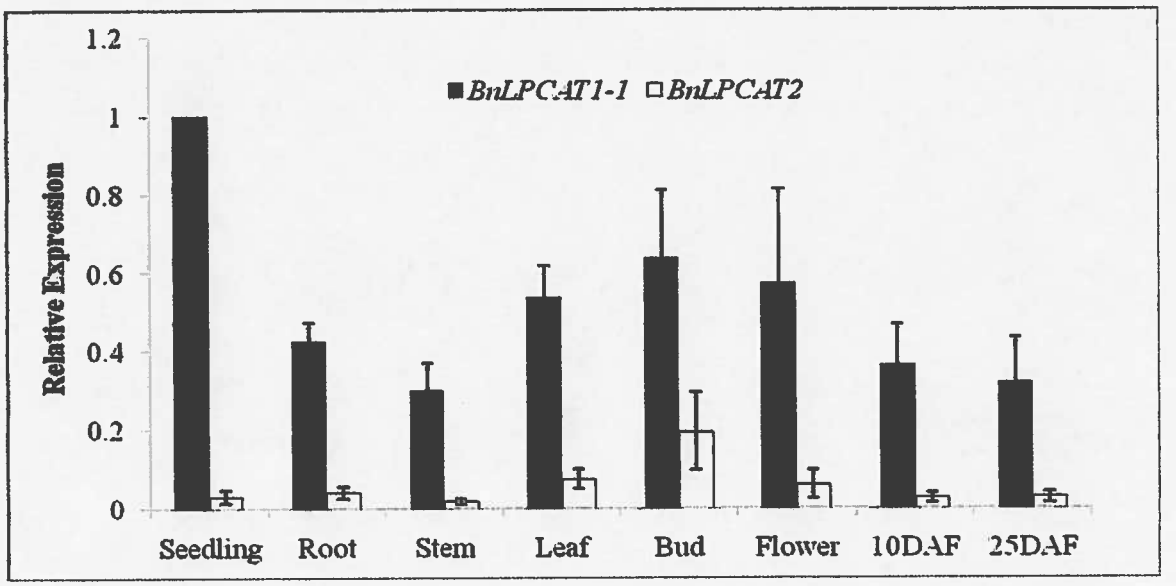
0
16:0
16:1
18:0
18:1
18:2
18:3
22:1
Acyl-CoA species

Figure 5


1.2
1
0.8
0.6
0.4
0.2
0
Relative Expression
BnLPCAT1-1
BnLPCAT2
Seedling
Root
Stem
Leaf
Bud
Flower
10DAF
25DAF

Supplementary data

Supplemental Table

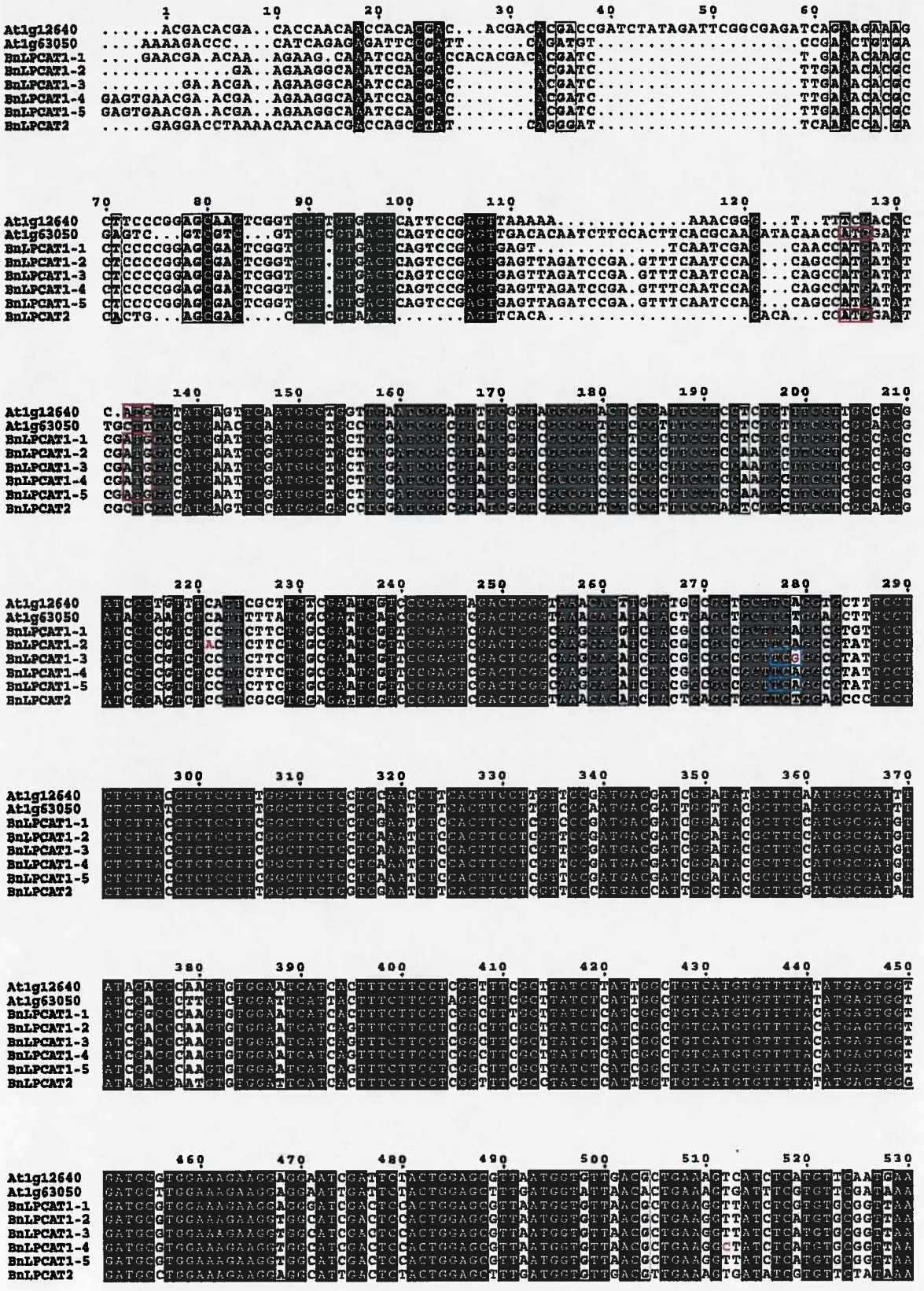
Supplemental Table 1. Primers pairs used for quantitative Real-Time RT-PCR analysis.

Gene Name	Forward primer (5' - 3')	Reverse primer (5' - 3')
BnActin7	ACGAGCTACCTGACGGACAAG	GAGCGACGGCTGGAAGAGTA
BnLPCAT1-F1	ATCCACGACCACACGACAC	GGTTGCTCGATTGAACTCAC
BnLPCAT2-F1	GCCTATCAGGGATTCAAAC	CCATGGAACTCATGTCGAG

Supplemental Figure Legend

Supplemental Figure 1. Alignment of cDNA sequences of BnLPCATs and AtLPCATs. Highly conserved sequences are shown on a black background. The predicted inition codon and termination codon of *BnLPCAT* cDNAs and *AtLPCAT* cDNAs are indicated in red box. Single nucleotide differences between *BnLPCAT1-2*, *1-3*, *1-4*, *1-5* were highlighted in red. The single nucleotide difference between *BnLPCAT 1-3* and *BnLPCAT 1-5* locates in a degenerate codon, which is denoted in blue box.

Supplemental Figure 1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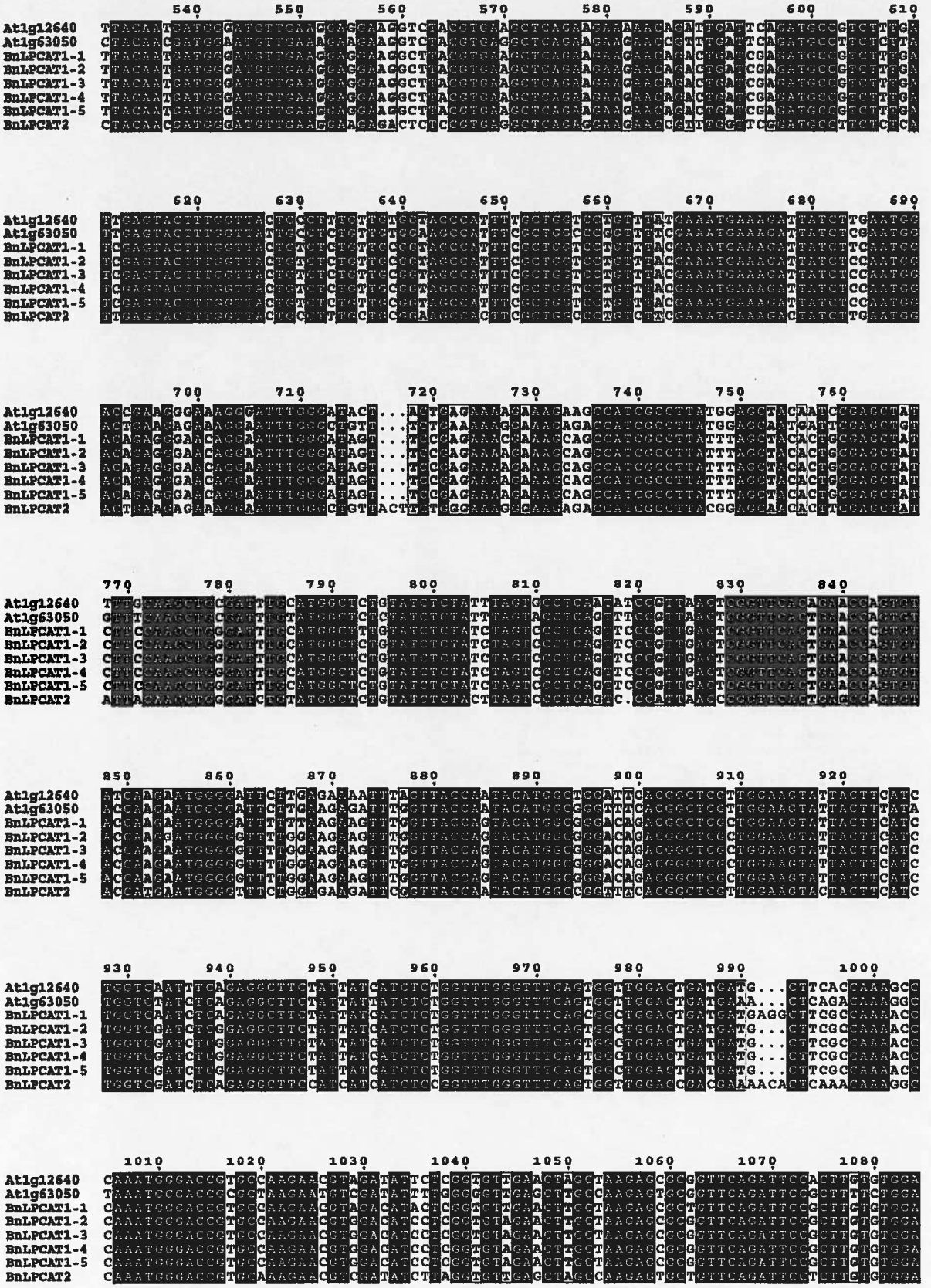
BnLPCAT1-3
BnLPCAT1-4
BnLPCAT1-5
BnLPCAT2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BnLPCAT1-2
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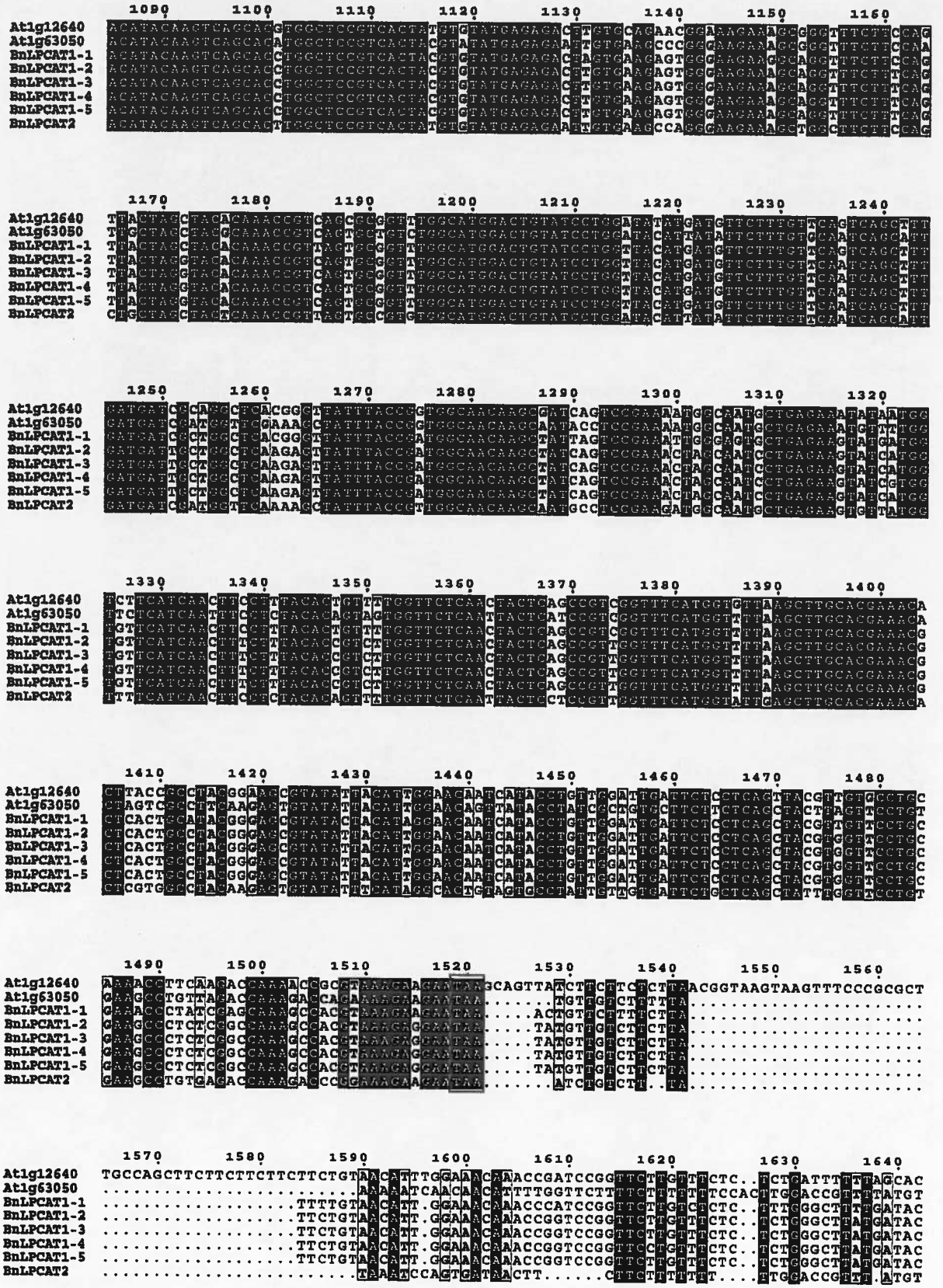
BnLPCAT1-5
BnLPCAT2


At1g12640
At1g63050
BnLPCAT1-1
BnLPCAT1-2
BnLPCAT1-3
BnLPCAT1-4
BnLPCAT1-5
BnLPCAT2

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                          1650       1660       1670       1680       1690       1700       1710
At1g12640   CGA...........TATTTTTTTTGTATTTGTTGCTTATAAATCTTATTTTTCACACTTCTTTTTTTTAATTAGTATTGG
At1g63050   AAAACAAGAGAAATCAAGATTTGAGGTTTTATT.............CTTCTTCT.........................C
BnLPCAT1-1  AGA...........TTTTTTTCC....TTTTTT.............TATTTTTAA.......................TGT
BnLPCAT1-2  AGA...........TTTTATT......TTCGTT.............TATTTTTGA.......................TGT
BnLPCAT1-3  AGA...........TTTTATT......TTCGTT.............TATTTTTGA.......................TGT
BnLPCAT1-4  AGA...........TTTTATT......TTCGTT.............TATTTTTGA.......................TGT
BnLPCAT1-5  AGA...........TTTTATT......TTCGTT.............TATTTTTGA.......................TGT
BnLPCAT2    ACACCACGG..AACTAATATTTTGGGTTTTATT.............CTTCTTC.............................

                 1720       1730       1740       1750       1760         1770       1780
At1g12640   ATTTGCAATTATATAGACAATAAGTATAAATATGTAACTGTAAATTGCAAATGGGAA..AAAATAGTAGTGTTTATGTTT
At1g63050   CTTCCCAATTTT............CGAAAATGATT..TTATTTTTTCTGATATATATCTAAGCTAGTCCAAAGTCAACTC
BnLPCAT1-1  ATTTGTGATGCT............TATAAAT......CT..TTTTTTCA..............TAGTTCTTTTTACAG..
BnLPCAT1-2  ATTTGTGATTCT............TATAAAT......CTGGGTTTTCCA..............................
BnLPCAT1-3  ATTTGTGATTCT............TATAAAT......CTGGGTTTTCCA..............................
BnLPCAT1-4  ATTTGTGATTCT............TATAAAT......CTGGGTTTTCCA..............TAGTTCTTTTT......
BnLPCAT1-5  ATTTGTGATTCT............TATAAAT......CTGGGTTTTCCA..............TAGTTCTTTTTA.....
BnLPCAT2    ..........................................................AGTTC............

            1790
At1g12640   GATGTT
At1g63050   G.....
BnLPCAT1-1  ......
BnLPCAT1-2  ......
BnLPCAT1-3  ......
BnLPCAT1-4  ......
BnLPCAT1-5  ......
BnLPCAT2    ......
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