Identification of lysophospholipid acyltransferases in the diatom *Phaeodactylum tricornutum* and characterization of the identified ones, with particular emphasis on the lysophosphatidylcholine acyltransferase

mgr Ada Połońska

Enzymes from the group of acyl-CoA:lysophospholipid acyltransferases (LPLAT) are common in plants, animals and microorganisms. They participate in remodeling the fatty acid composition of phospholipids and the cytoplasmic acyl-CoA pool by catalyzing the so-called forward and backward reactions. The forward reaction involves the attachment of an appropriate fatty acid (coming from the acyl-CoA pool) to a specific lysophospholipid, resulting in synthesis of the corresponding phospholipid. In the backward reaction, the fatty acid is detached from the phospholipid, attached to CoA and directed to the cytoplasmic pool of acyl-CoA, and the resulting lysophospholipid can be used to synthesize the appropriate phospholipid with a modified (compared to the phospholipid from which it was created) fatty acid composition.

The main aim of this research was to characterize the enzymatic reactions carried out by LPCATs (acyl-CoA:lysophosphatidylcholine acyltransferases) derived from the diatom *Phaeodactylum tricronutum*. Research on LPCATs originating from *P. tricornutum* and other microalgae is important due to current assumptions that these enzymes may be involved in the biosynthesis of very long-chain polyunsaturated fatty acids (VLC-PUFA). So far, there has been no such characterization of LPCATs from microalgae, including *P. tricornutum*.

Initial *in vitro* enzymatic assays showed that the enzyme encoded by the Phatr3_J20460 gene has a high preference for LPC, and it was named as *Pt*LPCAT1. Further search for genes from *P. tricornutum* encoding enzymes with LPCAT-type activity led to the identification of two enzymes encoded by the Phatr3_J11916 and Phatr3_J43099 genes as enzymes with LPAAT (acyl-CoA:lysophospholipid acyltransferase) activity, named *Pt*LPAAT1 and *Pt*LPAAT2, respectively. However, these studies did not lead to the identification of further genes encoding enzymes with LPCAT-type activity. The next stage of the research was the biochemical characterization and substrate specificity studies of the only identified enzyme with LPCAT activity (*Pt*LPCAT1) both in the reactions leading to the synthesis of appropriate phospholipids (forward reactions) and in the process of phospholipid remodeling (backward reactions and other types of reactions involved in the formation of LPC). Additionally, preliminary characterization of the identified enzymes *Pt*LPAAT1 and *Pt*LPAAT2 was performed. The last stage of the research was an attempt to obtain transgenic *A. thaliana* plants producing VLC-PUFA omega-3.

The obtained results of *in vitro* enzymatic assays allowed for determination of the biochemical properties of *Pt*LPCAT1. In the PC remodeling process, the highest *Pt*LPCAT1 activity was recorded at 40 °C, and in forward reactions at 30 °C. The *Pt*LPCAT1 enzyme showed the highest activity at alkaline pH (8.0 – 11.0) both in forward reactions and in the process of microsomal PC remodeling. However, the latter process also took place quite intensively in a slightly acidic environment (pH 5-6), in which forward reactions took place with just a trace intensity. Calcium and magnesium ions at concentrations of 0.05–0.5 mM stimulated forward reactions catalyzed by *Pt*LPCAT1. However, in microsomal PC remodeling reactions, the lowest tested concentrations of these ions resulted in inhibition of the reaction, and as the ion concentration increased, the reaction inhibition disappeared. The influence of potassium ions was examined only in forward reactions. In these reactions, potassium ions slightly inhibited the activity of *Pt*LPCAT1.

The substrate specificity of *Pt*LPCAT1 was investigated both in forward reactions and in the process of microsomal PC remodeling. This specificity was tested both for various fatty acid donors (acyl-CoA) and for their various acceptors (different lysophospholipids in forward reactions and different PC "species" in remodeling reactions). In most variants of the enzymatic assays, *Pt*LPCAT1 showed higher activity towards acyl-CoAs containing unsaturated fatty acids compared to those containing saturated fatty acids. However, in forward reactions in which its activity towards virtually all potential acyl-CoAs from the eicosapentaenoic acid biosynthetic pathway (EPA, $20{:}5^{\Delta5,8,11,14,17}$) was checked, not all acyl-CoAs containing unsaturated fatty acids (potential intermediates of this biosynthesis) were equally accepted. For example, high activity of *Pt*LPCAT1 in relation to 20:4-CoA n-3 and low activity in relation to 20:4-CoA n-6 was demonstrated, which suggests a better use of the former for EPA biosynthesis (results consistent with the currently proposed EPA biosynthesis pathway in *P. tricornutum*). Among the lysophospholipids used in the assays, *Pt*LPCAT1, in addition to LPC (the highest activity), was also able to acylate LPE, LPS and LPG. Of the examined LPC "species", 16:0-LPC, 18:0-LPC, and 18:1-LPC were used relatively well. However, *Pt*LPCAT1 showed trace activity towards 20:0-LPC. The activity of *Pt*LPCAT1 towards the *sn*-2 position was approximately 11 times higher than towards the *sn*-1 position. *Pt*LPCAT1 was able to remodel the fatty acid composition of PC, PE and PA of the tested microsomal fractions (obtained from yeast overexpressing *Pt*LPCAT1), however, the PC of these fractions was remodeled most intensively. The intensity of PC remodeling was influenced by both the type of fatty acid donor (in assays with acyl-CoA containing unsaturated fatty acids, the intensity was

usually higher than in their counterparts with saturated fatty acids) and the type of remodeled PC. The effect of the latter factor was examined by enriching the PC of the tested microsomal fractions with "new" PC molecules synthesized *de novo* by endogenous *Pt*LPCAT1 in forward reactions from the supplied exogenous substrates immediately before assays checking the intensity of PC remodeling of such modified microsomal fractions. In this way, microsomal fractions enriched with PC containing fatty acids from the EPA biosynthetic pathway were obtained (they constituted approximately 50% of the PC contained in such modified microsomes). Among such modified microsomal fractions, the PC of the microsomal fraction enriched with *sn*-1-18:1-*sn*-2-18:3(n-6)-PC was subject to the most intensive remodeling, followed by the PC of microsomal fractions enriched with: *sn*-1-18:1-*sn*-2-18:4(n-3)-PC, *sn*-1-18:1-*sn*-2-20:5(n-3)-PC, *sn*-1-18:1-*sn*-2-18:3(n-3)-PC and *sn*-1-18:1-*sn*-2-20:4(n-3)-PC. However, the PC remodeling intensity of all these modified microsomal fractions was higher than the PC remodeling intensity of the microsomal fraction from yeast overexpressing *Pt*LPCAT1 containing only fatty acids naturally occurring in yeast. The demonstrated substrate specificity of *Pt*LPCAT1 towards PC with various fatty acids from the EPA biosynthetic pathway does not exclude any of the "branches" of this pathway, although it suggests that its biosynthesis via 18:3(n-6) may be favored.

Substrate specificity assays of *Pt*LPAAT1 and *Pt*LPAAT2 showed that these enzymes have different preferences for different acyl-CoAs, with the *Pt*LPAAT1 enzyme best accepting 18:4-CoA n-3 and *Pt*LPAAT2 best accepting 18:1-CoA. Both enzymes, *Pt*LPAAT1 and *Pt*LPAAT2, showed the highest activity at 23 ^{o}C. However, *Pt*LPAAT1 showed relatively high sensitivity to temperature changes in the range of 10 - 60 ^{o}C. The activity of *Pt*LPAAT2 was subject to smaller fluctuations in the tested temperature range. *Pt*LPAAT1 showed the highest activity at pH 9.0 and *Pt*LPAAT2 at pH 10.0. Calcium ions inhibited the activity of both tested LPAAT-type enzymes. However, the influence of magnesium ions on their activity depended on magnesium concentration; a concentration of 0.05 mM of Mg^{+2} increased the activity of both enzymes, and concentrations of 0.5 mM and 1 mM inhibited their activity.

The transformation of *A. thaliana* plants with genes of the EPA biosynthetic pathway did not lead to obtaining transgenic plants containing fatty acids from the EPA biosynthetic pathway in the lipids of both the above-ground parts (leaves, stems, flowers, pods) and in the seeds. The cause of that remains currently unknown.