

**Molecular Analysis of Genes Involved in the Biosynthesis of  
Very Long Chain Polyunsaturated Fatty Acids (VLCPUFAs)**

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## ABSTRACT

The effective acyl flux between phospholipids and neutral lipids is critical for a high level of the biosynthesis of very long chain polyunsaturated fatty acids (VLCPUFAs) such as arachidonic acid (ARA, 20:4–5,8,11,14), eicosapentaenoic acid (EPA, 20:5–5,8,11,14,17) and docosahexaenoic acid (DHA, 22:6–4,7,10,13,16,19) which are essential for human health and wellbeing. Three membrane-bound enzymes, phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT), cholinephosphotransferase (CPT) and ethanolaminephosphotransferase (EPT) from VLCPUFA-producing fungi were selected as candidates for my thesis research based on the hypothesis that these enzymes play important roles in acyl trafficking between phosphatidylcholine (PCs) and diacylglycerols (DAGs) during the biosynthesis of VLCPUFAs. Two putative *PDCT* cDNAs (*PiPDCT1* and *PiPDCT2*) were cloned from *Phytophthora infestans* which encode polypeptides with two conserved domains and about 15% of amino acid identity to an *Arabidopsis* PDCT. However, *in vitro* assays in yeast *Saccharomyces cerevisiae* showed they did not have any PDCT activity. Four putative *CPT* and *EPT* cDNAs (*PiCPT1*, *PiCPT2*, *PiEPT* and *TaCPT*) were cloned from *P. infestans* and *Thraustochytrium aureum* which encode proteins with a conserved CDP-alcohol phosphotransferase motif and 22% to 26% of amino acid identity to the yeast CPT. *In vitro* assays indicated *PiCPT1* and *TaCPT* had CPT activity, *PiEPT* had EPT activity and *PiCPT2* did not have either activity. Substrate specificity assays showed that all the three functional CPT and EPT preferred VLCPUFA-containing DAGs as substrates with *PiCPT1* being the most specific towards ARA-DAG and DHA-DAG. Real-time qPCR analysis revealed that the expression of *PiCPT1* was up-regulated in *P. infestans* fed with exogenous VLCPUFAs. These results lead us to conclude that *PiCPT1* is a VLCPUFAs-specific CPT which may play an important role in shuffling VLCPUFAs from phospholipids to storage neutral lipids, would thus have potential use in metabolic engineering of VLCPUFAs in heterologous systems including oilseed crops.

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## List of Abbreviations

AAPT: aminoalcoholphosphotransferase

ARA: arachidonic acid

CE: crude extract

CIAP: calf intestinal alkaline phosphatase

CK: choline kinase

CPT: cholinephosphotransferase

CT: CTP/phosphocholine cytidyltransferase

DAG: diacylglycerol

DGAT: diacylglycerol acyltransferase

DHA: docosahexaenoic acid

EK: ethanolamine kinase

EPA: eicosapentaenoic acid

EPT: ethanolaminephosphotransferase

EST: expressed sequence tags

ET: CTP/phosphoethanolamine cytidyltransferase

FAMEs: fatty acid methyl esters

G3P: glycerol-3-phosphate

GLM: general linear model

GPAT: acyl-CoA: glycerol-3-phosphate acyltransferase

LPA: lyso-phosphatidic acid

LPAT: acyl-CoA: lyso-phosphatidic acid acyltransferase

LPCAT: lysophosphatidylcholine acyltransferase

MP: microsomal protein

PA: phosphatidic acid

PAP: phosphatidic acid phosphatase

PC: phosphatidylcholine

PDAT: phosphocholine diacylglycerol acyltransferase

PDCT: phosphatidylcholine: diacylglycerol cholinephosphotransferase

PE: phosphatidylethanolamine

PEMT: phosphatidylethanolamine methyl transferase

PKS: polyketide synthase

SMS: sphingomyelin synthase

TAG: triacylglycerol

TLC: thin layer chromatography

UFAs: unusual fatty acids

VLCPUFA: very long chain polyunsaturated fatty acid

## **1.0 PROJECT SURVEY**

### **1.1 Introduction**

Very long chain polyunsaturated fatty acids (VLCPUFAs) such as arachidonic acid (ARA, 20:4  $\Delta$ 5, 8, 11, 14), eicosapentaenoic acid (EPA, 20:5  $\Delta$ 5, 8, 11, 14, 17) and docosahexaenoic acid (DHA, 22:6  $\Delta$ 4, 7, 10, 13, 16, 19) play important roles in the maintenance of human health (Benatti *et al.*, 2004; Nettleton and Katz, 2005). Plant oils with a high level of VLCPUFAs have been proposed to be a potential alternative source as dietary supplements of VLCPUFAs. Detections of DHA and EPA in oilseed plants with the heterologous expression of desaturase and elongase from VLCPUFA-producing microorganisms in oilseed plants have proven the concept (Abbadi *et al.*, 2004; Wu *et al.*, 2005). However, the yield of VLCPUFAs in transgenics is low and a certain amount of VLCPUFAs remain in phospholipids such as phosphatidylcholine (PC) other than triacylglycerol (TAG). This suggests that additional factors involving the transfer of VLCPUFAs between polar lipids and neutral lipids might be required for a high level of VLCPUFA production in plant. This bottleneck triggered me to seek effective enzymes that can specifically channel VLCPUFAs between PC and diacylglycerol (DAG). Three alcoholphosphotransferases are selected as targeted enzymes based on following observations: 1) Phosphatidylcholine: diacylglycerol cholinephosphotransferase (PDCT), a membrane-associated enzyme, involves transferring of polyunsaturated fatty acids (PUFAs) from PC to DAG, a precursor for TAG (Lu *et al.*, 2009). PDCT can be a promising candidate to improve the flux of VLCPUFAs from PC to DAG. 2) Cholinephosphotransferase (CPT) converts DAG and CDP-choline into PC, which is the dominant substrate for most of PUFA desaturases. VLCPUFAs-specific CPT should add values to the movement of acyl-group from DAG to PC, maximizing the PUFA desaturation. 3) Ethanolaminephosphotransferase (EPT) is an enzyme synthesizing PE from DAG and CDP-ethanolamine, but it often has CPT activity.

### **1.2 Hypothesis and objectives**

I hypothesize PDCT, CPT and EPT from VLCPUFA-producing microorganisms play important roles in VLCPUFA trafficking between phospholipids and neutral lipids,

thereby will affect the synthesis and accumulation of VLCPUFAs in oleaginous eukaryotes. Therefore, molecular cloning and functional characterization of *PDCT*, *CPT* and *EPT* genes from VLCPUFA-producing microbes would benefit our understanding of the biosynthesis of VLCPUFAs and our ability to engineer these fatty acids in plants.

The objectives of this study are:

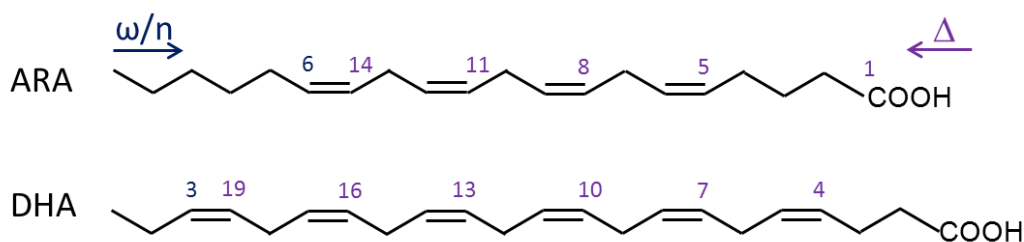
- 1) Molecular cloning of *PDCT*, *CPT* and *EPT* genes from VLCPUFA-producing microorganisms.
- 2) Functional analysis of *PDCT*, *CPT* and *EPT* genes in yeast.

## 2.0 LITERATURE REVIEW

### 2.1 An introduction of VLCPUFAs

Very long chain polyunsaturated fatty acids (VLCPUFAs) are defined as fatty acids with 20 or more carbons and two or more double bonds. They are classified into two families,  $\omega$ 6 and  $\omega$ 3 fatty acids, according to the positions of the last double bond towards the methyl end (Figure 1) (Abadi *et al.*, 2004). VLCPUFAs are important structural components of membrane phospholipids and precursors to eicosanoids and docosanoids, which are hormone-like bioactive compounds that help to maintain cell homeostasis (Tapiero *et al.*, 2002; Uauy-Dagach and Valenzuela, 1992). It has also been reported that VLCPUFAs play very important roles in brain development and in the prevention of cardiovascular and immunological diseases (Benatti *et al.*, 2004; Crawford *et al.*, 1997).

Among the VLCPUFAs, arachidonic acid (ARA, 20:4-5,8,11,14), eicosapentaenoic acid (EPA, 20:5-5,8,11,14,17) and docosahexaenoic acid (DHA, 22:6-4,7,10,13,16,19) attracted more attention from researchers than others because of their appealing benefits to human health. ARA, an  $\omega$ 6 VLCPUFA, has been recognized for many years for its ability to enhance the visual and cognitive abilities of infants (Hoffman *et al.*, 2009). In human body, ARA is found to be largely deposited in the brain as well as other growing tissues. It is also present in breast milk and contributes to the biosynthesis of eicosanoids in infants (Koletzko *et al.*, 1996). EPA, an  $\omega$ 3 VLCPUFA, shows anti-inflammatory and anticachectic properties and prevents many human ailments such as cardiovascular disease and cancer (Babcock *et al.*, 2000; Beck *et al.*, 1991). DHA is another important  $\omega$ 3 VLCPUFA that extensively involves in the development of the brain and retina where a high level of DHA is accumulated (Koletzko, 1992; SanGiovanni *et al.*, 2000) and its health benefits have been recognized for several decades. Clinical research has shown that DHA can decrease fatty acid synthesis in the liver, increase red blood cell membrane fluidity and reduce blood pressure (Ikeda *et al.*, 1998). Recently, it is discovered that DHA can protect neural cells from stress-induced apoptosis and possess anticancer properties (Gleissman *et al.*, 2010).



$\omega$ 6 family	Nomenclature	Double bond location
Dihomo- $\gamma$ -linolenic acid (DGLA)	20:3n-6	$\Delta$ 8,11,14
Arachidonic acid (ARA)	20:4n-6	$\Delta$ 5,8,11,14
$\omega$ 3 family	Nomenclature	Double bond location
Eicosatetraenoic acid (ETA)	20:4n-3	$\Delta$ 8,11,14,17
Eicosapentaenoic acid (EPA)	20:5n-3	$\Delta$ 5,8,11,14,17
Docosapentaenoic acid (DPA)	22:5n-3	$\Delta$ 7,10,13,16,19
Docosahexaenoic acid (DHA)	22:6n-3	$\Delta$ 4,7,10,13,16,19

**Figure 1.** Nomenclature of VLCPUFAs (Adapted from Truksa *et al.*, 2009).  $\Delta$ -designation, carbon numbering starts from carboxyl group;  $\omega$ /n-designation, carbon numbering starts from methyl end.  $\omega$ 3 family, fatty acids with the first double bond located three carbons from the methyl end;  $\omega$ 6 family, fatty acids with the first double bond located six carbons from the methyl end. 20:3n-6, a fatty acid contains 20 carbons with 3 double bonds at position 8, 11, 14 counting from the carboxyl end. The first double bond is located at position 6 from the methyl end.

Although VLCPUFAs, especially ARA, EPA and DHA are clearly beneficial to human health, human beings cannot *de novo* synthesize VLCPUFAs due to the absence of two important enzymes,  $\Delta 12$  desaturase and  $\Delta 15$  desaturase (Ruiz-Lopez *et al.*, 2012). The  $\Delta 12$  desaturase introduces a double bond at the 12<sup>th</sup> carbon from the carboxyl end of oleic acid (OA, 18:1  $\Delta 9$ ) to form linoleic acid (LA, 18:2  $\Delta 9,12$ ), while  $\Delta 15$  desaturase is responsible for the insertion of a double bond at the 15<sup>th</sup> carbon of LA to form  $\alpha$ -linolenic acid (ALA, 18:3  $\Delta 9,12,15$ ) (Figure 2). Therefore, LA and ALA are defined as the dietary essential fatty acids (EFAs), which are precursors for VLCPUFAs. Due to a limited capacity to convert two EFAs to their corresponding VLCPUFAs in humans, humans are encouraged to be supplemented with these VLCPUFAs through diets (Ganapathy, 2009; Hornung *et al.*, 2005). The major organisms accumulating VLCPUFAs are marine microorganisms (Domergue *et al.*, 2005b). Because the aquatic food chain leads to these fatty acids eventually being accumulated in fish oil, fish becomes our primary dietary source of VLCPUFAs for human nutrition (Domergue *et al.*, 2005a). However, this source of VLCPUFAs is not sustainable due to recently dramatic dwindling of wild fish stock in oceans.

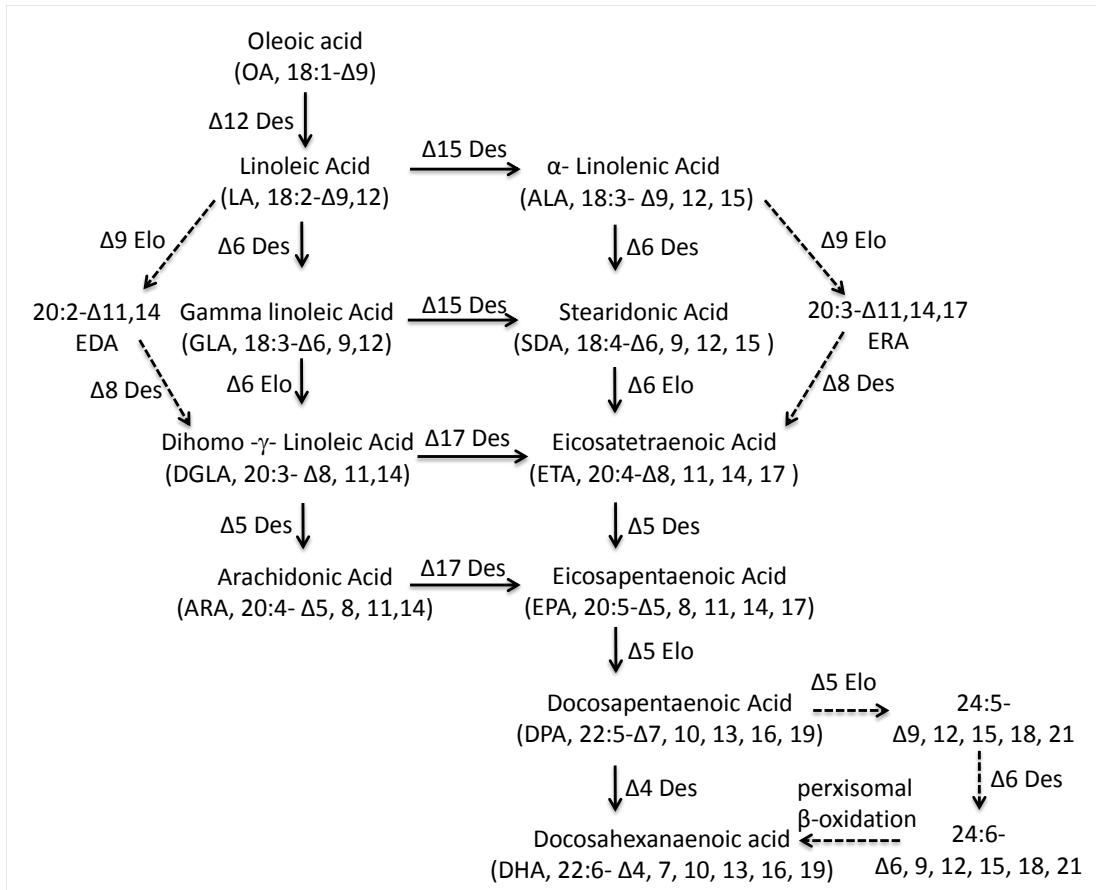
## **2.2 Biosynthesis of VLCPUFAs in microorganisms**

### **2.2.1 VLCPUFA-biosynthetic pathways**

There are two predominate pathways to synthesize VLCPUFAs in nature. The first pathway involves alternating series of desaturation and elongation, known as the aerobic pathway, which widely occurs in animals and most eukaryotic microorganisms. The second pathway, known as the anaerobic polyketide synthase (PKS) pathway, is only found in bacteria and lower eukaryotes (Damude and Kinney, 2007; Qiu, 2003).

As shown in Figure 2, the aerobic biosynthesis of VLCPUFAs is accomplished with alternating reactions of desaturation, introducing double bonds to fatty acid chain, and elongation, condensing a malonyl-CoA and the fatty acyl moiety to lengthen two carbon units. In the conventional  $\Delta 6$  pathway,  $\omega 6$ -VLCPUFA, ARA, and  $\omega 3$ -VLCPUFA, EPA, are synthesized by utilizing LA and ALA as precursor substrates, respectively.





**Figure 2.** The aerobic biosynthetic pathway of VLCPUFAs (Adapted from Damude and Kinney, 2008). The  $\Delta$ 6 pathway is shown with a  $\Delta$ 6 desaturase ( $\Delta$ 6 Des) and  $\Delta$ 6 elongase ( $\Delta$ 6 Elo) and the  $\Delta$ 8 pathway is shown with a  $\Delta$ 9 elongase ( $\Delta$ 9 Elo) and a  $\Delta$ 8 desaturase ( $\Delta$ 8 Des). Both pathways use a  $\Delta$ 5 desaturase ( $\Delta$ 5 Des). All pathway enzymes use both  $\omega$ 6 (LA, EDA, GLA, DGLA, ARA) and  $\omega$ 3 fatty acid substrates (ALA, ERA, SDA, ETA, EPA and DPA), respectively. Conversion of 18-carbon and 20-carbon  $\omega$ 6 fatty acids to  $\omega$ 3 fatty acids is catalyzed by the  $\Delta$ 15 desaturase ( $\Delta$ 15 Des) and  $\Delta$ 17 desaturase ( $\Delta$ 17 Des), respectively. Elongation of EPA is catalyzed by the  $\Delta$ 5 elongase ( $\Delta$ 5 Elo) and further  $\Delta$ 4 desaturation by the  $\Delta$ 4 desaturase ( $\Delta$ 4 Des). In the Sprecher pathway, elongation of DPA to tetracosahexaenoic acid (24:5) is shown catalyzed by a  $\Delta$ 5 elongase ( $\Delta$ 5 Elo) and desaturation occurs by the  $\Delta$ 6 desaturase ( $\Delta$ 6 Des). Conversion of tetracosahexaenoic acid to DHA by the Sprecher pathway occurs in the peroxisome.

ARA synthesis starts with a  $\Delta 6$  desaturation of LA into  $\gamma$ -linoleic acid (GLA, 18:3), followed by a  $\Delta 6$ -specific C2 elongation of GLA into dihomo  $\gamma$ -linoleic acid (DGLA, 20:3), and ends with  $\Delta 5$  desaturation of DGLA into ARA. Alternatively, ARA can also be synthesized from a  $\Delta 8$  pathway with sequential enzymatic reactions of  $\Delta 9$  elongase,  $\Delta 8$  desaturase and  $\Delta 5$  desaturase. Similarly, ALA is converted to EPA through  $\Delta 6$  or  $\Delta 8$  biosynthetic pathways, as in the ARA biosynthesis. Besides, EPA can be converted from ARA with  $\omega 3$  desaturase, also known as  $\Delta 17$  desaturase. With the availability of EPA, DHA can be synthesized in two different ways in eukaryotes. In a  $\Delta 4$  desaturation dependent pathway (microbial  $\Delta 4$  pathway),  $\Delta 5$  elongase and  $\Delta 4$  desaturase are required to convert EPA to DHA. This pathway generally occurs in certain VLCPUFA-producing microorganisms, such as *Thraustochytrium* (Qiu *et al.*, 2001). Whereas,  $\Delta 4$  desaturation independent pathway found in mammals, involves retro-conversion of a 24-carbon  $\Delta 6$  fatty acid via a peroxisomal  $\beta$ -oxidation (Qiu, 2003). Besides, an alternative DHA biosynthesis reaction catalyzed by PKS pathway instead of alternating desaturation and elongation steps has been reported in *Schizochytrium sp.* (Metz *et al.*, 2001).

Among various VLCPUFA-biosynthetic pathways mentioned above, the pathway involving alternation of desaturation and elongation has attracted more research attention than others. Genes involved in these steps have been cloned from many different species (Napier, 2007), making profound enzymatic studies of desaturases and elongases possible. Desaturases are oxygen dependent and contain a cytochrome b5 fusion domain at their N-terminus for receiving the electrons (Napier *et al.*, 1999; Sperling and Heinz, 2001). On the other hand, the elongation requires an elongase complex which consists of four discrete enzymatic subunits including a condensing enzyme, so called elongase, a ketoacyl-CoA reductase, a hydroxyl acyl-CoA dehydratase and an enoyl-reductase (Leonard *et al.*, 2004). Although some indirect evidences suggest that the rate-limiting step in elongation reaction is the activity of condensing enzyme (elongase) rather than other three enzymes, the detailed mechanism still remains to be elucidated (Leonard *et al.*, 2004). In terms of their substrate specificity, it is generally believed that the fatty acid desaturases in mammals prefer to use acyl-CoAs as substrates, while the fatty acid

desaturases from fungus tend to use PC-linked substrates. Unlike desaturation, elongation reaction occurs in the acyl-CoA pool.

### **2.2.2 VLCPUFA-producing microorganisms**

Various microorganisms such as fungus, marine protists, micro-algae and moss can effectively synthesize and accumulate VLCPUFAs so that these microbes are termed as VLCPUFA-producing microorganisms. A large number of desaturase and elongase genes from these microorganisms have been cloned and utilized for producing VLCPUFAs in yeast and plants.

#### **2.2.2.1 Fungi**

Many fungi are capable of producing VLCPUFAs. The filamentous fungus *Mertierella alpina* is commercially utilized in production of VLCPUFAs, since it can produce up to 50% of its biomass as TAG rich in ARA (Sakuradani, 2010; Zeng *et al.*, 2011). This fungus has been used as an important VLCPUFA gene resource. Genes encoding  $\Delta 5$ ,  $\Delta 6$  and  $\Delta 12$  desaturases have been isolated from this organism and utilized in the reconstitution of VLCPUFAs biosynthesis (Knutzon *et al.*, 1998; Nykiforuk *et al.*, 2012). *Conidiobolus* species are another group of fungi can accumulate high amount of ARA in TAG and phospholipids (Miura *et al.*, 1983).  $\Delta 6$ -elongase gene from *Conidiobolus thromboides* was utilized for the over-expression in yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) resulting in the production of DGLA, an elongated product of substrate GLA (Tan *et al.*, 2011). In addition, *Phytophthora infestans* which is a potato pathogenic fungus, can produce a considerable amount of EPA (Bostock *et al.*, 1981). Fungus *Entomophthora obscura* has also been reported to accumulate 24% of its total lipids as DHA (Yongmanitchai and Ward, 1989).

#### **2.2.2.2 Marine protists**

*Thraustochytrids* is a group of marine protists which are capable of heterotrophic growth and are taxonomically aligned with heterokont algae (Bowles *et al.*, 1999). Many

species in this family have been reported to produce a significant amount of DHA. The study of *Thraustochytrium sp.* ATCC 26185 revealed that the cells from liquid culture contain 32% of lipids, and up to 25% of which is DHA (Weete *et al.*, 1997). With a optimization of culture conditions, *Thraustochytrium roseum* ATCC 28210 could produce up to 52% DHA in its TAGs (Singh and Ward, 1996). In *Thraustochytrium sp.* 26185, DHA was reported to make up 32% of the total fatty acids (Weete *et al.*, 1997). *Thraustochytrium sp.* KK17-3 could produce 52.1% of DHA, while *Thraustochytrium sp.*, KH154 and KH155 produced even higher amounts of DHA, representing 58.7% and 59.7%, respectively (Huang *et al.*, 2001).

The sterling accumulation of DHA in *Thraustochytrids* triggered the discovery of two different DHA *de novo* biosynthesis pathway, alternating desaturation/elongation pathway and PSK pathway (Qiu, 2003). The first  $\Delta 4$  fatty acid desaturase was cloned from *Thraustochytrium sp.* 26185 as a vital enzyme for DHA synthesis. The identification of this enzyme elucidates the mechanism underlying the biosynthesis of DHA through the  $\Delta 4$  desaturation in *Thraustochytrium*. This discovery also resolves the controversial  $\Delta 4$  desaturase pathway and opens up the possibility of DHA productions in transgenic plants using this simple pathway (Qiu *et al.*, 2001).

### **2.2.2.3 Microalgae**

$\omega$ -3 VLCPUFAs have been found in various microalgae, such as *Monodus subterraneus* (Liu and Lin, 2005), *Phaedactylum tricornutum* (Yongmanitchai and Ward, 1991) and *Porphuridium cruentum* (Cohen and McLeod, 1988) producing a high level of EPA, and *Isochrysis galbana* (Boussiba *et al.*, 1988) producing a high level of DHA. In microalgae *Glossomastix chrysoplata*, the EPA content represents up to 46% of the total fatty acids (Hsiao *et al.*, 2007). An *Isochrysis galbana*  $\Delta 9$  elongase involved in the  $\Delta 8$  pathway has been identified and utilized in the reconstitution of VLCPUFA synthesis in plants (Qi *et al.*, 2004).

*Thalassiosira pseudonana* is another microalgae, which could accumulate 16.7% and 4.5% of EPA and DHA, respectively at the exponential growth phase (Tonon *et al.*, 2002). Several VLCPUFA enzymes were identified from this algae including two C20 VLCPUFA-specific elongases (Meyer *et al.*, 2004),  $\Delta 6$ ,  $\Delta 5$  and  $\Delta 4$  desaturases (Tonon *et al.*, 2005b). In addition, a VLCPUFA acyl-coenzyme A synthetase was also cloned recently from *T. pseudonana* (Tonon *et al.*, 2005a).

#### **2.2.2.4 Mosses**

The moss *Physcomitrella patens* contains high proportions of ARA and some EPA along with other 20-carbons unsaturated fatty acids (Grimsley *et al.*, 1981), which are synthesized by the desaturation/elongation pathway. Recently, a  $\Delta 6$  desaturase and a  $\Delta 6$  elongase (PSE1) have been isolated from this organism (Girke *et al.*, 1998; Zank *et al.*, 2002).

### **2.3 Metabolic engineering of VLCPUFA biosynthesis**

#### **2.3.1 Reconstitution of VLCPUFA synthetic pathways in plants**

Although progresses are made in commercially scaled up VLCPUFA production in some of the abovementioned microorganisms, attempts of producing VLCPUFAs in oilseed plants through metabolic engineering become an alternative approach to meet the market needs for VLCPUFAs. The first “proof-of-concept” study resulted in the accumulation of EPA and ARA to 3 and 6.6% of the total fatty acids in leaf tissues via overexpression of three enzymes (*Isochrysis galbana*  $\Delta 9$  elongase, a *Euglena gracilis*  $\Delta 8$  desaturase and a *Mortierella. alpina*  $\Delta 5$  desaturase) in *Arabidopsis thaliana* (*A. thaliana*) (Qi *et al.*, 2004). However, the attempt to produce C20 in seed oil was less promising that only a small amount of C20 fatty acids were observed when  $\Delta 9$ -elongase was constitutively expressed in *A. thaliana* (Fraser *et al.*, 2004). Later, seed-specific expression of  $\Delta 5$ ,  $\Delta 6$  desaturases from diatom *Phaeodactylum tricorutum* and a  $\Delta 6$  elongase from moss *Physcomitrella patens* in tobacco and linseed resulted in a low accumulation of EPA (1.6%) and ARA (2.7%) in the seed oil, but a high level of  $\Delta 6$  desaturated C18 fatty acids such as GLA and stearidonic acid (SDA, 18:4) (totally 33%)

(Abbadì *et al.*, 2004). Using the similar approach, Kinney *et al.*, (2004) reconstituted the EPA pathway in soybean using five different enzymes, a  $\Delta 6$  desaturase, a  $\Delta 6$  elongase and a  $\Delta 5$  desaturase from *M. alpina*, a  $\Delta 5$  desaturase from *Arabidopsis* and a  $\Delta 17$  desaturase from *Saprolegnia diclina*. Transgenic somatic embryos accumulated 19.6% EPA. In addition, they also first reconstituted the DHA pathway using an additional  $\Delta 4$  desaturase from *Schizochytrium aggregatum* and  $\Delta 5$  elongase from *Pavlova salina*. Transgenic somatic embryos produced a low level of DHA (2.0-3.3% of total fatty acids).

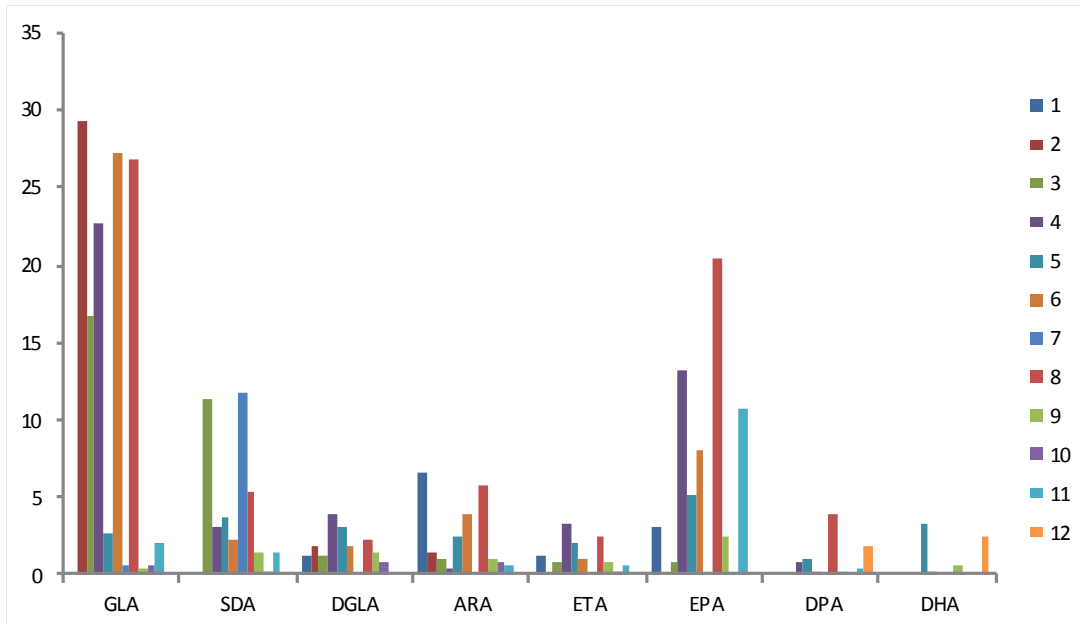
The entire DHA biosynthetic pathway was also reconstituted in canola *Brassica juncea* by stepwise metabolic engineering. Transgenic plants produced up to 25% ARA and 15% EPA, as well as up to 1.5% DHA in seeds (Wu *et al.*, 2005). Another attempt to produce DHA in plants made by Robert *et al.* (2005) introduced four genes in *Arabidopsis*, which resulted in production of 0.2-0.5% of DHA in seeds.

Researchers have been focusing on the increase of VLCPUFAs in transgenic plants for many years but the achievement was little. Recently, Kajikawa *et al.* (2008) co-expressed a  $\Delta 6$ -desaturase, a  $\Delta 6$ -elongase and a  $\Delta 5$ -desaturase from *Marchantia polymorpha* in tobacco, which resulted in an accumulation of 15.5% ARA and 4.9% EPA in transgenics (Kajikawa *et al.*, 2008). Hoffmann *et al.* (2008) reconstituted the EPA pathway in *Arabidopsis* using two acyl-CoA desaturases from *Mantoniella squamata* and an elongase from *Physcomitrella patens*, however the yield of EPA in transgenic T2 seeds reached only 0.45% on average. Cheng *et al.* (2009) co-expressed a 18C  $\omega 3$  desaturase from *Claviceps purpurea* and a 20C  $\omega 3$  desaturase from *Pythium irregular* along with a  $\Delta 6$  desaturase, a  $\Delta 6$  elongase and a  $\Delta 5$  elongase in zero-erucic acid *Brassica carinata* (Cheng *et al.*, 2009). Transgenic plants produced up to 25% of EPA in seeds. A diagrammatic summary of the transgenic results obtained from some of these studies is shown in Table 1 and Figure 3.

In summary, although the biosynthesis of VLCPUFAs has been successfully reconstituted in plants, the level of these fatty acids, particularly for DHA, is still low. Therefore, to further increase the production of these fatty acids in plants, additional

**Table 1.** Comparative analysis of VLCPUFA accumulation in transgenic plants from various studies. The different pathway or strategy, plant speices and tissues used are indicated. For clarity, VLCPUFAs accumulation in different transgenic plants in each study is shown in Figure 3.

Strategy	Reference	Plant species	Tissue	No.
$\Delta 8$ alternative pathway	Qi <i>et al.</i> (2004)	<i>Arabidopsis thaliana</i>	Leaves	1
	Abbadi <i>et al.</i> (2004)	<i>Nicotiana tabacum</i>	Seed	2
$\Delta 6$ conventional pathway	Abbadi <i>et al.</i> (2004)	<i>Linum usitatissimum</i>	Seed	3
	Kinney <i>et al.</i> (2004)	<i>Glycine max</i>	Embryo	4
	Kinney <i>et al.</i> (2004)	<i>Glycine max</i>	Embryo	5
	Wu <i>et al.</i> (2005)	<i>Brassica juncea</i>	Seed	6
	Ruiz-Lopez <i>et al.</i> (2009)	<i>Linum usitatissimum</i>	Seed	7
	Cheng <i>et al.</i> (2010)	<i>Brassica carinata</i>	Seed	8
acyl-CoA desaturase	Robert <i>et al.</i> (2005)	<i>Arabidopsis thaliana</i>	Seed	9
	Hoffmann <i>et al.</i> (2008)	<i>Arabidopsis thaliana</i>	Seed	10
	Petrie <i>et al.</i> (2009)	<i>Nicotiana benthamiana</i>	Leaves	11
PKS system	Metz <i>et al.</i> (2006)	<i>Arabidopsis thaliana</i>	Seed	12



**Figure 3.** Comparative analysis of VLCPUFA accumulation in transgenic plants from various studies (Adapted from Venegas-Caleron *et al.*, 2010). Overview of fatty acid composition in published transgenic lines is indicated. The study according to each number is shown in Table 1. The studies compared are: 1) Qi *et al.* (2004); 2,3) Abbadi *et al.* (2004); 4, 5) Kinney *et al.* (2004); 6) Wu *et al.* (2005); 7) Ruiz-Lopez *et al.* (2009); 8) Cheng *et al.* (2010); 9) Robert *et al.* (2005); 10) Hoffmann *et al.* (2008); 11) Petrie *et al.* (2009); 12) Metz *et al.* (2006).



factors besides desaturases and elongases need to be considered and identified in the biosynthesis and accumulation of VLCPUFAs.

### **2.3.2 Bottlenecks and potential approaches for increasing VLCPUFA biosynthesis**

As mentioned above, the yield of some VLCPUFAs in transgenic plants is still low for the viable commercialization, although there have been several successful cases reporting the production of VLCPUFAs in oilseed crops (Figure 3). The underlying reasons could be complicated since reconstitution of VLCPUFAs synthesis and channelling of these fatty acids to TAGs involve many different enzymes. These enzymes can be located in different domains of an organelle or in different organelles. The understanding of bottlenecks in the reconstituted pathway is paramount to guide producing high level VLCPUFAs in transgenic oilseeds.

The first bottleneck in the biosynthesis of VLCPUFAs in plants is the substrate dichotomy, which results from the difference of acyl-substrate requirement between two key enzymes, fatty acid desaturase and elongase in VLCPUFA biosynthesis (Napier *et al.*, 2004). It is believed that most PUFA desaturases prefer acyl-PC as their dominant substrate, while elongases can only use acyl-CoAs as their substrates (Domergue *et al.*, 2003). To apprehend the underlying mechanism causing the low accumulation of C20 fatty acids in transgenic studies, a detail metabolic analysis showed that after desaturation on PC,  $\Delta 6$  desaturated C18 fatty acids were effectively channeled to TAGs rather than to the acyl-CoA pool and the absence of  $\Delta 6$  desaturated acyl-CoA substrate for further elongation resulted in the limited synthesis of elongated C20 fatty acids (Abadi *et al.*, 2004). The inefficiency of acyl exchanges between PCs and acyl-CoA pool could limit the sequential elongation or desaturation and has a significant influence on the yield of VLCPUFAs. In transgenic oilseed plants with a reconstituted  $\Delta 6$  pathway of VLCPUFAs, a high level of  $\Delta 6$  desaturated products, GLA or SDA, found in PCs or TAGs rather than in the acyl-CoA pool implied that the elongation step was limited due to absence of substrates (Abadi *et al.*, 2004; Cheng *et al.*, 2009; Domergue *et al.*, 2003; Wu *et al.*, 2005). On the other hand, the reconstitution of a  $\Delta 8$  alternative pathway of VLCPUFAs

showed that a significant amount of elongated C20 fatty acid products (20:2 and 20:3) were found in the acyl-CoA pool of transgenic *Arabidopsis* lines (Sayanova *et al.*, 2006). All together it is evident that acyl exchange between PCs and acyl-CoAs is the major limiting step in the biosynthesis of VLCPUFAs in transgenic plant.

Utilization of acyl-CoA dependent desaturases is believed as one of effective approaches to overcome this bottleneck (Graham *et al.*, 2007). An acyl-CoA desaturase that can use acyl-CoA as substrate has recently been identified in microalgae, *Ostreococcus tauri* (Domergue *et al.*, 2005b). Co-expression of this desaturase with  $\Delta 6$  elongase and  $\Delta 5$  desaturase in yeast resulted in production of 4.5% of ARA and 4.7% of EPA in yeast transformants, which was 20 times higher than their previous experiments using PC-preferred  $\Delta 6$  desaturase (Domergue *et al.*, 2005b). A similar acyl-CoA dependent  $\Delta 6$  desaturase was identified from *Micromonas pusilla* and co-expression of this desaturase with  $\Delta 6$  elongase and  $\Delta 5$  desaturase resulted in accumulation of 26% EPA in leaves of *Nicotiana benthamiana* (Petrie *et al.*, 2009). However, even with the introduction of acyl-CoA dependent  $\Delta 6$  desaturase, the production of VLCPUFAs in transgenics was still limited.

The other proposed approach to overcome this bottleneck is to identify new acyl-exchange mechanics. Lysophosphatidylcholine acyltransferase (LPCAT) has been viewed as a potential target for this purpose, since it can not only catalyze the fatty acid incorporation into PC from acyl-CoA, but also remove fatty acids from the sn-2 position of PC and release them to an acyl-CoA in its reverse reaction (Domergue *et al.*, 2005a).

Another bottleneck in the VLCPUFA biosynthetic pathway is the channelling of VLCPUFAs from the acyl-PC and acyl-CoA pools to DAGs and TAGs. Ideally, all the forms of VLCPUFAs such as VLCPUFA-CoA and VLCPUFA-PC should be efficiently used for TAG synthesis. However, the fact that a certain amount of VLCPUFAs remains in the PCs and other phospholipid classes (Abbadì *et al.*, 2004; Wu *et al.*, 2005) suggests that the VLCPUFA flux from PC to TAG might be also important in VLCPUFAs production in transgenics.

A possible solution to this might be the use of TAG biosynthetic enzymes such as glycerol-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAT) and diacylglycerol acyltransferase (DGAT) from VLCPUFA-producing microorganisms in transgenic plants which have a substrate preference towards VLCPUFAs (Vogel and Browse, 1996). These acyltransferase activities are generally acyl-CoA-dependent, meaning the VLCPUFA substrate must be present in the acyl-CoA form. An alternative solution is the use of acyl-CoA-independent enzyme such as phospholipid: diacylglycerol acyltransferase (PDAT) (Dahlqvist *et al.*, 2000; Stahl *et al.*, 2004). Moreover, PC biosynthesis and the acyl-trafficking between polar and neutral lipid classes could also be important aspects in biosynthesis and accumulation of VLCPUFAs. Identification of a PDCT with substrate preference to VLCPUFAs for the acyl-CoA independent TAG biosynthesis might be worth pursuing. Furthermore, two major enzymes in PC biosynthesis, CPT and EPT, involve in the synthesis of PC from DAG in the forward reaction and also the movement of acyl-group moiety from PC to DAG and further to TAG in the reverse reaction (Stymne and Stobart, 1984), might play a role in the VLCPUFAs biosynthesis and accumulation.

#### **2.4 Importance of PDCT, CPT and EPT in TAG biosynthesis**

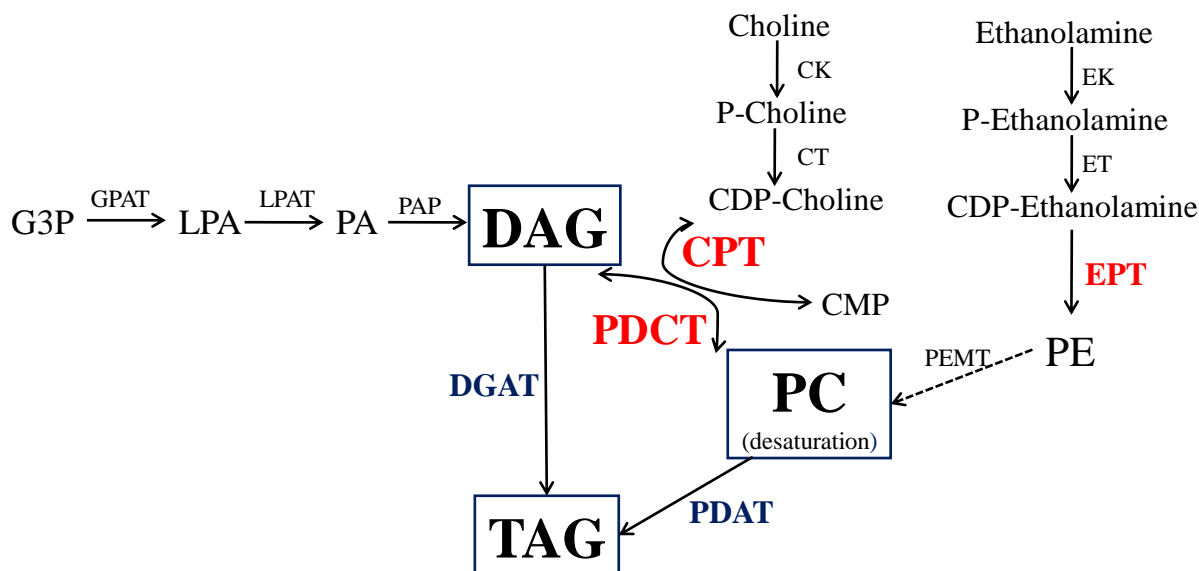
Analysis of acyl fluxes through multiple pathways of TAG synthesis revealed that DAG used for TAG synthesis is mostly derived from PC, while *de novo* DAG is mostly used for *de novo* PC synthesis (Bates *et al.*, 2009). Therefore, the fatty acid composition in transgenic seeds depends on the ratio of *de novo* DAG to PC-derived DAG. It further depends on the efficient interconversion of DAG and PC for both PC synthesis and derivation of DAG from PC. CPT and EPT play such a role in this interconversion between DAG and PC.

It is revealed that acyl-lipid metabolism in *Arabidopsis* requires more than 120 enzymatic reactions and more than 600 genes which encode the proteins and regulatory factors (Li-Beisson *et al.*, 2010). To understand the accumulation of unusual fatty acid (UFAs) in TAGs in transgenic plants, it is essential to know the difference between the

host plants and natural unusual FAs-producing plants as well as the enzymes involved in the TAG biosynthesis.

Firstly, it is notable that there are some differences in the metabolic pathways affecting the accumulation of UFAs in TAGs between natural plants and transgenic plants. For example, ricinoleate (hydroxyl fatty acid, HFA, 18:1-OH) can accumulate up to 90% of FAs in TAG in castor seed and over 70% of seed TAG contain three ricinoleates, but there is only about 5% of ricinoleate in total FAs in PC (Lin *et al.*, 2003). However, when the hydroxylase gene identified from castor bean (*Ricinus communis*), *RcFAH12*, was expressed in transgenic *Arabidopsis*, HFAs accumulated up to 17% in the mature seed TAG and 12% in developing seed PC. In addition, half of the TAGs contain only a single HFA, over 70% of which is located at in the sn-2 position (van Erp *et al.*, 2011).

Secondly, in TAG synthesis, DGAT catalyzes the terminal and only committed step, by using DAG and fatty acyl CoA as substrates (Cases *et al.*, 1998). Therefore, DGAT is a key enzyme and DAG is a crucial intermediate to synthesize TAG. There are two main pathways to produce DAG (Figure 4). The first pathway is *de novo* synthesis of DAG, called Kennedy pathway. It involves the sequential action of GPAT and LPAT to esterify FA onto the sn-1 and sn-2 positions of G3P producing LPA, PA respectively. Phosphate group on PA is finally removed by PAP to produce *de novo* DAG. DAG can also be synthesized through PC by the removal of phosphocholine group on sn-3 position of PC. However, the last step of *de novo* PC synthesis involves the utilization of *de novo* DAG as substrate and catalytic reaction of CPT and EPT. Because PC is the site of fatty acid desaturation, the fatty acids (FAs) esterified with *de novo* DAG are different molecular species from those with PC-derived DAG. Thus, the production of DAG through PC requires *de novo* DAG synthesis first, and conversion to PC, after desaturation, then conversion of PC back to DAG. PC-derived DAG can be produced either by the reverse action of the enzymes CPT or the catalysis of PDCT or phospholipase C.



**Figure 4.** Interconnection of phospholipid and neutral lipid biosynthesis. TAG biosynthesis follows Kennedy pathway, starting from G3P (Kennedy and Weiss, 1956). Biosyntheses of phospholipids, PC and PE, are catalyzed by three enzymes, CK/EK, CT/ET and CPT/EPT. G3P, glycerol-3-phosphate; LPA, lyso- phosphatidic acid; PA, phosphatidic acid; GPAT, acyl-CoA:glycerol-3-phosphate acyltransferase; LPAT, acyl-CoA: lyso-phosphatidic acid acyltransferase; PAP, phosphatidic acid phosphatase; DGAT, diacylglycerol acyltransferase; CK, Choline kinase; CT, CTP/phosphocholine cytidyltransferase; EK, ethanolamine kinase; ET, CTP/phosphoethanolamine cytidyltransferase; PEMT, phosphatidylethanolamine methyl transferase; PDAT, phosphocholine diacylglycerol acyltransferase

It has been proven that the engineering of oilseed plants to accumulate UFAs in seed oil (TAG) requires not only the biosynthetic enzymes which can catalyze the synthesis of UFAs but also efficient pathways for the flux of UFAs to TAG in the host plants. Recent metabolic labeling experiments demonstrated that the relative flux of *de novo* DAG transfer into PC is over 14 times the rate of the direct conversion from *de novo* DAG to TAG in wild type *Arabidopsis* and the major bottleneck for the accumulation of UFAs in TAG is the flux of DAG to PC (Bates and Browse, 2011). This result implies that factors involved in the flux of special fatty acids to TAG may be also very important in increasing VLCPUFAs accumulation in TAG in transgenic plants. However, so far there has been little data on coexpression of VLCPUFA-producing genes and genes involved in the TAG biosynthesis pathway in transgenic plants. Recently, characterization of *PDCT* (*AtRODI*) from *A. thaliana* *rod1* mutant revealed that 40% of PUFAs (20% of total FAs) in TAG are derived from interconversion between DAG and PC (Lu *et al.*, 2009). CPT and EPT activities have been well known for their essential role in the *de novo* synthesis of PC and PE. Thus, it is important to clone *PDCT*, *CPT* and *EPT* genes from VLCPUFA-producing microorganisms and to analyze their activities in using VLCPUFAs as substrates. The information on these enzymes would facilitate our understanding of mechanisms on the accumulation of VLCPUFAs in TAGs and our designing of strategies to engineer high level VLCPUFAs in transgenic plants.

## **2.5 Phosphatidylcholine: diacylglycerol cholinephosphotransferase (PDCT)**

The PDCT (EC 2.7.8) mutant was first identified from *Arabidopsis* with a reduced oleate desaturation (*rod1*) 20 years ago (Lemieux *et al.*, 1990). The gene, *AtRODI*, responsible for the phenotype, has not been cloned from *Arabidopsis* until 2009 (Lu *et al.*, 2009). The availability of the *AtRODI* allows the functional study on PDCT which catalyzes the reaction between 18:2/3-PC and 18:1-DAG resulting in production of 18:2/3-DAG and 18:1-PC. The catalytic mechanism of PDCT is similar to that of phosphatidylcholine: ceramide cholinephosphotransferase, also known as sphingomyelin synthase (SMS) found in animals for the synthesis of sphingomyelin (SM), a product of transferring the phosphocholine head group from PC onto the hydroxyl group of

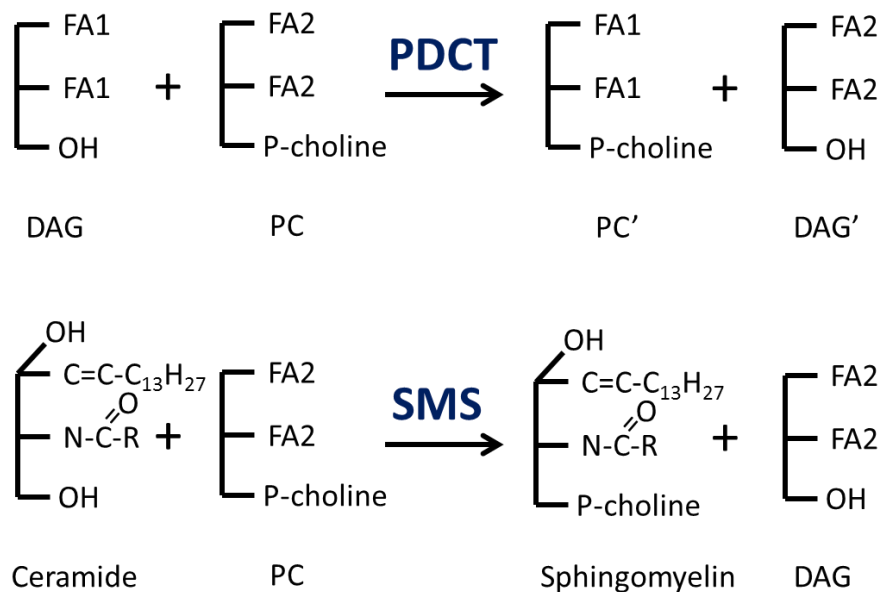
ceramide (Marggraf and Kanfer, 1984; Ullman and Radin, 1974), as shown in Figure 5. A comparative analysis of PDCT from *Arabidopsis* and SMS-related proteins from mice, *C. elegans*, *P. falciparum*, *D. melanogaster* and human revealed that there are four conserved motifs and five highly conserved residues in the primary sequence (Huiteima *et al.*, 2004; Lu *et al.*, 2009). A *PDCT* gene was identified from castor, *RcROD1*, whose deduced protein shows 70% identical residues with AtROD1 (Hu *et al.*, 2012). The putative transmembrane domains and the catalytic triad (His, His and Asp) are also conserved in *RcROD1* sequence. When *RcROD1* is coexpressed with fatty acid hydroxylase 12 (*RcFAH12*) in seed, hydroxy fatty acids (HFAs) was increased from 17% to 28% in PDCT-deficient mutant *Arabidopsis*. Analysis of regiochemistry and fatty acid profile in TAG and PC indicates that *RcPDCT* enhances HFAs through PC to DAG, and thus in TAG (Hu *et al.*, 2012). Therefore, studies on AtPDCT and *RcPDCT* support that PDCT should be recruited to the metabolic engineering toolbox for producing UFAs such as VLCPUFAs in transgenic oilseeds.

## **2.6 Cholinephosphotransferase (CPT) and Ethanolaminephosphotransferase (EPT) enzymes**

### **2.6.1 Functional and biochemical studies of CPT and EPT enzymes**

As mentioned in section 2.3, PC is an important phospholipid donating fatty acids to different pools such as acyl-CoA, other phospholipids and neutral lipids. To better understand the underlying mechanism of these complicated acyl trafficking processes, studies on PC and PE synthesis become imperative.

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the two major phospholipids constituents of most eukaryotic cells, which account for 50% and 25% of cellular membrane mass, respectively (Raetz, 1986). The fatty acyl groups of PC determine the membrane structure and function and act as a reservoir for numerous signaling molecules (Exton, 1990; Funk, 2001). In addition to its role as a structure component of membranes, PE can also be modified to form functional compounds and signaling molecules (Dewey and Goode, 1999; Dewey *et al.*, 1994; Qi *et al.*, 2003).



**Figure 5.** Catalytic reactions of PDCT and SMS (Adapted from Huitema *et al.*, 2004 and Lu *et al.*, 2009). PDCT transfers the phosphocholine moiety from phosphatidylcholine (PC) onto the hydroxyl of diacylglycerol (DAG) and generates a new PC (PC') and a new DAG (DAG'). SMS (or phosphatidylcholine: ceramide cholinephosphotransferase) transfers the phosphocholine moiety from PC onto the primary hydroxyl of ceramide, thus generating Sphingomyelin and DAG.

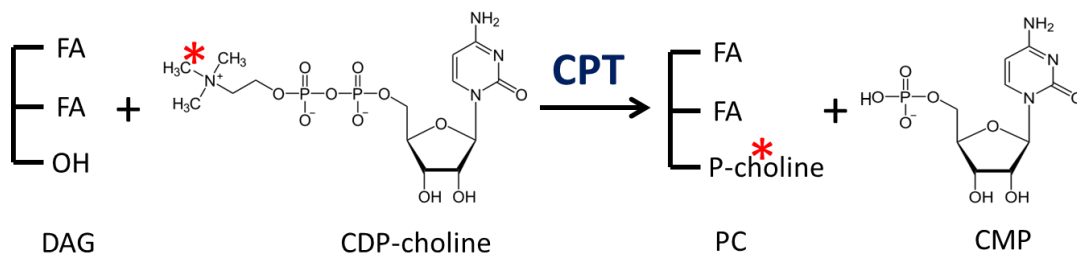


PC and PE are mainly synthesized *de novo* by homologous nucleotide pathways both of which consist of three consecutive reactions (Figure 4). The final step of each pathway involves the catalyzation of CPT and EPT, respectively. CPT (EC 2.7.8.2) and EPT (EC 2.7.8.1) were first described in the study that revealed the control role of cytidine nucleotides for phospholipids synthesis (Kennedy and Weiss, 1956). CPT catalyzes the final reaction of CDP-choline pathway for *de novo* synthesis of PC, and this reaction involves the formation of phosphoester via the transferring of a phosphocholine moiety of CDP-choline to DAG resulting in the formation of PC and CMP (Figure 6). Similarly, EPT catalyzes an analogous reaction by using CDP-ethanolamine as the substrate for the synthesis of PE (Figure 7).

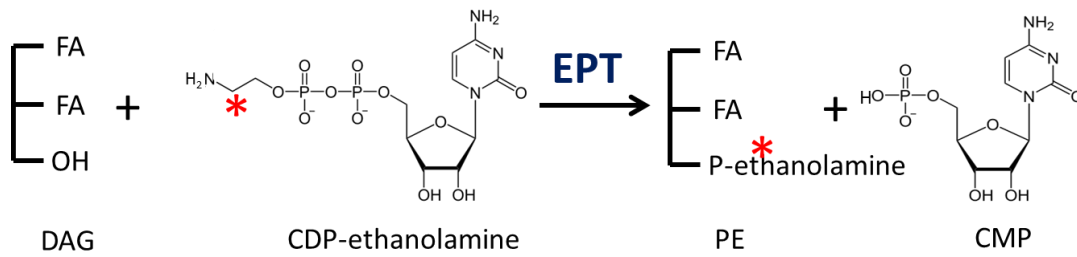
In mammals, CPT and EPT activities essentially account for *de novo* synthesis of PC and PE and their activities were widely detected in the tissues. In contrast, the formation of PC/PE via CDP-DAG pathway was only observed in certain tissues of other species (Cui *et al.*, 1999; McMaster and Choy, 1992; Ridgway *et al.*, 1989). This might explain why inactivation of CDP-choline and CDP-ethanolamine pathways in mammalian cells would be lethal. Yeast cells with the CDP-choline pathway being disrupted were still viable (Hjelmstad and Bell, 1987; 1988); PC could also be synthesized via the PE methylation pathway (Cui *et al.*, 1996; Henneberry *et al.*, 2002; McMaster and Bell, 1997). Very few prokaryotes contain PC and this small amount of PC is synthesized through methylation of PE (Arondel *et al.*, 1993).

### **2.6.2 Genetic studies of *CPT* and *EPT* genes**

The CPT and EPT activities were first observed in microsomal proteins of fat cells from female rat (Coleman and Bell, 1977). However, genes coding for CPT and EPT activities were first isolated from yeast through complementation screening of a genomic library in yeast mutants deficient in CPT and EPT activities (Hjelmstad and Bell, 1987; 1988). A comparison of *S. cerevisiae* CPT1 (ScCPT1) and *S. cerevisiae* EPT1 (ScCET1) protein sequences revealed over 50% identity at the amino acid level. The functional analysis of the two enzymes indicated that ScCPT1 strictly utilized CDP-



**Figure 6.** Catalytic reaction of CPT. CPT catalyzes the final reaction of CDP-choline pathway for *de novo* synthesis of PC. This reaction involves the formation of phosphoester via the transferring of a phosphocholine moiety of CDP-choline to DAG resulting in the formation of PC and CMP. DAG, diacylglycerol; CDP-choline, cytidine diphosphate-choline; PC, phosphatidylcholine; CMP, cytidine monophosphate.



**Figure 7.** Catalytic reaction of EPT. EPT catalyzes the final reaction of *de novo* synthesis of PE, which involves the formation of phosphoester via the transferring of a phosphoethanolamine moiety of CDP-ethanolamine to DAG resulting in the formation of PE and CMP. DAG, diacylglycerol; CDP-ethanolamine, cytidine diphosphate-ethanolamine; PE, phosphatidylethanolamine; CMP, cytidine monophosphate.

choline as the substrate while ScEPT1 was capable of using both CDP-choline and CDP-ethanolamine as the substrates to synthesize PC and PE, respectively (Hjelmstad and Bell, 1991).

In higher plant, a soybean cDNA encoding an aminoalcoholphosphotransferase (AAPT) which appears to possess both CPT and EPT activities was cloned by complementation of a yeast  $\Delta CPT/\Delta EPT$  mutant (Dewey *et al.*, 1994). By using this soybean (*Glycine max*) AAPT cDNA (*GmAAPT1*) as a heterologous hybridization probe, *AtAAPT1/AtAPPT2* from *A. thaliana* has been isolated (Dewey and Goode, 1999; Dewey *et al.*, 1994; Qi *et al.*, 2003). Besides, AAPT cDNA was cloned from Chinese cabbage, *Crassica napus* and wheat (Choi *et al.*, 2000; Qi *et al.*, 2003; Sutoh *et al.*, 2009). An alignment of these AAPTs reveals that as high as 70% amino acids are conserved (Sutoh *et al.*, 2009).

Human *CPT1/CEPT1* genes were identified from an analysis of the expressed sequence tags (EST) and homologous search for clones with significant similarity to yeast ScCPT1 protein (Henneberry and McMaster, 1999; Henneberry *et al.*, 2000). *In vitro* and *in vivo* functional analysis of these two genes demonstrated that human *CPT1* gene product catalyzes CPT reaction, whereas human *CEPT1* gene product utilizes both CDP-choline and CDP-ethanolamine as substrates (Henneberry and McMaster, 1999).

The reactions catalyzed by CPT and EPT enzymes are believed to be reversible. The *in vitro* studies showed that CDP-choline and CDP-ethanolamine could be synthesized in rat liver tissue by using phospholipids and CMP as substrates (Kano and Ono, 1973; Roberti *et al.*, 1992). Moreover, it was demonstrated that the reversibility of both enzymes occurring in brain tissue resulted in accumulation of DAG generated from PC and PE (Goracci *et al.*, 1986; Goracci *et al.*, 1977). In plants, the reverse reaction of CPT and EPT enzymes observed in cotyledons of safflower and linseed (Slack *et al.*, 1983; Slack *et al.*, 1985) are closely associated with the movement of glycerol and/or acyl-group moiety of PC to DAG and to TAG in developing seeds during TAG production (Stobart and Stymne, 1985). After three cDNAs encoding AAPT enzyme

cloned from *Arabidopsis* and soybean, their products were confirmed to be capable of catalyzing the reverse reaction by overexpressing these genes in yeast mutant and the *in vitro* assay (Dewey and Goode, 1999; Dewey *et al.*, 1994; Qi *et al.*, 2003).

DAG substrate preference of CPT and EPT enzymes from eukaryotic species has been widely investigated. In yeast, the forward reaction of CPT enzyme preferred di16:1-DAG, while EPT enzyme favoured di18:1-DAG (Hjelmstad *et al.*, 1994). Besides medium chain and long chain fatty acyl-DAG, CPT and EPT enzymes were also able to utilize VLCPUFA-containing DAGs (C16:0/C20:4-DAG and C18:0/C20:4-DAG) as substrates, although at a lower preference (Hjelmstad *et al.*, 1994). In human, CPT1 has highest activity by using di18:1-DAG as substrate but also works on VLCPUFA-DAG (C16:0/C22:6-DAG and C18:0/C20:4-DAG) (Henneberry *et al.*, 2000). CEPT1 protein prefers long chain PUFA-DAGs as substrate and can also use VLCPUFAs-DAGs substrate in both CPT and EPT reactions (Henneberry *et al.*, 2000; Henneberry *et al.*, 2002). However, the study in developing seeds of safflower and rapeseed showed that CPT enzyme has no or little substrate preference across a range of different DAG substrates (Vogel and Browse, 1996).

### **2.6.3 Structure and function relationship of CPT and EPT proteins**

The analysis of hydropathy plots for ScCPT1, ScEPT1, soybean AAPT1 and human CPT1/CEPT1 proteins identified a conserved region containing seven membrane-spanning domains (Dewey *et al.*, 1994; Henneberry and McMaster, 1999; Henneberry *et al.*, 2000). Comparison of CDP-alcohol (CDP-choline and CDP-ethanolamine) specificity between parental CPT protein and CPT and EPT chimeric enzymes resulted in the identification of a CDP-choline binding domain (79-186 residues of ScCPT1) (Hjelmstad *et al.*, 1994). Database searches (BLAST algorithm) indicated that the CDP-choline binding domain found in ScCPT1 was conserved in phospholipids-synthesizing enzymes, such as EPT, phosphatidylinositol (PI) synthase and phosphatidylserine (PS) synthase in yeast and mammals; as well as prokaryotic PS synthase and plant AAPT1. All of these enzymes were able to catalyze a CDP-aminoalcoholphosphotransferase reaction

(McMaster and Bell, 1997). The conserved domain consists of residues Asp-Gly-(X)<sub>2</sub>-Ala-Arg-(X)<sub>8</sub>-Gly-(X)<sub>3</sub>-Asp-(X)<sub>3</sub>-Asp, named CDP-alcoholphosphotransferase motif .

Besides, comparison of substrate specificity of parental CPT and EPT and their chimeric enzymes revealed a region of 218 amino acids (46-265 residues of ScCPT1) containing the first three predicted membrane-spanning domains confers the DAG specificity (Hjelmstad *et al.*, 1994). Scanning alanine mutagenesis of the conserved amino acid within the conserved motif of ScCPT1 protein revealed that two final aspartates within this motif are critical for the enzymatic activities (Williams and McMaster, 1998). In addition, scanning alanine site-directed mutagenesis of BnAAPT1 also indicated that last two Asp residues at positions 117 and 120 are crucial for *Brassica napus* BnAAPT1 activity (Qi *et al.*, 2003). Sited-directed mutagenesis of human *CEPT1* genes in DAG binding domain altered substrate preference for DAG (Henneberry *et al.*, 2002). These structural studies provide insights on further cloning *CPT* and *EPT* from other species and performing mutagenesis to best suit the purpose of metabolic engineering.

Therefore, cloning genes such as *PDCT*, *CPT* and *EPT* is promising to use in transgenic plants to solve the bottleneck of acyl-trafficking between neutral lipids and phospholipids, thus help increase VLCPUFAs accumulation in plants.

### **3.0 RESEARCH STUDIES**

#### **3.1 Study 1: Fatty acid analysis of VLCPUFA-producing microorganisms *P. infestans* and *T. aureum***

##### **3.1.1 Abstract**

*Phytophthora infestans* is a potato pathogenic fungus, which can produce a considerable amount of EPA (Bostock *et al.*, 1981). *Thraustochytrium aureum*, marine fungoid protists, has attracted a lot of attention recently because of their ability to accumulate large quantities of VLCPUFAs (Huang and Bao, 2002). In this study, fatty acid analysis of these two species was conducted, which confirmed that *P. infestans* could produce 10.4% of EPA in the total fatty acids, while *T. aureum* could produce 35% of DHA in the total fatty acids. Therefore, these two VLCPUFA-producing microorganisms are good candidates for genes sources of PDCT, CPT and EPT.

##### **3.1.2 Experimental approach**

A *Phytophthora infestans* strain was kindly provided by Lorne Adam, University of Manitoba. *P. infestans* was cultured in pea broth, shaking at 20 °C, 200 rpm for 2 weeks. Pea broth was made by 1) autoclave 120g frozen peas in 1 liter distilled water at 121 °C for 15 min; 2) strain peas from pea broth using 4 layers of cheese cloth squeezing gently to remove all excess liquid from peas; 3) bring volume of broth up to 1 liter; 4) autoclave at 121 °C for 20 min. Cells were harvested by vacuum filtration.

*Thraustochytrium aureum* was cultured in GPY medium, shaking at 28 °C, 200 rpm for 3 days. GPY medium was made by 1) mix 2% (w/v) glucose, 1% (w/v) polypeptone, 0.5% (w/v) yeast extract, 40 g/L sea salts; 2) adjust pH to 6.0-6.5; 3) autoclave at 121 °C for 20 min. Cells were harvested by centrifugation.

One gram cells of each was added with 2 mL of methanolic HCl (3 N) (Invitrogen Canada Inc., Burlington, Ontario) and heated at 80 °C for 1 hour. After transmethylation process, the sample was cooled down on the ice before 1 mL of 0.9% (w/v) NaCl and 2 mL of hexane were added to it. After vortexing, the mixture was centrifuged down for 10

min at 2,000 rpm. Hexane phase containing fatty acid methyl esters (FAMES) were collected into a new glass tube and dried under nitrogen gas. The FAMES were then resuspended in a small volume (50-200  $\mu$ L) of hexane and placed in a GC auto-sampler vial for gas chromatograph (GC) analysis.

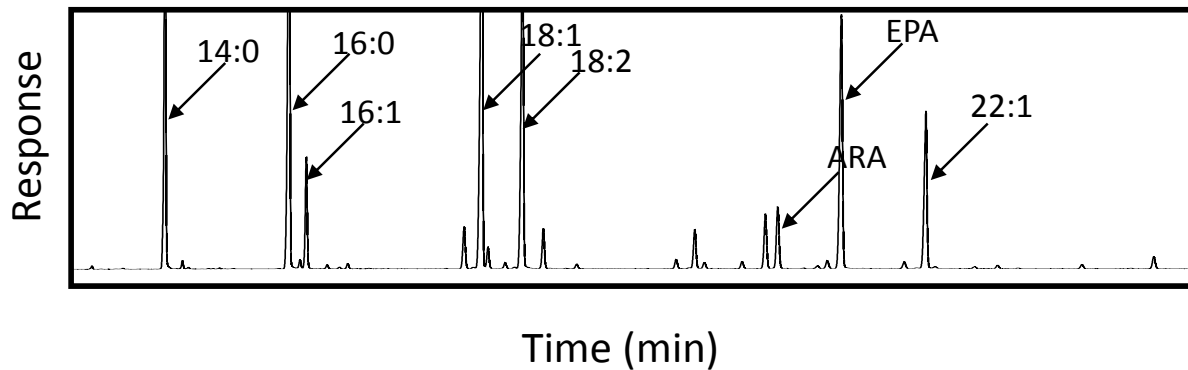
Two  $\mu$ L of the sample total FAMES was analyzed by using a GC equipped with DB-23 fused silica column (30 m  $\times$  0.25 mm) with 0.25  $\mu$ m film thickness (J&W Scientific, Mississauga, Ontario). The column temperature was monitored at 180  $^{\circ}$ C for 1 min, then raised at rate of 4  $^{\circ}$ C/min to 240  $^{\circ}$ C held for 15 min. Individual fatty acid was identified by comparing the retention times and mass spectra (MS) with that of authentic fatty acid standards (Nu-chek prep, Inc. Elysian, USA ). GC/MS analysis was carried out in standard EI mode using a Fisons VG TRIO 2000 mass spectrometer (VG Analytical, Manchester, UK) controlled by Masslynx version 2.0 software and coupled to a GC 8000 Series gas chromatograph.

### 3.1.3 Results

As shown in Figure 8, *P. infestans* produced 10.4% EPA in its total fatty acids and a small amount of ARA (3%). Other major fatty acids produced in *P. infestans* were 14:0, 16:0, 16:1-9, 18:1-9, 18:2-9,12 and 22:1-13 (Table 2). Figure 9 showed the fatty acid profile of *T. aureum*. It produced 35% of DHA, 11.7% of 22:5n-6, 7.0% of EPA and 2.4% of ARA (Table 3). Most of other fatty acids are saturated and monounsaturated fatty acids.

The whole genome of *P. infestans* has been sequenced and annotated, which is available on the NCBI website. An EST database of *T. aureum* was previously generated in our lab. These DNA sequence databases provide opportunity for us to clone *PDCT*, *CPT* and *EPT* genes from the two VLCPUFA-producing microorganisms simply by homology search and specific PCR amplification.

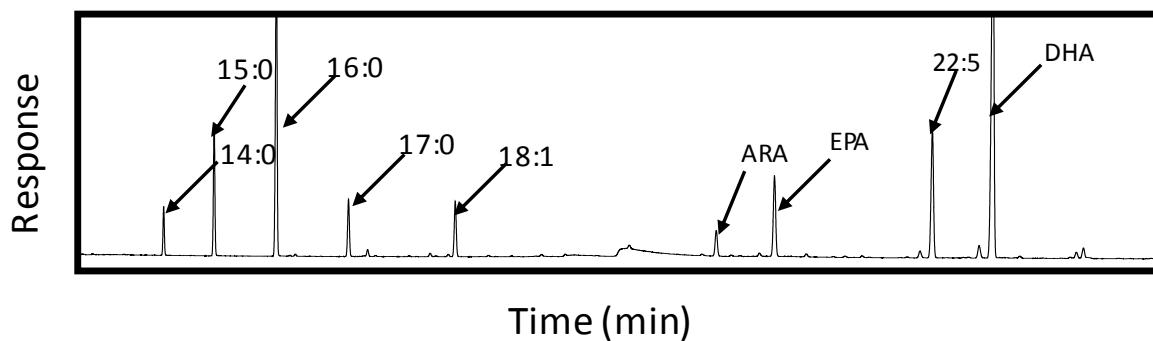




**Figure 8.** GC analysis of fatty acid methyl esters prepared from *P. infestans* cells. Identity of major fatty acids is indicated in the gas chromatogram. This experiment was performed once.

**Table 2.** Fatty acid composition (Mol %) of *P. infestans*. Data were calculated from Figure 8.

<b>Fatty acid</b>	<b>14:0</b>	<b>16:0</b>	<b>16:1</b>	<b>18:0</b>	<b>18:1</b>	<b>18:2</b>	<b>18:3</b>	<b>20:1</b>	<b>20:3</b>	<b>ARA</b>	<b>EPA</b>	<b>22:1</b>
<b>Mol %</b>	16.8	21.1	3.7	1.6	17.6	13.6	1.6	1.4	2.0	2.4	10.4	5.6



**Figure 9.** GC analysis of fatty acid methyl esters prepared from *T. aureum* cells. Identity of major fatty acids is indicated in the gas chromatogram. This experiment was performed once.

**Table 3.** Fatty acid composition (Mol %) of *T. aureum*. Data were calculated from Figure 9.

<b>Fatty Acid</b>	<b>14:0</b>	<b>15:0</b>	<b>16:0</b>	<b>17:0</b>	<b>18:1</b>	<b>ARA</b>	<b>EPA</b>	<b>22:5</b>	<b>DHA</b>
<b>Mol %</b>	3.2	9.7	23.3	4.5	4.0	2.4	7.0	11.7	35.0

## 3.2 Study 2: Cloning and functional characterization of *PDCT* from *P. infestans*

### 3.2.1 Abstract

PDCT is a novel transferase enzyme, catalyzing the transfer of phosphocholine head group from PC to DAG. This study aims to clone and characterize *PDCT* genes from VLCPUFA-producing microorganism, *P. infestans*. Identification of *PDCT* genes from this species was undertaken using *Arabidopsis* PDCT (AtPDCT) and related sphingomyelin synthase (SMS) amino acid sequences as queries to search the *P. infestans* genomic sequence database. Two putative *PDCT*s were identified and cloned from *P. infestans*.

For the functional analysis of two putative *PDCT* genes from *P. infestans*, the open reading frames of the candidate genes were inserted in a yeast expression vector, pESC-HIS under a *GALI* promoter, and the resultant plasmids were transformed into a yeast *CPT* and *EPT* double mutant. *In vitro* enzyme assays were conducted using the microsomal fraction of transformants expressing the putative *PDCT*; however, no activity was found with the genes.

To examine the possible effect of PDCT on the biosynthesis of VLCPUFAs, each putative *PiPDCT* was co-expressed with a  $\Delta 5$  desaturase gene in wild type yeast in the presence of DGLA (20:3), a precursor fatty acid for the  $\Delta 5$  desaturation, similarly, no effect was found on the production of  $\Delta 5$  desaturated fatty acids in the co-expression yeast.

### 3.2.2 Hypothesis

PDCT catalyzes acyl exchange between PC and DAG. VLCPUFAs synthesized on PC through desaturation can be channelled to DAG by this enzyme. Therefore, PDCT might play a role in the biosynthesis and accumulation of VLCPUFAs in the microorganisms such as *P. infestans*. Furthermore, the function of putative *PiPDCT*s cloned from the VLCPUFA-producing microbe could be determined in yeast and *in vitro* assays would be able to provide functional properties of cloned *PDCT*s from *P. infestans*

and help elucidate the role of PDCT in acyl exchange between PC and DAG during the biosynthesis of VLCPUFAs. Co-expression of *PiPDCTs* and VLCPUFA synthetic genes in yeast would examine the possible effect of PDCTs on the biosynthesis of VLCPUFAs.

### **3.2.3 Experimental approach**

#### **3.2.3.1 Homology search and sequence analysis**

AtPDCT (called AtROD1 in Lu *et al.*, 2009) protein sequence from *Arabidopsis* was used as a query sequence to search the sequence database. Since PDCT is related to SMS (Huitema *et al.*, 2004), SMS protein sequences from human have also been used as query sequences to BLAST search *P. infestans* genome sequence database ([http://www.broadinstitute.org/annotation/genome/phytophthora\\_infestans/MultiHome.html](http://www.broadinstitute.org/annotation/genome/phytophthora_infestans/MultiHome.html)) (Altschul *et al.*, 1997). Genes encoding protein sequences that share amino acid similarity with AtPDCT and SMS, and contains two conserved domains were identified and designed as putative *PiPDCTs* (*PiPDCT1* and *PiPDCT2*).

DNASTAR program (Lasergene 9 Core Suite, Madison, USA) was used for the alignment of putative PDCT proteins from *P. infestans* and related sequences. Hydrophathy analysis was used to predict topology of these putative PDCT from *P. infestans* (<http://www.vivo.colostate.edu/molkit/hydrophathy/>). Transmembrane domains of these putative *PiPDCT* were predicted by TMHMM program (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>).

#### **3.2.3.2 Cloning of PDCT from *P. infestans* and *A. thaliana***

Total RNA from 0.5 g *P. infestans* cells from 2 weeks culture was extracted using TRIzol reagent (Invitrogen Canada Inc., Burlington, Ontario). The RNA concentration was measured with DU730 Spectrophotometer by the absorbance at 260 nm using Nucleic acid analysis-RNA program (Beckman Coulter Canada, Inc., Mississauga, Ontario). Five  $\mu$ L RNA was denatured at 65°C for 5 min, cooled down on ice for 2 min before loaded into 50 mL 1.2% (w/v) agarose gel with 2  $\mu$ L ethidium bromide (10

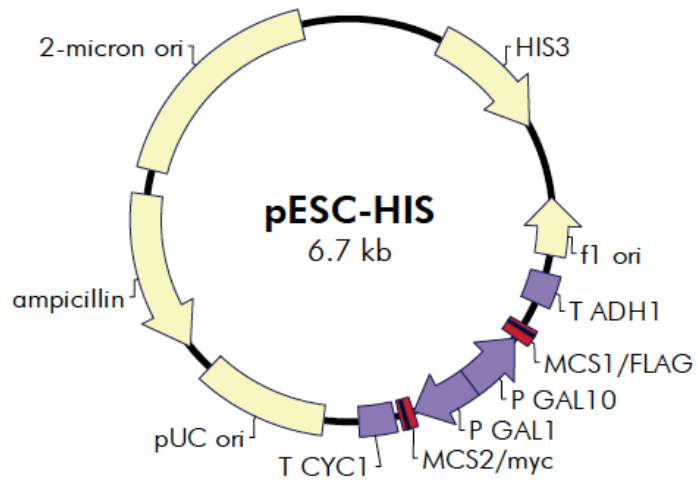
mg/mL) and checked the RNA quality under UV light by Alpha Imager HP (Alpha Innotech Corp., Ottawa, Ontario). First-strand cDNA was synthesized using the SuperScript III first-strand synthesis system (Invitrogen Canada Inc., Burlington, Ontario) following the instruction. Gene specific primers, which are shown in Table 4, were designed based on the results of homologous search of the genome sequences by using DNASTAR-PrimerSelect program (Lasergene 9 Core Suite, Madison, USA). Primers lengths are 18-25 bp. Primers melting temperatures are in the range of 52-58°C. The GC contents of primers are 40-60%. 3' end of primers ends with G(s) or C(s). Restriction enzyme whose recognition sites were found in the multiple cloning site 2 (MCS2) of yeast expression vector, pESC-HIS (Figure 10) but not found in target gene sequences was chosen for subcloning target genes into pESC-HIS. *Bam*HI was selected and its recognition cutting sites were added to 5' ends of the gene specific primers. In each forward primer, three nucleotides with G(s) or C(s), which were found in 5' untranslated region (5'UTR) of each gene, were inserted between enzyme cutting sites and start codon for efficient digestion.

Gene specific primers were used to retrieve the full-length cDNAs using specific reverse transcriptase-PCR (Invitrogen Canada Inc., Burlington, Ontario). Phusion DNA polymerase (0.25  $\mu$ L) (Finnzymes Canada Inc., Ottawa, Ontario) was used in 25  $\mu$ L PCR amplification reaction. Other components were: 5  $\mu$ L 5  $\times$  Phusion HF buffer, 0.5  $\mu$ L 10 mM dNTPs, 1.25  $\mu$ L of each 10 mM primer, 2  $\mu$ L template cDNA, add ddH<sub>2</sub>O to 25  $\mu$ L. PCR conditions were: 98°C for 30 seconds, 5 cycles of 98°C for 10 seconds, 55°C for 30 seconds, 72°C for 45 seconds, 25 cycles of 98°C for 10 seconds, 62°C for 30 seconds, 72°C for 45 seconds, and 72°C for 5 minutes. PCR reaction was then loaded on 50 mL 1.0% (w/v) agarose gel with 2  $\mu$ L ethidium bromide (10 mg/mL) and checked under UV light by Alpha Imager HP. The amplified fragment was cut and isolated from agarose gel using EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic Canada Inc., Mississauga, Ontario) and then sent for direct sequencing by using gene specific primers to generally confirm the right clone (Plant Biotechnology Institute, National Research Council, Saskatoon). The gene sequencing results were confirmed by comparing with the

**Table 4.** Gene specific primers for amplifying open reading frames of putative *PDCTs* from *P. infestans* and *AtPDCT* from *Arabidopsis*. F, forward primer; R, reverse primer; Both forward and reverse primers are shown from 5' ends to 3' ends. Restriction enzyme (*Bam*HI) cutting sites were added in primers for subcloning into yeast expression vector, shown in small letters. In each forward primer, three nucleotides with Gs or Cs, which were found in 5' untranslated region (5'UTR) of each gene, were inserted between enzyme cutting sites and start codon for efficient digestion.

<b>Gene name</b>	<b>Primer name and sequence</b>	<b>Restriction enzyme</b>	<b>Fragment size (bp)</b>
<i>PiPDCT1</i>	YC1-F: 5'g gatccGCGATGACGTCGTACCTGCC3'	<i>Bam</i> HI	1287
	YC2-R: 5'g gatccCTAGCAGATGAGCTGCACTTGG3'		
<i>PiPDCT2</i>	YC3-F: 5'g gatccGAGATGGAGAGCTGGACGGAC3'	<i>Bam</i> HI	1236
	YC4-R: 5'g gatccCTAGTCGATCTTTTCAATTCGATC3'		
<i>AtPDCT</i>	YC9-F: 5'g gatccGCCATGTCAGCCGCCGCAGC3'	<i>Bam</i> HI	921
	YC10-R: 5'g gatccTTAATTGACTAGAGAGTCTTTCG3'		





**Figure 10.** The pESC-HIS vector map (Reproduced from instruction manual of pESC yeast epitope tagging vectors, Agilent Technologies Canada Inc.).

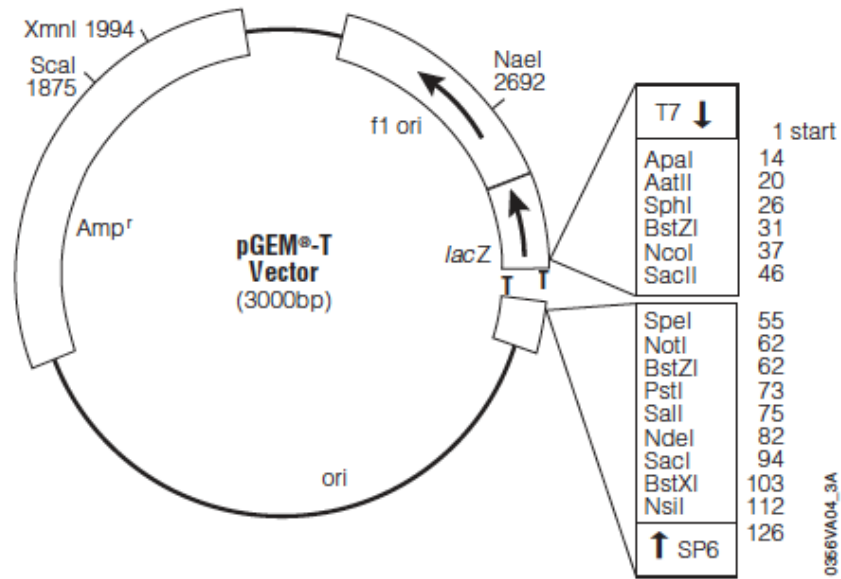
sequences obtained from the database.

As a positive control, *AtPDCT* (GenBank accession number: AEE75730) was cloned by using gene specific primers based on the public gene sequence (<http://www.ncbi.nlm.nih.gov/>). *A. thaliana* cDNA synthesized from developing seeds was kindly provided by Dr. Maozhi Ren (Plant Biotechnology Institute, Saskatoon). The cloning step was the same as the cloning of putative *PDCTs* from *P. infestans*. The gene specific primers are shown in Table 4. After aligned the cloned gene sequence with *AtPDCT* sequence in the database, it was found that the gene cloned from developing seeds had one amino acid substitution. This clone was designated as *AtPDCT'* and used in some experiments as the substitution was not within an important motif. Subsequently, *AtPDCT* was cloned from an *Arabidopsis* silique cDNA library and this clone demonstrated a 100% amino acid identity with the database sequence.

### 3.2.3.3 Cloning of putative *PiPDCTs* and *AtPDCT* into pGEM-T vector

Before cloning *PDCT* genes into yeast expression vector for the functional study, the gene fragments, which were obtained from PCR reaction using cDNA as template, were added with A tail using *Taq* DNA polymerase (Invitrogen Canada Inc., Burlington, Ontario). The components in 50  $\mu\text{L}$  reaction were: 0.125  $\mu\text{L}$  *Taq* DNA polymerase, 5  $\mu\text{L}$  10  $\times$  buffer, 0.5  $\mu\text{L}$  10 mM dNTPs, 25  $\mu\text{L}$  template DNA, 19.875  $\mu\text{L}$  ddH<sub>2</sub>O. The reaction was incubated at 72°C for 25 minutes and hold at 4°C. The A overhang DNAs were cloned into the intermediate vector pGEM-T (Promega cooperation, Madison, USA), shown in Figure 11. The constructs were transformed into *E. coli* Top 10 (Invitrogen Canada Inc., Burlington, Ontario) using electroporation method by Electroporator 2510 (Eppendorf Canada Inc., Mississauga, Ontario) and the transformed *E. coli* was selected on blue-white screening plate (Luria-Bertani (LB) agar medium + 100  $\mu\text{g}/\text{mL}$  ampicillin + 40  $\mu\text{L}$  40 mg/mL X-gal + 40  $\mu\text{L}$  100 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG)).

The white colonies were checked by PCR with the gene specific primers and HP *Taq* DNA polymerase to confirm the genes were inserted in pGEM-T successfully (UBI,



**Figure 11.** The pGEM-T vector map (Reproduced from technical manual of pGEM-T vector systems, Promega cooperation, Madison, USA).

Saskatoon, Saskatchewan). For colony PCR, 25  $\mu\text{L}$  PCR reaction contained: 0.125  $\mu\text{L}$  HP *Taq* DNA polymerase, 2.5  $\mu\text{L}$  10  $\times$  *Taq* reaction buffer, 2.5  $\mu\text{L}$  20 mM  $\text{MgSO}_4$ , 0.5  $\mu\text{L}$  10 mM dNTPs, 0.625  $\mu\text{L}$  of both 10 mM gene specific forward and reverse primers, and 18.125  $\mu\text{L}$  ddH<sub>2</sub>O. PCR conditions were: 95°C for minutes, 30 cycles of 95°C for 30 seconds, 64°C for 30 seconds, 72°C for 1 minute, 72°C for 5 minutes, holding at 16°C. After confirmed the insert by running PCR mixture on agarose gel with 2  $\mu\text{L}$  ethidium bromide (10 mg/mL), four positive colonies for each *PDCT* were selected and cultured in 5 mL LB with 100  $\mu\text{g}/\text{mL}$  ampicillin medium. After grown at 37°C for 16 h, plasmid DNAs were isolated from *E. coli* cells. The cells were centrifugated at 3,000 rpm for 10 min and the supernatant was decanted. Then cells were resuspended in 250  $\mu\text{L}$  of resuspension buffer P1 (50 mM Tris-HCl, 10 mM EDTA, 0.8% RNase) and then 250  $\mu\text{L}$  of lysis buffer P2 (200 mM NaOH, 1% (w/v) sodium dodecyl sulfate (SDS)), inverted 3 times before adding 350  $\mu\text{L}$  of neutralization buffer N3 (Qiagen, Toronto, Ontario). After inverted 3 times, mixture was centrifuged at 13,000 rpm for 10 min. 750  $\mu\text{L}$  of the supernatant was removed to a new tube and mixed with 750  $\mu\text{L}$  of isopropyl alcohol, followed by the centrifugation at 4°C for 10 min. Supernatant was decanted and the pellet was washed with 350  $\mu\text{L}$  75% (v/v) ethanol. Ethanol was then immediately poured out and briefly dried by air. The pellets were resuspended in 100  $\mu\text{L}$  H<sub>2</sub>O. After plasmids were isolated, they were digested with the restriction enzymes (*SalI*) which have recognition sites within both pGEM-T vector and inserted genes. After the digestion checking by running out the digestion mixture on a 1% (w/v) agarose gel, two plasmids of each gene were chosen and sent for sequencing using the pGEM-T vector primers (SP6 and T7).

#### **3.2.3.4 Cloning of putative *PiPDCT* genes and *AtPDCT* in yeast expression vector**

After confirm the plasmids by sequencing, one plasmid of each gene with right inserted gene was selected to do further cloning. Ten  $\mu\text{g}$  pGEM-T with insert genes were digested by 1  $\mu\text{L}$  *BamHI* in 50  $\mu\text{L}$  mixture containing 5  $\mu\text{L}$  10  $\times$  React3 and ddH<sub>2</sub>O (Invitrogen Canada Inc., Burlington, Ontario). pESC-HIS, which contains *GAL1* and *GAL10* promoter, was also digested with *BamHI* whose recognition site found in the

multiple cloning site 2 (MCS2) (Figure 10) of pESC-HIS. In 50  $\mu\text{L}$  mixture, there were three  $\mu\text{g}$  pESC-HIS plasmids, 1  $\mu\text{L}$  *Bam*HI, 5  $\mu\text{L}$  10  $\times$  REact3 and ddH<sub>2</sub>O. After the overnight digestion at 37°C, linear pESC-HIS vector was added with 1 unit calf intestinal alkaline phosphatase (CIAP), which removed 5'-phosphate groups of linear pESC-HIS vector, and incubated at 37°C for 10 min. Digested gene fragments and pESC-HIS vector were separated on the agarose gel and isolated by EZ-10 Spin Column DNA Gel Extraction Kit. The purified vector and DNA insert of *PiPDCT1*, *PiPDCT2* and *AtPDCT* were ligated by T4 DNA ligase at 16°C overnight. Two  $\mu\text{L}$  of ligation was transformed into *E.coli* through electroporation and the transformants were selected on LB with 100  $\mu\text{g}/\text{mL}$  ampicillin plates. After PCR-checking the orientation of 12 colonies by using one gene specific primer and *GAL1* forward primer, two clones with right orientation was sent for sequencing using *GAL1* forward primer and *GAL1* reverse primer (Plant Biotechnology Institute, National Research Council, Saskatoon).

Following sequenced *AtPDCT* was found to contain a nucleotide substitution leading to a one amino acid substitution. This clone was designated *AtPDCT'* and cloned into a different expression vector (pYES2.0) as described above. *PiPDCT1* and *PiPDCT2* were also cloned into the pYES2.0 expression vector.

### 3.2.3.5 Yeast transformation

Yeast *CPT* and *EPT* double mutant (DBY747- $\Delta\text{CPT}/\Delta\text{EPT}$ : Mata $\alpha$ ; *his3- $\Delta$ 1*, *leu2-3*, *leu2-112*, *ura3-52*, *trp1-289*, *cpt1::LEU2*, *ept1::URA3*), which was previously constructed in our lab, was grown on amino acid dropout plates, SD-LEU-URA [synthetic defined yeast media with all necessary compounds except leucine and uracil, to make it, mix DOB powder (26.7 g/L (1.7 g yeast nitrogen base, 5 g ammonium sulphate, 20 g glucose)) and CSM (complete supplement mixture) powder (0.6 g/L CSM-LEU-URA supplement)] for 2 days at 30°C. The plasmids of pESC-*AtPDCT* (positive control) and pESC-*PiPDCTs* with correct orientation, as well as pESC-HIS empty vector (negative control) were transformed into DBY747- $\Delta\text{CPT}/\Delta\text{EPT}$  according to Gietz and Schiestl (2007). The transformed yeast was cultured on amino acid dropout plates, SD-

HIS (26.7g/L DOB and 0.6 g/L CSM-HIS supplement) for 2 days at 30°C. To screen transformants, colonies were selected by toothpick and dipped in 20  $\mu$ L 0.02 N NaOH at room temperature for 10 min, followed by heating at 95°C for 10 min. Three  $\mu$ L of yeast lysate was used in a 25  $\mu$ L PCR reaction with the gene specific primers and HP *Taq* DNA polymerase (as described in section 3.2.3.3) using the *GALI* forward primer and gene specific reverse primer for each gene.

When initially DBY747- $\Delta$ CPT/ $\Delta$ EPT was used as a host and pESC-His as an expression vector to express the *PDCT* genes, no activity was detected with either two putative *PiPDCTs* as well as the positive control, *AtPDCT'* where the occurrence of a single nucleotide substitution results in switching serine by glycine at position 184. Also, no activity was observed when *AtPDCT'* was expressed using pYES2.0. Constructed plasmid with *AtPDCT'* was then transformed into HJ091 (Mata; *his3- $\Delta$ 1 leu2-3, leu2-112 ura3-52 trp1-289 cpt1::LEU2 ept1- $\Delta$ 1*), another yeast *CPT* and *EPT* double mutant used in Lu *et al.* (2009) and INVSc1 (Mata; *his3- $\Delta$ 1 leu2 trp1-289 ura3-52*) (Invitrogen Canada Inc., Burlington, Ontario), a diploid wild type yeast to see whether the host strain would make difference. In addition, *AtPDCT* was re-amplified with no nucleotide errors and cloned into pYES2.0, and expressed in HJ091 and INVSc1. Also, the two putative *PiPDCTs* in pYES2.0 were expressed in INVSc1. Meanwhile, the yeast strain HJ091 expressing Arabidopsis *AtPDCT* (*AtRODI*) in p424GPD under a constitutive promoter (GPD) was obtained from Dr. Lu who published the first *AtRODI* (Lu *et al.*, 2009).

### 3.2.3.6 Microsomal preparation

Microsomal preparation was essentially according to Richard and McMaster (1998). Yeast cells from overnight cultures in SD-HIS selection medium were inoculated to the inducing SD-HIS media containing 2% (w/v) galactose, grown four hours to mid-log phase (OD<sub>600</sub> 2-2.5) by rotary shaking at 30 °C. Experiments were also performed by extending induction time to one day, two days and four days to check the effect of induction time on the enzyme specific activity. Cells were pelleted by centrifugation at 3,000 rpm for 5 min, washed once with ice-cold GTE buffer (20% (v/v) glycerol, 50 mM

Tris-HCl pH 7.4, 1 mM EDTA), and resuspended in 1 mL GTE buffer. Cells were disrupted by vortexing in the presence of 0.5 mm glass beads for five bursts of 30 seconds separated by 30-second incubations on ice. The supernatant was transferred to a new tube, and the beads were rinsed twice with 0.5 mL GTE. The pooled sample was centrifuged at  $5,000 \times g$  for 10 min at 4 °C. The resulting supernatant was crude extract (CE). CE was then centrifuged at  $100,000 \times g$  for 90 min to get microsomal protein (MP). The protein pellet was resuspended in 250  $\mu$ L GTE and the protein concentration was determined using Bio-Rad protein assay according to the manufacturer's instructions (Bio-Rad Laboratories (Canada) Ltd, Mississauga, Ontario). The membranes were then stored at -80 °C.

### **3.2.3.7 *In vitro* enzymatic assays**

The PDCT activities in membrane preparations of yeast cells, transformed with pESC-HIS (negative control), pESC-*AtPDCT* (positive control) or pESC-*PiPDCTs*, were determined as the amount of [ $^{14}$ C] dioleoyl-PC produced from [ $^{14}$ C-glycerol] diolein. The substrates of 1.8 nmol (200,000 cpm) [ $^{14}$ C-glycerol] diolein (American Radiolabeled Chemicals Inc, St Louis, USA) and 0.1  $\mu$ mol dilinoleoyl-PC were dried under nitrogen gas and resuspended in 50  $\mu$ L of 4  $\times$  reaction buffer [final concentrations: 50 mM 3-(N-morpholino) propanesulfonic acid (MOPS)/NaOH (pH 7.5), 20 mM MgCl<sub>2</sub>, 0.45% (v/v) Triton X-100] with the aid of a sonication bath. Reactions (200  $\mu$ L) were started by adding 100  $\mu$ g of microsomal proteins unless indicated otherwise. Assays were incubated at room temperature for 15 min and were terminated by the addition of 700  $\mu$ L of chloroform/methanol (2:1, v/v) followed by 300  $\mu$ L of 0.9% (w/v) NaCl. Tubes were mixed by vortexing, and phase separation was facilitated by centrifugation at  $2,000 \times g$  for 2 min. Organic phase was analyzed by silica G-25 thin layer chromatography (TLC) on silica gel plates in a solvent system of chloroform/methanol/water (65:25:4, v/v/v), followed by radioautography with AR-2000 radio-TLC imaging scanner (Bioscan, Washington, USA). Corresponding bands were scraped, and radioactivity was determined by Beckman LS 6500 Scintillation Counter (Beckman, Mississauga, Ontario). DPM was then converted to the unit of specific activity by using the equation 1.

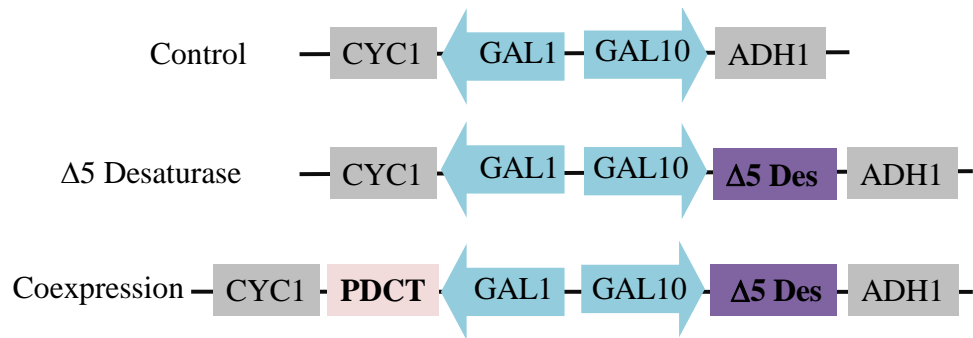
### Equation 1

$$\frac{DPM/(2.22 \times 10^6) \cdot 1\mu Ci}{55\mu Ci/\mu mol} \cdot \frac{1}{0.05mg \times 15min} = \frac{DPM}{91575} nmol \cdot mg^{-1} \cdot min^{-1}$$

### 3.2.3.8 Co-expression of *PDCTs* and $\Delta 5$ desaturase

The *PDCT* genes were cloned into the pESC-HIS yeast expression vector under the *GALI0* promoter. A  $\Delta 5$  desaturase gene from *Thraustochytrium* (Qiu *et al.*, 2001) was cloned by using pHC5 plasmid from our lab as template. *SpeI* recognition cutting sites were added to both forward and reverse primers. Phusion DNA polymerase was used and PCR condition and fragment purification were same as described in section 3.2.3.2.  $\Delta 5$  desaturase gene was cloned into pGEM-T vector before digested by *SpeI* whose cutting sites were found in MCS1 in pESC-HIS (Figure 10). Plasmids of pESC-HIS with *PDCT* gene inserted under the *GALI* promoter cloned into were also digested by *SpeI*. Digested  $\Delta 5$  desaturase gene was ligated with linear pESC-HIS with *PDCT* insert. Therefore, the coexpression plasmid contains *PiPDCT1*, *PiPDCT2* or *AtPDCT* open reading frame under the guidance of the *GALI* promoter and the  $\Delta 5$  desaturase open reading frame under the guidance of the *GALI0* promoter (Figure 12). The control plasmid is pESC-HIS empty vector. Another control plasmid contains the  $\Delta 5$  desaturase open reading frame under the guidance of *GALI0* promoter only. All these plasmids were transformed into a wild type yeast strain DBY747 (Mata; *his3- $\Delta 1$* , *leu2-3*, *leu2-112*, *ura3-52*, *trp1-289*). The transformants were selected on the selection SD-HIS plate. The expression of transgenes was induced using the synthetic dropout medium with 2% (w/v) galactose (SG-HIS) in presence of DGLA and grown at 20 °C for 2 days. After that, the cells were collected for lipid class analysis and fatty acid profiling as described below.





**Figure 12.** Plasmids for the coexpression study. The control plasmid contains the *GAL1* promoter and *CYC1* terminator, *GAL10* promoter and *ADH1* terminator only. The  $\Delta 5$  desaturase plasmid contains the  $\Delta 5$  desaturase open reading frame under the guidance of *GAL10* promoter. The coexpression plasmid contains *PiPDCT1*, *PiPDCT2* or *AtPDCT* open reading frame under the guidance of the *GAL1* promoter and the  $\Delta 5$  desaturase open reading frame under the guidance of the *GAL10* promoter.

### **3.2.3.9 Fatty acid analysis and lipid class analysis**

Yeast transformants cultured in SG-HIS liquid media at 20 °C for 2 days were pelleted by centrifugation (4,000 ×g, 10 min) and cell pellets were washed once with 10 mL of 1% (w/v) tergitol solution and twice with 10 mL water. FAME analysis followed the protocol described in 3.1.2.

For lipid class analysis, the total lipids were extracted from yeast cell cultures according to Wu *et al.* (2005). Yeast cultures were precipitated by centrifugation (4,000 ×g, 10 min) and cell pellets were washed once with 10 mL of 1% (w/v) tergitol solution and twice with 10 mL water. The yeast cell pellet was then dried under vacuum at ambient temperature. Five mL of chloroform/methanol 2:1(v/v) was added to the yeast samples in a glass tube and homogenized by a high speed homogenizer. After the centrifugation at 2,000 rpm for 10 min, the resulting organic phase was collected and dried with nitrogen gas. The sample was then dissolved in chloroform, representing the total lipid extract. The lipid extracts were resolved on TLC plates. Neutral lipids were developed with hexane/diethyl ether/acetic acid (70:30:1, v/v/v), and phospholipids with chloroform/methanol/water (65:25:4, v/v/v). The individual lipid class was identified by dying with iodine and each lipid class was scraped and used for direct transmethylation of fatty acid analysis as described above.

## **3.2.4 Results**

### **3.2.4.1 Homology search and sequence analysis of putative *PDCT* genes**

Phylogenetic analysis, topology prediction as well as amino acid sequence alignment all pointed to that PDCT is related to SMS (Lu *et al.*, 2009). Therefore, SMS protein sequences from human as well as AtPDCT protein sequence from *Arabidopsis* were used as query sequences to search *P. infestans* genomic database. Two homologous sequences from *P. infestans* were identified (*PiPDCT1*, GeneBank accession number: XM\_002899047 and *PiPDCT2*, GeneBank accession number: XM\_002907783) by the search. Two full-length cDNAs of *PiPDCT* from *P. infestans* are 1,272 bp and 1,221 bp

in length, which encode polypeptides of 424 and 407 amino acids, respectively. Both PiPDCT1 and PiPDCT2 protein sequences contain two conserved domains found in AtPDCT and human SMSs (Figure 13) and share 12.6% and 13.3% amino acid identity to AtPDCT protein, respectively.

Hydropathy profile analysis showed that the putative PDCTs from *P. infestans* are integral-membrane proteins. PiPDCT1 and PiPDCT2 have 8 and 7 putative transmembrane domains, respectively.

#### **3.2.4.2 Amplification of putative *PDCT* genes from *P. infestans***

The total RNA was isolated from the *P. infestans* mycelium. As shown in Figure 14, the good quality RNA was obtained with no obvious degradation occurring in the sample. The spectrophotometer measurement showed that the OD 260/280 ratio of the RNA sample was 1.714 and the concentration was 839.63 µg/mL. The first-strand cDNA was then synthesized from the RNA sample and used as template for the PCR amplification of putative *PDCT* genes. The PCR product was electrophoresized on agarose gel. As shown in the Figure 15, a major band with the expected size was observed in the PCR amplification of *PiPDCT1* and *PiPDCT2*, respectively.

As a positive control, *AtPDCT* was also amplified from *A. thaliana*. The PCR amplification product was examined on an agarose gel. A major band with the expected size (906 bp) was observed (Figure 15).

#### **3.2.4.3 Cloning of putative *PiPDCTs* and *AtPDCT* into intermediate pGEM-T vector**

After the amplification, putative *PDCT* cDNA fragments were added with A tail, and ligated with pGEM-T vector. The recombinant plasmids were transformed into *E. coli*. The transformants were screened on blue-white plates as well as by colony PCR using gene specific primers.

```

AtPDCT      -----MSFAAETDVSLERRSNSINGNE TNG
SMS1        MLSASTMKEVVYWSPKKVADWLEENAMPEYCEPLEHFTGQDLINLTQEDFKKPLCRVSSDNGQRITLIMTEFLKMEHRIE
SMS2        -----MIIETAPKLEFRIE
PiPDCT1     -----MTSYLPFAQSNLFRTRKRKMSGFVGTWGLSDEKNMVS RADINSTPVS VGGGFS PRHWQI SVDYREVASFVL
PiPDCT2     -----MESWTDYFTSHFYAMKARPVSAFDPAGFIIVISAFLLLVGVVAITAFALVSRG LLLLRSRVEKNIQ

AtPDCT      VVLDCTLDNNR---RVGDTNTHMDISAKKTDNRYANGVGGGG-WRSKASFTIWTARIVYVVRVYHWIPCMFA----AGL
SMS1        AHKNCHANGHIN--ICVDIPTPDCSFSIKIKPNCMPNRYR---EMTKIPMPELEFSQYEMEWKRTFLAFLIA----LSC
SMS2        NQPSDPTNTYARPAEPVEEENKNGKPKSLSSLRKCTKKYP-DYICAMPEESENKFELEWKKIGIAFLIA----VFN
PiPDCT1     FFLYMWEDVGLGVMVWVTFKFIIEPDQHRSEATWGGIWLFLAGTEIVRRLVRSFVIHIRGMMDHAEIFVZ---MSLV
PiPDCT2     DMSVYSDQRTF--VGENLQYIKRAIDHSGFQFIAEMAS---VAYLSRLAEGLSIFAIASYLNTLFAVITGWRTPNV

AtPDCT      LFFMGVEYTLQMI PARSEPFLLCFVVTSLNRLVASSD LNTMLAALNTVEMCMQTHYVVTW----LVECFARATLAAI
SMS1        FVLTITVMISVVEHVEEK--EVQPELEETEEEFNRYQRFESICETNGMLIVGLWLIGWLLIKYKSTLSEEFQIVGTI
SMS2        IVLITVMITVVEHVEEK--ELSPLEEDREEDYIDRVKRAFSSSEINGIIVGLWITGWLIFERKYKLVGRSECFIIGTI
PiPDCT1     MVFSVNI AFYLEVPA TMLPKDLICFMI IPEQGLESKWRVSDILTAG---VPMFLLQTFMTEPNRCKIVSSFRLATI C
PiPDCT2     VILDLAKESTDEKTLIDLGHLLFRFVMTIYQTTYIEFDLPDEF--LAAMGLTSEFLLIHPRRLILGSECFIFAI I

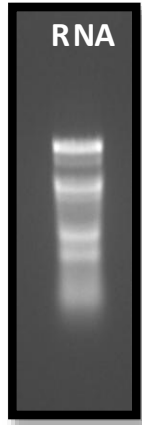
AtPDCT      FMFTGRGILG-----YSTQPLPQDFLGS CVDFPVGN---VSFFIFESCHVAGSMIASIDMRRM
SMS1        NLYRCHTMVMTILE--VEGMHEGNSPKLFGDWEZQLRRTMKLACCCGSLTIGS--FNMGGDYLVSCHEVMTITYLEKEL
SMS2        NLYRCHTMVMTILE--VEGMHEGCAFKLNGLSQAKVQRLRLISCCGSLTIGS--FIIQGFILSCHEVMTITYLEKEY
PiPDCT1     YFLRMLIVSMISLEGPAEHCRAGSS---LYFPPTTWIDIVTEVCPIYGNYN---SQGDIIESCHMAYTISAVILYLR T
PiPDCT2     NVMRACCVAVHSLDASEMCISCFDSKERENYKSLPIFPKAFEFKAWKVIREFQFITGGLMIESCHVAVLILCCMARTY

AtPDCT      QRLR-----LAMVFDTINVLQSRILICTRCHMIDLAVG---VGAGILFDSIAG-----
SMS1        SER-----RIMMHWLIGWLLSVVCTFQHLLAHDEMTVDMVVEVMITTRILEWVYETMANQQVLKEASQMNLLA
SMS2        SER-----HFTWVHLICWLLSAACITICHTVAHEHMTIDVITAYNITTELEWVYEMENKLNKLVSSQTNFLS
PiPDCT1     LDRNFAG-----RTASKIRWACGMAYLTIIAALCTAG--RKHMTVIVN LGLIISTLVGFHFHESWTPLCFQVPDGLLPVG
PiPDCT2     CVRSELNTPFTRRFPCLMIRIYYNYILSACCFATVCTRIHFTLLMLIIMTIIQVWFTHWLTNHP-----

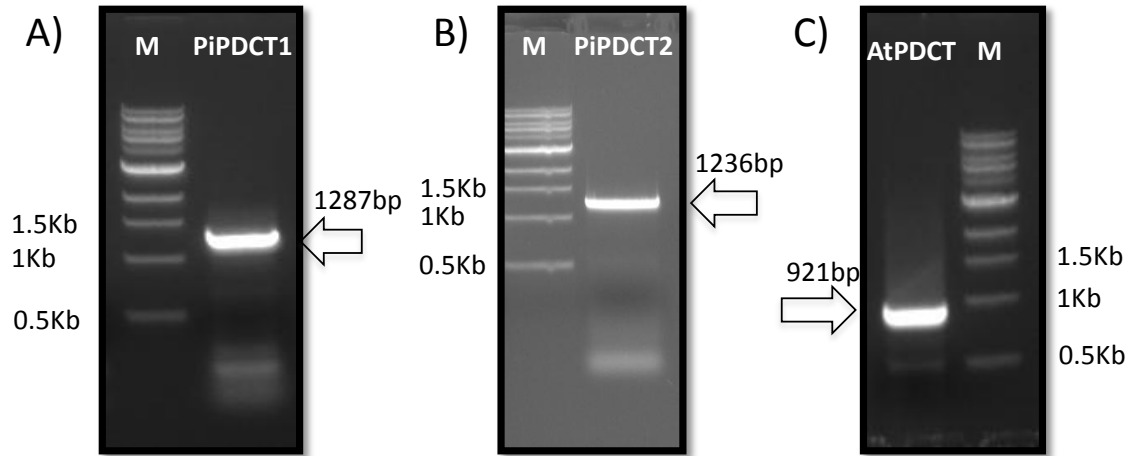
AtPDCT      -----KYEFMMSKRHLGTGSLISKDS-----LVN
SMS1        RVWVYRPECYHEKNVCCIMPRSYHWEFPPV-VVHLSRQVYKSELVNDT
SMS2        RAWVHEIEYFHEKNVCCSIPCCSWEELSPPGCFKSSCKKMSRVQKIGEDNEKST
PiPDCT1     ELQRIYGSQFRKRFMSMDVPDEALNELDDTEVDEEARRLIKTNASTYNQVQLIC
PiPDCT2     -ENSRVINWLFHAEVHLMDHNAYRKARRS-----G--SQSDRIEKTD

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**Figure 13.** Alignment of AtPDCT, human SMS1, SMS2 and putative PDCTs identified from *P. infestans*. GenBank accession number and source of each protein: AtPDCT, AEE75730, *Arabidopsis thaliana*; SMS1, Q86VZ5, *Homo sapiens* (human); SMS2, Q8NHU3, *Homo sapiens* (human). PiPDCT1, XM\_002899047, *P. infestans*; PiPDCT2, XM\_002907783, *P. infestans*; Two conserved motifs are highlighted by blue frames. Shaded in the dark black are the regions of the protein sequences that are highly conserved among the sequences.



**Figure 14.** Agarose gel electrophoresis of the total RNAs isolated from *P. infestans*. Five  $\mu\text{L}$  RNA was denatured at  $65^{\circ}\text{C}$  for 5 min, cooled down on ice for 2 min before loaded into 1.2% agarose gel.



**Figure 15.** Amplification of the open reading frames of putative *PDCT*s from *P. infestans* and *AtPDCT* from *Arabidopsis* with specific primers. M, 1 kb ladder marker. A, *PiPDCT1*; B, *PiPDCT2*; C, *AtPDCT*.

Four positive colonies of each gene were selected for plasmid digestion analysis by restriction enzyme *SalI* whose cutting site was found in both the pGEM-T vector and the *PiPDCT1* insert. After digestion step, two plasmids of each gene were then subjected to further sequencing analysis with two primers in pGEM-T vector. Comparison of the sequencing results with sequences in the database showed that no nucleotide error has occurred during the amplification of *PiPDCT1*. Plasmids of pGEM-*PiPDCT2* and pGEM-*AtPDCT* were constructed by the same procedures.

#### **3.2.4.4 Cloning of *PiPDCTs* in yeast pESC-HIS vector**

The oligo-nucleotides with restriction enzyme *BamHI* were added on 5' ends of gene specific primers to facilitate the cloning process. The digestion of pESC-HIS with *BamHI* resulted in a linear vector, while the digestion of pGEM-*PiPDCT1* resulted in linear pGEM-T vector and *PiPDCT1* insert. The DNA fragments of linear pESC-HIS and *PiPDCT1* were isolated from agarose gel and ligated. The ligated plasmid was transformed into *E. coli*, and the transformants were selected on a LB ampicillin plate and screened by PCR using two specific primers. Plasmids of two positive colonies for each gene that might contain the insert in the right orientation were extracted and sequenced. The sequencing result confirmed the right sequence and orientation in the plasmid which was then used for the yeast transformation.

To study the function of the putative *PDCT*, the plasmid with the right insert was introduced into the yeast *CPT* and *EPT* double mutant. The transformants were selected on SD-HIS plates and confirmed by PCR screening. One colony for each gene confirmed with the plasmid was used in *in vitro* assay. *PiPDCT2* and *AtPDCT* were cloned into pESC-HIS and transformed into the yeast double mutant using the same procedures.

#### **3.2.4.5 *In vitro* PDCT assays**

The first experiment of enzymatic assays was conducted using 100 µg microsomal proteins of the yeast *CPT* and *EPT* double mutant expressing pESC-HIS empty vector (EV), pESC-*AtPDCT'* (one amino acid substitution from the wild type

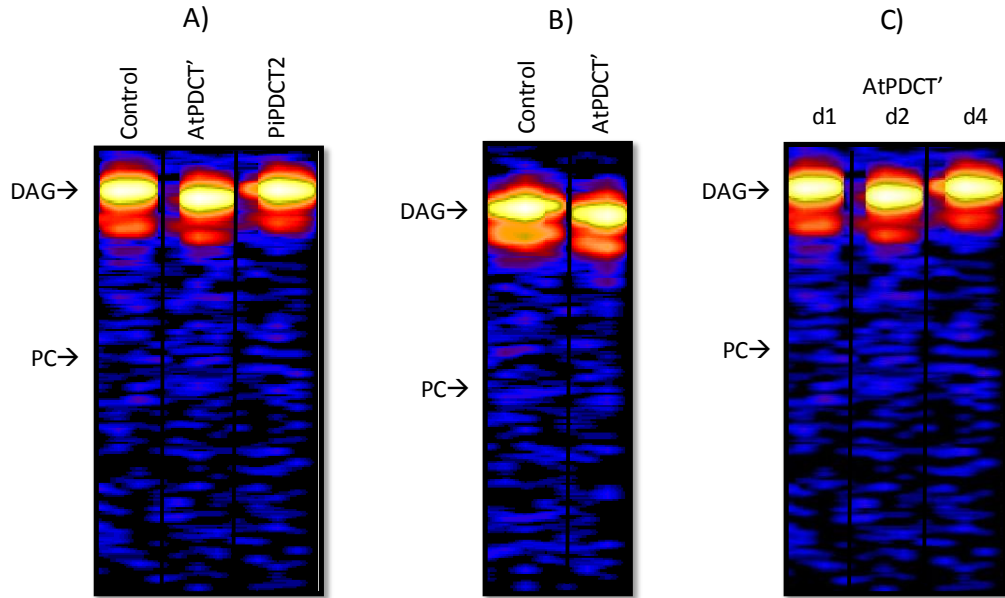
*Arabidopsis* PDCT found by sequencing), and pESC-*PiPDCT2*, respectively. However, there was no radioactive-labelled PC produced in all the reactions when [<sup>14</sup>C-glycerol] diolein and dioleoyl-PC were used in the assay. This result implies that either the assay protocol we adopted did not work as the positive control AtPDCT' did not produce radioactive PC or one amino acid substitution in AtPDCT' might affect the function (Figure 16A). To improve the assay condition, the amount of the microsomal protein was increased from 100 to 600 µg in the assay. However, still no product was produced in the reaction with the positive control (Figure 16B). Considering the *GALI* promoter is an induction promoter and the induction time might be critical for gene expression, the induction time was thus extended from normal four hours to one day, two days and four days, respectively. However, the radioautography of the reactions with the positive control AtPDCT' still did not detect any activity (Figure 16C).

We also did assays by switching the yeast host strain from the DBY747- $\Delta$ *CPT*/ $\Delta$ *EPT* to HJ091 and INVSc1 to see whether the host strain would make difference. Both CE and MP of yeast cells were used for the PDCT assay and there was still no radio-labeled PC produced in the reactions (Figure 17).

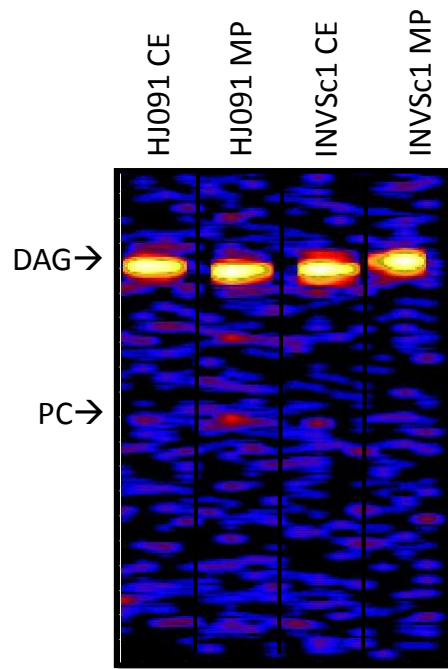
As the positive control AtPDCT' did not show any activity in our assays, I wondered whether the single substitution at position 184 of AtPDCT', where serine is replaced by glycine, would be the cause of the result although it occurs outside the two conserved domain regions. Therefore, I cloned a new *AtPDCT* gene from an *Arabidopsis* silique cDNA library which encodes a polypeptide with 100% amino acid identity to the published sequence. The enzymatic assay with this new gene in pYES2.0 produced weak signal at the PC position of radioautography, indicating the positive control has weak PDCT activity in this expression system. Meanwhile, using microsomal protein isolated from HJ091 expressing *AtRODI* from Dr. Lu, AtRODI showed slightly higher PDCT activity than AtPDCT (Figure 18).

Since the positive control worked, I then preceded the enzymatic assays with the two putative PDCTs, PiPDCT1 and PiPDCT2. The results showed repetitively that no

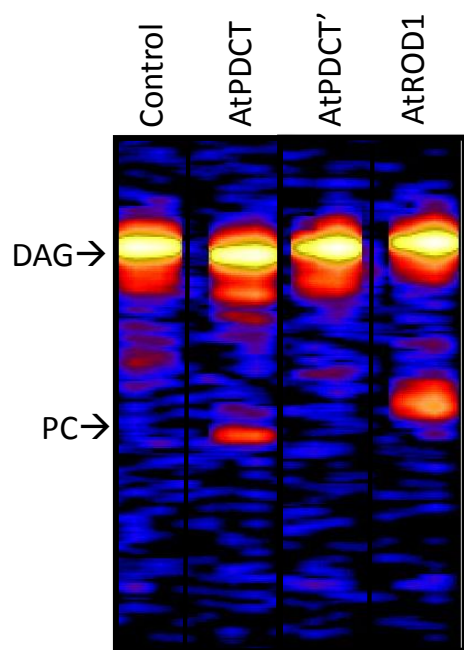




**Figure 16.** Radioautography of PDCT assays with DBY747- $\Delta$ CPT/ $\Delta$ EPT expressing *AtPDCT'* and *PiPDCT2*, respectively. Lipids were separated in TLC plate developed with chloroform/methanol/water (65:25:4, v/v/v) system. Positions of DAG and PC were indicated in radioautogram. A, 100  $\mu$ g microsomal proteins of control (pESC-HIS empty vector), *AtPDCT'* and *PiPDCT2* used in the assays; B, 600  $\mu$ g proteins of control (pESC-HIS empty vector) and *AtPDCT'* used in the assay; C, 100  $\mu$ g microsomal proteins of *AtPDCT'* induced for one day, two days and four days, respectively, and used in the assay. This experiment was performed once.



**Figure 17.** Radioautography of PDCT assays of HJ091 and INVSc1 expressing *AtPDCT*. Lipids were separated in TLC plate developed with chloroform/methanol/water (65:25:4, v/v/v) system. Positions of DAG and PC were indicated in radioautogram. *AtPDCT* with one amino acid difference in pESC-HIS vector was used to transform into yeast strains, HJ091 and INVSc1. Enzyme sources are crude extract and microsomal protein from transformed yeast cells, respectively. CE, crude extract; MP, microsomal protein. This experiment was performed once.



**Figure 18.** Radioautography of PDCT assays with different *Arabidopsis PDCT* genes. Lipids were separated in TLC plate developed with chloroform/methanol/water (65:25:4, v/v/v) system. Positions of DAG and PC were indicated in radioautogram. Control, the yeast strain HJ091 with pYES2.0 empty vector; AtPDCT, the yeast strain HJ091 with newly cloned *PDCT* gene in pYES2.0 vector; AtPDCT', the yeast strain HJ091 with *AtPDCT* containing one amino acid difference in pYES2.0 vector; AtROD1, the yeast strain HJ091 with *AtPDCT* gene in p424GPD, obtained from Dr. Lu. This experiment was performed once.

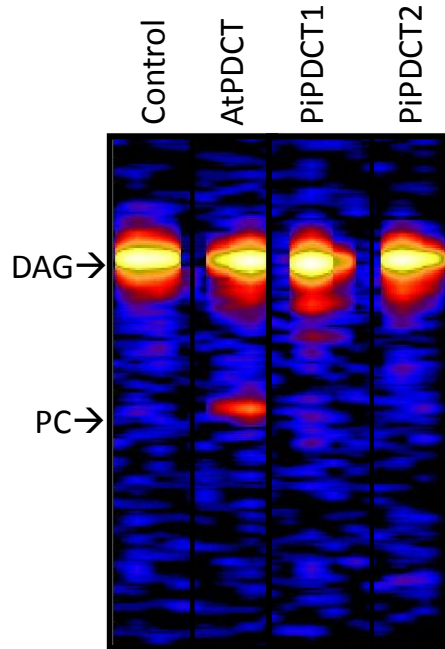
PDCT activities were observed in the enzymatic assays with either of putative PiPDCTs, while the positive control showed the activity, albeit weak (Figure 19). Dr. Lu from Montana State University (Lu *et al.*, 2009) also helped examine the activity of these two putative PDCTs from *P. infestans* in his lab and the same result was obtained.

#### 3.2.4.6 Co-expression of putative *PiPDCT* with $\Delta 5$ desaturase in yeast

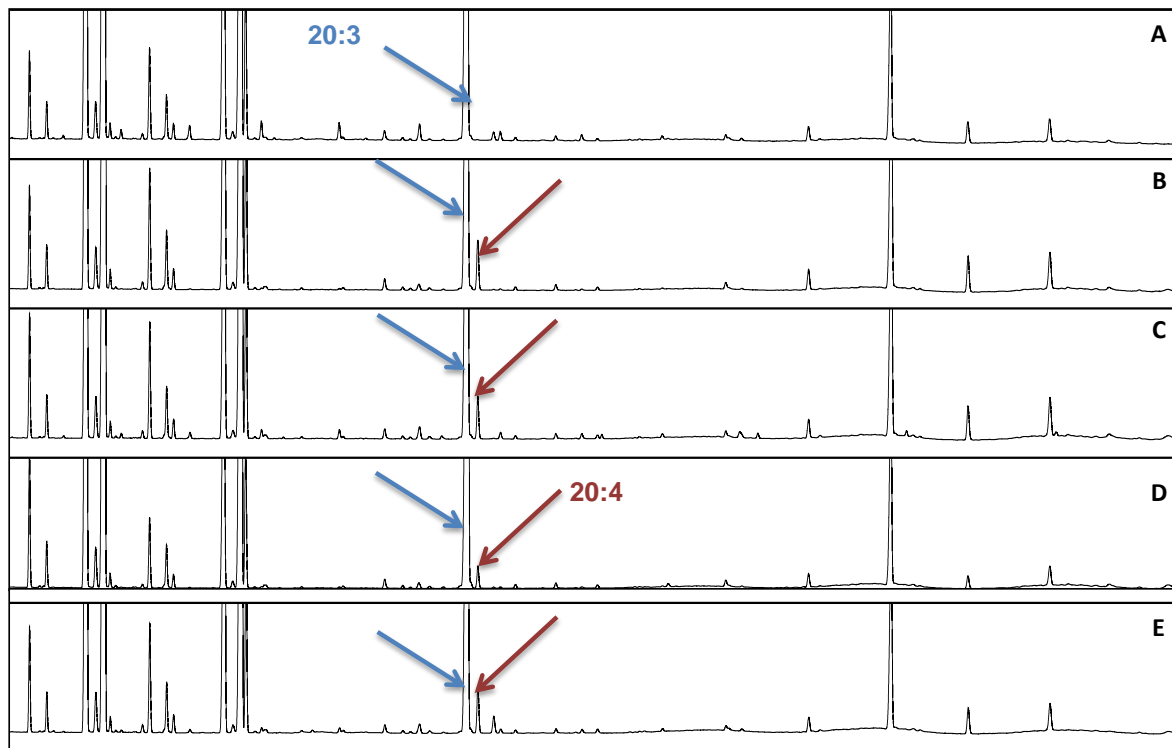
In this co-expression study, *PiPDCTs* or *AtPDCT* was cloned along with  $\Delta 5$  desaturase in pESC-HIS for the examination of the possible effect of PDCTs on the biosynthesis of VLCPUFAs. The rationale behind the experiment is that exogenously fed DGLA (20:3) could be readily acylated to PC via acyl-editing process (Bates *et al.*, 2007) where  $\Delta 5$  desaturase would convert 20:3-PC to 20:4-PC. Functional PDCT would then produce 20:4-DAG from 20:4-PC which would lead to the increased synthesis of 20:4-TAG.

Figure 20 shows the total fatty acid profile of the co-expression yeast. The control yeast had no  $\Delta 5$  desaturase, thus no 20:4 was produced when fed with 20:3. In the yeasts transformed with the  $\Delta 5$  desaturase gene, 20:4 was detected, albeit in a small amount, indicating the  $\Delta 5$  desaturase was functional in yeast. However, no difference in the amount of 20:4 was observed between the yeast strains expressing the  $\Delta 5$  desaturase alone and expressing the  $\Delta 5$  desaturase and the putative *PiPDCT* simultaneously (Figure 20).

To examine if there is difference in the amount of 20:4 in different lipid classes, PC, DAG and TAG were fractionated from the total lipids from the strains for their fatty acid composition analysis. Again, no difference in the amount of 20:4 was observed among the lipid classes. This co-expression result confirmed, although indirectly, the two putative PDCT genes do not encode functional PDCT.



**Figure 19.** Radioautography of PDCT assays with AtPDCT and PiPDCTs. Lipids were separated in TLC plate developed with chloroform/methanol/water (65:25:4, v/v/v) system. Positions of DAG and PC were indicated in radioautogram. Control, the yeast strain INVSc1 with empty vector pYES2.0. AtPDCT, the yeast strain INVSc1 with *AtPDCT* in pYES2.0. PiPDCT1, the yeast strain INVSc1 with *PiPDCT1* in pYES2.0. PiPDCT2, the yeast strain INVSc1 with *PiPDCT2* in pYES2.0. This experiment was performed once.



**Figure 20.** Co-expression of *PiPDCT* and a  $\Delta 5$  desaturase from *Thraustochytrium sp.*. Fatty acid methyl esters were prepared from the coexpression yeast. A, the control plasmid (pESC-HIS vector only); B,  $\Delta 5$  desaturase only plasmid,  $\Delta 5$  desaturase open reading frame under the guidance of *GAL10* promoter in pESC-HIS; C, coexpression plasmid,  $\Delta 5$  desaturase open reading frame under the guidance of the *GAL10* promoter and *PiPDCT1* open reading frame under the guidance of the *GAL1* promoter in pESC-HIS; D, coexpression plasmid,  $\Delta 5$  desaturase open reading frame under the guidance of the *GAL10* promoter with *PiPDCT2* open reading frame under the guidance of the *GAL1* promoter in pESC-HIS; E, coexpression plasmid,  $\Delta 5$  desaturase open reading frame under the guidance of the *GAL10* promoter with *AtPDCT* open reading frame under the guidance of the *GAL1* promoter in pESC-HIS. This experiment was performed once.

### 3.2.5 Discussion

Function of PDCT is to convert PUFA-PC into PUFA-DAG (Lu *et al.*, 2009). This enzyme is thus expected to be able to transfer VLCPUFAs from PC to DAG in VLCPUFA-producing microbes, which can be valuable for solving the bottleneck of limited VLCPUFAs accumulation in transgenic oilseed plants. One of the attempts in this study was to identify a functional PDCT from *P. infestans*, which accumulates 10.4% EPA. Identification of PDCTs from *P. infestans* is straightforward because its genome sequence is available. Two putative PDCT genes were found in the genome of *P. infestans* by using homologous search using AtPDCT and SMS as query sequences. These two genes were then cloned from the cDNAs of *P. infestans* and ligated into a yeast expression vector pESC-HIS. As a positive control, the AtPDCT cDNA from *Arabidopsis* was also cloned into pESC-HIS for the comparison. All these constructs were transformed into a yeast double mutant and the microsomal proteins of transformants were used for the enzyme assays.

The yeast expression system has well been established in our lab to study the function of heterologous lipid enzymes. Following the published protocol (Lu *et al.*, 2009), the *in vitro* assays were conducted to provide functional properties of cloned putative PDCTs from VLCPUFA-producing microbes. This information would help us elucidate the role of PDCT in acyl exchange between PC and DAG during the biosynthesis of VLCPUFAs.

When I initially used DBY747- $\Delta$ CPT/ $\Delta$ EPT as a host and pESC-His as an expression vector to express the PDCT genes, no activity was detected with the positive control, AtPDCT'. I then re-amplified the positive control gene AtPDCT and cloned it into two vectors, pESC-HIS and pYES2.0, and expressed them in different yeast hosts. By using this new gene as the positive control for the assays, still no product was detected in the assay with either of putative PiPDCTs, while the assays with the positive control showed the activity, albeit weak.

Since I could not detect any PDCT activity with the two putative PiPDCTs, I then sent the yeast strains containing the genes to Dr. Lu's lab in USA for their help to examine the activity. Again, no activity was detected with the genes in his lab by using his *AtROD1* in pYES2 vector as the control. Since both labs could not detect the PDCT activity with the *Phytophthora* genes, I believe that the putative *PDCT* genes I cloned from *P. infestans* do not encode active PDCT enzymes. It is noteworthy that At3g15830, an *Arabidopsis* homolog which shares very high sequence similarity to *AtROD1*, was also observed with no PDCT activity (Chaofu Lu, personal communication). In addition, it is noted that although the putative PiPDCTs contain the conserved domains shared with *AtPDCT*, they actually have only about 13% amino acid identity throughout the entire sequence with *AtPDCT*. Thus, this result along with those of the co-expressions indicates that putative *PiPDCT1* and *PiPDCT2* genes I cloned from *P. infestans* are related to *Arabidopsis AtPDCT*, but do not encode functional PDCT.



### 3.3 Study 3: Cloning and functional characterization of *CPT* and *EPT* from VLCPUFA-producing microorganisms

#### 3.3.1 Abstract

Four putative *CPT* and *EPT* genes (*PiCPT1*, *PiCPT2*, *PiEPT* and *TaCPT*) from VLCPUFA-producing microorganisms such as *P. infestans* or *T. aureum* have been identified. Sequence analysis of these sequences reveals structural features of these proteins, such as hydropathy profile and conserved domains, which are indicative of possible function of these putative genes.

The microsomal fractions prepared from a yeast  $\Delta CPT/\Delta EPT$  double mutant expressing putative *CPT* and *EPT* genes from VLCPUFA-producing microorganisms were used to evaluate enzymatic activity of these putative genes. The result shows that *PiCPT1* and *TaCPT* have *CPT* activity and *PiEPT* has *EPT* activity, while *PiCPT2* has neither activity.

To determine substrate specificity of functional *CPT* and *EPT* genes cloned from VLCPUFA-producing microorganisms, different DAG species were employed in *in vitro* assays using microsomal fractions prepared from the double mutant expressing these genes. The result shows that *PiCPT1* has strong substrate preference to VLCPUFA-containing DAG species such as 18:0/20:4-DAG and 18:0/22:6-DAG. *PiEPT* has slight substrate preference to VLCPUFA-containing DAG species.

The transcript level of *PiCPT1* was analyzed in *P. infestans* grown in presence of exogenous PUFAs by quantitative real-time PCR. The result indicates that exogenous supplementation of VLCPUFAs such as ARA could significantly induce the expression of *PiCPT1*.

#### 3.3.2 Hypothesis

*CPT* and *EPT* catalyze the final reaction of *de novo* synthesis of phospholipid, PC and PE, by using DAG as substrate. Genes encoding *CPT* and *EPT* enzymes have been

identified in model eukaryotes such as yeast, plants and mammals, but not in any VLCPUFA-producing organisms. It is my hypothesis that CPT and EPT from VLCPUFA-producing microorganisms might play a role in acyl-trafficking between phospholipids and neutral lipids during the biosynthesis of VLCPUFAs. Specifically, these gene products would have capacity to efficiently use VLCPUFA-DAGs as substrates to synthesize VLCPUFA-PCs. Furthermore, the transcript level of *CPT* and *EPT* genes in VLCPUFA-producing microorganisms would respond to changes of fatty acid profiles.

### **3.3.3 Experimental approach**

#### **3.3.3.1 Sequence analysis**

Putative *CPT* and *EPT* genes (*PiCPT1*, *PiCPT2*, *PiEPT* and *TaCPT*) were found in *P. infestans* genome database and *T. aureum* EST database by homology search using yeast ScCPT1 (GenBank accession number: AAA63571) and ScEPT1 (GenBank accession number: AAA63572) protein sequences as query sequences. DNASTAR program was used for the alignment of putative CPT and EPT proteins from VLCPUFA-producing microorganisms and related sequences. Hydropathy analysis was used to predict topology of these putative CPT and EPT from VLCPUFA-producing microorganisms (<http://www.vivo.colostate.edu/molkit/hydropathy/>). Phylogenetic tree of CPT and EPT sequences from diverse organisms was constructed by the program MEGA5.1 Beta3 (Tamura *et al.*, 2011). Window size was set as 19. Peaks greater than 1.6 in vertical axis indicate the presence of a hydrophobic domain.

#### **3.3.3.2 Cloning of putative *CPT* and *EPT* genes**

Putative genes as well as positive control genes (*ScCPT1* and *ScEPT1*) were previously cloned by RT-PCR as described in section 3.2.3.2. All the putative genes and positive control genes were first cloned into intermediate vector, pGEM-T (as described in section 3.2.3.3) and then cloned into a yeast expression vector pSCW231 (the plasmid contains a tryptophan selection marker) under the control of the ADH1 promoter, respectively. Four putative *CPT* and *EPT* genes (*PiCPT1*, *PiCPT2*, *PiEPT* and *TaCPT*)

and positive control genes (*ScCPT1* and *ScEPT1*) were successfully cloned from VLCPUFA-producing microorganisms and *S. cerevisiae* and the plasmids containing each gene were successfully constructed in our lab (Table 5).

### **3.3.3.3 Yeast transformation and microsomal preparation**

Plasmids were transformed to yeast *CPT* and *EPT* double mutant (DBY747- $\Delta CPT/\Delta EPT$ ) followed section 3.2.3.5 with the exception that yeast transformants were grown on amino acid dropout plates (SD-TRP). Transformed colonies were picked up to do PCR as described in section 3.2.3.5 except using the ADH1 forward primer and the gene specific reverse primer (Table 5).

The confirmed yeast transformants were grown at 30 °C in liquid SD-TRP. The overnight cultures were used to inoculate 50 mL of SD-TRP to give an initial OD<sub>600</sub> at 0.4-0.5 and grow at 30 °C one day to an OD<sub>600</sub> at 2.5-3. The cells were harvested and microsomal proteins were prepared as described in section 3.2.3.6.

### **3.3.3.4 Activity assays of CPT and EPT**

Because of the similarity between *de novo* synthesis of PC and PE, some EPTs have CPT activity (Hjelmstad and Bell, 1991; Qi *et al.*, 2003; Henneberry *et al.*, 2000). Therefore, putative *CPT* and *EPT* gene products were used for both CPT assay and EPT assay. An emulsion-based method described by Henneberry (2000) was adopted for the initial CPT and EPT activity assay with some modifications. 1, 2-dioleoyl-sn-glycerol (di18:1-DAG, 600  $\mu$ M) was dried under nitrogen gas and resuspended in 50  $\mu$ L of 0.016% (w/v) Tween 20 by sonication. The tube was then added with the assay buffer at the final concentration of 100 mM Tris-HCl (pH 7.5), 20 mM MgCl<sub>2</sub> and 50  $\mu$ g of microsomal protein in a total volume of 200  $\mu$ L. The mixture was incubated at room temperature for 5 min to allow for the lipids to be incorporated into the microsomal membrane and this was followed by the addition of [<sup>14</sup>C] CDP-choline or [<sup>14</sup>C] CDP-ethanolamine (American Radiolabeled Chemicals Inc, St Louis, USA) at the final concentration of 4.05  $\mu$ M. The mixture was incubated at 23 °C for 15 min.

**Table 5.** Sequence information of the open reading frames of putative *CPT* and *EPT* genes from VLCPUFA-producing microorganisms and *CPT* and *EPT* genes from *S. cerevisiae*.

<b>Gene Name</b>	<b>Source</b>	<b>DNA (bp)</b>	<b>Gene reverse primer</b>
<i>ScCPT1</i>	<i>S. cerevisiae</i>	1182	HC46: 5'CGCGGATCCCTAAATTCTTTTGG3'
<i>ScEPT1</i>	<i>S. cerevisiae</i>	1176	HC48: 5'CGCGGATCCTTATGTCAGCTTGG3'
<i>PiCPT1</i>	<i>P. infestans</i>	1182	HC19: 5'CCGCTCGAGCTACTTCGCCTTTTCAG3'
<i>PiCPT2</i>	<i>P. infestans</i>	1224	HC21: 5'CCGCTCGAGCTATCCTTGCTTCACC3'
<i>PiEPT</i>	<i>P. infestans</i>	1281	HC23: 5'CCGCTCGAGTCACGATGACTTTGCTGC3'
<i>TaCPT</i>	<i>T. aureum</i>	1239	HC50: 5'CCGGAATTCTCACGCATCGTCGATTTG3'

The reaction was terminated by the addition of 1 mL CHCl<sub>3</sub>/CH<sub>3</sub>OH (2:1, v/v) and 300 µL 0.9% (w/v) KCl. The phase separation was facilitated by centrifugation at 2,000 ×g for 2 min. The aqueous phase was removed and the organic phase was washed twice with 500 µL of CH<sub>3</sub>CH<sub>2</sub>OH/H<sub>2</sub>O (2:3, v/v). An aliquot of washed organic phase was dried and dissolved in 50 µL CHCl<sub>3</sub>/CH<sub>3</sub>OH (2:1, v/v). The results were detected as described in section 3.2.3.7.

### **3.3.3.5 Substrate specificity of the CPT**

Microsomal fraction preparation and enzyme activity assays followed the procedures as described in section 3.3.3.4. The DAG species containing di16:0, di16:1, di18:0, di18:1, di18:2, 18:0/20:4 and 18:0/22:6 fatty acids (Nu-chek prep, Inc. Elysian, USA and Sigma-Aldrich, St. Louis, USA ), which have been found in the *P. infestans* and *T. aureum*, were used as the substrates for studying the substrate preference of the *CPT* and *EPT* genes. The other substrate in the assays was [<sup>14</sup>C] CDP-choline or [<sup>14</sup>C] CDP-ethanolamine. Reactions with only fatty acid substrate but no [<sup>14</sup>C] CDP-choline or [<sup>14</sup>C] CDP-ethanolamine substrate were used as negative control.

### **3.3.3.6 Kinetic study of PiCPT1**

For the kinetic study of PiCPT1, the sufficient amount of 18:0/20:4-DAG (1.2 mM) along with different concentrations of CDP-choline, 0 µM, 10 µM, 20 µM, 40 µM, 80 µM, 160 µM, 320 µM were used in the *in vitro* assays described in section 3.3.3.4. The kinetic constants were estimated from Lineweaver-Burke plots by Microsoft-Excel (<http://www.biotech.sfasu.edu/bt/BTC560/HOW%20TO.htm>) using the average of triplicate measurements.

### **3.3.3.7 Gene expression analysis of *P. infestans* grown in presence of exogenous PUFAs**

*P. infestans* was cultured in pea broth, shaking at 20 °C, 200rpm for 4 weeks. Cells were harvested by vacuum filtration. The biomass of 0.12 g was inoculated to fresh

media fed with 200  $\mu\text{M}$  of OA (18:1), LA (18:2), ALA (18:3), ARA (20:4), EPA (20:5), DHA (22:6), separately, along with 0.1% (v/v) tergitol. Biomass not fed with any fatty acid was used as negative control. The cultures were grown at 20  $^{\circ}\text{C}$  for 24 hours. After that, biomass was quickly harvested by vacuum filtration, washed with 1% (v/v) tergitol once and  $\text{H}_2\text{O}$  twice, frozen in liquid nitrogen and stored at -80  $^{\circ}\text{C}$ . The biomass of 0.02 g was used for FAME analysis as described in section 3.1.2.

Total RNA was isolated from 0.1 g biomass using RNeasy Plant Mini Kit (Qiagen, Toronto, Ontario). The possible DNA contamination was removed by treating the RNA sample with RNase-free DNase Set (Qiagen, Toronto, Ontario). The cDNA was synthesized with 1  $\mu\text{g}$  purified RNA as template using qScript cDNA SuperMix according to the manufacturer's directions (Quanta, Gaithersburg, MD, USA).

### 3.3.3.8 Real-time qPCR amplification

Actin A (Avrova *et al.*, 2003; Grenville-Briggs *et al.*, 2005; GenBank Accession number: M59715.1), actin B (Avrova *et al.*, 2003; GenBank Accession number: M59716.1) as well as elongation factor 2 (Grenville-Briggs *et al.*, 2005; Llorente *et al.*, 2010; GenBank Accession number: EEY57133.1) were employed as housekeeping genes. *PiCPT1* were target gene. Primers were designed by Primer3Plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>, Untergasser, *et al.*, 2007, parameters were set as: design amplicon, 80-160 bp; primer size, 18-23; GC%, 50-60%; melting temperature ( $T_m$ ), between 50  $^{\circ}\text{C}$  and 65  $^{\circ}\text{C}$ ) and are listed in Table 6. Real-time PCR assays were carried out in 96-well plates. Each sample was analyzed in a total reaction volume of 20  $\mu\text{L}$  consisting of 50 ng cDNA as templates, 10  $\mu\text{L}$   $2 \times$  intercalating dye SsoFast EvaGreen and 500 nM of forward and reverse primers (Table 6). Reactions were run on a Bio-Rad CFX Real-time PCR Systems (Bio-Rad, Mississauga, Ontario) using the following cycling conditions: enzyme activation at 95  $^{\circ}\text{C}$  for 30 seconds and 45 cycles of denaturation at 95  $^{\circ}\text{C}$  for 5 seconds and annealing/extension at 60  $^{\circ}\text{C}$  for 5 seconds. The specificity of the PCR reactions was confirmed by melting curves analysis of the products in 0.5  $^{\circ}\text{C}$  increasing from 65 to 95  $^{\circ}\text{C}$ , 5 sec/step. The threshold cycle

**Table 6.** Primers used in quantitative Real-Time PCR. F, forward primer; R, reverse primer.

<b>Gene name</b>	<b>Primer name and sequence</b>	<b>Amplicon size (bp)</b>
<i>PiCPT1</i>	YC27-F: 5'ACCCGGTGTGTTCTTGTTC3'	134
	YC28-R: 5'GAAGATCACGAAGCCGAAAG3'	
<i>Actin A</i>	YC29-F: 5'CGTCCACCATGAAGATCAAG3'	112 bp
	YC30-R: 5'TTCGAGATCCACATCTGCTG3'	
<i>Actin B</i>	YC31-F: 5'CTGATGGGAACGTCATTGTG3'	154 bp
	YC32-R: 5'TACAAGTCCCGGCGAATATC3'	
<i>Elongation factor 2</i>	YC33-F: 5'CGTAACATGTCCGTGATTGC3'	92 bp
	YC34-R: 5'TGCTTGGCCGAGATAAATACC3'	

values (Ct) were determined by the cycles that reach the fluorescence threshold line for each gene by CFX Manager Version 2.1 software (Bio-Rad, Mississauga, Ontario). Three technical replicates and three biological replicates were performed and the values of Ct were recorded for both *PiCPT1* and all three reference genes for further analysis. Fold changes of transcript level were calculated as described by Livak and Schmittgen, 2001.

### 3.3.3.9 Statistical analysis

In order to compare among different samples, variance analysis of the *PiCPT1* expression was carried out with the general liner model (GLM) procedure of the SAS statistical package (SAS institute, Cary, NC, USA). *PiCPT1* expression values were submitted to the GLM procedure and Duncan hypothetical test in SAS statistical package. A probability of  $P < 0.01$  was chosen as the level of significance for the statistical tests.

## 3.3.4 Results

### 3.3.4.1 Sequence analysis of putative *CPT* and *EPT* genes

By using *S. cerevisiae* CPT (ScCPT1) and EPT (ScEPT1) as query sequences, three putative *CPT* and *EPT* genes from *P. infestans* and one from *T. aureum* were identified in the databases (*PiCPT1*, *PiCPT2*, *PiEPT* and *TaCPT*). The full-length open reading frames of *PiCPT1*, *PiCPT2*, *PiEPT* from *P. infestans* are 1,182, 1,224, 1,281 bp in length, encoding polypeptides of 394, 408, 427 amino acids, respectively (Table 5). The full-length open reading frame of a putative *TaCPT* is 1239 bp in length coding for a polypeptide of 413 amino acids.

Deduced amino acid sequences of these putative *CPT* and *EPT* genes all contain a CDP-alcoholphosphotransferase conserved domain (Figure 21). Two *PiCPTs*, *PiEPT* and *TaCPT* share 25.5%, 23.5%, 22% and 22.3% amino acid identities to ScCPT1 protein, respectively. A relatively high homology was found at the proximity to the N-terminus of the sequences where the conserved CDP-alcoholphosphotransferase motif was located.



```

ScCPT1 -----MCFEIQSSLGNIKLYKQSDRSEFISNHVLRERKRFAT-IFE
ScEPT1 -----MCFYVEDSHIENIKSKYKQSEDRSLVSKYFLKFEWQRFCF-IFE
PiCPT1 -----MLGKKAIFGIAAKYKACSYTYLDN-VLNHFWTYSAQ-FIF
PiCPT2 -MVLTRSAAAAAPSPTEAEPGAPKQPEPKTKQRNY-RVVTEDCVKRIILYHYSGLASLLYSNVISFLAQLVDHVLVS
PiEPT MSNATYSESETAVEASQIHALKQRVRRHSIGDGKSTYFCQYVSECESEKLRIMFYHGADNSLIVYKHVLTPEMNNFLVK-LIF
TaCPT -----MLVHDYIERSGLDARLNYKYKSCSEYSWIDL-QLNNEFWCAE-QLF

ScCPT1 LWAAPNIIVLLGFCFIIFENVLTTLYYDFYDQESRRTWYHESYALGHELYQTFEACDCMHARRTCQQGFICGLLEDFHCILSIF
ScEPT1 TWAAENIITLSCFAFIVINVLTVFYMDENLNTDTERWTYHESYALGVHLYQTFEGDCDVHARRINQSGPFCGLLEDFHSTLMI
PiCPT1 IYWAENIIVMLICTGVMMFTTVVQLVYAFHFSEICFAWVYILSALGHEFYQIMDADGKQARRFCASSPFCGLLEDFHGCDAV
PiCPT2 PRLAENAIITIGALSLLVILSHVIMLWYSENMVEEAERWVYANAGFSLLFYQVLEVADGKQARRTKNSPFCGLLEDFHGCDAV
PiEPT IWLAEPIITLIGLILVGGSHLFLVFLCFELVGDAEWVAVVAALAEHLYQTFENLDGKQARRTKSSPFCGLLEDFHGCDAV
TaCPT RSWAENIIVLLICTXHLFVIAFLAEVMSGLEKSEAWVYLVNAWCLLHLYQIMDAVDGKQARRTKSSPFCGLLEDFHGCDAV

ScCPT1 NAILLSMIPVCSMTGCGYHYMIFISQFATLCSFYLSSTWEEVYHHTKLYLA-EFCGEVEGHIIVLCISFIAVGTLYCFQTIWHTK
ScEPT1 NSILSIFTHASETCMGFSYNLMLSQFAMLTNEYLSSTWEEVYHHTKLYLS-EFCGEVEGHIIVCVSLIILGTYCKQVIWHTY
PiCPT1 CAVFNVLASATCQACVGLRYPYIALSSVSTAFYLAQWEEVYHHTGVMSCGNGEFCVTEGQITLVAVHLVAAFPCAG-FWTAF
PiCPT2 NVVVSACIFASITLVLCPTIYWSLIMFLPAMVFFMATWEEVYHHTGTAALF-TVNGENEGLFIYSVYIGTAVGEN-VWTPQ
PiEPT NVSVGTMMASILQMGTIWRTLGFVLSGHFVIFATWEEVYSGSL-ELF-LINGETECHITLGIALKLVTAVVG-VGNQE
TaCPT GATFIAIGLCSLMCFGGSVHTAIALGTVQLPEYMLCQWEEVYHHTVHVLRQVGNFCVTEGQYLSMSLNLAHAXLCSA-FWKMT

ScCPT1 VAQESWQDEVFDV---ETVHLMYAFCTGALIFNIVTAHTNIVRYRYEQQSTKSATPSKTAENISKAVNGLILEEFAYFSSIF
ScEPT1 LFTITVGDVIVDV---DTLDIVFSLAVHGLVMALSARENVDKYRNRN-STSSAN-NITQIEQDSIKCLILEEFAYYASTA
PiCPT1 IPIPTPPFVTMTD---VLIGALVASNVLAYGNISNVLRAAPDAIPRDELGN-----KHISKHLALIQILEIGILLVIGS
PiCPT2 NIVEPQINNGH---VVVLSLTTGIAQCQVFSAVIAIRSMERKAKD-----GAATFCITIEFVALVILSG
PiEPT MIEGVQNNS-----LFVIVTMISSCHTLLVVRNALHAWRLNQDS-----VLVAFTRILIEFVVINTIAC
TaCPT VAELLLTVGLAALPLPPLVSGTELRVASVFIGGFFPFMLGASTVVSVARSG-----TNFARATLLTAELVXQQGQI

ScCPT1 TIVLIQES-----EISLALILSICFSVAEIVGRMTIAHETMQPPEMVNFPPLEETIQLVLYA-----FMVYVLDYQKESL
ScEPT1 IIVWMOES-----EITLSFILLSVGFTRGRTVGRITVCHLTKQSEEMENAPMTEELCQIVLYK-----ICSLWGTESNKT
PiCPT1 IIVICGPDAE-NYANYEVLFLFPIGIGYVFEFSTVILVSEMGKIEFTPQLR--VLIPEGFVIFN-----AYGPVAGLIFSKEL
PiCPT2 IIVLWSES-D-VFTDHERILLWTVCLLFAKMMVHMVLSMSEMGDEPYWLLRK-ITFIQLVVS-----EILVVGIVPWGH
PiEPT IIVALYSES-D-IFSTHERMFLWMLGLLNSKLVHLMLLAHICGEEYHFERK-ITVVEIFVYAGHC-----AFCMVEGTYDAIN
TaEPT TTCLVSEGKEVFAHEEVPLIVVQCIVLHSLHANKIIVASVGLLRVSNRAQDPVPSASPDGAHLGAAAGRGRVSAACLGDEER

ScCPT1 VSAIIVW--MGLGLTLAIGHMFTNDIYDITTELDIYALSIRKPKKEI.
ScEPT1 VFALSW--IGFGLSLGVHIMEMNDIIEFTEYLDVYALSIRKSKLT.
PiCPT1 IPTIVASSMYVVFVAVYLEHVFVGVKDIICDEFNIFLFLKIKPVATESEKAK.
PiCPT2 ESSVYQ--IFEMISLAAYLEMIYFLSTELATILGHIHFKVYKQG.
PiEPT EELIVR--EFFFLSRAAYVIVITVWVEVKNVIGVSVFTIFESSKFKLKTNSKPAKSS.
TaCPT PRPFCR--IRSCDRVSVCALRRQRHHRDYASPGHPLLSREAAQQVGDLOXRSPOIDDA.

```

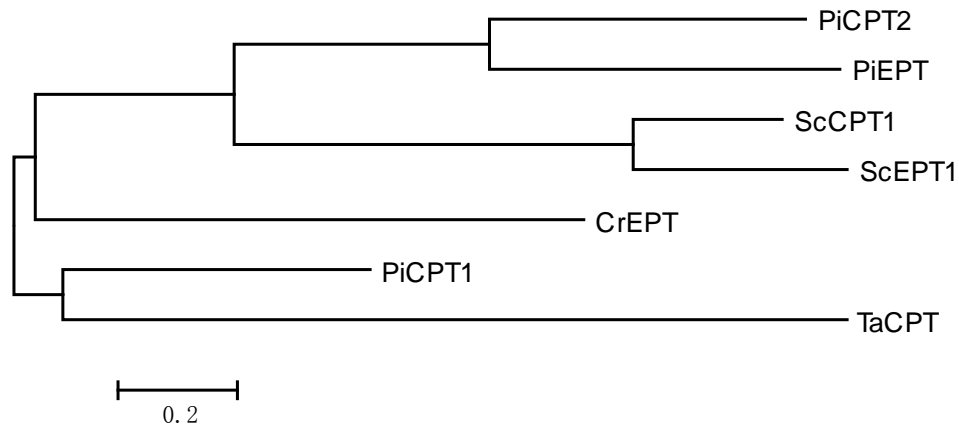
**Figure 21.** Alignment of putative CPT and EPT from *P. infestans* and *T. aureum*, and ScCPT1 and ScEPT1. GenBank accession number and source of each protein sequence: ScCPT1, AAA63571, *S. cerevisiae*; ScEPT1, AAA63572, *S. cerevisiae*. PiCPT1, XM\_002900684, *P. infestans*. PiCPT2, XM\_002997893, *P. infestans*. PiEPT, XM\_002907680, *P. infestans*. The conserved CDP-alcoholphosphotransferase motif is highlighted by the blue frame. Shaded in the dark black are the regions of the protein sequences that are highly conserved among the sequences.

The phylogenic analysis of putative CPT and EPT sequences from *P. infestans* and *T. aureum* and functionally characterized CPT and EPT sequences from other microbial species showed that two functional CPTs (PiCPT and TaCPT, see below) from *P. infestans* and *T. aureum* were tightly clustered together which were distantly related to the group that contains ScCPT1 and ScEPT1, a functional EPT (PiEPT) and nonfunctional CPT (PiCPT2) from *P. infestans*. Within the group, *S. cerevisiae* CPT and EPT formed a subgroup which is closely related with the subgroup comprising PiCPT2 and PiEPT. CrEPT from *Chlamydomonas reinhardtii*, a single cellular green algae, formed separately a single member group (Figure 22). This result indicates that the prototype of alcoholphosphotransferase might be the progenitor of both CPT and EPT in microbial species as these enzymes were not always clustered together according to their catalytic mode and origin. During the evolution, CPT and EPT could evolve independently from the progenitor through gene duplication and catalytic diversification.

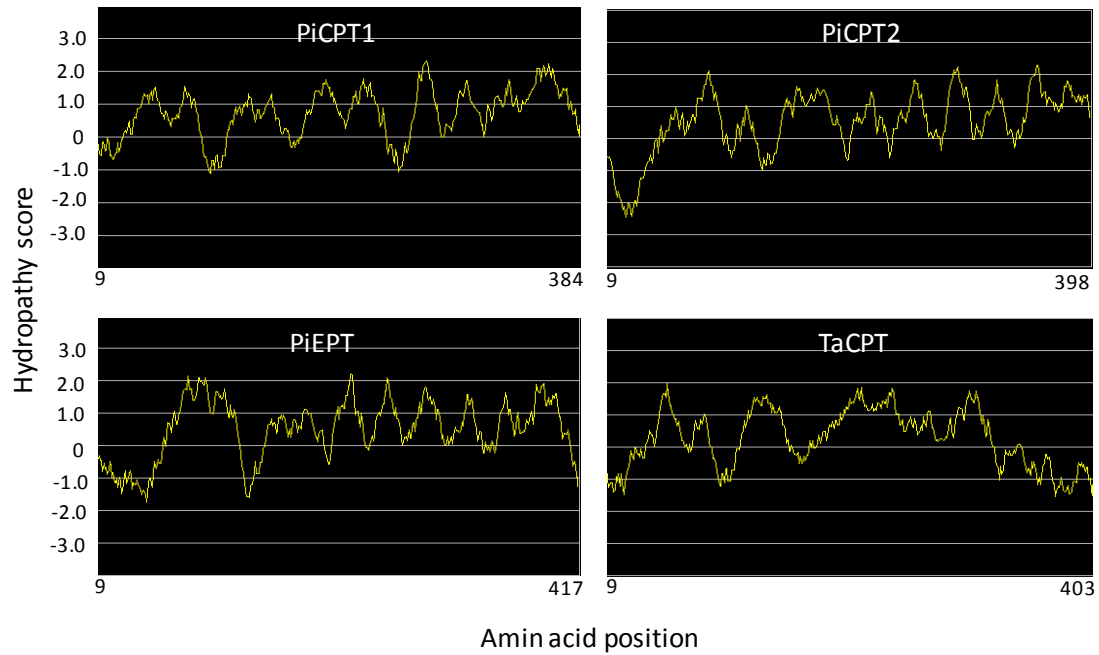
Hydropathy plot analysis of PiCPT1, PiCPT2, PiEPT and TaCPT revealed that these putative proteins are very hydrophobic and contain 5 to 7 highly hydrophobic domains presumably associated with membrane. These hydrophobic domains are localized throughout the entire sequences except for the N-terminus and the C-terminus regions which are hydrophilic (Figure 23). This result is consistent with notion that CPT and EPT are endoplasmic reticulum-based proteins (Henneberry *et al.*, 2002).

#### **3.3.4.2 Cloning of putative CPT and EPT genes**

All these putative CPT and EPT genes (*PiCPT1*, *PiCPT2*, *PiEPT* and *TaCPT*) were successfully amplified using specific primers and cloned into pGEM-T vector. To express them in yeast system, the full length cDNAs were subcloned into a yeast vector pSCW231, respectively. The recombinant plasmids were introduced into *E. coli* and the transformants were selected on LB plate with ampicillin and screened by colony PCR using ADH1 and gene specific primers. The colony that contains the insert in the right orientation from each construct was cultured in the selection medium (LB + ampicillin). The plasmids from the colony were extracted and sequenced with ADH1 and gene



**Figure 22.** Cluster analysis of CPT and EPT sequences from microbial species. The scale represents evolutionary distance expressed in the number of substitution per amino acid. The GenBank accession number and source of the different protein sequences are as follows: CrEPT, AAQ83686, *Chlamydomonas reinhardtii*; ScCPT1, AAA63571, *Saccharomyces cerevisiae*; ScEPT1, AAA63572, *Saccharomyces cerevisiae*; PiCPT1, XM\_002900684, *Phytophthora infestans*. PiCPT2, XM\_002997893, *Phytophthora infestans*. PiEPT, XM\_002907680, *Phytophthora infestans*. The unrooted phylogram was constructed by the Maximum Likelihood Tree method using MEGA5.1 Beta3.



**Figure 23.** Kyte-Doolittle hydropathy plots of PiCPTs, PiEPT and TaCPT. ProtScale was used to determine the hydrophobic profile of the polypeptide based on Kyte-Doolittle parameters (Kyte and Doolittle, 1982). Window size, 19. Peaks greater than 1.6 in vertical axis indicate the presence of a hydrophobic domain.

specific primers. The sequencing results confirmed that the plasmids contain the right sequence.

The confirmed constructs were then introduced into DBY747- $\Delta CPT/\Delta EPT$  that lacks both *CPT* and *EPT* genes generated previously by our lab (unpublished data) and the transformants were grown on selection plates (SD-TRP) and confirmed by colony PCR screening using ADH1 and gene specific primers.

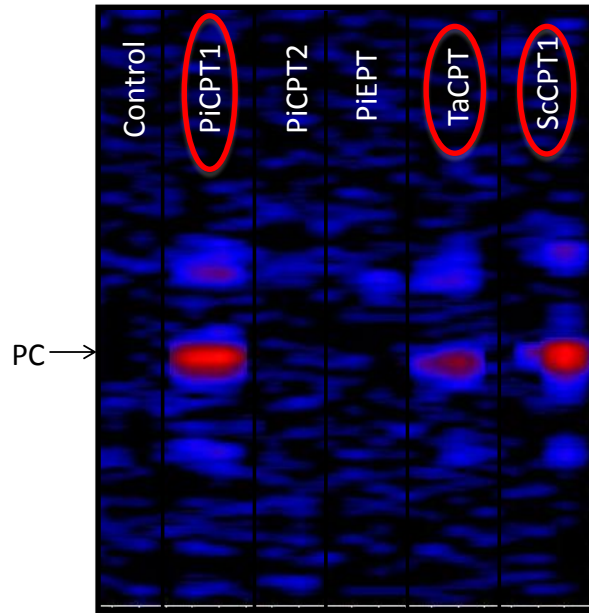
### **3.3.4.3 In vitro assays**

#### **3.3.4.3.1 CPT assays**

Figure 24 shows the representative result of the TLC image of the CPT assays of four putative *CPT* and *EPT* genes using the microsomal fraction of yeast transformants. Compared with the negative control (the yeast  $\Delta CPT/\Delta EPT$  with the empty vector, pSCW231), the  $\Delta CPT/\Delta EPT$  yeasts expressing *PiCPT1*, *TaCPT*, as well as the positive control *ScCPT1* produced new radioactive signals in the PC position. To quantify the enzyme activity, PC spots were scraped from the TLC plate and the radioactive amount was counted by a scintillation counter (Table 7). The result showed that *PiCPT1* from *P. infestans* had the highest CPT activity among the samples with appropriately 1.8 fold higher than that of the positive control, *ScCPT1*. The activity of *TaCPT* from *T. aureum* was lower than that of the positive control. Activities of *PiCPT2* and *PiEPT* from *P. infestans* were at the level similar to the background (the negative control), indicating they do not have CPT activity.

#### **3.3.4.3.2 EPT assays**

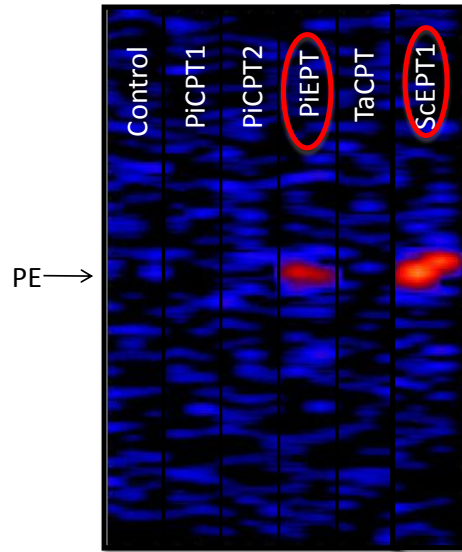
To determine whether these putative genes had any EPT activities, *in vitro* EPT assays were conducted using the *S. cerevisiae* *EPT* gene (*ScEPT1*) as a positive control. The result showed that out of four genes, only *PiEPT* showed the EPT activity (Figure 25). To quantify the activity level of this gene, the PE spot was scraped from the TLC plate and counted by a scintillation counter as described in the CPT assays. It appeared



**Figure 24.** The TLC plate showing the radioactive lipids in the CPT assays. Substrates used in this experiment were di18:1-DAG and [ $^{14}\text{C}$ ] CDP-choline. Lipids were separated in TLC plate developed with chloroform/methanol/water (65:25:4, v/v/v) system. Position of radiolabelled PC was indicated in radioautogram. Control, negative control, DBY747- $\Delta\text{CPT}/\Delta\text{EPT}$  with empty vector pSCW231. PiCPT1, DBY747- $\Delta\text{CPT}/\Delta\text{EPT}$  with *PiCPT1* in pSCW231 vector. PiCPT2, DBY747- $\Delta\text{CPT}/\Delta\text{EPT}$  with *PiCPT2* in pSCW231. PiEPT, DBY747- $\Delta\text{CPT}/\Delta\text{EPT}$  with *PiEPT* in pSCW231. TaCPT, DBY747- $\Delta\text{CPT}/\Delta\text{EPT}$  with *TaCPT* in pSCW231. ScCPT1, positive control, DBY747- $\Delta\text{CPT}/\Delta\text{EPT}$  with *ScCPT1* in pSCW231. This experiment was performed once.

**Table 7.** The CPT activity of four putative genes product as well as negative and positive control on di18:1-DAG. The specific activities were calculated according to equation 1 in section 3.2.3.7. Data were calculated from Figure 24. Control is the negative control, ScCPT1 is the positive control.

<b>Name</b>	<b>Control</b>	<b>PiCPT1</b>	<b>PiCPT2</b>	<b>PiEPT</b>	<b>TaCPT</b>	<b>ScCPT1</b>
<b>Specific activity (pmol/min/mg)</b>	0.27	117.13	0.55	0.39	31.83	75.52



**Figure 25.** The TLC plate shows the radioactive lipids in EPT assay. Substrates used in this experiment were di18:1-DAG and [ $^{14}\text{C}$ ] CDP-ethanolamine. Lipids were separated in TLC plate developed with chloroform/methanol/water (65:25:4, v/v/v/) system. Position of radiolabelled PE was indicated in radioautogram. Control, negative control, DBY747- $\Delta\text{CPT}/\Delta\text{EPT}$  with empty vector pSCW231. PiCPT1, DBY747- $\Delta\text{CPT}/\Delta\text{EPT}$  with *PiCPT1* in pSCW231 vector. PiCPT2, DBY747- $\Delta\text{CPT}/\Delta\text{EPT}$  with *PiCPT2* in pSCW231. PiEPT, DBY747- $\Delta\text{CPT}/\Delta\text{EPT}$  with *PiEPT* in pSCW231. TaCPT, DBY747- $\Delta\text{CPT}/\Delta\text{EPT}$  with *TaCPT* in pSCW231. ScEPT1, positive control, DBY747- $\Delta\text{CPT}/\Delta\text{EPT}$  with *ScEPT1* in pSCW231. This experiment was performed once.



that the activity level of PiEPT was relative low to that of the positive control, which reached about 20% of the ScEPT activity (Table 8).

Taken together, the *in vitro* assays show that gene products of *PiCPT1* and *TaCPT* have CPT activities, but no EPT activity when di18:1-DAG and [<sup>14</sup>C] CDP-choline were used as substrates, *PiEPT* gene product has EPT activity, but no CPT activity when di18:1-DAG and [<sup>14</sup>C] CDP-ethanolamine were used in the assay.

#### **3.3.4.3.3 Substrate specificity of PiCPT1**

To determine the substrate specificity of PiCPT1, the highest active CPT from *P. infestans*, seven different species of DAGs (di16:0, di16:1, di18:0, di18:1, di18:2, 18:0/20:4, 18:0/22:6) were used in the *in vitro* CPT assays. The result showed that the most preferred DAG substrate for PiCPT1 was 18:0/20:4-DAG, followed by 18:0/22:6-DAG. The specific activities towards 18:0/20:4-DAG and 18:0/22:6-DAG were 26 and 14 folds higher than those of other DAG species (Figure 26).

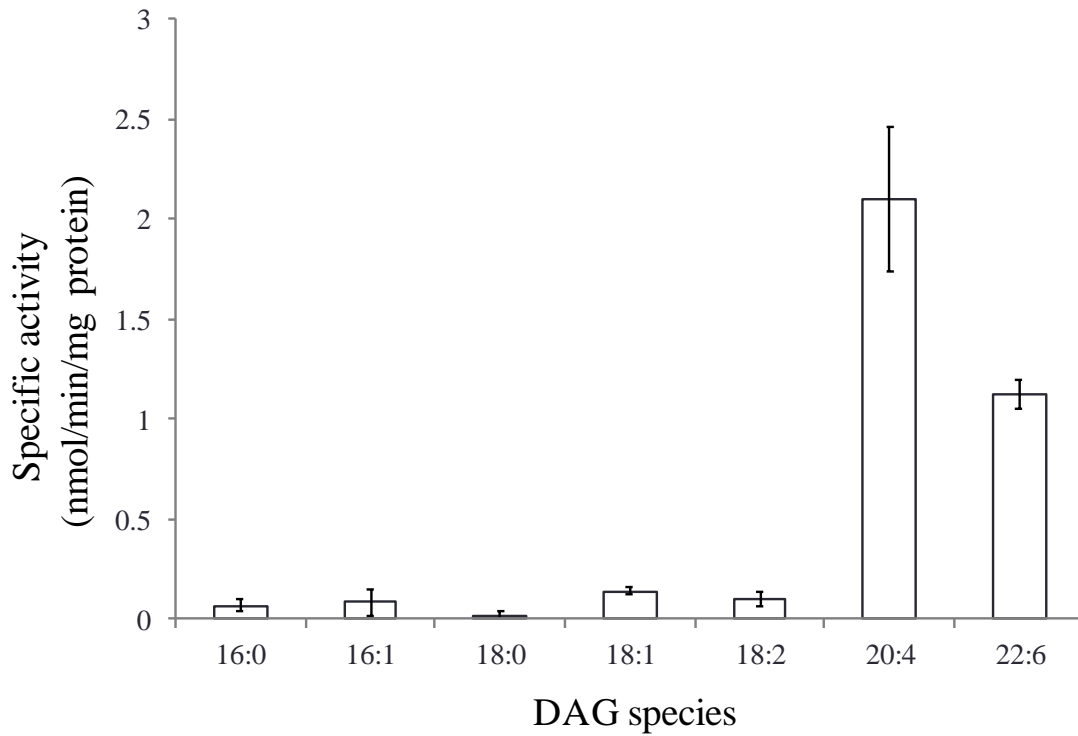
To confirm the CDP-amino alcohol specificity of PiCPT1, 18:0/20:4-DAG was employed as the substrate for the kinetic study using different concentrations of [<sup>14</sup>C] CDP-choline and [<sup>14</sup>C] CDP-ethanolamine. The result showed that the apparent  $K_m$  was 20.98  $\mu$ M and the  $V_{max}$  was 3.23 nmol/min/mg for CDP-choline (Figure 27). The kinetic constants were estimated from Lineweaver-Burke plots using the average of triplicate measurements. The low  $K_m$  value implies PiCPT1 has high affinity towards the CDP-choline substrate. On the contrary, PiCPT1 could not use CDP-ethanolamine as a substrate to synthesize PE. This result confirmed that PiCPT1 functions solely as CPT with CDP-choline being the CDP-amino alcohol substrate.

#### **3.3.4.3.4 Substrate specificity of TaCPT**

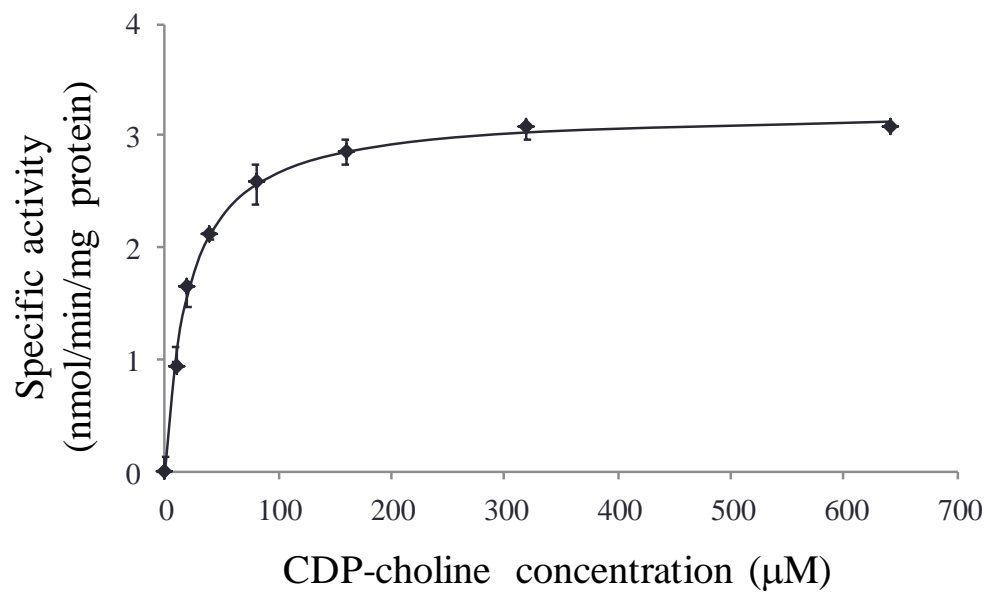
The result of the substrate specificity assays of TaCPT was shown in Table 9. It appeared that the activities of TaCPT on the substrates were relatively low compared to that of PiCPT1 and there was no significant substrate preference shown for TaCPT to

**Table 8.** The EPT activity of four putative genes product as well as negative and positive control on di18:1-DAG. The specific activities were calculated according to equation 1 described in section 3.2.3.7. Data were calculated from Figure 25. Control is the negative control, ScEPT1 is the positive control.

<b>Name</b>	<b>Control</b>	<b>PiCPT1</b>	<b>PiCPT2</b>	<b>PiEPT</b>	<b>TaCPT</b>	<b>ScEPT1</b>
<b>Specific activity (pmol/min/mg)</b>	0.30	0.37	0.55	8.50	0.17	40.71



**Figure 26.** Substrate specificity of PiCPT1 on different DAGs. Mean  $\pm$  SE values based on three biological replicates were used. Seven different species of DAGs (di16:0, di16:1, di18:0, di18:1, di18:2, 18:0/20:4, 18:0/22:6) were used in the *in vitro* CPT assays. The specific activities were calculated according to equation 1 described in section 3.2.3.7.



**Figure 27.** Kinetic parameters of PiCPT1 on 18:0/20:4-DAG. Mean  $\pm$  SE values based on three biological replicates were used. The kinetic constants were estimated from Lineweaver-Burke plots using the average of triplicate measurements by Microsoft-Excel software.

**Table 9.** Specific activities of TaCPT on different DAGs. Seven different species of DAGs (di16:0, di16:1, di18:0, di18:1, di18:2, 18:0/20:4, 18:0/22:6) were used in the TaCPT assays. The specific activities were calculated according to equation 1 described in section 3.2.3.7. This experiment was performed once. Reaction with only di18:1-DAG but no [<sup>14</sup>C] CDP-choline was used as negative control.

<b>DAG species</b>	<b>Control</b>	<b>16:0</b>	<b>16:1</b>	<b>18:0</b>	<b>18:1</b>	<b>18:2</b>	<b>20:4</b>	<b>22:6</b>
<b>Specific activity (pmol/min/mg)</b>	0.03	0.78	0.76	0.69	0.86	0.56	1.09	1.06

substrates tested although the specific activities were slightly higher to two VLCPUFA-containing DAGs.

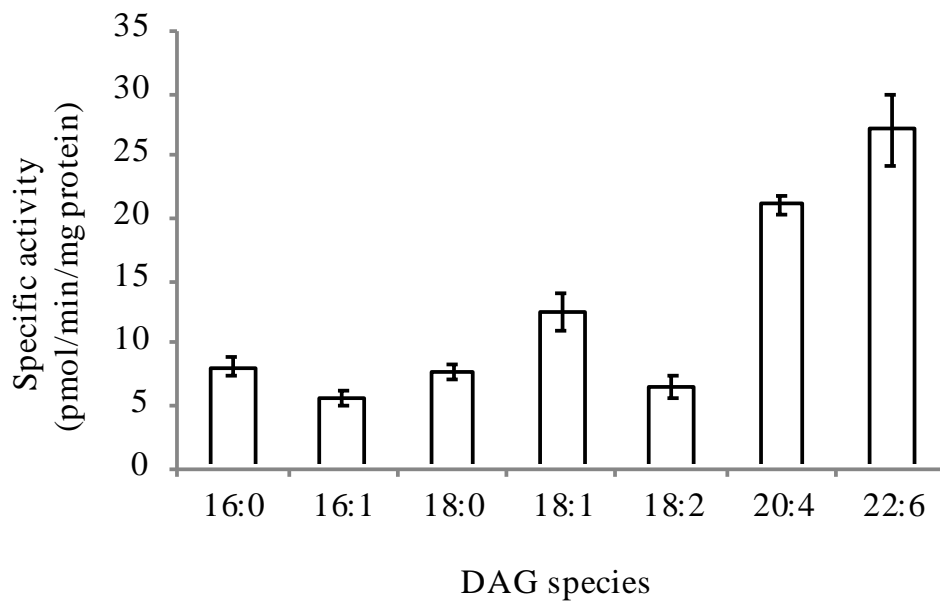
#### **3.3.4.3.5 Substrate specificity of PiEPT**

To study substrate specificity of PiEPT, again seven different DAG species, di16:0, di16:1, di18:0, di18:1, di18:2, 18:0/20:4, 18:0/22:6 were used in the EPT assays. The result showed that the most preferred substrate for PiEPT was 18:0/22:6-DAG, followed by 18:0/20:4-DAG. The DAG species of di16:0, di16:1, di18:0 and di18:2 were among the least preferred substrate (Figure 28).

#### **3.3.4.4 Effect of exogenous fatty acid supplementation on the gene expression**

Under the control condition (without any fatty acid supplementation), *P. infestans* could produce both 18C unsaturated fatty acids and 20C unsaturated fatty acids. Among 18C unsaturated fatty acids, OA was the most abundant which accounted for about 16%, followed by LA which accounted for about 15%, and ALA which accounted for only 0.1%. EPA is a major 20C polyunsaturated fatty acid representing about 11%, while ARA, another 20C polyunsaturated fatty acid, accounted for about 2% of the total fatty acids (Table 10). Feeding *P. infestans* with 18C unsaturated fatty acids did not dramatically alter the fatty acid profile of the fungus although the level of fed fatty acid was slightly increased. Feeding EPA did not observe similar changes. *P. infestans* did not produce DHA under the control condition, feeding the fungus with DHA indeed resulted in accumulation of DHA inside the cells, accounting for 9.5%. Overall, feeding with different fatty acids does not dramatically change the fatty acid profiles of *P. infestans* except for the fed one (Table 10).

To investigate whether supplementation of exogenous fatty acids could affect the expression of *CPT* genes, transcript levels of *PiCPT1* in *P. infestans* fed with different fatty acids were analyzed by real-time quantitative PCR. The experimental samples yielded a sharp peak at the melting temperature of the amplicons in melting curve analysis which indicates that the products are specific. The Ct values were recorded by



**Figure 28.** Substrate specificity of PiEPT on different DAGs. Mean  $\pm$  SE values based on three biological replicates were used. Seven different species of DAGs (di16:0, di16:1, di18:0, di18:1, di18:2, 18:0/20:4, 18:0/22:6) and [ $^{14}$ C] CDP-ethanolamine were used in the PiEPT assays. The specific activities were calculated according to equation 1 described in section 3.2.3.7.

**Table 10.** Fatty acid composition of *P. infestans* grown in the presence of exogenous fatty acids. Six target fatty acids were listed. Mean  $\pm$  SE values based on three biological replicates were used. Biomass not fed with any fatty acid was used as negative control.

<b>Fatty acid (mol %)</b>	<b>18:1</b>	<b>18:2</b>	<b>18:3</b>	<b>20:4</b>	<b>20:5</b>	<b>22:6</b>
<b>Control</b>	16.20 $\pm$ 1.53	14.54 $\pm$ 1.31	0.14 $\pm$ 0.10	2.28 $\pm$ 0.26	11.13 $\pm$ 1.05	-
<b>18:1 feeding</b>	19.38 $\pm$ 0.61	14.46 $\pm$ 1.23	0.1 $\pm$ 0.09	2.56 $\pm$ 0.15	11.4 $\pm$ 0.54	-
<b>18:2 feeding</b>	14.43 $\pm$ 2.13	15.86 $\pm$ 1.19	0.69 $\pm$ 0.05	2.23 $\pm$ 0.18	10.52 $\pm$ 1.87	-
<b>18:3 feeding</b>	16.31 $\pm$ 2.75	13.11 $\pm$ 1.66	3.70 $\pm$ 1.26	2.44 $\pm$ 0.17	10.08 $\pm$ 0.47	-
<b>20:4 feeding</b>	17.12 $\pm$ 1.53	12.76 $\pm$ 0.39	0.12 $\pm$ 0.10	7.58 $\pm$ 1.39	10.48 $\pm$ 0.77	-
<b>20:5 feeding</b>	16.11 $\pm$ 0.87	13.72 $\pm$ 1.72	0.17 $\pm$ 0.02	2.27 $\pm$ 0.08	11.96 $\pm$ 0.70	-
<b>22:6 feeding</b>	14.71 $\pm$ 0.76	13.65 $\pm$ 0.87	0.12 $\pm$ 0.02	2.07 $\pm$ 0.12	10.84 $\pm$ 0.91	9.49 $\pm$ 2.46

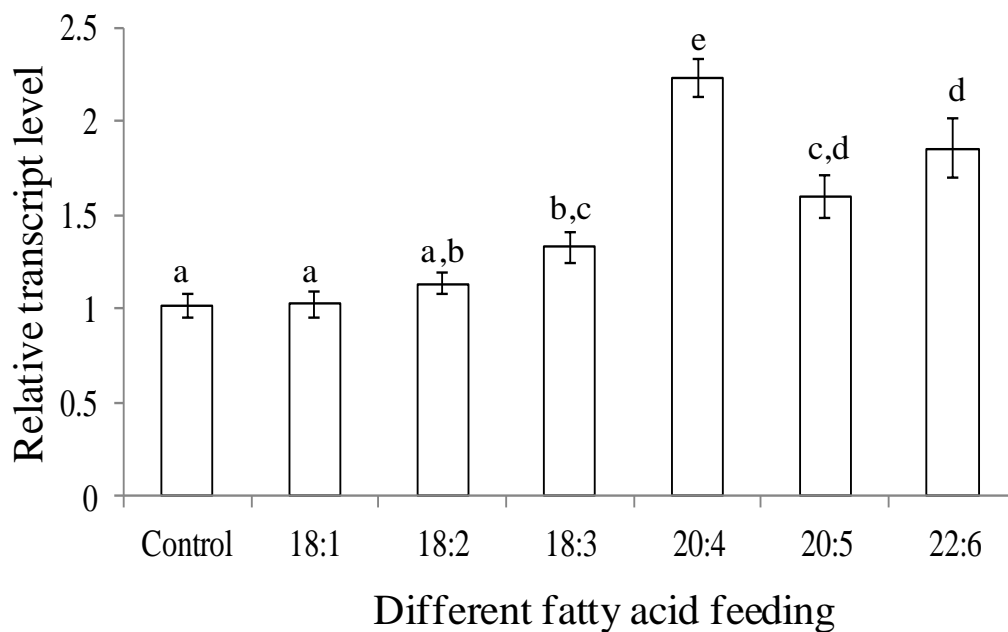


the software the fold changes of transcript level of *PiCPT1* and housekeeping genes were calculated. The expression values were then put into SAS to calculate the standard error (Figure 29). The result showed that feeding 18C unsaturated fatty acids such as OA and LA did not significantly change the expression of *PiCPT1*. However, the expression of *PiCPT1* was significantly up-regulated when the fungus was fed with VLCPUFAs such as ARA, EPA and DHA, especially with ARA which induced the expression at the level 2.2 times that of OA feeding. This result indicates that expression of *PiCPT1* could be up-regulated by VLCPUFAs.

### 3.3.5 Discussion

Although several *CPT* and *EPT* genes from model organisms have been studied, no *CPT* and *EPT* gene has been cloned and characterized from any VLCPUFA-producing microorganisms. In this study, four putative *CPT* and *EPT* genes were identified from VLCPUFA-producing microorganisms: *P. infestans* and *T. aureum*. Their deduced protein sequences (PiCPT1, PiCPT2, PiEPT and TaCPT) shared around 26% amino acid identities to ScCPT1, a *CPT* from *S. cerevisiae*. They all contain a CDP-alcoholphosphotransferase domain as seen in previously characterized *CPT* and *EPT* enzymes from other species. The phylogenetic analysis shows that PiCPT1 and TaCPT form a clade which is distantly related to the clade comprising yeast ScCPT1 and ScEPT1. Putative *CPT* and *EPT* exhibit distinct hydropathy profiles and location patterns of predicted transmembrane domains, indicating that these proteins from VLCPUFA-producing microorganisms would have discrete catalytic activity and substrate specificity.

*CPT* is a membrane-bound enzyme located in endoplasmic reticulum, catalyzing the synthesis of PC from CDP-choline and DAG (Henneberry *et al.*, 2000; Hjelmstad *et al.*, 1994). As both insoluble (DAG) and soluble (CDP-choline) substrates are involved in the reaction, the *in vitro* assay of this enzyme is challenging (McMaster and Bell, 1997). Based on the published assay methods from previous studies, a modified assay system was effectively established in this study using a low concentration of a surfactant. Using this assay method, we showed that PiCPT1 and PiEPT from *P. infestans* have *CPT* and



**Figure 29.** Transcript levels of *PiCPT1* in *P. infestans* cultured in presence of exogenous fatty acids. Values are the mean of three replicates  $\pm$  standard error (SE). cDNA from the biomass not fed with any fatty acid was used as negative control. Values followed by a different letter are significantly different using Duncan hypothetical test ( $P < 0.01$ ).

EPT activities, respectively, and TaCPT from *T. aureum* has CPT activity. Although PiCPT2 has similar amino acid similarity as PiCPT1 to ScCPT1 and ScEPT1 and contains the conserved CDP-alcoholphosphotransferase domain, there was no CPT or EPT activity detected with PiCPT2, indicating *PiCPT2* does not encode either a functional CPT or a functional EPT. However, whether it has any activity of other alcoholphosphotransferase such as PI synthase or PS synthase remains to be determined as some of these enzymes also share the conserved CDP-alcohol phosphotransferase domain.

One of the main objectives of this study is to examine the substrate specificity of CPT and EPT derived from VLCPUFA-producing microorganisms. Substrate specificity of CPT and EPT enzymes from other species has been investigated. *S. cerevisiae* CPT and EPT preferred di16:1-DAG and di18:1-DAG, respectively, even though they could also use medium and very long chain fatty acids-DAG as substrates, but with lower efficiency (Hjelmstad *et al.*, 1994). CPT activities have been examined from *C. lanceolata* (C10:0), castor bean (C18:1-OH), safflower (normal C18) and rapeseed (C22:1), each species produces different predominate fatty acids in seeds. However, these CPTs showed little or no substrate preference towards a range of DAG species (Vogel and Browse, 1996). Human CPT1 and CEPT1 have the highest activity to di18:1-DAG, but can also work on VLCPUFA-DAGs (C16:0/C22:6-DAG and C18:0/C20:4-DAG) (Henneberry *et al.*, 2000). In this study, CPT and EPT activities of three genes from *P. infestans* and *T. aureum* were examined by *in vitro* assays. The results show PiCPT1 strongly prefers VLCPUFA-containing DAG species 18:0/20:4-DAG and 18:0/22:6-DAG as substrates. PiEPT has slightly higher EPT activity towards VLCPUFA-DAGs.

To ascertain the CDP-amino alcohol specificity of PiCPT1, the highest active CPT from *P. infestans*, the kinetic study was performed to investigate its  $K_m$  and  $V_{max}$  values for CDP-amino alcohols. The result showed PiCPT1 displays an apparent  $K_m$  at 20.98  $\mu\text{M}$  and  $V_{max}/K_m$  at 154.0 pmol/min/mg/ $\mu\text{M}$  for CDP-choline with 16:0/20:4-DAG as substrate. PiCPT1 does not use CDP-ethanolamine as substrate. With the similar method, the  $V_{max}/K_m$  of ScCPT1 for CDP-choline is 94.5 pmol/min/mg/ $\mu\text{M}$  with di18:1-

DAG as substrate (Williams and McMaster, 1998). *B. napus* CPT (BnAAPT1) exhibits the  $V_{max}/K_m$  at 18.2 and 1.7 pmol/min/mg/ $\mu$ M for CDP-choline and CDP-ethanolamine, respectively, with di18:1-DAG as substrate (Qi *et al.*, 2003). Human CEPT1 enzyme displays  $V_{max}/K_m$  at 397.2 and 83.7 pmol/min/mg/ $\mu$ M for CDP-choline and CDP-ethanolamine, respectively (Wright and McMaster, 2002). Although kinetic parameters such as  $K_m$  and  $V_{max}$  vary on the assay conditions, the relative high ratio of  $V_{max}/K_m$  on CDP-choline and no activity on CDP-ethanolamine for PiCPT1 indicates that PiCPT1 from *P. infestans* functions as CPT with high affinity for CDP-choline.

If PiCPT1 indeed prefers to using VLCPUFA-DAGs to synthesize PC in *P. infestans*, the expression of this gene should respond to the presence of VLCPUFAs. To test this hypothesis, transcript levels of *PiCPT1* in *P. infestans* in the presence of exogenous fatty acids with various chain length and double bonds were analyzed by real-time quantitative PCR. The results show that feeding the fungus with VLCPUFAs such as ARA, EPA and DHA significantly induces the expression of this gene, while feeding 18C unsaturated fatty acids does not have the same effect. CPT is an enzyme involved in the synthesis of PC from DAG and CDP-choline, thus possibly, when the fungus is fed with VLCPUFAs, higher *PiCPT1* expression is required for specific channelling of these fatty acids to the phospholipids via DAG intermediates.

#### 4.0 GENERAL DISSUSSION AND FUTURE DIRECTIONS

Increasing demand of VLCPUFAs such as ARA, EPA and DHA in the market place is due to the public awareness of potential health benefits of these fatty acids. Traditional sources for VLCPUFAs are oceanic fish and oleaginous microbes. However, these sources are either unsustainable due to declining of the fish population or expensive due to the high cost in growing and extracting oil from VLCPUFA-producing microbes. Producing VLCPUFAs in plants by metabolic engineering is viewed as an attractive alternative which has recently drawn much attention from lipid research community and nutraceutical industries. Since plants are unable to synthesize *de novo* VLCPUFAs, the genetic engineering approach has to be employed to produce these fatty acids in plants based on our understanding of fatty acid and TAG biosynthesis in plants as well as in VLCPUFA-producing microorganisms. Many desaturases and elongases involved in the biosynthesis of VLCPUFAs have been cloned from microbial species and successfully introduced into oilseed plants to produce VLCPUFAs (Qiu *et al.* 2005). However, the level of the fatty acids in transgenics is still low for the viable commercialization. To increase the VLCPUFA production, limiting factors or bottlenecks in the metabolic pathway reconstituted in transgenic plants have to be thoroughly examined. As mentioned in the literature review section, one of the main issues in effectively reconstituting the VLCPUFA pathway in plant is how to increase VLCPUFA trafficking between PC and DAG. PC is the substrate for most desaturases in the VLCPUFAs synthesis while DAG is the precursor for TAG synthesis by DGAT. Recent labeling studies demonstrate DAG used for TAG synthesis is mostly derived from PC, while *de novo* DAG is mostly used for *de novo* PC synthesis (Bates *et al.*, 2009), the relative flux of *de novo* DAG transfer into PC is over 14 times the rate of the direct conversion from *de novo* DAG to TAG and the major bottleneck for the accumulation of UFAs in TAG is the flux of DAG to PC (Bates and Browse, 2011). These results imply that factors involved in the flux of specialty fatty acids from DAG to PC and vice versa are extremely important in VLCPUFA accumulation in TAG in transgenic plants. PDCT, CPT and EPT are alcoholphosphotransferase involved in the acyl flux between PC and DAG. Therefore, studying PDCT, CPT and EPT from VLCPUFA-producing microbes such as

*P. infestans* and *T. aureum* is very important for us to understand the shuffling process between PC and DAG during the biosynthesis of VLCPUFAs and to design a strategy to increase the production of VLCPUFAs in plants.

Commercial oilseed crops produce only simple fatty acid profile in their oils and may not be able to effectively handle unusual VLCPUFAs synthesized by heterologous genes. Firstly, UFAs in PC where the desaturation occurs may affect the integrity of the membrane structure in host plants. Secondly, oilseed crops may lack effective mechanisms to channel VLCPUFAs to TAGs, as during evolution, the oilseed crops have developed the system where the enzymes involved in the TAG synthesis have functional selectivity only on their indigenous fatty acids. For example, in *C. lanceolata* that accumulates oil containing saturated fatty acids with 10 carbons (C10:0), the DGAT prefers di10:0-DAG as substrate over DAGs with C18 chain fatty acids. In castor bean that accumulates a high amount of ricinoleic acid (C18:1-OH) in its oil, the DGAT exhibited higher activity on diricinoleoyl-DAG than other DAG species (Vogel and Browse, 1996). Moreover, both PDCT and PDAT in castor bean are found to be efficiently using C18:1-OH containing substrates (Hu *et al.*, 2012; van Erp *et al.*, 2011). However, interestingly, CPTs from plants such as *C. lanceolata*, castor bean, safflower and rapeseed (each produces an unique fatty acid profile in its TAG) show little or no substrate preference across a range of different DAG substrates (Vogel and Browse, 1996). Yet, this study clearly shows that PiCPT, a CPT from VLCPUFA-producing *P. infestans* has strong substrate specificity towards VLCPUFA-DAGs. To my knowledge, this is the first report describing a CPT with high specificity to convert VLCPUFA-DAGs to VLCPUFA-PCs. As CPT plays an important role in acyl-trafficking between phospholipids and neutral lipids during the biosynthesis of PUFAs, this gene and other CPT genes with similar enzymatic properties might be good candidates to be included in the heterologous expression with VLCPUFA genes for increased production of VLCPUFAs in transgenics in the future.

CPT and EPT are hydrophobic enzymes with several trans-membrane domains. Previous studies indicates first three membrane-spanning domains of these enzymes

might be involved in binding the DAG substrate thereby determining substrate specificity (Hjelmstad *et al.*, 1994). The site-directed mutagenesis of human CEPT1 reveals that only the third predicted transmembrane domain is responsible for DAG substrate specificity for common DAGs (Henneberry *et al.*, 2002). In this study, PiCPT1 from *P. infestans* has been proven by the *in vitro* assays to have high substrate preference to unusual VLCPUFA-DAGs. Thus, this enzyme would be an ideal candidate for mutagenesis analysis of the structure responsible for VLCPUFA-DAGs substrate specificity.

The reactions catalyzed by CPT and EPT enzymes are believed to be reversible. (Goracci *et al.*, 1986; Goracci *et al.*, 1977; Kano and Ono, 1973; Roberti *et al.*, 1992; Stobart and Stymne, 1985). The reverse reaction from PC and CMP to DAG and CDP-choline has been established using microsomal fractions of yeast mutant cells expressing AAPT genes from soybean and *Arabidopsis*. (Dewey and Goode, 1999). However, our preliminary experiment using [<sup>14</sup>C] PC and regular CMP in the reaction could not detect the reverse reaction using *PiCPT1* expressed yeast (data not shown). This experiment should be repeated under different assay conditions in order to draw the affirmative conclusion.

This is the first time to report that *CPT* and *EPT* genes (*PiCPT1*, *PiEPT* and *TaCPT*) cloned from VLCPUFA-producing microorganisms prefer VLCPUFA-containing substrates. By expressing these genes in transgenic plants would alleviate the bottleneck of acyl-trafficking between neutral lipids and phospholipids; therefore, this promising finding may help increase VLCPUFAs accumulation in transgenic plants in the future.

## 5.0 REFERENCES

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