Investigation of potential roles of Phospholipase D 
in *Arabidopsis thaliana* seed oil accumulation

by

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Abstract

Most plants depend on the production of storage compounds in the seed to provide nutrients during germination. These storage compounds have been exploited by man for a variety of purposes. For example vegetable oil is an important lipid source for cooking and the production of lubricants, inks, paints, and biodiesels. Almost all seed oil is stored in the chemical form of triacylglycerol (TAG), and in mature seed, TAG is stored in densely packed oil bodies. Genetic engineering offers great potential to increase the amount and/or manipulate the type of oil produced. There are two routes to this goal: first, the development of existing plant species that already synthesize the desired novel oil into “alternative oilseed’ crops; second, the engineering of existing crops through the cloning of genes which determine the synthesis of novel oils and their introduction into existing crops such as oilseed rape (Brassica napus) by genetic transformation. Whichever approach is adopted we need to know how storage oil is accumulated in order to optimize yield.

My research is part of a broader effort to explore the possibility of increasing oil content in Brassica species using Arabidopsis thaliana as a model system. My specific project examines whether the phospholipase D zeta (PLDZ) enzyme can be used to control oil production in Arabidopsis thaliana. In plants, PLDZ hydrolyzes membrane phospholipids to form phosphatidic acid, which is the branch point for the synthesis of diacylglycerol that is an immediate precursor of storage oil. In addition, previous research had shown that the PLDZ gene was negatively regulated in the leaves by GLABRA 2 (GL2), a transcription factor that was also known to negatively regulate oil content in the seed. In this thesis I show that the pldz1pldz2 double mutant accumulates 11% less oil in the seed than did wild type, without differences in seed size, plant growth or development. Both PLDZ genes localize to the embryo and funiculus during the later stages of seed development. In the gl2 mutant background, PLDZ transcript levels in the seeds are lower
than in the wild type; besides the \textit{pldz1pldz2gl2} triple mutant shows intermediate oil content which is unlike neither \textit{pldz1pldz2} double mutant nor \textit{gl2} mutant phenotype, suggesting that \textit{GL2} is not a negative regulator of \textit{PLDZ} genes during oil production process.
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Abbreviations

35S Cauliflower mosaic virus 35S promoter
aa Amino acid
ABA Abscisic acid
ACCase Acetyl-CoA carboxylase
ACP Acyl carrier protein
ACS Acetyl-CoA synthetase
ARF Adenosine diphosphate-ribosylation factor
ATP Adenosine triphosphate
BLAST Basic Local Alignment Search Tool
bp DNA base pair
C16; C18 Fatty acids and derivatives with the specified number of carbon atoms
cDNA Complementary DNA
CAPS Calcium-activated protein for secretion
CoA Coenzyme A
Col Columbia ecotype, Arabidopsis
COPI Coat protein I
CPT Choline phosphotransferase
DAG Diacylglycerol
DGAT Diacylglycerol acyltransferase
DPA Days post anthesis
ER Endoplasmic reticulum
Fad Fatty acid desaturase
G-3-P Glycerol-3-phosphate
GC Gas chromatography
GFP Green fluorescence protein
GUS β-glucuronidase
KAS 3-ketoacyl-ACP synthetase
kb kilo base (1000 bp)
LB Luria-Bertani bacterial growth medium formulation
LPA Lyso-phosphatidic acid
LPAT Lyso-phosphatidic acid acyltransferase
mTOR Mammalian target of rapamycin
NADH Nicotinamide adenine dinucleotide
ORF Open reading frame
PA Phosphatidic acid
PC Phosphatidylcholine
PCR Polymerase chain reaction
PDC Pyruvate dehydrogenase complex
PE Phosphatidylethanolamine
PEP Phosphoenolpyruvate
PI Phosphoinositide
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>PIP</td>
<td>Phosphatidylinositol phosphates</td>
</tr>
<tr>
<td>PIPK</td>
<td>Phosphatidylinositol phosphate kinases</td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>PSV</td>
<td>Protein storage vacuoles</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse-transcriptase PCR</td>
</tr>
<tr>
<td>SUS</td>
<td>Sucrose synthase</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetate EDTA buffer</td>
</tr>
<tr>
<td>TAG</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>TAIR</td>
<td>The Arabidopsis Information Resource</td>
</tr>
<tr>
<td>TE</td>
<td>Tris EDTA buffer</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2-hydroxymethyl-1,3-propanediol</td>
</tr>
<tr>
<td>VLDLs</td>
<td>Very low density lipoproteins</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>X-gluc</td>
<td>5-bromo-4-chloro-3-indolyl- β-D-glucuronide</td>
</tr>
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Nomenclature

Throughout the thesis, standard Arabidopsis nomenclature (Meinke and Koornneef, 1997) has been used. The following is a brief overview of the naming rules:

All the Arabidopsis mutants, genes and proteins are identified with a 4-letter symbol corresponding to the full name.

Genes (wild type alleles) are italicized and capitalized (Example: *PLDZ1*).

Proteins are capitalized and non-italicized (Example: PLDZ1).

Mutant alleles are lowercase, italicized (Example: *pldz1*).

If multiple alleles exist, then the mutant name is followed by a hyphen and the allele designation (Example: *pldz1-1, pldz2-1*).

Multiple mutants are indicated with a space separating each individual mutant symbol (Example: *pldz1 pldz2* double mutant).
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Chapter 1

Introduction and literature review

The extraction of oil from plant seeds has occurred for thousands of years and seed oil has become an important daily product for human consumption. In recent years, seed oil has also been used to make biodiesel which has the potential to reduce greenhouse gas emissions and replace fossil fuel. Oilseed rape (*Brassica napus*) is the second largest oil crop in the world, contributing 13% of total oil production. Cultivars of *B. napus* that produce less than 2% erucic acid (22:1), such that it is more suitable for human consumption, have been bred in Canada and named “canola”. Canola has become a major “cash crop” in Canada. Canadian canola plantings are increased every year, reaching 15.8 million acres by 2008, and the markets for canola oil contribute billions of dollars every year to Canadian economy.

*Arabidopsis thaliana* is a useful genetic model plant owing to its small size, short reproductive cycle and low complexity of its genome. Importantly, Arabidopsis is closely related to canola, and represents an excellent model oilseed for investigating storage compound accumulation. The major goal of my research is to investigate the mechanisms involved in the regulation of seed oil biosynthesis. In this chapter of my thesis, I will review the current knowledge of seed oil production and accumulation in Arabidopsis and end this chapter by presenting my research goals and thesis objectives.

1.1 Fatty acid biosynthesis and regulation in *Arabidopsis thaliana*

Oils are made from two kinds of molecules: glycerol and fatty acids. Biosynthesis of fatty acids in plants takes place within the plastids through the activity of fatty acid synthetase (Harwood et al., 1998). In certain plant tissues, these fatty acids are used to form the storage oils (TAG: triacylglycerols). All the nutrients needed for this process are
commonly delivered from the maternal tissues of the plants. However, our understanding of the process of carbon supply to storage oil synthesis in the seed is still poor.

In the photosynthetic organs, exogenous carbon is fixed in the chloroplasts. This carbon can be used for the synthesis of acetyl-CoA which participates in the very first step of fatty acid synthesis. For tissues like developing seeds, photosynthesis can't provide enough material for storage lipid synthesis. So the plastid is dependent upon import of carbon from the mother plant in order to synthesize acetyl-CoA. Sucrose is known to be the major carbon compound transported to the reproductive sink tissues. Cleavage of sucrose can be used to produce glucose-6-phosphate (G-6-P) which later will be converted into phosphoenolpyruvate (PEP), the preferred substrate for lipid synthesis in the seed (Kang and Rawsthorne, 2000) (Fig. 1.1). Sucrose synthase (SUS) and invertase participate in this reaction; their activity is the major point for carbon entry and metabolism (Koch, 2004). For example, the ability to uptake sucrose is decreased in 7-day-old tomato fruit of an sus mutant, resulting in small unhealthy fruit, which suggests that SUS activity is a determinant for young fruit development (D'Aoust et al., 1999).

Formation of malonyl-CoA from acetyl-CoA in the plastid is the first committed step for fatty acid synthesis, because malonyl-CoA is the central carbon donor for fatty acid synthesis. In this reaction, malonyl-CoA is formed from acetyl-CoA and CO₂ by acetyl-CoA carboxylase. Before the fatty acid synthesis begins, acetyl-CoA and malonyl-CoA are linked to an acyl carrier protein (ACP) to form acetyl-ACP and malonyl-ACP. Acetyl transacylase and malonyl transacylase catalyzes the reactions. Once the fatty acid synthesis is complete, a thioesterase removes the fatty acid from acyl carrier protein, transferred to CoA in the cytoplasm by Acyl-CoA synthases and transported to the ER mn,(Fig. 1.1). The established pathway for TAG biosynthesis is the Kennedy pathway (Kennedy, 1961). In the ER, fatty acids are transferred from CoA to each of the three hydroxyl groups of the glycerol backbone. In the mature embryo, oil is mostly stored in the sub-cellular organelle called oil bodies. These organelles consist of a matrix of TAGs surrounded by a phospholipid monolayer (Herman, 1995).
1.1.1 Seed development

The seeds of Arabidopsis are produced in fruits called siliques. Reproductive development from fertilization to seed desiccation usually takes 2 weeks under normal growing conditions in the lab (see Chapter 2). Within the silique, seeds are attached to the septum through a funicular. The funicular guides the pollen tube during fertilization (Shimizu and Okada, 2000) and provides a pipeline for nutrients from the maternal plant. Double fertilization occurs as soon as the pollen tube reaches to the embryo sac and two sperm cells are released: one of them fuses with the central cell and gives rise to the endosperm (3n) which later becomes the nutrient supply organ for seed development; the other sperm cell fuses with the egg cell and becomes a diploid zygote.
The embryo and endosperm are covered by a maternal seed coat. The seed coat consists of several cell layers and is used to protect the seed from physical injuries. Arabidopsis embryogenesis development can be divided into two broad stages: embryo morphogenesis and maturation. Embryo morphogenesis is initiated by fertilization. Through a series of cell divisions, the zygote gives rise to the embryo and its suspensor with morphogenesis ending approximately 6 days post anthesis (DPA). The suspensor transports nutrient from maternal tissues to the developing embryo and it is also a source of growth factors required for continued growth of the embryo. Later, during the maturation phase, the embryo grows rapidly, allowing it to fill the seed while the endosperm is degraded to one cell layer between the embryo and seed coat (during 7-10 DPA). Around the same time, the seed starts to accumulate storage compounds. From 7 to 10DPA, the starch level decreases while oil and protein synthesis increases resulting in an increase in seed weight. Endosperm development is different from that of the embryo, and can be divided into 3 steps: syncytial phase, cellularization and degeneration (Fig1.2). During the first phase, the endosperm cells undergo rapid cell division, resulting in 3 types of endosperm cells. The micropylar endosperm surrounds the embryo, while the peripheral endosperm surrounds the developing embryo sac, and the chalazal endosperm is adjacent to the suspensor. When the embryo reaches maturation; the endosperm degenerates, providing storage reserves to the developing embryo. In Arabidopsis and oilseed rape (Brassica napus), the embryo absorbs these reserves from degenerated endosperm and stores them in the cotyledons (Olsen, 2004).

In the mature embryo cotyledons, storage proteins and lipids are stored in the protein storage vacuoles (PSVs) and oil bodies. The seed oil, in the form of TAGs, comprises 33%-43% of the Arabidopsis seed’s dry weight while proteins represent up to one third (O’Neil et al., 2003; Baud et al., 2002).
Fig 1.2 An overview of seed development in Arabidopsis. The diploid embryo and the triploid endosperm are generated from double fertilization. Embryo organization is completed during the embryogenesis phase and reserve accumulation mainly takes place during the maturation phase.

1.1.2 Import of carbon for fatty acid synthesis in developing seeds

Almost 80 percent of the sugar from photosynthetic tissues is transported through the phloem to various sink tissues (like seeds). The high concentration of sugar inside the phloem attracts water, increasing the hydrostatic pressure until it is delivered into the sink tissues (Fig 1.3 a). In Arabidopsis, the vascular tissue terminates in the funiculus with no direct connection to the seed (Lepiniec et al., 2006). Therefore, all nutrients destined for the embryo must first go through the funiculus, seed coat and endosperm. The Arabidopsis seed coat has two integuments: the inner integument which consists of three cell layers and the outer integument which has two layer of cells (Lepiniec et al., 2006). The outer cell layers can upload nutrients and represent a symplastic extension of the funicular phloem. In this way, nutrients flow from cytoplasm to cytoplasm. Later, nutrients are also retrieved, with the help of membrane transporters, from the seed coat apoplast and moved apoplastically to the storage site. In contrast to the symplastic flow, the apoplastic route allows free diffusion within the cell wall (Kim and Zambryski, 2005).
During the morphogenesis phase of seed development, sucrose is first converted into hexoses (glucose and fructose). The hexoses move across the apoplastic space and get into the endosperm and embryo by hexose transporters located in epidermal cells (Weber et al., 1997). These hexoses can be stored in either embryo or endosperm during the early developmental stages. It is believed that initially the endosperm contains most of the sugars. Later in embryogenesis, hexoses represent the major reserve accumulated in the embryo. In the maturation phase, sucrose is directly delivered into the embryo through the activity of a sucrose transporter located in the epidermis of the embryo (Fig 1.3b). During the time that the endosperm is degraded, the embryo grows, and nutrients recycled from the endosperm are taken up by the embryo to be used in part for storage compounds (Offler and Patrick, 1993). In oil seeds, sucrose can be used to generate the substrates for fatty acid biosynthesis needed for oil metabolism (Ruuska et al., 2002).
Fig 1.3 Sucrose transport in Arabidopsis.

a) Sucrose is loaded into the phloem of leaves (source) and unloaded from the phloem in sink tissues. This process is shown by red arrows. At the same time, water returns to the parent plant through the xylem (shown in black arrows).
**Fig 1.3** Sucrose transport in Arabidopsis.

b) The upper half demonstrates the pathway of sucrose import during the morphogenesis phase. At this time sucrose is taken-up from the mother plant through the vascular system in the seed coat and converted into hexoses. The lower part represents the pathway of sucrose import during maturation phase. In this pathway, sucrose is used directly by the embryo without conversion to hexoses. V: Vascular system.

### 1.1.3 Synthesis of Triacylglycerols

Triacylglycerols (TAGs) consist of glycerol-3-phosphate (G-3-P) backbone and fatty acids which are both synthesized in the plastid and later transported into the ER to form TAGs. Fatty acids are produced from acetyl-coenzyme-A (acetyl-CoA) and malonyl-CoA (Fig 1.4).

There are 4 possible pathways for acetyl-CoA synthesis. First the free acetate can be used to form acetyl-CoA by acetyl-CoA synthetase (ACS) in an ATP-dependent manner; the second route involves the acetyltransferase reaction in which acetate is transferred from acetyl-carnitine to CoA; a third possible way is through the ATP-citrate lyase reaction;
the last but the most important way is from pyruvate through the activity of pyruvate dehydrogenase complex (PDC), and this pathway has been proposed to be the major pathway of acetyl-CoA pool synthesis in oil seeds (Lernmark and Gardestriim, 1994; Roughan and Slack, 1982; Roughan, and Ohlrogge, 1994; Smith et al., 1992; Kang and Rawsthorne, 1994). Acetyl-CoA is the precursor of fatty acid synthesis.

An activated CO₂ group is transferred to acetyl-CoA by acetyl-CoA carboxylase to form malonyl-CoA which is the major carbon donor in the fatty acid synthesis pathway. This reaction is the first committed step of fatty acid synthesis and also is rate-limiting. A great deal is known about the process of fatty acid synthesis; however the knowledge about this pathway has relied on the analysis of intact plastids in vitro. Biosynthesis of fatty acids in the plastid requires attachment to acyl carrier protein (ACP). So, before fatty acid synthesis begins, the acetyl group is transferred from CoA to ACP by acetyl CoA-ACP transacylase, to form acetyl-ACP and malonyl-ACP. ACP is a small protein which can bind the growing acetyl chain as a thiol ester at the distal thiol of a 4'-phosphopantethiene moiety. All the fatty acids in these reactions carry ACP until they are ready for export from the plastid.

The initial condensation reaction in fatty acid biosynthesis utilizes acetyl-CoA and malonyl-ACP to form 3-ketoacyl-ACP. In this process a molecule of CO₂ is lost from the α-carbon of the malonyl-ACP in a reaction catalyzed by 3-ketoacyl-ACP synthetase III (KAS III). Following this condensation reaction a series of reactions occurs (Fig 1.4 left): First the ketone is reduced to the alcohol by the enzyme 3-ketoacyl-ACP reductase, then the alcohol is dehydrated by 3-hydroxyacyl-ACP dehydratase to form a 2,3 double bond. Last, a second reduction removes the double bond to form butyryl-ACP by enoyl-ACP reductase. The first series of reactions results in the formation of a four-carbon chain attached to ACP. In the next round of fatty acid synthesis, a new malonyl-ACP is utilized, extending the acyl chain by 2 carbon molecules. The series of cyclical reactions does not terminate until the number of carbons in the acyl chain reaches an appropriate length. The major products of this process are 16:0-ACP and 18:1-ACP which are exported to the ER.
The condensation reactions of the fatty acid biosynthetic pathway are catalyzed by 3-ketoacyl-ACP synthases (KASs). All plants contain three KAS isoenzymes: KAS I, KAS II and KAS III, distinguished by substrate specificity (Somerville et al., 2000). The first condensation reaction which is catalyzed by KAS III has been mentioned above. KAS I is responsible for producing chain lengths from C4 to C16 and the elongation from C16 to C18 requires KAS II (Fig. 1.4 right) (Ohlrogge and Browse, 1995).

### Fig. 1.4
The reactions of fatty acid biosynthesis. The number represents the enzyme in each reaction. 1: acetyl-CoA carboxylase; 2: acetyl-CoA-ACP transacylase; 3: malonyl-CoA-ACP transferase; 4: 3-ketoacyl-ACP synthetase; 5: 3-ketoacyl-ACP reductase; 6: 3-hydroxyacyl-ACP dehydratase; 7: enoyl-ACP reductase. FAE: fatty acid elongation.
(Modified from Ohlrogge and Browse: Lipid Biosynthesis. 1995)

The major products of fatty acid synthesis are 16:0-ACP and 18:0ACP. By the time the acyl group is removed from the ACP, 18:0-ACP is already desaturated to 18:1-ACP by stearoyl-ACP desaturase. There are two types of enzymes involved in releasing the fatty acid from ACP: thioesterase and acyltransferase. The choice of enzyme determines the fate of fatty acids. Acyltransferase moves the fatty acid from the ACP directly to the glycerol-3-phosphoethanolamine in the plastid which later can be used as substrates for more complex lipid biosynthesis (Somerville et al., 2000). Alternatively, the fatty acid
can be released by the thioesterase and later exported from the plastid. Those exported fatty acids are used for TAG biosynthesis on the ER. There are two types of thioesterases in plants: FatA and FatB. FatA is active mostly with 18:1-ACP, FatB, on the other hand, prefers shorter chains and saturated acyl-ACP substrates (Fig1.5). Subsequent desaturation of these lipids to highly unsaturated forms is carried out by fatty acid desaturases (Fad) in the chloroplast and the ER.

Another important precursor of TAG is G-3-P which is also synthesized in the plastid. In seeds, dihydroxyacetone phosphate and NADH can be converted into G-3-P by G-3-P dehydrogenase. Alternatively another enzyme, glycerol kinase, can convert glycerol into G-3-P as well (Sadava and Moore, 1987; Kirsch et al., 1992). Within the ER, G-3-P and acyl-CoAs represent the primary substrates for TAG assembly (Stymne and Stobart, 1986). TAG biosynthesis takes place via the Kennedy pathway (Fig1.6) in the ER (Kennedy, 1961). This pathway is initiated by transferring the acyl chain from CoA to the sn-1 position of G-3-P by G-3-P acyltransferase, resulting in lysophosphatidic acid. Then the lyso-phosphatidic acid acyltransferase (LPAT) catalyzes the transfer of acyl-chain from the CoA ester to sn-2, creating phosphatidic acid (PA). Alternatively, PA can also be synthesized from phosphatidylcholine (PC) by the activity of phospholipase D (PLD) which releases diacylglycerol (DAG). The final enzyme, diacyl-glycerol acyltransferase (DGAT) transfers the third acyl group to DAG and forms TAG. Alternatively, membrane lipids (PC, PE or PI) can be used to generate DAG through the activity of choline phosphotransferase (CPT). Fatty acids produced in the plastid are not always immediately

Fig1.5 Fatty acid desaturation and transportation.
available for TAG biosynthesis, instead they may enter into PC, PE or PI, in which form they can be stored within the membrane temporarily. Thus, formation of TAG can be achieved in different ways.

Seed oil is accumulated in organelles called oil bodies which are spherical structures, comprised of an oil droplet of TAGs surrounded by a monolayer of phospholipids. Usually the surface of these oil bodies is covered by a unique class of protein called oleosins (Yatsu and Jacks, 1972). There is still much debate about oil body biogenesis. It has been proposed that the highly hydrophobic TAG would accumulate between the two leaflets of the ER membrane until an oil-filled vesicle forms and separates from the ER. An alternative hypothesis is that TAGs are synthesized as naked oil droplets in a granular area of cytoplasm and encased with phospholipids and oleosins at a later stage of development (Murphy, 1990; Huang, 1992).

Fig 1.6 Triacylglycerol biosynthesis by the Kennedy pathway in developing oilseeds. G-3-P, glycerol-3-phosphate; G-3-P AT, glycerol-3-phosphate acyltransferase; LPAT, lysophosphatic acid acyltransferase; PA, phosphatidic acid; PC, Phosphatidylcholine; PE, phosphatidylethanolamine; PAP, PA phosphatase; PI, phosphatidylinositol; DAG, diacylglycerol; DGAT, diacyl-glycerol acyltransferase; TAG, triacylglycerol; CPT, sn-1,2-diacylglycerol cholinephosphotransferase. (Modified from Stymne and Stobart 1986).
1.2 Roles of Phospholipase D (PLD) in storage oil production

Phospholipase D (PLD) is a ubiquitous enzyme participating in various cellular processes (Liscovitch et al. 2000; Wang, 2000). This enzyme is widespread in animals, fungi and plants. It was first discovered in plants several years ago but only received renewed attention in recent years. PLD catalyzes the hydrolysis of glycerol phospholipids, such as PC, to produce PA and a free hydrophilic head group. PA is an intracellular lipid messenger and can also be further metabolized to two other lipid mediators, diacylglycerol and lyso phosphatidic acid. All these activities potentially affect membrane structure and lipid degradation in the cells. Since the PLD characterized in this thesis belongs to the PLD family, this section of the literature review focuses on the various properties of PLD enzymes found in mammals and plants.

1.2.1 Molecular diversity of Phospholipase D

Many PLD isoforms have been cloned from plants, yeast and mammalian sources. All the PLDs share a highly conserved HxK(x)4D(x)6GSxN motif (Ponting et al. 1996).

A cDNA encoding a 1072-amino-acid protein that was isolated from HeLa cells was named PLD1. Following the cloning of PLD1, a second cDNA, encoding a 932-amino-acid protein with 51% amino acid sequence to PLD1, was found in a mouse embryo cDNA library. This new PLD was recognized as a distinct mammalian isoform (PLD2) with the most notable homology within the central catalytic core (Colley et al. 1997).

Plant PLD genes have also been described for several years. The first cDNA coding for plant PLD was isolated from castor bean (Wang et al., 1994). Later, expression of this PLD protein in Escherichia coli suggested it possessed both hydrolytic and transphosphatidylation activities using PC as a substrate (Wang et al. 1994). Three Arabidopsis EST cDNA clones were found to share a significant sequence similarity to the cloned castor bean PLD, and named PLDo, β, and γ. PLD cDNAs have subsequently been cloned from many other plant species, including Pimpinella brachycarpa (Cha et al. 1998), rice, maize (Ueki et al., 1996), cabbage, cow-pea, and tobacco (Pappan et al., 1999).
As a widely used model system, Arabidopsis has been used extensively to study PLD function. While humans only have two PLD isoforms and yeast one (SPO14), Arabidopsis contains 12 PLD genes which can be grouped into 2 classes, based on their lipid-binding domains. Those with highest sequence similarity to the mammalian and yeast PLDs including PX and PH domains are called PLDζ (PLDZ). The other class includes 10 isoforms: PLDα(1,2,3,4), β(1,2), γ (1,2,3) and δ.

1.2.2 Domain structure and catalytic mechanism of Phospholipase D

PLD enzymes have a unique HKD catalytic motif, representing HxK(x)4D(x)6GSxN sequence, where H, K and D represent histidine, lysine and aspartic acid and x represents any amino acid residue (Ponting et al. 1996). Two nonadjacent HKD domains are present in each protein (Fig 1.7). Mutations in either motif can abolish the enzyme activity, suggesting that the duplicated motifs cannot function independently (Sung et al. 1997). Other defining features of PLD enzymes are the phox consensus sequence (PX) and the plekstrin homology (PH) domain (Fig 1.7). PLD1 and PLD2 mainly differ in the "loop" region, PLD1 has a 116-amino acid loop region inserted immediately following the first HKD domain (Fig 1.7). Deletion of this loop region increases PLD1 activity threefold (Sung et al. 1999), indicating it may function as a negative regulatory element. Otherwise, PLD1 and PLD2 are quite similar. Point mutations in the PH domain cause mislocalization of the PLD protein within the cells, suggesting that the domain is involved in protein translocation. Indeed, when the entire PH domain is removed, PLD still retains enzymatic activity in vitro (Sciourra et al. 2002; Sugars et al. 2002). Mutagenesis of PLD1 suggested that the PX domain is also not essential for the catalysis in vitro (Sung et al. 1999). However, the PX domain was found to bind phosphatidylinositol phosphate (PIP) signaling molecules, including PI4,5P2, PI3,4P2, PI3,5P2, and PI3,4,5P2 (Xu et al. 2001), but the function is still in debate.
All Arabidopsis PLDs contain two HKD motifs but they differ by the presence of distinct N-terminal phospholipid-binding domains—C2 vs. PX/PH (Fig 1.8). In Arabidopsis, there are 10 PLD isoforms that have a C2 domain. The C2 domain which can bind Ca2+ or other effectors (including phospholipids, inositolphosphates and proteins) is unique to plant PLDs. Only PLDZ1 and Z2 bear two phospholipid-binding domains, the PX and PH domain.

Radiolabel exchange experiments using a plant PLD suggested that the catalytic mechanism of PLD involves a “two step” reaction. In the first step of the reaction, PLD removes the head group of a structural, phospholipid, such as PC, and forms a covalent bond with the resulting phosphatidyl moiety, the PLD-PA intermediate. In the second step, PLD transfers the phosphatidyl moiety to a nucleophile. Under physiological conditions, this is water, representing the hydrolysis of PC to generate PA. Primary alcohols, such as 1-butanol, can also be used as acceptors, resulting in the formation of PBut, a reaction that is used to measure PLD activity in vivo and in vitro (Fig 1.9).
PA has been implicated in various cellular processes including signal transduction, membrane trafficking, secretion, and cytoskeletal rearrangement. So understanding how PLD is regulated in the cell will help us know more about PA metabolism.

![Diagram of PLD's catalytic activity](image)

Fig1.9 PLD's catalytic activity.
PLD removes the head-group of PC resulting in the formation of PA or PBut.
(Modified from Munnik and Musgrave: Phospholipid Signaling in Plants: Holding On to Phospholipase D. 2001)

1.2.3 Functional properties of Phospholipase D

PLD enzymatic action produces PA which is a potential lipid “second” messenger. PA can also be metabolized to form lyso-PA (LPA) or DAG. Early studies on PLD focused on the role in PLD-derived signaling networks. Recently, more attention has been paid to plant PLDs, centering on their participation in metabolism during different stresses (like aging, wounding, freezing or water deficit (Sang et al. 2001; Katagiri et al. 2001; Wang et al. 2001)). In particular, this section will describe several cellular functions of PLD, as well as its role in defense responses in plants.

1.2.3.1 Phospholipase D in signal transduction

The direct products of PLD hydrolysis are PA and a free head group. PA has been linked to many events of intercellular signal transduction in mammals. These pathways include signaling through phosphatidylinositol 4-phosphate 5-kinase (PI4P5-kinase) and activation of the mammalian serine threonine kinase (mTOR).
The phosphatidylinositol phosphate kinases (PIPK) use phosphatidylinositol phosphate (PI4P and PI5P) as the substrates to generate the important second lipid messenger, PI(4,5)P2 (Di Paolo et al. 2006). PI(4,5)P2 is involved in the regulation of a number of cellular activities, including gating of ion channels, recruitment of signaling complexes and vesicular trafficking (Moritz et al., 1992). PA stimulates PI4P5-kinase activity for PI(4,5)P2 production. On the other hand, PI(4,5)P2 itself can activate PLD activity, thereby increasing the PA concentration (Liscovitch et al., 1994). These results suggest the role of PA and PI(4,5)P2 in cellular signaling pathway is likely regulated through a PLD/PI4P5-kinase feedback loop.

mTOR is a serinethreonine kinase which is also the mammalian target of rapamycin. Rapamycin acts as an inhibitor of mTOR function. Recently, using an in vitro binding assay, PA was found to directly bind to mTOR at the rapamycin-binding site (Chen et al., 2002). Pretreatment with rapamycin blocked PA interaction with mTOR, suggesting that rapamycin and PA compete for this binding site (Greene et al., 1993). In support of this idea, overexpression of PLD2 conferred resistance to rapamycin while in a pld2 mutant, the sensitivity to rapamycin was increased (Chen et al., 2003). In addition, the use of interfering RNA (siRNA) against PLD1 almost completely inhibited S6 kinase1 (which is activated by mTOR) activity (Chen et al. 2003). All together these data suggest that PLD and PA are both important in the mTOR signaling network and the biological processes it governs.

1.2.3.2 Phospholipase D in membrane traffic

PLD enzyme is believed to function in vesicle trafficking, secretion and endocytosis. PLD was first associated with membrane traffic through its activation by adenosine diphosphate (ADP)-ribosylation factor (ARF). The ARF protein family functions in vesicle formation and transport between the endoplasmic reticulum (ER) and Golgi apparatus, suggesting that activation of PLD plays a critical role in these processes. One example is that mammalian PLDs can stimulate coat protein I (COPI) formation in vitro
COPI is an ARF-dependent protein functioning in membrane trafficking. In this secretory pathway, PLD product PA can be rapidly converted to DAG and DAG is required for membrane traffic in this pathway. Recent evidence showed the mechanism by which DAG might function in membrane traffic. When PA was depleted from the cell, the DAG in the Golgi decreased by 65% and COPI-coated vesicles were frozen in a late stage of budding. This suggests that DAG is required for the formation of COPI vesicles for transport between the Golgi and the ER.

Recent studies using PLD1-and PLD2-selective antibodies to explore the subcellular localization of PLD1 and PLD2 have shown that 25-30% of both enzymes localize to the Golgi apparatus (Freyberg et al. 2001). However, the role for PLD in vesicle formation is unclear. It was hypothesized that PA, which is the major product of PLD, functions to lower the energy barrier to fusion, acts as the binding sites for many proteins on membranes or it can be a precursor for diacylglycerol.

In contrast, the role of PLD in secretion and endocytosis is much better studied. The use of primary alcohols, including ethanol, blocked protein transport from ER to Golgi while the block was alleviated by exogenous liposomes containing PA, suggesting that PLD activity and its ability to generate PA were required for structural integrity of the Golgi. During the vesicle fusion, PLD attaches to the membrane and converts the membrane PC, a cylindrical, non-fusogenic lipid, to cone-shaped, fusogenic PA which promotes negative curvature in bilayer membranes. At this point, PA may also activate PI4P5-kinase and increase the level of singling PI(4,5)P₂, resulting in recruiting of the calcium-activated protein for secretion (CAPS) which is essential for Ca²⁺-regulated dense-core vesicle exocytosis (Grishanin et al. 2004; Olsen et al. 2003). It is reported that PLD plays a role in endocytosis/exocytosis in an ARF-dependent manner (Jones et al. 1999) and recently the PH domain of PLD1 has been shown to be required for regulated exocytosis of neuroendocrine cells (Du et al. 2004).

PLD may also be a player in the translocation of GLUT4 glucose transporter from intracellular membranes to the plasma membranes which is stimulated by insulin (de...
Torres Zabela et al. 2002; Emoto, 2000). PLD1 has been found to co-localize with GLUT4-containing structures and PLD activation has been observed in cells which express high levels of insulin receptors. Another membrane trafficking event that requires PLD activity is in the human liver (Munnik et al. 1995; Asp et al. 2000). The major function of very low density lipoproteins (VLDLs) is to deliver triacylglycerol from the liver to peripheral tissues. The assembly of VLDLs containing apolipoprotein B is a complex process that occurs in the lumen of the secretory pathway. Recent observations indicate the conversion of the apolipoprotein B-containing precursor to VLDL is dependent on the activation of PLD (Munnik, 2001; Olofsson et al., 1999).

1.2.4 Biological functions of Phospholipase D enzymes in Arabidopsis thaliana

PLD has been implicated in many plant processes which include responses to abiotic and biotic stresses, such as low temperature, salts, nutrient starvation, ROS and wounding. It also function in specific steps in growth and development such as seed germination, seed aging and root hair formation, root growth and pollen germination. PLD has been demonstrated to affect plant physiology through its enzymatic production of PA, which has a role in hormone signaling responses and downstream kinase activation.

1.2.4.1 PLD in abscisic acid (ABA) signaling responses

Abscisic acid (ABA) is an isoprenoid plant hormone involved in diverse plant processes, including stomatal movement, seed and bud dormancy, and plant responses to drought, salinity and low temperature stresses. In Arabidopsis, the stomata of PLDα1-deficient plants fail to close in response to ABA, whereas added PA promotes the closure (Zhang et al. 2004), suggesting that ABA signal transduction is defective in PLDα1-deficient plants. On the other hand, overexpression of PLDα1 makes the plant more sensitive to ABA and reduces water loss (Sang et al. 2001). Together, these data indicate that PLDα1 and its enzymatic product PA play an important role in ABA-induced stomatal closure. Additionally, recent studies have shown that mutants with defective PA phosphatase genes, are hypersensitive to ABA during germination (Katagiri et al. 2005). PA phosphatase catalyzes the conversion of PA to DAG and loss of this activity may
attenuate PA degradation and increases PA accumulation, making seeds more sensitive to ABA.

For years, the mechanism by which PLD mediates ABA signaling cascades remained a mystery until the identification of the ABI (ABA insensitive) protein which is a negative regulator of ABA responses in Arabidopsis. ABI can bind to PA and the binding decreases the activity of ABI, thereby promoting ABA signaling (Zhang et al 2004). This finding provides a direct connection between the PLD family and the important ABA signaling pathway.

1.2.4.2 PLD in the response to reactive oxygen species (ROS) and cell death

ROS are thought to damage proteins, DNA, and lipids and eventually cause cell death in plants. However, the mechanism by which the toxic molecules injure the cells is still unknown. Nevertheless, what we do know that the level of ROS in cells needs to be tightly regulated. In Arabidopsis there are more than one hundred genes involved in controlling the level of ROS. This network is complex and dynamic, but recent work revealed several “key players” in this pathway, including PLD.

In Arabidopsis, PLDα1 has been implicated in increasing NADPH oxidase activity which stimulates the production of ROS (Sang et al. 2001). Unlike PLDα1, PLDδ is reported to stimulate a decrease in ROS-promoted programmed cell death (Zhang et al. 2003). These results suggest that different PLDs function differently in response to ROS. However, it is reported that when PA is added to Arabidopsis leaves, the level of ROS increases, as does the cell death rate (Zhang et al. 2005). The explanation is still in debate.

1.2.4.3 PLD in stress tolerance

In several stress conditions, such as dehydration and high salt, and during cold acclimation, the expression of PLDδ increases. Deletion of PLDδ in Arabidopsis decreases the tolerance to freezing, and overexpression of PLDδ increases cold tolerance (Li et al., 2004). In contrast to PLDδ suppression of the most abundant plant PLD,
PLDa1, makes plants more tolerant to freezing. The increase in PA during freezing is accompanied by a decrease in PC. PC is a bilayer-stabilizing lipid, whereas PA has the tendency to form hexagonal phase that destabilizes membranes and increases leakage, the key events in freezing injury (Welti et al. 2002). Thus, suppression of PLDa1 may prevent the membrane from wounding transition from lamellar to hexagonal phase.

1.2.4.4 PLD in plant-pathogen interaction

PLD has been suggested to be involved in plant-pathogen interactions. In Arabidopsis, there are four PLDs (α1, β1, γ1 and γ2) that exhibit different temporal patterns of expression after infiltration of leaves with a pathogen. Before inoculation, PLDs are distributed evenly along the plasma membrane; however, they become clustered at the site of pathogen attachment (de Torres Zabela et al. 2002). This clustering could be related to the generation of signaling messengers such as PA, free fatty acid and DAG in response to pathogen attack.

1.2.4.5 PLD in root growth and root hair patterning

Root hair initiation and development is controlled by both genetic and environmental factors, such as the transcription factor GLABRA2 (GL2), Ca2+ ROS, light and rhizobium nodulation factors (Charron et al. 2004). GL2 is a negative regulator of root hair development. Deletion of GL2 causes massive root-hair growth in Arabidopsis. During this process, GL2 was found to bind the promoter of PLDZ1 directly. In addition, overexpression of PLDZ1 induces branched and swollen hair-roots. This phenotype is similar to the gl2 mutant, suggesting that GL2 is a negative regulator of PLDZ1 in root hair pattern formation. Arabidopsis plants doubly mutant for PLDZ1 and Z2 do not show an obvious root phenotype except that root elongation during phosphate-limited conditions is decreased (Li et al., 2006). On the other hand, PLDs may affect root growth and development through their effects on membrane-trafficking, exocytosis and cytoskeletal rearrangements. PLDZ1 fused to GFP is found mainly in vesicles in root-hair apices. This location suggest that PLDZ1 may play a role in vesicle trafficking during root hair initiation and growth (Ohashi et al. 2003).
1.3 Roles of GLABRA2 in storage oil production

A number of regulatory proteins that are required for oil accumulation in the Arabidopsis seed, including GLABRA2 (GL2), have been identified. In this section, I focus on GL2 both because a loss-of-function mutant in the GL2 gene has a high-oil phenotype (Shen et al, 2006), and because GL2 directly regulates PLDZ in root hair pattern formation (Ohashi et al, 2003).

1.3.1 GLABRA2 is a transcription factor

GL2 is a transcription factor that belongs to the homeodomain-leucine zipper (HD-Zip) family which has only been identified in plants (Rerie et al., 1994). There are four classes of the HD-Zip proteins in Arabidopsis, and GL2 belongs to class IV (Ruberti et al, 1991; Schena and Davis, 1992). As suggested by the name, the GL2 protein consists of a typical leucine zipper that binds to DNA as a homodimeric complex. Deletion of the leucine zipper motif or the HD domain significantly reduces binding affinity, suggesting that these two parts are important for function.

1.3.2 Biological functions of GLABRA2 in trichome patterning, morphogenesis and differentiation in Arabidopsis

Trichomes are present on the surfaces of leaves and stems in most plants. They are thought to function in plant resistance to insect herbivores, reduce water loss, increase freezing tolerance and protect plants from UV light. Trichomes are large, single polyploid cells sticking out from the epidermis. Trichome development starts with an enlargement of the nucleus and an increase in cell size. The cell then elongates and finally branches. Secondary branching occurs perpendicularly to the primary branch plane, and a thick secondary cell wall is produced resulting in a mature trichome decorated with cell surface papillae. (Hulskamp et al., 1994)

Mutations causing abnormal trichome phynotypes have been identified during the last decade. A large number of genes were reported to be involved in the trichome formation
and development. Most of the genes have been cloned and the regulatory network they establish is well understood (Fig1.10).

The gl2 mutant was first identified based on its trichome phenotype (Koornneef et al., 1982) Loss-of-function gl2 mutants produce trichomes that expand aberrantly on the surface of leaf (Rerie et al., 1994). In leaves, GL2 is expressed in trichomes at all stages of their development, and it always has a lower expression level in the cells surrounding the trichome.

A report from Ohashi suggested that GL2 positively regulates trichome initiation. Overexpression of GL2 leads to increased trichome initiations as well as trichome clusters. Also, GL2 heterozygotes were reported to have reduced number of trichomes on leaves (Ohashi et al., 2002).

The genetic and molecular analysis of the trichome-patterning identified a feedback loop including several positive and negative regulators. It has been shown that GL2 is a downstream target of the patterning genes GLABRA1(GL1), TRANSPARENT TESTA GLABRA 1(TTG1), ENHANCER OF GL3 (EGL3) and GLABRA3(GL3) as its expression is dependent on them (Szymanski and Marks, 1998; Hung et al., 1998; Lin and Schiefelbein, 2001). In addition, GL2 is also a downstream target of the negative regulators of trichome development like TRIPTYCHON (TRY), CAPRICE (CPC) and ENHANCER CAPRICE TRIPTYCHON1 (ETC1). All three of these belong to a small family of single-repeat MYB proteins. Mutations in these genes result in trichome clusters or increased number of trichomes (Wada et al. 2002). At first, GL2 and TRY/CPC/ETC1 are equally present in all epidermal cells. Then, small changes in their concentration can be amplified, inhibitors like CPC can diffuse faster than GL2 to neighboring cells and inhibit GL2 production there (non-trichome cells), while the GL2 level in the less inhibited cells (trichome cells) keeps increasing due to a positive feedback loop by GL1/TTG1/GL3/EGL3 complex (Fig1.10).
The GL1/TTG1/GL3/EGL3 complex is thought to activate both GL2 and the negative regulators TRY/CPC/ETC1. Later, these negative regulators move into the neighbouring cells where they compete with GL1 for binding to the GL1/TTG1/GL3/EGL3 complex, which results in inactivation of the complex.
(Modified from Hülskamp: Plant trichomes: a model for cell differentiation. 2004)

1.3.3 GLABRA2 is required for root hair patterning and specification in Arabidopsis

Roots are used to absorb water and nutrients from the environment. Root epidermal cells have one of two fates: development into a trichoblast (or root-hair forming cell) or development into an atrichoblast (root-hairless cell). Root hair growth may be considered as two separate phases: initiation and elongation. In Arabidopsis epidermal cells, the site of initiation for a developing root hair normally occurs in the apical end, which suggests that trichoblasts are polarized (Schiefelbein and Somerville, 1990; Masucci and Schiefelbein, 1994). Genetic analysis has revealed that GL2 is one the genes involved in root hair initiation and development.

Normally, the root epidermis of Arabidopsis contains root-hair-bearing or root-hairless cells. Loss-of-function mutation of gl2 results in plants with hairs throughout
the epidermis. These root-hair cells in the \( gl2 \) background are similar to differentiating hairless cells in their reduced cytoplasmic density, advanced cell vacuolation and extensive cell elongation. They also differ from normal root-hair cells in delayed root-hair outgrowth (James D. Masucci et al., 1996). Together, all these data suggest a role for GL2 in controlling the inhibition of root hair formation, however, other genes may act earlier than \( GL2 \) to control other aspects of root epidermal cell development.

\( TTG1 \) is a gene coding for a WD40 protein involved in root hair development, and its loss-of-function mutation, like \( gl2 \) mutants, have hairs throughout the epidermis. \( TTG1 \) is one of the important activators of GL2 (Di Cristina, 1996; Hung et al., 1998), but it is not the only regulator known. As in the trichome developmental regulation, CPC is also a negative regulator of GL2 in root hair formation.

Previous study shows that a \( WER/GL3/EGL3/TTG1 \) complex promotes expression of GL2 and CPC. One possible model can explain how this complex works: accumulation of GL2 in the cells leads to the specification of non-hair cell fate while CPC moves to the neighboring cells to form the inactive complex \( TTG1/CPC/GL3/EGL3 \), which prevents expression of GL2 in the future hair cells. The presence of CPC also leads to activation of GL3 and EGL3 expression. In a lateral feedback loop, GL3/EGL3 protein moves back to the no root-hair cells and participate in the \( WER/GL3/EGL3/TTG \) complex which activates GL2 and CPC. (Fig1.11).
Fig 1.11 The proposed models for root-hair patterning. Epidermal cells that express GL2 do not form root hairs. Most of the genes (or their homologues) that are involved in trichome development are also involved in root-hair patterning formation. (Modified from Hülskamp: Plant trichomes: a model for cell differentiation. 2004)

1.3.4 GLABRA2 is required for seed coat development in Arabidopsis

In the ovule, the gametophyte is surrounded by the nucellus and integuments. Following fertilization, the ovule develops into a seed, with the seed coat differentiating from the integuments. The seed coat consists of several layers of specialized cell types. In Arabidopsis, the innermost cell layer specializes in tannin biosynthesis while the outmost layer differentiates into the epidermal cells containing mucilage. The mucilage is composed of pectin, a highly hydrophilic polysaccharide that has a gel-like consistency when hydrated, so the mucilage is thought to protect the seed during germination. The mucilage accumulates in the apoplastic space of the epidermal cells. Subsequently a thick secondary cell wall, the columella, is formed trapping the mucilage between the primary and secondary walls. (Windsor et al., 2000, Western et al 2004).

GL2 is known as a regulator of mucilage production. In gl2 loss-of-function mutants there is no mucilage deposited in the seed. The MUCILAGE-MODIFIED4 (MUM4) gene, encoding a rhamnose synthase required for mucilage synthesis, was shown to be a
downstream target of the GL2 transcription factor. In one possible pathway, the GL2 is under the control of a complex composed of TRANSPARENT TESTA8 (TT8), ENHANCER OF GLABRA 3 (EGL3), TTG1 and APETALA2 (AP2), this complex activates \textit{GL2} and GL2 then activates \textit{MUM4} to enhance mucilage biosynthesis (Haughn and Chaudhury, 2005; Ramsay and Glover, 2005; Western, 2006; Payne et al., 2000; Johnson et al., 2002; Schiefelbein, 2003).

1.3.5 \textit{GLABRA2} affects storage oil content in Arabidopsis seed

As mentioned before, the major storage component in Arabidopsis seeds is oil which is accumulates in the form of TAG. In a recent study, a high-oil mutant in Arabidopsis was isolated. The high-oil phenotype was found to be caused by a disruption of \textit{GL2}. Upstream regulators of \textit{GL2} in trichome formation, root hair differentiation and mucilage synthesis, like GL1 and GL3, were tested to see whether they have a role in seed oil accumulation but the evidence did not support this hypothesis. These results suggest that the effect of \textit{GL2} on seed oil may be independent of trichome formation. A similar result was shown for MUM4 and TTG2, which both function upstream of GL2 in mucilage synthesis but mutations in these genes did not result in changes in seed oil content compared to the wild type, indicating that the lack of mucilage alone is not related to high-oil content in \textit{gl2} (Shen et al., 2006). The mechanism by which GL2 affects oil accumulation in the seed remains unclear. A previous study showed that GL2 protein was specifically bound to a 303-base pair (bp)-long DNA fragment of Phospholipase D zeta 1 (\textit{PLDZ1}) promoter (Ohashi et al, 2003). PLD, which cleaves phospholipids to produce phosphatidic acid (PA) and a free head group, has been implicated in root hair growth and patterning (Ohashi et al, 2003). This suggests that PLDZ under the control of GL2 may also be involved in seed oil accumulation.
Although 12 PLD genes have been identified in the Arabidopsis genome, there is little information about the contributions of individual PLD enzymes to specific fatty acid metabolic pathways. Based on previous papers, we know that GL2 negatively regulates seed oil accumulation and it also negatively regulates PLDZ1 gene. I hypothesize that GL2 negatively regulates PLDZ1 and PLDZ2 genes and that these genes together positively regulate seed oil accumulation (Fig 1.12).

**Fig 1.12 Research hypothesis**

### 1.4.2 Thesis objectives

The primary goal of my research was to functionally characterize PLDZ enzyme function in seed oil accumulation and to test their functional relationship with GL2. Amino acid sequence comparison suggests that PLDZ1 and PLDZ2 are very similar to each other and might be redundant in function. Therefore, loss of both gene functions in the seeds might lead to a visible phenotype. Furthermore, previous research has indicated that GL2 is a negative regulator of PLDZ1 in the root hair pathway. The same regulatory pattern may
also exist in the seed oil production pathway. Thus, the high oil content in the *gl2* mutant might be the consequence of increased *PLDZ* activity.

I had the following specific objectives for my Master project:

1. **To determine the tissue specificity of the expression of the *PLDZ1* and *PLDZ2* genes**

2. **To determine the function of *PLDZ1* and *PLDZ2* using reverse genetic analyses**

3. **To examine functional interactions of *PLDZ* genes and *GL2***
Chapter 2

Materials and Methods

2.1 Bioinformatics

12 PLD family proteins of Arabidopsis were chosen from the National Centre for Biotechnology Information (NCBI [http://www.ncbi.nlm.nih.gov]) database to perform full-length protein alignments.

In order to construct the maximum likelihood phylogram, the PLD amino acid sequences were first aligned with the ClustalX programme (Thompson et al., 1997), regions that could not be aligned unambiguously or contained deletions/insertions were removed prior to alignment. The program PHYML version 2.4.4 and Bayesian version 3.1.2 were used to generate a maximum likelihood-based tree. The tree was visualized using Treeview and processed with Photoshop (Adobe System Incorporated, Mountain View, CA).

2.2 General Arabidopsis Methodologies

2.2.1 Plant material

The Arabidopsis ecotype Columbia 0 (Col-0) was used as a WT control. \textit{GL2::GUS} was a gift from Shucai Wang (Department of Botany, UBC). All Salk T-DNA insertion lines were ordered from the Arabidopsis Biological Recourse Center (ABRC, Columbus, OH). There, six T-DNA insertion lines were available for the \textit{PLDZ1} gene and three for the \textit{PLDZ2} gene.

2.2.2 Growth conditions

Seeds were sown on culture plates containing AT-agar medium (Table 2.1; 3.5 g/500 mL) (Somerville and Ogren, 1982) and the appropriate antibiotics for selection (for transgenic seeds only). The seeds were sown on plates, stratified at 4°C for 3-4 days,
germinated in continuous light at 20°C for 7-9 days and transplanted into soil (Sunshine mix 5, SunGro Horticulture, Seba Beach, AB). Before planting, the soil was saturated with tap water. After transplanting, pots were covered with plastic wrap (Resinite, AEP Canada Inc., Westhill, ON) for 5 days after which the plastic was slit with a razor blade, and then removed 2 days later. Either 8-inch pots or 72-well potting trays were used for growing the plants. Growth chambers were maintained at 20°C with continuous light (90-120 uEin/m²sec PAR) for all experiments.

Table 2.1 Composition of AT medium.

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃</td>
<td>5(mM)</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>2.5(mM)</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>2(mM)</td>
</tr>
<tr>
<td>Ca(NO₃)₂</td>
<td>2(mM)</td>
</tr>
<tr>
<td>FeEDTA</td>
<td>0.05(mM)</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>70(μM)</td>
</tr>
<tr>
<td>Mn₄H₂O</td>
<td>14(μM)</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>0.5(μM)</td>
</tr>
<tr>
<td>ZnSO₄7H₂O</td>
<td>1(μM)</td>
</tr>
<tr>
<td>NaMoO₄2H₂O</td>
<td>0.2(μM)</td>
</tr>
<tr>
<td>NaCl</td>
<td>10(μM)</td>
</tr>
<tr>
<td>CoCl₂6H₂O</td>
<td>0.01(μM)</td>
</tr>
</tbody>
</table>

2.2.3 Plant Transformation
2.2.3.1 Floral dip

*Arabidopsis* plants were usually transformed using the *Agrobacterium tumefaciens*-mediated floral dip method (Clough and Bent, 1998). The plants were grown in 8-inch pots with 6-8 plants per pot. Once the plants had bolted, the primary bolts were cut off. The plants were used for transformation when the secondary bolts had at least a few open flowers. Single colonies of *Agrobacterium* strains containing the appropriate binary vector were inoculated into 25 mL of LB (Luria-Bertani) medium and cultured at 28°C overnight. 5mL of the overnight culture was then used to inoculate 250mL of LB broth in 1 L flasks that were then incubated on a shaker for approximately 24 hr. at room temperature. The *Agrobacterium* culture was transferred to sterile centrifuge tubes and
spun for 20 min at 4°C at 4000 rpm in a Beckmann J2-21 centrifuge with a JA 14 rotor, to pellet the cells. The supernatant was discarded and the pellet was resuspended by pipetting in a solution containing 5 % (w/v) sucrose and 0.02 % (v/v) Silwet-L77 (Lehle Seeds, Round Rock, TX).

This bacterial suspension was placed in a shallow container. Pots containing Arabidopsis plants were inverted and the flowers were immersed in the bacterial suspension for 20 to 30 seconds with gentle swirling. Pots were then placed on their sides, covered with black plastic bags to generate low light conditions and left overnight. Plants were then returned to their normal upright positions and grown until senescence and the seeds harvested.

2.2.3.2 Spraying

Arabidopsis was also occasionally transformed by spraying the plants with an Agrobacterium suspension (Chung et al., 2000). In this case, Agrobacterium was grown to the stationary phase, and then pelleted at approximately 8,000 RPM in the centrifuge (Beckman model J2-21, Rotor JA-14) for 5 min. at room temperature. Harvested cells were resuspended in 5% sucrose for washing and centrifuged again. Finally two volumes of 5% (w/v) sucrose with 0.02 % (v/v) Silwet L-77 (Lehle Seeds, Round Rock, TX) was used to suspend Agrobacterium. The bacterial suspension was applied to flowering plants (pots containing 150-200 flowers) at weekly intervals using a spray bottle. Transformed plants were placed in covered flats for 1 day before returning to normal growing conditions.

2.2.3.3 Screening for transgenic Arabidopsis in the T1 generation

Harvested seeds were germinated on AT medium containing agar (3.5 g/500 mL) and appropriate antibiotics. Depending on the transgenic construct, the selection agent was ampicillin (100 mg/L), hygromycin (50 mg/L) or kanamycin (50 mg/L).

Seedlings that have acquired the transgene were selected based on their resistance to a specific antibiotic. After 14 days, resistant seedlings were transplanted into soil. Along with transgenic plants, untransformed WT plants (ecotype Columbia-0) and WT
Columbia-0 plants transformed with an empty vector were always planted as controls for comparison.

2.2.4 Double and triple mutant construction

The double mutant \textit{pldz1pldz2} was obtained by crossing the \textit{pldz1} and \textit{pldz2} single mutants and screening the \textit{F2} population. In order to reduce the possibility of self-fertilization during crosses, flowers from the female parents were used before the anthers began to shed pollen onto the stigma. The oldest female parent flowers where petal tissue was not visible were emasculated and the stigma pollinated with pollen of mature stamens from the male parent. Once the siliques had senesced (approximately 2-3 weeks) the seeds were collected and dried at room temperature for 2 weeks before planting. The triple mutant \textit{gl2pldz1pldz2} was obtained by crossing the \textit{pldz1pldz2} double mutant and \textit{gl2} in the same way. Double or triple mutants were identified from the \textit{F2} generation by PCR analysis.

2.3 Nucleic acid analysis

2.3.1 Isolation of genomic DNA

One young rosette leaf from each seedling was removed and stored at -80°C in a 1.5 mL Eppendorf tube. After all samples were collected, 50 μL of 0.5 N NaOH was added to each tube. The tissue was ground using a plastic pestle and the tube was vortexed briefly and centrifuged for 2 min. at maximum speed in a microcentrifuge. 2 μL of supernatant was diluted in 198 μL of 100 mM Tris-HCl (pH 7.9) and an aliquot was used for genotyping (Alonso et al., 2003).

2.3.2 Isolation of total RNA

Developing seeds were harvested and immediately frozen in 1.5 mL Eppendorf tubes by immersing the tubes into a liquid nitrogen bath. Total RNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA) according to manufacturer’s protocol. Briefly, a method involving acid phenol-LiCl (Downing et al., 1992) was applied to extract RNA from seeds. 3M sodium acetate (pH 5.2) was added to the sample to remove excess polysaccharides from the RNA precipitation.
2.3.3 RNA qualification and reverse transcriptase reaction

RNA concentration was quantified by using BioSpec-1601 B UV/visible spectrophotometer (Mandel Scientific Co. Ltd, Shimadzu, Japan). The concentration and purity of RNA was determined by measuring the absorbance values at 260 nm (A260) and 280 nm (A280). After quantification, 1 ug of total RNA was used for first-strand cDNA synthesis by Superscript III reverse transcriptase according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA).

Template concentrations and PCR cycle number were optimized for all fragments amplified, in order to equalize cDNA concentrations in the samples and to avoid DNA amplification. The primers designed for RT are shown in Table 2.2. General PCR conditions were 94°C for 3 min, followed by the number of cycles optimized for the particular primer pair, denaturation at 94°C for 15 s; annealing at 56°C for 30 s; and extension at 72°C for 30 s. PCR products were resolved by electrophoresis in a 0.8% agarose-TAE gel containing 5 ug/mL of ethidium bromide and visualized and photographed using the Alphalmager 1220 UV transilluminator equipped with a digital camera.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLDZ1</td>
<td>CGGCCGATATTAGCCCTGTACTCTTG</td>
<td>GCCATTCTTTAACCTGTTCCTGCT</td>
</tr>
<tr>
<td>PLDZ2</td>
<td>AGACAGGAGAAAGTACCAGCGAAT</td>
<td>TGTGGTGGTGAGGCATCAACAAATG</td>
</tr>
<tr>
<td>GL2</td>
<td>GCGCAAGCCTTCCGTCTTTC</td>
<td>GTCTTTGATGGGGACGTAG</td>
</tr>
<tr>
<td>GAPC</td>
<td>CAGATTGCCCTCAGGGCTTCCGCG</td>
<td>TTCACGCACCCTGTGGCGAGTCC</td>
</tr>
</tbody>
</table>

2.4 Analysis of Salk T-DNA insertion lines

The Salk Institute Genomic Analysis Laboratory (SIGnAL; http://signal.salk.edu) database was searched for all T-DNA-mutagenized lines with insertions in the PLDZ1, PLDZ2 and GL2 genes. Relevant lines were ordered from the ABRC and used for PCR-based genotyping to identify plants that were homozygous for the insertions. To genotype the plants, DNA was isolated from fresh leaves using the method described above. This DNA was used for PCR reactions with genomic DNA-specific forward and reverse
primers (Table 2.3) for specific target genes. The T-DNA-specific LBb1 forward primer (5’GCGTGGACCGCTTGCTGCAACT 3’) was used with a gene specific reverse primer to identify the presence of an insertion.

Table 2.3  Forward and reverse primers used for Salk line genotyping.

<table>
<thead>
<tr>
<th>Mutant allele</th>
<th>Salk designation</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLDZ1</td>
<td>S_089098</td>
<td>TCACACTTCCCAATTTTGGAG</td>
<td>GCGAGAGACAGAGACGATCAC</td>
</tr>
<tr>
<td>PLDZ2</td>
<td>S_094369</td>
<td>CCGGAGATGAAGAAATGTTC</td>
<td>TCGCACCACGAGAAAATAGT</td>
</tr>
<tr>
<td>GL2</td>
<td>S_130213</td>
<td>ACCACCGATGACGATGGATC</td>
<td>GACCTGAACATGCCAGTTTCTC</td>
</tr>
</tbody>
</table>

2.5  Analysis of Gene Expression
2.5.1  Real-Time PCR
The first-strand cDNA used for Real-Time PCR was obtained as described above (2.3.2 and 2.3.3). Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA) was used to perform qPCR in a MJ Mini Opticon Personal Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) according to manufacturer’s protocols. The primers designed for Real Time PCR are listed in Table 2.4.

Table 2.4  Primers used for Real Time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLDZ1</td>
<td>GCTGATGAAGTTCCGTCTACA</td>
<td>GCATTGCATGCTTTCCCTCTA</td>
</tr>
<tr>
<td>PLDZ2</td>
<td>GGAAACTTCCAGAGAAAGCG</td>
<td>TCACCTGCTTCCCAATCTG</td>
</tr>
<tr>
<td>Actin</td>
<td>GGGAAATCGTGCGTGACATC</td>
<td>CCAAGAAGGAAGGCTTGAAAG</td>
</tr>
</tbody>
</table>

2.5.2  GUS analysis
2.5.2.1  Generation of promoter::GUS constructs
For all the promoter::GUS fusion constructs, PCR fragments were amplified using Platinum Pfx DNA polymerase (Invitrogen, Carlsbad, CA) from Col-0 genomic DNA. For the promoter-GUS constructs, the promoter regions of PLDZ1 (Fig 2.1) and PLDZ2 (Fig 2.2) were amplified using gene specific primers (Table 2.5). The PLDZ promoters were introduced by BP recombination into the Gateway entry vector pDONR207 (Invitrogen Carlsbad, CA) using BP ClonaseTM II Enzyme Mix (Invitrogen, Carlsbad, CA) and transformed into chemically competent E. coli cells (DH5α). Following the selection on Gentamicin (50 mg/mL), sequencing of an individual clone confirmed that there were no errors in the PLDZ cDNA fragment. The insert was then subcloned into
Gateway-compatible cloning vector pMDC163 (Provided by Peter Wu, Kunst Lab) using the LP ClonaseTM II Enzyme Mix (Invitrogen, Carlsbad, CA) separately by an LR recombination reaction to obtain a translational fusion between the PLDZ gene and the GUS gene according to the manufacturer’s direction. Colonies were selected on LB medium supplemented with Hygromycin (25 mg/mL).

**Fig 2.1 PLDZ1 promoter::GUS construct.**
A 1986-bp-long PLDZ1 fragment including the 5’ upstream regulatory region, a part of the coding region of the upstream gene, the 5' untranslated region of PLDZ1, the first intron (denoted I), and a part of the PLDZ1 open reading frame (including one intron that is denoted II) was used for promoter-reporter gene fusion constructs. Introns are shown as thin lines, untranslated regions as hatched boxes, and coding regions as open boxes. Numbers shown are relative to the PLDZ1 translational start codon. attB1 and attB2 refer to the corresponding Gateway recombination sequences.
Fig 2.2 PLDZ2 promoter::GUS construct.
A 1886-bp-long PLDZ2 fragment including the 5’ upstream regulatory region, a part of the coding region of the upstream gene, the 5’ untranslated region of PLDZ2, the first intron (denoted I), and a part of the PLDZ2 open reading frame (including one intron that is denoted II) was used for promoter-reporter gene fusion constructs. Introns are shown as thin lines, untranslated regions as hatched boxes, and coding regions as open boxes. Numbers shown are relative to the PLDZ2 translational start codon. attB1 and attB2 refer to the corresponding Gateway recombination sequences.

Table 2.5 Primers used to amplify promoter fragments to generate Promoter::GUS constructs

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Fragment length(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLDZ1</td>
<td>GGGGACAAAGTTTGTACAAA AAAGCAGGCTCCGGTAACC AAGAAAGAAGTCGA</td>
<td>GGGGACCACCTTTGTACA AGAAAGCTGGGTCGTTA CATAAAGCGCTAAAGT</td>
<td>1753</td>
</tr>
<tr>
<td>PLDZ2</td>
<td>GGGGACAAAGTTTGTACAAA AAAGCAGGCTCCGGTAACC CCTATAGCATA</td>
<td>GGGGACCACTTTGTACA AGAAAGCTGGGTCGTTA CATATACAAAAACAG</td>
<td>2068</td>
</tr>
</tbody>
</table>
2.5.2.2 GUS histochemical assay

GUS histochemical assays were performed on freshly isolated plant seeds or whole siliques. The staining solution (100 mM NaPO₄, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.2 % Triton-X-100, and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc)) (Jefferson, 1987) was added and the samples were incubated at 37°C for between 2 hr. to overnight. The reaction was stopped by removal of the staining buffer and addition of 70% ethanol. The samples were destained by incubation in ethanol overnight at room temperature.

2.5.3 In situ hybridization analysis

In order to synthesize the probes for in situ hybridization analysis of PLDZ1 and PLDZ2, templates were amplified from first-strand cDNA (described in 2.3.2) by using primers that can incorporate the T7 RNA polymerase binding site at the 5' end (Table 2.6). These products were used for in vitro transcription to produce RNA by adding 2 μL of 10X DIG mix (Roche), 2 μL of 10X T7 polymerase transcription buffer (Roche), 0.5 μL of RNase inhibitor (Roche), 2 μL of T7 RNA polymerase (Roche) and 11.2 μL of sterile distilled water. After a 2-hour incubation at 37°C, the RNA produced was precipitated by adding 2.5 μL of 4 M LiCl and 75 μL of 100 % ethanol, and incubating overnight at -20°C. RNA was pelleted by spinning at maximal speed at 4°C in a refrigerated centrifuge (Effendorf centrifuge model 5415C, rotor F-45-18-11) and resuspended in 100 μL of DEPC treated water. The probe was then hydrolyzed into fragments between 75 and 150 base pairs long by adding 60 μL 200 mM Na2CO3 and 40 μL 200 mM NaHCO3 followed by incubation at 60°C for 87 min. The resulting reaction was neutralized with 10 μL 20 % acetic acid and the probe precipitated with 21 μL of 3 M NaOAC, 2 volumes of 100 % ethanol and 1 μL of 20 mg/ml oyster glycogen as carrier at -20°C for 2-3 hr. Probe pellet was dissolved in 100 μL of 50 % deionized formamide and then quantified against DIG standard according to manufacturer’s instructions.

Wild type seeds (Col-0) were fixed with FAA (3.7 % formaldehyde, 5 % acetic acid, 50 % ethanol) for 1.5 hour, then dehydrated through an ethanol series (60 %, 70 %, 85 %, 95 %, 100 %, and 100 %) for 30 min each. After ethano! dehydration, the samples were
treated with 75:25, 50:50, 25:75 ethanol/Histoclear, and two times 100% Histoclear. At last, the seeds were filled with half Histoclear and topped up with paraffin (Paraplast Plus; Sigma) in a 20 ml scintillation vial and left at 58°C overnight. Molten paraffin was subsequently used to replace the Histoclear/paraffin solution at least 4 times. Embedded tissues were sectioned (12 μm sections) with a microtome and floated onto microscope slides using double-distilled water, dried at 42°C overnight, and affixed to the slides by heating to 56°C for 4 hr. (Coen et al., 1990).

According to the protocol specified in Coen et al. (1990), paraffin was removed by dipping slides in 100 % xylene twice, and 100 % ethanol twice for 5 min. each. Following this, the samples were treated with 95, 90, 80, 60, and 30 % ethanol 0.85%NaCl, and 1X PBS (0.13 M NaCl, 3 mM NaH2PO4, 7 mM Na2HPO4 mM) for 5 min. each, incubated for exactly 30 min. at 37°C with 1 μg/mL proteinase K in 100 mM Tris-HCl, pH 7.5, and 50 mM EDTA, and washed with 1X PBS. Then slides were dehydrated in 0.85% NaCl, 30, 60, 80, 90, 95, 100 and 100 % ethanol for 5 min. each and stored at 4°C in a closed box with a few drops of ethanol. As soon as the samples were ready, digoxigenin-labeled RNA probes (10-50 ng) were diluted into 150 μL of hybridization buffer (10 mM Tris-HCl, pH7.5, 1 mM NaCl, 50 % formamide, 7 % dextran sulfate, 1 X Denhardt’s solution [0.02 % Ficoll type 400, 0.02 % polyvinylpyrrolidone, 0.02 % BSA], 500 μg/mL rRNA, and 250 μg/mL poly (A) RNA) and incubated overnight at 55°C. The next day, slides were washed in 2X SSC (1X SSC = 0.15 M NaCl, 0.015 M sodium citrate) for 5 min. to remove the coverslips, followed by washing four times in 0.2 X SSC at 55°C for 30 min., then one time at 37°C and once at room temperature. The slides now could be stored at 4°C or subject to immunological detection immediately. Immunological detection involved treating with 1 mL of 1 % blocking reagent (Boehringer Mannheim) in 100 mM maleic acid, pH 7.5, and 150 mM NaCl for 45 min and incubating for 45 min. in 1 mL of buffer A (1 % BSA (Sigma), 0.3 % Triton X-100 (Sigma), 100 mM Tris-HCl, pH 7.5, and 150 mM NaCl). After treating
with 1-2 mL fresh BSA solution, the slides were covered with cover slips and exposed to the antibody which recongnized the label on the probes. For the color reaction, slides were incubated overnight with 0.25 mL of 0.34 mg/mL nitroblue tetrazolium salt and 0.175 mg/mL 5-bromo-4-chloro-3-indolyl-phosphate p-toluidine salt in substrate buffer in the dark. The color reaction was stopped by dipping the slides in 10 mM Tris-HCl, pH 8.0, and 5 mM EDTA. The samples were then visualized and photographed under a compound light microscope. The photographs were processed using Adobe Photoshop (Adobe System Inc).

| Table 2.6 Primers used for in situ probe synthesis |
|-----------------------------|-----------------------------|
| **Gene** | **Forward primer** | **Reverse primer** |
| PLDZ1 | CATAATACGACTCACTATAGGCC | TGAAAGTAGCGTCCACCACCA |
| | CGACTTTTGAATTTTCCC | |
| PLDZ2 | CATAATACGACTCACTATAGGTT | ATTTGACACCGGCAATCACT |
| | GTTGTACCTACCCACCA | |

### 2.6 Fatty acid analysis

#### 2.6.1 Preparation of fatty acid methyl esters (FAMEs)

In order to measure the total fatty acids in seeds, 2.5 mg dry seed was transferred into 1cmX10cm glass tube with a Teflon screw cap. 1ml of freshly prepared 5% (v/v) sulfuric acid in methanol was added to each tube, followed by 25 ul of 0.2% (w/v) butylated hydroxy toluene in methanol and 75 ul of 12.5 µg/300 µL triheptadecin (17:0 methyl ester ) as the internal standard. Samples were incubated at 90°C for 1.5 h with vortexing every 20 min. After that, samples were placed on ice for 20 min. and 1.5 ml of 0.9% NaCl (w/v) and 2 ml hexane was added. Centrifugation at 2500 rpm was used to separate the different phases, with FAMEs in the top (hexane) phase. This phase was transferred into a new 1cmX10cm glass tube and dried under N2. 50 ul hexane was added to the dry tube and the dissolved FAMEs used for GC analysis.

#### 2.6.2 Single seed analysis

A single seed was placed individually into screw top Pyrex tubes and 0.5 mL 1N methanolic-HCl (Supelco) and 300µL of hexane were added to each tube, followed by 10
μL of 0.1 mg/ml triheptadecnoin as internal standard. After incubation at 80°C overnight, samples were cooled on ice for 20 min and 0.5 mL of 0.9 % NaCl was added. Approximately 200-250 μL of the top phase was extracted after centrifugation. Samples were dried completely under N₂, 20 μL of hexane was added to each vial, vortexed and transferred into a conical glass GC vial (Agilent Technologies, Waldbronn, Germany).

2.6.3 Gas Chromatographic analysis FAMEs

Each sample was placed on a 30 m x 0.25 mm DB-23 capillary column and analyzed by the Hewlett Packard 6890 GC machine. Initial temperature of 180°C was used for 1 min. in the GC analysis, followed by an increase of 4°C min⁻¹ to 240°C until a further 3 min. was maintained. A temperature of 250°C was used for injector and flame ionization detector, and the gas flow rates applied were as follows: H₂ 30 ml min⁻¹, air 300 ml min⁻¹, N₂ makeup 30 ml min⁻¹ and He 23 cm sec⁻¹ at 150°C. Retention times of reference standards were recorded and used for identifying the components of the FAMEs from each sample.
Chapter 3

Results

3.1 Phylogenetic analysis of the PLD family

Apart from being a signaling messenger, PA can be used as a precursor for storage lipid biosynthesis. As mentioned in the literature review, PLD catalyzes the hydrolysis of structural phospholipids, like PC, to produce PA. In Arabidopsis, there are 12 PLD genes that are grouped into six classes based on sequence similarity and biochemical properties (Wang et al., 2006). Phylogenetic analysis indicates that all 12 Arabidopsis PLDs can be classified into two evolutionarily divergent groups. I constructed the phylogenetic tree based on the maximum likelihood method using an amino acid alignment. This tree reveals that C2-domain containing proteins PLDα, PLDβ, PLDδ and PLDγ group together, whereas PLDZ proteins are distinctly different and have unique N-terminal phox homology (PX) and pleckstrin homology (PH) domains which are also conserved in mammalian homologs (Fig 3.1). Since these two subfamilies differ by the presence of distinct N-terminal phospholipid-binding domains (C2 vs. PX-PH), I will denote them as C2-PLDs and PX/PH-PLDs in this thesis. In keeping with the established Arabidopsis gene nomenclature I designate the genes encoding PLDZ1 and PLDZ2 as PLDZ1 and PLDZ2, respectively. I focused my thesis research on the reverse genetic analysis of both PLDZ1 and PLDZ2 as they most likely have overlapping functions.
Table 3.1: Phospholipase D genes of *Arabidopsis thaliana*.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Proposed gene name</th>
<th>Original gene name</th>
<th>Genomic locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>PXPH-PLD</td>
<td>PLDZ1</td>
<td>PLDζ</td>
<td>At3g16790</td>
</tr>
<tr>
<td></td>
<td>PLDZ2</td>
<td></td>
<td>At3g05630</td>
</tr>
<tr>
<td>C2-PLD</td>
<td>PLDA1</td>
<td>PLDα</td>
<td>At3g15730</td>
</tr>
<tr>
<td></td>
<td>PLDA2</td>
<td></td>
<td>At1g52570</td>
</tr>
<tr>
<td></td>
<td>PLDA3</td>
<td>PLDε</td>
<td>At1g55180</td>
</tr>
<tr>
<td></td>
<td>PLDA4</td>
<td>PLDδ</td>
<td>At4g35790</td>
</tr>
<tr>
<td></td>
<td>PLDB1</td>
<td>PLDβ</td>
<td>At2g42010</td>
</tr>
<tr>
<td></td>
<td>PLDB2</td>
<td></td>
<td>At4g00240</td>
</tr>
<tr>
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<td>PLDG1</td>
<td>PLDγ</td>
<td>At4g11850</td>
</tr>
<tr>
<td></td>
<td>PLDG2</td>
<td></td>
<td>At4g11830</td>
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<tr>
<td></td>
<td>PLDG3</td>
<td></td>
<td>At4g11840</td>
</tr>
<tr>
<td></td>
<td>PLDD</td>
<td>PLD8</td>
<td>At5g25370</td>
</tr>
</tbody>
</table>

Protein sequences of PLD were from the National Centre for Biotechnology Information (NCBI). All sequences databases containing Arabidopsis data were exploited.
Fig 3.1 An unrooted amino-acid Bayesian tree was generated by using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) with the following parameters: rate=invgamma; amodelpr=mixed; ngen=500000; nchains=4.25%.
3.2  *PLDZ* gene expression analysis

3.2.1 Expression of *PLDZ* promoter::*GUS* fusion in transgenic *Arabidopsis thaliana*

Although RT-PCR and promoter::GUS analyses have been used to determine *PLDZ2* expression in a variety of different tissues (Li and Xue, 2007), at the start of my thesis research nothing was known about *PLDZ1* or *PLDZ2* expression in the seed. To determine the expression pattern within the seed, I used the GUS reporter system. The *GUS* (β-glucuronidase) reporter gene, driven by the *PLDZ1* and *PLDZ2* promoters was transformed into plants and the standard GUS histochemical assay (Jefferson et al., 1987) was used to detect cells expressing *GUS*. The *PLDZ* promoter::*GUS* transgenic lines for both genes showed similar expression in the seed funiculus, embryo and in the vascular bundle of the silique wall (Fig. 3.2 & Fig. 3.3).

The expression pattern of *PLDZ1* and *PLDZ2* overlapped extensively. Both genes were expressed in the funiculus at 7 DPA (Fig. 3.2 d,e & Fig. 3.3 d,e). Both *PLDZ1::GUS* and *PLDZ2::GUS* were expressed in the embryo beginning at 7DPA (Fig. 3.2 f,g & Fig. 3.3f,g), coincident with the production and accumulation of storage oil within the cotyledon. *PLDZ* expression was absent in the seed coat during all developmental stages.
Fig 3.2 Expression analysis of the PLDZ1 gene.

a. Siliques of 6 developmental stages, from left to right 3, 5, 7, 10, 13, 15 DPA siliques.
b. 3 DPA seeds showing lack of expression of PLDZ1::GUS. c. 5 DPA old seeds showing lack of expression of PLDZ1::GUS.
d. 7 DPA old seeds showing PLDZ1::GUS expression in the funiculus.
e. 10 DPA old seeds showing PLDZ1::GUS expression in the funiculus and vascular bundle of the silique wall.
f. No PLDZ1::GUS activity was detected in the 3 DPA old embryo.
g. The 7 DPA old embryo showing PLDZ1::GUS expression throughout the embryo.
h. The 10 DPA old embryo showing PLDZ1::GUS expression throughout the embryo.
Fig 3.3 Expression analysis of the *PLDZ2* gene.

a. Siliques of 6 development stages, from left to right 3,5,7,10,13,15 DPA siliques.
b. 3 DPA seeds showing lack of expression of *PLD2::GUS*. c. 5 DPA old seeds showing lack of expression of *PLDZ2::GUS*. d. 7 DPA old seeds showing *PLDZ2::GUS* expression in the funiculus. e. 10 DPA old seeds showing *PLDZ2::GUS* expression in the funiculus and vascular bundle of the silique wall.
f. No *PLDZ2::GUS* expression was detected in the 3 DPA old embryo. g. The 7 DPA old embryo showing *PLDZ2::GUS* expression throughout the embryo. h. The 10 DPA old embryo showing *PLDZ2::GUS* expression throughout the embryo.

3.2.2 In situ hybridization of *PLDZ* in the embryo

In situ hybridization assay was used to confirm the expression of *PLDZ1* and *PLDZ2* in developing Arabidopsis seeds. In situ hybridization was performed on seeds at 7 and 10 DPA, representing mid and late stages of seed development in Arabidopsis. An antisense probe raised against *PLDZ1* (or *PLDZ2*) mRNA was used to detect *PLDZ* expression within the seed. Since *PLDZ1* and *PLDZ2* share high sequence similarity, the probes were designed to detect a sequence unique to each one of them (data not shown).
Consistent with my promoter-GUS results both genes are expressed at 7 and 10 DPA, (Fig3.4&Fig3.5).

**Fig 3.4** Accumulation of PLDZ1 transcripts during embryo development. Median longitudinal sections of developing seeds were hybridized to PLDZ1 antisense or sense RNA probes.

**Fig 3.5** Accumulation of PLDZ2 transcripts during embryo development. Median longitudinal sections of developing seeds were hybridized to PLDZ2 antisense or sense RNA probes.
3.3 Phenotypic characterization of PLDZ1 and PLDZ2 mutants

3.3.1 Identification of homozygous pldz mutants

Insertional mutagenesis by Agrobacterium T-DNA plays an important role in plant functional genomics. It is a powerful tool for obtaining loss-of-function mutants for a cloned gene. I used T-DNA insertional lines of PLDZ1 and PLDZ2 from The Arabidopsis Information Resource (TAIR) to identify homozygous mutants for both PLDZ genes. Table 3.2 lists the lines identified for PLDZ1 and PLDZ2. Two independent T-DNA insertional lines were identified for the PLDZ1 gene and were designated pldz1-1 and pldz1-2. The T-DNA in the pldz1-1 allele was inserted into the end of the 1st exon and the pldz1-2 allele has an insertion in the region upstream from the putative start codon of the opening reading frame ATG. For PLDZ2, two insertions were also found, designated pldz2-1 and pldz2-2. The pldz2-1 line has an insertion in the 9th exon while pldz2-2 line was identified in the 3rd intron (Fig 3.6 a).

Table 3.2 T-DNA insertion lines in PLDZ genes in Arabidopsis thaliana.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>AGI Number</th>
<th>Salk line name</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLDZ1</td>
<td>At3g16785</td>
<td>SALK_000939</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SALK_067875</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(pldz1-2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SALK_083090</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(pldz1-1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SALK_089098</td>
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<tr>
<td></td>
<td></td>
<td>SALK_122157</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SALK_083090C</td>
</tr>
<tr>
<td>PLDZ2</td>
<td>At3g05630</td>
<td>SALK_094369</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(pldz2-2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SALK_119084</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(pldz2-1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SALK_012466</td>
</tr>
</tbody>
</table>

Since PLDZ1 and PLDZ2 were reported to have overlapping functions in primary root hair elongation (Wang et al., 2006), and have high sequence similarity, I suspected that the two genes might be functionally redundant in the seed. For this reason, I decided to create pldz1pldz2 double mutants and examine its oil content, as well as the oil content of single mutants. I crossed pldz1-1 with pldz2-1 and genotyped individual F2 progeny using PCR to determine the presence of WT or mutant amplified fragments. Since
pldz1/ pldz2 double mutants were previously shown to have a short root phenotype under phosphate starvation (Wang et al., 2006), all pldz1/ pldz2 double mutant plants selected by PCR were germinated under phosphate-limited conditions and the elongation of primary roots in F3 pldz1/ pldz2 double mutant plants was examined. All putative double mutants had shorter roots suggesting that these F3 plants are indeed pldz1/ pldz2 double mutants.

3.3.2 RT-PCR analysis of homozygous pldz mutants

T-DNA insertions may cause altered phenotypes by eliminating or reducing the function of the gene they interrupt. Frequently, T-DNA insertions result in low levels of gene transcript accumulation. Thus RT-PCR was used to examine transcript abundance in the pldz1 and pldz2 homozygous lines. (Fig3.6 a) Low levels of PLDZ1 transcript were found in the pldz1-2 mutant but the pldz1-1 mutant had wild type transcript levels (Fig3.6 b). The transcript of PLDZ2 was not detectable in the pldz2-2 mutant, but wild type transcript levels were observed in the pldz2-1 mutant (Fig3.6 b). These data indicate that pldz1-2 and pldz2-2 could be strong mutant alleles.
Fig 3.6 *pldz* loss-of-function mutants.

**a.** Graphical depiction of the transcribed regions of the *PLDZ1* gene. The blue box and lines between them indicate exon and introns, respectively. The position of the insertion in the mutant allele is marked.

**b.** RT-PCR analysis of *PLDZ* expression in the seeds of wild type (Col) and *pldz1* mutants. The embryo was harvested from 10-13 DPA seeds for RNA extraction and cDNA synthesis. GAPC (cytosolic-glyceraldehyde-3-phosphate dehydrogenase) was used as a loading control. *PLDZ*, GAPC: 35 cycles.

### 3.3.3 Fatty acid compositions of seeds of single and double *pldz* mutants

If *PLDZ* is a positive regulator of seed oil biosynthesis, the seed oil content in *pldz1*, *pldz2* and *pldz1pldz2* single and double mutants should decrease compared to the wild type. In three independent experiments, I noticed that the *pldz1pldz2* double mutant exhibited an average decrease in seed oil content of 11%, and either *pldz1* or *pldz2* mutant showed an average decrease in seed oil content of 4% compared to wild type (Fig 3.7). The fatty acid composition of *pldz1*, *pldz2* or *pldz1pldz2* double mutant showed no difference from that of wild type (data not shown).
Seed oil was isolated from 2.3-3.0mg dry seeds and oil content is shown as percentage of dry seed weight. The mean from three independent GC analyses with standard error is shown. Asterisks represent a statistically significant difference between WT and pldz mutants.

The fact that the seed oil content in pldz1pldz2 double mutants is lower than the combined phenotypes of the single knockout mutants suggests that PLDZ1 and PLDZ2 have overlapping functions in seed oil biosynthesis.

### 3.3.4 PLDZ is required for normal mucilage in Arabidopsis seed coats

The Arabidopsis seed coat consists of several cell layers and provides protection to the embryo. The epidermal cells of the seed coat contain a large quantity of polysaccharide mucilage which is extruded when the seeds are placed in an aqueous environment. The major component of mucilage is pectin which is synthesized from UDP-sugars deposited within the seed coat during seed development.

When wild-type seed is exposed to water, the mucilage is extruded and envelops the seed within a minute. The mucilage capsule can easily be observed by treating with Ruthenium red (a dye that stains acidic polysaccharides) (Fig 3.8). In the pldz1pldz2
double mutant, only the micropylar half of the seed is encapsulated by mucilage. This result suggests that PLDZ may be involved in seed coat mucilage biosynthesis as well as oil synthesis, but the exact nature of the defect and its relationship to seed oil remains to be elucidated.

![Image](image_url)

**Fig 3.8** Mucilage extrusion is altered in the pldz1pldz2 double mutant. Left: Mature seeds of wild type (Col); Middle: pldz1pldz2; Right: gl2. All seeds were stained with 0.05% ruthenium red for 20 min without shaking.

### 3.4 Relationship between PLDZ and GL2 during oil production

Since GL2 has been reported to be a negative regulator of PLDZ1 in root hair pattern formation (Ohashi et al., 2003) and is a negative regulator of oil biosynthesis, I hypothesized that GL2 might function in oil biosynthesis by negatively regulating PLDZ. In this section I characterize the relationship between PLDZ and GL2 in seed oil production.

#### 3.4.1 Identification of homozygous gl2 mutants

Loss-of-function mutations of GL2 have been reported to cause a high-oil phenotype in seeds (Shen et al., 2006) and negatively regulate PLDZ in the roots (Ohashi et al., 2003). As a first step toward investigating whether GL2 controls oil biosynthesis by regulating PLDZ, I examined putative gl2 mutant alleles obtained from TAIR. Table 3.3 lists these lines. For each Salk T-DNA insertional line I ordered, I screened 16 individual plants for homozygosity of the T-DNA insertion using PCR. Plants homozygous for a T-DNA insertion in the GL2 gene were identified for each of the two lines, designated gl2-7 and
Both of them have an insertion in the 2nd intron that I confirmed by sequencing (Fig 3.9 a).

Table 3.3 T-DNA insertion lines in the GL2 gene of Arabidopsis thaliana

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>AGI Number</th>
<th>Salk line name</th>
<th>Mutant Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLABRA2 (GL2)</td>
<td>At1g79840</td>
<td>SALK_039825</td>
<td>High oil content</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(gl2-8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>SALK_130213</td>
<td>High oil content</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(gl2-7)</td>
<td></td>
</tr>
</tbody>
</table>

3.4.2 RT-PCR identification of homozygous mutants

RT-PCR was used to examine transcript abundance in the gl2 homozygous lines (Fig 3.9 b). The transcript of GL2 was undetectable in gl2-7 mutant and reduced in gl2-8 mutant in comparison to the WT level (Fig 3.9 b).
Fig 3.9 GL2 loss-of-function mutants.

a. Graphical representations of the transcribed regions of the GL2 gene. The blue boxes and lines between them indicate exons and introns, respectively. The position of insertion in the mutant allele is marked by lines.

b. RT-PCR analysis of GL2 expression in the seeds of wild type (Col) and gl2 mutants. The embryo was harvested from 10-13 DPA seeds for RNA extraction and cDNA synthesis. GL2 expression is considerably reduced in the gl2-8 mutant compared to wild type, and there is no detectable GL2 expression in the gl2-7 mutant. GAPC (cytosolic-glyceraldehyde-3-phosphate dehydrogenase) was used as a loading control. GL2, GAPC: 35 cycles.

3.4.3 Fatty acid compositions of gl2 mutants

Seed oil content was reported to be increased in gl2 mutants (Shen et al., 2006). To confirm that the same phenotype is observed in the gl2-7 and gl2-8 mutants, I used gas chromatography (GC) to obtain the information on seed oil content. In three independent experiments, gl2-7 mutant plants from a segregating population exhibited an average 8% increase and gl2-8 mutant showed an average 4% increase in seed oil content compared to wild type plants from the same population (Fig 3.10). The fatty acid
composition of \textit{gl2-7} or \textit{gl2-8} showed no difference from that of wild type (data not shown). In addition to the change in oil content, the leaf surface of \textit{gl2-7} and \textit{gl2-8} mutants was glabrous, a phenotype common with all other \textit{gl2} mutant lines.

![oil content (% dry seed weight)](image)

**Fig 3.10** Increased oil content in \textit{gl2} mutants.

Total seed oil was extracted from \textit{gl2} mutants and wild type plants from populations segregating for specific \textit{gl2} alleles and oil content determine by GC. Oil content is shown as percentage of dry seed weight. The mean from three independent GC analyses with standard error is shown. Asterisks represent a statistically significant difference between WT and \textit{gl2} mutants.

### 3.4.4 Expression of \textit{GL2} promoter-GUS fusion during oil accumulation

Previous study showed that \textit{GL2} is a negative regulator of seed oil accumulation (Shen et al., 2006). \textit{In situ} hybridization was used to show that \textit{GL2} is expressed during the early-heart stage as well as in hypocotyl cells and epidermal cells on the cotyledon margins in later stages (Lin et al., 2001). In order to confirm and extend these results, I introduced the \textit{GL2} promoter::\textit{GUS} transgene into wild type plants. As expected, the GUS activity
was detected in the embryo throughout seed development in a pattern similar to what was described previously (Fig3.11a-c). Besides the expression in the developing embryo, GUS assays also revealed that GL2 is expressed in the seed coat (Fig3.11 d). GL2 expression is in the seed coat (beginning at 5 DPA) and its expression patterns overlap with PLDZ in the embryo in late stages (after 7DPA), but cells expressing GL2 represent only a subset of those expressing PLDZ.

Fig 3.11 Expression analysis of the GL2 gene.

a. GL2::GUS was detected in 3 DPA old embryos.
b. 7 DPA old embryos showed GL2::GUS expression in hypocotyl cells and epidermal cells of the cotyledons.
c. The 10 DPA old embryo showed GL2::GUS expression throughout the embryo.
d. At 7 DPA, GL2 was expressed in the seed coat but not in the funiculus.

3.4.5 Real-Time PCR analysis of PLDZ expression

Real-time RT-PCR was used to determine whether GL2 is a negative regulator of PLDZ expression in the seed. I compared the expression of PLDZ genes in wild type seed (Col-0) with that in the gl2 mutant. Consistent with my PLDZ-GUS results, the expression of both PLDZ1 and PLDZ2 was markedly increased from early (3-7 DPA) to late (7-13
DPA) stages in wild type seeds while in the gl2 mutant background the expression of both PLDZ genes were significantly reduced compared with the wild type (Fig3.12). These results suggest that GL2 regulates the expression of PLDZ in a positive manner during seed oil production.

![Diagram of PLDZ1 and PLDZ2 expression](image)

**Fig 3.12** Quantitative detection of PLDZ expression by real-time PCR. Expression level was calculated using actin expression for normalization. RNA was extracted from pooled seeds at early (3-7 DPA) or late (7-13 DPA) developmental stages.
3.4.6 Seed oil content of gl2pldz1pldz2 triple mutant seeds

The analysis of the phenotype of plants homozygous for multiple mutations represents a powerful tool for defining the regulatory role of multiple genes in development. Since pldz and gl2 mutants have opposite phenotypes with respect to oil content, the phenotype of the gl2pldz1pldz2 triple mutant should help define their regulatory relationship. If GL2 functions as a negative regulator of PLDZ1 and PLDZ2 the gl2pldz1pldz2 triple mutant should have a pldz phenotype. As shown in Fig3.12, the gl2pldz1pldz2 triple mutant displays a phenotype intermediate between that of gl2 and pldz1pldz2 suggesting that PLDZ and GL2 function independently of each other in controlling seed oil levels.

Fig 3.13 The gl2pldz1pldz2 triple mutant displays an intermediate oil phenotype. Oil content is shown as percentage of dry seed weight. The mean from three independent GC analyses with standard error is shown. Asterisks represent statistically a significant difference between the gl2pldz1pldz2 and gl2 mutants.
3.4.7 Analysis of the maternal versus embryonic contributions of GL2 to seed oil accumulation

Since GL2 is expressed in both the maternal seed coat and the embryo, I wanted to determine whether GL2 functions to negatively regulate oil in one or both of these tissues. The seed coat as a maternal tissue has the same genotype as the female parent whereas the genotype of the embryo is dependent on contributions of both parents. Consequently, it is possible to generate a seed where the seed coat and embryo have different genotypes. For example, if the female parent is heterozygous for gl2 while the male parent is homozygous for gl2, the F1 seed embryo will have 50% chance to be homozygous for gl2 or heterozygous (GL2 is a recessive gene), but the seed coat will always be heterozygous (Fig3.14 cross A). Alternatively, when the mother plant is homozygous for gl2 and the male parent is a wild type plant, all their progeny in F1 will have a gl2 mutant seed coat and a heterozygous embryo. Analysis of the oil content from these F1 seeds can give us information concerning whether GL2 affects seed oil accumulation through the seed coat, embryo or both.

GC analysis of single seed oil content revealed that in cross A where half of the seeds lacked GL2 only in the embryo, half of the seeds showed a gl2-like oil content. In contrast, when GL2 function was absent in only the seed coat (cross B), all the seeds showed a high oil phenotype (Fig 3.15). This result suggests that GL2 regulates seed oil content through both the embryo and the seed coat.
Fig 3.14 Crossing schemes for generating seeds with different genotypes in the embryo and seed coat. Heterozygous GL2/gl2 plants were used as the female parent while homozygous gl2 plants were used as the male parent in cross A; in cross B the female parent is homozygous gl2 and the male parent was wild type.

<table>
<thead>
<tr>
<th>Cross</th>
<th>WT oil</th>
<th>gl2 oil</th>
<th>ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8</td>
<td>11</td>
<td>0.7</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

Fig 3.15 Single seed oil content of F1 progeny.
Chapter 4

Discussion

4.1 PLDZ proteins are positive regulators of *Arabidopsis thaliana* seed oil accumulation

I have presented two lines of evidence that, taken together, suggest that *PLDZ1* and *PLDZ2* function to positively regulate seed oil production and accumulation in *Arabidopsis*. First, *pldz1* and *pldz2* loss-of-function single mutants, and *pldz1pldz2* double mutants, have significantly lower seed oil content compared to wild type. Second, *PLDZ1* and *PLDZ2* are expressed late in embryo development. These two *PLDZ* genes are believed to have redundant function. They share similar protein structure, both single mutants have a phenotype, but the double mutant phenotype is stronger than expected for an additive effect. This suggests that the two genes are partially redundant in function.

PLDZ may function by suppling PA to the oil production pathway as PLDZ activity is known to generate PA. We know that PC is the most abundant membrane phosphoglycerol lipid in the cell and PLDZ selectively hydrolyzes PC to generate PA. In plants, the Kennedy Pathway is recognized as the major oil production pathway in which PA serves as the precursor of storage TAG. Much of the PA needed for seed oil biosynthesis is derived from LPA, but PLDZ activity represents an additional source. Alternatively, PLDZ derived PA may affect oil accumulation through its role in producing a lipid signaling messenger. In plants, PA acts as a central signaling lipid that is required for many cellular/physiological functions, such as polarized cell growth, ionic fluxes, vesicular trafficking, ABA response, pathogen defense and cold tolerance (Wang et al., 2006). During oil accumulation, PA may be involved in activation/inactivation of oil-biosynthesis-related proteins, such as its role in ABA response; alternatively it may
function during vesicle fusion/fission process, transporting oil-biosynthesis related proteins within the cell.

In addition to the specific effects of PLDZ function on seed oil content, loss of both PLDZ genes appears to affect seed mucilage production/secretion since when exposed to water, mucilage is released from only half of the seed coat. Because there is no expression of PLDZ in the seed coat, this effect on mucilage must be indirect and based on a non-cell-autonomous effect of PLDZ. During the early seed development stages, sucrose is delivered through the funiculus, where PLDZ is expressed, into the seed coat and endosperm. The sucrose in the seed coat is catabolized by sucrose synthase (SUS) to produce starch which is used, in part, for cell wall biosynthesis. Studies in maize indicated that SUS provides monosaccharide precursors for mucilage synthesis in the seed coat (Kladnik et al, 2004). It is possible therefore that PLDZ is needed in the funiculus to supply carbon to the developing seed.

PLDZ1 and PLDZ2 are expressed in a number of organs for which there is no obvious phenotype in the pldz1pldzp2 double mutant. It is possible that redundancy with other gene members in PLD family obscures PLDZ function in these parts of the plant or that phenotypes exist but remain to be detected in the double mutant.

4.2 GL2 affects Arabidopsis thaliana seed oil production independently from PLDZ

The analysis of gl2 loss-of-function mutants indicates that GL2 acts as a negative regulator of seed oil accumulation. In 2003, Ohashi and his colleagues proposed that GL2 acts as a transcriptional repressor of the PLDZ1 gene based their analysis of root hair phenotypes and the fact that GL2 was found to bind the PLDZ1 promoter region in vitro. These data led me to hypothesize that GL2 might function in oil biosynthesis by negatively regulating PLDZ function. However, several lines of evidence suggest that contrary to this hypothesis, GL2 and PLDZ exert their effect on seed oil levels independently of one another. First, analysis of gl2pldz1pldz2 triple mutants revealed a different oil phenotype from either gl2 or pldz1pldz2, as evidenced by the intermediate oil
content. If GL2 functions as a negative regulator I expected that the phenotype of the triple mutant would be similar to the pldz double mutant. Second, in the absence of GL2 function, PLDZ transcript level decreased indicating that GL2 is a positive not a negative regulator of PLDZ in the seed. The fact that PLDZ, a positive regulator of oil biosynthesis, decreases in a gl2 mutant background, yet the oil content of the seed increases seems contradictory but can be explained if the regulatory effects of GL2 on PLDZ are indirect (see below). Third, it is interesting to note that GL2 is expressed in the seed coat and embryo epidermis but not in the funiculus while PLDZ1 and PLDZ2 are expressed throughout the embryo and the funiculus but not the seed coat. Thus the expression patterns of GL2 and PLDZ are complementary and mostly non-overlapping and are unlikely to be direct regulators of one another. The increase in oil biosynthetic activity in the gl2 mutant may result in a downregulation of PLDZ expression since the PLDZ contribution to oil biosynthesis may no longer be needed. This hypothesis would explain the apparent positive control of GL2 on PLDZ expression. As for PLDZ, the mechanisms by which GL2 influence levels of seed oil remains to be determined but are likely to be indirect since GL2 is not expressed in the embryonic cells that store oil (Fig4.1).

Fig4.1  Proposed model for seed oil biosynthesis regulated by GL2 and PLDZ. GL2 may be a positive regulator of PLDZ or it may also regulate other unknown factors and affects seed oil accumulation. PLDZ activity contributes to seed oil biosynthesis by providing PA.
4.3 Summary and Future directions

Arabidopsis seed oil is the major storage component which is important for seed germination and development. In this study, I have characterized two functionally redundant proteins, *PLDZ1* and *PLDZ2*, whose function is to promote oil production/accumulation during embryogenesis. It would be interesting to see if oil content could be increased by over-expression of *PLDZ* in seeds. Although the mechanism by which PLDZ influences oil content is unknown, insight into its function might be achieved by determining its subcellular location. Genetic analyses have indicated that *GL2* does not act as a negative regulator of *PLDZ*. A better understanding of GL2 function might be achieved by determining its downstream targets.
LITERATURE CITED


Zhang W.H., Qin C.B., Zhao J. and Wang X.M. 2004. Phospholipase D alpha 1-