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CHLOROPLAST ANIONIC LIPID BIOSYNTHESIS AND FUNCTION

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Major Professor's Signature

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Date

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CHLOROPLAST ANIONIC LIPID BIOSYNTHESIS AND FUNCTION

By

Bin Yu

A DISSERTATION

Submitted to
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ABSTRACT

CHLOROPLAST ANIONIC LIPID BIOSYNTHESIS AND FUNCTION

By Bin Yu

The thylakoid membranes of photosynthetic organisms contain four major glycerolipids, mono- and digalactosyldiacylglycerol (MGDG and DGDG), phosphatidylglycerol (PG), and the sulfolipid sulfoquinovosyldiacylglycerol (SQDG). These lipids are critical for maintaining chloroplast structure and function. This dissertation focuses on dissecting the biosynthesis and function of the two anionic lipids.

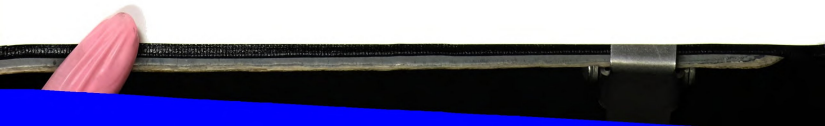
SQDG is anionic at physiological pH because of its 6-deoxy-6-sulfonate-glucose (sulfoquinovose) head group. Using a reverse genetic approach, I identified the sulfoquinovosyltransferase, designated as SQD2, which catalyzes the transfer of the sulfoquinovose moiety from UDP-sulfoquinovose to diacylglycerol. Insertion of a transfer DNA into this gene in *Arabidopsis* led to complete lack of sulfolipid in the respective *sqd2* mutant. This mutant showed reduced growth under phosphate-limited growth conditions. These results support the hypothesis that sulfolipid can function as a substitute for anionic phospholipids in order to maintain constant amounts of anionic lipids under phosphate-limited growth conditions.

PG is the only major phospholipid in the thylakoid membranes. Using a forward genetic approach, we isolated a PG-deficient mutant of Arabidopsis, *pgp1-1*, in which the overall content of PG was reduced by 30%. The *pgp1-1* mutant is a pale green plant with impaired photosynthetic activity. Further analysis suggested that this mutant carries a point mutation in the CDP-alcohol phosphotransferase motif of the phosphatidylglycerol-

phosphate synthase (EC 2.7.8.5) isoform. The mutant showed an 80% reduction in plastidic phosphatidylglycerolphosphate synthase activity consistent with the plastidic location of this particular isoform.

To test the SQDG/PG substitution hypothesis, a double mutant, *sqd2 pgp1-1* was constructed. The double mutant exhibited an approximately one-third reduction in total anionic lipids, which resulted in pale yellow cotyledons and leaves with reduced chlorophyll content. The double mutant showed compromised photoautotrophic growth and impaired photosynthetic activity. In particular, the photosynthetic electron transfer at the level of photosystem II (PSII) was affected. Furthermore, it was observed that the double mutant had a reduced number of mesophyll cells and chloroplasts with altered ultrastructure. Observations of the *sqd2 pgp1-1* mutant led to the conclusion that the total content of anionic thylakoid lipids is limiting for chloroplast structure and function, and is critical for overall photoautrophic growth and plant development.

Phosphatidic acid is a key intermediate for chloroplast membrane lipid biosynthesis. Using the combination of a reverse genetic approach and heterozygous complementation, I identified a plastidic acylglycerolphosphate acyltransferase, ATS2, catalyzing the acylation of the *sn*-2 position of lysophosphatidic acid. A green-fluorescent protein fusion with ATS2 localized to the chloroplast. Disruption of the *ATS2* gene of Arabidopsis by T-DNA insertion caused embryo lethality. Development of the embryos was arrested at the globular stage. Therefore, plastidic LPAAT appears to be essential for embryo development in Arabidopsis. Furthermore, the mutant shed light on the origin and function of phosphatidic acid in plastids.



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ABBREVIATIONS

APS	adenosine 5'-phosphosulfate
ASQD	2'-O-acyl-sulfoquinovosyldiacylglycerol
CDS	cytdine triphosphate: diaylglycerol cytidylyltransferase
DAG	diaclyglycerol
DCMU	3(3,4-dichlorophenyl)-1,1-dimethyurea
DGDG	digalactosyldiacylglycerol
DHA	dihydroxyactone
ER	endoplasmic reticulum
FAB-MS	fast atom bombardment mass spectroscopy
FADs	fatty acid desaturases
GGGT	processive galactolipid: galactolipid galactosyltransferase
GPAT	glycerol-3-phosphate acyltransferase
LPAAT	lyso-PA acyltransferase
LTP	lipid transfer proteins
MGDG	monogalactosyldiacylglycerol
ORF	open reading frame
PA	phosphatidic acid
PAPS	3'-phosphoadenosine-5'-phosphosulfate
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol

PGPS	phosphatidylglycerol phosphate synthase
pLPAAT	plastidic lyso-PA acyltransferase
PSII	photosystem II
PSI	photosystem I
SQDG	sulfolipid sulfoquinovosyldiacylglycerol
SQ-DHA	sulfoquinvose-1-O-dihydroxyactone
UDP-SQ	sulfoquinovose 6'-deoxy- α -D-glucosyl-6'-sulfonic acid

CHAPTER 1

Introduction: Lipid Biosynthesis, Function and Regulation

Lipids are, loosely defined, structurally divergent compounds that are preferentially soluble in non-polar organic solvents such as chloroform. The most abundant lipids in cells are fatty acid derived compounds (Ohlrogge and Browse, 1995). Other lipids include many pigments and some secondary metabolites that are not related to fatty acids. These lipids are essential for living organisms. A *sheet-like* bilayer of polar lipids and associated proteins are the main components of the biological membranes critical to life. The hydrophobic nature of the lipid bilayer ensures that the membrane is a barrier to water-soluble compounds. They delineate the cell boundary and therefore facilitate the creation and maintenance of distinct environments between the inside and outside of cells. Lipid membranes also establish the eukaryotic organelles such as nucleus, mitochondria, and chloroplast. In addition, membranes are the sites where the two most important energy conversion processes occur: Photosynthesis in which light energy is converted into the energy of chemical-bonds is carried out in the chloroplast. Oxidative phosphorylation, in which adenine triphosphate is formed by oxidation of fuel molecules, takes place at the inner mitochondria membrane. Lipids themselves, such as triacyl-glyerol, also serve as a substantial chemical reserve of energy.

In plants, the major membrane lipids are glycerolipids, in which fatty acids are esterified to the *sn*-1 and *sn*-2 position of the glycerol backbone and a polar head group attached to the *sn*-3 positions (Figure 1.1, below). Due to the presence of the hydrophobic fatty acids and the polar head group, glycerolipids have amphipathic physical properties essential for forming the bilayer structure of membranes. Like in membranes of animal cells, phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) are the most abundant membrane lipids in extraplastidic

plant membranes. In the chloroplast where oxygenic photosynthesis occurs, PC is only found in the cytosolic leaflet of the outer envelope of chloroplasts (Dorne *et al.*, 1985; Joyard *et al.*, 1998) and PG is the only major phospholipid found in thylakoids (Marechal *et al.*, 1997). Beyond PG, chloroplast membranes chiefly consist of glycolipids, the galactolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) and the sulfolipid sulfoquinovosyldiacyglycerol (SQDG) (Joyard *et al.*, 1998; Slabas, 1997). MGDG and DGDG represent up to 80% of the thylakoid membrane glycerolipids, of which MGDG constitutes 50% (Joyard *et al.*, 1998b). PG and SQDG are acidic anionic lipids. Beyond their role as membrane building blocks, anionic lipids have specific roles in chloroplast development and function. It is hypothesized that under severe phosphate limitation, SQDG substitutes for PG ensuring a constant amount of anionic lipids even under adverse conditions (Benning *et al.*, 1993; Güler *et al.*, 1996). Biochemical and genetic approaches have been used to understand the biosynthesis of glycerolipids in plants, focusing on the biosynthesis of the diaclyglycerol (DAG) backbone, the head group, and the assembly of backbone and head group (Browse and Somerville, 1991; Ohlrogge and Browse, 1995; Joyard *et al.*, 1998; Dörmann and Benning, 2002).

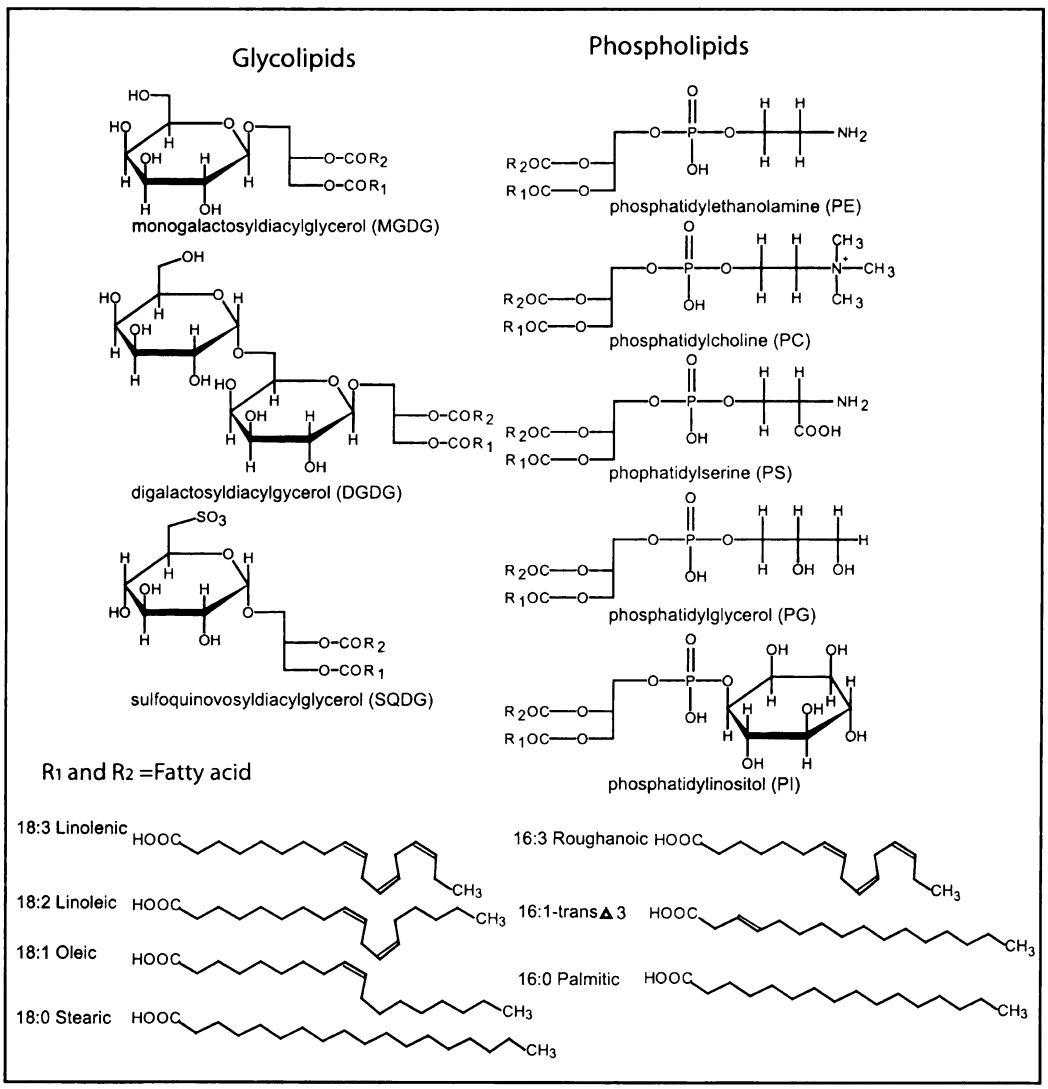
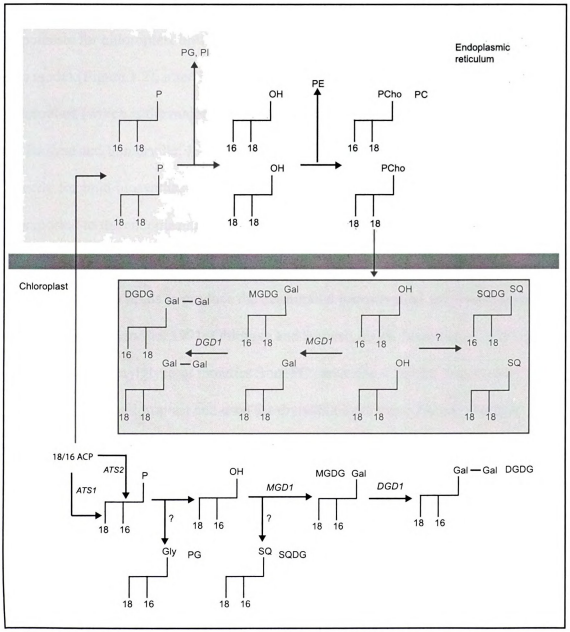


Figure 1.1. Structure of the major fatty acids and glycerolipid in plant cell membranes. Note that the fatty acids are referred to by the number of carbon atoms, colon, followed by the number of double bonds.

Figure 1.2 Schematic representation of the pathways of glycerolipids biosynthesis discussed in the text. The glycerol backbones with the typical carbon length of fatty acid in the C-1 and C-2 positions are indicated to illustrate the main molecular species derived from each pathway. The two pathways for chloroplast lipid biosynthesis are indicated, (I): the prokaryotic pathway; (II): the eukaryotic pathway. DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; SQDG, sulfoquinovosyldiacylglycerol.


Endoplasmic reticulum
PG, PI
PE
P
OH
PCho
PC
16 18
18 18
Chloroplast
DGDG
Gal — Gal
MGDG
Gal
SQDG
SQ
DGD1
MGD1
?
18/16 ACP
ATS2
ATS1
Gly
PG
SQDG

1.1. The "eukaryotic" and "prokaryotic" pathway hypothesis for chloroplast lipids biosynthesis

One of the most intriguing hypotheses in plant lipid metabolism is the two pathway hypothesis for chloroplast lipid biosynthesis (Roughan and Slack, 1982). According to this model (Figure 1.2), after 16:0 and 18:1 fatty acids are synthesized *de novo* in the chloroplast (which is the major site for fatty acid biosynthesis in seed plants, see review by Browse and Somerville, 1991; Ohlrogge and Browse, 1995), they may be used directly for lipid biosynthesis *via* the "prokaryotic" pathway in the chloroplast envelope, or exported to the endoplasmic reticulum (ER) where the "eukaryotic" pathway of lipid biosynthesis occurs. Subsequently, lipid linked fatty acid desaturases (FADs) introduce double bonds into lipids to produce the desaturated forms typical for plant membranes (Browse and Somerville, 1991; Ohlrogge and Browse, 1995, Joyard *et al.,* 1998 a, b). A fraction of the diacylglycerol moieties from PC synthesized *via* the "eukaryotic" pathway is returned to the chloroplast and used for thylakoid lipid biosynthesis (Roughan and Slack, 1982). Due to the substrate specificity of the acyltransferases in some plants, phosphatidic acid (PA) produced *via* the "prokaryotic" pathway has 16:0 at the *sn*-2 position and 18:1 at the *sn*-1 position and PA from the "eukaryotic" pathway has 18:1 at the *sn*-2 position and 16:0 when present, is at the *sn*-1 position. In some species, the "prokaryotic" pathway only contributes to PG biosynthesis, and the "eukaryotic" pathway is responsible for the rest of membrane lipid biosynthesis. In some other species, both pathways contribute to MGDG, DGDG and SQDG biosynthesis. Because MGDG synthesized from the "prokaryotic" pathway contains a substantial amount of 16:3 at the *sn*-2 position, e.g. in Arabidopsis and spinach, these plant species are called "16:3" plants

to distinguish from other species (18:3 plants) whose galactolipids are synthesized completely by the eukaryotic pathway.

1.1.1. The Prokaryotic Pathway

In the plastid of all plants, an acyl-ACP: glycerol-3-P acyltransferase (ACT) catalyzes the biosynthesis of lyso-PA, the first step of the "prokaryotic" pathway. cDNAs encoding this enzyme have been cloned from many plant species (Ishizaki *et al.*, 1988; Bhella and Mackenzie 1994; Liu *et al.*, 1999; Manaf and Harwood 2000; Weber *et al.*, 1991; Nishida *et al.*, 1993; Ishizaki-Nishizawa *et al.*, 1995). In some species, this enzyme is highly specific for 18:1-ACP. Inactivation of plastid glycerol-3-P acyltransferase in the Arabidopsis *act1* mutant leads to the loss of the "prokaryotic" pathway for glycolipid biosynthesis (Kunst *et al.*, 1988). This deficiency is compensated by increased flux from the eukaryotic pathway and therefore the *act1* mutant is changed from a 16:3 to an 18:3 plant. In all seed plants, the prokaryotic pathway is responsible for chloroplast PG biosynthesis. However, the *act1* mutant still contains 70% of the wild type level of PG suggesting the existence of an alternative pathway or altered regulation of that pathway (Kunst et al., 1988). Based on this fact, one may hypothesize that the putative alternative pathway or a lyso-PA pool from the "eukaryotic" pathway is used to synthesize "prokaryotic" PA molecular species utilized specifically for PG biosynthesis. How plants control PA partitioning between PG and glycolipids is an intriguing question.

Following the first step of lyso-PA formation, a lyso-PA acyltransferase (LPAAT), highly specific for 16:0-ACP, completes the "prokaryotic" PA biosynthesis. A plastidic LPAAT, *BAT2*, from *Brassica napus*, has been isolated by functional complementation of

an E. *coli LPAAT* deficient mutant *plsC*. This enzyme is chloroplast-localized and shows 16:0-ACP preference to 18:1-ACP (Bourgis *et al.,* 1999). However, the *in vivo* role and the specific sub-organelle localization of this protein are still in need of further investigation. PA synthesized *via* the prokaryotic pathway enters PG biosynthesis in all seed plants. In 16:3 plants, it is converted to DAG by a PA-phosphatase located at the inner membrane. This DAG pool is used to synthesize glycolipids such as MGDG, DGDG and SQDG (Joyard, 1993). The DAG pool can't be formed in 18:3 plants due to the absence of a PA-phosphatase (Heinz and Roughan, 1983) and thus DAG precursor entirely derived from the eukaryotic pathway is used for glycolipid biosynthesis.

The biosynthesis of MGDG is catalyzed by UDP-galactose:DAG galactosyl-transferase (EC 2.4.1.46). MGDG synthase was first cloned from cucumber (Shimojima *et al.,* 1997) and subsequently from Arabidopsis. Arabidopsis contains three MGDG synthases, MGD1, MGD2 and MGD3 belonging to the glycosyltransferase gene family GT28 (http://afmb.cnrs-mrs.fr/CAZY/) (Miège *et al.*, 1999; Jorasch *et al.*, 2000; Jarvis *et al.*, 2000; Awai *et al.*, 2001). MGD1 is located at the inner chloroplast envelope and proposed to be the major enzyme for MGDG synthesis, because a 75% reduction in MGDG synthase activity resulted in the 42% reduction of MGDG content (Jarvis *et al.*, 2000). Among these three genes, MGD2 and MGD3 are more closely related to each other based on sequence similarity. They are located at the outer envelope of the chloroplast and are suggested to be involved in phosphate-stress regulated glycolipid formation (Awai *et al.*, 2001). In Arabidopsis, the DGDG synthase gene *DGD1* was isolated by map-based cloning in the DGDG-deficient mutant *dgd1*, in which the amount of DGDG is reduced by 90% (Dörmann *et al.*, 1999). In agreement with previous

biochemical studies, the DGD1 protein is associated with the outer chloroplast envelope. The protein lacks a cleavable-chloroplast targeting sequence, and is inserted into the outer envelope by a mechanism independent of the general import pathway (Froehlich *et al.,* 2001). The C-terminal end of DGD1 is proposed to contain the enzymatic domain required for galactosylation based on its similarity to the plant and bacterial glycosyltransferase family GT4. The function of the N-terminal domain of DGD1 is unknown (Dörmann and Benning 2002). Utilizing a reverse genetic approach, a second DGDG synthase, *DGD2,* was isolated in Arabidopsis and other plant species (Kelly and Dörmann, 2002). It was proposed that this enzyme activity is responsible for the synthesis of DGDG under specific conditions such as phosphate-limitation (Kelly and Dörmann, 2002). It was found that DGD1 and DGD2 use the UDP-Galactose as galactose donor to synthesize DGDG and oligogalactosyldiacylglycerol trigalactosyl-diacylglycerol and tetragalacosyldiaylglycerol (Kelly *et al.,* 2003; Kelly and Dörmann, 2002). The Galactosyltransferase activity reponsible for biosynthesis of DGDG and oligogalactosyldiacylglycerol is still present in the *dgd1 dgd 2* double null mutant, suggesting the existence of a progressive galactolipid synthase independent from DGD1 and DGD2 in Arabidopsis. This enzyme activity has been proposed to be induced in the *tgd1* mutant carrying a mutation in a permease-like gene presumably involved in lipid trafficking between the ER and chloroplast (Xu *et al.,* 2003). This galactolipid synthase was thought to be related to a processive galactolipid:galactolipid galactosyltransferase (GGGT) catalyzing the galactosylation of MGDG using a second MGDG molecule as the galactose donor (Kelly *et al.,* 2003, Xu *et al.,* 2003). This GGGT was previously proposed to be the DGDG synthase that synthesizes both DGDG and the unusual

oligogalactosyldiacylglycerol (Dorne *et al.*, 1982; Cline and Keegstra. 1983; van Besouw and Wintermans, 1978). The biosynthesis of SQDG and PG will be addressed below in detail.

1.1.2. The eukaryotic pathway

After the fatty acids are exported to the ER as acyl-CoA from the chloroplast, they are incorporated into PA to give rises to molecular species with 18:1 at the *sn*-2 position and18:1 or 16:0 at the *sn*-1 positon by two ER acyltransferases. This PA then is incorporated into PC, PE, PI and extraplastidic PG (review by Browse and Somerville, 1991; Ohlrogge and Browse, 1995, Joyard *et al.,* 1998 a, b). Experiments show that PC synthesized in the ER gives rise to PC in the chloroplast, which lacks the enzymes for *de novo* PC biosynthesis (Joyard *et al.*, 1991), thus the intact diacylglycerol moiety from PC can be returned to the chloroplast envelope and contribute to chloroplast lipids (Slack *et al.*, 1977). For instance, pulse-chase labeling experiments using ^{14}[C] acetate on intact leaves showed that PC was labeled first and subsequently the label from PC is incorporated into MGDG and later into DGDG (Slack *et al.*, 1977). However when the incorporation of label into fatty acids at each position of the DAG moiety is analyzed, differences between the *sn*-1 and the *sn*-2 position of PC and MGDG is revealed (Mongrand *et al.*, 1997, 2000). Based on these results, it was postulated that lyso-PC rather than PC is returned into the chloroplast (Mongrand *et al.,* 1997, 2000). Similar experiments also indicated that an extensive modification of the DAG moiety of PC occurs and that only a specific metabolic pool of PC is used to supply chloroplast lipid biosynthesis (Williams *et al.,* 2000). Thus, the molecule transported and the transport

mechanism itself remains unknown. Lipid transfer proteins (LTP) have been postulated to be involved in the lipid transporting process between ER and chloroplast. However, the expected ER or secretory pathway localization of LTP from maize, spinach and barley are inconsistent with a role in intracellular lipid trafficking (Browse and Somerville, 1991). Recently, a mutant of Arabidopsis, *tgd1,* was isolated by *Xu* et al (2003). Several pieces of evidences suggest *tgd1* is deficient in ER to plastid lipid trafficking. First, in contrast to the *act1* mutant, the proportion of chloroplast lipids derived from the ER pathway in *tgd1* is reduced to below 20%. Second, pulse chase experiments showed that the transfer of ER-derived PC to the plastid or the incorporation of diacylglycerol backbones derived from PC into the thylakoid lipids MGDG and DGDG is impaired in the mutant. Furthermore, the TGD1 protein is similar to the permease subunit of some bacterial ATP binding cassette (ABC) transporters. Understanding the biochemical function of TGD1 and the identification of the components of the TGD1 complex will provide important insights in understanding the lipid trafficking between ER and chloroplast.

1.2. The biosynthesis and functions of PG

Phosphatidylglycerol (PG) is a common membrane lipid present in animals, plants and microorganisms. In all organisms, the biosynthesis of PG starts with the biosynthesis of phophatidylglycerol phosphate by a reaction of CDP-diacylglycerol with glycerol-phosphate, followed by cleavage of phosphate by a phosphatase to produce PG (Moore, 1982; Andrews and Mudd, 1986; Kinney, 1993). Genetic disruption of the PG biosynthesis pathway and therefore depletion of PG is detrimental to *E. coli* (Kikuchi *et*

al., 2000), in which PG is the major anionic lipid and *Saccharomydes cerevisiae* (Ostrander *et al.*, 2001), where PG is predominantly associated with mitochondria, suggesting essential roles for PG in non-photosynthetic organisms. In photosynthetic organisms, PG is mainly associated with the photosynthetic membrane, and it is the predominant phospholipid present in thylakoid membrane, although it also exists in extraplastidic membranes. PG is the only phospholipid present in cyanobacteria, and PG typically constitutes 8-10% of chloroplast lipids (Browse and Somerville, 1993). Thus the biosynthesis and function of PG in photosynthetic organism is an interesting question.

1.2.1. The biosynthesis of PG

Three enzymes are involved in PG biosynthesis. The first is cytdine triphosphate: diaylglycerol cytidylyltransferase (CDS) catalyzing the biosynthesis of CDP-diacylglycerol, the precursor of the minor lipids PI, PG, cardiolipin, and possibly phosphatidylserine (Moore, 1982) and therefore is not specific for PG biosynthesis. The next two enzymes in the pathway are phosphatidylglycerol phosphate synthase (PGP) and phosphatidylglycerol phosphate phosphatase (Joyard et al., 1998b).

In the anoxgenic photosynthetic bacterium, *Rhodobacteria sphaeroides,* CDS enzyme activity has been shown to co-localize with the phosphatdiylglycerol phosphate synthase activity at the cytoplasmic membrane consistent with the idea that phospholipids are synthesized there and then transferred to the intracytoplasmic membrane system (Cain *et al.*, 1984; Radcliffe *et al.*, 1985). In cyanobacteria, the open reading frame (ORF) encoding this enzyme has been identified based on a similarity search with CDS rom other organisms. The mutant defective in this gene requires the supplementation of

PG to the medium for growth, suggesting that this gene indeed encodes CDP-DAG synthase and is essential for PG biosynthesis (Sato N *et al.*, 2000). In higher plants, CDS activity is localized in microsomal membranes which might account for PI biosynthesis (Moore, 1982), the inner membrane of mitochondria where CDP-DAG is the substrate for PG and cardiolipin biosynthesis (Frentzen and Griebau, 1994), and the inner membrane of the chloroplast , which is the major site for PG biosynthesis in leaves (Andrews and Mudd, 1985). The CDS gene has been cloned from Arabidopsis and Potato *(Solanum tuberosum*) and has been characterized in some detail (Kopka *et al.*, 1997). However, the localization and *in vivo* roles of the enzyme still need further investigation.

The committed step for the PG biosynthesis pathway is the biosynthesis of phosphatidylglycerol phosphate (PGP) catalyzed by the phosphatidylglycerol phosphate synthase (PGPS). There are two phylogeneticially distinct groups of PGP synthases, the bacterial PGP synthase of about 20 KDa containing the typical CDP-alchohol binding motif (Müller and Frentzen, 2001; Hagio *et al.*, 2000; Dryden and Dowhan., 1996; Jiang *et al.*, 1997; Tuller *et al.*, 1998; Ohta *et al.*, 1981), and the mitochondrial PGP synthase of about 60 KDa which, like the bacterial cardiolipin synthases, belongs to a conserved protein family defined by an invariant motif, HXK(X)4D, denoted as HKD (Ponting and Kerr, 1996; Müller and Frentzen, 2001; Kawasaki *et al.*, 1999; Chang *et al.*, 1998; Tropp, 1997). In plants, labeling studies indicate that enzymatic activity involved in PG biosynthesis is associated with the inner mitochondrial membrane, the endoplasmic reticulum, and the chloroplast (Moore, 1982; Kinney, 1993), suggesting that at least three isoforms of PGP synthases and PGP phosphatases might exist in plant cells. However, little is known about plant PGP synthases other than their cellular compartmentation.

Recently, two isoforms of PGP synthases, PGP1 and PGP2, have been identified and characterized from Arabidopsis (Müller and Frentzen, 2001). Both PGP1 and PGP2 belong to the bacterial PGP synthases group. PGP1 contains a cleavable transit peptide for import into plastids and with a lower probability for import into mitochondria. Consistent with this hypothesis, PGP1, which is not active in *E. coli*, can be imported into the yeast mitochondria and processed to a catalytically active protein (Müller and Frentzen, 2001). PGP2 has 10-times higher activity in *E. coli* than in yeast where the PGP2 activity is predominantly located in the microsomal fraction. Thus, PGP2 is possibly the microsomal enzyme (Müller and Frentzen, 2001). The *R. sphaeroides* PGP synthase, *pgsA,* has been identified. Overexpression of this gene in both *R. spaeroides* and *E. coli* do not have dramatic effects on the phospholipid composition of either organism indicating that this enzyme activity is tightly regulated, although the mechamism is unknown (Dryden and Dowhan 1996).

1.2.2. The function of PG in photosynthetic organisms

As the major anionic phospholipid, PG has critical roles for the proper function of photosynthetic membranes. It is also thought to play a critical role in cold acclimation of plants. Based on a survey of lipids present in the chloroplast membranes from a variety of plant species representing both chilling-sensitive and chilling-resistant types, chilling sensitivity is proposed to closely correlate with the proportion of specific molecular species of PG, specifically disaturated PG, containing only 16:0, 18:0 or 16:1 trans, which has physical properties similar to that of a saturated fatty acid (Murata, 1983). The identification and analysis of genetic mutants that are impaired in PG biosynthesis, their

biochemical and physiological analysis, and the analysis of transgenic plants with an altered ftraction of PG has began to offer new insights into the functions of PG in photosynthetic organism (Murata, 1983; Murata and Nishida, 1990; Murata and Yamaya,1984; Sato *et al.,* 2000).

Several biochemical studies have demonstrated the essential roles of PG in photo-synthesis. *In vitro* experiments showed that PG is enriched in the light-harvesting pigment-protein complexes of photosystem II (PSII) (Murata *et al.*, 1990; Tremolieres *et al.*, 1994) and is essential for the dimerization of the PSII reaction center core pigment-protein complex (Kruse *et al.*, 2000). Furthermore, the recently published crystal structure of photosystem I (PSI) contains three molecules of PG in tight coordination with the core and the antenna complex (Jordan *et al.*, 2001) and PG is required for the *in vitro* reconstitution of the light-harvesting pigment-protein complexes of PSI (Schmid *et al.,* 1997). Thylakoid membranes treated with phospholipase A_2 were PG depleted and were inhibited in their photosynthetic electron transport activities (Jordan *et al.,* 1983; Siegenthaler *et al.,* 1987). The function of PG in photosynthesis has been studied with a mutant of the cyanobacterium *Synechocystis* sp. PCC6803, which is defective in the biosynthesis of PG. The growth of this mutant requires supplementation with PG and the photosynthetic activity of the mutant has been shown to decrease with a concomitant decrease in the PG content in thylakoid membranes primarily due to a decrease in the PSII activity. These results provided the first *in vivo* evidence for an essential function of PG in PSII. Further analysis of this mutant indicated that PG is indispensable for the PSII reaction center complex, and that PG is required to maintain the structural integrity of the Q_B-binding site (Gombos *et al.,* 2002). In Arabidopsis, it has been known that the

relative proportion of PG is reduced in the *pho1* and *act1* mutants (Härtel *et al.*, 1998; Kunst *et al.*, 1988; Poirier *et al.*, 1991). However because both mutants are affected in multiple aspects of lipid metabolism, they are of limited usefulness for the investigation of specific functions of PG in the photosynthetic membranes of seed plants. Therefore, identification of mutants deficient in the PG biosynthetic pathway was expected to provide new insights into the specific functions of PG.

It has been proposed that one primary event during chilling injury is a Lα-to-Lβ lipid phase transition (liquid crystalline to gel phase) resulting in alterations in the metabolism of chilled cells that leads to injury and death of the chilling-sensitive plants (Lyons, 1973; Raison, 1973). It has been observed that PG is the only glycerolipid exhibiting a significant phase transition and PG prepared from chilling-sensitive plants has been shown to undergo this phase transition at 30 °C while PG purified from chilling-resistant plants underwent phase transition at 15 °C. Based on these data and the correlation between disaturated PG and chilling-sensitivity, it has been proposed that disaturated chloroplast phosphatidylglycerol (PG) confers chilling sensitivity to plants (Murata, 1983; Murata and Yamaya, 1984; Murata and Nishida, 1990).

The substrate specificity of the glycerol-3-phosphate acyltransferase of chloroplasts (GPAT) is proposed to be the dominant factor in determining the proportion of disaturated PG, because chloroplasts always contain 16:0 or trans 16:1 at the *sn*-2 position (Roughan, 1985). It has been observed that GPAT from chilling-resistant plants prefers 18:1-ACP to 16:0-ACP as a substrate, resulting in low level of disaturated PG in chloroplast membranes, while the enzyme from chilling-sensitive plants hardly distinguishes between 18:1-ACP and 16:0-ACP, resulting in a high proportion of

disaturated PG. To directly test the effect of disaturated PG on chilling sensitivity, the GPAT cDNAs from chilling resistant and chilling sensitive plants have been used in transgenic experiments. In transgenic tobacco (a chilling sensitive plant) expressing a GPAT cDNA from squash (a chilling sensitive plant) increased the proportion of disaturated PG (76%). In the converse experiment, disaturated PG is decreased (28%) in transgenic tobacco expressing GPAT cDNA from Arabidopsis, a chilling resistant plant. The increase of disaturated PG was positively correlated with low-temperature induced photoinhibition, an indication of chilling sensitivity, and the decreased amount of disaturated PG was correlated with decreased chilling sensitivity (Murata *et al.,* 1992). These results showed the first direct evidence for the causal correlation between the amount of disaturated PG and low-temprature induced injury. Further analysis of transgenic tobacco with increased amount of PG suggests that the variation of disaturated PG amount has no effect on the rate of low-temperature induced photoinhibition. Instead, the rate at which damaged PSII can be repaired is affected in the transgenic plants. It has been hypothesized that the replacement of damaged D1 protein at the photosystem II reaction center with newly synthesized D1 protein is effected by the amount of disaturated PG (Moon *et al.,* 1995). However, the possibility that reduced recovery from photoinhibition contributes to plant chilling sensitivity has been debated (Moon *et al.,* 1995; Somerville, 1995). For example, the analysis of transgenic Arabidopsis plants expressing the *E.coli plsB* gene suggests that an increase of disaturated PG has no effect on their ability to recover from photoinhibition (Wolter *et al.,* 1992, Bruggemann and Wolter, 1995). In agreement with this result, Vijayan and Browse (2002) found that there is no difference between wild-type and the *fab1* mutant in terms of the speed of recovery

from photoinhibition temperatures between 7°C and 27°C. The Arabidopsis *fab1* mutant is deficient in the elongation of palmitic acid which leads to leaf PG containing 70% high-melting-point fatty acids (Wu and Browse, 1995). The *fab1* mutant shows no obvious difference with wild-type in low temperature induced injury. This result is not necessarily in conflict with those of Moon *et al.*, (1995). Instead, it suggests that a critical threshold for disaturated PG is required to cause rapid chilling injury. Recently, transgenetic experiments with cDNAs of *GPAT* have been successfully used to improve the chilling sensitivity of rice (Yokoi *et al.*, 1998; Ariizumi *et al.*, 2002) suggesting a practical application of this research in the improvement of the chilling sensitivity of crops.

1.3 The biosynthesis and function of sulfoquinovsyldiacylglycerol (SQDG)

The sulfolipid sulfoquinovosyl diacylglycerol (SQDG) is an abundant sulfur containing bio-organic compound in nature. Its structure was elucidated by Benson and coworkers (1959) over 40 years ago, as 1'-O-(6'-deoxy-α-D-glucosyl-6'-sulfonic acid)-3-O-diacylglycerol. The head group of SQDG, referred to as sulfoquinovose (6'-deoxy- α -D-glucosyl-6'-sulfonic acid) is hydrophilic and negatively charged at biological pH value. As the diacylglycerol backbone is also hydrophobic, SQDG has strong amphipathic characteristics (Benning, 1998). SQDG has been found in most photo-synthetic organisms, including all seed plants, mosses, ferns, algae and photosynthetic bacteria (Haines 1973; Araki *et al.,* 1991; Dembitsky *et al.,* 1990, 1991). SQDG was also identified from the nonphotosynthetic bacteria *Rhizobium melitoti* (Cedergreen *et al.,* 1994) and *Bacillus acidocaldarius* (Langworthy *et al.,* 1976) showing that SQDG is not restricted to photosynthetic organisms. In seed plants, SQDG is exclusively associated with chloroplast membranes. In other photosynthetic organisms, SQDG is likewise found associated with photosynthetic membranes. Thus, SQDG has long been thought to play a role in photosynthesis (Barber and Gounaris, 1986).

Recently, the identification of genes essential for SQDG biosynthesis (Benning, 1998) (Figure 1.3) has provided strong evidence in support of the proposed sugar-nucleotide pathway (Barber, 1963; Pugh *et al.,* 1995; Benning, 1998) (Figure 1.4). Furthermore, the analyses of SQDG deficient mutants have provided new insight into the

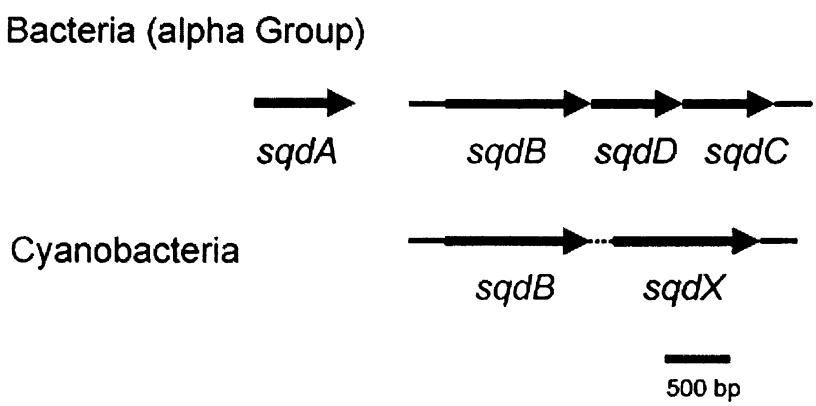


Figure 1.3 Organization of sulfolipid genes in purple bacteria and cyanobacteria. bp, base pairs. Adapted from (Benning C, 1998)

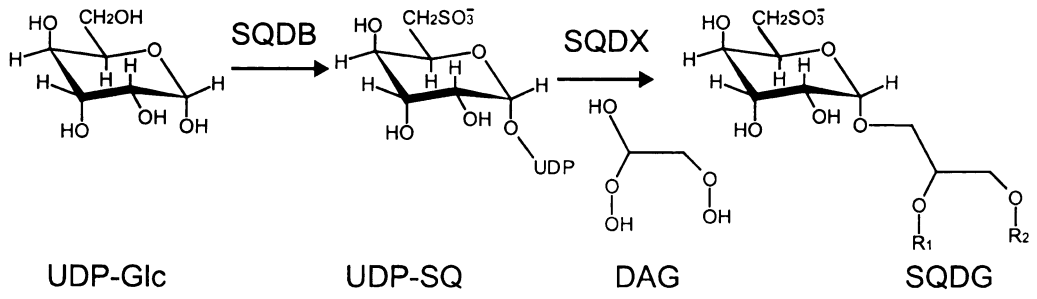


Figure 1.4. The pathways for sulfolipid biosynthesis in cyanobacteria. SQDB is specific to the first reaction. SQDX from cyanobacteria is the SQDG synthase. DAG, diaylglycerol; R, fatty acyl groups; UDP-Glc, UDP-glucose; SQDG, sulfoquinovosyldiacylglycerol; UDP-SQ, UDP-sulfoquinovose

functions of SQDG (Benning, 1998). However, in higher plants, the pathway for SQDG biosynthesis was still incomplete, because the gene, encoding SQDG synthase catalyzing the final assembly of SQDG, was not isolated. Also, the function of SQDG in higher plants was unclear due to the absence of an SQDG deficient mutant. The identification of SQDG synthase in Arabidopsis was necessary to verify the SQDG biosynthetic pathway in plants. In addition, by construction of an SQDG-deficient mutant, the function of SQDG could be tested.

1.3.1. The biosynthesis of SQDG

It has become clear that SQDG is synthesized via the sugar nucleotide pathway which is a two step reaction, initially utilizing UDP-glucose as the direct precursor of UDP-sulfoquinovose (UDP-SQ), followed by the transfer of sulfoquinovose to diacylglycerol (DAG) to complete the final assembly of SQDG (Pugh, 1995; Benning, 1998). The most elusive aspect in this pathway has been the origin of UDP-SQ, i.e. the nature of the sulfur-donor. How SQDG is assembled posed yet another question. In the past, progress toward answers to these questions has been hampered because of the lack of a suitable *in vitro* system for SQDG biosynthesis and a large number of sulfurous water-soluble compounds present in extracts of higher plants (Benning, 1998). But recently it became possible to solve these problems with the cloning of genes involved in SQDG biosynthesis and the isolation of SQDG deficient mutants in different organisms.

1.3.1.1. Genes encoding enzymes involved in SQDG biosynthesis

More than 12 years ago, a combination of biochemical and genetic approaches was used to identify genes essential for the biosynthesis of SQDG in various organisms. Initially, four genes involved in SQDG biosynthesis (*sqdA, sqdB, sqdC*, and *sqdD*)(Benning, 1992a, b; Rossak 1995), were isolated from the purple bacterium *R. sphaeroides*.The *sqdA, sqdB,* and *sqdC* genes were isolated by screening SQDG-deficient mutants and followed by complementation. The genes are organized in two transcriptional units as shown in Figure 1.3. Subsequently an SQDD gene was identified by insertional inactivation of an open reading frame flanked by *sqdB* and *sqdC* (Rossak *et al.,* 1995). The sequence of the *sqdB* product shows similarity to sugar nucleotide-modifying enzymes, UDP-glucose-4-epimerases and nucleotide-hexose-4, 6-dehydratases, suggesting that the gene product may be responsible for the conversion of UDP-Glc to UDP-SQ (Benning, 1992). The similarity of the *sqdD* sequence to glycosyltransferases suggests that it may be a SQDG synthase (UDP-sufquinovose: diacylglycerol sulfoquinovsyltransferase), an enzyme catalyzing the final assembly of SQDG (Rossak, 1995). It has been suggested that the *sqdC* protein provides substrate specificity to the *sqdD* gene product (Rossak *et al.,* 1995). In an *sqdC* mutant, the novel compound sulfoquinvose-1-O-dihydroxyactone (SQ-DHA) was identified (Rossak *et al.,* 1997). When added to the extracts of *R. sphaeroides* or spinach chloroplasts, labeled SQ-DHA was not incorporated into SQDG (Rossak *et al.,* 1997). Therefore it is still unclear whether SQ-DHA is a true intermediate of sulfolipid synthesis or whether it is a by-product that is only present in the mutant (Rossak, 1997). Because no water-soluble

sulfur-containing compounds accumulated in the *sqdA* mutant, the biochemical function of the *sqdA* gene product remains unclear (Benning, 1998).

Orthologs of the *sqdB* gene subsequently were cloned from cyanobacteria *Synechococcus* sp. PCC7942, and synechocystis sp PCC 6803 (Güler *et al.*, 1996), as well as Arabidopsis (Essigmann *et al.*, 1999), spinach (Shimojima and Benning, 2003) and the green alga *C. reinhardtii* (Riekhof *et al.*, 2003). All SQDB orthologs are highly conserved suggesting that the mechanism for UDP-SQ biosynthesis evolved during a single event (Benning, 1998). However no other sequences similar to the other *sqd* genes from *R. sphaeroides* have been found in organisms not belonging to α-group bacteria, indicating that some aspects of the pathway are evolutionarily more divergent than others. By analyzing the sequence flanking the *sqdB* in *Cyanobacteria*, a new gene involved in sulfolipid biosynthesis, *sqdX* was identified. The predicted *sqdX* product has a sequence similar to glycosyltransferases (Güler *et al.*, 2000). The insertional inactivation of *sqdX* caused sulfolipid dificiency. This gene product may therefore encode the SQDG synthase in *Cyanobacteria*.

1.3.1.2. The biosynthesis of UDP-sulfoquinovose

Based on the presence of a sulfur containing sugar nucleotide in extracts of *Chlorella* (Shibuya *et al.*, 1963), UDP-sulfoquinovose (UDP-SQ) was proposed to be the head group donor for SQDG. Recently, more evidence has arisen in support of this hypothesis. For example, chemically synthesized UDP-SQ could stimulate the SQDG biosynthesis by membranes of spinach chloroplasts (Heinz *et al.*, 1989). More direct

evidence was obtained from SQDG-deficient mutants. The accumulation of UDP-SQ was observed in mutants of *R. sphaeroides* inactivated in the *sqdD* gene (Rossak *et al.,* 1995). UDP-SQ has also been identified in extracts of different unicellular algae and mosses (Benning, 1998). The biosynthesis of UDP-SQ starts from UDP-glucose and a sulfur donor. The involvement of UDP-glucose is based on the stimulation of the incorporation of $[^{35}S]SO_4^{2-}$ into SQDG by UTP and the observation that UDP-[^{14}C]glucose is effectively incorporated into sulfolipid. In addition, the *sqdB* mutant of *R. sphaeroides* did not accumulate a sulfur-labeled water soluble compound suggesting that the SQDB protein catalyzes the reaction before or during the incorporation of sulfur into UDP-SQ (Benning, 1993). Indeed, the SQDB protein can accept UDP-glucose and convert it to a new compound *in vitro*. The similarity of the SQDB protein to sugar nucleotide-modifying enzymes, UDP-glucose-4-epimerases and nucleotide-hexose-4, 6-dehydratrases suggest that the intermediate, UDP-4-ketoglucose-5-ene, could serve as a sulfur acceptor. The production of UDP-SQ from this intermediate was first suggested by Barber (1963). Furthermore, the incorporation of UDP-[^{14}C]glucose to SQDG was stimulated by the addition of methyl alpha-glucose enide or by addition of an enzyme system known to be forming (although not accumulating) UDP-4-ketoglucose-5-ene.The cloning of the *SQD1* gene from Arabidopsis made it possible to study the biosynthesis of UDP-SQ in detail. SQD1 contains conserved Y-XXX-K and glycine-rich (G-XX-G-XX-G) sequence patterns and appears to be a member of the short-chain dehydrogenase-/reductase (SDR) family (Mulichak *et al.,* 1999). It has been proposed that SQD1 catalyzes the change from UDP-glucose to UDP-4-keto-glucose, followed by the formation UDP-4-keto-5,6-glucosene which serves as an acceptor for a sulfur donor

(Essigmann et al., 1999). The SQD1 protein has been crystalized at 1.6 Å (Mulichak *et al.,* 1999). Recombinant SQD1 lacking the chloroplast transit peptide forms a dimer, and contains a buried active site with tightly bound NAD^+. Co-crystallization with UDP-Glucose (UDP-Glc) demonstrated directly the binding of this presumed substrate in the active site (Mulichak *et al.,* 1999). Another intriguing question regarding UDP-SQ biosynthesis was the identity of the sulfur donor. Labeled sulfate can be incorporated into SQDG by isolated chloroplasts, indicating that the sulfur donor is derived from the sulfate reduction pathway in the chloroplast (Haas *et al.,* 1980; Joyard *et al.,* 1986; Kleppinger-Sparace *et al.,* 1985). Intermediates of this pathway, adenosine 5'-phosphosulfate (APS) and 3'-phosphoadenosine-5'-phosphosulfate (PAPS), were indeed incorporated into SQDG by isolated chloroplasts (Kleppinger-Sparace et al; 1990). Another intermediate of the sulfate reduction pathway, sulfite, was shown to be incorporated into SQDG by extracts of *Chlamydomonas reinhardtii* (Hoppe and Schwenn, 1981). However this reaction was linear over time and thus was thought to be nonenzymatic. An *in vitro* assay for the recombinant SQD1 protein has been developed to find the sulfur donor (Sanda *et al.,* 2001). The result showed that the recombinant SQD1 protein uses sulfite but not APS to synthesize UDP-SQ, demonstrating that sulfite rather than APS is the sulfur donor (Sanda *et al.,* 2001). Recently, Shimojima and Benning (2003) showed that the Km for sulfite of the native enzyme purified from isolated spinach chloroplasts is at least four times lower than that of the recombinant spinach enzyme. Additionally, size determination by gel filtration indicated that the native form of SQD1 is purified as a large complex of approximately 250 kDa, which was more than twice as large as the calculated size for the homodimer, suggesting a multimeric complex in association with

SQD1 is required for UDP-SQ biosynthesis. It has been proposed that the function of this complex is to cope with low sulfite concentrations in the chloroplast by increasing the affinity for free sulfite, or substrate channeling (Shimojima and Benning, 2003).

1.3.1.3. The assembly of SQDG

The final step of SQDG biosynthesis is catalyzed by a UDP-α-sulfoquinovose:1, 2-diacylglycerol 3-O-α-D-sulfoquinovosyltransferase designated SQDG synthase (Heinz *et al.*, 1989; Seifert and Heinz, 1992; Rossak *et al.*, 1997; Güler *et al.*, 2000; Benning, 1998). In spinach, this enzyme activity has been characterized in some detail using intact chloroplasts into which radio labeled DAG had been biochemically preloaded as the acceptor for the sulfoquionovose moiety (Heinz *et al.*, 1989; Seifert and Heinz, 1992). Among the different nucleotide sulfoquinovoses tested, UDP-SQ was the best substrate while GDP-SQ resulted in a lower enzyme activity. This enzyme showed the maximum activity at pH 7.5 with a Km for UDP-SQ of 10μM in the presence of 5 mM Mg^{2+}. The enzyme activity was only observed for osmotically ruptured chloroplasts following the addition of UDP-SQ. This fact together with the co-purification of enzyme activity with the chloroplast inner envelope suggested that SQDG synthase is associated with the inside of the inner envelope (Heinz *et al.*, 1989; Seifert and Heinz, 1992). The *R. sphaeroides sqdD* gene belongs to glycosyltransferase gene family GT8 and was proposed to encode a potential SQDG synthase. The accumulation of UDP-SQ in the *sqdD*-inactivated mutant is consistent with the anticipated effect of blocking the assembly of SQDG (Rossak *et al.*, 1995). The proposed function of SQDC is to provide

substrate specifity for SQDD based on accumulation of SQ-DHA in the *sqdC* mutant (Rossak et al., 1997). Alternatively, the function of SQDD is to synthesize SQ-DHA followed by the reduction of SQ-DHA and acylation at the *sn-1* and *sn-2* position of glycerol catalyzed by SQDC and the SQDA product. The involvement of SQDC and SQDD in SQDG assembly suggests that the fully functional SQDG synthase is a protein complex in some organisms. In cyanobaceteria, the SQDG synthase is encoded by the SQDX gene and the gene product belongs to the glycosyltransferase family GT4 (Güler *et al.,* 2000). The SQDX protein contains a putative membrane spanning domain. The cyanobaterial SQDX is not similar to the α-group bacterial SQDD protein, suggesting that SQDG assembly may have evolved independently more than one time, while UDP-SQ biosynthesis evolved only once based on the conservation of the SQDB protein in all organisms. However, both GT4 and GT8 enzymes are retaining glycosyltransferases maintaining the anomeric proton configuration. Mechanistically, a two-step processes is involved, beginning with the formation of a glycosyl-enzyme intermediate, followed by the release of the nucleotide diphosphate and the subsequent attack of the glycosyl-enzyme by the acceptor (Sinnott, 1991; McCarter and Withers, 1994). Therefore SQDX and SQDD probably have similar catalytic mechamsims despite their divergent amino acid sequences. In higher plants, much was known about UDP-SQ biosynthesis. On the contrary, although the activity of the sulfolipid synthase catalyzing the last step of sulfolipid biosynthesis had been fairly well characterized in spinach chloroplast extracts , the protein had not been isolated and the respective gene remained unidentified.

1.3.1.4. The biosynthesis of acyl-SQDG

Recently, 2'-O-acyl-sulfoquinovosyldiacylglycerol (ASQD), an SQDG derivative, was identified in *C.reinhardtii* (Riekhof *et al.*, 2003). Unlike SQDG in this organisms, the acyl groups of ASQD predominantly are unsaturated fatty acids. Moreover, the18-carbon fatty acid with four double bonds directly attached to the head group of ASQD was the dominant acyl group. Inactivation of the *C. reinhardtii* SQD1 ortholog led to the absece of both lipids suggesting that ASQD is synthesized either by acylation of UDP-sulfoquinovose or by acylation of SQDG (Riekhof *et al.*, 2003). It was proposed that SQDG is the substrate for acylation, because UDP-sulfoquinovose synthesis occurs in the plastid and the acyl groups found attached to the sulfoquinovose head group of ASQD appeared to be of extraplastidic origin (Riekhof *et al.*, 2003).

1.3.2. The function of SQDG

Since the discovery of SQDG, speculations about its function focused on its role in photosynthesis, based on the association of SQDG and photosynthetic membranes in photosynthetic organisms, combined with the unusual structure of the head group (Barber and Gounaris, 1986; Benning, 1993, 1998). With the SQDG-deficient mutants constructed by insertional inactivation of the *sqd* genes (Benning, 1992, 1993), as well as a mutant from the unicellular algae *Chlamydomonas* (Sato *et al.*, 1995), the role of SQDG in photosynthetic organisms could be analyzed in detail. It was also found that SQDG has apparent antiviral and antitumor activities (Gustafson *et al.*, 1989; Shirahashi *et al.*, 1993). Recently, SQDG has been reported as a selective inhibitor of eukaryotic

DNA polymerases α and β (Ohta *et al.*, 1998), and as an immunosuppressive agent (Matsumoto *et al.*, 2002).

1.3.2.1. The function of SQDG in photosynthetic organisms

The availability of SQDG-deficient mutants permitted us to test the correlation between the primary phenotype, SQDG deficiency, and secondary phenotypes such as photo- synthetic activity, growth, and so on (Benning, 1998). The detailed analysis of SQDG -deficient mutants showed that SQDG plays different roles in different organisms. In the oxygenic bacteria *R. sphaeroides*, SQDG deficiency has no observed effect on growth of the bacterium under normal growth conditions (Benning et al., 1993). Photosynthetic electronic transfer was also not affected in these mutants. These results suggested that SQDG has no essential role in *R. sphaeroides* under conditions tested (Benning et al., 1993). In a SQDG-deficient mutants of the oxygenic cyanobacteria *Synechococcus* sp. PCC7942, subtle changes in the photosynthesis-related biochemistry of molecular oxygen were observed as well as a slight increase in the variable room temperature chlorophyll fluorescence yield, but the light response curves for oxygen evolution were not affected (Güler *et al.* 1996). The growth of this mutant was not affected either. Thus neither the overall electron transport rate nor the optical cross section of oxygen evolution were effected by the deficiency of SQDG (Güler *et al.* 1996). In comparision, SQDG seems to play essential roles in *Synechocystis*. sp PCC6803, (Aoki *et al.*, 2003). The SQDG-deficient mutant, named SD1, lacked the ability to synthesize SQDG and required SQDG supplementation for growth. After transfer from SQDG-supplemented to SQDG-free medium, SD1 showed decreased net photosynthetic

and PSII activity on a chlorophyll basis proportionate to the decrease in SQDG (Aoki *et al.,* 2003). The PSII activity of SD1 was more sensitive to 3(3,4-dichlorophenyl)-1,1-dimethyurea (DCMU), which is binding to the Q_B acceptor site of PSII and therefy blocks PSII activity (Aoki *et al.,* 2003). In Arabidopsis, transgenic plants with reduced amounts of SQDG expressing the SQD1 cDNA in antisense orientation showed no obvious growth impairment (Essigmann *et al.,* 1998). In the unicellular green alga *C. reinhardtii*, SQDG seems to play an intermediate role. The SQDG deficient mutant exhibited a reduced growth rate, impaired photosynthetic activity and was more sensitive to DCMU (Minoda *et al.,* 2002; Riekhof *et al.,* 2003; Sato *et al.,* 2003). The reasons for this discrepancy in the importance of SQDG among different organisms is not clear. One possible explanation is the evolutionary flexibility of photosynthetic membranes in terms of their lipid compositions, which could only require that a given combination of these lipids forms a functional photosynthetic membrane. Consistent with the analysis of SQDG deficient mutants, biochemical analysis also suggests that SQDG plays different roles in PSII of different organisms. In *C. reinhardtii,* SQDG is associated with the PSII core complex and is part of light harvesting complex II (LHCII) (Sato *et al.,* 1995; Sigris *et al.,* 1988). In tobacco, SQDG was found in the outer surface of the D1/D2 heterodimer where it was accessible to antibodies against SQDG (Radunz,and Schmid., 1992). It has also been found that SQDG is located in PSII core dimers in spinach (Murata *et al.,* 1992; Kruse *et al.,* 2000). But in preparations of light harvesting complex II trimers of pea chloroplast, PG in place of SQDG was found, and PG rather than SQDG was required for the crystallization of the complex (Nußberger *et al.,* 1993).

Because of the presence of plastids in eukaryotic photosynthetic organisms, SQDG may play additional roles during the development or assembly of the photosynthetic apparatus. In fact, the interaction between SQDG and the transit peptide of a chloroplast protein has been observed. This suggests that the negatively charged lipid SQDG may be essential for protein import into the chloroplast (van't Hof *et al.*, 1993).

It has been speculated that SQDG provided plant resistance to stress-factor action (Taran *et al.*, 2000). It has been found that SQDG accumulated in response to cold temperature in 1-year-old of cold temperature tolerant shoot bark of apple varieties and in Siberia apple. In response to high temperature, SQDG accumulated in resistant plants while the proportion of SQDG decreased in sensitive plants. It has been proposed that the role of SQDG in these phenomena could be the stabilization of photosynthetic processes, particularly ATP synthesis and light-harvesting complex II functioning, although the possibility that SQDG functions in signaling can not be ruled out either (Taran *et al.*, 2000).

1.3.2.2. Function of SQDG in non-photosynthetic organisms

SQDG is also present in non-photosynthetic organisms, such as *Bacillus acidocaldarius* (Langworthy *et al.*, 1976), *halophilic bacterium* (Sprott et al., 2003), several *Caulobacter* and *Brevundimonas ssp* and *Sinorhizobium meliloti* (Cedergreen and Hollingsworth, 1994). This raises the question of the function of SQDG in these organisms. Taking advantage of the cloning of genes involved in SQDG biosynthesis, a reverse genetic approach has been used to explore the function of SQDG in non-photosynthetic organisms. The inactivation of an *sqdB*-like gene in *Sinorchozobium*

meliloti led to SQDG deficiency (Weissenmayer *et al.,* 2000). However, the SQDG-deficient mutant was still capable of producing functional nodules and did not show an obvious disadvantage under different laboratory culture conditions. Therefore, no specific roles for SQDG could be asigned in *Sinorchizobium meliloti* and it was suggested that sulfolipid, may become critical only under growth conditions that still need to be discovered (Weissenmayer *et al.,* 2000).

1.3.3. Other potential applications for SQDG

SQDG also possesses biological activities outside its natural environment due to its unique head group and its excellent detergent properties. It has been reported that SQDG purified from cyanobacteria functions as an antiviral agent against HIV (Gustafson *et al.,* 1989) suggesting its potential application in medicine. SQDG also exhibits anti-tumor activities. The proliferation of human gastric cancer cell line SNU-1 cells by 72 hours of culture in the presence of 100 μM and 1 mM SQDG was inhibited 24 and 100%, respectively, as compared with the number of SNU-1 cells cultured in the absence of SQDG (Quasney *et al.,* 2001). Inhibition of cell proliferation by 100 μM sulfoquinovosyldiacylglycerol was in part associated with apoptotic cell death. These results suggested that sulfolipid has intriguing potential as a chemopreventive or chemotherapeutic agent (Quasney *et al.,* 2001). In addition, SQDG is a potential inhibitor of eukaryotic DNA polymerases and HIV-reverse transcriptase type 1. The inhibition was dose-dependent, and complete (more than 90%) inhibition of DNA polymerase α, DNA polymerase β and HIV-reverse transcriptase type 1 (HIV-RT) was observed at concentrations of 5, 10, and 30 μM, respectively (Ohta *et al.,* 1998). These results

suggested the potential application of SQDG as an inhibitor in DNA polymerase research. Recently Matsumoto *et al.*, (2002) reported the synthetic analogues of sulfolipids β-SQDG(sn-1, 18:0; sn-2, 18:0) could suppress the host's immunocompetence and prolonged the time before rat skin allograft rejection *in vivo*. There seemed to be no obvious adverse pathohistological effects in these rats. These results suggest that β-SQDG could act as a new immunosuppressive reagent.

1.4. Phosphate regulation of lipid biosynthesis

During their evolution, plants, like other organisms, have developed strategies to cope with various environmental stresses. The replacement of phospholipids by nonphosphorous lipids is one way by which plants deal with phosphate deprivation. This phenomena was first discovered in nonphotosynthetic bacteria and later in photo-synthetic organisms (Minnikin *et al.*, 1974; Benning *et al.*, 1993) and Arabidopsis (Essigmann *et al.*, 1998). It has been shown that the acidic lipid SQDG can replace the acidic thylakoid lipid PG. Analyzing the phosphate-deficient *pho1* mutant of *Arabidopsis*, it was found that not only SQDG but also DGDG increased after phosphate deprivation. Thus it was proposed that the substitution of phospholipids by nonphosphorous lipids is a more general phenomenon in plants. Consistent with the increase of glycolipids, the genes involved in these lipid's biosynthesis, are also induced by phosphate stress, indicating the presence of a signal transduction cascade to regulate lipid metabolism in response to phosphate-limiting conditions (Essigmann *et al.*, 1998; Kelly and Dormann, 2002). Further analysis showed that DGDG accumulated after phosphate-limitation, was also present in extraplastic membranes and possesed eukaryotic lipid structure (Härtel *et*

al., 2000). This also occurred in the *act1* mutant defection in the prokaryotic pathway (Härtel *et al.,* 2000). These results suggested an increased contribution of the eukaryotic pathway to DGDG biosynthesis, therefore, a general redirection of galactolipid synthesis from the plastid to the eukaryotic pathway was present in phosphate-deprived plants. Further understanding of the redirection and the regulation of glycolipid biosynthesis could provide new insight into the control of lipid metabolisim.

The observation that SQDG has no apparent essential role in some photosynthetic organisms gives rise to the question why the SQDG biosynthetic pathway is maintained in these organisms. One appealing idea is that SQDG is required to help these organisms to cope with specific environmental condition such as nutrient limitation (Benning, 1998). In support of this idea, it has been observed that the growth of SQDG-deficient mutants such as *R. sphaeroides* (Benning et al., 1993), cyanobacteria *Synechococcus* sp. PCC7942 (Güler *et al.,* 1996) and *C. reinhardtii (*Riekhof *et al.,* 2003) was reduced under phosphate-limitation. Drastic changes in lipid composition also occurred in response to phosphate-limitation in these mutant as compared to wild type. Particularly interesting was the inverse relationship between two acidic/anionic lipids SQDG and PG. In the wild type grown under phosphate-limiting condition, the relative amount of SQDG was increased, while the amount of PG was decreased; in SQDG-deficient mutants, the relative amount of PG was maintained at the same level as under optimal growth conditions, although the changes in the relative amount of lipids other than SQDG were comparable with those of the wild-type. Based on these observations, the sulfolipid-phospholipid substitution hypothesis was proposed (Benning *et al.,* 1993; ; Güler *et al.,* 1996; Benning, 1998; Riekhof *et al.,* 2003). The sum of the two major anionic lipids,

SQDG and PG, was proposed to be critical for photosynthetic membrane development and function. Photosynthetic organisms are able, within limitations, to maintain a constant amount of total anionic thylakoid lipids by reciprocally adjusting the sulfolipid and phosphatidylglycerol contents as phosphate availability changes. Considering that one third of organic phosphate is normally bound in the membrane in the wild-type (Poirier *et l.,* 1991), sulfolipid-deficient null mutants exhaust their phosphate reserves sooner than the corresponding wild types, resulting in reduced growth under these conditions (Benning, 1998). This reverse relationship is not only present under phosphate stress, it also happens under other conditions. For instance, under sulfur-deprived condition, the proportion of PG is increased while SQDG is decreased in C. *reinhardtii* (Sato *et al.,* 2000). These facts suggest that sulfolipid/phosphatidylglycerol substitution may be ubiquitous to photosynthetic organisms containing these lipids. It should be pointed out that SQDG can only partially substitute for PG which, unlike sulfolipid, is prominently present in the crystal structure of PSI (Jordan *et al.,* 2001). Recently, Aoki *et al.*, (2003) showed that the proportion of PG is increased in the *Synechocystis* sp PCC6803 SQDG deficient mutant SD1, but it can not fully complement the loss of SQDG suggesting that SQDG also has some unique function in this organism which can't be replaced by PG.

1.5 Overview

Many aspects of the biosynthesis and function of thylakoid lipids in higher plants are still unclear. In the current dissertation, efforts to use Arabidopsis as a model system for understanding the biosynthesis and function of two major anionic lipids, SQDG and PG, and for dissecting the role of plastidic lyso-PA acyltransferase (pLPAAT) in plants are described.

As discussed above, our understaning of the plant SQDG biosynthetic pathway was previously incomplete due to the unidentified SQDG synthase. Therefore my first goal was to identify the SQDG synthase from Arabidopsis and to propose a complete SQDG biosynthetic pathway. Because plant chloroplast shares a common ancestor with cyanobacteria, it seemed possible that plants possess an ortholog of SQDX, the SQDG synthase of cyanobacteria. Therefore, a combination of reverse genetics and heterozygous expression in *E.coli* was employed to identify the SQDG synthase in Arabidopsis as described in Chapter 2. It was shown that a reduced level of SQDG has no apparent effect on the growth of Arabidopsis. Thus I postulated that like in bacteria (Benning *et al.*, 1993; Güler *et al.*, 1996), SQDG may play an essential role under phosphate-limitation in plants as well. As described in Chapter 2, this hypothesis was tested utilizing an SQDG-deficient mutant.

At the beginning of my thesis work, most genes involved in chloroplast PG biosynthesis were not known, and the specific function of PG in photosynthesis was unclear. A forward genetic approach was used to screen for a mutant deficient in PG, which would presumably arise from disrruption of the PG biosynthetic pathway. In

Chapter 3, I describe the isolation of such a mutant. This mutant was further employed to dissect chloroplast PG biosynthesis as well as the function of PG in photosynthesis. The observation of the inverse relationship between SQDG and PG led to the hypothesis that the function of SQDG is to replace PG under phosphate-limitation to maintain a constant amount of anionic lipids. To test the SQDG/PG substitution hypothesis and to understand the essential role of a constant proportion of anionic lipids in photosynthesis and chloroplast development, an SQDG-deficient mutant described in Chapter 2 and a partially PG-deficient mutant, described in Chapter 3, were used to construct a double mutant, which was hypothesized to contain a reduced amounts of total anionic lipids . In Chapter 4, I describe the results of my work utilizing this double mutant to address the questions discussed above.

Phosphatidic acid is a key intermediate for chloroplast lipid biosynthesis. At the beginning of my thesis work, the gene for plastidic LPAAT had not been identified in Arabidopsis. The *in vivo* role of LPAAT was also unkown in plants. A reverse genetic approach was used to address this problem. In Chapter 5, the identification of a plastidic LPAAT, and a detailed analysis of the *in vivo* function of LPAAT are described. Inactivation of a plastidic G-3-P acyltransferase in the *ats1* (*act1*) mutant led to the loss of the "prokaryotic" pathway for glycolipid biosynthesis. However, despite the drastic effects on "prokaryotic"glycolipid biosynthesis, PG biosynthesis was only mildly impaired in the *ats1 (act1)* mutant. This observation made the origin of "prokaryotic" PA unclear. The LPAAT-deficient mutant was used to address the origin of "prokaryotic" PA as described in Chapter 5.

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Yu B, Xu C, Benning C. (2002) Arabidopsis disrupted in *SQD2* encoding sulfolipid synthase is impaired in phosphate-limited growth. Proc. Natl. Acad. Sci. USA 99:5732-5737.

Chapter 2

Arabidopsis disrupted in *SQD2* encoding sulfolipid synthase is impaired in phosphate-limited growth

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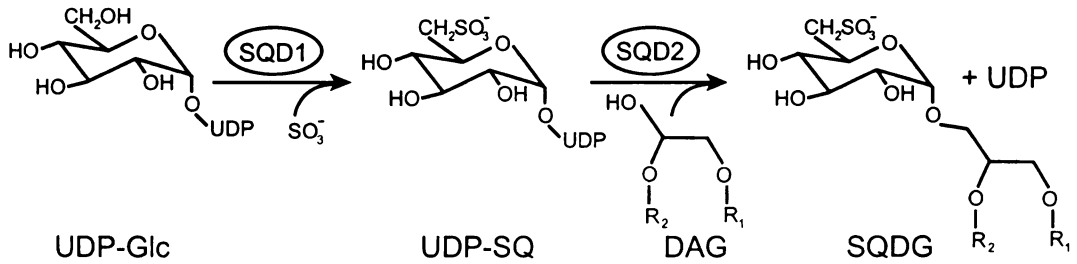
Abstract

The sulfolipid sulfoquinovosyldiacylglycerol is one of the three non-phosphorous glycolipids that provide the bulk of the structural lipids in photosynthetic membranes of seed plants. Unlike the galactolipids, sulfolipid is anionic at physiological pH due to its 6-deoxy-6-sulfonate glucosyl (sulfoquinovose) head group. The biosynthesis of this lipid proceeds in two steps, first, the assembly of UDP-sulfoquinovose from UDP-glucose and sulfite and second, the transfer of the sulfoquinovose moiety from UDP-sulfoquinovose to diacylglycerol. The first reaction is catalyzed by the SQD1 protein in *Arabidopsis*. Here we describe the identification of the *SQD2* gene of *Arabidopsis*. We propose that this gene encodes the sulfoquinovosyltransferase catalyzing the second step of sulfolipid biosynthesis. Expression of *SQD1* and *SQD2* in *Escherichia coli* reconstituted plant sulfolipid biosynthesis in this bacterium. Insertion of a T-DNA into this gene in *Arabidopsis* led to complete lack of sulfolipid in the respective *sqd2* mutant. This mutant showed reduced growth under phosphate-limited growth conditions. The results support the hypothesis that sulfolipid can function as a substitute of anionic phospholipids under phosphate-limited growth conditions. Along with phosphatidylglycerol, sulfolipid contributes to maintaining a negatively charged lipid-water interface, which is presumably required for proper function of photosynthetic membranes.

Introduction

The photosynthetic membranes of seed plants are rich in non-phosphorous glycolipids. These include the galactolipids mono- and digalactosyldiacylglycerol and the sulfolipid sulfoquinovosyldiacylglycerol. This lipid is unusual, because its polar head group consists of sulfoquinovose, a 6-deoxy-6-sulfonate-glucose. Much has been speculated about the possible function of this lipid in photosynthesis (Barbar and Gounaris, 1986; Benning, 1998), but in recent years the isolation of sulfolipid-deficient bacterial mutants (Benning *et al.,* 1993; Güler *et al.,* 1996) and a mutant of the unicellular algae *Chlamydomonas* has provided some novel clues (Sato *et al.,* 1995). It has become apparent from these mutants that there is no essential role for sulfolipid in photosynthetic bacteria with anoxygenic or oxygenic photosynthesis, or in eukaryotic cells containing chloroplasts, because all mutants were capable of photoautotrophic growth. The effects on photosynthesis are subtle under normal growth conditions, at best. However, if bacterial sulfolipid-deficient null mutants are starved for phosphate, they cease growth much earlier than the respective wild type (Benning *et al.,* 1993; Güler *et al.,* 1996). Thus, sulfolipid is of conditional importance in these bacteria. Like in bacteria, the ratio of non-phosphorous glycolipids-to-phospholipids drastically increases in *Arabidopsis* upon phosphate deprivation (Essigmann *et al.,* 1998; Härtel *et al.,* 2000). In particular, the relative amount of sulfolipid rises several fold due to an active process based on the increased expression of at least one of the sulfolipid genes, *SQD1* (Essigmann *et al.,* 1998). Therefore, it seems likely that sulfolipid is of conditional importance also in *Arabidopsis* and other plants.

Figure 2.1. The pathway for sulfolipid biosynthesis in *Arabidopsis*. Two enzymes, SQD1 and SQD2, are specific to this process and catalyze the reactions as indicated. DAG, diacylglycyerol; R, fatty acyl groups; SQDG, sulfoquinovosyldiacylglycerol; UDP-Glc, UDP-glucose; UDP-SQ, UDP-sulfoquinovose.


SQD1
SQD2
+ UDP
UDP-Glc
UDP-SQ
DAG
SQDG

The unique step in sulfolipid biosynthesis is the synthesis of UDP-sulfoquinovse, the sulfolipid head group donor (Fig. 2.1), catalyzed by the SQD1 protein in *Arabidopsis* (Sanda *et al.,* 2001). The crystal structure for this protein is known and a reaction mechanism has been proposed (Essigmann *et al.,* 1999; Mulichak *et al.,* 1999). On the contrary, although the activity of the sulfolipid synthase catalyzing the last step of sulfolipid biosynthesis has been fairly well characterized in spinach chloroplast extracts (Seifert and Heinz, 1992), the protein had not been isolated and the respective gene remained unidentified. Orthologs of the *Arabidopsis SQD1* gene are highly conserved in different groups of bacteria and even in plants (Benning, 1998). However, two unrelated classes of genes encode the UDP-sulfoquinovose-dependent glycosyltransferases responsible for the final assembly of sulfolipid in the bacteria of the a-group (Rossak *et al.,* 1995) and cyanobacteria (Güler *et al.,* 2000). Because it seemed possible that plants inherited their sulfolipid synthase from the postulated cyanobacterial ancestor of chloroplasts, we searched for an ortholog of the cyanobacterial *sqdX* gene in the fully sequenced *Arabidopsis* genome. As a result of this genomic approach, the correct *Arabidopsis* ortholog of *sqdX*, designated *SQD2,* was identified, characterized, and exploited to answer open questions about sulfolipid biosynthesis and function in seed plants.

Materials and methods

Plant Material and Plant Growth Experiments.

Surface sterilized seeds of *Arabidopsis* (*A. thaliana*, ecotype Wassilewska, WS) were germinated on 0.8% (w/v) agar-solified MS medium (Murashige and Skoog, 1962) supplemented with 1% (w/v) sucrose. The seedlings were kept on agar for 10 days prior to the transfer to pots containing a soil mixture as described (Estelle and Somerville, 1987), except that sphagnum was replaced with Bactomix (Michigan Peat Company, Houston, TX). Plants were grown in growth chambers (AR-75L, Percival Scientific Inc., Boone, IA) under light of a photosynthetic photon flux density of 80 μmol photons m^{-2} s^{-1}. A 14-h light/10-h dark regime was applied. The day/night temperature was controlled at 22/18°C. For growth under phosphate limitation, sterile *Arabidopsis* medium (Estelle and Somerville, 1987) was used, but at half concentration and containing 0.8% agarose, 1% sucrose and 20 mM MES (pH 6.0), and different concentrations of KH_2PO_4 as indicated. Plants were grown for 8 days on 0.8% (w/v) agar-solidified MS medium supplemented with 1% (w/v) sucrose and then transferred to the agar plates containing the phosphate-limited medium. For quantitative growth experiments, sucrose was left out of the medium following the transfer. Plant culture dishes for each experiment were placed on a single shelf of a CU-365L chamber (Percival Scientific Inc., Boone, IA). The dishes were covered with two layers of cheese cloth to diffuse the light and maintain a uniform photon flux density of 80 μmol photons m^{-2} s^{-1}. The dishes were carefully rotated over the shelf to ensure uniformity. At each time point, eight mutant and wild -type plants on matching dish positions were harvested and their aerial parts were weight. At least,

three independent biological repeats of the experiment were conducted with similar results.

Mutant Isolation.

A T-DNA insertion into the *SQD2* gene was isolated from the *Arabidopsis* knock-out collection at the University of Wisconsin (Logemann et al., 1987; Sambrook *et al.,* 1989). The gene-specific primers used for screening of insertions into the *SQD2* gene were 5'-AATCTCTCTATACCTCCTCATTTGCTTCC-3' and 5'-CACAAGCTCAATA-TAGGTTGACCCATAAG-3', the T-DNA-specific primer matching the left end of the T-DNA was 5'-CATTTTATAATAACGCTGCGGACATCT AC-3'. PCR fragments obtained using combinations of the T-DNA-specific primer and the two gene-specific primers were subcloned and sequenced at the MSU Genomics Facility to determine the insertion point for the 5.5 kb T-DNA.

Cloning of the *SQD2* cDNA.

The *SQD2* open reading frame corresponding to the protein (GeneBank accession CAB69850) predicted from BAC locus F7J8_200 (GenBank accession AL137189) was cloned by RT-PCR using the primers 5'-CGGGATCCCCATGACGACTCTTTCT-TCTATA-3' and 5'-AAGGTACCCTACACGTTACCTTCCGGTA-3'. Total leaf RNA was isolated from approximately 20 day old plants (Col-2) according to the method by Logemann et al. (Logemann *et al.,* 1987). Poly A^+ mRNA was purified using an oligotex kit from Qiagen (Valencia, CA) according to the instructions. RT-PCR reactions were performed using the ProSTAR HF single tube RT-PCR system from Stratagene (La Jolla,

CA). The PCR product was cloned into the ligation ready Stratagene vector pPCR-Script Amp SK^+ giving rise to pPCR-SQD2. The insert was sequenced and deposited at GenBank (Accession AF454354).

Northern blot Analysis.

Total RNA isolated as described above was separated by agarose gel electrophoresis and blotted onto Hybond N^+ (Amersham Pharmacia Biotech Inc., Piscataway, NJ) membranes using standard procedures (Bechtold and Pelletier, 1998). Hybridization was done under standard conditions (Sambrook *et al.,* 1989).

Complementation Analysis.

The insert of pPCR-SQD2 containing the full length coding sequence including the transit peptide was amplified by PCR using the primers 5'-AAGGTACCATGACGAC-TCTTTCTTCTATA-3' and 5'-AATCTAGACTACACGTTA CCTTCCGGTA-3'. This fragment was cloned into the binary vector pBINAR-Hyg (Dörmann and Benning, 1998) using the *Kpn*I and *Xba*I sites. The resulting plasmid pBINAR-Hyg/SQD2 was used to transform the *sqd2* mutant by vacuum infiltration (Bechtold and Pelletier, 1998). Transgenic plants were selected in the presence of kanamycin (60 μg/mL) and hygromycin B (25 μg/mL) on MS medium lacking sucrose.

Expression of *SQD1* and *SQD2* in *E. coli.*

To express *SQD2*, the *Bam*HI/*Kpn*I fragment of pPCR-*SQD2* containing the transit peptide encoding sequence was cloned in-frame into the expression vector pQE32 from

Qiagene (Valencia, CA) giving rise to p*SQD2*. The expression cassette encoding the SQD1 protein lacking the N-terminal 84 amino acid transit peptide was excised from pSQD1-TP (6) with *Xho*I/*Pvu*II, and was ligated into pACYC184 (Chang and Cohen, 1978) cut with *Sal*I/*Eco*RV giving rise to p*SQD1*, which is compatible with p*SQD2* in *E. coli*. Both plasmids were transferred to *E. coli* host XL1-Blue (Stratagene, La Jolla, CA) and selected for in the presence of 100 μg/mL ampicillin and 34 μg/mL chloramphenicol. Expression was induced according to the Qiagene protocol.

Lipid and Fatty Acid Analysis.

Lipids were extracted from frozen leaves or *E. coli* pellets as previously described (Dörmann *et al.*, 1999). Lipid extracts were analyzed as described (Essigmann et al., 2000) and lipids were visualized with iodine vapor or with sugar sensitive a-naphthol reagent (Gage *et al.*, 1992) and identified by co-chromatography with lipid extracts of known composition. For quantitative analysis methyl esters were prepared and quantified by GLC (Rossak *et al.*, 1997). For the analysis of ^{35}S-labeled lipids, four leaves of 3-week-old plants were cut midway through the petiole and immediately transferred petiole-down into a polypropylene microfuge tube containing 100 μL of an aqueous solution of carrier free ^{35}S-sulfate (3.7 MBq/mL; NEN Life Science Products, Boston, MA). Lipids were extracted following 1 hour of incubation and separated by two-dimensional TLC as previously described (Benning *et al.*, 1995). Labeled compounds were visualized by autoradiography. Fast atom bombardment mass spectroscopy (FAB-MS) measurements of sulfolipid (Gage *et al.*, 1992) were done at the MSU-Mass Spectrometry Facility.

Chlorophyll Fluorescence Measurements.

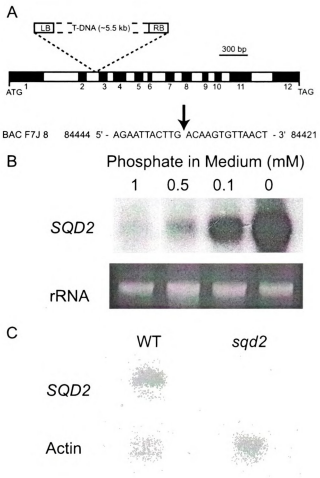
In vivo Chlorophyll fluorescence was measured at room temperature with an FMS2 fluorometer (Hansatech, King's Lynn, UK) after 20 min of dark adaptation. The maximal variable fluorescence (F_v) was obtained by subtraction of the initial fluorescence (F_o) from the maximum fluorescence (F_m). The ratio of F_v/F_m served as a measure of the maximal photochemical efficiency of PSII. The effective quantum yield of photochemical energy conversion in photosystem II, Φ_{PSII}, was determined as described by Härtel et al. (Härtel *et al.,* 1998). Calculation of Φ_{PSII} was according to Genty *et al.*(1989).

Results

Identification of an *Arabidopsis SQD2* Candidate Gene.

Taking advantage of the published *Arabidopsis* genome , we developed a set of criteria for the identification of an *SQD2* candidate gene encoding the sulfolipid synthase of *Arabidopsis*. First, given the ancestral relationship between chloroplasts and cyanobacteria it seemed likely that *SQD2* would be similar to the *sqdX* gene of the cyanobacterium *Synechococcus* strain sp. PCC7942, which was proposed to encode the sulfolipid synthase (Güler *et al.,* 2000). Indeed we, and recently Berg et al. (Berg *et al.,* 2000), identified *Arabidopsis* gene F7J8_200 (AT5g01220; GenBank accession AL137189.2) on chromosome five as a possible *sqdX* ortholog. The originally predicted protein (GenBank accession CAB69850) for this gene showed 37 % identity over 370 amino acid residues with the cyanobacterial SqdX protein, sufficient to be considered an ortholog. A wild-type cDNA corresponding to gene F7J8_200 was isolated by RT-PCR and sequenced (GenBank accession AF454354). The open reading frame for this cDNA disagreed with the current protein predictions for the F7J8_200 gene (GenBank accessions CAB69850, AAK76635) leading us to revise the exon-intron structure for F7J8_200 as shown in Figure 2.2A. Exon 6 was added and exons 7, 8, and 9 increased slightly in size. The revised protein sequence for the F7J8_200 open reading frame of 510 amino acids is 41% identical over 370 residues to the cyanobacterial SqdX protein. As a second criterium for the correct identification of an *SQD2* candidate, a protein domain search using the Pfam database (Betemann *et al.,* 2000) revealed a glycosyltransferase group 1 motif (Pfam accession 00534) from amino acids 294 to 458 of the putative SQD2

Figure 2.2. Structure of the *SQD2* gene carrying the T-DNA insertion (A), induction of *SQD2* expression following phosphate deprivation (B), and lack of expression of *SQD2* in the *sqd2* mutant (C). A, exons are numbered as referred to in the text. A small portion of the BAC F7J8 sequence is shown with nucleotide numbers referring to GenBank accession AL137189.2. The arrow indicates the T-DNA insertion point. B, plants were grown on agar solidified medium for approximately 20 days. As loading control one of the rRNA bands stained with ethidium bromide is shown. C, plants were grown on soil for approximately 25 days. An actin cDNA (GenBank accession M20016.1) was used as a probe for control purposes.


A
LB
T-DNA (~5.5 kb)
RB
300 bp
ATG
1
2
3
4
5
6
7
8
9
10
11
12
TAG
BAC F7J 8
84444
5' - AGAATTACTTG ACAAGTGTTAACT - 3'
84421
B
Phosphate in Medium (mM)
1
0.5
0.1
0
SQD2
rRNA
C
WT
sqd2
SQD2
Actin

protein encoded by the cDNA. According to the glycosyltransferase classification database CAZy (http://afmb.cnrs.mrs.fr/~pedro/ CAZY/db.html) (Herissat *et al.,* 2001), this protein is grouped in with glycosyltransferase family 4, which is mechanistically characterized by "retaining" glycosyltransferases. This classification would be in agreement with the a-anomeric proton configuration of the substrate and product of the sulfolipid synthase reaction. Third, sulfolipid synthase is an enzyme of the inner envelope in chloroplasts (Tieje and Heinz, 1998). As such, the translation product should contain a chloroplast transit peptide. According to an *in silico* analysis (Emanuelsson *et al.,* 1999), the putative SQD2 protein contains an 83 amino acid transit peptide. Fourth, we expected that the expression of the candidate *SQD2* gene would be induced as phosphate is depleted, because sulfolipid biosynthesis and the expression of *SQD1* is induced under these conditions (Essigmann *et al.,* 1998). As shown in Figure 2.2B, the expression of the candidate *SQD2* gene followed this prediction. With all four prediction criteria satisfied, a series of experiments was conducted to determine, whether *Arabidopsis* gene F7J8_200 indeed encodes a sulfolipid synthase.

Isolation of an *sqd2* T-DNA Insertion Mutant.

To obtain corroborating evidence that the gene F7J8_200 encodes a protein essential for sulfolipid biosynthesis in *Arabidopsis*, the isolation of a T-DNA insertion mutant from the University of Wisconsin-Madison collection (Sussman *et al.,* 2000) was pursued. One homozygous mutant line, which lacked sulfolipid was isolated. Sequencing PCR-products obtained using gene-specific and T-DNA specific primers, it was determined that the T-DNA was inserted after base pair 84434 of the F7J8 BAC sequence

(Gen Bank accession AL137189.2). This places the insertion point into the second intron of the predicted F7J8_200 gene as shown in Figure 2.2A. A comparative Northern analysis between the wild type and the homozygous *sqd2* mutant (Fig. 2.2C) suggested complete lack of expression. Homozygosity was determined at the molecular level using combinations of gene-specific and T-DNA-specific primers to generate PCR products diagnostic for mutant and wild-type chromosomes. Following a cross between the homozygous mutant and the wild type, genetic segregation was determined in the F_2 generation. Approximately one quarter of the plants were kanamycin sensitive (93 of 379 total) and approximately one third of the kanamycin resistant plants (31 of 100) were sulfolipid deficient (see below). The observed segregation ratios agreed with a single T-DNA insertion into the F7J8_200 locus, with kanamycin resistance scoring as a dominant and sulfolipid-deficiency as a recessive marker. To ensure that the observed T-DNA insertion was indeed causing the lipid phenotype, the cDNA derived from F7J8_200 was ligated into a binary expression vector, and transferred into the homozygous mutant (sulfolipid-deficient, kanamycin resistant) by *Agrobacterium*-mediated transformation. A hygromycin B (transgene T-DNA) and kanamycin resistant (Ko-T-DNA) transgenic line was recovered. This line produced sulfolipid (data not shown) and showed wild-type growth characteristics (see below), thereby demonstrating complementation. Therefore, disruption of the F7J8_200 locus was responsible for the sulfolipid lipid deficiency in the *sqd2* mutant and growth impairment as discussed below. This locus was designated *SULFOQUINOVOSYLDIACYLGLYCEROL 2 (SQD2)*.

The *sqd2* Mutant is Completely Devoid of Sulfolipid.

Lipids extracted from the *sqd2* mutant and the wild type were separated by two-dimensional TLC. No sulfolipid was detected in the mutant extracts by iodine staining (Fig. 2.3) or by highly sensitive isotope labeling (Fig. 2.3, inserts). Based on this result it appears that sulfolipid biosynthesis is completely inactivated in this mutant due to a *sqd2* null-allele. To investigate compensatory effects in the *sqd2* mutant, the leaf lipid composition was compared for extracts from mutant and wild-type (WS) plants grown in the presence and absence of phosphate (Table 2.1). Within the experimental error and besides the complete lack of sulfolipid, the leaf composition of the mutant showed no drastic differences compared to the wild type. Given that the relative amount of sulfolipid under phosphate-sufficient conditions does not exceed 2 mol%, this result was not surprising. However, under phosphate-deficient growth conditions, under which relative sulfolipid amounts increased and phosphatidylglycerol amounts decreased in the wild type, phosphatidylglycerol did not decrease in the sulfolipid-deficient *Arabidopsis* mutant. The same had been observed for sulfolipid-deficient bacterial mutants (Benning et al., 1993; Güler et al., 1996) and was interpreted as a requirement to maintain the overall amount of anionic lipids in the thylakoid membrane. Taking sulfolipid and phosphatidylglycerol out of consideration, the glycolipid-to-phospholipid ratio increased in similar ways in the *sqd2* mutant and the wild type in response to phosphate starvation (Tab. 2.1).

Figure 2.3. The *sqd2* mutant is SQDG deficient. Two dimensional separation of lipids extracted from wild-type (A) and *sqd2* mutant (B) leaves. The lipids were visualized by iodine. Inserts represent autoradiographs in the area of the sulfolipid spots. Leaves were incubated with ^{35}S-sulfate to label sulfolipid. Lipids were separated as in the main panel. Small circles in the lower right corners indicate the origins, the arrows point towards the position of the sulfolipid. DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; SQDG, sulfoquinovosyldiacylglycerol.

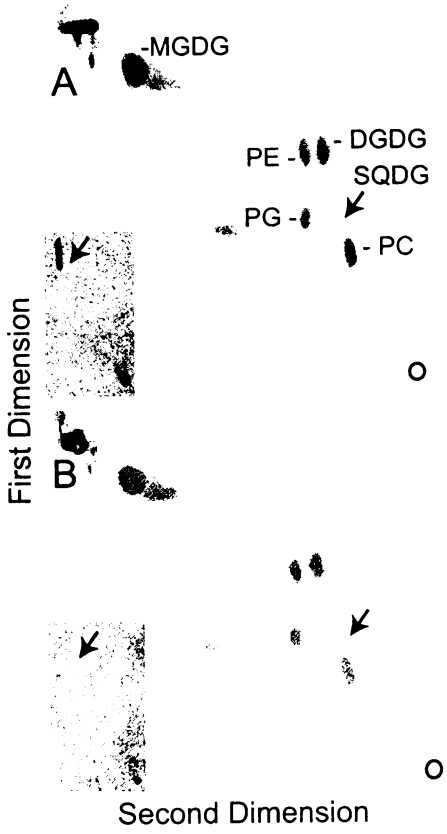

-MGDG
A
PE -
- DGDG
SQDG
PG -
- PC
O
First Dimension
B
O
Second Dimension

Table 2.1 Quantitative analysis of leaf lipid extracts from wild type (WS) and the *sqd2* mutant of *A. thaliana* grown on medium with or without phosphate for 20 days [a].

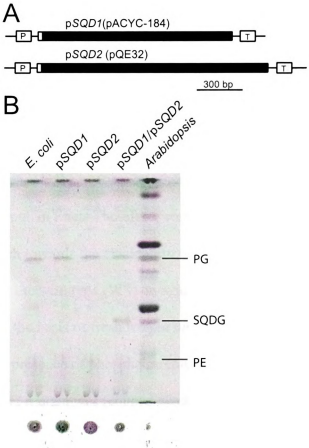
	1 mM Phosphate in medium		no Pi in medium	
Lipid	WT (WS)	*sqd2*	WT (WS)	*sqd2*
MGDG	34.8 ± 0.9	32.2 ± 3.3	38.4 ± 0.7	37.7 ± 0.2
PG	10.5 ± 0.5	12.8 ± 1.1	3.2 ± 0.3	11.2 ± 0.3
DGDG	16.5 ± 1.0	16.6 ± 0.7	39.9 ± 2.0	37.5 ± 1.0
SQDG	1.3 ± 0.2	n.d.	5.7 ± 0.7	n.d.
PI	2.0 ± 0.2	2.8 ± 0.2	0.8 ± 0.4	1.7 ± 1.1
PE	15.1 ± 0.1	15.6 ± 1.1	4.1 ± 0.3	4.9 ± 0.2
PC	19.8 ± 1.2	20.2 ± 0.6	7.6 ± 0.9	6.9 ± 0.4

[a] Values (mol %) represent means of three measurements. Lipid symbols as in Fig. 2.1 and Fig. 2.3; n.d. , not detected.

Expression of *Arabidopsis SQD1* and *SQD2* in *E. coli.*

To demonstrate directly that *SQD2* encodes the sulfolipid synthase and to test whether the two proteins SQD1 and SQD2 of *Arabidopsis* represent the biosynthetic machinery specific to sulfolipid biosynthesis in plants, these two genes were coexpressed in *E. coli.* This bacterium does not normally contain sulfolipid. A truncated version of the *SQD1* protein lacking the chloroplast transit peptide had been previously expressed in *E .coli*, and the purified recombinant protein was shown to catalyze the formation of UDP-sulfoquinovose from UDP-glucose and sulfite *in vitro* (Sanda *et al.,* 2001). To express both proteins, we followed a two plasmid strategy as was used for the reconstitution of plant galactolipid biosynthesis in *E. coli* (Chang and Cohen, 1978). Both constructs are shown in Figure 2.4A. No new glycolipid bands were visible by a-naphthol staining in lipid extracts from *E. coli* cells containing only one of each of the two plasmids. However, when induced *E. coli* cells were analyzed that contained both constructs, a new band appeared, which cochromatographed with authentic plant sulfolipid from an *Arabidopsis* leaf lipid extract (Fig. 2.4B). This new lipid was isolated from induced *E. coli* cultures using TLC purification and was analyzed by negative fast atom bombardment mass spectrometry. Two diagnostic molecular ion peaks (M-H) at 791 m/z and at 819 m/z were detected which agreed with the predicted M-H mass for sulfoquinovosyldiacylglycerol containing 16:0/16:1 and 16:0/18:1 fatty acids respectively. Gaschromatographic analysis of fatty acid methylesters derived from the newly produced lipid revealed 16:0, 16:1, and 18:1 fatty acids as the predominant fatty acids. Taken together, all data were in agreement with the formation of authentic sulfolipid in *E. coli* following the expression of the

Figure 2.4. Expression of Arabidopsis *SQD1* and *SQD2* in *E. coli*. Plasmid constructs used for the expression of the *SQD1* and *SQD2* genes in *E. coli* (A), and separation of lipids from different *E. coli* strains (B). A, the employed vector plasmid is indicated in brackets for each construct. The open boxes indicate the His tag, the black boxes plant sequences. P, promoter; T, terminator. B, the four left lanes represent extracts from *E. coli* harboring no plasmid or plasmids as indicated. An *Arabidopsis* leaf lipid extract was included for lipid identification purposes. The lipids were visualized by α-naphthol, which primarily stains glycolipids. Other lipids are visualized due to light charring. Abbreviations as in the legend of Fig. 2.3


A
pSQD1(pACYC-184)
P
T
pSQD2 (pQE32)
P
T
300 bp
B
E. coli
pSQD1
pSQD2
pSQD1/pSQD2
Arabidopsis
PG
SQDG
PE

Arabidopsis SQD1 and *SQD2* genes. These data also suggested that *SQD2* indeed encodes the UDP-sulfoquinovosyl:diacylglycerol sulfoquinovose transferase from *Arabidopsis*.

The effect of sulfolipid deficiency on the *sqd2* mutant.

The *sqd2* sulfolipid null-mutant of *Arabidopsis* described here, provides an excellent tool for the *in vivo* analysis of sulfolipid function in seed plants. Thus far, the molecular and genetic analysis of the mutant described above has not provided any evidence for multiple T-DNA insertions into the genome or other secondary mutations, which can occur in T-DNA tagged mutant lines. Therefore, comparison of mutant and wild type should be a valid approach towards the elucidation of sulfolipid function. Comparing the growth of the mutant and the wild type (WS) on agar plates in a controlled growth chamber environment on the basis of fresh weight did not reveal any difference, when the plants were grown in the presence of phosphate-replete medium (Fig. 2.5A). However, the growth rate of the *sqd2* mutant was reduced when plants were phosphate depleted during growth on agar medium lacking phosphate (Fig. 2.5B). To eliminate the possibility that this subtle difference in growth was due to a secondary mutation, we repeated one time point midway through the experiment comparing the homozygous mutant (*sqd2*) with the homozygous mutant line expressing the wild-type SQD2 cDNA (*sqd2/SQD2* cDNA), lacking and containing sulfolipid, respectively. These two lines are nearly isogenic, but the second line contains an additional mutation due to the insertion of an additional T-DNA. Again, no difference between the wild type and the mutant was observed under normal growth conditions (Fig. 2.5A, open symbols), although, the onset

Figure 2. 5. Growth curves for wild type (filled diamonds, solid lines) and ***sqd2* mutant** (filled circles, broken lines) on agar-solidified medium with 1 mM (A) or no (B) phosphate added. Eight plants were weight for each time point. To rule out effects of secondary mutations, one time point (12 days) was repeated with an *sqd2* mutant line transformed with the *SQD2* cDNA (open diamonds) containing sulfolipid and the original sulfolipid-deficient *sqd2* mutant (open circles). At least 15 plants are represented in each point in this experiment. Standard errors are indicate where they exceed the size of the symbols.

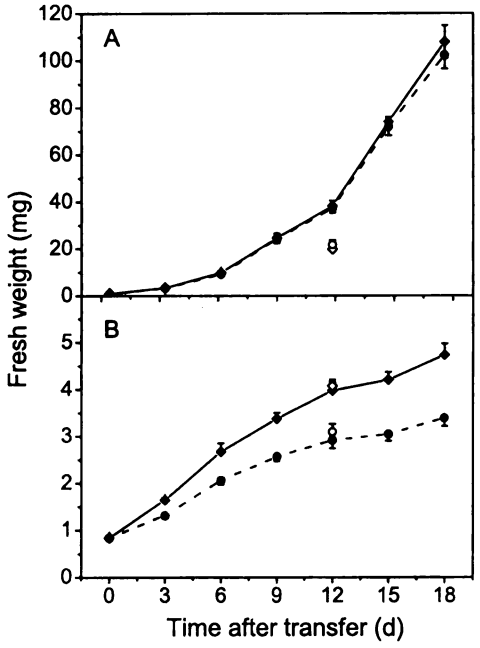

A
B
Fresh weight (mg)
Time after transfer (d)
0
20
40
60
80
100
120
1
2
3
4
5
0
3
6
9
12
15
18

Table 2.2. Pigment content (mg g^{-1} Fresh Weight) [a] and chlorophyll fluorescence [b] of wild type and *sqd2* mutant.

	WT	*sqd2*
Chlorophyll *a*	0.91 ± 0.06	0.93 ± 0.03
Chlorophyll *b*	0.29 ± 0.02	0.28 ± 0.02
Chlorophyll *a+b*	1.21 ± 0.07	1.21 ± 0.05
Chlorophyll *a/b*	3.14 ± 0.12	3.32 ± 0.14
Carotenoids	0.40 ± 0.02	0.42 ± 0.01
F_v/F_m	0.82 ± 0.01	0.80 ± 0.01
Φ_{PSII}	0.77 ± 0.01	0.70 ± 0.01

[a] Samples were taken from fully expanded leaves of 20-to-30 day old plants grown on agar solidified medium containing 1 mM phosphate. Pigment values represent means (± SE) of 3 independent determinations. [b] Actinic light for fluorescence measurements was 80 μmol photons m^{-2} s^{-1}, the same as used for raising the plants. Values represent means (± SE) of 10 independent determinations.

of the exponential growth phase in this completely independent experiment was delayed, as visible in the slight off-set between the two data point sets (Fig. 2.5A open and closed symbols). More importantly, the mutant lacked behind the complemented mutant line under phosphate-limited growth conditions (Fig. 2.5B, open symbols). Thus, secondary mutations as cause for the observed growth difference between the mutant and wild type under phosphate limiting conditions can be ruled out.

Using noninvasive chlorophyll fluorescence measurements it was determined that the maximum quantum yields for photosystem II photochemistry (F_v/F_m) were very similar for the mutant and the wild type (Table 2.2). The effective quantum yield of photochemical energy conversion in photosystem II (Φ_{PSII}) was slightly, but significantly reduced in the mutant grown under normal conditions (Table 2.2). Pigment content was not altered in the mutant (Table 2.2). Based on this basic analysis of photosynthesis, an essential function for sulfolipid in photosynthesis of seed plants can be ruled out. However, the current results do not exclude more subtle or conditional roles for sulfolipid in photosynthesis as were observed for Chlamydomonas and cyanobacteria (Güler *et al.*, 1996; Sato *et al.*, 1995). It should be pointed out that chlorophyll fluorescence parameters were indistinguishable for the mutant and the wild type under phosphate limitation (data not shown). Apparently, phosphate deficiency by itself has more pronounced effects on chlorophyll fluorescence (Härtel *et al.*, 1998) than sulfolipid-deficiency.

Discussion

More than 40 years after the discovery of the "Plant Sulfolipid" sulfoquinovosyl-diacylglycerol by A.A. Benson (1959), the quest for the enzymes involved in the biosynthesis of sulfolipid in seed plants has come to an end. Only two *Arabidopsis* proteins, SQD1 and SQD2 are required for sulfolipid biosynthesis (Fig. 2.1), when simultaneously produced in *E. coli.* The first, SQD1, has been shown to catalyze the biosynthesis of UDP-sulfoquinovose from UDP-glucose and sulfite (Sanda *et al.,* 2001) *in vitro.* The successful reconstitution of sulfolipid biosynthesis in *E. coli* suggests that the proper *in vivo* sulfolipid donor is present in this bacterium, but is not specific to organisms naturally capable of sulfolipid biosynthesis. Thus, this results supports the hypothesis that sulfite as product of the general sulfur assimilation pathway acts also as the *in vivo* sulfur donor. The second required plant enzyme, SQD2, is highly similar to glycosyltransferases (Berg *et al.,* 2001) and it is proposed that this protein represents the sulfolipid synthase. Thus far, this enzyme has only been described in envelope preparations from spinach chloroplasts (Seiftert and Heinze, 1992). The availability of an expression system for the production of SQD2 in *E. coli* provides the means to study the enzymatic reaction catalyzed by this protein in detail. The high similarity between the two *Arabidopsis* proteins SQD1 and SQD2, and the cyanobacterial sulfolipid proteins SqdB and SqdX (Güler *et al.,* 1996; Güler *et al.,* 2000) and their ability to functionally reconstitute sulfolipid biosynthesis in *E. coli* suggests that these are true orthologs.

The isolation of the sulfolipid-deficient *sqd2* null-mutant of *Arabidopsis*, allowed us to address another fundamental question, the role of sulfolipid in photosynthetic membranes of seed plants. Although the *sqd2* mutant has not yet been studied in greater

detail, our initial analysis of photosynthesis and growth suggests that sulfolipid does not have a crucial role in plant photosynthesis. The *sqd2* mutant did not show any obvious signs of altered morphology or growth and photosynthetic parameters were only mildly affected under normal laboratory growth conditions. A similar conclusion can be drawn for bacterial mutants (Benning *et al.,* 1993, Güler *et al.,* 1996) and a sulfolipid deficient mutant in *Chlamydomonas* (Sato *et al.,*1995). Although the later mutant appears to be more severely impaired in photosynthesis than the *Arabidopsis sqd2* mutant or the bacterial mutants, effects due to secondary mutations cannot be ruled out. Unlike the *Chlamydomonas* mutant, the *Arabidopsis sqd2* mutant and its nearly isogenic complementation line should provide the ideal material to probe further for specific roles of sulfolipid in chloroplastic photosynthesis.

If sulfolipid is not essential to photosynthesis, why did plants maintain this pathway during the course of evolution? One answer to this question is that sulfolipid is of importance under conditions under which phosphate becomes limiting for membrane biosynthesis. As in bacterial null-mutants (Benning *et al.,* 1993; Güler *et al.,* 1996), sulfolipid deficiency in the *Arabidopsis sqd2* mutant led to growth impairment under phosphate-limiting growth conditions (Fig.2.5). Although the effect was subtle, it was clearly measurable under highly controlled laboratory conditions and one would assume that sulfolipid-deficiency may be of even greater disadvantage under naturally occurring conditions. Remarkably, the wild type is able to maintain, within limitations, the relative amount of total anionic thylakoid lipids by reciprocally adjusting the sulfolipid and phosphatidylglycerol contents as phosphate availability changes (Essigmann *et al.,* 1998). The lipid profiles for the bacterial and *Arabidopsis* sulfolipid-deficient null-mutants share

a common feature, phosphatidylglycerol amounts are not decreasing under phosphate-limiting conditions. Therefore, sulfolipid-deficient null-mutants exhaust their phosphate reserves sooner than the corresponding wild types resulting in reduced growth under these conditions. The fact that this effect occurs in bacteria and plants suggests that sulfolipid/phosphatidylglycerol substitution may be ubiquitous to photosynthetic organisms containing these lipids. It should be pointed out, though, that sulfolipid cannot fully substitute phosphatidylglycerol, which unlike sulfolipid is prominently present in the crystal structure of photosystem I (Jordan *et al.,* 2001) and may have specific functions beyond sulfolipid. However, photosynthetic membranes generally seem to need a certain amount of anionic lipids for proper function and these can be provided either by phosphatidylglycerol or by sulfolipid. One component of the underlying regulatory process involves changes in the transcription of the *SQD1* (Essigmann *et al.,* 1998) and the *SQD2* genes (Fig. 2.1) in response to phosphate availability. It will be interesting to see, whether these lipid genes are controlled by a more general phosphate sensing regulatory pathway, or by a regulatory pathway sensing biophysical properties of membranes, such as the ratio of anionic to neutral lipids.

Acknowledgments

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Chapter 3

The pgp1 Mutant Locus of Arabidopsis Encodes a Phosphatidylglycerolphosphate Synthase with Impaired Activity

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Abstract

Phosphatidylglycerol is a ubiquitous phospholipid that is also present in the photosynthetic membranes of plants. Multiple independent lines of evidence suggest that this lipid plays a critical role for the proper function of photosynthetic membranes and cold acclimation. In eukaryotes, different subcellular compartments are competent for the biosynthesis of phosphatidylglycerol. Details on the plant-specific pathways in different organelles are scarce. Here we describe a phosphatidylglycerol biosynthesis-deficient mutant of Arabidopsis, *pgp1*. The overall content of phosphatidylglycerol is reduced by 30%. This mutant carries a point mutation in the CDP-alcohol phosphotransferase motif of the phosphatidylglycerolphosphate synthase (E.C.2.7.8.5) isoform encoded by a gene on chromosome 2. The mutant shows an 80% reduction in plastidic phosphatidylglycerol-phosphate synthase activity consistent with the plastidic location of this particular isoform. Mutant plants are pale green and their photosynthesis is impaired. This mutant provides a promising new tool to elucidate the biosynthesis and function of plastidic phosphatidylglycerol in seed plants.

Introduction

Phosphatidylglycerol (PG) is one of the most common phosphoglycero-lipids found in nature. It is the only major phospholipid present in the thylakoid membranes of plant chloroplasts (Marechal *et al.,* 1997) and the only phospholipid in cycanobacteria, which strikingly resemble plant chloroplasts in their lipid composition (Murata and Nishida, 1987). Indeed, a large body of correlative and direct evidence suggests that PG is critical for the structural and functional integrity of the thylakoid membrane. Thus, the presence of specific molecular species of PG in photosynthetic membranes correlates well with low-temperature-induced photoinhibition and chilling sensitivity of plants and cyanobacteria (Murata *et al.,* 1992; Somerville, 1995). Light harvesting pigment protein complexes of photosystem II are specifically enriched in PG (Murata *et al.,* 1990; Tremolieres *et al.,* 1994). Moreover, PG is crucial for the in vitro trimerization of the major peripheral light-harvesting pigment-protein complexes (Nußberger *et al.,* 1993; Hobe *et al.,* 1994; Kühlbrandt, 1994) and the dimerization of the reaction/center core pigment-protein complexes of photosystem II (Kruse *et al.,* 2000). It is also an integral component of the phototsystem I reaction center (Jordan *et al.,* 2001) and required for the in vitro reconstitution of the light harvesting pigment protein complexes of photosystem I (Schmid *et al.,* 1997). Thylakoid membranes treated with phospholipase A_2 are PG depleted and are inhibited in their photosynthetic electron transport activities (Jordan *et al.,* 1983; Siegenthaler *et al.,* 1987). Furthermore, the anionic lipid PG interacts with the transit peptide of chloroplast precursor proteins during protein import into chloroplasts (van't Hof *et al.,* 1993).

Recently, PG-deficient auxotrophic mutants of the cyanobacterium *Synechocystis* sp. PCC6803 have been isolated which are severely impaired in photosynthesis (Hagio *et al.*, 2000; Sato *et al.*, 2000). Two Arabidopsis mutants, *pho1* and *ats1*(*act1*), are known that show, among other phenotypes, a decreased relative amount of PG (Härtel *et al.*, 1998a; Kunst *et al.*, 1988; Poirier *et al.*, 1991). However, because both mutants are affected in multiple aspects of lipid metabolism, their usefulness for the investigation of specific PG functions in photosynthetic membranes of seed plants is limited.

Two enzymatic reactions are specific to PG biosynthesis in plants, the formation of phosphatidylglycerolphosphate (PGP) from CDP-diacylglycerol and glycerol-3-phosphate catalyzed by the PGP synthase (E.C. 2.7.8.5), and the subsequent dephosphorylation by PGP phosphatase (E.C. 3.1.3.27) (Moore, 1982; Kinney, 1993; Andrews and Mudd, 1986). Labeling studies indicate that enzymatic activity involved in PG biosynthesis is associated with the inner mitochondrial membrane, the endoplasmic reticulum, and the chloroplast (Moore, 1982; Kinney, 1993) suggesting that at least three isoforms of PGP synthase and PGP phosphatase exist in plant cells. None of them has been purified to homogeneity and studied in vitro. Here we describe a chemically induced, PG-deficient mutant of Arabidopsis, *pgp1*, with a mutation in one isoform of PGP synthase.

Materials and methods

Plant Material

Surface sterilized seeds of Arabidopsis (A. thaliana, ecotype Col-2, or Ler-0), the multiple-times-backcrossed *pgp1*, *ats1*(*act1*) (Kunst *et al.,* 1988), and *pho1* (Poirier *et al.,* 1991) mutants were germinated on 0.8% (w/v) agar-solidified MS medium (Murashige and Skoog, 1962) supplemented with 1% (w/v) sucrose. The seedlings were kept on agar for 10 days prior to the transfer to pots containing a standard soil mixture (equal parts of Bacto Soil, Michigan Peat Company, Houston, TX; medium vermiculite; and perlite) drenched with half-strength Arabidopsis nutrient solution (Estelle and Somerville, 1987). Plants were grown in growth chambers (AR-75L, Percival Scientific Inc., Boone, IA) under light of a photosynthetic photon flux density (400-700 nm) of 75 μmol m^{-2} s^{-1}. A 14-h light/10-h dark regime was applied. The day/night temperature was controlled at 23/18°C.

Genetic and Molecular Procedures

The *pgp1* mutant was isolated by TLC of lipid extracts of individual M_2 plants as previously described for the isolation of the *dgd1* mutant (Dörmann *et al.,* 1995). The *pgp1* mutant locus was mapped in a small F_2 population (42 homozygous *pgp1* plants) derived from a cross of *pgp1* (Col-2) to a standard Landsberg erecta (Ler-0) line employing single sequence polymorphic markers evenly spaced on the Arabidopsis genome map (http://www.arabidopsis.org) under conditions described by Bell and Ecker (1994). At least two markers per chromosome were tested. Standard sequence searching

tools (Altschul *et al.,* 1990) were used at the National Center for Biological Information web site (http://www.ncbi.nlm.nih.gov) to identify putative PGP synthase genes in the Arabidopsis genomic sequence (The Arabidopsis Genome Initiative, 2001). References to the respective DNA and amino acid sequences are provided as GenBank accession numbers throughout the text. New sequences were obtained at the MSU Genomics Facility by automated sequencing.

For Southern blot analysis, Arabidopsis DNA was extracted from 4-week-old plants (Rogers and Bendich, 1994). The digested DNA was separated on a 1% agarose gel and transferred onto a Hybond N^+ membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Blots were hybridized at 65°C in standard hybridization solution (250 mM sodium phosphate buffer, 7% (w/v) SDS, 1 mM EDTA, 1% (w/v) BSA, 10mg/mL autoclaved herring sperm DNA). Blots were repeatedly washed for 15 min in 2 X SSC and 0.1% SDS.

Cloning of the Mutant and Wild-type cDNAs and Expression in E. coli

The *PGP1* cDNA was amplified in three steps from an Arabidopsis λgt11 cDNA library derived from leaves. An internal fragment of the Arabidopsis *PGP1* cDNA was amplified by PCR using the primers 5'-CGCTGCAGGTCTGGCTTCGTTAAT-3' and 5'-CGCTGCAGCTACTTCATTAGTACTTT-3'. The 5'-terminal region of the cDNA was obtained by the 5'-rapid amplification of cDNA ends (5'-RACE) method (5'-Full RACE Core Set; Takara, Shiga) and the 3'-terminal region by amplification using an internal primer and oligo dT. The amplified cDNAs were subcloned into pCR2.1 (Original TA Cloning Kit; Invitrogen, Carlsbad, CA, USA) and their nucleotide sequences were

determined. The sequence fragments were combined using appropriate restriction sites to obtain the full-length cDNA as deposited in GenBank (AB048535).

The *E. coli* mutant YA5512 defective in PGP synthase (Asai *et al.*, 1989; Usui *et al.*, 1994) was provided by K. Matsumoto (Saitama University, Japan). A region of the Arabidopsis *PGP1* cDNA encoding the presumed mature form of PGP synthase (first 93 amino acids removed) was amplified by PCR using the primers 5'-AAGGTACCCTT CACCGCCTCCGT-3' and 5'-AACTGCAGCTACTTCATTAGTACTTTCCA-3'. The corresponding *pgp1* mutant sequence was cloned by RT-PCR using the same set of primers. For this purpose, total leaf RNA was isolated from approximately 20-day-old plants according to the method by Logemann *et al.* (1987). Poly A^+ mRNA was purified using an oligotex kit from Qiagen (Valencia, CA) according to the instructions. RT-PCR reactions were performed using the Omniscript RT-PCR system from Qiagen. The PCR product was cloned into the ligation ready Stratagene vector pPCR-Script Amp SK+ (La Jolla, CA). The presence of the C to T mutation in *pgp1* cDNA was confirmed by DNA sequencing. The amplified DNA fragments from the wild type and the mutant were digested with KpnI and PstI, and ligated into the KpnI and PstI sites of the expression vector pQE32 from Qiagen. The resulting plasmids expressing the wild-type (pQE32-*PGP1*) and mutated protein (pQE32-*pgp1*) were used to transform *E. coli* YA5512. The constructs were confirmed by sequencing. YA5512 was also transformed with the vector pQE32, for control purposes. The transformants were grown at 37 °C in LB medium supplemented with 100 μg mL^{-1} ampicillin. A 3-mL overnight culture was centrifuged and the cells were washed and resuspended in 10 mL of the same medium with antibiotic. The culture was incubated at 37°C for 3 h and then isopropyl-1-thio-β-galactoside was

added to a final concentration of 0.4 mM and the culture was further incubated for 3 h at 37°C. Cells were collected by centrifugation (10 min, 5000 g). The resulting cell pellet was resuspended in 50 mM Tris, pH7.2, 20 % (v/v) glycerol and stored at –80 °C. For enzyme assays, the cells were disrupted by one freeze/thaw cycle and ultrasonification (three times for 20 s). The sonicated suspension was centrifuged at 15,000g and the supernatant (cell-free extract) was used for the assay of PGP synthase activity as described below.

Complementation Analysis

The full length coding sequence of wild type PGP synthase was amplified by RT-PCR using the primer 5'-AAGGTACCATGCTCAGATCCGGTCTGGCT-3' and 5'-AACTGCAGCTACTTCATTAGTACTTTCCA-3' as described above for the *pgp1* mutant sequence. This fragment was ligated into the Stratagene pPCR-Script Amp SK^+ vector and the nucleotide sequence was determined. The insert was excised with KpnI taking advantage of the site in the vector and in one of the primers, and was subsequently cloned into the respective site of the binary vector pBINAR-Hyg (Dörmann and Benning, 1998). Arabidopsis wild-type (Col-2) plants were transformed in planta (Bechtold and Pelletier, 1998). Transformants were selected on agar-solidified MS medium containing 1% sucrose and 70 μg/mL hygromycin B. The lipid phenotype was determined by thin layer chromatographic analysis of lipid extracts (see below). The presence of the transgene construct was confirmed by PCR using the primers 5'-AAGGTACCATGCT CAGATCCGGTCTGGCT-3' and 5'-ACCATAAGCTTATCAGCAACTGGATTCAA-3'.

Lipid Analysis

Rosette leaves were immediately frozen in liquid nitrogen upon harvesting, and lipids were extracted as previously described (Dörmann *et al.*, 1995). Bacterial cells were extracted as described by Benning and Somerville (1992). Lipid extracts were analyzed on activated ammonium sulfate impregnated silica gel TLC plates using a solvent system of acetone/toluene/water (91:30:7, v/v/v). Lipids were visualized with iodine vapor and identified by co-chromatography with lipid extracts of known composition. For quantitative analysis, individual lipids were isolated from TLC plates and used to prepare fatty acid methyl esters. The methyl esters were quantified by GLC using myristic acid as the internal standard (Rossak *et al.*, 1997).

Chloroplast Isolation and PGP Synthase Assay

Chloroplasts were isolated according to Joy and Mills (1987) from 4-week-old Arabidopsis plants grown on agar-solidified MS medium supplied with 1% sucrose. Typically, the equivalent of 5 g of rosette leaves was used in a single preparation. The chloroplast PGP synthase activity was measured by the incorporation of radiolabled glycerol 3-phosphate into the chloroform-soluble product PGP according to Mudd and coworkers (Mudd *et al.*, 1987). The reaction mixture contained 25 mM HEPES (pH 7.3), 2 mM $MgCl_2$, 0.1 mM CDP-diacylglycerol prepared as described (Mudd *et al.*, 1987), 0.5 mM [^{14}C]-DL-glycerol 3-phosphate (1 μCi/150 nmol), 0.05% Triton X-100 (v/v) and chloroplast suspension equivalent to 150 μg chlorophyll in a final volume of 150 μL. The reaction mixture for the determination of galactolipid synthase activities in isolated chloroplasts contained the same buffer except that 0.5 μCi UDP-[^{14}C] galactose (325

mCi/mmol; Amersham) was added instead of CDP-diacylglycerol and labeled glycerol-3-phosphate. Furthermore, this assay was performed in a total volume of 120 μL containing chloroplasts equivalent to 150 μg chlorophyll. The incubations were performed at 27°C for 60 min, and terminated by the addition of 0.5 mL of 0.1 M HCl in methanol. The lipids were extracted with 2 mL of chloroform and 3 mL of 1 M $MgCl_2$. The chloroform phase was evaporated under a stream of nitrogen and then redissolved in 0.2 mL chloroform. A 25-μL aliquot was taken for the determination of radioactivity by scintillation counting. The remainder was used for lipid analysis by TLC as described above.

Chlorophyll Fluorescence Measurements and Pigment Analysis

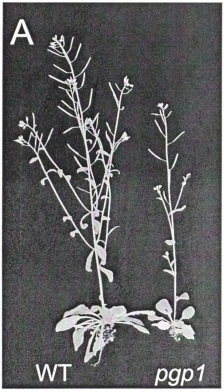
In vivo room temperature chlorophyll fluorescence was monitored with plants, which were dark adapted for 1 h as described (Härtel *et al.,* 1998c). The fluorescence parameters used in this study are as previously defined (Genty *et al.,* 1989; van Kooten and Snel, 1990). Pigments were determined as described by Lichtenthaler (1987).

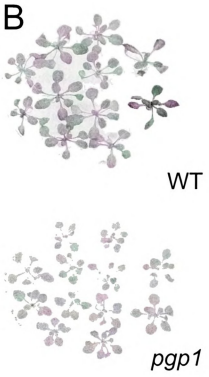
Results

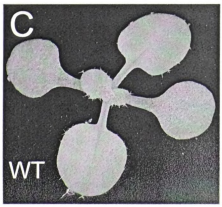
Isolation of a new phosphatidylglycerol-deficient mutant of Arabidopsis

To isolate mutants of Arabidopsis deficient in the biosynthesis of complex lipids, we designed a brute force screening procedure based on thin-layer-chromatographic analysis of lipid extracts from leaves of a chemically mutagenized Arabidopsis M_2 population. Screening 3,000 M_2-plants from 20 independent batches for lipid alterations by thin layer chromatography (TLC) of lipid extracts, we were not only able to isolate a galactolipid-deficient *dgd1* mutant as previously described (Dörmann *et al.,* 1995), but also two mutant lines with a slight reduction in PG content. One of these mutant lines was dark green, small, and resembled the *pho1* mutant in appearance, while one mutant was pale green in color. Due to phosphate deprivation of the *pho1* mutant, in which the primary defect is in the translocation of phosphate from the root to the shoot system (Poirier *et al.,* 1991), the relative content of phospholipids, including PG, is reduced while the content of non-phosphorous lipids is increased (Essigmann *et al.,* 1998; Härtel *et al.,* 1998a). Unlike the pale green mutant, the dark green mutant was reduced in leaf phosphate content and Yves Poirier confirmed by genetic complementation analysis that it carries a mutant allele of the *pho1* locus (Y. Poirier, personal communication). The pale green mutant, designated *pgp1* and backcrossed three times, is shown in Fig. 3.1. Its growth was slightly reduced and, particularly under photoautotrophic growth conditions, developing young leaves in the center of the mutant rosette were yellowish in color (Fig. 3.1 C). Thin layer chromatography of lipid extracts from expanded leaves followed by quantitative analysis of lipid composition of the *pgp1* mutant showed that the PG content

Figure 3.1. Growth and morphology of Arabidopsis wild type and *pgp1* mutant. A, six week old plants grown for 10 days on agar-solidified MS medium with 1% sucrose followed by photoautotrophic growth on soil. B, three week old plants grown on agar-solidified MS medium with 1% sucrose. C, three week old plants grown on soil. Note the yellow tissues in the center of the mutant rosette.


A
WT
pgp1


B
WT
pgp1


C
WT

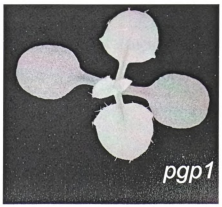

pgp1

Table 3.1. Leaf Lipid Composition (mol%) of Arabidopsis Wild Type and Mutants [a]

	WT	*pgp1*	*ats1(act1)*	*pho1*
MGDG	48.5 ± 1.3	45.9 ± 3.3	41.0 ± 0.9	48.4 ± 1.1
PG	8.4 ± 0.6	5.6 ± 0.8	5.5 ± 0.6	4.9 ± 0.3
DGDG	16.3 ± 0.8	14.7 ± 1.9	13.3 ± 1.7	28.6 ± 0.3
SQDG	1.2 ± 0.4	1.0 ± 0.3	1.1 ± 0.4	5.8 ± 0.3
PI	1.8 ± 0.5	1.5 ± 0.4	0.9 ± 0.4	0.7 ± 0.3
PE	9.1 ± 0.3	13.1 ± 1.2	13.8 ± 0.7	4.3 ± 1.2
PC	14.7 ± 1.4	18.3 ± 2.4	24.4 ± 0.7	7.3 ± 0.7

[a] Values represent means of three measurements. Plants were 25-to-30 day old. DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; SQDG, sulfoquinovosyldiacylglycerol.

was reduced by approximately 30% (Table 3.1). For comparison, we also included the analysis of the *pho1* and the *ats1*(*act1*) mutants. In all three cases the reduction in PG content was similar. However, contrary to *pgp1* and *ats1*(*act1*), the *pho1* mutant showed an increase in the relative amounts of the sulfolipid sulfoquinovosyldiacylglycerol and the galactolipid digalactosyldiacylglycerol, a phenotype characteristic for *pho1* (Härtel *et al.*, 1998a).

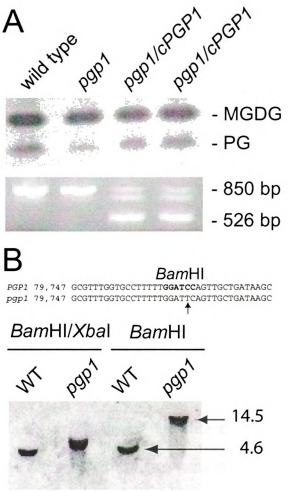
The primary defect in the *ats1*(*act1*) mutant is a reduction in the activity of the plastidic glycerol-3-phosphate:acyl-ACP acyltransferase (Kunst *et al.*, 1988). As a consequence, the plastid pathway of lipid assembly (Browse *et al.*, 1986; Browse and Somerville, 1991; Heinz and Roughan, 1983) in this plant is shut down and molecular species of monogalactosyldiacylglycerol containing 16:3 fatty acids (16 carbons, three double bonds) are strongly reduced. This phenotype was not visible in the fatty acid composition of monogalactosyldiacylglycerol of *pgp1*, which had an overall fatty acid composition similar to wild type (data not shown). Furthermore, morphology and growth of *ats1*(*act1*) are similar to wild type, unlike observed for *pgp1* (Fig. 3.1). This phenotypic analysis suggested that *pgp1* was not a new allele of *pho1* or *ats1*(*act1*), but a new locus affecting the biosynthesis of PG as was confirmed by genetic mapping (see below).

The *pgp1* locus maps in the proximity of a putative PGP synthase gene on chromosome 2

To narrow down the search for a candidate gene affected in *pgp1*, genetic mapping of the mutation leading to PG-deficiency in *pgp1* was employed. For this purpose we crossed *pgp1*, which is in the Col-2 genetic background, to a *PGP1* wild-type line in the Ler-0

genetic background. In the F_1 generation, all plants were phenotypically indistinguishable from wild type with regard to PG content based on semiquantitative TLC analysis with an example shown in Fig. 3.2A (top, left two lanes). However, in the F_2 generation of 233 plants screened, 54 (23%) plants were recovered with a reduced amount of PG, as shown for the homozygous pgp1 mutant in Fig. 3.2A, while lipid extracts from all other plants were indistinguishable from wild-type extracts. Without exception, only these 54 plants were also reduced in growth. The F_3 progeny of these plants did no longer segregate suggesting that they represent the homozygous mutant fraction. The nearly 3-to-1 segregation observed for the F_2 plants was in agreement with a single nuclear recessive mutation that is responsible for the PG-deficiency in the *pgp1* mutant. A small mapping population of 42 homozygous *pgp1* mutants derived from this cross was used to map simple sequence polymorphic markers (SSLPs) (Bell and Ecker, 1994) relative to the *pgp1* locus. Only markers on chromosome 2 at approximately 70 cM showed linkage to *pgp1*. Recombination frequencies and calculated map distances for this region are shown in Fig. 3.3A. Two minimally overlapping bacterial artificial chromosomes (BACs) sequenced by "The Arabidopsis Genome Initiative" (2001), one containing the most tightly linked SSLP marker nga168 as derived from "The Arabidopsis Information Resource" web page (www.arabidopsis.org), are shown in Fig. 3.3B. The nucleotide sequence of T16B24 (GenBank AC004697) contains an open reading frame (BAC locus T16B24.7, nucleotides 78,883-80,690; gene At2g3920) with a translated amino acid sequence (Genbank AAC28995) annotated as CDP-diacylglycerol glycerol-3-phosphate 3-phosphatidyltransferase (PGP synthase). The predicted intron-exon structure for this gene is shown in Fig. 3.3C and was later confirmed following the isolation of the cDNA

Figure 3.2. Lipid phenotype of *pgp1* and molecular defect. Sections of the lipid chromatogram (top panel) and the a DNA gel of PCR products (bottom panel) of wild type, the *pgp1* mutant and two independent transgenic *pgp1* lines transformed with the *PGP1* wild-type cDNA (*pgp1/cPGP1*) are shown. Lipids were analyzed by thin layer chromatography and stained by exposure to iodine. Monogalactosyldiacylglycerol (MGDG),serving as loading control, and phosphatidylglycerol (PG) are shown. B, restriction length polymorphism in the *pgp1* mutant locus. Sequence comparison of the wild-type (*PGP1*) and mutant (*pgp1*) locus showing the mutated *Bam*HI site (top). The nucleotide number refers to the GenBank accession number of the BAC clone T16B24 (AC004697). Genomic Southern blot of the wild type and the *pgp1* mutant probed with the *PGP1* gene (bottom). Restriction digests are indicated. Numbers indicate the size (kb) of the fragments marked by arrows.


A
wild type
pgp1
pgp1/cPGP1
pgp1/cPGP1
- MGDG
- PG
- 850 bp
- 526 bp
B
BamHI
PGP1 79,747 GCGTTTGGTGCCTTTTTGGATCCAGTTGCTGATAAGC
pgp1 79,747 GCGTTTGGTGCCTTTTTGGATTCAGTTGCTGATAAGC
BamHI/XbaI
BamHI
WT
pgp1
WT
pgp1
14.5
4.6

(see below). Searching the amino acid sequence (Altschul *et al.*, 1990) of PGP synthase from *E. coli* (GenBank P06978) against all amino acid sequences of Arabidopsis confirmed this open reading frame on chromosome 2 as putative PGP synthase and revealed two additional putative PGP synthase genes of Arabidopsis, one on chromosome 3 (BAC locus T15C9.30, gene At3g55030) and one on chromosome 4 (BAC locus T4B21.19, gene At4g04870).Targeting signal analysis (Emanuelsson *et al.*, 2000) suggested that of all three putative PGP synthases, only the one encoded on chromosome 2 contained a putative chloroplast transit peptide.

The *pgp1* mutant is recognizable by a distinct RFLP in BAC locus T16B24.7

To determine the exact molecular defect in the *pgp1* mutant, a detailed analysis of the presumed PGP synthase locus on chromosome 2 was conducted in the mutant and the wild type. By chance we discovered during the restriction analysis of cloned fragments derived from the mutant that the T16B24.7 equivalent locus in the *pgp1* mutant is missing a BamHI restriction site resulting in a restriction fragment length polymorphism (RFLP). Fig. 3.2B shows a comparison of wild type and *pgp1* genomic DNA restricted with BamHI/XbaI or just BamHI and probed with a 987 bp genomic DNA fragment from the beginning of the open reading frame to the BamHI site present in the wild type (GenBank AC004697, nucleotides 78,777 to 79,764). In both digests the size of the hybridizing fragment is increased in the *pgp1* sample as predicted from the genomic sequence for the loss of a specific BamHI site (GenBank AC004697, nucleotide 79,764). Sequence analysis of the entire coding region of the respective gene isolated by PCR

from wild-type and mutant genomic DNA confirmed a mutation altering the BamHI site (Fig. 3.2B).

Expression of a cDNA derived from wild-type locus T16B24.7 restores PG biosynthesis in the *pgp1* mutant background

To obtain conclusive evidence that the deficiency in PG biosynthesis in the *pgp1* mutant is indeed caused by the observed mutation in the T16B24.7 locus, the respective wild-type cDNA was isolated by RT-PCR and cloned into a T-DNA binary vector. This construct was used to transform *pgp1* mutant plants by vacuum infiltration. Several hygromycin B resistant transformants were isolated and their lipid composition was examined by semiquantitative TLC. Lipid extracts from two independent transformants are shown in Fig. 3.2A (top). Their lipid composition as well as their macroscopic appearance was indistinguishable from wild type. The presence of the transgene construct was confirmed by PCR as shown in Fig. 3.2A (bottom). The genomic sequence gives rise to a diagnostic fragment of 850 bp, while the transgene appears as 525 bp fragment. The intermediate size fragment was isolated and analyzed, but its sequence was unrelated to PGP1 or any of its paralogs (data not shown). Thus, introduction of the wild-type cDNA corresponding to T16B24.7 into the mutant background rescued lipid and morphological phenotypes, thereby confirming that the observed mutant phenotypes are caused by a single mutation in the T16B25.7 locus.

BAC locus T16B24.7 encodes a fully functional PGP synthase in the wild-type but not in the *pgp1* mutant

The mutation in *pgp1* that abolished the BamHI site mentioned above is located in the third exon of the gene (Fig. 3.3C). Accordingly, the predicted protein carries a Pro^{170} to Ser mutation. The alignment of the predicted protein with the two other Arabidopsis paralogs and known bacterial PGP synthases (Fig. 3.4A) revealed that this proline residue is part of a conserved active site motif for CDP-alcohol phosphotransferases (Williams and McMaster, 1998). At the beginning of this study, no experimental data were available for any of the three putative PGP synthase genes of Arabidopsis. To confirm that the wild-type locus T16B24.7 indeed encodes a fully functional PGP synthase, the respective full length wild type cDNA (GenBank AB048535) was isolated as described in the methods section. In addition, the corresponding cDNA from the *pgp1* mutant was cloned by RT-PCR. The 1210 bp long full-length wild-type cDNA encodes a 296 amino acid protein with a molecular mass of 32.2 kD. To test PGP synthase activity for the respective wild-type and mutant proteins, we expressed the cDNA for the predicted mature proteins (with the 93- N-terminal amino acids missing) in the *E. coli* mutant YA5512. This mutant is deficient in PGP synthase activity (Asai et al., 1989) and carries a Thr^{60} to Pro replacement in the coding region of the *pgsA* gene (Usui *et al.,* 1994). As a consequence this strain has very low residual PGP synthase activity leading to PG deficiency. In addition, the content of cardiolipin is very low because PG is a precursor for this lipid. Total lipids extracted from lines of YA5512 transformed with vector control (pQE32), the wild-type cDNA (pQE32-*PGP1*) or mutant cDNA (PQE32-*pgp1*) expression constructs were quantified. Both, wild type and mutant constructs partially

Figure 3.3. The *PGP1* locus on chromosome 2 of Arabidopsis. A, genetic map showing SSLP markers (positions on map), experimentally determined map distances, and recombinant chromosomes/total chromosomes analyzed for each marker. B, BACs from the genome sequencing project and markers. C, structure of the *PGP1* gene (top) and cDNA (bottom). The grey box represents the exon carrying the mutation in *pgp1*. A, poly A tail; ATG , start codon; TAG, stop codon; TP, transit peptide.

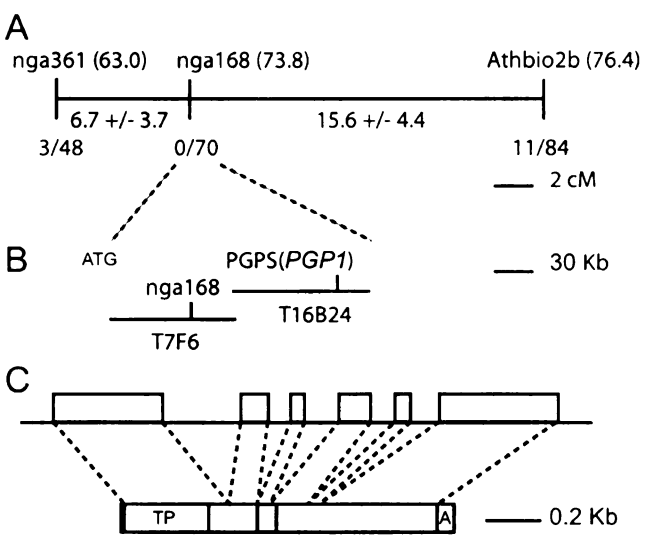
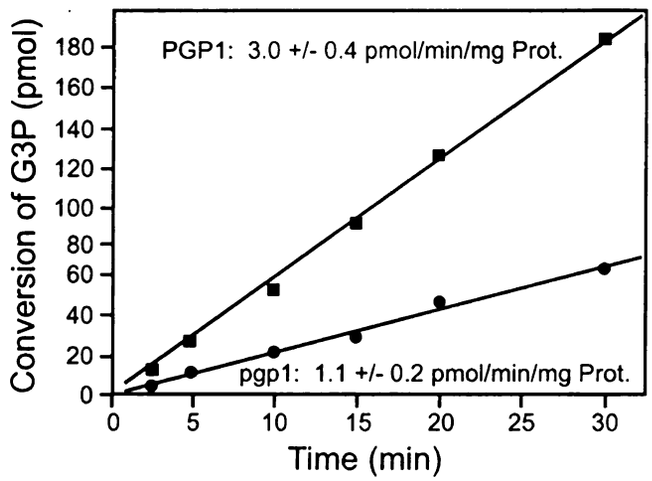

A
nga361 (63.0)
nga168 (73.8)
Athbio2b (76.4)
6.7 +/- 3.7
15.6 +/- 4.4
3/48
0/70
11/84
2 cM
B
ATG
PGPS(PGP1)
30 Kb
nga168
T16B24
T7F6
C
TP
A
0.2 Kb

Figure 3.4. Active site mutation of PGP synthase in the *pgp1* mutant. A, alignment of different Arabidopsis *PGP1* paralogs (*PGP2* and *PGP3*) and bacterial PGP synthase orthologs. GenBank accession numbers (Arabidopsis gene numbers) for the respective protein sequences are: *Arab-PGP1*, AAC28995 (At2g3920); *Arab-PGP2*, CAB82698 (At3g55030); *Arab-PGP3*, CAB80852 (At4g04870); *E. coli*, P06978; *Rhodobacter sph.*, AAC44003; *Bacillus sub.*, I39950; *Synechocystis*, S76208. The arrow indicates the proline (Pro^{170}) to Ser change in the *pgp1* mutant. B, reduced specific activity of the PGP1-S^{170} mutant protein. Total activity in extracts of the *E. coli* YA5512 PGP synthase-deficient mutant expressing wild type *PGP1* cDNA (squares) or mutant *pgp1* cDNA (circles) was determined. The two graphs represent single representative experiments. Equal amounts of protein were used. In addition, the mean specific activity (+/- SE) for three independent PCR constructs is provided. The PGP synthase activities in YA5512 and YA5512 containing pQE32 were not detectable.

A

```
                          ↓
Arab-pgp1           SAFGAFLDSVADKLMVAATLILLCTK
Arab-PGP1           SAFGAFLDPVADKLMVAATLILLCTK
Arab-PGP2           SEFGAFLDPVADKLMVAATLILLCTK
Arab-PGP3           SVVGSYLDPLADKVLIGCVAVAMVQK
E.coli              -RFGAFLDPVADKVLVAIAMVLVTEH
Rhodobacter sph.    SKFGAMLDPIADKAMVVIALVIITGY
Bacillus sub.       TNFGKFLDPLADKLLVSAALIILVQF
Synechocystis       ---GKFLDPLVDKLLVLAPLLILLTW
```

B



restored the lipid composition of the *E. coli* mutant, but the *pgp1* mutant construct to a lesser extent suggesting reduced activity (data not shown). Based on this result it was concluded that *PGP1* encodes a functional PGP synthase. Because mutant and wild proteins are expressed to the same extent in *E. coli* as confirmed by gel electrophoresis of total proteins (data not shown), the result suggested that the activity of the *pgp1* mutant is impaired, but not abolished. This was directly confirmed by enzymatic assays under saturating substrate conditions in the linear range of the assay of crude protein extracts from the two *E. coli* strains as shown in Fig. 3.4B. Three independent clones were tested for each construct and averaged. The specific activity of the mutant protein was reduced to approximately one third of wild type activity. The PGP synthase activity was not detectable in the crude protein extracts from YY5512 or YY5512 hosting the empty vector pQE32 (data not shown).

Plastidic PGP synthase activity is strongly reduced in the *pgp1* mutant

To determine the effect of the P^{170} to S mutation in the pgp1 mutant, PGP-synthase activity was determined in isolated chloroplasts. When extracts from isolated wild-type chloroplasts were incubated with CDP-diacylglycerol and labeled glycerol-3-phosphate in the dark, two major lipids were labeled, PGP and PG, which was presumably derived from PGP due to the action of PGP phosphatase (Fig. 3.5). In chloroplast extracts from the *pgp1* mutant, the incorporation of label into PGP was nearly abolished and the labeling of PG was markedly reduced (Fig. 3.5). In comparison, incorporation of labeled UDP-galactose into galactolipids was not affected in the *pgp1* mutant (Fig. 3.5). The labeling pattern was more complex when the PGP synthase assay

Figure 3.5. Chloroplastic PGP synthase and galactolipid biosynthetic activities in wild type and *pgp1* mutant. Isolated and ruptured chloroplast were either incubated with labeled glycerol-3-phosphate (Gro-3-P) and CDP-diacylglycerol (PGP synthase assay, left two lanes), or with labeled UDP-galactose (galactolipid biosynthesis, right two lanes). Shown is an autoradiograph of a thin layer chromatogram of labeled lipid extracts. Identified lipids were: DGDG, Digalactosyldiacylglycerol; MGDG, monogalactosyl-diacylglycerol; PG, phosphatidylglycerol; PGP, phosphatidylglycerolphosphate.

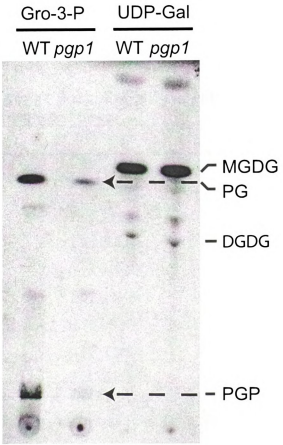

Gro-3-P
UDP-Gal
WT pgp1
WT pgp1
MGDG
PG
DGDG
PGP

Table 3.2 Pigment content (mg g^{-1} Fresh Weight)[a] and chlorophyll fluorescence[b] of wild-type and mutant fully expanded leaves

	WT	*pgp1*
Chlorophyll *a*	1.48 ± 0.08	1.17 ± 0.11
Chlorophyll *b*	0.49 ± 0.03	0.36 ± 0.03
Chlorophyll *a+b*	1.97 ± 0.11	1.55 ± 0.14
Chlorophyll *a/b*	3.01 ± 0.02	3.29 ± 0.07
Carotenoids	0.25 ± 0.02	0.19 ± 0.02
Chlorophyll/Carotenoid	7.77 ± 0.26	7.99 ± 0.27
F_v/F_m (n=25)	0.79 ± 0.02	0.74 ± 0.01
Φ_{PSII} (n=10)	0.55 ± 0.01	0.49 ± 0.01
F_v'/F_m' (n=10)	0.69 ± 0.01	0.60 ± 0.01

[a] Samples were taken from fully expanded leaves of 25-to-30 day old plants harvested after 8 h light exposure. Plants were grown under a 16-h-light/8-h-dark regime at a PPFD of 75 μmol m^{-2} s^{-1} in soil. Pigment values represent means (± SE) of six independent determinations. [b] Data were acquired at a PPFD of 75 μmol $m^{-2}s^{-2}$.

was performed in the light (data not shown). Apparently, fatty acid biosynthesis and de novo lipid biosynthesis are stimulated in the light and lead to the incorporation of label from glycerol-3-phosphate into the diacylglycerol moieties of other lipids. We therefore quantified the PGP synthase activity in dark incubated chloroplasts by determining the amount of label in the total lipid extracts derived from the assay mixture. In the *pgp1* mutant, the chloroplastic PGP synthase activity was reduced to approximately 19% of wild-type activity from 24.4 ± 4.4 μmol glycerol-3-phosphate min^{-1} mg^{-1} chlorophyll to 4.7 ± 1.7 μmol glycerol-3- phosphate min^{-1} mg^{-1} chlorophyll.

Photosynthesis is impaired in the *pgp1* mutant

Although the loss of total PG in leaves of the *pgp1* mutant does not exceed 30%, the growth of the mutant is reduced and the mutant plants appear pale green consistent with an impairment in photosynthesis (Fig. 3.1). This initial observation was confirmed by quantification of the pigment content of fully expanded leaves (Table 3.2). Chlorophyll a and b as well as carotenoid amounts were reduced in the mutant and the ratio of chlorophyll a/b was increased. This result points to an alteration in the structure of the photosynthetic apparatus in the mutant and explains the pale green color of *pgp1* leaves. To investigate the functionality of the photosynthetic apparatus in the *pgp1* mutant, we used non-invasive chlorophyll fluorescence measurements. This technology is well established and was successfully used to investigate photosynthetic competency and light utilization of other Arabidopsis lipid mutants (Härtel *et al.*, 1997; Härtel *et al.*, 1998a; Härtel *et al.*, 1998b). Measurements under steady state conditions at a photosynthetic photon fluence density comparable to that used for the growth of the

plants revealed a marked decrease in the quantum yield of linear electron transport through photosystem II (Φ_{PSII}) and in the efficiency of open photosystem II units (F_v'/F_m') (Table 3.2). A small (approximately 6%), but significant decrease in the maximum photochemical efficiency of photosystem II (F_v/F_m) was observed for the mutant following a 1 h dark adaptation period. For comparison, the initial F_v/F_m ratios in the *ats1*(*act1*) and pho1 mutant were 0.79±0.01 and 0.78±0.02, respectively. Taken together, these alterations in the pigment composition and photosynthetic light energy utilization in the *pgp1* mutant underline the importance of PG for the proper structure and function of photosynthetic membranes in seed plants.

Discussion

The majority of PG in green plant tissues is localized in the chloroplasts and the biosynthesis of PG in green plants has been studied in greatest detail using intact or broken chloroplasts from pea leaves (Andrews and Mudd, 1986). Based on pulse chase labeling experiments, it was proposed that PG biosynthesis proceeds in chloroplasts as worked out for bacteria and yeast. First, CDP-diacylglycerol is formed from CTP and phosphatidic acid through the action of CDP-diacylglycerol synthase (E.C. 3.1.3.4). Plant cDNAs encoding this enzyme have been recently identified and expressed in E. coli (Kopka *et al.*, 1997). Although this reaction is required for PG biosynthesis, it is not specific for this process, because CDP-diacylglycerol also serves as precursor in the biosynthesis of other phospholipids (Kinney, 1993).The second step of PG biosynthesis involves the transfer of glycerol-3-phosphate onto CDP-diayclglyerol giving rise to PGP and CMP. This reaction is catalyzed by PGP synthase (E. C. 2.7.8.5). Third, PGP is hydrolized to PG and inorganic phosphate by PGP phosphatase (E.C. 3.1.3.27). While the latter two reactions had been observed in pea chloroplasts (Andrews and Mudd, 1986), no further biochemical analysis of the respective proteins has been performed. A mutant deficient in one of the two enzymes involved in plastidic PG biosynthesis would serve at least two purposes. First, it would permit the study of PG function in plastid membranes, and second, it would provide experimental evidence that the pathway described above indeed represents the major route of PG biosynthesis in plastids. Based on our analysis, *pgp1* is just such a mutant, because it severely affects plastidic PGP synthase activity. The experimental evidence for this conclusion can be summarized as follows: first, *pgp1* maps within one cM of BAC T16B24.7 annotated as chloroplast targeted PGP synthase;

second, complementation analysis employing a PGP synthase deficient *E. coli* mutant confirmed the gene product of BAC locus T16B24.7 is indeed a PGP synthase; third, introduction of wild type cDNA corresponding to T16B24.7 into the mutant background rescued lipid and morphological phenotypes; forth, a mutation in the third exon of T16B24.7 in *pgp1* gives rise to an RFLP at the DNA level, and at the protein level to a Pro^{170} to Ser substitution in a highly conserved motif characteristic for CDP-alcohol phosphotransferase; and fifth, PGP synthase activity in isolated chloroplasts of *pgp1* is markedly reduced consistent with the other findings. Taken together, these observations provided an unambiguous logical link between the molecular defect and the biochemical phenotype in *pgp1*, as well as a firm gene-product relationship between T16B24.7 and the encoded PGP synthase.

While the Pro^{170} to Ser mutation in *pgp1* affects a highly conserved proline in the active site (Williams and McMaster, 1998), the activity of the recombinant mutant protein was only reduced by approximately two thirds. Thus, the mutation in *pgp1* is "leaky". Despite the fact that the relative amount of PG was only reduced by 30% in the *pgp1* mutant, this biochemical defect led to a visible phenotype. More detailed analysis revealed that pigment composition and photosynthetic light utilization are affected in this mutant, thereby explaining the slow growth and the pale green appearance of *pgp1*. Chlorophyll b is exclusively located in the light-harvesting pigment-protein complexes of photosystem II and photosystem I. Therefore, a decrease of total chlorophyll content associated with an increase in the chlorophyll a/b ratios in the pgp1 mutant (Table 3.2) indicates both, a decrease in the total number of photosystems and a stronger decline in light-harvesting antenna complexes relative to the chlorophyll a-containing reaction

center/core complexes. Chlorophyll fluorescence measurements on intact leaves revealed that the chlorophyll loss was associated with a decline in the quantum efficiency of photosystem II photochemistry. In particular, Φ_{PSII} and F_v'/F_m' were markedly reduced in the mutant. Taken together, the chlorophyll fluorescence and pigment data for the *pgp1* mutant most likely reflect the importance of PG in photosynthesis of seed plants. This is consistent with previous observations on cyanobacterial mutants which are deficient in PG biosynthesis (Hagio *et al.*, 2000; Sato *et al.*, 2000). These cyanobacterial mutants have an absolute requirement for PG supplementation in their medium and deficiencies in photosynthetic competence correlate in severity with the amount of PG in cell membranes, which can be manipulated in this system through external supply. What is surprising about the *pgp1* mutant in comparison to *ats1*(*act1*), is the fact that the latter mutant does not show a striking effect on growth and a less severe effect on photosynthesis in spite of a similar reduction in overall PG content (Kunst *et al.*, 1988). While we currently do not know the basis for this difference, we expect that the detailed analysis of the *pgp1* mutant and a future reexamination of *ats1* (*act1*) mutant may provide clues towards a deeper understanding of PG biosynthesis and function in plant chloroplasts.

While this work was under review, Müller and Frentzen (2001) published the expression of the *PGP1* cDNA in yeast and demonstrated a mitochondrial localization of the enzyme in this heterologous system. Although the data presented here, clearly show that PGP1 is the major chloroplastic isoform of phosphatidylglycerolphosphate synthase, we currently cannot rule out that *PGP1* is also associated with mitochondria in Arabidopsis cells.

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Chapter 4

Anionic lipids are required for chloroplast structure and function in Arabidopsis

The work presented in this chapter is in press:

Bin Yu and Benning. The Plant Journal

Abstract

Photosynthetic membranes o f plants primarily contain non-phosphorous glycolipids. The exception is phosphatidylglycyerol (PG), which is an acidic/anionic phospholipid. A second major anionic lipid in chloroplasts is the sulfolipid sulfoquinovosyldiacylglycerol (SQDG). It is hypothesized that under severe phosphate limitation SQDG substitutes for PG, ensuring a constant proportion of anionic lipids even under adverse conditions. A newly constructed SQDG and PG-deficient double mutant supports this hypothesis. This mutant, *sqd2 pgp1-1*, carries a T-DNA insertion in the structural gene for SQDG synthase (SQD2) and a point mutation in the structural gene for phosphatidylglycerolphosphate synthase (PGP1). In the *sqd2 pgp1-1* double mutant the fraction of total anionic lipids is reduced by approximately one third resulting in pale yellow cotyledons and leaves with reduced chlorophyll content. Photoautotrophic growth of the double mutant is severely compromised and its photosynthetic capacity is impaired. In particular, photosynthetic electron transfer at the level of photosystem II is affected. Besides these physiological changes, the mutant shows altered leaf structure, a reduced number of mesophyll cells and ultrastructural change of the chloroplasts. All observations on the *sqd2 pgp1-1* mutant lead to the conclusion that the total content of anionic thylakoid lipids is limiting for chloroplast structure and function, and is critical for overall photoautotrophic growth and plant development.

Introduction

The two major anionic/acidic lipids of photosynthetic membranes are phosphatidyl-glycerol (PG) and the non-phosphorous glycolipid sulfoquinovosyldiacylglycerol (SQDG). The phospholipid PG is common in the biological membranes of animals, plants and microorganisms and was found to be essential in photosynthetic organisms (Hagio *et al.*, 2000; Sato *et al.*, 2000; Hagio *et al.*, 2002; Babiychuk *et al.*, 2003). *In vitro* experiments showed that PG is enriched in light-harvesting pigment-protein complexes of photosystem II (PSII) (Murata *et al.*, 1990; Tremolieres *et al.*, 1994) and that it is essential for the dimerization of the PSII reaction center core pigment-protein complex (Kruse *et al.*, 2000). Furthermore, the recently published crystal structure of photosystem I contained three molecules of PG in tight coordination with the core and the antenna complex (Jordan *et al.*, 2001). A PG-deficient mutant of the cyanobacterium *Synechocystis* sp. PCC6803 is no longer able to grow photoheterotrophically and PSII activity was severely compromised (Hagio *et al.*, 2000; Sato *et al.*, 2000). In addition, a pale green mutant of Arabidopsis, *pgp1-1*, carrying a leaky mutation in the gene for phosphatidylglycerolphosphate synthase (PGP1) was recently described (Xu *et al.*, 2002). In this mutant, a reduction in the content of PG of only 30% resulted in the impairment of photosynthesis. Moreover, complete inactivation of the *PGP1* gene by T-DNA insertion led to the abolishment of chloroplasts and loss of photoautotrophy (Hagio *et al.*, 2002; Babiychuk *et al.*, 2003). These results suggested that PG was essential for chloroplast development and function in seeds plants.

The second anionic lipid found in thylakoid membranes, the sulfolipid SQDG, is not essential under normal growth conditions. Mutants of photosynthetic bacteria and Arabidopsis completely lacking this lipid showed only subtle impairments in photosynthesis and growth unless they are phosphate starved (Benning *et al.*, 1993; Güler *et al.*, 1996; Yu *et al.*, 2002). As a common phenomenon in photosynthetic organisms, the relative amount of total anionic thylakoid lipids is maintained by reciprocally adjusting SQDG and PG contents as phosphate availability changes. Typically the relative content of PG decreases and that of SQDG increases following phosphate limitation. In SQDG-deficient mutants, the proportion of PG does not decrease under phosphate-limitation and these mutants become phosphate starved sooner than the respective wild types. Based on these observations, it was suggested that one of the main function for SQDG is to substitute for PG under phosphate limitation to maintain the proper balance of anionic charge in the thylakoid membrane (Benning *et al.*, 1993; Güler *et al.*, 1996; Yu *et al.*, 2002). To further test this SQDG/PG substitution hypothesis by endogenously manipulating the total amount of anionic/acidic thylakoid lipids, we constructed an *sqd2 pgp1-1* double mutant of Arabidopsis lacking SQDG completely and PG partially. The *sqd2* mutant carries a T-DNA insertion into the sulfolipid synthase gene *SQD2* (Yu *et al.*, 2002). The weak allele, *pgp1-1*, for the PGP synthase gene *PGP1* was preferred in this study, because plants carrying stronger *pgp1* homozygous alleles are already completely non-photosynthetic (Hagio *et al.*, 2002; Babiychuk *et al.*, 2003) and additive effects of SQDG-deficiency cannot be revealed. Here we describe the construction and characterization of the *sqd2 pgp1-1* double mutant.

Materials and methods

Plant material, growth experiments and DCMU treatment

Arabidopsis wild-type and mutants were grown under a photosynthetic photon flux density (PPFD) of 70-80 :mol $m^{-2}s^{-1}$ at 22/18°C(day/night) with a 14-h light/10-h dark period. Surface-sterilized seeds were germinated on 0.8% (w/v) agar-solidified Murashige and Skoog (MS) medium (Murashige *et al.*, 1962) supplemented with 1% sucrose. Seedlings were grown for 8 days on agar before transfer to soil or plates containing Arabidopsis medium (half strength without sucrose) (Estelle *et al.*, 1987). For phosphate limitation experiments, KH_2PO_4 was omitted from the medium and substituted by MES buffer (Härtel *et al.*, 2000). Quantitative growth experiments were done as described (Yu *et al.*, 2002). Briefly, plants were grown under a uniform PPFD of 80 :mol $m^{-2} s^{-1}$. At each time point 7-to-10 plants per line were harvested and their aerial parts were weighed. For DCMU treatment, half strength Arabidopsis medium without sucrose was used with addition of different concentrations of DCMU as indicated. Plants were grown on MS medium for 8 days and then transferred to the DCMU containing agar plates. After 7 days of growth the fresh weight of 4-to-5 plants was averaged and relative growth was plotted as the ratio of the fresh weight per plant of DCMU untreated-to-DCMU treated samples.

Construction of the *sqd2 pgp1-1* double mutant

To generate the *sqd2 pgp1-1* double mutant, *sqd2* (Yu *et al.*, 2002) was used as the pollen donor in a cross to *pgp1-1* (Xu *et al.*, 2002). The ecotypes were Wassilewskija for

the *sqd2* mutant and Columbia-2 for *pgp1-1*. Nearly isogenic lines were generated by inbreeding through 8 generations. Following each round of selfing, seeds of 20 individual plants were collected, 100-200 seeds per plant were plated and the segregation of young seedlings was phenotypically scored. This was accomplished by visual examination of the plants, as homozygous *pgp1-1* seedlings, double homozygous mutants and wild type were clearly distinguishable from each other and other genotypes based on their tint and size (Fig. 4.1). Descendants from a double heterozygous line were analyzed again in the next generation until the four required lines were recovered from a single F_7 double heterozygous plant.

Analysis of lipids from leaves and chloroplasts

Leaves were harvested and frozen immediately in liquid nitrogen, and lipids were extracted as previously described (Dörmann *et al.*, 1995). Lipid extracts were analyzed on activated, ammonium sulfate-impregnated silica gel TLC plates (Härtel *et al.*, 2000) using running solvent of acetone/toluene/water (93:30:8 v/v/v). Lipids were visualized with iodine vapor and identified by cochromatography with lipid extracts of known composition. For quantitative analysis, methylesters were prepared and quantified by GLC (Rossak *et al.*, 1997). For chloroplast lipid analysis, plastids were isolated as described (Härtel *et al.*, 2000). The chloroplasts were immediately resuspended in 200 :l chloroform/methanol/formic acid (10:10:1, v/v/v), followed by the addition of 100 :l 0.2 M H_3PO_4 and 1 M KCl. In all quantitative experiments Student's *t*-test was used to assess the significance of differences between samples.

Transmission electron microscopy and light microscopy

Three leaves from each line were prepared for light and transmission electron microscop-y. The leaves were fixed with 2.5% (v/v) glutaraldehyde and 2% paraformaldehyde (v/v) in 0.1 M sodium phosphate buffer (PH 7.4) by infiltration under vacuum and continually incubated at ambient pressure and 4 °C for 24 h. The samples were post fixed with1% osmium tetroxide in the same buffer for 2 h at room temperature. This was followed by a serial dehydration with 50%, 70%, 80%, 88%, 95%, and 100% (v/v) of acetone in water. The specimens were infiltrated with a series of 33%, 50%, 75%, and 100% (v/v) of poly/bed resin (Polyscience Inc., Warrington, PA) in acetone. After embedding in poly/bed resin, three blocks each of wild-type and mutants were sectioned and the sections stained with 1% toluidine blue and examined with a Zeiss Pascal confocal laser scanning microscope (Carl Zeiss, Germany). The thin sections were stained with 2% uranyl acetate and lead citrate before viewing in a JEOL 100CX transmission electron microscope (JEOL Inc., Peabody, MA).

ChlorophyII Fluorescence and pigments measurements

Pigments were determined as described (Lichtenthaler, 1987). In *vivo* chlorophyll fluorescence at room temperature was determined with an FMS2 fluorometer (Hansatech Instruments, Pentney King's Lynn, UK) as previously described (Härtel *et al.*, 1998). Fluorescence parameters used were as defined by Genty et al., and van Koten and Snel (Genty *et al.*, 1989; van Kooten *et al.*, 1990).

Results

Growth of the *sqd2 pgp1-1* double mutant is reduced

The *sqd2 pgp1-1* double mutant was constructed by crossing the two respective single mutant lines (Yu *et al.*, 2002; *Xu et al.*, 2002). These lines were of different ecotypes (*sqd2*, Wassilewskija and *pgp1-1*, Columbia-2), a fact which might have posed a serious problem for the interpretation of comparative data as discussed below. To create nearly isogenic lines for the four genotypes compared in this study, *sqd2/sqd2 PGP1/PGP1* (*sqd2* mutant), *SQD2/SQD2 pgp1-1/pgp1-1* (*pgp1-1* mutant), *SQD2/SQD2 PGP1/PGP1* (wild type), and *sqd2/sqd2 pgp1-1/pgp1-1* (homozygous double mutant), a recombinant inbred line was generated by repeated selfing of single double heterozygous descendants from the original cross through 8 generations. According to theory (Reiter *et al.*, 1992), the four different homozygous lines isolated in the F_8 generation should be nearly (99.2%) isogenic, eliminating effects from genetic variations at loci different from those under direct consideration. In agreement with recessive mutations, the F_1 progeny were phenotypically wild-type. Thin-layer chromatography (TLC) of lipid extracts from 315 segregating F_2 plants showed that 64 (approximately 3/16) had a reduced proportion of PG (homozygous for *pgp1-1*), 60 were SQDG-deficient (homozygous for *sqd2*), and 20 (approximately1/16) lacked SQDG and showed a reduced proportion of PG. These latter were the expected homozygous *sqd2 pgp1-1* double mutants. The cotyledons and true leaves of *sqd2 pgp1-1* double mutants were paler green than those of the *pgp1-1* mutants as shown in Figure 4.1. The true leaves of the double mutant were smaller

Figure 4.1. Appearance and growth of wild-type and mutant lines. (a), six week-old plants grown in soil under standard conditions; (b), plants grown for 8 d on agar-solidified MS medium with 1% (w/v) sucrose followed by 12 d of growth on a half concentration Arabidopsis medium without sucrose. (c), close up of leaves from plants shown in (a); (d), seedlings 2-3 days after germination grown on agar-solidified MS medium with 1% (w/v) sucrose.

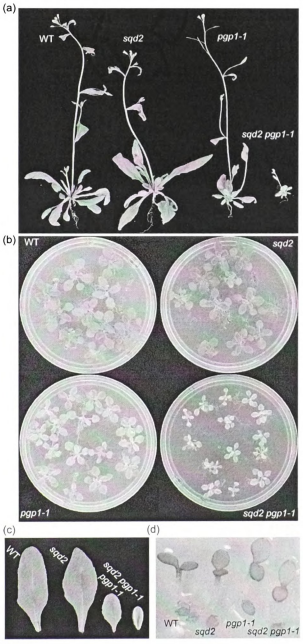
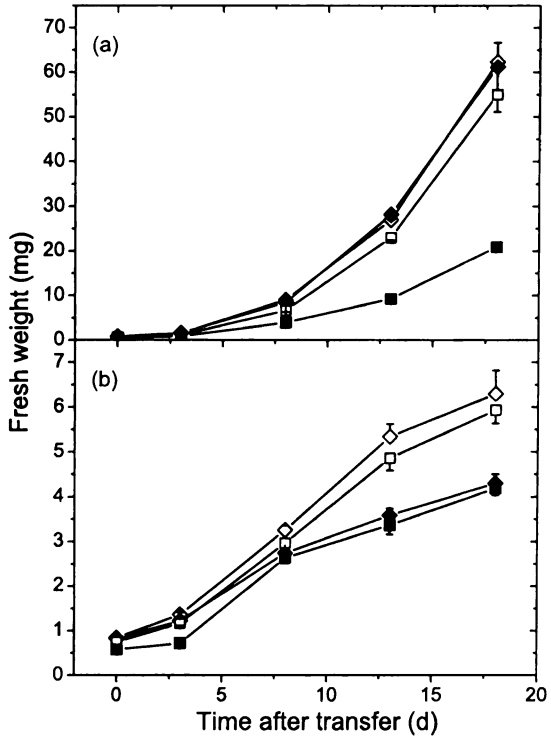

(a)
WT
sqd2
pgp1-1
sqd2 pgp1-1
(b)
WT
sqd2
pgp1-1
sqd2 pgp1-1
(c)
WT
sqd2
sqd2 pgp1-1
pgp1-1
(d)
WT
sqd2
pgp1-1
sqd2 pgp1-1

Figure 4.2. Growth curves for wild-type (open diamond), ***sqd2*** (filled diamond)**, *pgp1-1*** (open square), and ***sqd2 pgp1-1*** (filled square) lines on agar-solidified half-strength Arabidopsis medium with 1mM(a) or no (b) phosphate added. At each time point, the aerial part fresh weights of at least 7 plants were determined and averaged (±SE).

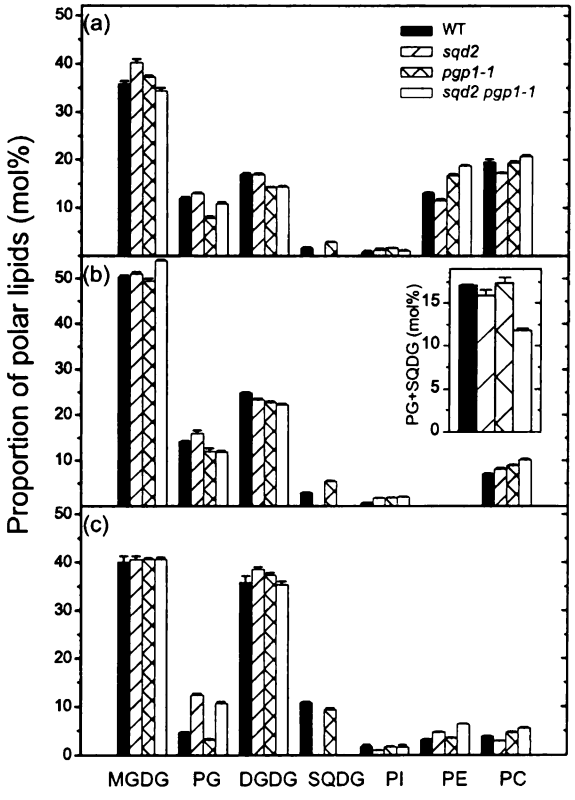

(a)
(b)
Fresh weight (mg)
Time after transfer (d)
0
10
20
30
40
50
60
70
1
2
3
4
5
6
7
0
5
10
15
20

and its growth rate was reduced. As shown in Figure 4.2a, the fresh weight of the aerial part of the double mutant was reduced to approximately one third compared to the other lines at three weeks of growth on phosphate-replete medium. The double mutant was able to survive on soil and on agar-solidified medium lacking sucrose, but the growth rate was greatly reduced compared to plants grown in the presence of sucrose (data not shown). These results suggested that the *sqd2 pgp1-1* double mutant is capable of photoautotrophic growth, but its growth is severely limited by a compromised photosynthetic apparatus. Under phosphate-limited conditions, the growth of the double mutant was reduced compared to the wild-type and the *pgp1* mutant, but to nearly the same extent as observed for the *sqd2* mutant (Fig. 4.2b).

The proportion of total anionic lipids in the double mutant is decreased

Total leaf lipid analysis of the *sqd2 pgp1-1* double mutant as shown in Figure 4.3a revealed that the relative proportion of PG was approximately 11 mol%, higher than in the *pgp1-1* mutant. Because PG is also found in extraplastidic membranes, this result might reflect a decrease in the absolute amount of chloroplast membranes. Indeed, the relative proportion of the exclusively extraplastidic lipid phosphatidylethanolamine (PE), was also increased in the double mutant compared to the *pgp1-1* mutant, in agreement with an overall increase in the ratio of extraplastidic-to-plastidic membrane lipids. Comparing the lipid composition of isolated chloroplasts from the four different lines as shown in Figure 4.3b, a similar decrease in the relative amount of PG was observed for the *pgp1-1* mutant and the *sqd2 pgp1-1* double mutant corroborating the

Figure 4.3. Quantitative analysis of polar lipids. Lipids were extracted from leaves (a), or isolated chloroplast (b). Plants in (a) and (b) were grown on medium with 1mM phosphate. Leaf lipid extracts of plants grown on medium without phosphate are shown in (c). Values represent means (±SE) of three independent measurements. DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; SQDG, sulfoquinovosyldiacylglycerol.


(a)
(b)
(c)
WT
sqd2
pgp1-1
sqd2 pgp1-1
Proportion of polar lipids (mol%)
PG+SQDG (mol%)
MGDG
PG
DGDG
SQDG
PI
PE
PC

hypothesis that the ratio of extraplastidic-to-plastid lipids is increased in the double mutant. Summing the amount of the anionic lipids SQDG and PG in chloroplast extracts (Figure 4.3b, inset), it was apparent that the total amount of anionic lipids in the two single mutants remained similar to that of wild type, but was reduced in the *sqd2 pgp1-1* double mutant by approximately one third (99% confidence level based on Student's *t*-test). In the *pgp1-1* mutant the relative increase in SQDG and in the *sqd2* single mutant the relative increase in PG appeared to reciprocally compensate the respective deficiency in the alternate anionic lipid. These compensatory mechanisms were also visible when total leaf lipid extracts were examined following phosphate-limited growth (Figure 4.3c). The relative amount of PG in the two SQDG-deficient lines was similarly maintained under these conditions, explaining the similar growth reduction of the *sqd2* and *sqd2 pgp1-1* double mutant following phosphate deprivation (Figure 4.2b). In addition to these changesin relative anionic lipid content, the proportion of the galactolipid DGDG increased following phosphate limitation as was previously observed for Arabidopsis (Härtel *et al.*, 2000).

Photosynthetic activity is compromised in the double mutant

The pale green leaves of the *sqd2 pgp1-1* double mutant suggested a reduction in chlorophyll content and a possible impairment in photosynthetic activity. Indeed, the amounts of chlorophylls a and b, and carotenoids were decreased in the double mutant to approximately one third of that observed for wild type and *sqd2* mutant, and to one half of that of the *pgp1-1* mutant (Table 4.1). The chlorophyll a/b ratio was higher than for wild type and *sqd2*, but was similar to that of the *pgp1-1* mutant. Taken together, these

Table 4.1 Pigment content (mg g^{-1} fresh weight)* of wild-type and mutant lines

WT		*sqd2*	*pgp1-1*	*sqd2 pgp1-1*
chlorophyll *a*	0.86 ± 0.04	0.99 ± 0.07	0.61 ± 0.07	0.32 ± 0.03
chlorophyll *b*	0.29 ± 0.01	0.36 ± 0.02	0.18 ± 0.02	0.09 ± 0.01
chlorophyll *a+b*	1.14 ± 0.05	1.34 ± 0.1	0.80 ± 0.08	0.42 ± 0.04
chlorophyll *a/b*	2.98 ± 0.03	2.76 ± 0.03	3.3 ± 0.1	3.54 ± 0.16
Carotenoids	0.25 ± 0.01	0.27 ± 0.02	0.18 ± 0.02	0.10 ± 0.08

*Samples were taken from fully expanded leaves of 20-30 day-old plants grown on agar-solidified medium containing 1 mM phosphate. Pigment values represent means (± SE) of at least four independent determinations.

results suggested that the ratio of light-harvesting antenna-to-reaction core complex was similarly altered in the *pgp1-1* single and the *sqd2 pgp1-1* double mutant, but that the total amount of pigment protein complexes and therefore the extent of total thylakoid membrane in the double mutant was strongly reduced. Photosynthesis in plants of the four lines included in this study was investigated in greater detail using pulse amplitude-modulated chlorophyll fluorescence analysis, a sensitive and non-invasive method (Krause *et al.*, 1991). This method provides an indication of the overall photosynthetic competence of leaves and indirectly permits conclusions with regard to the electron-transport reactions at the thylakoid membranes. As shown in Figure 4.4a and b, there was no obvious difference in the maximum intrinsic efficiency of PSII in the light adapted state (F_V'/F_M') over a wide range of photosynthetic photon flux densities (PPFD), but the quantum yield of linear electron transfer through PSII (Φ_{PSII}) was reduced in the double mutant (95% confidence level based on *t*-test), especially in the low PPFD range as compared to wild type, *sqd2*, and *pgp1-1*. The deduced fluorescence parameter $1-q_P$, which reflects the fraction of "open" PSII reaction center provides an indication of the reduction state of the primary electron acceptor of PSII, Q_A. This parameter was increased in the *sqd2 pgp1-1* double mutant (99% confidence level), while no obvious differences were visible for the wild type and the two single mutant lines *sqd2* and *pgp1-1*. Therefore, it seemed likely that PSII is more reduced in the *sqd2 pgp1-1* double mutant in accordance with a decreased quantum yield of PSII (Φ_{PSII}). To verify this hypothesis using an independent approach, we tested the effect of 3 (3, 4-dichlorophenyl)-1, 1-dimethylurea (DCMU) on the growth of plants. This herbicide binds at the secondary electron acceptor (Q_B) site of PSII, and blocks the electron transfer from Q_A to Q_B

Figure 4.4. Light dependence of steady-state chlorophyll fluorescence in fully expanded leaves of the wild-type (open diamond), ***sqd2*** (filled diamond), ***pgp1-1*** (open square), and ***sqd2 pgp1-1*** (filled square). (a), Φ_{PSII}, effective quantum yield of linear electron transport through PSII calculated as $(F_m'-F_s')/F_m'$; (b) F_v'/F_m', maximum photochemical efficiency of PSII calculated as $(F_m'-F_o')/F_m'$; (c), Q_A photoreduction state $(1-q_p)$ estimated as $1-(F_s'-F_o')/(F_m'-F_o')$. Values represent means (±SE) of 6 independent determinations.

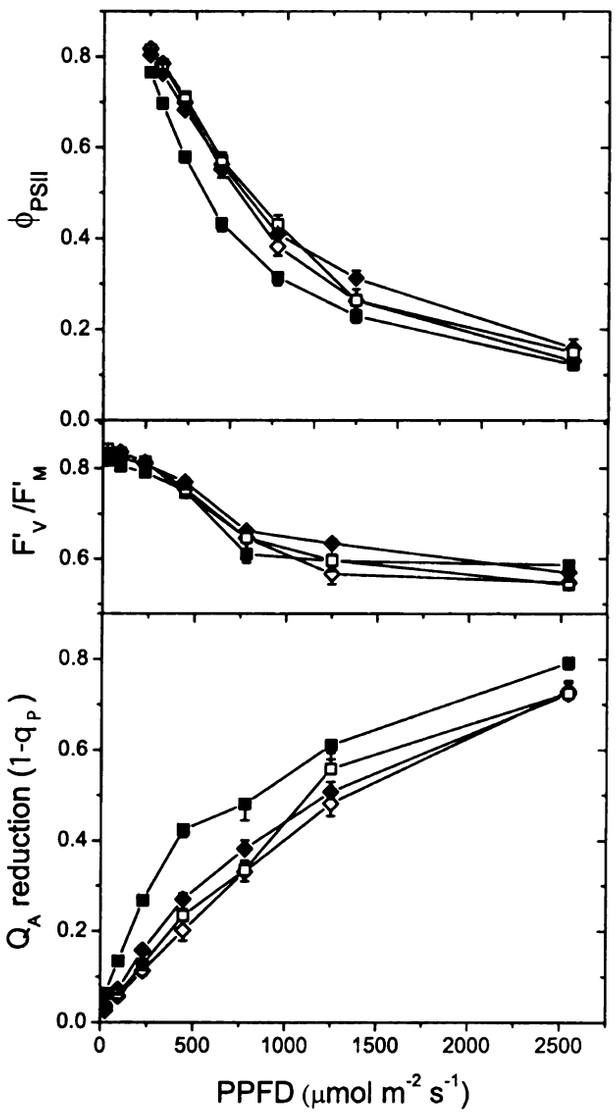
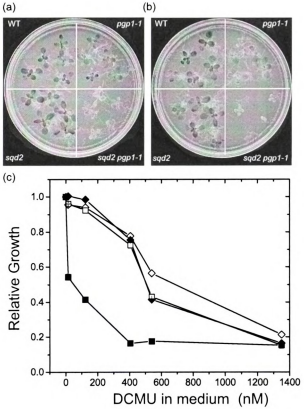

0.8
0.6
0.4
0.2
0.0
ϕ_{PSII}
0.8
0.6
F'_v/F'_M
0.8
0.6
0.4
0.2
0.0
Q_A reduction $(1-q_p)$
0
500
1000
1500
2000
2500
PPFD (μmol m^{-2} s^{-1})

Figure 4.5. Sensitivity to DCMU of wild-type and mutant lines. Plants were grown for 12 days on MS medium followed by 7 days on plates with half strength Arabidopsis medium containing the herbicide; (a) no DCMU; (b) 121 nm DCMU. (c), relative growth of plants on agar with different DCMU concentrations. Relative growth was calculated as fresh weight of the aerial part of plants at a specific DCMU concentration divided by fresh weight of the aerial part of plants grown in the absence of DCMU. Wild type (open diamond), *sqd2* (filled diamond), *pgp1-1* (open square), and *sqd2 pgp1-1* (filled square).


(a)
(b)
WT
pgp1-1
sqd2
sqd2 pgp1-1
WT
pgp1-1
sqd2
sqd2 pgp1-1
(c)
Relative Growth
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
1000
1200
1400
DCMU in medium (nM)

(Oettmeier *et al.*, 1983). As shown in Figure 4.5, the *sqd2 pgp1-1* double mutant was considerably more sensitive to DCMU than the two single mutant lines and the wild type in agreement with an increased reduction pressure at the Q_A site of PSII in the double mutant. After one week in the presence of 13.5 nM DCMU, the relative growth of the double mutant was reduced to approximately 50% (99% confidence level), while the other three lines were still growing normally. A concentration of 405 nM DCMU led to a complete cessation of growth of the *sqd2 pgp1-1* double mutant. At even higher concentrations of DCMU, the single mutants also showed increased sensitivity to the herbicide compared to the wild type, suggesting that complete SQDG deficiency or a decrease of the PG content by 30% were sufficient to cause subtle changes in PS II activity, however, with no apparent effect on growth under normal laboratory conditions (Figure 4.2a).

Leaf structure and chloroplast ultrastructure are altered in the double mutant

The biochemical analysis of the *sqd2 pgp1-1* double mutant described above provided indirect evidence for an increased ratio of extraplastidic-to-plastidic lipids. To corroborate this hypothesis in more direct ways, the leaf structure of the different lines was compared by light microscopy of thick sections (Figure 4.6). The *sqd2 pgp1-1* double mutant showed larger intercellular spaces and a reduction in mesophyll cell numbers. The epidermal cells and mesophyll cells were enlarged. Mesophyll cells with chloroplasts were only present adjacent to vascular structures. Chloroplast numbers per cell cross section were reduced in the *sqd2 pgp1-1* double mutant to approximately 50% of wild type. Averaging at least 30 sections per sample, numbers of chloroplasts per cell cross

Figure 4. 6. Light microscopy of the leaf sections of wild-type (a), ***sqd2*** (b), ***pgp1-1*** (c) and ***sqd2 pgp1-1*** (d). Bar = 100 :m. Sections of the first true leaves of two week-old plants are shown. The sections were stained with 1% toluidine blue

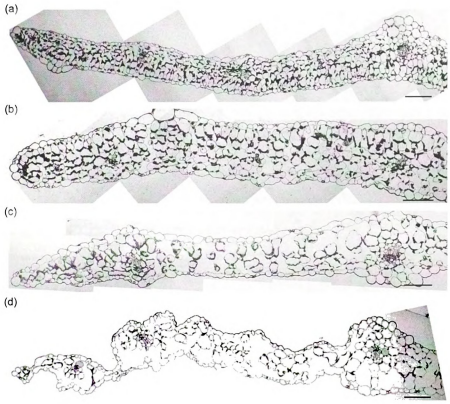
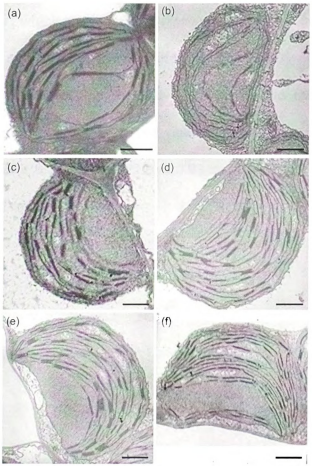

(a)
(b)
(c)
(d)

Figure 4.7. Ultrastructure of chloroplasts from mesophyll cells. Wild type (a), and *sqd2 pgp1-1* (b) from emerging (youngest true) leaves. Wild type (c), *sqd2* (d), *pgp1-1* (e), *sqd2 pgp1-1* (f) from first true leaves. The plants were grown for two weeks in soil. Bar=1μm.


(a)
(b)
(c)
(d)
(e)
(f)

section ± SD were for wild type, 8.0 ± 0.3; *sqd2*, 7.9 ± 0.2; *pgp1-1*, 8.0 ± 0.3, and *sqd2 pgp1-1*, 3.9 ± 0.3. The ultrastructures of chloroplasts in emerging (not yet expanded) leaves of the wild type and the double mutant are shown in Figure 4.7a and b. The wild-type chloroplasts contained well developed thylakoid membranes contrary to the double mutant chloroplasts , which nearly lacked grana stacks. Figure 4.7 panels c, d and e depict chloroplasts of the expanded first true leaves from wild-type, *sqd2,* and *pgp1-1*. Chloroplasts in these samples were generally indistinguishable. However, in chloroplasts of the double mutant, the thickness of the thylakoid grana stacks (Figure 4.7f) appeared to be reduced(WT, 0.22 ± 0.03 :m; *sqd2*, 0.20 ± 0.03 :m; *pgp1-1*, 0.21 ± 0.03 :m; *sqd2 pgp1-1*, 0.11 ± 0.02 :m; n=25).

Discussion

The analysis of genetic mutants with biochemical defects provides a powerful tool to understand the function of the affected pathway and the molecule in question. As more biochemical mutants of Arabidopsis become available, opportunities arise to combine these mutants in single lines, thereby revealing the interaction of two and more pathways and compounds. Combining mutations in different pathways in a single line might reveal redundancies and limit the plant's adaptive responses. An example is the *dgd1 act1* double mutant, which is unable to grow photoautotrophically, while the parental mutants are much less affected in their growth (Dörmann *et al.*, 1999). The phenotype was interpreted as a reduction of flux through the endoplasmic reticulum and plastid-based pathways of thylakoid lipid biosynthesis, which are partially redundant in Arabidopsis (Klaus *et al.*, 2002).

Plants are well known for their broad biochemical repertoire enabling them to adapt to and survive under adverse environmental conditions. A mutation in a single biochemical pathway of mostly conditional importance might present no phenotype at all, or might have only mild effects on the development or physiology of the plant under standard laboratory conditions. Sulfolipid-deficient mutants are a particularly good example. When the first sulfolipid-deficient mutants of bacteria became available, their growth was generally not affected by the mutation under optimal growth conditions (Benning *et al.*, 1993; Güler *et al.*, 1996). In the case of the cyanobacterium *Synechococcus* sp. PCC7942, subtle changes in PSII photochemistry were observed. However, these changes were not growth-limiting (Güler *et al.*, 2000). Arabidopsis completely lacking sulfolipid also did not show a growth impairment under standard

laboratory growth conditions (Yu *et al.*, 2002), which may not necessarily duplicate conditions encountered by wild plants in nature. However, when additional metabolic pressure was applied to sulfolipid-deficient mutants, they began to lag behind wild type in growth. Phosphate-limitation brings about a metabolic impasse, to which bacteria and plants respond by decreasing the ratio of phosphorous-to-non-phosphorous lipids (Benning *et al.*, 1995; Härtel *et al.*, 2000). Sulfolipid-deficiency limits the ability of photosynthetic organisms to remodel their thylakoid membrane, partially replacing PG with SQDG. This inability to adjust was proposed as the primary cause of reduced growth of sulfolipid-deficient mutants under phosphate limitation (Benning *et al.*, 1993; Güler *et al.*, 1996; Yu *et al.*, 2002).

A different independent way of demonstrating the conditional importance of sulfolipid is to study SQDG-deficiency in a double mutant impaired also in the biosynthesis of PG. Two issues had to be addressed for the construction of such a double mutant and the corresponding control lines. First, severe PG-deficiency leads to a complete lack of photosynthesis making it impossible to study the more subtle effects of SQDG in such a line (Hagio *et al.*, 2002). This issue was overcome by choosing the weak allele, *pgp1-1*, which causes only a 30% reduction in PG content. However, the choice of this allele posed a second problem, namely that the sulfolipid-deficient mutant of Arabidospis, *sqd2*, carrying a null allele caused by T-DNA insertion was of a different ecotype. Because the conclusions of this study were expected to be based on subtle differences between the double mutant, the two single mutants, and the wild type, it was prudent to generate nearly isogenic lines by inbreeding through 8 generations. This

strategy resulted in four strains with the required homozygous *sqd2* or *pgp1-1* alleles in a 99.2% identical genetic background in all four lines.

The *sqd2 pgp1-1* double mutant analysis demonstrated that SQDG-deficiency can be growth-limiting if the PG-based compensatory mechanism is eliminated. The two biochemical defects in this mutant caused a reduction in the overall anionic thylakoid lipid content. This was not the case for the single mutants, which compensate by adjusting the amount of the alternate anionic lipid (Fig. 4.3b). It is proposed that the overall reduction in anionic lipids limits the development of fully functional photosynthetic membranes, and therefore photoautotrophic growth. Different independent phenotypes of the double mutant were consistent with a limitation in thylakoid membrane lipid biosynthesis leading to an increase in the ratio of extraplastidic-to-plastidic lipids in the *sqd2 pgp1-1* double mutant: 1. The relative proportions of PE, which exclusively occurs in extraplastidic membranes, and of PG present in plastidic and extraplastidic membranes were increased (Fig. 4.3); 2. Pigments exclusively associated with the photosynthetic membranes were reduced on a fresh weight basis (Tab.4. 1), and most directly, mesophyll cell numbers, chloroplasts per cell and the amount of thylakoid membranes within chloroplasts were reduced (Fig. 4.6, 4.7).

Beyond the inability of the *sqd2 pgp1-1* double mutant to assemble thylakoid membranes at a rate required for normal growth, lack of SQDG and reduction in PG appeared to have also more direct effects on the photosynthetic apparatus. The double mutant clearly showed altered chlorophyll fluorescence consistent with a reduced electron transport rate through PSII in the double mutant, as compared to the single mutants or the wild type (Fig. 4.4). This effect was further aggravated by additions of the

herbicide DCMU, which blocks the electron transfer from PSII to the Q_B plastoquinone acceptor (Oettmeier *et al.*, 1983). Contrary to the control lines, growth of the double mutant was 3 orders of magnitude more sensitive to DCMU (Fig. 4.5). Studying a sulfolipid-deficient mutant of *Chlamydomonas reinhardtii*, Sato et al. and Minoda et al., (Minoda *et al.*, 2002; Sato *et al.*, 2003) have observed that this mutant is sensitive to DCMU and shows other signs of photosynthetic impairment. In the case of Arabidopsis as studied here, there is only a slight increase in sensitivity towards DCMU in the SQDG-deficient and PG-deficient mutants suggesting that the sensitivity of the double mutant is not specifically due to SQDG-deficiency, but a consequence of the reduction of anionic lipids in general. The alga *C. reinhardtii* contains a relatively high proportion (~12 mol%)of SQDG(Sato *et al.*, 1995), compared to Arabidopsis (~3 mol%), and SQDG-deficiency may sufficiently reduce the amount of anionic thylakoid lipids to cause an impairment in photosynthesis by itself. This effect became more aggravated at elevated temperature (Sato *et al.*, 2003) suggesting structural roles for SQDG in association with pigment protein complexes of the photosynthetic membrane. In general, the findings made with the *C. reinhardtii* mutant and the Arabidopsis *sqd2 pgp1-1* double mutant agree, suggesting a role of SQDG as an anionic lipid in the assembly and proper function of the photosynthetic membrane. In the case of Arabidopsis, which contains only small amounts of SQDG, it takes the additional reduction in PG content to clearly bring about the effects of sulfolipid deficiency under normal growth conditions. However, under phosphate-limited growth conditions, when the relative amount of SQDG is increased in the wild type, the inability to synthesize SQDG as a substitute for PG quickly becomes limiting for growth in an SQDG-deficient mutant. At first glance it seemed puzzling that

under phosphate-limited conditions, the *sqd2 pgp1-1* double mutant showed no additional reduction in growth in comparison to *sqd2* (Fig. 4.2). Upon closer examination of the lipid composition in the double mutant and *sqd2* (Fig. 4.3), it is apparent that the relative PG content in these two lines under phosphate limitation was similar, and therefore no further reduction of anionic lipids in the double mutant occurred. Presumably, the PG-biosynthetic activity provided by the enzyme encoded by the *pgp1-1* allele was sufficient under these conditions to keep up with the demand of PG biosynthesis.

Whatever the specific biochemical or molecular roles that anionic lipids might fill in the photosynthetic membrane, i.e. providing a proton conducting pathway at the surface of the thylakoid membrane (Haines, 1983), or as boundary lipids and integral components of photosynthetic complexes (Barber *et al.*, 1986), the double mutant analysis described here led to the conclusion that the total amount of the anionic lipids PG and SQDG is most critical. These findings also support the general concept of substituting one anionic lipid for another under phosphate-limited conditions. While SQDG and PG both play an important role in this adaptive mechanism to maintain the proper balance of anionic lipids in the thylakoid membrane, PG might have additional and more specific functions, as the greater impairment of the PG-deficient single mutants suggests (Hagio *et al.*, 2002; Babiychuk *et al.*, 2003).

Acknowledgments

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Chapter 5

Loss of plastidic lysophosphatidic acid acyltransferase causes embryo-lethality in Arabidopsis

The work presented here has been submitted to The plant Journal:

Bin Yu, Setsuko Wakao, Jilian Fan, Christoph Benning (2003)

Abstract

Phosphatidic acid is a key intermediate for chloroplast membrane lipid biosynthesis. *De novo* phosphatidic acid biosynthesis in plants occurs in two steps: First the acylation of the *sn*-1 position of glycerol-3-phosphate giving rise to lysophosphatidic acid; Second, the acylation of the *sn*-2 position of lysophosphatidic acid to form phosphatidic acid. The second step is catalyzed by a lysophosphatidic acid acyltransferase (LPAAT). Here we describe the identification of the *ATS2* gene of Arabidopsis encoding the plastidic isoform of this enzyme. Introduction of the *ATS2* cDNA into *E. coli* JC 201, which is temperature-sensitive and carries a mutation in its LPAAT gene *plsC*, restored this mutant to nearly wild-type at high temperature. A green-flourescent protein fusion with ATS2 localized to the chloroplast. Disruption of the *ATS2* gene of Arabidopsis by T-DNA insertion caused embryo lethality. The development of the embryos was arrested at the globular stage concomitant with a transient increase in *ATS2* gene expression. Apparently, plastidic LPAAT is essential for embryo development in Arabidopsis during the transition from the globular to the heart stage when chloroplasts begin to form.

Introduction

The thylakoid membrane of photosynthetic organisms contains four major glycerolipids, mono- and digalactosyldiacylglycerol (MGDG and DGDG), phosphatidylglycerol (PG), and the sulfolipid sulfoquinovosyldiacylglycerol (SQDG). Two pathways contribute to the biosynthesis of membrane lipids in many plants including Arabidopsis (Roughan and Slack, 1982). According to this "two pathway hypothesis", fatty acids *de novo* synthesized in the chloroplast and bound to the acyl carrier protein (acyl-ACP) are either directly incorporated into glycerolipids in the chloroplast envelopes or are exported to the endoplasmic reticulum (ER). At the ER, they serve in the form of acyl-CoAs as precursors for extraplastidic glycerolipids. A fraction of the diacylglycerol moieties derived from phosphatidylcholine is returned to the chloroplast and enters thylakoid lipid biosynthesis. As a consequence, diacylglycerol (DAG) moieties of plastidic and extra-plastidic origin are found in the glycerolipids of the thylakoid membranes of Arabidopsis.

A critical intermediate of both pathways in plants is phosphatidic acid (PA), which is also essential for the biosynthesis of glycerolipids and triacylglycerol in bacteria, yeast and animals. A deficiency for PA biosynthesis is lethal in *E. coli* consistent with an essential role for PA in this bacterium (Coleman, 1990). Unlike animals and yeast, in plants the glycerol-3-phosphate (G3P) pathway is thought to be the only pathway for the *de novo* PA biosynthesis (Athenstaedt and Daum, 1999). Two acylations are involved: First the transfer of an acyl group from either acyl-ACP or acyl-CoA to the *sn*-1 position of G3P catalyzed by a G3P acyltransferase leading to the formation of 1-acyl-*sn*-G3P (lysophosphatidic acid, LPA). This intermediate is further acylated to PA by an 1-acyl-*sn*-G3P acyltransferase (LPAAT). In plants, LPAAT activity is associated with multiple

membrane systems, including chloroplasts, ER, and the outer membrane of mitochondria, suggesting the presence of several different isoforms.

Genes encoding the G3P acyltransferase have been isolated from different plant species. Generally, the *sn*-1 position of PA typically contains 16 or 18 carbons indicating that this enzyme does not discriminate between these two fatty acid substrate classes. However, in some plant species, G3P acyltransferase seems to be more specific. For instance, in Arabidopsis, the ATS1(ACT1) protein, the plastidic isoform of G-3-P acyltransferase, has considerable substrate preference for 18-carbon fatty acids (Yokoi *et al.*, 1998). Of the enzymes acting on LPA, the plastidic LPAAT prefers 16-carbon saturated fatty acids and the ER form 18-carbon unsaturated fatty acids. This substrate specificity provides the means to distinguish thylakoid lipid species derived from the plastid or ER-pathways based on the fatty acids at the *sn*-2 position. Furthermore, the distinct substrate specificities of the different LPAAT isoforms are a critical factor in determining the overall lipid acyl composition in plants. To date, several cDNAs encoding plant LPAATs have been isolated from coconut (Davies *et al.*, 1995; Knutzon *et al.*, 1995), the immature embryo of meadow foam (Brown *et al.*, 1995; Hanke *et al.*, 1995), the maize endosperm (Brown *et al.*, 1994) and from *Brassica napus* (Bourgis *et al.*, 1999). Among these enzymes, BAT2 of *B. napus* has been shown to be a plastid-localized isoform. However, the *in vivo* roles for this enzyme are still unknown due to the lack of lines with altered activity.

In Arabidopsis, inactivation of the plastidic G3P acyltransferase in the *ats1* (*act1*) mutant led to the loss of the plastid pathway for glycolipid biosynthesis (Kunst *et al.*, 1988). A surprising observation was that despite the drastic effects on plastidic glycolipid

biosynthesis, PG biosynthesis was only mildly impaired in the *ats1* (*act1*) mutant. However, it is not clear whether the currently described alleles for *ats1* (*act1*) are leaky, a caveat that makes it difficult to draw definitive conclusions regarding the function of the enzyme and leaves the interpretation of the mutant phenotype ambiguous. This study was conducted to gain a better understanding of the origin of PA in plants and to determine the *in vivo* function of plastidic LPAAT. As will be described in detail, the respective null-mutant leads to embryo-lethality, an unequivocal demonstration of the essential function of plastidic LPAAT in Arabidopsis.

Materials and methods

Plant materials and growth

Surface-sterilized seeds were germinated on 0.8% (w/v) agar-solidified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 1% sucrose. Seedlings (10-days-old) of Arabidopsis wild-type and mutants were transferred to soil drenched with half-strength Arabidopsis nutrient solution (Estelle and Somerville, 1987), and grown under a photosynthetic photon flux density (PPFD) of 70-80 mol m^{-2} s^{-1} at 22/18 ^{o}C (day/night) with a 14-h light/10-h dark period.

Bioinformatics

For routine sequence comparison, BLAST2 was used (Altschul *et al.*, 1997). Multiple sequence alignment was done by CLUSTALW software (Thompson *et al.*, 1994) at The Biology Work Bench (http://workbench.sdsc.edu/). Prediction of chloroplast transit peptides was accomplished using ChloroP (Emanuelsson *et al.*, 1999). Transmembrane spanning helices were predicted using TMHMM (Sonnhammer *et al.*, 1998).

T-DNA insertion analysis, ATS2 cDNA cloning and expression in transgenic plants

The T-DNA insertional mutants, *ats2-1* (SALK_073445) and *ats2-2* (SALK_108812) were obtained from the Arabidopsis Stock Center (www.arabidopsis.org). The T-DNA left border primer P1 5'-GTTCACGTAGTGGGCCATCG-3', and gene-specific primers P2 5'-CAG G T ACCTTAG A G A TCCATTGATTCTGC-3' and P3 5'-GAGGATC-CAGTGAAAAATTTATGGGCGA-3' were used to screen the plants for T-DNA

insertions (cf. Fig. 4). The T-DNA-bordering DNA fragments were ligated into the plasmid pPCR-script AMP SK(+) (Stratagene, La Jolla, CA), and sequenced to determine the localization of the T-DNA insertion. The *ATS2* open reading frame corresponding to Arabidopsis gene At4g30580 (GenBank accession no. NP_194787) was predicted from the DNA sequence of BAC F17I23_80 (GenBank accession no. AF160182) and amplified by reverse transcription-PCR using the primers 5'-GAGGATCCATGGA-TGTCG-CTTCTGCTCG-3' and 5'-CAGGTACCTTAGAGATCCATTGATTCTGC-3'. For this purpose, total leaf RNA was isolated from 20-day-old plants (Col-2) according to the method by Logemann *et al.* (Logemann *et al.*, 1987). Reverse transcription-PCR was performed by using the ProSTAR HF system from Stratagene (La Jolla, CA). The PCR product was inserted into the ligation-ready Stratagene vector pPCR-Script Amp SK(+) giving rise to pATS2. For plant transformation, the insert of pATS2 containing the full-length coding sequence of *ATS2* including the transit peptide was amplified by PCR using the primers 5'-GGACTAGTGATGTCGCTTCTGCTCGGA-3' and 5'-GGACTAGT-GAGATCCATTGATTCTGCA-3'. This fragment was inserted into the binary vector pCAMBIA1304 (www.cambia.org) using the *Spe*I restriction site to give plasmid pCAT-S2. Based on the nature of pCAMBIA1304, this construct led to the expression of an ATS2-GFP (green fluorescent protein) fusion protein and the resulting plants were used in the complementation and localization analysis. Stable transformation of Arabidopsis was achieved using the vacuum infiltration method (Bechtold and Pelletier, 1998). Transgenic plants were selected in the presence of hygromycinB (25μg/ml) on MS medium lacking sucrose.

Semi-quantitative RT-PCR

Total RNA was extracted from plant tissues other than developing siliques with an RNA extraction kit (RNeasy, QIAGEN, Valencia, CA). Silique RNA was extracted from frozen tissue (50-100 mg) wich was ground in 1.5mL tubes. To the powder 350 μL of pre-heated (80 °C) extraction buffer (0.02 M sodium borate, 30 mM EDTA, 30 mM EGTA, 1% sodium dodecylsulfate, 2% deoxycholate, 2% polyvinylpolypyrrolidone, 2% polyvinylpyrrolidone 40K, 100 mM dithiothreitol, 100 mM α-mercaptoethanol) were added. This mixture was incubated at 80 °C for 1min, then chilled on ice. After addition of 0.15 mg of Proteinase K, the extract was incubated at 37 °C for 1hr. Following the clearing by centrifugation, the supernatant was extracted twice with phenol/choloroform (1:1), and once with chloroform/isoamyl (24:1). Total RNA was precipitated with isopropanol, resolved in water, then precipitated with 2 M LiCl overnight, and washed with 70% ethanol. Following DNase treatment (DNase I, Roche, Indianapolis, IN), cDNA was synthesized from 300 ng of RNA using a reverse transcriptase kit (Omnis-cript, QIAGEN, Valencia, CA) and 1U of Taq DNA polymerase (Roche, Indianapolis, IN). The 3' region of the *ATS2* gene was amplified for gene expression analysis using the primers 5'-ACGCTAATGGGAACAGGCA-3' and 5'-AAGATCTCAACATTTAATTC-TTC-3'. The coding region of a translation elongation factor EF1α (GenBank Accesion no. NM_125432) was used as a control (primers: 5'-ATGCCCCAGGACATCGTGATT-TCAT-3' and 5'-TTGGCGGCACCCTTAGCTGGATCA-3') (Boisson *et al.*, 2001). Cycle numbers of 28 and 30 were applied for detection of *EF1α* and *ATS2* respectively. The amplified fragments were quantified using Quantity One (BioRad, Hercules, CA). Each *ATS2* signal was normalized to the *EF1* • signal for calculation of relative amounts.

Heterologous complementation

The *ATS2* open reading frame lacking the predicted transit peptide was generated by RT-PCR using the primers 5'-GAGGATCCAGTGAAAAATTTATGGGCGA-3' and 5'-CAGGTACCTTAGAGATCCATTGATTCTGC-3'. The open reading frame was fused by blunt end ligation to the *lacZ* gene under the control of an IPTG-inducible promoter in the predigested plasmid pPCR-script AMP SK(+) (Stratagene, La Jolla, CA) giving rise to plasmid pATS2-s. The plasmid pATS2-s was used to complement the temperature-sensitive phenotype of *E. coli* JC201 ((Coleman, 1990); genotype: *plsC thr-1 ara-14* Δ *(gal-attλ)-99 hisG4 rpsL136 xyl-5 mtl-1 lacY1 tsx-78 eda-50 rfbD1 thi-1*).This strain is unable to grow at 42°C due to the deficiency in lysophosphatidic acid acyltransferase encoded in the wild type by *plsC*, but can grow at 30°C. The control strain JC200 was isogenic to JC201 with exception of the *plsC* wild-type allele in JC200.

Microscopy

For the GFP fusion protein localization study, leaf samples of transgenic lines were directly examined using a Meridian Instruments Insight confocal laser scanning microscope (Okemos, MI). Excitation light was provided by an argon laser at 488 nm. GFP fluorescence was obsered with a band-pass filter of 520-560 nm and chlorophyll fluorescence with a 670 nm cut-off filter. Siliques of different developmental stages from heterozygous *ATS2/ats2* plants were dissected with hypodermic needles. Ovules from individual siliques were mounted on microscope slides in a clearing solution (chloral hydrate, water, glycerol, 8:2:1 v/v) and cleared for 1 hour at 4°C before microscopy. The ovule was observed with a Leica DMLB microscope (Leica Microsystems, Wetzlar, Germany).

RESULTS

Identification of an Arabidopsis ATS2 candidate gene

Taking advantage of the published *Arabidopsis* genome, we identified an Arabidopsis gene At4g30580 (GenBank accession no. NP_194787) on chromosome 4 as a putative ortholog of *BAT2* from *B. napus*. A wild-type cDNA corresponding to gene At4g30580 was isolated by reverse transcription-PCR and sequenced. This cDNA encoded 356 amino acids in agreement with the protein predicted from the genomic sequence. The protein was designated ATS2 following the gene designation at TAIR (www.Arabidopsis.org) for the plastidic G3P acyltransferase in Arabidopsis, ATS1(ACT1). Using the BLAST2 sequence alignment software (Altschul *et al.*, 1997), it showed 80% identity, and 85% similarity over 344 amino acid residues with BAT2 (Fig.5.1a). Like for BAT2, three putative membrane spanning domains were predicted for ATS2 by the TMHMM software (Sonnhammer *et al.*, 1998).

To explore the evolutionary origin of LPAATs, we performed a phylogenetic analysis with ATS2 and other putative and *bona fide* LPAATs. The experimentally verified LPAATs included enzymes from *Escherichia coli* (Coleman, 1992), human (AGPAT1, AGPAT2) (Eberhardt *et al.*, 1997; West *et al.*, 1997), *Limnanthes douglasii* (LAT1, LAT2) (Brown *et al.*, 1995) and *L. alba* (meadowfoam) (Lassner *et al.*, 1995), coconut (Knutzon *et al.*, 1995), *B. napus* (BAT1 and BAT2*)* (Bourgis *et al.*, 1999), Yeast (Nagiec *et al.*, 1993), and maize (Brown *et al.*, 1994). Predicted LPAATs were those from *A. thaliana* (LPAT1-4), *Oryza sativa* (LPAT1-2), *B. napus* (BAT3), *Schizosaccharomyces pombe*, the bacteria *Rhodobacter sphaeroides* and *Sinorhizobium*

Figure 5.1. Evolutionary relationship between LPAATs. Sequence alignment of BAT2 and ATS2 (a). Identical amino acids are indicated by black boxes, similar ones are shaded grey. (b) Phylogenetic relationship of LPAATs. The following proteins were included in the analysis (GenBank accession no.): *Arabidopsis. thaliana ATS2* (NP_194787); *A. thalianaLPAT1* (NP_567052); *A. thaliana* LPAT2 (NP_175537); *A. thalianaLPAT3* (NP_565098); *A.thaliana* LPAT4 (NP_188515); *Brassica napus* BAT1 (CAA90019); *Brassica napus* BAT2(AF111161); Brassica napus BAT3 (CAB09138); *Oryza sativa* LPAT1 (CAE03516); Oryza sativa LPAT2 (AAL58271); Limnanthes alba (Q42868); *Limnanthes douglasii* LAT2 (CAA86877); *Limnanthes douglasii* LAT1 (CAA88620); *Cocos nucifera* (Q42670); *Synechococcus sp.* WH 8102 (NP_898339); *Nostoc sp.* PCC 7120 (NP_484285); Synechocystis sp. PCC 6803 (NP_441924); *Saccharomyces cerevisiae* (NP_010231); *Schizosaccharomyces pombe* (NP_595192); *Escherichia coli* (NP_417490); *Homo sapiens* AGPAT1 (AAH03007); *Homo sapiens* AGPAT2 (AAH07269); *Sinorhizobium meliloti* (NP_386764); *Rhodobacter sphaeroides* (ZP_00008109).

(a)

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BAT2    1 MDVASARGVSSHPPYYSKPICSSQSSLIRIPISKGCCFKRSSNLVTSLHA
ATS2    1 MDVASARSISSHPSYYGKPICSSQSSLIRISRDKVCCFGRISNGMTSFTT

BAT2   51 ASRGVTR-------RTSGVQWCYRSIRFDPFKVNDKNS-------RTVTV
ATS2   51 SLHAVPSEKFMGETRRTGIQWSNRSLRHDPYRFLDKKSPRSSQLARDITV

BAT2   87 RSDLSGAATPESTYPEPEIKLSSRLRGICFCLVAGISAIVLIVLMIIGHP
ATS2  101 RADLSGAATPDSSFPEPEIKLSSRLRGIFFCVVAGISATFLIVLMIIGHP

BAT2  137 FVLLFDRYRRKFHHFIAKLWASISIYPFYKTDIQGLENLPSSDTPCVYVS
ATS2  151 FVLLFDPYRRKFHHFIAKLWASISIYPFYKINIEGLENLPSSDTPAVYVS

BAT2  187 NHQSFLDIYTLLSLGQSYKFISKTGIFVIPVIGWAMSMMGVVPLKRMDPR
ATS2  201 NHQSFLDIYTLLSLGKSFKFISKTGIFVIPIIGWAMSMMGVVPLKRMDPR

BAT2  237 SQVDCLKRCMELVKKGASVFFFPEGTRSKDGRLGPFKKGAFTIAAKTGVP
ATs2  251 SQVDCLKRCMELLKKGASVFFFPEGTRSKDGRLGSFKKGAFTVAAKTGVA

BAT2  287 VVPITLMGTGKIMPTGSEGILNHGDVRVIIHKPIYGSKADVLCEEARNKI
ATS2  301 VVPITLMGTGKIMPTGSEGILNHGNVRVIIHKPIHGSKADVLCNEARSKI

BAT2  337 AESMNLLS
ATS2  351 AESMDL--
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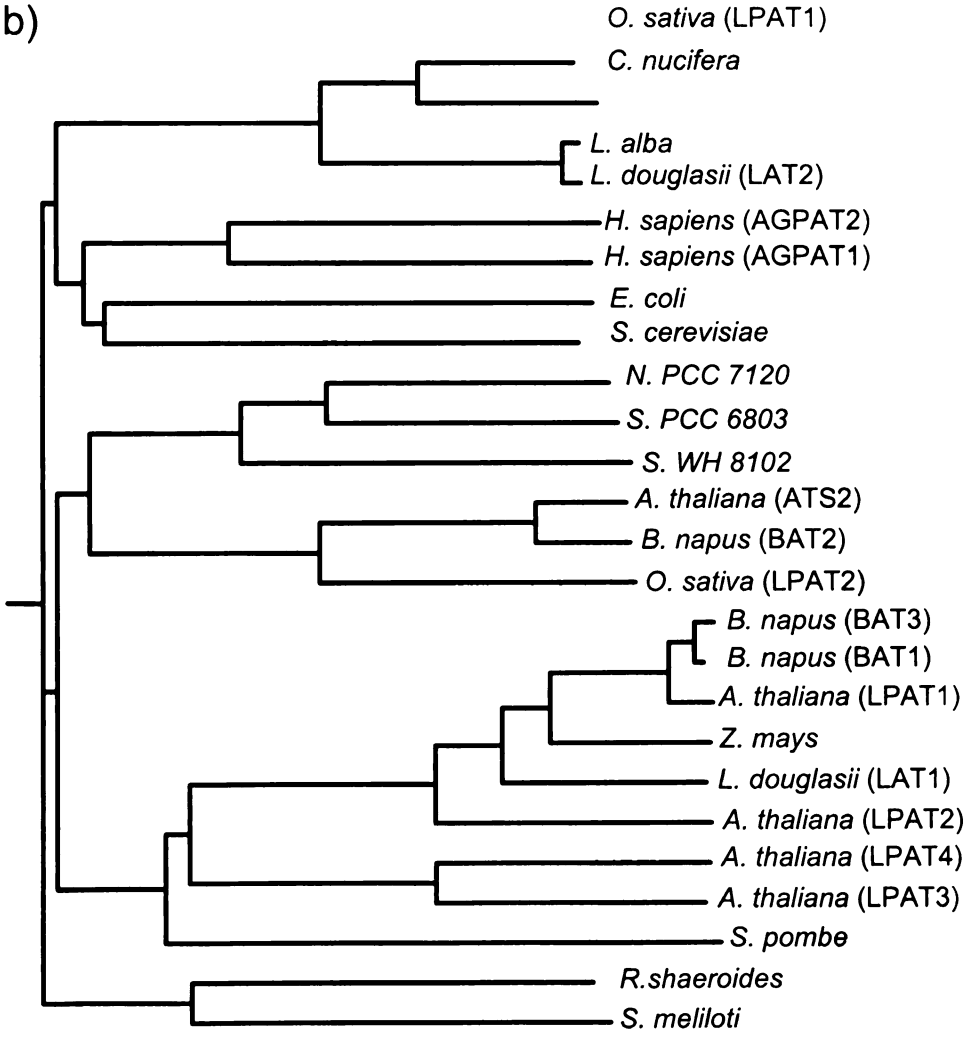
(b)



meliloti and different cyanobacteria as indicated. Of these LPAATs, LPAT2 from *O. sativa* is a putative plastidic isoform based on the predicted presence of a chloroplast transit peptide. Figure 5.1b shows a phylogenetic tree derived from protein sequence alignments. The ATS2 protein clustered with two plastidic LPAATs on a branch alongside the cyanobacterial cluster. ATS2 appears to represent a highly conserved plastidic LPAAT. It shared 68% and 91% identity, respectively, with *O. sativa* and *B. napus* over 207amino acids representing the domain aligning with the bacterial sequence encoded by *plsC*.

Expression of the ATS2 cDNA complements an E. coli LPAAT mutant

To demonstrate directly that *ATS2* encodes an LPAAT, we tested if the expression of the *ATS2* cDNA rescued the temperature-sensitive phenotype of *E. coli* strain JC201. This strain is unable to grow at 42°C but grows well at 30°C due to the deficiency of LPAAT activity (Coleman, 1990). Thus, the test is based on the restoration of its growth at the non-permissive temperature. This strategy was successfully used to isolate LPAATs from many other organisms (Brown *et al.*, 1994; Davies *et al.*, 1995; Eberhardt *et al.*, 1997; West *et al.*, 1997; Bourgis *et al.*, 1999). A truncated version of the *ATS2* cDNA lacking the sequence encoding the putative chloroplast transit peptide was fused to the *lacZ* gene which was under the control of an inducible promoter. The result shown in Fig. 5.2 indicates that the vector expressing *ATS2*, but not the empty vector rescued the temperature sensitivity of *E. coli* JC201. This result suggested that *ATS2* of Arabidopsis indeed encodes an LPAAT that can substitute for the inactive bacterial LPAAT in the mutant.

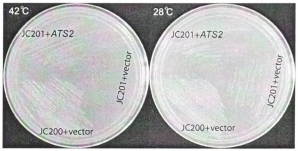


Figure 5.2. Heterologous complementation of *E. coli* JC201 by expression of *ATS2* of Arabidopsis. Strain JC200 is the *E. coli* wild-type *plsC* control strain isogenic to JC201.

Localization of the ATS2 protein

The ATS2 protein sequence has a predicted 56 amino acid N-terminal chloroplast transit peptide (ChloroP) (Emanuelsson *et al.*, 1999). To experimentally verify the subcellular localization of ATS2, the *ATS2* cDNA was fused to the N- terminus of the green fluorescent protein (GFP) cDNA. The resulting construct was expressed in transgenic wild-type plants under the control of the 35S cauliflower mosaic virus (CMV) promoter. The GFP fluorescence in transgenic plants was observed using confocal microscopy. As shown in Figure 5.3, the GFP fluorescence was exclusively associated with chloroplasts. In control plants expressing only the GFP cDNA, green fluorescence was not associated with chloroplasts but appeared to be diffuse in the cell. Based on these results, it was concluded that ATS2 is localized in the plastid. These results were also consistent with findings of proteomics studies detecting the presence of this protein in chloroplast preparations (Ferro *et al.*, 2002; Ferro *et al.*, 2003).

Isolation of mutants with T-DNA insertion into ATS2

In an attempt to investigate the function of ATS2 in the chloroplast, two T-DNA insertion lines, SALK_073445 and SALK_108812, (alleles *ats2-1* and *ats2-2* respectively) were identified in theSalk T-DNA insertion population (Alonso *et al.*, 2003). The T-DNA insertion sites were determined by sequencing PCR products obtained with a combination of gene-and T-DNA-specific primers as shown in Figure 5.4. The *ats2-1* allele carried a T-DNA insertion in the fourth exon of the predicted At4g30580 gene (Fig. 5.4a) at base pair 22022 of the sequence of BAC F17I23 (GenBank accession no. AF160182) and the *ats2-2* allele in the fifth exon at base pair 22390 (Fig. 5.4b). Despite extensive

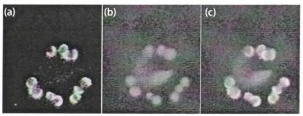


Figure 5.3. Subcellular localization of the ATS2 protein.

(a) ATS2-GFPF fusion protein, (b) chlorophyll fluorescence, (c) overlay of (a) and (b).

Figure 5.4. Isolation and characterization of *ATS2* T-DNA insertion lines.

(a) Insertion into the *ATS2* gene in the heterozygous *ATS2-1/ats2-1* allele and (b) into the *ATS2-2/ats2-2* allele. Black boxes represent exons, open boxes introns. The exact insertion site (based on the sequence of BAC F17I23 GenBank Accession no. AF160182) is indicated by a vertical arrow. Horizontal arrows labeled P1, P2, P3 represent the binding site locations for PCR primers. (c) PCR results for genomic DNA isolated from the wild type (*ATS2/ATS2*) and the two allelic heterozygous mutant lines (*ATS2-1/ats2-1* and *ATS2-2/ats2-2*). The primer combination P2-P3 is diagnostic for the endogenous gene, the combination P1-P3 for the T-DNA flanking genomic DNA as indicated in (a) and (b). (d) Dissected green siliques of heterozygous mutant lines (alleles as indicated) showing the segregation of clear white seeds.

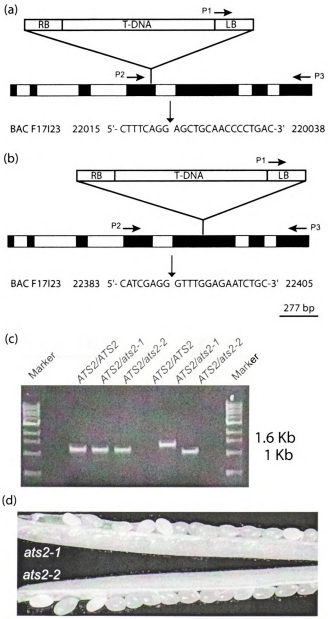

(a)
P1
RB
T-DNA
LB
P2
P3
BAC F17I23 22015 5'- CTTTCAGG AGCTGCAACCCCTGAC-3' 220038
(b)
P1
RB
T-DNA
LB
P2
P3
BAC F17I23 22383 5'- CATCGAGG GTTTGGAGAATCTGC-3' 22405
277 bp
(c)
Marker
ATS2/ATS2
ATS2/ats2-1
ATS2/ats2-2
ATS2/ATS2
ATS2/ats2-1
ATS2/ats2-2
Marker
1.6 Kb
1 Kb
(d)
ats2-1
ats2-2

Table5.1. Segregation and complementation analysis of the two allelic heterozygous *ATS2/ats2* lines. Multiple siliques from single plants were analyzed. The heterozygous state of *ATS2/ats2* was confirmed by genotyping using PCR (cf. Fig. 5.4c).

	Green seeds	White seeds	χ^2(P< 0.1)
ATS2-1/ats2-1	413	135	0.04 (3:1)
ATS2-2/ats2-2	353	120	0.03 (3:1)
ATS2-1/ats2-1 (*ATS2 cDNA*)	498	35	0.09 (15:1)
ATS2-2/ats2-2 (*ATS2 cDNA*)	540	38	0.10 (15:1)

screening, no homozygous plants could be identified. Combinations of gene-specific and T-DNA-specific primers to generate PCR products diagnostic for mutant and wild-type chromosomes and a typical result for wild-type and *ATS2/ats2-1* and *ATS2/ats2-2* heterozygous lines are shown in Figure 5.4c. Both mutant lines showed insert-specific as well as wild-type genomic DNA fragments. Furthermore, when immature siliques were opened which grew on heterozygous plants as confirmed by genotyping, approximately 25% of the seeds were clear white (Fig. 5.4d), indicative for the arrest of embryo development at an early stage. This observation raised the possibility that a homozygous *ats2* null-mutant was embryo-lethal. To quantify this phenomenon, one or two siliques from 10 heterozygous plants were opened and the ratio of viable to aborted seeds in all the siliques examined was obtained as shown in Table 5.1. These data were consistent with a 3:1 segregation ratio as expected for a recessive mutation in an essential nuclear gene. When viable plants derived from *ATS2/ats2-1* heterozygous lines or *ATS2/ats2-2* were analyzed, approximately two thirds (39 out of 61 for *ATS2/ats2-1* and 42 out of 62 for *ATS2/ats2-2*) produced aborted seeds consistent with a 2-to-1 ratio of heterozygous to wild-type genotypes among the surviving plants. Complementation of the embryo-lethal phenotype was tested by introduction of the wild-type *ATS2* cDNA under the control of the 35S-CMV promoter into *ATS2/ats2* heterozygous plants. In the T1 generation, transgenic plants were selected on hygromycinB containing medium and tested for GFP expression. Three classes were expected among the transgenic plants: homozygous mutants (*ats2/ats2),* heterozygous plants (*ATS2/ats2*) and homozygous wild-type plants (*ATS2/ATS2*). Because a single copy of the transgene would behave like a second (dominant) genetic marker in this experiment, complementation would be in effect if

homozygous mutant lines segregated 1/4 white seeds, heterozygous lines 1/16 white seeds, and homozygous wild-type lines no white seeds. All three classes were observed among 10 transgenic plants tested, indicating complementation. Because transgenic heterozygous plants were most informative, one plant for each allele was analyzed in detail. Genotyping by PCR (cf. Fig. 5.4c) confirmed these plants to be *ATS2/ats2* heterozygous and analysis of seeds in immature siliques indicated the presence of 1/16 white seeds (Tab.5.1) as expected for complementation.

Because of the embryo lethality, no further phenotypic analysis was feasible on homozygous lines. When heterozygous lines were analyzed, no changes in growth or leaf fatty acid composition were observed (data not shown).

A transient increase in ATS2 expression in developing siliques in the wild type corresponds to the arrest of embryo development in the mutant

Analyzing the expression of the *ATS2* gene, the mRNA was detected in all tissues tested (Fig. 5.5), as would be expected for a gene involved in an essential process. Interestingly, *ATS2* mRNA abundance was reduced in silique walls compared to leaves and the abundance was higher in RNA samples from intact siliques. This result suggested an increased expression of *ATS2* in developing seeds. Because it is exceedingly difficult to obtain sufficient amounts of mRNA from very young embryos, a time course of developing intact siliques was analyzed. Comparison to the silique wall sample (5 days after flowering) should provide an indication of mRNA abundance in the developing seed. Interestingly, *ATS2* expression transiently increased in siliques four days after flowering (Fig. 5.5).

Figure 5.5. Expression of *ATS2* in various tissues and during silique development. Semi-quantitative RT-PCR was used and products for *ATS2* are shown in comparison to the control gene *EF1α* (GenBank accession no. NM_125432). Relative levels of *ATS2* normalized to *EF1α* are shown in the bottom panel. Lanes from left to right: L, leaf; S, stem; SW, silique wall (age 5 days after flowering); R, root; B, bud; F, flower; 2-12, siliques of different development as indicated (days after flowering).

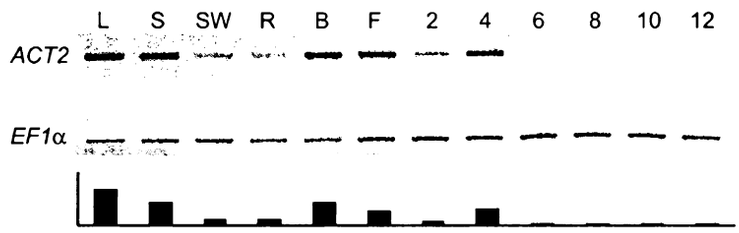
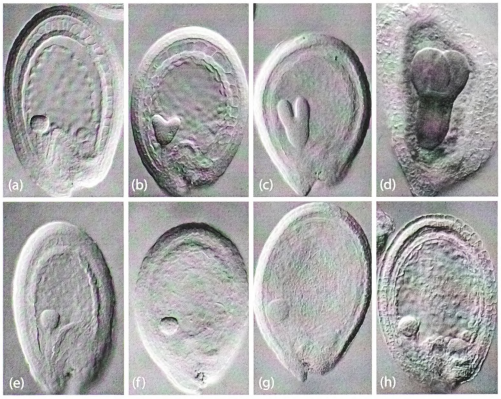

L
S
SW
R
B
F
2
4
6
8
10
12
ACT2
EF1α

Figure 5.6. Arrest of embryo development. Four siliques of different developmental age growing on heterozygous *ATS2/ats2-1-1* plants were dissected. (a, e), two representative embryos from the youngest silique, in which all embryos were at the globular stage and indistinguishable; (b, f), older silique with most embryos at the heart stage (b) and with about 25 % of the embryos arrested at the globular stage (f); (c) developing early torpedo stage embryo and (g) arrested embryo from the same silique at the globular stage; (d), oldest silique with developing embryo at the late torpedo stage and (h) globular arrested embryo from the same silique.


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)

Embryo development was compared by Nomarski microscopy in different developing seeds of siliques growing on heterozygous plants (Fig. 5.6). Before the onset of visible chlorophyll accumulation at the transition from globular to the early heart stage (Mansfield *et al.*, 1991), the embryos were indistinguishable. However in older siliques, seeds with arrested development became visible to the naked eye and closer examination revealed that the clear white seeds contained embryos arrested at the globular stage. Presumably, these seeds corresponded to dark brown aborted seeds apparent in mature siliques. It should be noted that the transient increase in *ATS2* expression observed for the wild type four days after flowering (Fig. 5.5) corresponded to the transition in embryo development from the globular to the heart stage, when the developmental arrest occurs in a quarter of the seeds developing in siliques on heterozygous plants.

Discussion

With the availability of the Arabidopsis genome sequence (The Arabidopsis Genome Initiative, 2001) it has become feasible to predict genes encoding enzymes involved in lipid metabolisms in Arabidopsis based on sequence similarity to enzymes with known functions from bacteria and other plants. Ohlrogge and coworkers have used this approach to assemble a catalog of lipid metabolism genes in Arabidopsis (Beisson *et al.*, 2003). As we now know based on the present study, they correctly annotated Arabidopsis gene At4g30580 to encode a plastidic LPAAT. Functional proof was provided by heterologous complementation of the *E. coli* LPAAT-deficient *plsC* mutant (Fig. 5.2). Furthermore, subcellular localization of the ATS2-GFP fusion (Fig. 5.3) agreed with a plastidic localization of the protein as predicted. Independent experimental evidence for a plastid localization of ATS2 was also obtained by Ferro and colleagues (Ferro *et al.*, 2002; Ferro *et al.*, 2003). When confirmed and putative LPAAT protein sequences from different organism were aligned and grouped based on the amino acid sequence similarity, ATS2 of Arabidopsis clustered with BAT2 from *B. napus* and LPAT2 from *O. sativa*, both plastidic LPAATs (Fig. 5.3). Furthermore, these plastidic LPAATs formed a subcluster with cyanobacterial LPAATs providing supportive evidence for the cyanbacterial endosymbiont origin of chloroplasts. Four other putative Arabidopsis LPAATs were grouped in a distinct cluster representing microsomal LPAATs (Fig. 5.1) suggesting that their evolutionary origin is different from that of plastidic isoforms.

Although in case of the well studied class of LPAATs the analysis of genomic information led to precise predictions, for which the experimental verification as shown seemed trivial, one should keep in mind that most of the information in current genome

databases is based only on conjecture. Corroborating genomic predictions is only the beginning to a broader understanding of the biological (physiological) function of gene products, in this case ATS2. One of the most versatile resources to shed light on the function of a particular gene in Arabidopsis is the broad availability of sequenced T-DNA insertion lines (Alonso *et al.*, 2003). In case of *ATS2*, two independent alleles were available, both of which gave rise to a striking phenotype, embryo-lethality. The presence of two independent alleles and restoration of embryo development following the transgenic expression of the *ATS2* cDNA (genetic complementation) linked the embryo defect to the T-DNA inactivation of the gene encoding plastidic LPAAT in the two allelic mutants. The result was surprising, because a mutant in the plastidic G3P acyltransferase gene, *ATS1*(*ACT1*), affecting the step prior to ATS2 in the pathway, had no effect on embryo development, despite the fact that the plastid pathway of thylakoid lipid biosynthesis was strongly impaired in this mutant (Kunst *et al.*, 1988). However, it should be noted that it is not clear at this time to which extent the *ats1*(*act1*) mutants is "leaky", if at all, because plastidic PG, which is assumed to be synthesized by the plastid pathway of membrane lipid biosynthesis, was only moderately reduced in the mutant. The complete loss of PG in the *ats2* mutants could not be the cause for the observed embryo arrest, because homozygous null-alleles of the *PGP1* gene encoding plastidic phosphatidylglycerolphosphate synthase gave rise to seedlings lacking plastidic PG (Hagio *et al.*, 2002; Babiychuk *et al.*, 2003). However, these mutant seedlings were completely white and non-photosynthetic. Therefore, either the absence of plastidic PA as a critical intermediate or signaling molecule, or the accumulation of lyso-PA harmful to membrane integrity might be the cause of the observed embryo lethality in the *ats2*

mutants. The fact that embryo development was arrested just at the transition from the globular to the heart stage, when thylakoid membranes start to develop and a corresponding peak in *ATS2* gene expression was observed (Fig.5.5), suggests that the lack of PA as a central intermediate of lipid metabolism might be the main cause of embryo arrest in the *ats2* mutants. However, other possibilities cannot be ruled out at this time. While embryo-lethality is an easily observable phenotype, the respective null-mutants provide no detailed information on the role of plastidic ATS2, because homozygous lines could not be further analyzed and heterozygous lines lacked any phenotype. Mutations with leaky alleles in *ATS2* giving rise to viable plants with milder phenotypes will have to be isolated and studied to better understand the role of PA in plant metabolism.

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Chapter 6

Conclusions and Future Directions

While our knowledge of chloroplast lipid biosynthesis has been advanced in the past several decades by biochemical and genetic analysis (Browse and Somerville, 1991; Dormann and Benning 2002; Ohlrogge and Browse, 1995, Joyard *et al.,* 1998 a, b), many aspects still need further investigation. The current dissertation, with the use of Arabidopsis (*A. thaliana*) as a model system, is mainly focused on identification of enzymes involved in SQDG and PG biosynthesis, using reverse or forward genetic approaches, and on dissecting the functions of these two anionic lipids by analyzing the corresponding single or double mutants. During the course of this research, effort was also put on the isolation of the plastidic LPAAT responsible for PA biosynthesis, and the elucidation of the function of this LPAAT in lipid biosynthesis.

A gene, *SQD2* involved in SQDG biosynthesis, was identified (Chapter 2). Several lines of evidence indicated that *SQD2* encodes the SQDG synthase catalyzing the transfer of SQ from UDP-SQ to DAG. First, the deduced SQD2 protein sequence showed 41% similarity to that of SQDX, a cyanobacterial SQDG synthase. Second, inactivation of *SQD2* led to the deficiency of SQDG in Arabidopsis. Third, stable transformation of *sqd2* with *SQD2* cDNA restored the biosynthesis of SQDG. Finally, the biosynthetic pathway of SQDG was reconstituted in *E. coli* by co-expressing SQD1 and SQD2. The isolation of the sulfolipid-deficient *sqd2* null mutant of *Arabidopsis* allowed me to address another fundamental question about the role of sulfolipid in photosynthetic membranes of seed plants. My initial analysis of photosynthesis and growth suggests that sulfolipid does not have a crucial role in plant photosynthesis. The *sqd2* mutant did not show any obvious signs of altered morphology or growth and photosynthetic parameters were affected only mildly under normal laboratory growth conditions. Like some bacteria, SQDG deficiency

in the *Arabidopsis sqd2* mutant led to growth impairment under phosphate-limiting growth conditions, indicating the conditional importance of SQDG in seed plants. The wild type is able, within limitations, to maintain the relative amount of total anionic thylakoid lipids by increasing its sulfolipid content, when phosphatidylglycerol content is decreased by reduced phosphate availability. In comparison, phosphatidylglycerol amounts are not decreased under phosphate-limiting conditions in the *Arabidopsis* sulfolipid-deficient mutant, due to a requirement for the constant amount of anionic lipid. Considering that one-third of the organic phosphate is normally bound in the membranes of wild-type plants, the conclusion can be drawn that a sulfolipid-deficient null mutant exhausts its phosphate reserves sooner than the corresponding wild type, resulting in reduced growth under these conditions. Based on these observations, I concluded that one function of SQDG is to help plants to cope with phosphate-limited growth conditions. Analyzing the correlation between the relative amount of SQDG of different plant species and phosphate availability in different geographic areas should provide verification of this conclusion.

SQDG obviously plays different roles in different cyanobacterial species (Aoki et al., 2003). This phenomenon gives rise to the question of whether SQDG also has different function in different higher plants. Clarifying this question will not only increase our understanding of the evolution and the function of SQDG, but also shed new light on how different plants maintain a functional photosynthetic membrane by adjusting their lipid composition. An approach to solve this question is to analyze SQDG-deficient mutants either artificially constructed or naturally occurring in other species.

The SQD2 protein belongs to the glycosyltransferase gene family GT4, which represents retaining glycosyltransferases. The catalytic mechanism for these glycosyltransferases is still unclear. Further experiments toward understanding the SQD2 catalytic mechanism will provide new insights into SQDG biosynthesis, and will providing understanding of the GT4 family enzyme in general. An attractive approach is to determine the protein's three-dimensional structure by X-ray diffraction using a crystallized SQD2 protein. Some enzymes in this gene family have been studied focusing on the substrate-binding and catalytic sites (Edman *et al.,* 2003; Abdian *et al.,* 2000). Utilizing information from these studies, I would predict the amino acids of the SQD2 protein involved in substrate-binding and catalytic reaction, followed by experimental verification of the function by site-directed mutagenesis and enzyme activity assays.

Using a forward approach, we isolated a PG deficient mutant, *pgp1* (Xu *et al.,* 2002), and identified the phosphatidylglycerolphosphate synthase 1 (*PGP1*). Three facts confirmed that PGP1 encodes phosphatidylglycerolphosphate synthase. The first was that the *pgp1* mutant has a reduced PGP synthase activity in isolated chloroplasts. Second, stable transformation of PGP cDNA into the *pgp1* mutant rescued the *pgp1* mutant phenotype. Third, the expression of *PGP1* complemented the *E.coli* mutant YA5512 deficient in PGP synthase activity (Asai et al., 1989). The *pgp1* mutant plant was pale green, and its photosynthesis was impaired. In addition, the complete inactivation of *PGP1* by T-DNA knockout led to the arrest of chloroplast development, and the mutant plants could not survive on soil (Hagio *et al.,* 2002; Babiychuk *et al.,* 2003). These facts suggested that phosphatidyl glycerol is essential for the development of the photosynthetic thylakoid membranes in *A. thaliana.*

Similar to *pgp1-1*, the overall content of PG in the *act1* mutant is also reduced by 30%. However, unlike *pgp1-1*, the *act1* mutant showed no obvious growth phenotype. One possible explanation for this difference is that *pgp1-1* is a leaky mutant. The activity of the PGP1 enzyme is reduced by 70%, therefore it is possible that PG content is reduced more than 30% at the beginning of chloroplast development, which may lead to the phenotype of *pgp1-1*. This hypothesis is supported by the fact that developing leaves of *pgp1-1* have more severe phenotype than mature leaves. To further verify this possibility, the overall content of PG in *pgp1-1* and *act1* can be compared at different developmental stages. Alternatively, the phenotype of *pgp1-1* could be caused by the accumulation of PA, which is known to be a signaling molecule, or lyso-PA, which is toxic, due to the deficiency of PGP1. The *act1 pgp1* double mutant showed the phenotype of *act1*, suggesting this possibility (Xu and Benning, unpublished data). Further comparision of the amount of PA or lyso-PA of *pgp1-1* and *act1* may provide some clues regarding to this hypothesis.

Photosynthesis is impaired in the *pgp1-1* mutant. An obvious reason for this is the decrease of PG, which has been shown to play an important role in photosynthesis. However, the reduction of mesophyll cell numbers in *pgp1-1* suggested that the decrease in photosynthetic activity might be a secondary effect. Further study of the photosynthesis with isolated chloroplast may provide some clue as to which is the correct interpretation. The reduction of mesophyll cell numbers in *pgp1-1* also suggested a possible function of PG in development, although this function may be indirect, i.e. caused by chloroplast impairment. One interesting question to be addressed in further studies is how PG affects cell number. One approach would be to look for suppressors or

enhancers of *pgp1-1* in terms of cell number by screening EMS-mutagenised *pgp1-1* seeds.

It was hypothesized that under severe phosphate limitation SQDG substitutes for PG, ensuring a constant amount of anionic lipids even under adverse conditions (Benning, 1998). Taking advantage of the SQDG deficient-mutant, *sqd2*, and partially PG- deficient mutant, *pgp1-1*, an SQDG and PG-deficient double mutant was constructed to test this hypothesis (Chapter 4; Yu et al., 2003). In the *sqd2 pgp1-1* double mutant, the fraction of total anionic lipids was reduced by approximately one third resulting in pale yellow cotyledons and leaves with reduced chlorophyll content. Photoautotrophic growth of the double mutant was severely compromised and its photosynthetic capacity was impaired. In particular, photosynthetic electron transfer at the level of photosystem II was affected. Besides these physiological changes, an altered leaf structure, a reduced number of mesophyll cells and ultrastructural changes in the chloroplasts were observed. All observations on the *sqd2 pgp1-1* mutant led to the conclusion that the total content of anionic thylakoid lipids is limiting for chloroplast structure and function, and is critical for overall photoautotrophic growth and plant development.

The reciprocal regulation of SQDG and PG gives rise to questions of how the plant senses the decrease of anionic lipid, and what the signal transduction network is, which is involved in this reciprocal regulation. Therefore, future experiments should be focused on the identification of genes involved in the regulation of PG and SQDG biosynthesis. For instance, a forward screening of EMS mutagenized *sqd2* can be conducted to look for mutants, which have reduced PG content under phosphate stress. Alternatively, one can look for mutants in which SQDG does not respond to phosphate regulation by screening

EMS mutagenized wild-type. Isolation of genes in the corresponding mutants by map-based cloning should provide new insight into the regulation of lipid biosynthesis.

Phosphatidic acid (PA) is a key intermediate for chloroplast lipid biosynthesis. The *de novo* PA biosynthesis ocurres by a two-step reaction: The first step is the *sn*-1 acylation of G-3-P by glycerol-3-phosphate acyltransferase leading to the formation of lyso-phosphatidic acid (LPA), the second is the *sn-2* acylation of LPA to form PA catalyzed by 1-acylglycerolphosphate acyltransferase (LPAAT) (Athenstaedt and Daum, 1999). I confirmed the *ATS2* gene of Arabidopsis and determined that *ATS2* encodes a LPAAT by heterozygous complementation of *E. coli* mutant JC 201, which is a temperature sensitive mutant and deficient in the LPAAT, encoded by *plsC* (Coleman, 1990, 1992). A green fluorescent protein fusion with ATS2 localized to the chloroplast. Thus, *ATS2* encodes a plastidic isoform of LPAAT of Arabidopsis. Insertion of a transfer DNA into this gene in Arabidopsis led to embryo lethality. The development of the embryo was arrested at the globular stage. These results indicated the essential role of *de novo* plastidic PA biosynthesis in embryo development. One possible reason for the lethality is that PA is required for chloroplast development. Due to the embryo lethality, the functions of ATS2 in chloroplast development and chloroplast lipid biosynthesis could not be further analyzed using the null mutants. Partial down regulation of *ATS2* in wild-type plants by RNAi or antisense approach may give rise to viable plants, which can be studied in great detail to clarify the function of PA in chloroplast biogenesis.

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