THESIS

pect1-4 Mutation Downregulates Mitochondrial Phosphatidylethanolamine Levels and Leaf Respiration Capacity in *Arabidopsis thaliana*

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Contents

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Abbreviations

ADP	adenosine diphosphate
AOP	alternative oxidase pathway
AOX	alternative oxidase
ATP	adenosine-5'-triphosphate
BSA	bovine serum albumin
CCT	CTP:phosphorylcholine cytidylyltransferase
CDP-Cho	cytidine 5’-diphosphete-choline
CDP-Etn	cytidine 5’-diphosphate-ethanolamine
Cho	choline
Cho-P	phosphorylcholine
CKI	choline kinase
CL	cardiolipin
COP	cytochrome oxidase pathway
COX	cytochrome *c* oxidase
CPT	cholinephosphotransferase
DAG	diacylglycerol
DGDG	digalactosyldiacylglycerol
DTT	dithiothreitol
EDTA	ethylenediamine-N,N,N',N'-tetraacetic acid
EKI	ethanolamine kinase

EPT	CDP-Etn:diacylglycerol ethanolaminephosphotransferase
ER	endoplasmic reticulum
Etn	ethanolamine
Etn-P	phosphorylethanolamine
FW	fresh weight
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
MES	2-(N-morpholino)ethanesulfonic acid
MGDG	monogalactosyldiacylglycerol
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
PA	phosphatidic acid
PAGE	polyacrylamide gel electrophoresis
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PECT	CTP:phosphorylethanolamine cytidylyltransferase
PEM	phosphatidylethanolamine N-methyltransferase
PG	phosphatidylglycerol
PI	phosphatidylinositol
PS	phosphatidylserine
PSD	phosphatidylserine decarboxylase
PSS	phosphatidylserine synthase

SDS	sodium dodecyl sulfate
SHAM	salicylhydroxamic acid
SQDG	sulfoquinovosyldiacylglycerol
TES	N-tris [hydroxymethyl] methyl-2- aminoethane -sulfonic acid
TPP	thiamine pyrophosphate
TR	total respiration

Chapter I

General Introduction

Biological membranes are important for various cellular functions, such as energy conversion, signal perception, and nutrient translocation. The biological membranes consist chiefly of proteins and lipids, the latter of which form lipid bilayer for membrane protein functions. The lipid matrix contains many kinds of lipid classes and molecular species. Why are there so many lipids in the biological membranes and what is the function of individual lipids? This subject has been extensively studied in the past 50 years.

To reveal the importance of each lipid, the best way is to use genetics; under the circumstances of extensive genome disclosure and availability of genomic resources, it is advantageous to adopt a reverse genetic approach in addition to forward genetics. Forward genetics depends on EMS-mutagenesis and screenings for mutants with discernible phenotypes, whereas reverse genetics depends on mutagenesis by an insertion of exogenous DNA, such as transposon or T-DNA. A Tilling search is a unique method based on EMS-mutagenesis and allows us to screen for mutations within a 1 kb region of our interest (Till et al., 2003). Biochemical pathways to glycerolipids, the major class of membrane polar lipids, have been almost identified to date, and this also accelerates the identification of the genes that encode enzymes responsible for lipid biosynthesis. However, in any of these approaches, a single gene mutation often does not show any discernible phenotype due to the presence of redundant homologous genes.

Several organisms have been used for studying the function of lipids. The most

extensive researches have been done in *Escherichia coli* and *Saccharomyces cerevisiae*. In higher eukaryotes, Chinese hamster ovary (CHO) cells provide a useful system for mammalian lipid functions. In the past 20 years since the disclosure of genome information, *Synechocystis* sp. PCC 6803 provides most powerful system for studying the lipid functions in photosynthesis. In higher plants, *Arabidopsis thaliana* is the most frequently used system for lipid researches, and an array of fatty acid desaturation mutants has been isolated by a forward genetics method in the pioneering works by a group of C. Somerville (Hwang et al., 1991). However, the forward genetics of plant lipids required laborious lipid analyses for finding mutants with altered lipid compositions, and an altered lipid composition does not necessarily link to remarkable mutant phenotype. In this respect, a method taken by a group of Christoph Benning, using bulk lipid analysis using high-throughput micro TLC analysis, is unique and successful in isolating mutants defective in lipid transfer functions between different subcellular compartments in Arabidopsis. There are a number of excellent reviews or books on this subject (Kent, 1995; Dowhan, 1997; Matsumoto, 2001; Carman and Zeimetz, 1996; Kuge and Nishijima, 2003; Benning and Ohta, 2005; Gibellini and Smith, 2010).

Table I-1 summarizes the genes and their mutant phenotypes studied to date. These studies have been mostly successful in identifying the functions of fatty acid desaturases; or the specific function of acidic lipids, such as phosphatidylglycerol (Frentzen, 2004; Wada and Murata, 2007; Wada and Mizusawa, 2009), cardiolipin (Katayama et al., 2004; Katayama et al., 2012), phosphatidylserine (Yamaoka et al. 2011) and phosphatidylinositol derivatives (Kusano et al., 2008; Luo et al., 2011), but the importance of relatively abundant lipids, such as phosphatidylcholine (PC) or phosphatidylethanolamine (PE), or polar lipid compositions is far from comprehensive

Table I-1. Phenotypes of mutants of glycerophospholipid synthesis in various organisms

organisms	Lipid	Gene	Mutant phenotype	Reference
Escherichia coli	PE	*PSS*	Lethal without divalent cations	DeChavigny et al. (1992)
	PG (CL)	*PGSA*	Lethal without *lpp* mutation	Kikuchi et al. (2000)
Saccharomyces cerevisiae	PC	*PEM1*, *PEM2*	Choline auxotroph	Kodaki and Yamashita (1987)
	PE	*PSD1*, *PSD2*	Ethanolamine auxotroph	Storey et al. (2001)
	PG	*PGS1*	Mitochondrial dysfunction	Kawasaki et al. (1999)
	PI	*PIS*	Lethal	Nikawa et al. (1987)
	CL	*CRD1*	Mitochondrial dysfunction	Jiang et al. (2000)
Chinese Hamster Ovary cells	PE		Defect in cytokinesis	Emoto and Umeda (2000)
	PG	*PGS1*	Mitochondrial dysfunction	Ostrander et al. (2001)
	PS	*PSA*	PS auxotroph	Kuge et al. (1986)
	CL		Mitochondrial dysfunction	Ohtsuka et al. (1993)
Arabidopsis thaliana	PE	*PECT1*	Lethal	Mizoi et al. (2006)
	PG	*PGP1*	Sucrose auxotroph; Defect in thylakoid development	Hagio et al. (2002)
	PS	*PSS1*	Dwarf, Sterility	Yamaoka et al. (2011)
	CL	*CLS*	Growth retardation	Katayama et al. (2012)
Synechocystis sp. PCC 6803	PG	*CDSA*, *PGSA*	PG auxotroph; Defect in photosynthesis	Hagio et al. (2000), Sato et al. (2000)

understanding.

In this thesis, I am going to describe that mitochondrial PE levels or PE:PC ratios are important for the maintenance of respiration capacity, especially the cytochrome oxidase pathway (COP) capacity in Arabidopsis rosette leaves. PC and PE are abundant phospholipids in eukaryotic organelle membranes. PC is a typical bilayer-forming lipid, whereas PE is a non-bilayer lipid that occurring as comparable quantity as PC in biological membranes. Why do biological membranes require such a large amount of non-bilayer lipids, which might promote destabilization of the bilayer structure of biomembranes?

In the following sections, before going into details my studies, I would like to introduce basic knowledge in the structure and functions of glycerolipids and the respiratory systems in higher plants, which may help readers understand the importance and interests of the present research submitted for a PhD thesis.

The classes and properties of major glycerolipids in biological membranes

When lipids are extracted from cells, tissues and organs, the lipid extracts usually contain nonpolar lipids and pigments as well as polar lipids. Non-polar lipids include triacylglycerols and diacylglycerols, both of which are usually the major storage lipids in seed and other storage tissues. Pigments are often bound to membrane proteins or floated in lipid bilayer matrix for some functions. Polar lipids are essential for constructing the matrix of biomembranes or lipid bilayer.

Glycerolipids and sphingolipids are the representative polar lipids.

Sphingolipids are localized in vacuolar and plasma membranes (Yoshida and Uemura, 1986) and constitute 6.8 % of the total cellular lipids, whereas glycerolipids account for 49.9 % of the total cellular lipids.

Glycerolipids are a large group of membrane polar lipids that account for 49.9 % of the total cellar lipids. They commonly have a glycerol backbone that is esterified with fatty acids in the *sn* (*sn* for *s*ystematic *n*umber)-1 and *sn*-2 positions and different kinds of polar head groups in the *sn*-3 position. Glycerolipids are divided into two major types, glycerophospholipids and glyceroglycolipids. Glycerophospholipids include PC, PE, PG, PI, phosphatidylserine (PS), cardiolipin (CL), and phosphatidic acid (PA), which are widely conserved among the eukaryotes. Plant cells include additional three glyceroglycolipids, monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG).

Biosynthetic pathways to glycerolipids in different organisms

In bacteria, PE is synthesized via decarboxylation of PS, which is catalyzed by bacterial PS decarboxylase (Kanfer and Kennedy, 1964; Kanfer and Kennedy, 1961). PS is the sole precursor for synthesizing PE, and normally PS is not detectable in bacterial cells.

In yeast, PC and PE are synthesized from PS as follows: CDP-DAG + Ser $\rightarrow$ PS $\rightarrow$ PE $\rightarrow\rightarrow\rightarrow$ PC. In the absence of PS synthesis (as in *cho1/pss* mutants, Atkinson et al., 1980a; Hikiji et al., 1988), cells are auxotrophic for Cho or Etn, which are converted to CDP-Cho and CDP-Etn, respectively, for PC and PE synthesis by CPT and EPT, respectively (Atkinson et al., 1980b).

In mammals, however, no CDP-DG-dependent PS synthesis has been identified.

Although a lack of PS decarboxylase causes embryotic lethality (Steenbergen et al., 2005), the CDP-Cho and CDP-Etn pathways are thought to be the major pathways to PC and PE biosynthesis (Zelinski and Choy, 1982; Tijburg et al., 1989). PC and PE are converted to PS by the base-exchange-type PS synthase PSS1 and PSS2, respectively (Kuge et al., 1986; Suzuki and Kanfer, 1985; Kuge et al., 1985; Voelker and Frazier, 1986).

In photosynthetic organisms, the glycerolipid synthesis has been summarized (Murata and Nishida, 1987; Ohlrogge and Browse, 1995; Wada and Murata, 1998; Murata and Siegenthaler, 1998; Wada and Murata, 2009; Shimojima et al., 2009; Sadre and Frentzen, 2009; Moellering et al., 2009; Sato and Wada, 2009; Awai et al., 2009; Dörmann and Hölzl, 2009). In higher-plants, ethanolamine (Etn) is phosphorylated to phosphorylethanolamine (Etn-P) by ethanolamine kinase (EKI). In the next step, CDP-Etn is synthesized from Etn-P by CTP:phosphorylethanolamine cytidylyltransferase (PECT) which is considered as the rate-limiting enzyme of the CDP-Etn pathway. In the last step, CDP-Etn: 1,2-diacylglycerol ethanolaminephosphotransferase (EPT) synthesizes PE from CDP-Etn and diacylglycerol (DAG). The CDP-Cho pathway uses choline instead of ethanolamine to synthesize PC.

PE is also synthesized by decarboxylation of PS (PS decarboxylation pathway), and PC is also synthesized by methylation of PE (Bremer and Greenberg, 1961). In mammalian and plant cells, the exchange of polar head groups between PE and PS is reported (Base exchange pathway; Vance and Vance, 2004; Yamaoka et al., 2011). In mammalian cells, another Base exchange pathway between PC and PS is also reported (Hubscher et al., 1959).

The phospholipid compositions are unique in each organelle

Biological membranes include different types of organelle membranes, such as plasma membrane, vacuolar membranes, endoplasmic reticulum, and plastids and mitochondrial membranes. According to Margulis (1970), each organelle has evolutionary ancestors: plastids are from cyanobacteria and mitochondria from α-proteobacteria like a *Rickettsia prowazekii* (the endosymbiont hypothesis). Thus, the fact that each organelle displays unique lipid composition reflect the evolutionary fact that different organisms contained different lipid compositions.

Table I-2 shows glycerolipid compositions of organelles from cauliflower inflorescence and spinach leaves. Mitochondrial membranes contain PC, PE, PI, PG and CL. Mitochondrial inner membranes contain larger amounts of PE and CL than the outer membranes. Plasma membranes and peroxisomes include only three phospholipids, PC, PE and PI, and microsomes include PC, PE, PI and CL. Plasma membranes contained a very minor proportion of glyceroglycolipids, MGDG and DGDG, the functions of which remain to be elucidated (Yoshida and Uemura, 1986). On the other hand, chloroplasts include PC, PI, PG, MGDG, DGDG and SQDG, but not PE and CL. Chloroplast envelopes include PC, MGDG and DGDG. Thylakoid membranes include large amounts of MGDG and DGDG compared to phospholipids. Because each organelle has specific functions, the unique lipid composition should have an important meaning in each organelle.

Importance of phase proportions in lipid functions

As described above, each organelle has unique lipid compositions. To understand the functions of lipids in organelle membranes, the concepts of lipid phases

Table I-2 Lipid composition of several organelle membranes.

	Lipid composition (mol %)							
	PC	PE	PI	PG	CL	MGDG	DGDG	SQDG
Cauliflower inflorescence								
Mitochondrial outer membrane	42	24	21	10	3			
Mitochondrial inner membrane	42	37	5	3	14			
Plasma membrane	66	21	13					
Peroxisome	48	46	6					
Microsome	50	36	6		8			
Spinach leaf								
Chloroplast envelope	27		1	8		22	32	5
Thylakoid	3		1	9		51	26	7

Mazliak (1980)

and phase transitions are important. I aqueous suspensions within physiological temperature changes and under atmospheric pressures, glycerolipids display several different phases or lipid polymorphism; lipids can change from one phase to another by a process called phase transition. Phase transition is driven by several parameters, among which temperature and ionic strength of solution are important. The former is referred to as thermotropic phase transition, whereas the latter is referred to as lyotropic phase transition.

The thermotropic phase transition refers to a transition driven by temperature changes. A transition between the fluid liquid crystalline phase (L_α) and the ordered gel phase (L_β) is a typical example (Fig. I-2A), and the formation of the L_β is promoted at low temperatures. On the other hand, the lyotropic phase transition refers to a transition driven by changes in a degree of hydration of the polar head group. Under physiological conditions, PC, PI, PS, CL and DGDG are bilayer-forming lipids (Fig. I-2B right). On the other hand, PE, MGDG, and PA are non-bilayer-forming lipids (Fig. I-2B left). CL and PA form non-bilayer structures in the presence of divalent cations such as Mg^{2+}, Ca^{2+} or Sr^{2+}, but not Ba^{2+} (Cullis and de Kruijiff, 1979). Non-bilayer lipids are contained at comparatively high levels in the biological membranes. These non-bilayer lipids are thought to be essential for dynamic aspects of membrane biology such as formation of membrane curvature and fission or fusion of membranes.

The importance of lipids in mitochondrial membranes and proteins

Mitochondria are thought to have evolved from a symbiont similar to the extant α-proteobacteria such as *Salmonella typhimurium* and *E. coli* (Yang et al., 1985; Gray et al., 1999). In these bacteria, PE constitutes 69–78 % of the total phospholipids in the plasma membrane (Ames, 1968). On the other hand, mitochondria from eukaryotes

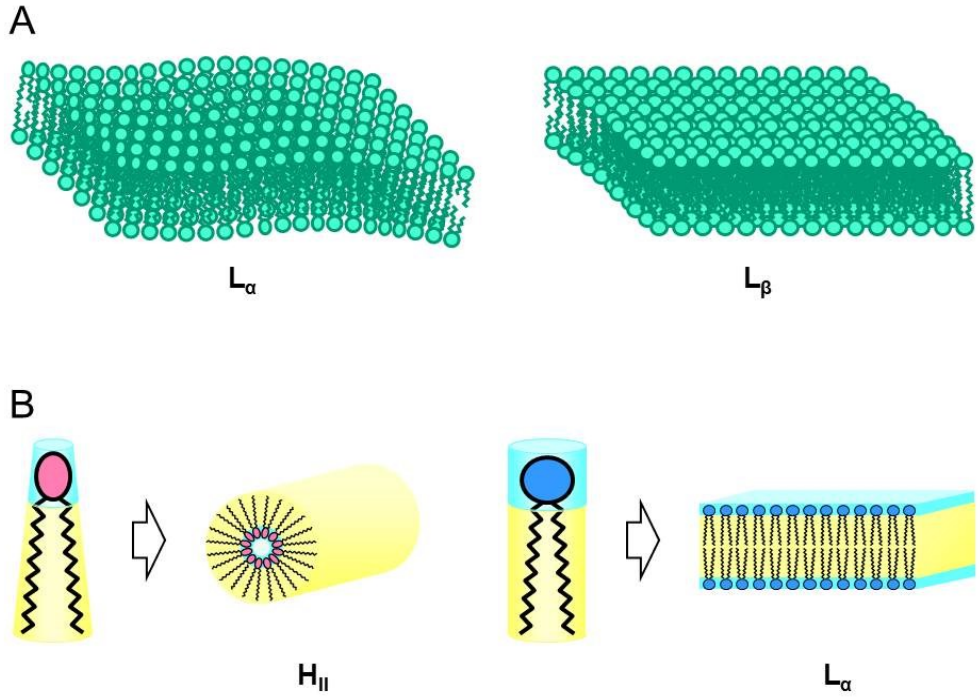


Fig. I-1 Structural representation of phospholipids in biological membranes.

A. thermotropic phase transition.

B. Shape of lipid molecules

L_α, fluid liquid crystalline phase; L_β, ordered gel phase; H_{II}, hexagonal II phase.

contain PE and PC as the major phospholipids, the sum of which constitutes ~78% of the phospholipids in plant mitochondria (Douce, 1985).

In *E. coli*, *pss* mutants that lack a functional enzyme for PS synthase show extremely low PE levels (0.007 % of the total phospholipids) because PE is solely synthesized from PS by PS decarboxylase (DeChavigny et al., 1991). *pss* mutants are only viable in the presence of divalent cations, which are thought to form a complex with CL and transform this acidic lipid into a non-bilayer-forming lipid (DeChavigny et al., 1991). *pss* mutants have normal ability to oxidize succinate and lactate, hydrolyze ATP, and generate proton gradients. However, these mutants have reduced capacity to oxidize NADH owing to a lower rate of electron transfer from the site of NADH oxidation to terminal oxidases. Thus, PE is dispensable for cellular respiration but has specific roles in electron transfer associated with NADH oxidation (Mileykovskaya and Dowhan, 1993).

In yeast and mammalian mitochondrial membranes, PE and PC are most abundant phospholipids and, to some extent, CL and PI are also contained. Using CL deficient mutants of yeast and mammalian cells, the function of CL in mitochondria has been studied. In yeast, *crd1* mutants have no CL in mitochondrial membranes, and the mutant show pleiotropic defects in mitochondrial function such as mitochondrial enzyme activities, maximum respiration capacity and protein import. Because of the inhibition of the respiratory complex activities, the mutants cannot maintain proton gradient across the mitochondrial inner membranes. Thus, CL is required to maintain the mitochondrial membrane potentials. In mammalian cells, CL is associated with the major respiratory complexes. *In vitro* experiments using isolated major complexes suggest that CL might be important in electron transfer process. These studies have

provided evidence that CL is required for proper biogenesis and function of mitochondria.

In many plant species, mitochondria have been reported to adjust their respiration capacity at low temperatures by increasing the levels of unsaturated phospholipids (Talts et al., 2004; Armstrong et al., 2008). Genetic evidence using Arabidopsis *fad2* mutants that are defective in 12-oleate desaturase demonstrates that membrane lipid unsaturation is important for maintaining proton gradient across the inner membrane at low temperature (Matos et al., 2007). On the other hand, the role of each phospholipid in the maintenance of mitochondrial function remains to be elucidated.

As described above, although the relationship between lipids and mitochondrial membranes or proteins has been shown in several organisms, little information has been reported in higher plants.

Respiration chain in plant mitochondria

Plants have two pathways for mitochondrial electron transport, namely the cytochrome oxidase pathway (COP) and the alternative oxidase pathway (AOP). Fig. I-3 summarizes the mitochondrial electron transport chains.

1. Cytochrome oxidase pathway (COP)

COP is a major electron transport pathway and is coupled with ATP production system. During glucose oxidation via glycolysis, pyruvate is incorporated into the matrix of mitochondria and oxidized to acetyl-CoA and CO2 by pyruvate dehydrogenase complexes. Acetyl-CoA is then oxidized by the tricarboxylic acid (TCA)

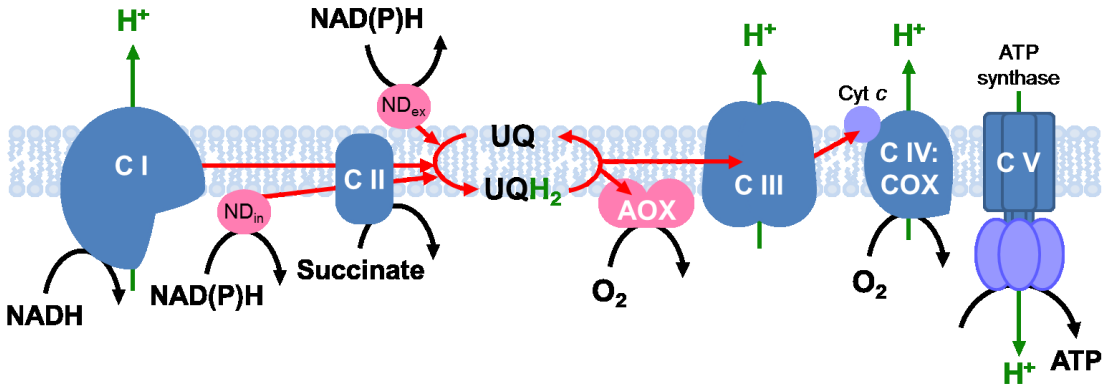


Fig. I-2 The electron transport chain in higher plants.

Red arrows indicate the electron flow.

C I–V, complex I–V; UQ, ubiquinone; UQH_2, ubiquinol; Cyt *c*, cytochrome *c*; ND_{ex}, external NAD(P)H dehydrogenase; ND_{in}, internal NAD(P)H dehydrogenase.

cycle or citric acid cycle, and the co-enzymes NAD^+ and FAD are reduced to NADH + H^+ and $FADH_2$, respectively. In mitochondria, the electrons from the reduced form of NAD^+ are transferred to the electron carrier proteins, complex I–IV. When the electrons move from complex to complex, protons are carried from the matrix to the inner membrane space through complex I, complex III and complex IV. The transfer of protons generates a proton gradient across the inner mitochondrial membranes.

The cytochrome *c* oxidase (COX, complex IV) is a terminal oxidase in COP. COX catalyzes the oxidation of reduced cytochrome *c*, and electrons are transferred to oxygen to produce water.

After making the proton gradient by proton carrier proteins, protons diffuse from the intermembrane space (the area of the high proton concentration) to the matrix (the area of the low proton concentration) through the inner mitochondrial membranes. When the protons reenter the matrix pass through ATP synthase (complex V), the proton movement causes proton motive force. The power is used for producing ATP from ADP and phosphate (Mitchell, 1961).

2. Alternative oxidase pathway (AOP)

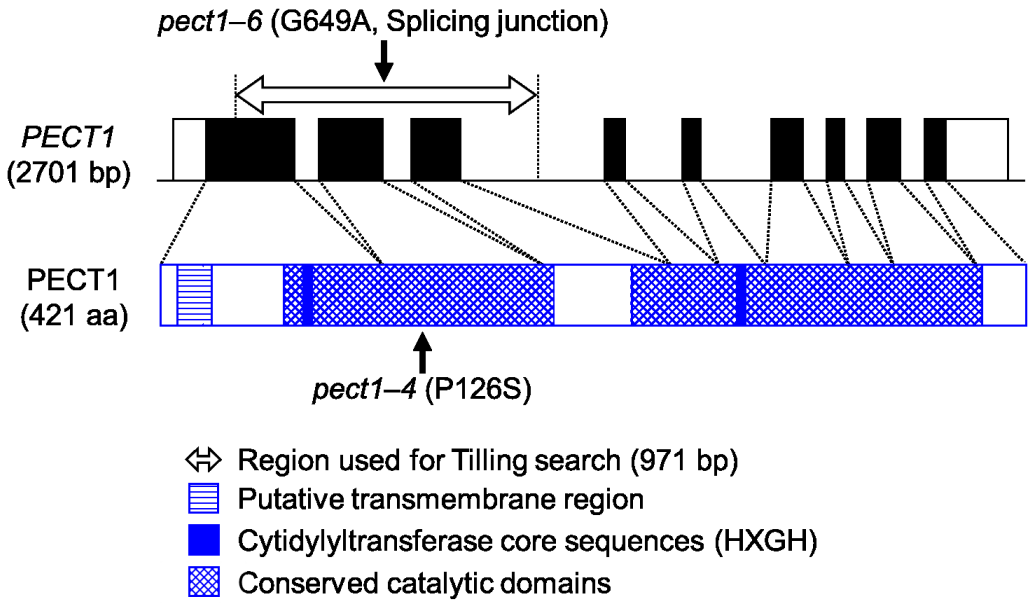
Unlike the case of COP, AOP is not coupled with the production of ATP. The alternative oxidase (AOX), a terminal oxidase in AOP, is found in plants as well as a number of fungi, several yeast and some protozoans, but not in mammals. The plant AOX is a functional dimer (Umbach and Siedow, 1993) and is located on the matrix side of inner mitochondrial membrane (Rasmusson et al., 1990). The oxidase directly catalyzes the oxidation of ubiquinol, by reducing oxygen to water as a product without proton translocation.

Wagner and Krab (1995) showed that partial inhibitions of COP are known to induce synthesis of AOX in plants. Furthermore, many researchers reported that the one of the AOP functions is to keep the low ubiquinone reduction levels in order to minimize the generation of reactive oxygen species when COP is inhibited by various stresses such as cold stress.

The aim of this study

Many researchers reported the importance of PE in biological membranes using several mutants lacking PE. However little is known about the importance of PE in higher plants.

Mizoi et al. (2006) have isolated several *pect1* mutant alleles. Among these mutant alleles, *pect1–4* and *pect1–6* show their visible phenotypes. *pect1–6* mutant appears to be null allele due to the mutation of splicing junction (Fig. I-3). *pect1–6* mutant shows embryonic lethality, demonstrating that the CDP-ethanolamine pathway is essential for PE biosynthesis in the plant. In *pect1–4* mutant, a single base substitution causes the amino acid substitutions (Fig. I-3). *pect1–4* is a mild mutant that exhibit 25.9 % of wild-type PECT1 activity and shows pleiotropic mutant phenotypes. *pect1–4/pect1–6* mutants show more severe phenotypes such as enhanced dwarfism or lower fertility compared to *pect1–4*. However, because of its heterozygotic property and severity of phenotypes, it is difficult to study the role of PE using this heterozygous mutant. On the other hand, *pect1–4* shows pleiotropic phenotypes and produces relatively fertile seeds. Mizoi et al. (2006) also showed that PECT1-EYFP is associated with mitochondrial outer surface. Because PE is a major component of mitochondrial membrane, and *pect1–4* decreases cellular PE levels compared to the wild type, I supposed that *pect1–4* might decrease mitochondrial PE levels and affect respiratory



Modified from Mizoi et al. (2006)

Fig. I-3 Structure of the *PECT1* gene and the PECT1 protein.

The *PECT1* gene structure (top) and the PECT1 protein structure (bottom). Top, the gene structure includes untranslated regions (open boxes), coding exons (closed boxes), and introns (lines between exons). An arrow indicates the site of a single base substitution in *pect1–6* mutant. Bottom, an arrow indicates the site of an amino acid substitution in *pect1–4* mutant.

aa, amino acids.

functions, which could be related to pleiotropic *pect1–4* phenotypes such as lower fertility or growth retardation. Respiratory capacity is highly correlated with the growth of an organism (Semikhatova et al., 1992). Thus, to investigate the importance of PECT1 in regulation of mitochondrial lipid compositions and of PE in mitochondrial respiratory capacity, I herein focus on the respiration capacity of Arabidopsis rosette plants, which extensively develop rosette leaves under short-day conditions.

In Chapter II, I show that total respiration capacity of rosette leaves changes with the development of rosette plants. I also show that respiration capacity decrease in *pect1–4* rosette leaves. In Chapter III, I show that reduction of total respiration capacity is due to low mitochondrial COP capacity in *pect1–4* rosette plants. One of the reasons for decreased COP capacity is due to partly inhibited COX activity in *pect1–4* mitochondria. In Chapter IV, I show that *pect1–4* mutation causes low PE levels in mitochondrial membranes at 5 weeks, whereas PE levels are comparable at 3 weeks.

In summary, I found that *pect1–4* leaves are unable to increase mitochondrial PE levels and COP respiration capacity in coordination with an increase in COX protein levels accompanied by rosette leaf development. I thus conclude that PECT1 is essential for keeping mitochondrial PE levels, which are important for maintaining respiration capacity in Arabidopsis leaves during prolonged growth under short-day conditions.

Chapter II

Respiration in Arabidopsis rosette leaves and *pect1–4* rosette leaves

Introduction

Plant mitochondria play a central role in respiration and biosynthesis of many essential biomolecules (Millar et al., 2008). Mitochondria also play important roles in cellular redox regulation and signaling (Rhoads and Subbaiah, 2007; Suzuki et al., 2012). However, comprehensive understanding of mitochondrial functions in growth and cellular homeostasis requires further studies.

As shown in Chapter I, plants have two pathways of mitochondrial electron transport, COP and AOP. The electron flow through the COP is coupled to ATP production and its terminal oxidase is COX. In contrast, the electron flow through the AOP is not coupled to ATP production and its terminal oxidase is AOX (Day et al., 1980).The respiration capacities can be measured using inhibitors with a Clark-type oxygen electrode. KCN and salicylhydroxamate (SHAM) are most commonly used inhibitors of COX and AOX, respectively (Wagner and Krab, 1995). Using these inhibitors, respiration capacity has been measured in various tissues. (Noguchi et al., 2005; Hachiya et al., 2010).

Mizoi et al. (2006) showed that *pect1–4* rosette plants exhibit 26% of wild-type PECT activity and cause pleiotropic phenotypes, such as seedling dwarfism at 8°C, low fertility and delayed embryo maturation. However, the relationship between these phenotypes and *pect1–4* mutation remains unclear.

In plants, mitochondrial inner membranes, which contain respiratory electron transport chains, include higher levels of PE than mitochondrial outer membranes (Mazliak et al., 1980). Because *pect1–4* rosette plants display reduced PE levels compared to the wild type by 7.9 % (Mizoi et al., 2006), it seems that *pect1–4* plants

might include lower levels of PE in mitochondrial membranes and hence affect mitochondrial respiratory functions.

I herein focus on the respiratory capacity of Arabidopsis plants grown under short-day conditions and examine the effect of *pect1–4* on leaf respiration. In rosette plants grown for 2 to 7 weeks under short-day conditions, I found that leaf respiration capacity changed with growth stages. When grown for more than 3 weeks under short-day conditions, *pect1–4* rosette plants significantly decreased the leaf respiration capacity caused by reduced COP capacity.

Materials and Methods

Plant material and growth conditions

Wild-type seeds of *Arabidopsis thaliana* (ecotype Columbia) were obtained from Lehle Seeds (http://www.arabidopsis.com/). Wild-type, *pect1–4* and *transPECT1* seeds (Mizoi et al., 2006) were sown on peat sheets (Sakata Seed, Yokohama, Japan) irrigated with water and vernalized at 4 °C for 2 days in darkness. Seeds were germinated and plants were grown in a climate chamber for up to 2–7 weeks at day/night temperatures of 23 °C/18 °C under a day/night light regime with an 8-h photoperiod at a photon flux density of 75 μmol m^{-2} s^{-1}.

Determination of chlorophyll content

The chlorophyll content in wild-type and *pect1–4* rosette leaves was determined according to Porra et al. (1989). Rosette plants were harvested at 2 to 5 weeks each and ground with a mortar and pestle. Chlorophylls were extracted in 80 % acetone. After centrifugation, the absorption of the supernatant was measured by spectrophotometer (UV-1800, UV spectrometer, Shimadzu, Kyoto, Japan). The chlorophyll contents were calculated according to Porra et al. (1989).

Measurement of leaf respiration capacity

Leaf respiration capacity was measured according to Hachiya et al. (2010). Rosette leaves (50–80 mg fresh weight) were sandwiched between nylon netting and inserted in a Clark-type oxygen electrode (6-ml chamber volume; Rank Brothers, Cambridge, UK) containing 4 ml assay buffer comprised of 50 mM HEPES (pH 6.6), 10 mM MES, and 0.2 mM $CaCl_2$. Total respiration (TR) capacity was defined as an

O_2-consumption rate in the dark in the absence of any inhibitor. COP capacity was defined as an O_2-consumption rate in the dark in the presence of 15 mM salicylhydroxamate (SHAM, 1 M stock in dimethyl sulfoxide) that was sensitive to 2 mM KCN (1 M stock in assay buffer). AOP capacity that is sensitive to 15 mM SHAM was defined as an O_2-consumption rate in the dark in the presence of 2 mM KCN. The O_2 concentration in air-saturated water at 25°C was assumed to be 253 mM (Truesdale and Downing, 1954), and the null oxygen concentration was adjusted by adding $Na_2S_2O_4$.

Statistical analysis

Statistical analysis was conducted using R version 2.12.1 (R Development Core Team 2010).

Results

Growth of wild-type and *pect1–4* rosettes under short-day conditions

Before measuring the respiration capacity of detached rosette leaves, I compared the growth profile of wild-type and *pect1–4* plants grown at 23 °C/18 °C for up to 7 weeks under 8 h/16 h light/dark cycles. Under these conditions, both wild-type and *pect1–4* rosette plants grew similarly with no visible symptoms of stress until 5 weeks (Fig. II-1A), but the only *pect1–4* plants showed leaf yellowing at 7 weeks (Fig. II-1B). The chlorophyll content was comparable in wild-type and *pect1–4* leaves at 3 weeks but was significantly lower in *pect1–4* leaves than in wild-type leaves at 5 weeks (Fig. II-2). These results suggested that *pect1–4* rosettes senesce earlier than wild-type rosettes.

The total respiration (TR) capacity changes with the growth of rosette plants

After 2 weeks of growth, wild-type rosettes exhibited an average TR rate of 193.8 ± 7.7 nmol O_2 min^{-1} mg fresh $weight^{-1}$ (FW^{-1}). However, the TR rates transiently decreased by 41% at 3 weeks and increased afterwards 2-fold by 5 weeks (Fig. II-3, Total, blue line). These results demonstrated that the TR capacity is developmentally regulated.

TR capacity is lower in *pect1–4* rosette plants

After 2 weeks of growth, *pect1–4* rosettes exhibited an average TR rate of 172.8 ± 3.7 nmol O_2 min^{-1} mg FW^{-1}, and this value was not significantly lower than that of wild-type leaves (Fig. II-3, Total, red line). The TR rates of *pect1–4* rosettes became comparable to those of wild-type rosettes at 3 weeks but increased only

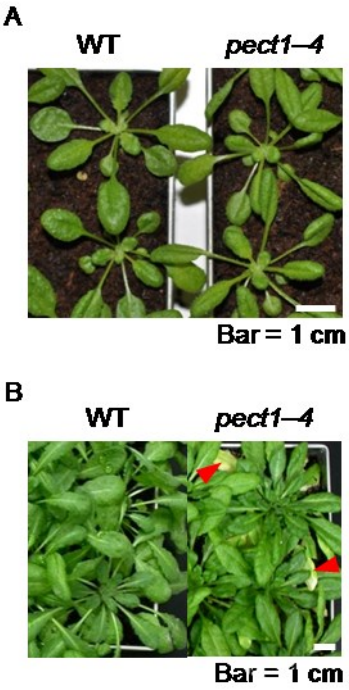


Fig. II-1 Growth of wild-type and *pect1–4* rosette plants under short-day conditions.

(A) Wild-type and *pect1–4* rosette plants grown for 5 weeks under short-day condition.

(B) Wild-type and *pect1–4* rosette plants grown for 7 weeks under short-day condition. Arrow heads indicate yellowing leaves.

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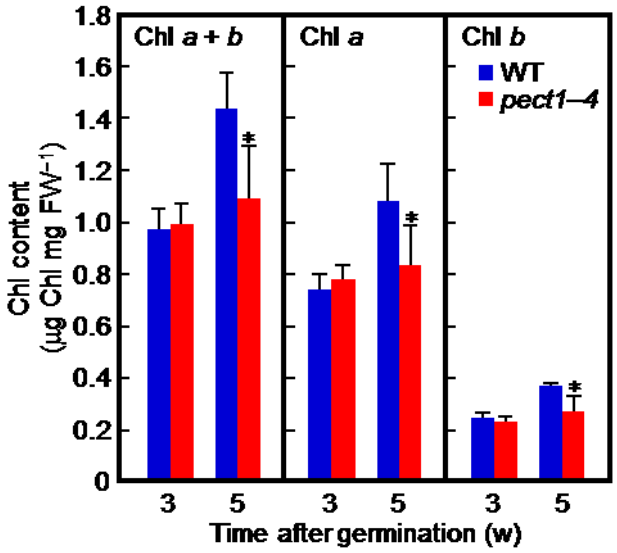

Fig. II-2 Chlorophyll (Chl) content in wild-type and *pect1–4* rosette plants.

Chl content (Chl *a* and Chl *b*) in rosette leaves grown under short-day condition was measured at 3 and 5 weeks. Bars indicate the mean ± SD (n = 9–10). **P* < 0.01, comparing mutant to wild type using Tukey's multiple comparison test.

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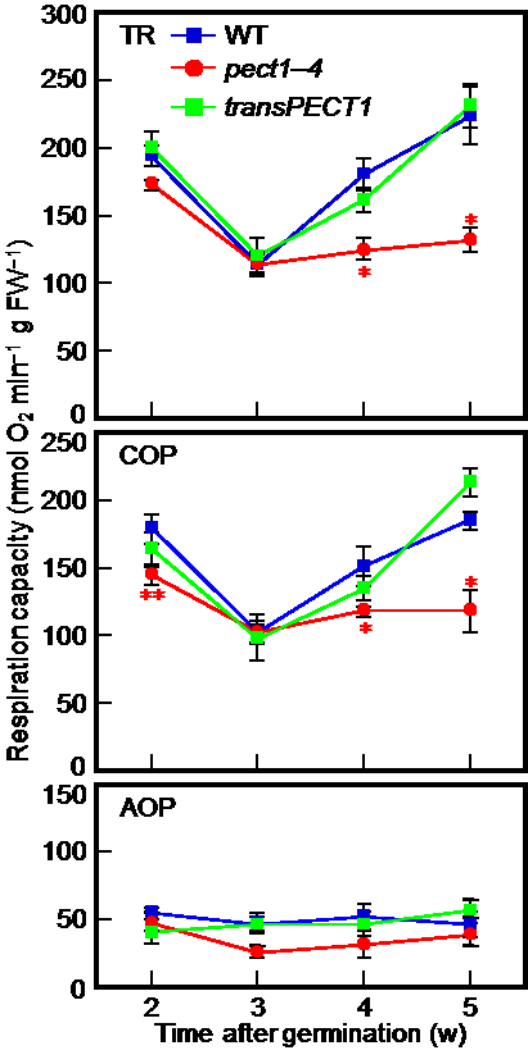

Fig. II-3 Respiration capacity in wild-type (WT), *pect1-4* and *transPECT1-4* rosette plants grown for 2 to 5 weeks.

The TR capacity was measured at 25 °C in the absence of any inhibitors, whereas COP and AOP respiration capacities were measured in the presence of 15 mM SHAM and 2 mM KCN, respectively. Bars indicate the mean ± SD (n = 3–10). **P* < 0.01, ***P* < 0.05, comparing mutant to wild type using Tukey's multiple comparison test.

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1.1-folds by 5 weeks, resulting in a 41% relative decrease at 5 weeks compared to the wild type. These results demonstrated that the TR capacity is lower in *pect1–4* rosettes compared with that in the wild type at growth stages later than 3 weeks.

COP respiration capacity is lower in *pect1–4* leaves

The COP and AOP respiration capacities were measured in the presence of 15 mM SHAM (for COP) and 2 mM KCN (for AOP) using wild-type and *pect1–4* plants grown for 2–5 weeks. COP respiration rates were lower in *pect1–4* leaves than in wild-type leaves at all growth stages except 3 weeks (Fig. II-3, COP). In contrast, AOP respiration rates were indistinguishable in wild-type and *pect1–4* leaves at all growth stages (Fig. II-3, AOP). These results suggested that the reduced TR capacity of *pect1–4* leaves is attributed to downregulation of COP capacity. Both TR and COP capacities were restored in transgenic *pect1–4* plants expressing genomic *PECT1* (Fig. II-3, *transPECT1*, green line). These results demonstrated that *pect1–4* is responsible for the observed lower leaf respiration capacity.

Discussion

In wild-type rosette plants grown under short-day conditions, the leaf TR capacity decreased transiently at 3 weeks but increased afterwards. Arabidopsis plants continuously develop new rosette leaves under short-day conditions, whereas the onset of bolting around 3 weeks inhibits new rosette leaf development under long-day conditions (Mozley and Thomas, 1995). Thus, the increase in leaf respiration capacity after 3 weeks under short-day conditions apparently correlates with extensive vegetative growth of the wild type after 3 weeks.

pect1–4 plants maintain a respiration capacity that is similar to that in wild-type plants until 3 weeks. However, *pect1–4* plants are unable to maintain both TR and the COP capacity to the extent found in the wild type until 5 weeks. I noted that *pect1–4* rosette plants showed slightly reduced leaf chlorophyll levels at 5 weeks and showed leaf yellowing at 7 weeks. Furthermore, *pect1–4* plants showed early flowering under long-day (16 h/8 h) conditions (data not shown). Thus, maintenance of the leaf respiration capacity after 5 weeks could be important for the longevity of rosette leaves under the growth conditions examined in the present study.

In addition, TR capacity decreased due to the reduced COP capacity in *pect1–4* rosette leaves, suggesting that mitochondrial COP capacity is decreased in *pect1–4* plants. In the next Chapter, I attempted to confirm that the COP capacity might be reduced in *pect1–4* mitochondria.

Chapter III

Mitochondrial COP capacity and COX activity in *pect1–4* rosettes

Introduction

In Chapter II, I showed that the TR capacity decreases in *pect1–4* rosette leaves due to reduced COP capacity. This result suggested that *pect1–4* plants might have some defects in mitochondrial COP capacity. Accordingly, I isolated mitochondria from rosette leaves grown under short-day conditions, and compared the respiration capacity between wild-type and *pect1–4* mitochondria.

Protocols for isolating highly purified and functional mitochondria have been described for many species, including spinach (Bergman et al., 1980; Gardeström et al., 1978), pea (Day et al., 1985), soybean (Gerard and Dizengremel, 1988), barley (Lernmark et al., 1991) and potato (Bykova et al., 2005). Arabidopsis is an invaluable tool for genomic and proteomic studies. However, Keech et al. (2005) reported two methods, one is to isolate crude mitochondria and the other is to isolate purified mitochondria, which improved the points such as contaminations and limited quantity. According to their methods, I was able to obtain high quality mitochondria from Arabidopsis rosette leaves.

In this chapter, I found that *pect1–4* mitochondria exhibit decreased COP capacity due to COX inhibition in *pect1–4* mitochondria.

Materials and Methods

Plant material and growth conditions

Plants were grown as described in Chapter II.

Preparation of *A. thaliana* mitochondria from rosette leaves

Purified mitochondria were prepared according to Keech et al. (2005). Briefly, rosette leaves (10–12 g) were ground with a mortar and pestle, together with 0.5 g quartz (Merck KGaA, Darmstadt, Germany) and 15 ml grinding buffer consisting of 0.3 M sucrose, 60 mM TES (pH 8.0), 10 mM KH_2PO_4, 25 mM tetrasodium pyrophosphate, 50 mM sodium ascorbate, 20 mM cysteine, 2 mM EDTA, 1 mM glycine, 1 % polyvinylpyrrolidone-40, and 1 % defatted bovine serum albumin (BSA). Tissue homogenates were filtered through a nylon mesh (mesh size, 20 microns; Kyoshin Rikoh Inc., Tokyo, Japan) together with a rinse with 20 ml fresh grinding buffer. The resultant filtrate (designated homogenate) was centrifuged at 2,500 × *g* for 5 min at 4 °C to remove thylakoid membranes and then at 15,000 × *g* for 15 min at 4 °C to recover the crude mitochondrial pellet. The pellet was then resuspended in 0.5 ml suspension buffer (pH 7.5) consisting of 0.3 M sucrose, 10 mM TES, and 10 mM KH_2PO_4, using a chilled 5-ml glass homogenizer and a Teflon pestle. For purification of mitochondria, a Percoll gradient (6 ml each) was made in 10-ml centrifugation tubes (13PET TUBE ASSY, HITACHI Koki Co., Japan) by centrifugation of a solution (pH 7.5) containing 50 % (v/v) Percoll, 300 mM sucrose, 10 mM TES, 1 mM EDTA, 10 mM KH_2PO_4, and 1 mM glycine at 39,000 × *g* for 40 min at 4 °C. Then, 0.5–0.7 ml each suspension of crude mitochondria was laid over the top of the Percoll gradient. After centrifugation at 15,000 × *g* for 15 min at 4 °C, mitochondria that formed a whitish band close to the tube

bottom were recovered, diluted 20-fold with suspension buffer, and centrifuged again at 15,000 × *g* for 20 min at 4 °C. The pellet was resuspended in 0.2–0.5 ml suspension buffer (1.5–7.5 μg protein ml^{-1}). The protein content was determined according to Bradford (1976) using γ-globulin as a standard.

Integrity and purity of isolated mitochondria

Mitochondrial integrity was determined as described by Neuburger et al. (1982) by evaluating the latency of COX activity. The purity of mitochondria was estimated as described by Keech et al. (2005). Briefly, peroxisomal HPR activity was measured according to Winter et al. (1982) by measuring the decrease in absorbance at 340 nm (reduction of NAD^+) in 1 ml assay medium. Cytosolic PEPC activity was measured according to Gardeström and Edwards (1983) by measuring the increase in absorbance at 340 nm (oxidation of NADH) owing to a coupled reaction with malate dehydrogenase.

Measurement of mitochondrial respiration capacity

Mitochondrial respiration capacity was measured according to Robson and Vanlerberghe (2002). Immediately after mitochondria were isolated, O_2 consumption in the dark was measured in a Clark-type oxygen electrode (1-ml chamber volume; Rank Brothers) in 0.4 ml reaction medium containing 10 mM TES (pH 7.5), 300 mM sucrose, 5 mM KH_2PO_4, 2 mM $MgSO_4$, 0.1 % (w/v) BSA, and 0.1 mM each NAD, NADP, ATP, and thiamine pyrophosphate. To initiate the measurement, 44 μl of a substrate mixture was added to make final concentrations of the following substrates: 2 mM ADP, 2 mM NADH, 10 mM each succinate, malate, and glutamate, 1 mM pyruvate, and 10 mM dithiothreitol (Vanlerberghe et al., 1998; Robson and Vanlerberghe, 2002).

Approximately 0.15–0.25 mg mitochondrial protein per milliliter was used in each measurement. COP and AOP capacities were measured in the presence of 2 mM SHAM or 1 mM KCN, respectively. Stock solutions of pyruvate, NADH, and dithiothreitol were freshly made before use, and other chemicals were stored frozen at −80 °C.

SDS–PAGE analysis of mitochondrial proteins

SDS–PAGE was performed according to Laemmli (1970). Mitochondrial proteins (20 μg) were loaded in each lane of a 12.5 % (w/v) polyacrylamide gel. After electrophoresis, the gel was stained with a silver staining kit (2D–SILVER STAIN II, DAIICHI, Tokyo, Japan).

Immunoblot analysis

Mitochondria equivalent to 20 mg protein were suspended in sample buffer containing 50 mM Tris-HCl (pH 6.8), 2 mM EDTA, 1 % SDS, 0.05 % bromophenol blue, and 0.1 M dithiothreitol. Proteins were separated with SDS-PAGE on a 12.5 % (w/v) polyacrylamide gel and electroblotted onto a polyvinylidene difluoride membrane (Immobilon™, Millipore) at 60 V for 30 min using a Trans-Blot Wet Electrophoretic Transfer Cell (SYSTEM INSTRUMENTS Co., Ltd., MARYSOL, Tokyo, Japan) and blotting buffer containing 25 mM Tris, 192 mM glycine (pH 8.3) and 20 % (v/v) methanol. Protein bands detected immunochemically using a Can Get Signal System (TOYOBO, Osaka, Japan) and 2 % ECL Advance Blocking reagent (GE Healthcare, Amersham, UK). Rabbit polyclonal anti-COX II (Agricera, Vännäs, SWEDEN) was diluted 1:100,000. Goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate (Bio-Rad Laboratories, USA; diluted 1:200,000) was used as the secondary antibody. Protein bands were detected with a chemiluminescence detection kit (GE Healthcare).

Membranes were exposed to X-ray film (FUJIFILM Corporation, Tokyo, Japan) and visualized using Fuji Medical Film Processor FPM100 (FUJIFILM Corporation).

Measurement of COX activity

COX activity was measured at 25 °C according to Noguchi et al. (2005). Each reaction (800 μl) contained 10 mM TES (pH 7.5), 300 mM sucrose, 10 mM NaCl, 5 mM KH_2PO_4, 2 mM $MgSO_4$, and 0.1 % (w/v) BSA. The reaction was initiated by adding 20 μl purified mitochondria (20 μg protein), 10 mM sodium ascorbate, 25 mM reduced cytochrome *c*, and 0.05 % (w/v) Triton X-100.

Statistical analysis

Statistical analysis was conducted using R version 2.12.1 (R Development Core Team 2010).

Results

COP respiration capacity decreased in *pect1–4* mitochondria

Because leaf TR capacity differed most significantly at 5 weeks between wild-type and *pect1–4* rosettes, I compared the respiration capacity of mitochondria isolated from 5-week-old plants according to Keech et al. (2005). The integrity of the outer membrane of isolated mitochondria was ~92 % as estimated by the latency of COX (Table III-1). The purity of isolated mitochondria was comparable to that of the original method (Keech et al. 2005) as estimated by the co-purified activity of peroxisomal hydroxypyruvate reductase (HPR) and cytosolic phosphoenolpyruvate carboxylase (PEPC) (Table III-1). Protein profiles were indistinguishable between wild-type and *pect1–4* mitochondria (Fig. III-1).

Fig. III-2 shows the TR of mitochondria isolated from wild-type plants (206.6 ± 13.9 nmol O_2 min^{-1} mg $protein^{-1}$) and *pect1–4* plants (144.5 ± 8.0 nmol O_2 min^{-1} mg $protein^{-1}$) at 5 weeks, demonstrating a 30 % decrease in TR capacity in *pect1–4* mitochondria ($P < 0.01$). In the presence of 2 mM SHAM, wild-type and *pect1–4* mitochondria exhibited average COP respiration rates of 129.0 ± 21.4 and 73.8 ± 9.6 nmol O_2 min^{-1} mg $protein^{-1}$, respectively, demonstrating a 43 % decrease in COP respiration capacity in *pect1–4* mitochondria ($P < 0.01$).

COX activity but not COX II protein level was lower in *pect1–4* mitochondria

Because *pect1–4* mitochondria showed a lower COP respiration capacity compared with wild-type mitochondria, we examined cytochrome *c* oxidase subunit II (COX II) protein levels in 3- and 5-week-old plants, respectively (Fig. III-3A) and maximum COX activity (Fig. III-3B) in mitochondria purified from 5-week-old plants.

Table III-1. The integrity and purity of isolated mitochondria

	Purity (nmol NAD min^{-1} mg $protein^{-1}$)				Integrity (%)	Yield of mitochondria (μg protein g FW^{-1})
	HPR[a]		PEPC[b]			
	Purified	Crude	Purified	Crude		
WT	6.2 ± 2.1	74.7 ± 2.0	N.D.[c]	4.3 ± 0.4	92.9 ± 2.9	82.1 ± 11.0
pect1–4	6.9 ± 1.7	86.6 ± 7.2	N.D.	5.1 ± 0.5	92.2 ± 0.9	64.6 ± 6.7

[a]HPR, peroxisomal hydroxypyruvate reductase
[b]PEPC, cytosolic phospho*enol*pyruvate carboxylase
[c]N.D., not detected

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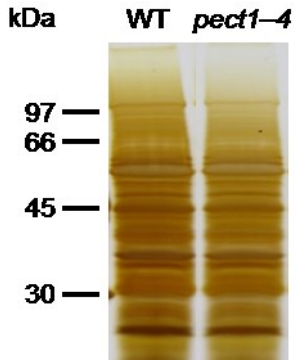

Fig. III-1 SDS-PAGE analysis and silver staining of mitochondrial proteins.

Mitochondrial proteins from 5-week-old plants equivalent to 20 μg protein were loaded in each lane.

Taken from Otsuru et al. (2013) with permission.

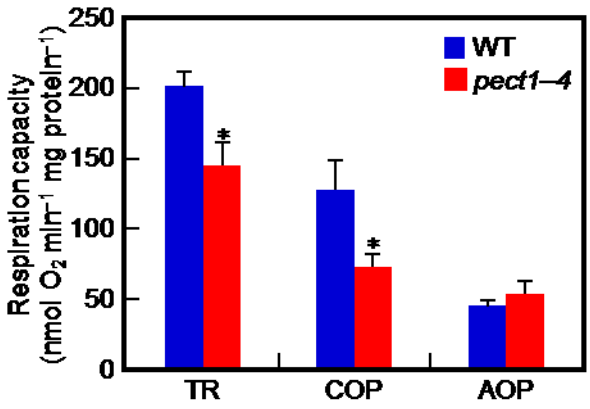

Fig. III-2 Respiration capacity in mitochondria isolated from wild-type (WT) and *pect1–4* plants at 5 weeks.

TR and the COP and AOP respiration capacities were measured using purified mitochondria. Bars indicate the mean ± SD (n = 3–4). $*P < 0.01$, comparing mutant to wild type using Tukey's multiple comparison test.

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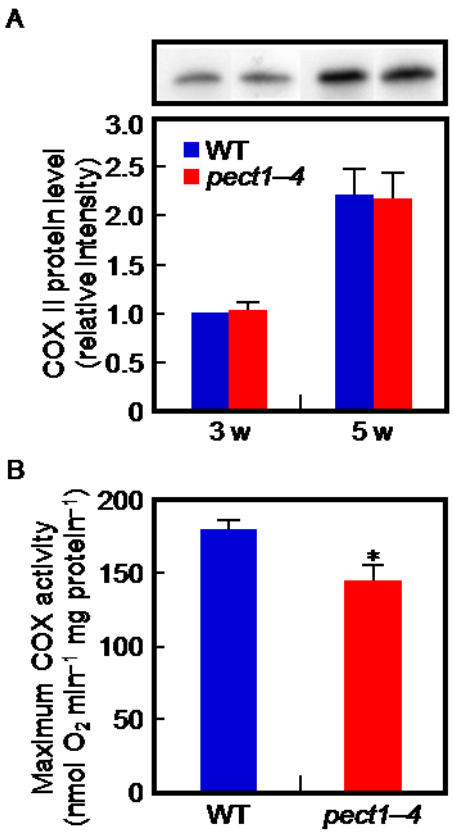

Fig. III-3 COX II protein level and COX activity in wild-type (WT) and *pect1–4* mitochondria.

(A) Immunoblot analysis of COX II using anti-COX II polyclonal antibody (top). Mitochondrial proteins from 3- and 5-week-old plants equivalent to 20 μg protein were loaded in each lane. The band intensity was quantified using ImageJ software. Bars indicate the mean ± SD (n = 3–5).

(B) COX activity in mitochondria purified from 5-week-old plants is expressed as O_2 uptake rate during oxidation of reduced cytochrome *c*. $*P < 0.01$, comparing mutant to wild type using Tukey's multiple comparison test.

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The maximum COX activity in *pect1–4* mitochondria was 20 % lower than that in wild-type mitochondria (Fig. III-3B, $P < 0.01$), whereas COX II protein levels were comparable in wild-type and *pect1–4* mitochondria (Fig. III-3A). Furthermore, COX II protein levels increased 2-fold in both wild-type and *pect1–4* mitochondria from 3 to 5 weeks (Fig. III-3A). These results suggested that *pect1–4* did not affect *COX* expression, at least between 3 and 5 weeks, but did affect COX activity in plants grown longer than 3 weeks under this growth conditions.

Discussion

Wild-type plants increased leaf TR capacity at 5 weeks compared to at 3 weeks, which is due to an increased mitochondrial COP capacity that is attributable to a higher COX II protein level. In contrast, *pect1–4* plants were unable to increase their leaf TR capacity as much as the wild type from 3 to 5 weeks, which is due to a relatively lower mitochondrial COP capacity after 3 weeks. Because COX II protein level was found to be similar between wild-type and *pect1–4* mitochondria, a reduction in COP capacity in *pect1–4* mitochondria is likely due to the relative reduction in COX activity.

In wild-type mitochondria, COX II protein levels increase from 3 to 5 weeks, which parallels with a COP capacity increases. Increased mitochondrial COP capacity suggested that higher ATP production is probably required to develop rosette leaves under short-day conditions. To investigate why COP capacity was reduced in *pect1–4* mitochondria, I examined whether COX protein levels and/or maximal COX activity decrease in *pect1–4* mitochondria. *pect1–4* mitochondria have comparable COX II protein levels but lower COX activity compared to the wild type.

Since PECT1 is the key enzyme for PE biosynthesis, which is localized in mitochondrial outer membrane, the decreased COX activity might be correlated with alteration of mitochondrial lipid composition in *pect1–4*. Therefore, I compared lipid composition between wild-type and *pect1–4* mitochondria in Chapter IV.

Chapter IV

Lipid composition in *pect1–4* mitochondria

Introduction

In Chapter III, I showed that respiration capacity decreased in *pect1–4* mitochondria because of the low COX activity. Why does the COX activity decrease in *pect1–4* mitochondria? Because *pect1–4* is a mutation in PE biosynthesis, I hypothesized that *pect1–4* might affect mitochondrial lipid composition, thereby affecting COX activity. In Chapter II, I showed that leaf COP capacity is comparable at 3 weeks but decreases at 5 weeks in *pect1–4* rosettes compared to the wild type. Thus, I suspected that PE levels might be comparable at 3 weeks but decrease at 5 weeks in *pect1–4* mitochondria compared to the wild type.

Mitochondrial membranes contain two major phospholipids, PE and PC, and three minor acidic lipids, CL, PG, and PI. In addition, PS is present at very low levels, because mitochondria appear to contain PS decarboxylase that rapidly converts PS to PE (Rontein et al., 2003). These phospholipids are important to maintain the structure and function of mitochondria (Douce, 1985).

PE, a nonbilayer-forming lipid in extraplastidial membranes (Block et al., 1983), is enriched in mitochondrial membranes (Douce et al., 1973). In Arabidopsis, mitochondria appear to uptake PE from the endoplasmic reticulum, where PE is synthesized from diacylglycerol and CDP-ethanolamine by aminoalcoholphosphotransferase (EC 2.7.8.1 and EC 2.7.8.2). On the other hand, CTP:phosphorylethanolamine cytidylyltransferase (PECT1) synthesizes CDP-ethanolamine and regulates PE biosynthesis in the endoplasmic reticulum associated with the mitochondrial outer membrane (Heazlewood et al., 2004; Millar et al., 2005; Taylor et al., 2005; Mizoi et al., 2006). Although PE is major phospholipid in

mitochondrial membranes, the importance of PE in plant mitochondrial functions in higher plants is not clear.

In this Chapter, I isolated mitochondria from 3- and 5-week-old rosette leaves and conducted lipid analysis. I found that PE levels decreased in *pect1–4* mitochondria isolated from 5-week-old rosette leaves. I found that PECT1 is important for maintaining mitochondrial PE levels, and the mitochondrial PE levels are important for COX activity and hence the COP capacity.

Materials and Methods

Plant material and growth conditions

Plants were grown as described in Chapter II.

Preparation of *A. thaliana* mitochondria from rosette leaves

Mitochondria were isolated from rosette leaves as described in Chapter III.

Analysis of mitochondrial lipids

Lipids were extracted from purified mitochondria (0.8–1.5 mg protein) and quantified with a gas-liquid column chromatograph (Inatsugi et al., 2002).

After purified mitochondria were obtained from rosette leaves, they were immediately immersed in 5 ml 2-propanol with 0.01 % 2,6-di-*t*-butyl-4-methylphenol (BHT) at 80 °C for 5 min and then chilled on ice. Lipids were then extracted according to Bligh and Dyer (1959). Total lipid extracts were separated into representative lipid classes by two-dimensional silica gel thin layer chromatography. For the first and second development, acetone:toluene:methanol:water = 8:3:2:1 (by volume) (Sato et al., 1988) and chloroform:methanol:acetic acid:water = 170:25:25:4 (by volume) (Whitaker, 1991) were used, respectively. Lipid spots were viewed under ultraviolet light after spraying with primuline (0.01 % in 80 % acetone). Each lipid class was subjected to methanolysis at 80 °C for 3 h in 3 ml of 5 % (w/v) HCl in methanol, with an additional 50 nmol pentadecanoic acid (15:0) as an internal standard and 10 nmol BHT as an antioxidant, and the resultant fatty acid methyl esters were extracted with 3 ml of distilled hexane. Lipid content was determined by quantifying fatty acid methyl esters with a gas chromatograph (GC-18A, Shimadzu, Kyoto, Japan) equipped with a fused

silica capillary column (0.25 mm ø × 50 m, HR-SS-10, Shinwa Chemical Industries, Kyoto, Japan) and a data processor (C-R6A, Shimadzu). Temperatures for the injector, column and detector chambers were set at 250 °C, 170 °C and 250 °C, respectively.

Statistical analysis

Statistical analysis was conducted using R version 2.12.1 (R Development Core Team 2010).

Results

pect1–4 has a lower PE level in mitochondria isolated from 5-week-old plants

I examined whether *pect1–4* affects the PE level in mitochondria (Fig. IV-1). In mitochondria isolated from 5-week-old plants, no significant difference was found in the total amount of lipids per mitochondrial protein between the wild type (0.87 ± 0.25 mg lipid/mg protein) and *pect1-4* mutant (0.91 ± 0.39 mg lipid/mg protein). In wild-type mitochondria, PE and PC constituted 46.9 ± 4.1% and 33.9 ± 3.6 % of the total phospholipids, respectively. CL, PI, and PG accounted for 11.7 ± 0.7 %, 4.6 ± 1.5 %, and 2.8 ± 1.7 %, respectively. In *pect1–4* mitochondria, PE levels were lower than those in the wild type by 24 %, accounting for 35.7 ± 2.2 % of the total phospholipids; PC levels were higher than those in the wild type by 26 %, accounting for 42.8 ± 0.5 % of the total phospholipids. CL, PG, and PI levels were comparable to those found in wild-type mitochondria (Fig. IV-1A). In mitochondria isolated from 3-week-old plants, however, no significant difference was observed in major phospholipid compositions between wild-type and *pect1–4* (Fig. IV-1B). These results demonstrated that *pect1–4* is unable to maintain mitochondrial PE levels as in the wild type when grown for longer than 3 weeks under short-day conditions.

No significant difference in the compositions of major unsaturated fatty acids in *pect1–4*

At 5 weeks, PE and PC levels were significantly different between *pect1–4* and wild-type mitochondria. I therefore determined whether fatty acid compositions of each lipid class in *pect1–4* mitochondria are changed in *pect1–4* mitochondria.

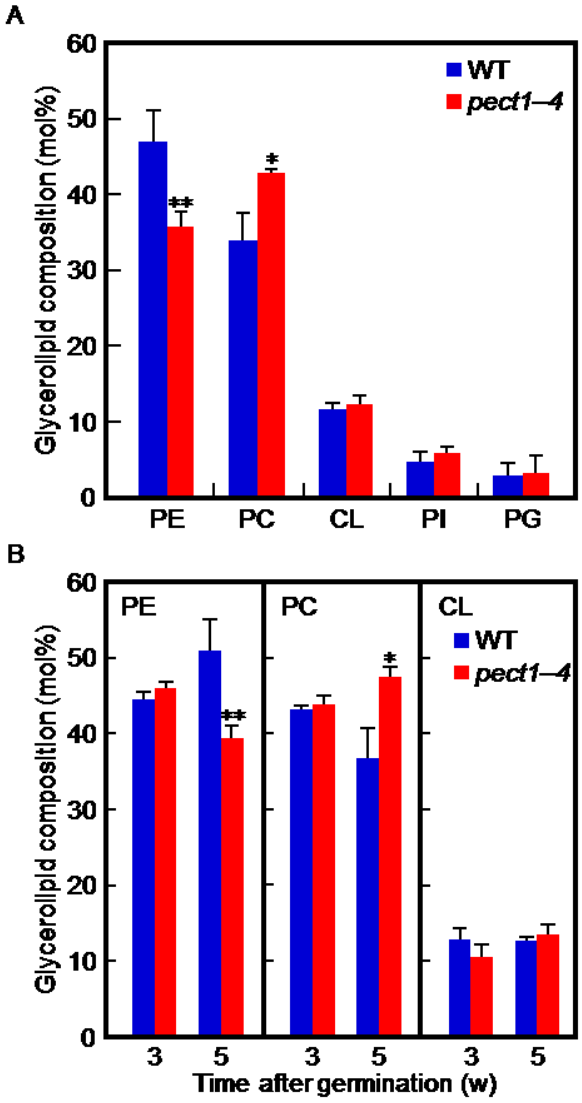

Fig. IV-1 Glycerolipid composition in mitochondria purified from wild-type (WT) and *pect1–4* rosettes.

Total lipid was extracted from mitochondria. PE, PC, CL, PI, and PG were separated with thin-layer chromatography and quantified by gas chromatography.

(A) Lipid composition in mitochondria isolated from 5-week-old plants.

(B) Comparison of PE, PC, and CL levels in mitochondria between 3 and 5 weeks.
$*P < 0.01$, $**P < 0.05$, comparing mutant to wild type using Tukey's multiple comparison test.
Taken from Otsuru et al. (2013) with permission.

The compositions of major unsaturated fatty acids were equivalent between wild-type and *pect1–4* mitochondria (Fig. IV-2); PE and PC included 16:0, 18:0, $18:1^{\Delta 9}$, $18:1^{\Delta 11}$, 18:2 and 18:3 fatty acids, and CL, PI and PG included 16:0, 18:0, $18:1^{\Delta 9}$, 18:2 and 18:3 fatty acids. PG included 16:0, 16:1, 18:0, $18:1^{\Delta 9}$, 18:2 and 18:3 fatty acids in wild-type mitochondria, but not detected 16:1 fatty acid in *pect1–4* mitochondria.

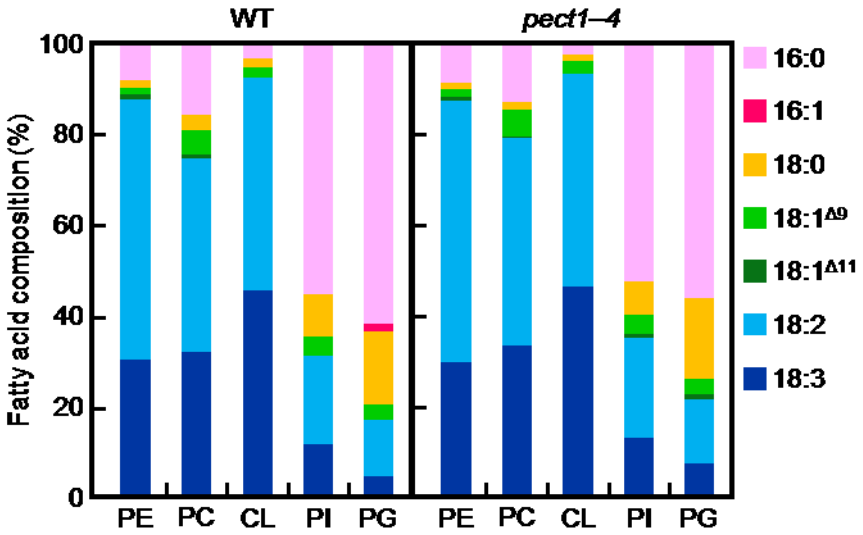

Fig. IV-2 Fatty acid compositions in mitochondria isolated from 5-week-old wild-type and *pect1–4* rosette plants.

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Discussion

Wild-type and *pect1–4* mitochondria had similar PE levels at 3 weeks, accounting for 45–46 % of the major phospholipids (PE, PC, and CL; Fig. 5B). Then, PE levels increased in wild-type mitochondria (from 44 % at 3 weeks to 51 % at 5 weeks relative to the sum of PE, PC, and CL), whereas PE levels decreased in *pect1–4* mitochondria (from 46 % at 3 weeks to 39 % at 5 weeks relative to the sum of PE, PC, and CL). Increase of PC levels compensated for decrease of PE levels. Because no significant difference was found in the compositions of major unsaturated fatty acids between wild-type and *pect1–4* mitochondria (Fig. IV-2), our results suggest that the relative reduction in maximum COX activity at 5 weeks may be primarily attributed to the decrease in PE levels and/or decreased PE:PC ratios in *pect1–4* mitochondria. Although the maximum COX activity was 20 % lower in *pect1–4* mitochondria compared with wild-type mitochondria (Chapter III, Fig. III-3B), a 43 % reduction in COP capacity was observed in *pect1–4* mitochondria (Chapter III, Fig. III-2). Therefore, COP capacity was decreased due to not only reduction of COX activity. Thus, a decreased PE level (and/or decreased PE:PC ratios) may have additional negative effects on the respiration chain of COP.

Increases of COX protein levels from 3 to 5 weeks are comparable between wild-type and *pect1–4* mitochondria, whereas PE levels decrease from 3 to 5 weeks in *pect1–4* mitochondria. These results suggest that decrease of COX activity is not due to decreased COX protein levels, but due to low PE levels in *pect1–4* mitochondria. The decrease of COP capacity in *pect1–4* mitochondria was correlated with a decrease of PE levels in mitochondrial membranes.

Chapter V

General Discussion and Future Perspectives

Biological membranes contain many kinds of glycerolipids with different head groups and fatty acids. The composition of glycerolipids in membranes may be important for the activities and functions of organelle membranes. Glycerolipids are known to determine biophysical properties of membranes such as fluidity and propensity to thermotropic phase transition. Genetic studies using mutants defective in the biosynthesis of specific classes of glycerolipids have revealed physiological roles of the lipid classes in different organisms.

In this thesis, I have conducted my study for elucidating the significance of PE in mitochondrial respiration. I first investigated the leaf respiration capacity in Arabidopsis rosettes and found that the leaf respiration capacity increases with the growth of rosette plants under short-day conditions, which may be required for a sustained high metabolic rate in rosette leaves (Chapter II). I also showed that PECT1 regulates the mitochondrial PE level, which is important for the maintenance of maximal COX activity and hence COP capacity (Chapters III and IV). These findings gave me an opportunity to study the unique role of PE in higher plants.

In this chapter, I would like to discuss unanswered questions and future perspectives as well as unexpected findings that I obtained during the course of this study.

The relationship between PE and respiration capacity has been investigated in various organisms. In mice, *psd* mutants with defects in mitochondrial PE synthesis show embryonic lethality and abnormal mitochondrial morphology (Steenbergen et al.,

2005). PE is required for COX dimer formation in bovine heart mitochondria (Shinzawa-Itoh et al., 2007). In yeast, mitochondrial PE is synthesized by mitochondrial PS decarboxylase 1 (Horvath et al., 2012). Recently, Böttinger et al. (2012) reported that *psd1* mutants display a ~90 % decrease in PE in mitochondrial inner membrane and ~50 % decrease in both O_2 consumption and COX activity compared with the wild type. These mutants have a decreased membrane potential and hence decreased protein import activity across the inner membrane, which correlates with a slight decrease in COX protein levels.

In the present study, I showed that COX activity was reduced in *pect1–4* mitochondria from 5-week-old plants, whose mitochondria contain lower PE levels and higher PC levels than wild-type mitochondria. These results provide additional evidence to support my hypothesis that mitochondrial PE levels are important for COX activity in higher eukaryotes. However, how is the COX activity affected in *pect1–4* mitochondria remains to be elucidated in future studies. PE might be required for COX dimer formation in plant mitochondria as is the case with bovine heart mitochondria. Thus, it is interesting to analyze the composition of respiratory chain complexes by blue-native PAGE.

I showed that COP capacity decrease due to COX activity in *pect1–4* plants and discussed additional negative effects on the respiration chain of COP (Chapter IV, Discussion). What is the additional negative effect? Because AOP capacity is comparable between wild-type and *pect1–4* mitochondria, decreased COP capacity in *pect1–4* mitochondria might be due to reduced electron flow. If electron flow is reduced, there are two possibilities: other respiratory complex activities might be affected in *pect1–4* mitochondria; and secondly, levels of co-enzymes, NADH and $FADH_2$, might

be decreased due to downregulation of the citric acid cycle or glycolysis activities by *pect1–4*. In this study, I measured mitochondrial respiration capacities using mixed substrates. Thus, I need to test the possibility of these cases in future studies, using a single respiratory substrate.

I showed that PE levels decrease and PC levels increase in *pect1–4* mitochondria. Because PE and PC are abundant lipids, the mitochondrial structure might be affected in *pect1–4* mitochondria. Transmission electron microscopic observation of *pect1–4* mitochondria is important to understand if the lipid changes influence mitochondrial structures.

pect1–4 plants show dwarf phenotype at 8 °C under long-day conditions (Mizoi et al., 2006). The cold-sensitive character of soybean is reflected by the lipid changes in mitochondrial membranes. When exposed to an increase of temperature (from 25 to 35 °C), mitochondrial membranes were characterized by a higher PC:PE ratio and a lower content in 18:3 fatty acids. After a reduction of temperature (from 25 to 18 °C), the opposite changes were found (Davy de Virville et al., 2002). The changes in lipid composition may imply that different mechanisms operate in adaptation to decreased and increased temperatures. The molar ratio of the two main phospholipids, PC and PE, was affected by the temperature, with PE becoming the predominant species at lower temperature (Wodtke, 1978). These changes may affect many cellular compartments including the mitochondria. It is important to examine if the lipid composition of *pect1–4* mitochondrial membranes might be affected by low temperature and if the respiration capacities might be altered in correlation with the lipid composition changes.

These studies should enhance the importance of PE in the mitochondrial membranes.

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