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CR Somerville
Major professor

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MUTANTS OF *ARABIDOPSIS THALIANA* (L.) HEYNH. WITH ALTERED LEAF MEMBRANE LIPID COMPOSITION

By

Ljerka Kunst

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ABSTRACT

MUTANTS OF ARABIDOPSIS THALIANA (L.) HEYNH. WITH ALTERED LEAF MEMBRANE LIPID COMPOSITION

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In plant cells each membrane has a characteristic lipid composition. However, the specific roles of lipids and fatty acids in proper functioning of membranes are not well understood. In an attempt to examine the functional significance of chloroplast fatty acid composition, a collection of mutants of the crucifer Arabidopsis thaliana (L.) Heynh. was isolated from an ethyl methane sulfonate mutagenized population. The mutants were selected by direct analysis of leaf fatty acid composition using gas chromatography. This dissertation describes biochemical and physiological characterization of two of these mutants.

The first mutant analyzed was deficient in the activity of the chloroplast glycerol-3-phosphate acyltransferase, due to a single nuclear mutation at a locus designated act1. This lesion in the prokaryotic pathway of glycerolipid biosynthesis results in a redirection of fatty acids towards the eukaryotic pathway in the endoplasmic reticulum. The increased synthesis of lipids by the eukaryotic pathway provides, with the exception of phosphatidylglycerol, almost normal amounts of lipids required for chloroplast biogenesis. Since the acyltransferases associated with the two lipid biosynthetic pathways exhibit different substrate

specificities, the fatty acid composition of chloroplast membrane lipids is altered. As a consequence, pronounced changes in chloroplast ultrastructure were observed. The number of stacked membrane regions per chloroplast is increased, but there is a corresponding reduction in the average number of thylakoid membranes in the appressed regions. The analysis of Chl fluorescence emission spectra of the *actl* mutant revealed a slight decline in the excitation energy transfer from the light harvesting Chl a/b protein complex to PSII and PSI. However, the changes in chloroplast ultrastructure and Chl fluorescence emission do not affect the overall photosynthetic performance of the mutant.

The second mutant studied lacks polyunsaturated 16-carbon fatty acids, and shows a corresponding increase in the levels of the 16:0 acyl group. These changes suggest that a single nuclear mutation at the *fadB* locus causes a specific deficiency in the activity of a chloroplast n-9 desaturase. The mutation affects the fatty acid composition of both chloroplast and extrachloroplast lipids, but it does not appear to have any major functional effects on photosynthesis. However, *fadB*-related changes in leaf lipid composition seem to confer an enhanced thermal stability upon chloroplast membranes of the mutant.

To George and my family

for their love, patience and support

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LIST OF ABBREVIATIONS

ACP	acyl carrier protein
actl	symbol for a gene controlling the activity of glycerol-3-phosphate acyltransferase
BSA	bovine serum albumine
Chl	chlorophyll
CoA	coenzyme A
CP1	PSI reaction center Chl-protein complex
CP1a	oligomer of CP1
CPa	PSII reaction center Chl-protein complex
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DGD	digalactosyldiacylglycerol
DPH	1,6-diphenyl-1,3,5-hexatriene
EDTA	ethylenediaminetetraacetic acid
fadB	symbol for a gene controlling the activity of an n-9 fatty acid desaturase
F_o	initial fluorescence
F_v	variable fluorescence
F_m	maximum fluorescence
GLC	gas liquid chromatography
G3P	glycerol-3-phosphate
LHCP	light harvesting Chl a/b protein complex
$LHCP^1$ and $LHCP^2$	oligomeric forms of LHCP
$LHCP^3$	monomeric form of LHCP
LPA	lysophosphatidic acid

MES	2[N-morpholino]-ethane sulfonic acid
MOPS	3[N-morpholino]-propane sulfonic acid
MGD	monogalactosyldiacylglycerol
MV	methyl viologen
n:x	fatty acid containing n carbons and x double bonds
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEP	phosphoenolpyruvate
PG	phosphatidylglycerol
PI	phosphatidylinositol
PS	photosystem
SDS	sodium dodecyl sulfate
SL	sulfolipid
WT	wild type

CHAPTER 1

LITERATURE REVIEW

Lipid structure

The largest group of acyl lipids present in the photosynthetic tissue is based on glycerol, and includes acyl glycerols, glycoglycerolipids and phosphoglycerolipids. In all cases, sn-1 and sn-2 positions of the glycerol backbone are esterified with fatty acids, while the sn-3 position contains sugar or phosphate moieties, known as the head group. The head group is the polar part of the lipid molecule and faces out into the aqueous environment when in a bilayer.

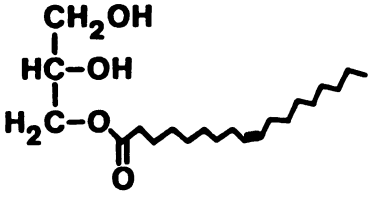
The major fatty acids in leaves are even numbered, unbranched, monocarboxylic acids, which can be classified into two distinct groups: the saturated and unsaturated fatty acids. Saturated fatty acids contain no double bonds in their hydrocarbon chain. The most common representatives of this group are palmitic (hexadecanoic) acid and stearic (octadecanoic) acid, comprising approximately 12% and 3 %, respectively, of the total fatty acid content of the leaf (Harwood, 1980; Table 1-I). On the other hand, unsaturated fatty acids contain one or more double bonds, and are by far the most abundant leaf fatty acids. For example, linolenic (cis 9,12,15 octadecanoic) acid, with

Table 1-I. The major fatty acids in leaves.

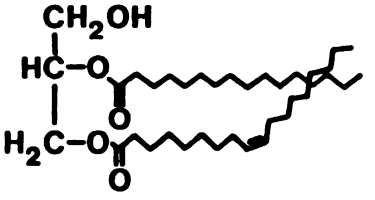
	Symbol	Systematic name	Common name
Saturated	16:0	Hexadecanoic acid	Palmitic acid
	18:0	Octadecanoic acid	Stearic acid
Unsaturated	16:1 (7c)	Hexadecenoic acid	Palmitoleic acid
	16:1 (3t)	trans-hexadecenoic acid	
	16:2 (7c 10c)	Hexadecadienoic acid	
	16:3 (7c 10c 13c)	Hexadecatrienoic acid	
	18:1 (9c)	Octadecenoic acid	Oleic acid
	18:2 (9c 12c)	Octadecadienoic acid	Linoleic acid
	18:3 (9c 12c 15c)	Octadecatrienoic acid	Linolenic acid

three double bonds between the 9^{th} and 10^{th}, 12^{th} and 13^{th} and 15^{th} and 16^{th} carbons of the chain, counting from the carboxyl end, may comprise up to 80% of total fatty acids of the leaf in some plant species. A shorthand nomenclature is commonly used for fatty acids. It consists of two numbers separated by a colon. The first number corresponds to the carbon chain length, while the number after the colon denotes the number of double bonds (Table 1-I). Unless otherwise specified it is assumed that the double bonds are cis.

On the basis of the head group attached to position sn-3 of glycerol, we can distinguish three major categories of leaf lipids: acyl glycerols, with unesterified sn-3 hydroxyl groups, glycolipids, whose third position is occupied with a sugar residue, and phospholipids, containing a phosphatidic acid derivative for a head group. Acyl glycerols, 1,2-diacylglycerol (DAG) and monoacylglycerol (Figure 1-1), are only minor constituents of the photosynthetic tissue and do not accumulate to any significant amount. However, they are important metabolic intermediates. One of the most striking features of leaf lipid composition is an extremely high proportion of glycolipids. The three principal glycolipids, monogalactosyldiacylglycerol (MGD), digalactosyldiacylglycerol (DGD) and sulfoquinovosyldiacylglycerol (SL) (Figure 1-2), are predominantly found in chloroplasts where they account for more than 70% of total lipids of the leaf cells (Harwood, 1980; Barber and Gounaris, 1986). The commonly occurring phospholipids in plant tissues include: phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) Their structures are shown in Figure 1-3. PC is the most important phospholipid in the

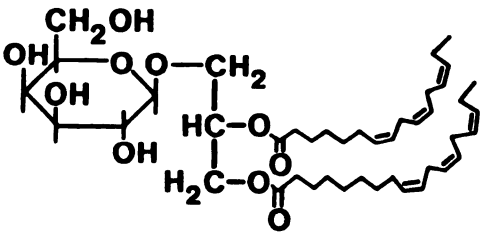


1-monoacylglycerol

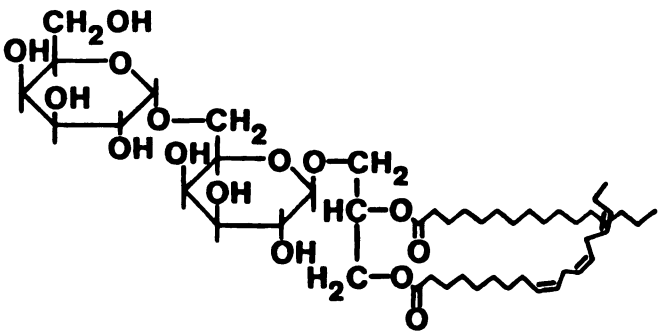


1,2-diacylglycerol (DAG)

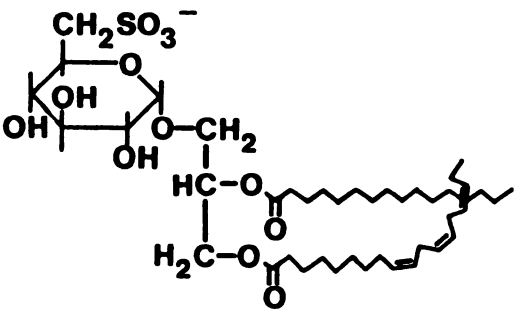
Figure 1-1. Acyl glycerols of leaf membranes.



Monogalactosyldiacylglycerol (MGD)

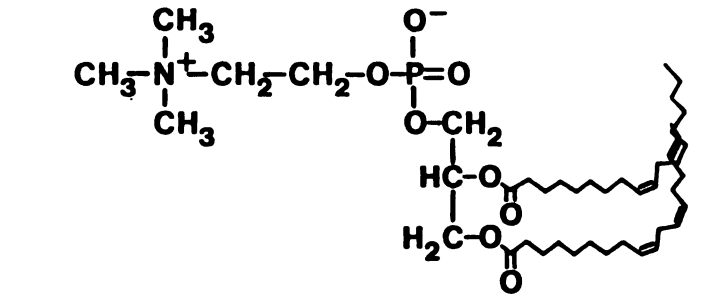


Digalactosyldiacylglycerol (DGD)

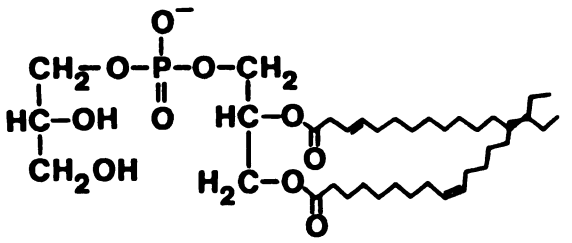


Sulfoquinovosyldiacylglycerol (SL)

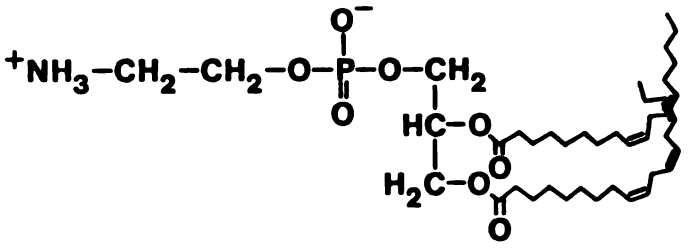
Figure 1-2. Glycolipids of leaf membranes.



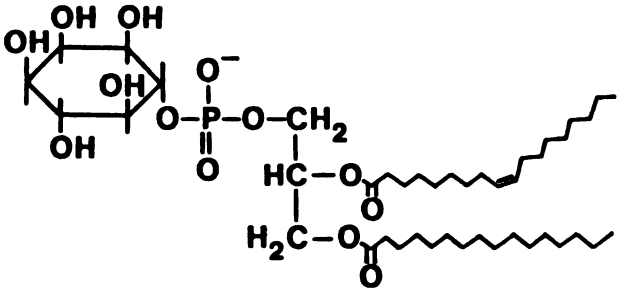
Phosphatidylcholine (PC)



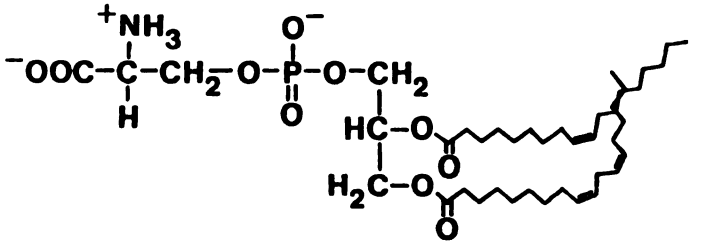
Phosphatidylglycerol (PG)



Phosphatidylethanol-amine (PE)



Phosphatidylinositol (PI)



Phosphatidylserine (PS)

Figure 1-3. Major phospholipids of leaf membranes.

majority of plant membranes, except the thylakoid membranes, where PG predominates. PC and PI that are found in the chloroplast are located only in the outer chloroplast envelope. On the other hand, PE and PS have not been detected in the chloroplast, and are primarily found in the mitochondrion (Harwood, 1980).

Glycerolipid synthesis in leaf cells

Isolated intact chloroplasts readily incorporate added acetate into long chain fatty acids in the light (Slack, 1977; Roughan et al. 1979; Roughan and Slack, 1982). This observation, in conjunction with the localization of acyl carrier protein (ACP) (Ohlrogge et al., 1979) and acetyl-CoA synthetase (Kuhn et al., 1981) exclusively within chloroplasts, led to the conclusion that these organelles are the only sites of fatty acid synthesis in the photosynthetic tissue. The mechanism of fatty acid synthesis involves three steps: (1) the carboxylation of acetyl-CoA to form malonyl-CoA, (2) the repeated condensation of malonyl-CoAs with a growing acyl chain attached to ACP to make 16:0-ACP, and (3) the elongation of 16:0-ACP to 18:0-ACP. The condensation reactions are catalyzed by fatty acid synthase (FAS), that consists of six loosely associated enzymes, partially purified and characterized by Shimakata and Stumpf (1982). In contrast, fatty acid synthase activity in animal tissues is localized in the cytosol, and it is associated with a single enzyme, a homodimer with 6-7 active site domains (Stumpf, 1981). Most of the 18:0-ACP produced in the plant chloroplast is desaturated to 18:1-ACP by a highly active ferredoxin

dependent stromal desaturase (McKeon and Stumpf, 1982). Therefore, the main products of fatty acid synthesis are 16:0- and 18:1-ACPs. These thioesters can be used directly in the chloroplast by the prokaryotic pathway of lipid synthesis, or may be hydrolyzed to free fatty acids and exported to the cytoplasm and eventually to the endoplasmic reticulum (ER), where the enzymes of the eukaryotic pathway are located. The partitioning of fatty acids between these cell compartments, and their respective pathways of lipid synthesis, depends on the plant species and results in differences in lipid composition of their chloroplast membranes. Detailed analyses of leaf lipid composition of a variety of plants has led to their classification into two major groups: those containing hexadecatrienoic acid ("16:3 plants"), and those that do not contain 16:3 acyl groups ("18:3 plants"; Roughan and Slack, 1984).

16:3 plants

16:3 plants include families like: Solanaceae, Brassicaceae, Chenopodiaceae and Apiaceae, and typically contain up to 20% 16:3 fatty acid in their leaf lipids. This acyl group is found only on MGD and DGD molecules synthesized in the chloroplast through the prokaryotic pathway. The prokaryotic pathway is initiated by the sequential acylation of glycerol-3-phosphate (G3P) using acyl-ACPs (Figure 1-4). The final product of these reactions, catalyzed by two acyl transferases, is phosphatidic acid (PA). The sn-1 specific acyl transferase is a soluble stromal protein which preferentially utilizes 18:1-ACP as a substrate (compared to 16:1-ACP) (Frentzen et al., 1983) and generates lysophosphatidic acid (LPA). This lipid enters the inner

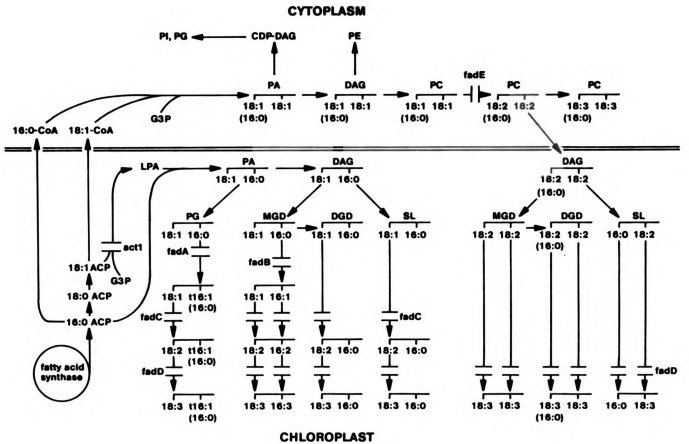


Figure 1-4. Lipid biosynthesis in 16:3 plants.

envelope of the chloroplast membrane where it is converted to PA by an sn-2 specific membrane bound acyl transferase (Frentzen et al., 1983). The second acyl transferase uses only a 16:0 acyl group for the esterification reaction, hence PA and all the lipids made from it by the prokaryotic pathway can easily be distinguished due to the presence of a 16-carbon fatty acid at their sn-2 position. PA gives rise to PG, the most important chloroplast phospholipid. PG synthesis takes place in the inner envelope, and involves condensation of PA and CTP to produce CDP-diacylglycerol. This in turn reacts with G3P to form PG (Andrews and Mudd, 1985). Alternatively, prokaryotic PA can be converted to DAG by a PA phosphatase (Bloch et al., 1983; Andrews et al., 1985), also located in the inner chloroplast envelope. The DAG pool then acts as a precursor for the synthesis of thylakoid glycolipids: MGD, DGD and SL (Heemskerk et al., 1985; Kleppinger-Sparace et al., 1985; Coves et al., 1986).

Besides the prokaryotic pathway, 16:3 plants can synthesize chloroplast lipids through the eukaryotic pathway in the ER (Figure 1-4). Acyl groups made in the chloroplast are cleaved from ACP and diffuse into the lipid bilayer, where they are esterified to CoA by the acyl-CoA synthetase in the outer envelope (Andrews and Keegstra, 1983). This makes the acyl chains soluble for the cytoplasmic transport to the ER, where they are used by the eukaryotic acyl transferases for PA synthesis. In contrast to the chloroplast, PA produced in the ER always has an 18-carbon fatty acid esterified to the sn-2 position, while 16:0, when present, is confined to the sn-1 position (Frentzen et al., 1984). This PA is used for the synthesis of phospholipids: PC, PE, PI and PG, which are characteristic of the various extrachloroplast

membranes (Moore, 1982). In addition, the DAG moiety of PC is returned to the chloroplast and contributes to the synthesis of thylakoid lipids (Slack et al., 1977; Roughan and Slack, 1982). The mechanism of DAG transport from the ER to the chloroplast has not been elucidated. The discovery of phospholipid exchange proteins in plants (Ohnishi and Yamada, 1982) introduced a possible mechanism of PC transport to the chloroplast. However, the absence of PC phosphatase activity in the chloroplast envelope, as well as evidence that PC is probably converted to DAG in the ER (Roughan and Slack, 1984) would argue against this possibility.

In summary, 16:3 plants synthesize two distinct populations of PA and DAG, due to simultaneous operation of prokaryotic and eukaryotic pathways. The two DAG types readily equilibrate in the chloroplast envelope and are further metabolized to MGD through the action of a chloroplast-specific galactosyl transferase (Heemskerk et al., 1985). UDP-galactose for the galactosylation reaction is contributed by the cytoplasm. The mechanism of DGD formation has not been resolved. In principle, MGD could give rise to DGD by the addition of another galactose molecule (Neufeld and Hall, 1964; Ongun and Mudd, 1968). However, the nature of the galactose donor is not known, and the enzyme(s) involved have not been fully characterized. Two distinct pathways for DGD synthesis have been proposed so far. The former is based on results of Ongun and Mudd (1968), and Siebertz and Heinz (1977), and suggests a stepwise addition of galactosyl groups from UDP-Gal to DAG:

$$DAG + UDP\text{-}Gal \longrightarrow MGD + UDP$$

$$MGD + UDP\text{-}Gal \longrightarrow DGD + UDP.$$

The enzyme responsible for DGD formation would be UDP-Gal:MGD galactosyltransferase. However, this enzyme has not been identified in isolated chloroplasts, envelope membranes or microsomes. The latter pathway was proposed by Van Besouw and Wintermans (1978), who discovered another enzymatic activity in the spinach chloroplast envelope, producing DGD in the absence of UDP-Gal. This galactolipid:galactolipid galactosyltransferase transfers the galactose moiety between two MGD molecules to produce DGD and DAG:

$$MGD + MGD \longrightarrow DGD + DAG.$$

This enzyme was localized on the outer surface of the outer envelope membrane, and it is, at the moment, a more likely candidate for DGD synthesis. Despite considerable attention, the biosynthetic pathway of SL also remains uncertain (Barber and Gounaris, 1986). When spinach leaves were labeled with $^{14}CO_2$, ^{14}C was found in 16:0 at position sn-2, and in both 16:0 and 18-carbon fatty acids at position sn-1 (Siebertz et al., 1979). These features, shared with prokaryotic DAG, suggest a biosynthetic relationship *in vivo*. The incorporation of ^{14}C-acetate into SL by isolated chloroplasts (Roughan et al., 1979, Roughan et al., 1980) also indicates that chloroplasts are probably autonomous in SL synthesis.

18:3 plants

Short term labeling experiments of intact leaves with $^{14}CO_2$ and ^{14}C-acetate (Roughan, 1970; Slack and Roughan, 1975; Williams et al., 1976) have shown that plants of certain plant families such as: Cucurbitaceae, Fabaceae, Asteraceae and Poaceae distribute their newly synthesized fatty acids predominantly into PC, and only a minor proportion into PG (Figure 1-5). Since PC is exclusively made by the eukaryotic pathway, this labeling pattern indicated that the ER is the major site of glycerolipid synthesis in these plants. A detailed analysis of leaf fatty acid composition demonstrated that they do not contain a 16:3 acyl group, which is a specific marker of MGD and DGD made through the prokaryotic pathway. It is replaced mostly by linolenic acid (18:3), hence these species were named 18:3 plants (Heinz and Roughan, 1983). Direct assays of chloroplast enzymes confirmed a relatively low activity of prokaryotic acyl transferases (Heinz and Roughan, 1983) and PA phosphatase, and led to the conclusion that the only product of the prokaryotic pathway in 18:3 plants is PG (Andrews and Mudd, 1985).

Fatty acid desaturation

Each lipid class constituent of higher plant membranes has a characteristic acyl group composition in terms of chain length, as well as degree, position and stereochemistry of unsaturation. Typically, plant fatty acids contain 0-3 cis double bonds (Frentzen, 1986). The first double bond is always introduced at position 9, counting from the carboxyl end of the acyl group, the second at position 12, while the third occurs at position 15. The most common exception to this general

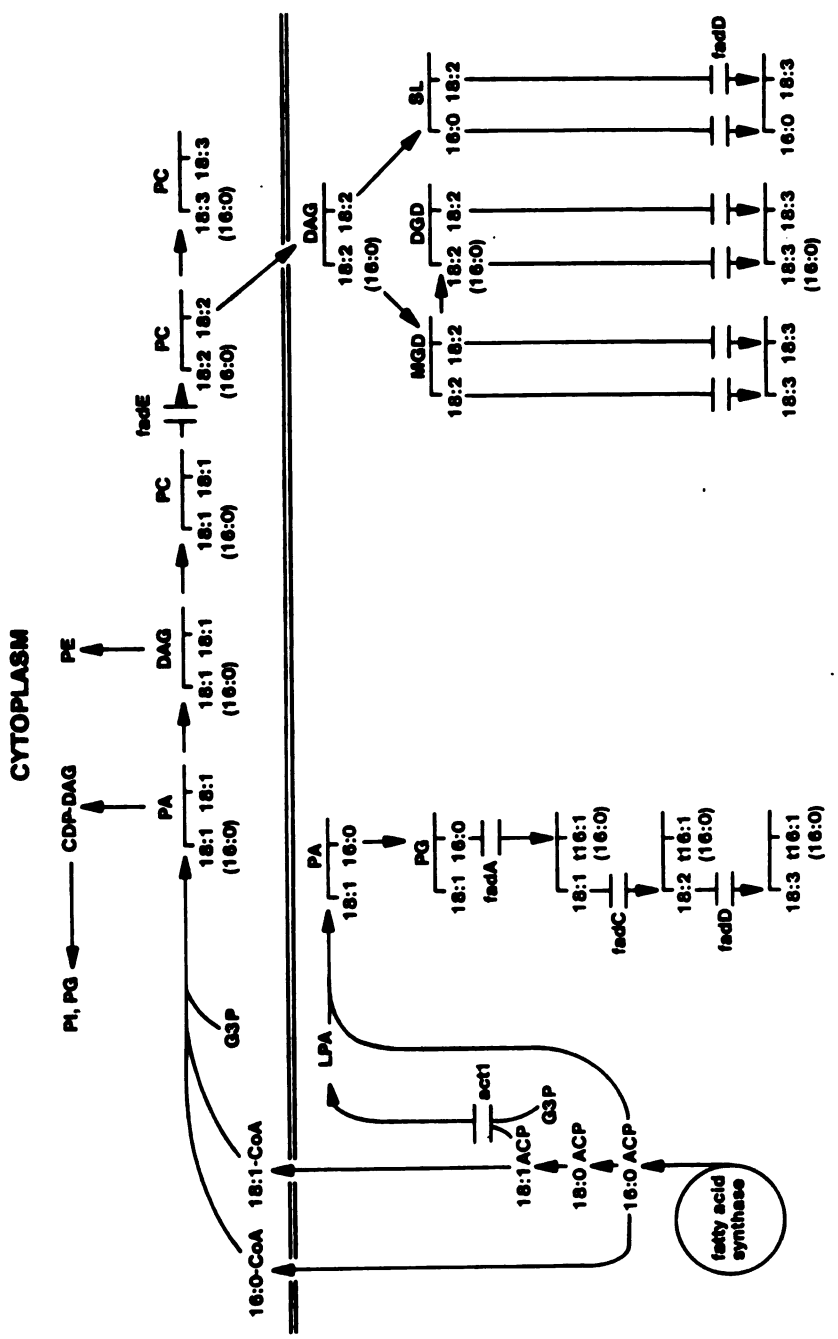


Figure 1-5. Lipid biosynthesis in 18:3 plants.

rule is the acyl group 3- trans 16:1 which is always esterified to the sn-2 position of PG (Dubacq and Tremolieres, 1983). Besides these observations, the desaturation reactions in plants are not well understood. It is not known, for example, how many desaturase enzymes are involved in the desaturation process, and their compartmentation has not been precisely established. The only desaturase partially purified and characterized in some detail so far, is the 18:0-ACP desaturase, a soluble stromal enzyme (McKeon and Stumpf, 1982). All the other attempts to isolate chloroplast desaturases have not been successful, since chloroplasts lose the ability to synthesize polyunsaturated fatty acids when broken (Roughan, 1979), or when they are exposed to hypotonic conditions (Andrews and Heinz, 1987). Microsomal desaturases have not been purified either, but it is possible to measure the 18:1 and 18:2 desaturase activity in microsomal preparations (Browse and Slack, 1981).

Despite the described difficulties associated with the characterization of plant desaturases, substantial information concerning fatty acid desaturation is available from in vivo labeling experiments. Analysis of ^{14}C labeled acyl groups suggested that PC is the major site of 18:1 desaturation for lipids made by the eukaryotic pathway (Slack et al., 1977). This conclusion was confirmed by showing that ^{14}C-oleoyl-CoA is first esterified to lyso-PC before desaturation occurs (Murphy et al., 1983). On the other hand, the major substrate for 18:2 desaturation for eukaryotic lipids seems to be MGD (Hawke and Stumpf, 1979; Roughan and Slack, 1982). This lipid also serves as a major site for 18:1 and 18:2 desaturation of prokaryotic lipids. However, the desaturation reactions of lipids made through the

prokaryotic pathway are not confined to MGD. For example, ^{14}C-18:1-PG synthesized by the chloroplast is sequentially converted to labeled 18:2- and 18:3-PG (Roughan, 1985). A similar conclusion was reached by analyzing the fadD mutant of Arabidopsis (Browse et al., 1986). This mutant is deficient in the activity of a chloroplast 18:2 desaturase, due to a single nuclear mutation. The observation that all the chloroplast polar lipids are affected by the fadD mutation, clearly indicates that they are all substrates for 18:2 desaturation, or that substantial transfer of acyl groups occurs between these lipids. In addition, the fadD mutant provided important, although indirect information concerning 18:2 desaturase function. This enzyme introduces the final double bond in acyl chains with no apparent specificity for the chain length (16- or 18-carbon), or their point of attachment to the glycerol backbone (sn-1 or sn-2).

Lipid composition of chloroplast membranes

A detailed survey of lipid composition of various plant tissues revealed major differences among membranes of different cell types, and even organelles within a single cell type. Basically, every single membrane in the cell has a unique lipid composition with respect to both the head group and the acyl chains (Harwood, 1980). However, the functional significance of lipid diversity has not been established.

In comparison with other eukaryotic membranes, the acyl lipid composition of the photosynthetic membrane is remarkably constant in a wide variety of species (Table 1-II; Harwood, 1980; Douce and Joyard,

Table 1-II. Polar lipid composition of thylakoid membranes. Values are weight %.

Plant	MGD	DGD	SL	PG	PC	Source
Spinach	52	26	7	10	5	1
Wheat	45	35	8	10	2	1
Barley	54	24	8	9	5	2
Tomato	50	25	3	22	0	2
White clover	48	28	4	21	0	1
Broad bean	52	28	5	9	6	1
Arabidopsis	48	28	6	13	5	3

1 Harwood JL 1980

2 Douce R, J Joyard 1980

3 This dissertation

1980). The principal chloroplast polar lipids are MGD and DGD, accounting for more than 70 % of total lipid fraction of this organelle. The additional 30 % is made up mostly of PG, PC and SL. Another interesting feature of thylakoid membranes is an unusually high proportion of polyunsaturated fatty acids. Depending on the plant species, trienoic acids (18:3 and 16:3) represent up to 80 % of fatty acyl groups of this membrane.

In an attempt to examine the structural and functional roles of individual lipid classes, and distinct fatty acid composition of chloroplast membranes, we have isolated a number of mutants of Arabidopsis thaliana (L.) Heynh. with altered leaf lipid metabolism. Two of the mutants have already been investigated in detail. One is deficient in trans-16:1 synthesis (Browse et al., 1985, McCourt et al., 1985), while the other lacks a specific desaturase responsible for introducing n-3 double bond in both 18-carbon and 16-carbon fatty acids (Browse et al., 1986, McCourt et al., 1987). This work describes the genetic and biochemical characterization of the two additional mutants, as well as the consequences of specific changes in fatty acid composition on photosynthetic properties of these plants.

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CHAPTER 2

MUTANT ISOLATION AND GENETIC CHARACTERIZATION

Introduction

The analysis of microbial mutants has shown that there are many changes in lipid composition which are incompatible with the survival of the organism (Clark and Cronan, 1981; Rock and Cronan, 1985). In particular, major changes in the amounts of different head groups are severely deleterious, hence all of these mutants have a detectable phenotype. On the other hand, some E. coli mutants are completely defective in the synthesis of cyclopropane fatty acids, but show no impairment of growth under a variety of environmental conditions (Taylor and Cronan, 1976; Gorgan and Cronan, 1986). This observation suggested that fatty acid composition of membrane lipids might not be essential for the organism. Therefore, a rapid direct screening method was designed that involves the preparation of fatty acid methyl esters (FAME) from single leaves followed by gas chromatography (GC) (Browse et al., 1985b). A typical GC tracing of fatty acids obtained from a wild-type leaf is shown in Figure 2-1. This method was employed to analyze leaf fatty acid composition of individual mutagenized Arabidopsis plants. From approximately 2000 plants examined in the

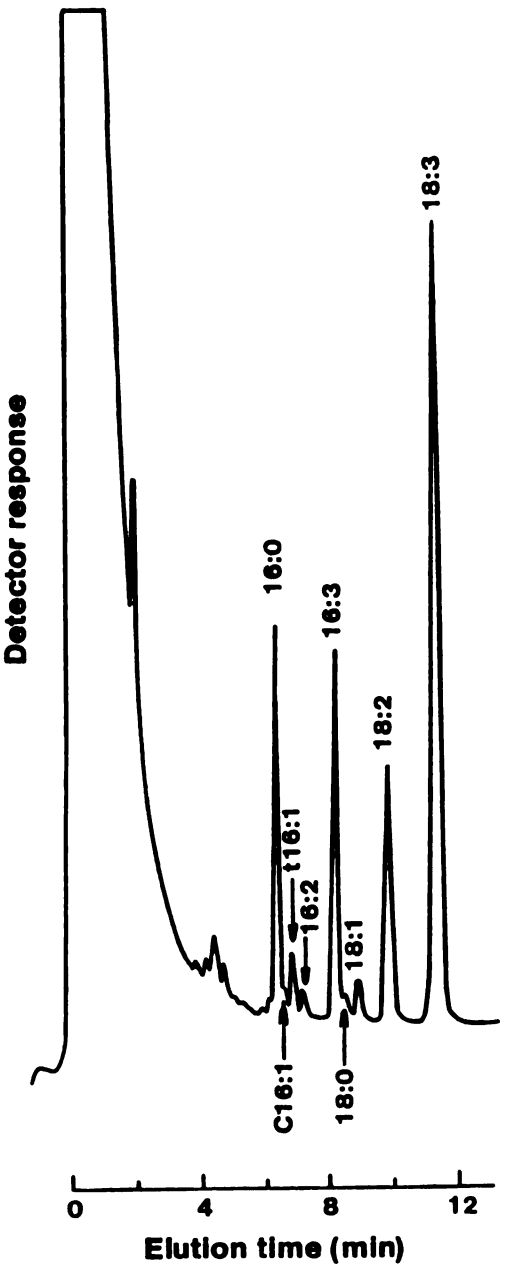

Figure 2-1. Gas chromatography tracing of fatty acid methyl esters from a wild type leaf.

initial screen, and 2000 analyzed in several subsequent searches, we isolated 9 lines with stably inherited changes in fatty acid composition of leaf glycerolipids (Table 2-I).

These mutants provide an opportunity to directly address questions concerning the enzymology, regulation and functional significance of desaturation, as well as the control of cellular lipid metabolism. Furthermore, since 75 % of all fatty acyl groups in Arabidopsis leaves are constituents of chloroplast membranes, mutant analysis can contrubute to our understanding of the relationship between specific fatty acid composition of thylakoids and chloroplast structure and function.

Act1 mutants

Four mutant lines (JB3, JB25, JB28 and LK8) were isolated from two independently mutagenized populations because of the deficiency in 16:3 acyl group. The absence of 16:3 is compensated for by increases in 18:1, 18:2 and 18:3 fatty acids (Table 2-II). Otherwise the mutants were indistinguishable from the wild type in appearence.

Complementation analysis has shown that the four lines have a lesion at the same locus designated act1 (acyl transferase 1). Therefore, the genetic basis of the phenotype was determined only for the line JB25 by crossing the mutant with the wild type as maternal parent. The leaf fatty acid composition of F_1 progeny, measured by GC, was identical to the wild type, suggesting a recessive mutation (Table 2-II). The frequency of the homozygous mutant phenotype in the F_2

Table 2-I. Fatty acid composition of total leaf lipids of *Arabidopsis* mutants at 25°C. Each value is a mean of 10 plants.

		Fatty acid composition (mol %)									
Mutant line	Gene Symbol	16:0	c16:1	t16:1	16:2	16:3	18:0	18:1	18:2	18:3	Source
JB60	*fadA*	18	tr.	-	tr.	12	1	3	19	47	1
JB27	*fadA*	17	tr.	-	tr.	13	1	3	19	47	2
JB67	*fadB*	24	-	2	-	-	1	2	14	56	3
LK3	*fadC*	15	11	2	-	-	1	21	14	36	3
JB1	*fadD*	15	2	2	12	3	1	3	26	36	2
LK9	*fadD*	13	2	3	12	4	2	3	32	29	3
JB12	*fadE*	15	tr.	4	tr.	10	1	27	4	36	4
JB25	*act1*	13	tr.	2	tr.	tr.	1	8	23	53	3
LK8	*act1*	12	tr.	2	tr.	tr.	1	9	23	53	3
WT		15	tr.	2	tr.	12	1	3	18	48	3

1 Browse et al. 1985a

2 McCourt PJ, Ph. D. Thesis

3 This dissertation

4 Browse JA, personal communication

Table 2-II. Fatty acid composition of total leaf lipids of mutant and wild type *Arabidopsis* grown at 22°C. Values are mol % ± SD (n=10)

Fatty acid	Wild type	F1(WT x JB25)	JB25
16:0	14.1 ± 0.5	14.0 ± 0.5	12.6 ± 0.3
16:1 *cis*	1.6 ± 0.1	1.6 ± 0.5	1.4 ± 0.4
16:1 *trans*	1.6 ± 0.3	2.3 ± 0.4	2.0 ± 0.4
16:2	0.5 ± 0.1	0.9 ± 0.4	0.5 ± 0.3
16:3	11.4 ± 0.3	10.5 ± 0.6	1.5 ± 0.3
18:0	1.7 ± 0.1	1.6 ± 0.1	0.8 ± 0.2
18:1	3.0 ± 0.3	3.2 ± 0.4	8.4 ± 0.8
18:2	13.4 ± 0.5	14.2 ± 0.8	17.8 ± 0.8
18:3	52.5 ± 0.4	51.7 ± 1.7	55.1 ± 1.5

generation was also analyzed. Of 271 F_2 plants, 64 had no detectable levels of 16:3. This is a good fit to the 3:1 hypothesis (X^2=0.36, P>0.5) indicating that the alteration in fatty acid composition is due to a single nuclear mutation.

The *act1* mutation was mapped to a chromosome by F_2 mapping from a cross of the W-100 strain to JB25. W-100 contains 2 visible markers for each of the five *Arabidopsis* chromosomes. All the markers and the *act1* mutation segregated 3:1, as expected for a simple mendelian trait. *Act1* assorted independently of all markers, except for *an*. A significant departure from 9:3:3:1 ratio is indicated by a high X^2 value (Table 2-III). This aberrant independent assortment was used to assign *act1* mutation to chromosome 1, 34.8 map units (cM) away from the *an* marker (Figure 2-2).

Fad mutants

The majority of *Arabidopsis* mutants identified by direct GC assay of leaf fatty acid composition, showed specific changes in the levels of unsaturation of their acyl groups. The responsible mutations map to five different loci designated *fadA* i.e., *f*atty *a*cid *d*esaturation gene A, *fadB*, *fadC*, *fadD* and *fadE* (Table 2-I, Figure 1-4). In all the mutants described so far (*fadA*: Browse et al., 1985a, McCourt et al, 1985; *fadD*: Browse et al. 1986, McCourt et al., 1987) the enzymatic lesions were due to single nuclear mutations.

I have recently determined that the same is true for the *fadB* and *fadC* mutants. The lines JB67 carrying a *fadB* mutation, and LK3 with a

Table 2-III. F_2 linkage analysis of *actl*.

W-100 (*an* *apl* *py* *er* *hy2* *gll* *bp* *cer2* *tt3* *ms* +) x
JB25 (+ + + + + + + + + + *actl*)

(A) Single locus goodness of fit tests

	Obs	Exp[1]	$X^2(1)$		Obs	Exp	$X^2(1)$
an	90	74	4.61	*bp*	42	72	16.88
+	206	222		+	247	217	
apl	81	73	1.17	*cer2*	74	73	0.03
+	211	219		+	217	218	
py	53	74	7.78	*tt3*	67	73	0.61
+	242	221		+	224	218	
er	67	73	0.66	*ms*	100	73	13.91
+	225	219		+	190	217	
hy2	70	74	0.32	*actl*	60	74	3.42
+	227	223		+	235	221	
gll	67	74	0.94				
+	230	223					

[1] expected segregation for a single recessive nuclear trait (3:1)

(B) Joint segregation for pairs of loci

Chromosome			+/+	+/-	act1/+	act1/-	X^2
1	an	Obs	151	83	53	7	12.74*
		Exp[2]	167	56	56	19	
	apl	Obs	170	55	51	19	0.0003
		Exp	167	55	55	18	
2	py	Obs	193	41	48	11	0.04
		Exp	165	55	55	18	
	er	Obs	179	54	44	13	0.004
		Exp	163	54	54	18	
3	hy2	Obs	182	53	45	15	0.16
		Exp	166	55	55	18	
	gl1	Obs	183	52	46	14	0.04
		Exp	166	55	55	18	
4	bp	Obs	198	33	47	9	0.12
		Exp	161	54	54	18	
	cer2	Obs	171	61	44	13	0.29
		Exp	163	54	54	18	
5	tt3	Obs	175	57	48	9	2.00
		Exp	163	54	54	18	
	ms	Obs	146	85	43	14	3.03
		Exp	162	54	54	18	

[2] expected segregation for two recessive nuclear traits assorting independently (9:3:3:1)

* Indicates a significant difference

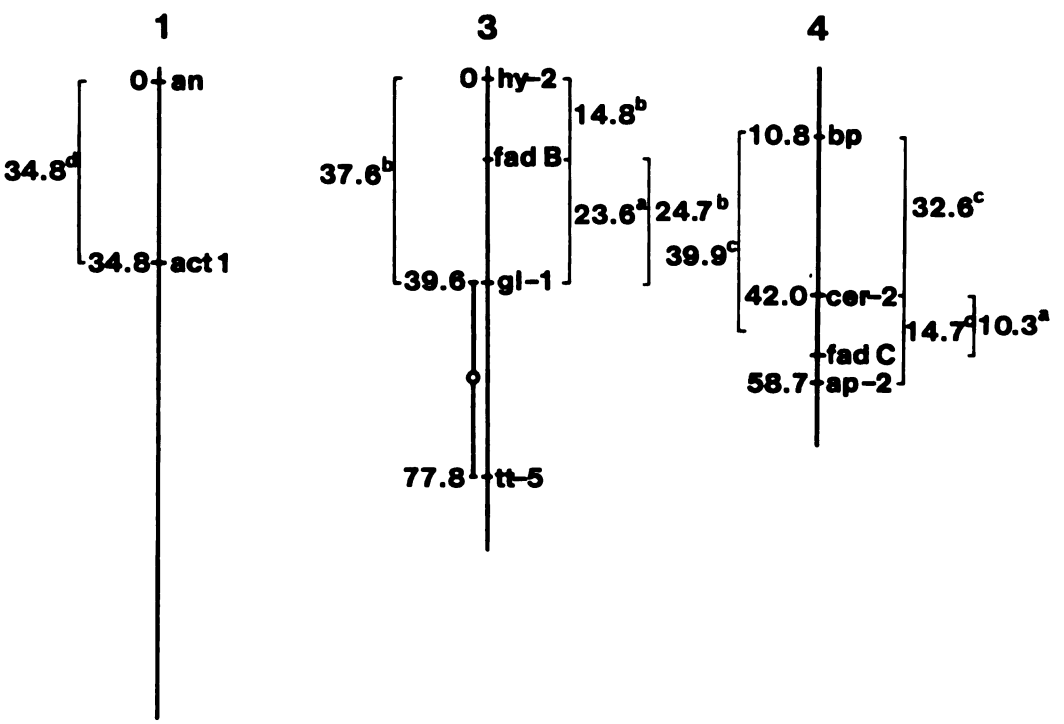

Figure 2-2. Position of act1, fadB and fadC loci on chromosomes 1, 3 and 4, respectively, with estimated recombination percentages. Circle with a range indicates the position of the centromere.

lesion at the fadC locus were crossed to the wild type as maternal parent, and the fatty acid composition of F_1 and F_2 progeny was analyzed. All the F_1 plants from the WT x JB67 cross had higher levels of 16:0 than the wild type (Table 2-IV), while F_1 progeny from the WT x LK3 cross had higher levels of 16:1 and 18:1 acyl groups than wild-type plants (Table 2-V). These results suggest codominant mutations and indicate that levels of c16:1, as well as 16:2 and 18:2 levels are probably regulated by the amount of active enzyme present. Leaf fatty acid composition of 308 F_2 plants from the WT x JB67 cross, and 221 plants from the WT x LK3 cross was also determined. The 3:1 segregation pattern (78 fadB : 230 WT, X^2=0.013, P>0.9; 51 fadC : 170 WT, X^2=0.44, P>0.4) obtained for both mutants is consistant with the presence of single nuclear mutations at the fadB (JB67) and fadC (LK3) loci.

JB67 and LK3 mutant lines were also crossed to line MK1 which carries 5 visible chromosome markers. Linkage analysis was carried out on 417 F_2 plants from the MK1 x JB67 cross, and 228 F_2 progeny from the MK1 x LK3 cross (Tables 2-VI and 2-VII). High X^2 value obtained for gl1, fadB pair of loci clearly indicated that these two mutations are closely linked on chromosome 3 (23.6 map units away, Figure 2-2). The same reasoning was used to assign the fadC allele to chromosome 4, 10.3 map units away from the cer2 marker (Figure 2-2).

In order to determine the position of fadB on the map more accurately, I analyzed the F_2 progeny of a cross between fadB/fadB and the strain MSU 22, homozygous recessive for 3 marker genes: hy-2/hy-2, gl-1/gl-1,and tt-5/tt-5 (Table 2-VIII). Joint segregation for pairs of loci (Table 2-VIII) and recombination frequencies (Figure 2-2) were obtained using a computer program (Linkage1) written by K.A. Suiter,

Table 2-IV. Fatty acid composition of total leaf lipids of mutant and wild type *Arabidopsis* grown at 22 C. Values are mol % ± SD (n=10).

Fatty acid	Wild type	F1(WT x JB67)	JB67
16:0	13.0 ± 0.8	16.4 ± 0.8	24.1 ± 0.9
16:1 *cis*	1.5 ± 0.2	1.1 ± 0.2	1.5 ± 0.3
16:1 *trans*	3.6 ± 0.4	2.8 ± 0.3	2.8 ± 0.4
16:2	1.7 ± 0.5	1.0 ± 0.3	0.3 ± 0.1
16:3	15.7 ± 1.0	12.5 ± 0.7	0.3 ± 0.2
18:0	0.7 ± 0.2	1.2 ± 0.2	1.2 ± 0.4
18:1	2.4 ± 0.4	3.1 ± 0.4	2.5 ± 0.5
18:2	12.3 ± 0.6	13.6 ± 0.3	17.1 ± 1.0
18:3	49.1 ± 1.4	48.2 ± 1.4	50.21 ± 1.5

Table 2-V. Fatty acid composition of total leaf lipids of mutant and wild type Arabidopsis grown at 22°C. Values are mol % ± SD (n=10)

Fatty acid	Wild type	F1(WT x LK3)	LK3
16:0	13.0 ± 0.8	12.3 ± 0.5	13.9 ± 0.3
16:1 cis	1.5 ± 0.2	3.7 ± 0.3	11.2 ± 0.7
16:1 trans	3.6 ± 0.4	2.5 ± 0.3	3.8 ± 0.5
16:2	1.7 ± 0.5	0.3 ± 0.1	0.5 ± 0.2
16:3	15.7 ± 1.0	14.0 ± 1.3	0.2 ± 0.1
18:0	0.7 ± 0.2	0.7 ± 0.2	0.8 ± 0.2
18:1	2.4 ± 0.4	5.8 ± 0.8	16.1 ± 1.0
18:2	12.3 ± 0.6	16.7 ± 0.5	16.5 ± 0.2
18:3	49.1 ± 1.4	43.9 ± 1.7	37.0 ± 1.5

Table 2-VI. F_2 linkage analysis of *fadB*.

MK1 (*an* *py* *gll* *cer2* *ms* +) x
JB67 (+ + + + + *fadB*)

(A) Single locus goodness of fit tests

	+	*an*	+	*py*	+	*gll*	+	*cer2*	+	*ms*	+	*fadB*
Obs	286	91	289	85	270	106	277	96	266	105	301	73
Exp[1]	283	94	281	94	282	94	280	93	278	93	280	94
X^2	0.15		1.03		2.04		0.11		2.16		5.99	

[1] expected segregation for a single recessive nuclear trait (3:1)

(B) Joint segregation for pairs of loci

Chromosome			+/+	+/-	fadB/+	fadB/-	X^2
1	*an*	Obs[2]	221	62	80	11	4.23
		Exp[2]	211	70	70	23	
2	*py*	Obs	232	55	68	17	0.03
		Exp	209	70	70	23	
3	*gll*	Obs	199	69	102	4	23.34*
		Exp	211	70	70	23	
4	*cer2*	Obs	223	54	77	18	0.01
		Exp	211	70	70	23	
5	*ms*	Obs	209	57	90	15	2.46
		Exp	208	70	70	23	

[2] Expected segregation for two recessive nuclear traits assorting independently (9:3:3:1)

* Indicates a significant difference

Table 2-VII. F_2 linkage analysis of fadC.

MK1 (an py gll cer2 ms +) x
LK3 (+ + + + + fadC)

(A) Single locus goodnes of fit tests

	+	an	+	py	+	gll	+	cer2	+	ms	+	actl
Obs[1]	179	42	169	46	173	48	159	56	151	56	177	44
Exp[1]	166	55	161	54	166	55	161	54	155	52	166	55
X^2	4.24		1.49		1.27		0.13		0.47		3.05	

[1] expected segregation for a single recessive nuclear trait (3:1)

(B) Joint segregation for pairs of loci

Chromosome			+/+	+/-	fadC/+	fadC/-	X^2
1	an	Obs[2]	142	36	34	8	0.03
		Exp[2]	124	41	41	14	
2	py	Obs	136	33	36	10	0.11
		Exp	121	40	40	13	
3	gll	Obs	134	39	43	5	3.47
		Exp	124	41	41	14	
4	cer2	Obs	90	56	68	1	30.89*
		Exp	121	40	40	13	
5	ms	Obs	121	30	46	10	0.11
		Exp	116	39	39	13	

[2] Expected segregation for two nuclear recessive traits assorting independently (9:3:3:1)

* Indicates a significant difference

Table 2-VIII. Localization of *fadB* on chromosome 3.

MSU 22 (*hy-2* *gl-1* *tt-5* +) x JB67 (+ + + *fadB*)

(A) Single locus goodness of fit tests

	+	*hy-2*	+	*gl-1*	+	*tt-5*	+	*fadB*
Obs	98	44	97	45	109	32	125	17
Exp[1]	106	36	106	36	106	35	106	36
X^2	2.71		3.39		0.40		12.85	

[1] Expected segregation for a single recessive nuclear trait (3:1)

(B) Joint segregation for pairs of loci

		+/+	+/-	*fadB*/+	*fadB*/-	X^2
hy-2	Obs	64	39	38	1	15.72*
	Exp[2]	80	27	27	9	
gl-1	Obs	83	42	17	1	4.60
	Exp	80	27	27	9	
tt-5	Obs	96	27	13	4	0.008
	Exp	79	26	26	9	

		+/+	+/-	*gl-1*/+	*gl-1*/-	
hy-2	Obs	76	21	22	23	11.13*
	Exp	80	27	27	9	

[2] Expected segregation for two nuclear recessive traits assorting independently (9:3:3:1)

* Indicates a significant difference

J.F. Wendel and J.S. Case (1983). All the values were corrected for double cross-overs with the Kosambi mapping function:

$$D = 25\ln (100+2r)/(100-2r)$$

where D = distance in centiMorgans (cM), and r = estimated recombination percentage (Koornneef et al., 1983). The precise location of the fadC locus was estimated by S. Hugly on the basis of the F_2 analysis resulting from a cross between fadC/fadC and the line MSU 15 carrying recessive marker genes cer-2/cer-2, bp/bp and ap-2/ap-2 (Table 2-IX). The linkage data (Table 2-IX) and recombination percentages (Figure 2-2) were obtained as described for fadB gene.

Double mutants

Double mutants were constructed for two reasons: determination of epistatic relationships between various mutations and generation of more severe phenotypes with respect to the overall changes in fatty acid composition. An epistatic relationship between the two mutations can provide detailed information about the sequence of steps affected by mutations in a biosynthetic pathway. Since both the line JB25 (act1) and the line JB1 (fadD) have altered 16:3 levels, it was of interest to check their epistatic relationship. To do this we have first crossed the two mutants, and analyzed 49 F_2 plants from the JB1 x JB25 cross. The mutations segregated independently (27 FADD -/- ACT1 : 5 FADD act1/- act1 : 16 fadD ACT1/fadD - : 1 fadD act1/fadD act1, $X^2(3)=5.59$

Table 2-IX. Localization of *fadC* on chromosome 4.

MSU 15 (*cer2* *bp* *ap-2* +) x LK3 (+ + + *fadC*)

(A) Single locus goodness of fit tests

	+	*cer-2*	+	*bp*	+	*ap-2*	+	*fadC*
Obs	123	38	125	36	122	39	127	34
Exp[1]	121	40	121	40	121	40	121	40
X^2	0.17		0.60		0.05		1.29	

[1] Expected segregation for a single recessive nuclear trait (3:1)

(B) Joint segregation for pairs of loci

		+/+	+/-	*fadC*/+	*fadC*/-	X^2
cer-2	Obs[2]	89	38	34	0	13.32*
	Exp[2]	91	30	30	10	
bp	Obs	94	33	31	3	4.55*
	Exp	91	30	30	10	
ap-2	Obs	88	39	34	0	13.77*
	Exp	91	30	30	10	

		+/+	+/-	*bp*/+	*bp*/-	X^2
cer-2	Obs	102	18	20	18	17.92*
	Exp	89	30	30	9	

		+/+	+/-	*ap-2*/+	*ap-2*/-	
cer-2	Obs	112	11	10	28	66.29*
	Exp	91	30	30	10	

[1] Expected segregation for two recessive nuclear traits assorting independently (9:3:3:1)

* Indicates a significant difference

P>0.9) confirming that act1 and fadD loci are unlinked. An individual designated LIP1 homozygous for fadD and act1 was identified from the F_2 population on the basis of fatty acid compositional analysis, and the result was verified by backcrossing the double mutant to both parents. As in the JB25 parent, LIP1 had no detectable 16:3 acyl group at all growth temperatures examined. The 16:3 fatty acids occur only on the sn-2 position of MGD made by the prokaryotic pathway. Therefore it can be concluded that the act1 mutation affects one of the steps of the prokaryotic pathway, and that it precedes the step marked by the fadD mutation. On the other hand, LIP1 also has reduced amounts of 18:3, but only at 27^{o}C, a trait described for mutant line JB1 (Table 2-X). If the act1 mutation is epistatic to fadD, one would not expect a deficiency in 18:3 levels, since the act1 mutation has no major effect on 18:3 synthesis. This result suggests the involvement of another (eukaryotic) pathway in 18:3 synthesis, that is affected only by the mutation at fadD locus.

Summary

The mapping of genes on a linkage map is becoming increasingly important with the development of molecular genetic procedures that allow the cloning of genes by chromosome walking if they are located in close proximity to those DNA sequences that are already available. Arabidopsis thaliana is especially suitable for this approach because of the small size of its genome (Meyerowitz and Pruitt, 1985). Therefore, the linkage data integrated in Figure 2-2, which show

Table 2-X. Fatty acid composition of total leaf lipids (in mol %) of wild type and mutant Arabidopsis plants grown at 22°C. *Plants grown at 27°C. Each value is a mean of 10 plants. ND, not detected.

Fatty acid	Wild type	JB25	JB1*	LIP1	LIP1*
16:0	13.6	13.3	13.4	12.3	13.6
16:1 cis	0.9	0.7	3.1	1.5	ND
16:1 trans	2.3	2.7	2.7	2.8	2.0
16:2	0.9	0.3	10.6	1.2	ND
16:3	15.7	1.5	2.0	ND	ND
18:0	1.1	0.8	1.4	1.6	1.1
18:1	2.2	6.2	9.3	9.4	6.0
18:2	13.3	19.7	38.3	31.0	58.2
18:3	50.0	54.8	19.2	40.2	19.1

estimated recombination percentages between different pairs of loci, might prove useful in future efforts to isolate the genes marked by act1, fadB and fadC mutations.

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CHAPTER 3

ALTERED REGULATION OF LIPID BIOSYNTHESIS IN A MUTANT OF *ARABIDOPSIS* DEFICIENT IN GLYCEROL PHOSPHATE ACYLTRANSFERASE ACTIVITY

Abstract

The leaf lipids of many plant species, including *Arabidopsis thaliana* (L.) Heynh., are synthesized by two complementary pathways which are located in the chloroplast and the endoplasmic reticulum. By screening directly for alterations in lipid acyl group composition we have identified several mutants of *Arabidopsis* which lack the plastid pathway because of a deficiency in activity of glycerol-3-phosphate acyltransferase, the first enzyme in the plastid pathway of glycerolipid synthesis. The lesion does not cause the accumulation of precursors within chloroplasts, but results in a redirection of fatty acids towards cytoplasmic sites of lipid synthesis. The increased synthesis of lipids by the cytoplasmic pathway compensates for the loss of the plastid pathway and provides, with the exception of phosphatidylglycerol, normal amounts of the various lipids required by the chloroplasts. However, the fatty acid composition of the membrane lipids of the mutant is altered because the acyltransferases associated with the two pathways normally exhibit different substrate

specificities. The remarkable flexibility of the system indicates the existence of regulatory mechanisms which allocate lipids for membrane biogenesis.

Introduction

In the present model of glycerolipid metabolism in higher plants, (Figure 3-1) two pathways contribute to the synthesis of chloroplast glycerolipids in leaf cells (Slack and Roughan, 1975; Roughan, 1975; Roughan et al., 1980; Roughan and Slack, 1982; Heinz and Roughan, 1983; Roughan and Slack, 1984; Gardiner et al., 1984). The chloroplast is the sole site of de novo fatty acid synthesis (Ohlrogge et al., 1979) and the main products of this process are 16:0- and 18:1-ACPs (Soll and Roughan, 1982). These fatty acids either enter the prokaryotic pathway through acylation of glycerol-3-phosphate within the chloroplast (Frentzen et al., 1983), or are exported as CoA thioesters (Roughan and Slack, 1982; Andrews and Keegstra, 1983) to enter the eukaryotic pathway at extrachloroplast sites, particularly in the endoplasmic reticulum (Roughan et al., 1980; Roughan and Slack, 1982). Most of the enzymes of the prokaryotic pathway are located in the inner membrane of the chloroplast envelope and can lead to the synthesis of PG, MGD, DGD and SL which are the major glycerolipids of the thylakoid membranes (Heinz, 1977; Joyard and Douce, 1977; Andrews ans Mudd, 1985). The eukaryotic pathway is responsible for the synthesis of the glycerolipids found in extrachloroplast membranes including PC, PE and PI (Roughan and Slack, 1984). In addition, however, diacylglycerol

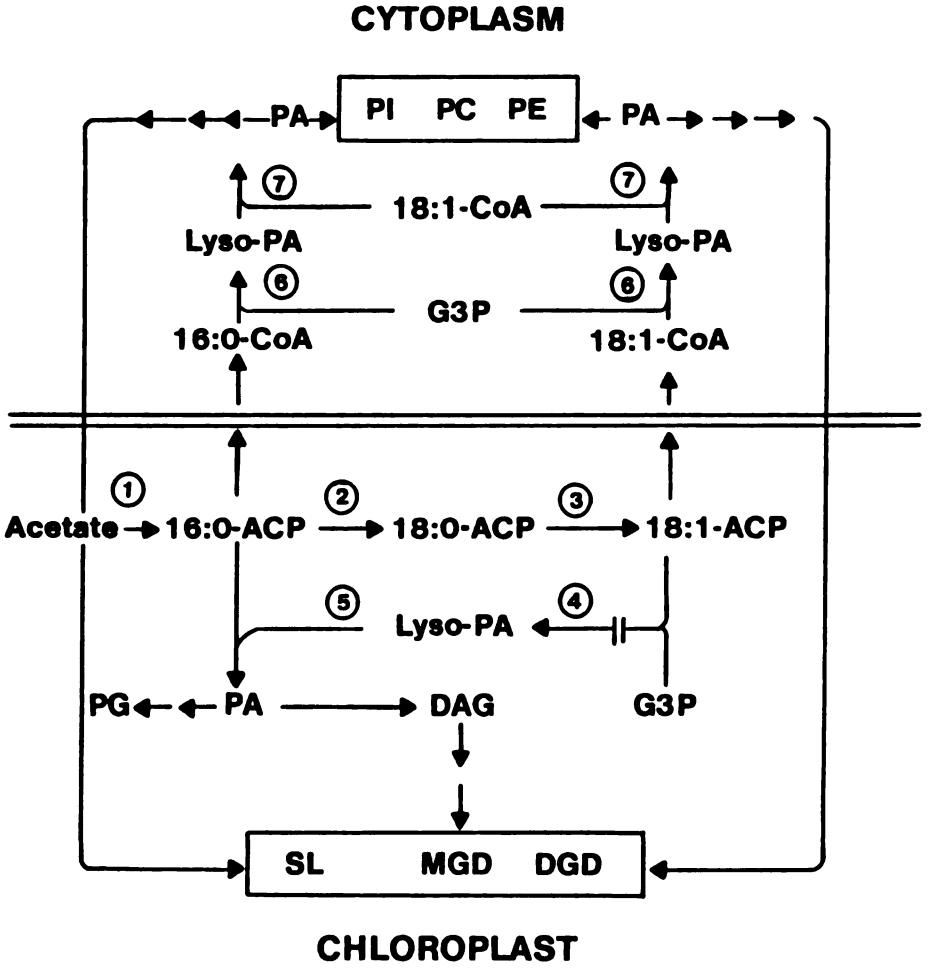

Figure 3-1. An abbreviated scheme for lipid biosynthesis in the leaves of a 16:3 species. The enzymes identified by numbers are [1] fatty acid synthetase; [2] elongase; [3] stearoyl-ACP desaturase; [4] G3P acyltransferase; [5] monoacyl-G3P acyltransferase; [6] G3P acyltransferase; [7] monoacyl-G3P acyltransferase. The enzymatic defect in the actl mutant is indicated by a break in the pathway at reaction 4.

moieties from PC are returned from the endoplasmic reticulum to the chloroplast where they are used for further production of MGD, DGD and SL (Douce and Joyard, 1979; Roughan et al., 1979; Murphy and Stumpf, 1980; Ohnishi and Yamada, 1980; Heinz and Roughan, 1983).

In the majority of higher plants PG is the only product of the prokaryotic pathway and the remaining chloroplast lipids are synthesized entirely by the eukaryotic pathway (Roughan and Slack, 1982; Roughan and Slack, 1984). These species are known as 18:3 plants. However, there are angiosperms such as Arabidopsis thaliana in which both pathways contribute to the synthesis of MGD, DGD and SL (Browse et al., 1986b). They characteristically contain substantial amounts of hexadecatrienoic acid (16:3) which is found only at the sn-2 position of MGD and DGD molecules produced by the prokaryotic pathway. These species have been termed 16:3 plants to distinguish them from 18:3 plants whose galactolipids contain predominantly α-linolenate (Roughan and Slack, 1982; Browse et al., 1986b).

It is not known what regulates the allocation of fatty acids between the two pathways in 16:3 plants. Cooperation between the two pathways of lipid biosynthesis is apparent from the results of labeling experiments with whole leaves which indicate that as much as half of the acyl groups exported to the cytoplasm are reimported into chloroplasts for MGD, DGD and SL biosynthesis (Roughan et al., 1979; Murphy and Stumpf, 1980; Ohnishi and Yamada, 1980). The G3P acyltransferases associated with the chloroplast and endoplasmic reticulum have different substrate specificities, resulting in the sn-2 position of the glycerolipid being occupied exclusively by 18- or 16-carbon fatty acids in eukaryotic or prokaryotic lipids, respectively

(Roughan and Slack, 1984). Thus, the origin of chloroplast glycolipids can be determined by the characteristic fatty acid positional distribution (Roughan and Slack, 1982; Heinz and Roughan, 1983). This has been exploited to compile a detailed account of the relative contribution of the two pathways in Arabidopsis (Browse et al., 1986b).

We have previously described the isolation of a number of mutants of Arabidopsis with altered fatty acid composition. The mutants were identified by direct analysis of leaf fatty acid composition of individual mutagenized plants by GLC. Several of these mutants have previously been characterized as being deficient in specific desaturases (Browse et al., 1985a; Browse et al., 1986a). Here we describe the biochemical characterization of a new class of mutants which lacks 16:3 acyl group due to a deficiency of chloroplast G3P acyltransferase. Because these mutants lack the activity of the first enzyme of the prokaryotic pathway, the mutation effectively converts a 16:3-plant into an 18:3 type. Thus, the mutant offers a unique opportunity to examine both the effects of this change on the regulation of glycerolipid metabolism and the physiological significance of the 16:3-18:3 dimorphism.

Materials and methods

Plant material

The lines of Arabidopsis thaliana (L.) Heynh. described here were descended from the Columbia wild type. The mutant lines JB3, JB25, JB28 and LK8 were isolated following mutagenesis with ethyl methane

sulfonate (Haughn and Somerville, 1986). Before being used for experiments, the line JB25 was backcrossed to the wild type at least three times and an individual with the mutant phenotype was reselected from a segregating population. Plants were grown under continuous fluorescent illumination (100-150 uE m^{-2} s^{-1}) at 22°C on a perlite : vermiculite : sphagnum (1:1:1) mixture irrigated with mineral nutrients (Haughn and Somerville, 1986).

Chemicals

Sodium (^{14}C)-acetate (54 mCi/mmol) and $NaH^{14}CO_3$ (55.5 mCi/mmol) were obtained from Research Products International Corporation, Mt. Prospect, IL, (^{14}C)16:0-CoA (58 mCi/mmol) and (^{14}C)18:1-CoA (53 mCi/mmol) were purchased from DuPont, Wilmington, DE, and (^{14}C)-glycerol-3-phosphate (30 mCi/mol) from ICN Radiochemicals, Irvine, CA. (^{14}C)16:0-ACP (55 mCi/mmol) and (^{14}C)18:1-ACP (55 mCi/mmol) were a generous gift from Dr. J.B. Ohlrogge, Department of Botany and Plant Pathology, Michigan State University. Sodium methoxide was prepared as described previously (23). Methanolic HCl reagent was prepared by diluting 3 M solution (Supelco) to 1 M with methanol (Browse et al., 1985b).

Lipid analysis

Plants were frozen in liquid N_2, then extracted with chloroform : methanol : formic acid (10:10:1 by vol.) (Browse et al., 1986b). Following centrifugation, the supernatant was decanted and the tissue reextracted with chloroform : methanol : water (5:5:1 by vol.). The extracts were combined and washed with 0.2 M H_3PO_4/2 M KCl (Hajra,

1974). Lipids were recovered in the chloroform phase, dried under N_2 and taken up in a small volume of chloroform.

Lipids were separated by thin layer chromatography on silica gel coated plates (Baker). For one dimensional chromatography a solvent system of chloroform : acetone : methanol : acetic acid : water (100:40:20:20:10 by vol.) was used. When the lipids were chromatographed in two dimensions a solvent system of chloroform : methanol : ammonia (65:25:2 by vol.) was used in the first development, and chloroform : methanol : acidic acid : water (85:15:10:3 by vol.) in the second development. Individual lipids were identified by comparing their R_f values with those of reference standards and transmethylated with either sodium methoxide or hot methanolic HCl after the addition of a known amount of $C_{17:0}$ methyl ester as an internal standard. The resulting methyl esters were quantified by gas chromatography as described (Browse et al., 1985b).

The kinetics of lipid biosynthesis was followed by labeling intact Arabidopsis plants with (^{14}C)-acetate as described previously (Browse et al., 1986b).

Chloroplast isolation

Chloroplasts used directly in labeling experiments with (^{14}C)-acetate and (^{14}C)-glycerol-3-phosphate were obtained by grinding 20 g of leaf tissue in 200 ml of homogenization medium containing 0.45 M sorbitol, 20 mM Tricine-KOH (pH 8.4), 10 mM EDTA, 10 mM $NaHCO_3$ and 0.1 % BSA. The extract was passed through Miracloth, centrifuged at 270 x g for 90 sec and resuspended in 0.3 M sorbitol, 20 mM Tricine-KOH (pH 7.6), 5 mM $MgCl_2$ and 2.5 mM EDTA. Chloroplast suspension was then

transferred to Percoll gradients prepared by centrifuging 50% Percoll in resuspension buffer at 43,000 x g for 30 min in a Sorvall SS-34 rotor (Cline et al., 1981). The overlayered gradients were centrifuged at 13,000 x g for 6 min in a Sorvall HB-4 rotor. Intact chloroplasts, which form a band near the bottom of the gradient, were recovered, diluted with resuspension buffer, pelleted at 3000 x g for 90 sec in a Sorvall HB-4 rotor and resuspended in the resuspension medium.

Chloroplasts used for fractionation and enzyme assays were isolated from protoplasts of Arabidopsis leaves by a modified procedure of Somerville et al. (1981). Intact leaves were vacuum infiltrated with a medium containing 0.5 M sorbitol, 10 mM MES-KOH (pH 6), 1 mM $CaCl_2$, 1.6 % (w/v) Macerase and 1.6 % (w/v) Cellulase, and incubated for one hour at room temperature in the same medium. Protoplasts were passed through several layers of cheesecloth to remove debris, harvested by centrifugation at 4^oC for 5 minutes at 100 x g, and resuspended in cold 0.5 M sorbitol, 10 mM MES-KOH (pH 6), 1 mM $CaCl_2$. The suspension was transferred into test tubes containing 50% Percoll (v/v), 0.5 M sorbitol, 10 mM MES-KOH (pH 6), 1 mM $CaCl_2$, and intact protoplasts were banded by centrifugation at 100 x g for 10 minutes. Protoplasts were resuspended in 0.3 M sorbitol, 20 mM Tricine-KOH (pH 8.4), 10 mM EDTA, and gently lysed by passing through a 15 um mesh net. Chloroplasts were centrifuged at 270 x g for 90 seconds and resuspended in cold 0.3 M sorbitol, 20 mM Tricine-KOH (pH 7.6), 5mM $MgCl_2$, 2.5 mM EDTA. The percentage of intact chloroplasts was determined by measuring oxygen evolution in the presence of ferricyanide before and after osmotic shock (Lilley et al., 1975).

Chloroplast labeling

Intact chloroplasts (400 ug chlorophyll/ml) were incubated with shaking at 25°C in a medium containing 0.33 M sorbitol, 25 mM Hepes-NaOH (pH 7.9), 10 mM $NaHCO^3$, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 0.4 mM sn-glycerol-3-phosphate with 0.15 mM (^{14}C)-acetate (54 mCi/mmol), or 0.4 mM (^{14}C)-glycerol-3-phosphate (30 mCi/mmol) with 0.15 mM Na-acetate, for 20 min under illumination (150 uE m^{-2} s^{-1}) and then in darkness for 20 min. Reactions were stopped by adding chloroform/methanol (1:1 v/v) and lipids were recovered from the chloroform layer after partitioning against 0.2 M H_3PO_4/2 M KCl (Hajra, 1974).

Enzyme assays

Crude leaf extracts were prepared by lysing intact protoplasts in 20 mM Tricine-KOH (pH 8.4) and 10 mM EDTA. Chloroplast stromal extracts were prepared by resuspending intact chloroplasts in 10 mM Tricine-KOH (pH 7.6), 1 mM $MgCl_2$. This causes rupture and detachment of envelope membranes and liberation of the stroma (Joyard and Douce, 1977). The chloroplast components were then layered on density step gradients composed of 0.93 M and 0.6 M sucrose in 10 mM Tricine-NaOH, pH 7.6/4 mM $MgCl_2$ and were centrifuged for 1 h in a swinging-bucket rotor at 72,000 x g. The envelopes were collected from the interface of the 0.93 M and 0.6 M sucrose layers. The fraction containing the chloroplast stromal components was recentrifuged at 130,000 x g for 2 h to remove any remaining membranes and was used immediately for enzyme assays.

G3P acyltransferase and monoacyl-G3P acyltransferase activities were assayed at 22°C essentially as described (Bertrams and Heinz,

1981, Frentzen et al., 1983), with [^{14}C]acyl CoA as the substrate for chloroplast extracts and [^{14}C]acyl-ACP for whole-cell extracts. The 80 ul G3P acyltransferase assay mixtures contained 250 mM Mops-NaOH (pH 7.4), 50 ug of bovine serum albumin, 5 uM acyl-ACP of acyl-CoA, 2 mM L-G3P, and 25-50 ug of chloroplast stromal protein or 250-300 ug of protein from whole-cell extracts. The same mixture was used for monoacyl-G3P acyltransferase assays except that 1-oleoyl-G3P was used instead of G3P and 50-80 ug of chloroplast envelope protein was used instead of the stromal extract. Ribulosebisphosphate carboxylase (Pierce et al., 1982) and phosphoenolpyruvate carboxylase (Stit et al., 1978) were assayed essentially as described. Protein concentrations were measured by the methods of Bradford (1976), or Markwell et al., (1981) with BSA as standard.

Results

Genetic analysis

A genetic analysis of the *act1* mutants is described in Chapter 2.

Biochemical characterization

On the basis of exploratory labeling studies with intact plants and with isolated chloroplasts using [^{14}C]-glycerol-3-phosphate (Figure 3-2A) we inferred that the mutant had a lesion at an early step of the prokaryotic pathway. However, the distribution of radioactivity among polar lipids from the mutant and wild type was identical in [^{14}C]-acetate labeled chloroplasts (Figure 3-2B). Therefore, in order

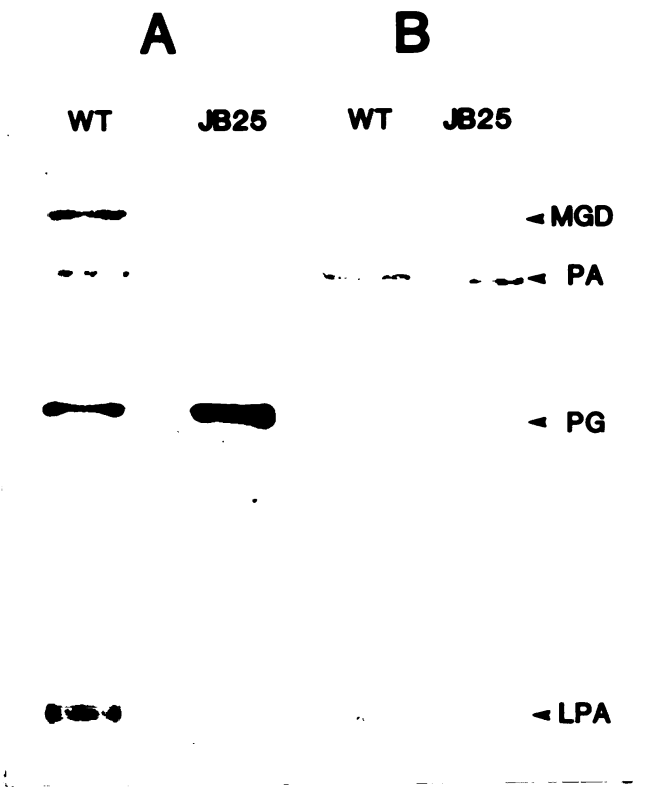

Figure 3-2. The distribution of radioactivity among the polar lipids of mutant JB25 and wild type Arabidopsis following A) (^{14}C)-G3P and B) (^{14}C)-acetate labeling of isolated chloroplasts. The same amount of radioactivity was applied to each lane.

to determine whether the prokaryotic pathway is affected by the mutation, we assayed the chloroplast enzymes involved in PA synthesis by measuring the incorporation by stromal extracts of (^{14}C)18:1-CoA and (^{14}C)16:0-CoA into lipids. Although acyl-ACP is the normal substrate for these reactions, the CoA esters are also readily accepted by the chloroplast acyltransferases (Bertrams and Heinz, 1981). Because of the presence in crude extracts of both chloroplast and microsomal acyltransferases we first purified chloroplasts and then assayed stromal extracts for activity. The results of this experiment (Table 3-I) indicated that the mutant exhibits only 3.8% of the wild type activity of glycerol-3-phosphate acyltransferase, the first enzyme of the prokaryotic pathway (Figure 3-1). Since the chloroplasts were slightly contaminated with protoplasts (1.9% PEP carboxylase activity was detected in JB25 stromal extract), some of the residual activity in the mutant is due to contamination of the chloroplast fraction by cytoplasmic enzymes. The mutant had wild-type levels of the chloroplast enzyme ribulosebisphosphate carboxylase (Table 3-I) and appears to have normal levels of monoacylglycerol-3-phosphate acyltransferase activity. The independently isolated mutant line LK8 also had a specific deficiency in G3P acyltransferase activity (Table 3-I). Therefore, it seems very likely that the *act1* locus specifically controls the activity of the plastid isozyme of glycerol-3-phosphate acyltransferase.

Chloroplast G3P acyltransferase can use acyl-ACP as well as acyl-CoA for the acylation reaction, but if both are present, ACP thioesters are exclusively used as substrate (Frentzen et al., 1983). Since ACP thioesters are confined to the chloroplasts, we wanted to

Table 3-I. Enzyme activities in the stromal fraction and leaf extracts of Arabidopsis chloroplasts. Values are the mean of 3-5 assays.

	Specific activity (nmol/mg protein/min)	% activity
STROMA (^{14}C)18:1-CoA labeling		
G3P acyltransferase		
WT	0.011	100
JB25	0.00042	3.8
Monoacyl-G3P acyl-4 transferase		
WT	0.17	100
JB25	0.20	118
RuBisCO		
WT	400	100
JB25	490	122.5
PEP carboxylase		
WT	0.224	100
JB25	0.239	106.7
LEAF EXTRACT (^{14}C)18:1-ACP labeling		
G3P acyltransferase		
WT	0.116	100
JB25	0.0057	4.9
WT + JB25	0.064	55.2
LK8	0.0039	3.4
Monoacyl-G3P acyl-transferase		
WT	0.0034	100
JB25	0.0035	102
LK8	0.0034	100
RuBisCO		
WT	570	100
JB25	550	97
PEP carboxylase		
WT	10.2	100
JB25	12.6	124

determine whether they can be used as acyl donors by the other leaf acyltransferases. Therefore, we assayed crude leaf extracts of the wild type and JB25 mutant by measuring the incorporation of (^{14}C)18:1-ACP into lipids. Parallel assays were performed in leaf extracts with (^{14}C)18:1-CoA as substrate. This experiment has shown that ACP thioesters in the wild type plants are predominantly incorporated in LPA, PA and PG, while the mutant exhibits less than 5% of G3P acyltransferase activity (Table 3-I). On the other hand, when CoA esters are used as donors, the total G3P acyltransferase activity in the mutant reaches 82% of the activity of wild-type plants. This result indicates that acyl-ACP thioesters are probably not the substrates for extraplastid acyltransferases.

Labeling of leaves

In order to investigate the consequences of the enzyme defect on lipid biosynthesis in the mutant (^{14}C)-acetate was applied to leaves of mutant and wild-type plants and the redistribution of radioactivity in polar lipids was followed during the subsequent 142 hours. From the results of this experiment it can be seen that the mutation causes dramatic differences in the pattern of (^{14}C)-acetate incorporation in JB25 when compared to the wild type (Figure 3-3). As we have discussed previously (Browse et al., 1986b) the labeling kinetics for wild-type plants demonstrate the parallel operation of the two pathways of lipid synthesis. Flux through the prokaryotic pathway leads to the substantial labeling of MGD at early times while the subsequent transfer of ^{14}C from PC to MGD and DGD occurs via the eukaryotic pathway. In contrast the mutant contained the label primarily in PC at

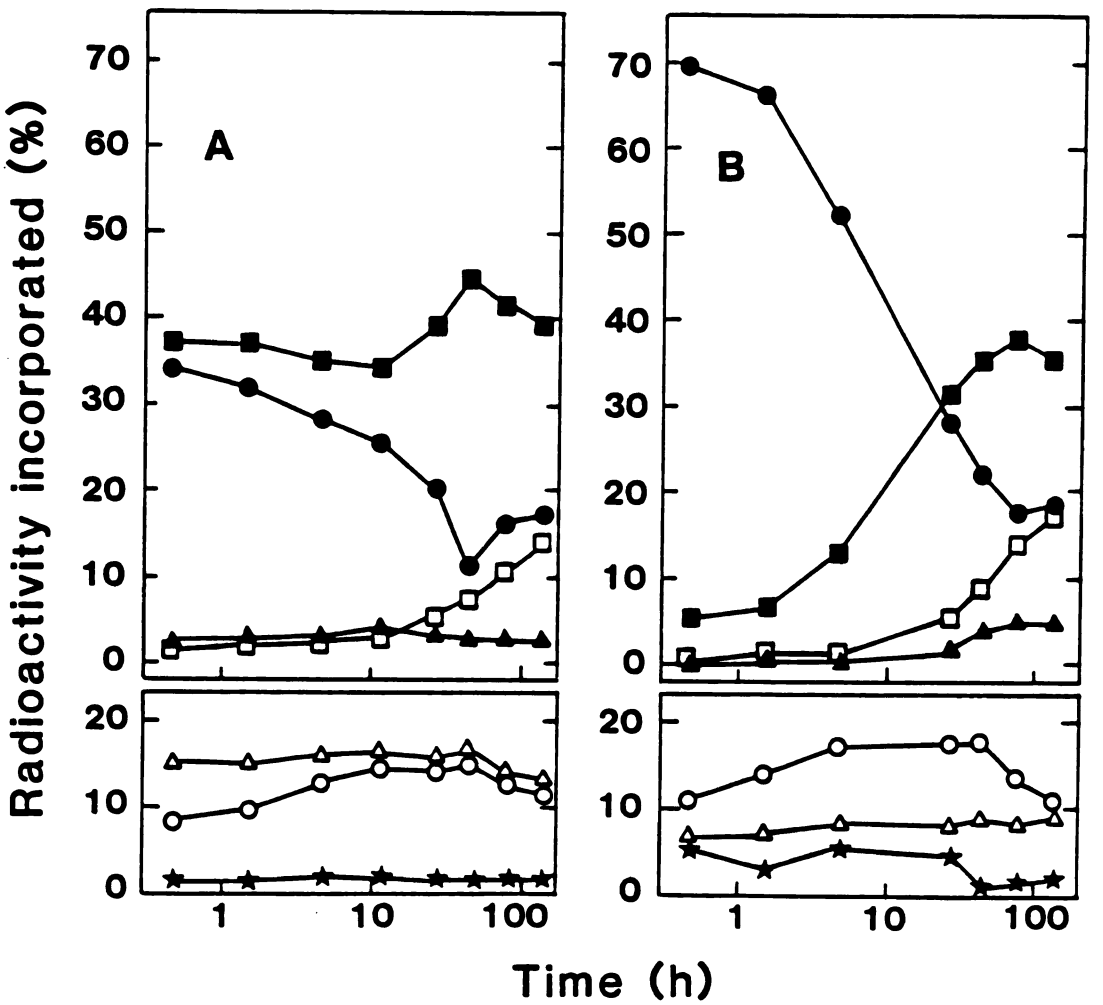

Figure 3-3. The distribution of radioactivity in leaf lipids of (A) wild type and (B) JB25 mutant Arabidopsis after labeling with ^{14}C-acetate. Symbols: ●, PC; ■, MGD; □, DGD; ▲, SL; △, PG; ○, PE; ★, PI.

short times, whereas MGD contained less than 3% of the total counts. During the course of the experiment there is a steady and substantial decline of radioactivity in PC, which is accompanied by increased label in MGD, DGD and SL so that by the end of the experiment the distribution of ^{14}C among the various polar lipids is similar to the wild type. These kinetics demonstrate a precursor-product relationship between PC and the chloroplast glycolipids and indicate that MGD in JB25 is made entirely by the eukaryotic pathway. In these respects the labeling kinetics are extremely similar to those observed in analogous experiments with 18:3 plants in which all the chloroplast glycerolipids except PG are derived from the eukaryotic pathway (Slack and Roughan, 1975; Roughan and Slack, 1982; Roughan and Slack, 1984). 18:3 plants synthesize PA and PG by the prokaryotic pathway but synthesis of other chloroplast lipids is precluded because chloroplast PA is not converted to diacylglycerol (Heinz and Roughan, 1983; Roughan and Slack, 1985).

The deficiency in the chloroplast acyl-ACP:glycerol-3-P acyltransferase found in JB25 would be expected to block PG synthesis by the prokaryotic pathway. However PG does become labeled in the mutant although the extent of ^{14}C incorporation into this lipid is only about half of that found in the wild type (Figure 3-3).

Lipid composition

In wild-type Arabidopsis the prokaryotic pathway is responsible for producing approximately 70% of the total leaf MGD, 12% of the DGD, 63% of the SL and 85% of the PG (Browse et al., 1986b). Nevertheless, the deficiency of acyl-ACP:glycerol-3-P acyltransferase in the mutant does not lead to a dramatic reduction in the amount of any of these

lipids. The total lipid content of leaves from the mutant was the same as the wild type on the basis of both fresh weight and chlorophyll (data not shown) and the most pronounced effect on any individual lipid was the 30% reduction in the proportion of PG in the mutant (Table 3-II). There is also a small decrease in the amount of MGD and a corresponding increase in the amount of PC which is the precursor of MGD in the eukaryotic pathway.

The similar proportions of each lipid in the mutant and wild type (Table 3-II) together with the data from the labeling experiment (Fig. 3-3), indicate that the lack of synthesis of MGD, DGD and SL by the prokaryotic pathway is compensated for by increased production of these lipids via the eukaryotic pathway. The differential effect of the *act1* mutation on the amounts of the various chloroplast-specific lipids reflects the various degrees to which these lipids are normally produced by the prokaryotic pathway (Browse at al., 1986b). The increased amounts of PC and PE are consistent with (but not proportional to) the increased flux through the eukaryotic pathway.

In order to determine if PG in the mutant had the characteristic structure of a product of the prokaryotic pathway, the purified lipid was digested with *Rhizopus* lipase and the fatty acid composition of the lyso-PG and free fatty acid was determined (Browse et al., 1986b). This analysis indicated that in the mutant 75% of the fatty acid at the *sn*-2 position of PG was C_{16}. In the wild type the *sn*-2 position was 83% C_{16}. In contrast, other polar lipids in the mutant contained more than 90% C_{18} fatty acids at *sn*-2 of the glycerol, indicating that they were produced by the eukaryotic pathway.

Table 3-II. Fatty acid composition of leaf lipids from wild-type and mutant Arabidopsis grown at 22°C. Values presented are mol %. Dashes indicate that the acyl group was not detected.

	MGD		DGD		SL		PG		PC		PE		PI	
	WT	JB25	WT	JB25	WT	JB25	WT	JB25	WT	JB25	WT	JB25	WT	JB25
16:0	1.1	1.1	12.0	7.6	43.3	26.5	32.0	35.0	27.8	20.1	31.6	29.2	47.5	47.9
16:1t	-	-	-	-	-	-	22.6	29.9	-	-	-	-	-	-
16:2	1.9	-	-	-	-	-	-	-	-	-	-	-	-	-
16:3	33.0	1.7	1.7	0.8	-	-	-	-	-	-	-	-	-	-
18:0	-	0.2	0.7	0.8	1.2	1.5	2.4	2.8	2.0	1.8	2.4	2.3	3.2	5.7
18:1	-	1.4	1.2	1.2	2.1	8.8	7.0	3.0	3.7	19.7	3.2	13.0	1.7	10.2
18:2	2.9	5.1	3.7	3.3	4.7	8.7	8.0	7.8	20.5	24.2	36.7	41.0	23.6	24.8
18:3	61.1	90.5	80.5	86.9	48.7	54.5	28.0	21.5	46.0	34.2	26.1	14.5	23.5	10.2
%	39.0	34.6	15.2	18.0	2.8	2.0	8.1	5.7	21.8	26.2	10.0	10.5	3.0	3.0

The chloroplast-specific acyl group 16:3 which is characteristic of prokaryotic MGD is virtually absent from the lipids of the mutant and is replaced by 18-carbon fatty acids (primarily 18:3). The 13-fold reduction in the amount of 16-carbon fatty acids in MGD is not accompanied by an increase in 16-carbon fatty acids in any other lipid. Thus the 16:0 excluded from the prokaryotic pathway appears to be elongated and desaturated to 18:1 before being exported from the chloroplast to enter the eukaryotic pathway. The implication is that the export of 16:0 is regulated not by the availability of 16:0-ACP but by some other mechanism.

In a detailed analysis of wild-type Arabidopsis we have previously shown (Browse et al., 1986b) that for every 1,000 fatty acids made in the chloroplast 615 enter the eukaryotic pathway (117 C_{16} + 498 C_{18}). A similar analysis on the mutant shows that the increase in flux through the eukaryotic pathway (to 950 per 1000) is made up almost entirely of C18 fatty acid chains (126 C16 + 824 C18). The ratio of C16:C18 fatty acids in PC, PE and PI are the same as in the corresponding lipids of the wild type (Table 3-II). In contrast the C16:C18 ratio in MGD, DGD and SL of the mutant is in each case less than the ratio calculated for these lipids synthesized by the eukaryotic pathway in wild-type Arabidopsis (Table 4, Browse et al., 1986b). Thus, the additional C18 fatty acids entering the eukaryotic pathway in the mutant are found specifically in the additional quantities of chloroplast lipids (MGD, DGD and SL) which are produced by the eukaryotic pathway in response to the loss of the prokaryotic pathway.

The mutation causes an increase in the amount of 18:1 fatty acids and a decrease in the amount of 18:3 in all of the extrachloroplast (PC, PE, PI) lipids of the mutant. There was little or no effect on the amount of 18:2 in these lipids (Table 3-II). The data indicate a 10-30% reduction in the extent of 18:1 desaturation in these lipids in the mutant relative to the wild type. It seems likely that this is caused by the inability of the endoplasmic reticulum $C_{18:1}$ desaturase to completely metabolize the increased flux of lipid through the eukaryotic pathway in the mutant.

Discussion

In the mutant JB25 a single recessive nuclear mutation at the actl locus causes a specific deficiency in the activity of the acyl-ACP:glycerol-3-P acyltransferase. Three other mutants, JB3, JB28 and LK8, are allelic to JB25. These other mutants show all of the changes in lipid and fatty acid composition which have been described here for JB25 indicating that all the changes are direct consequences of the deficiency in acyltransferase activity. Our analysis of the mutant provides a general outline of the features of the controls which regulate lipid metabolism to maintain suitable glycerolipid and fatty acid compositions in cellular membranes.

Synthesis of glycerolipids

Both the labeling data (Fig. 3-3) and the lipid analysis (Table 3-II) indicate that loss of the acyltransferase activity does not

result in the accumulation of precursors (18:1- and 16:0-ACPs) upstream of the block but in redirection of lipid metabolism so that the eukaryotic pathway predominates in the mutant. Suprisingly, this redirection has little effect on the proportion of each lipid synthesized or indeed on the total glycerolipid content of the tissue. Thus not only is the flux through the eukaryotic pathway increased in the mutant, but the proportion of individual lipids synthesized by this pathway changes to produce a complement of leaf lipids which is similar to that of wild-type plants. It would appear from these observations that demand for a particular lipid is a major factor regulating synthesis. There is, at present, no evidence pertaining to the mechanism by which this redistribution of acyl groups might be accomplished.

Altered ratio of 16C/18C fatty acids

In wild-type Arabidopsis, C16 fatty acids represent half of the acyl chains found in lipids made by the prokaryotic pathway. However, loss of the prokaryotic pathway in the mutant does not result merely in redirection of the C16 chains into the eukaryotic pathway. Instead the 16:0-ACP is elongated and desaturated to 18:1-ACP before export from the chloroplast. Thus, the overall ratio of C16 to C18 chains produced by fatty acid synthesis is reduced from 0.3 in the wild type to 0.18 in the mutant (Table 2-II). It is noteworthy in this respect that the primarily extrachloroplastic lipids (PC, PE and PI) have relatively normal levels of 16:0. The implication is that the amount of export of 16:0 is not regulated simply by the availability of 16:0. This suggests that elongase activity is regulated by availability of

substrate (16:0) and that this is determined by competition between alternative pathways of 16:0 utilization.

Synthetic capacity of the eukaryotic pathway.

The two-fold increase in the flux through the eukaryotic pathway obviously challenges the synthetic capabilities of the enzymes involved. Two sets of evidence suggest that in the mutant the eukaryotic pathway is operating near the limit of its ability to produce chloroplast lipids. First, the amount of PC is increased from 21.8 to 26.2% of the leaf lipids while the amount of MGD is slightly decreased. This suggests that transfer of acyl groups to the chloroplast, or further metabolism of PC may be limiting in the mutant. Secondly the extent of desaturation of 18:1 in PC and the other extrachloroplast lipids PE and PI is decreased by 10-20% compared with the wild type. This may well be caused by the inability of the endoplasmic reticulum 18:1 desaturase to match the increased throughput of the eukaryotic pathway.

Synthesis of PG.

Evidence from studies by Andrews and Mudd (1985) indicates that isolated chloroplasts are able to synthesize PG at rates which are sufficient to meet the requirements for chloroplast membrane biogenesis and that the pathway of synthesis of PG involves the same pools of LPA and PA used for the synthesis of prokaryotic MGD, DGD and SL. It is puzzling, therefore, that the mutant contains at least 75% as much PG as the wild type, even though it exhibits less than 4% of the wild-type level of G3P acyltransferase activity. One possibility is that the

amount of PA made in the mutant with the residual 4% of chloroplast acyltransferase activity is adequate to meet most of the requirement for PG synthesis and is utilized preferentially for PG synthesis. A reduction in the size of the LPA and PA pools might explain why these compounds did not accumulate radioactivity during [^{14}C]G3P labeling of isolated chloroplasts (Figure 3-2). Since only very small amounts of prokaryotic MGD are found in the mutant (Table 3-II) this explanation would require that PG synthesis from PA is efficiently maintained at the expense of DAG synthesis.

The other possibility seems to be that an alternative source of PA is used for PG synthesis. However, the predominance of 16-carbon fatty acids on the sn-2 position of PG in the mutant is most consistent with this lipid being derived from the prokaryotic pathway in the chloroplast rather than from any other source.

Evolutionary implications

A preliminary physiological characterization of the mutant suggests that the loss of the prokaryotic pathway is not deleterious. The general appearance of the mutant plant is similar to the wild type, and it is not impaired in growth or development under standard growth conditions. Thus, the question that inevitably arises concerns the dispensibility of the prokaryotic pathway for glycerolipid synthesis in the mutant, as well as in naturally occurring 18:3 plants and in the fruits of 16:3 species (Whitaker, 1986). In the course of evolution, the majority of higher plants have abandoned the prokaryotic pathway so that it persists to varying degrees only in less advanced genera (Jamieson and Reid, 1971). Since a single mutation can eliminate the

prokaryotic pathway, it seems reasonable to suggest that there must be both some physiological advantages and disadvantages associated with the loss of prokaryotic lipids from chloroplast membranes. We anticipate that further analysis of the mutants described here will provide unique insights into why both 16:3-plants and 18:3-plants coexist.

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CHAPTER 4

ALTERATIONS IN CHLOROPLAST ULTRASTRUCTURE CAUSED BY CHANGES IN MEMBRANE LIPID COMPOSITION IN A MUTANT OF ARABIDOPSIS DEFICIENT IN PLASTID GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE ACTIVITY

Abstract

A mutant of Arabidopsis thaliana has altered chloroplast membrane composition due to a deficiency in chloroplast glycerol-3-phosphate acyltransferase activity. The most pronounced effect of the mutation is an increase in the number of appressed regions per chloroplast and a corresponding decrease in the average number of thylakoid membranes in each appressed region. These changes were not associated with a significant alteration in the amount of Chl a/b binding proteins, suggesting that the model for membrane appression based on the properties of light harvesting Chl a/b protein complex (LHCP) is incorrect, or incomplete. The changes in leaf lipid composition do not affect growth or development of the mutant under standard conditions ($22^{o}C$, 100-150 uE m^{-2} s^{-1}). Similarly, photosynthetic electron transport, net CO_2 fixation and room temperature fluorescence of the mutant are comparable to wild-type plants. However, at temperatures above $28^{o}C$ the mutant grows more rapidly. Measurements of

temperature-induced fluorescence yield enhancement and the delayed inactivation of whole chain electron transport in isolated chloroplast membranes at high temperatures suggest an increased thermal stability of the photosynthetic apparatus of the mutant. A comparison of 77K fluorescence emission spectra of thylakoid membranes from the mutant and wild type indicated a slight decline in excitation energy transfer from LHCP to PSII and PSI in the mutant. These changes may be due to altered structural organization of _actl_ chloroplasts.

Introduction

Glycerolipid synthesis in leaves of higher plants is thought to involve two biosynthetic routes designated 'prokaryotic' and 'eukaryotic' pathways (Roughan and Slack, 1982). Fatty acids synthesized _de novo_ in the chloroplasts may either enter the prokaryotic pathway in the chloroplast envelope, or be exported to the endoplasmic reticulum where they are incorporated into lipids through the eukaryotic pathway. In '16:3 plants' such as _Arabidopsis thaliana_ both pathways are involved in the production of chloroplast lipids. However, the majority of higher plants uses the prokaryotic pathway only for the synthesis of phosphatidylglycerol (PG), while the remaining chloroplast lipids are made by the eukaryotic pathway. They include more advanced angiosperm genera and are known as '18:3 plants'.

Since '18:3 plants' have abandoned the prokaryotic pathway for the synthesis of chloroplast glycolipids, the question that arises concerns the role of this pathway in '16:3 plants'. It seems reasonable to

suggest that an operational prokaryotic pathway confers some selective advantage, because '16:3' and '18:3 plants' coexist. We have recently described the isolation and biochemical characterization of a class of mutants of Arabidopsis that lack the activity of the first enzyme of the prokaryotic pathway, glycerol-3-phosphate acyltransferase (Kunst et al. 1988). The mutation responsible (act1) effectively converts a '16:3' into an '18:3 plant' and offers a unique opportunity to examine the functional significance of the prokaryotic pathway.

As a direct consequence of the deficiency in the prokaryotic pathway, the mutant lines JB25 and LK8 show specific alterations in composition of leaf membrane lipids (Kunst et al. 1988). These involve a 15-20% reduction in PG, a 9% decrease in MGD, a 12% decrease in DGD, and a 30% decrease in SL content, while the amounts of PC and PE are increased 12% and 10%, respectively. The mutation also results in greatly reduced levels of 16:3 acyl group, characteristic of prokaryotic MGD, and a corresponding increase in 18-carbon fatty acids. Since the prokaryotic pathway provides lipids specifically for chloroplast biogenesis, we have investigated the effects of the changes in membrane composition in the act1 mutant on chloroplast structure and function.

Materials and methods

Plant material and growth conditions

All lines of Arabidopsis described here are descended from the Columbia wild type. The isolation and biochemical characterization of

the mutant lines JB25 and LK8 has been described (Kunst et al., 1988). Both lines carry a single recessive nuclear mutation at a locus designated *act1*. Before being used for experiments reported here, the lines JB25 and LK8 were backcrossed to the wild type five times and two times, respectively, and individuals with the mutant phenotype were reselected from segregating populations. Unless otherwise indicated, plants were grown at 22°C under continuous fluorescent illumination (100-150 uE m^{-2} s^{-1}) on a perlite : vermiculite : sphagnum mixture irrigated with mineral nutrients (Haughn and Somerville, 1986).

Measurements of growth rate

Plants were germinated at 22°C and grown as described above for seven days. After that the temperature was adjusted to that mentioned in the text. Samples of four plants were randomly harvested at three day intervals for the next 12 days, and their fresh weight was measured. The relative growth rate (w^{-1}) was determined as the slope of the natural logarithm of the average fresh weight (of 4 samples) plotted against days since the temperature adjustment.

Extraction and analysis of Chl, proteins and lipids

Leaves were harvested at the rosette stage (3 weeks old plants) and their fresh weight was determined prior to homogenization in cold 20 mM Tricine-KOH (pH 8.4), 5 mM $MgCl_2$ and 2.5 mM EDTA. Insoluble matter was removed by centrifugation at 100 x g for 10 min and aliquots of the extract were used for Chl, protein and lipid determinations. Chlorophyll was assayed by the method of MacKinney (1941), protein measurements were performed using a modified Lowry assay (1978), and

lipids were quantified by gas chromatography using a known amount of 14:0-methyl ester as an internal standard (Browse et al.,1985a). Fatty acid composition of total leaf lipids was determined after preparation of fatty acid methyl esters as described (Browse et al., 1985b.

Pigment-protein electrophoresis

Chloroplast membranes were isolated as described above. Pigment-protein electrophoresis was performed according to the method of Andersson et al. (1982), except that the sodium dodecyl sulfate (SDS, Sequanal Grade, Pierce, Rockford, IL) to Chl weight ratio was adjusted to 3.75 : 1.

L-[^{35}S]-Methionine labeling of thylakoid proteins and protein extraction

L-[^{35}S]-Methionine (DuPont, NEN, Boston, MA, 1083 Ci mmol^{-1}) was diluted to 0.5 mCi ml^{-1} with 0.025% Triton X-100 and applied onto both leaf surfaces of 15 days old plants. Twenty-four h after application of the label, aerial portions of 5 plants were harvested and homogenized in 30 ml of medium containing 450 mM sorbitol, 20 mM Tricine-KOH (pH 8.4), 10 mM NaCl, 10 mM EDTA and 0.1% (w/v) BSA. The extract was filtered through Miracloth (Calbiochem, La Jolla, CA), centrifuged for 10 min at 3000 x g, and resuspended in 1 ml of the homogenization buffer. The chloroplast suspensions were then transferred to Eppendorf tubes, repelleted, and suspended in 200 ul of a buffer containing 700 mM sucrose, 500 mM Tris-KOH (pH 9.4), 50 mM EDTA, 100 mM KCl, 2% 2-mercaptoethanol (v/v) and 2 mM phenylmethyl-sulfony fluoride (PMSF) (Hurkman and Tanaka, 1986). An equal volume of phenol was added to the

suspension, and proteins were extracted for 10 min. The phases were separated by centrifugation and the phenol phase was recovered. Proteins were precipitated from the phenol phase by the addition of 1 ml of 100 mM ammonium acetate in methanol and incubation at -20^{o}C overnight. The precipitate was washed twice with 20% acetone and dried for 1 h at room temperature.

Two-dimensional gel electrophoresis

Isoelectric focusing tube gels (3 mm x 120 mm) contained 9.5 M urea, 3% Triton X-100 (w/v), 2.24% ampholines (4-6), 0.96% ampholines (5-8), 0.8% ampholines (3-10), 4% acrylamide (w/v), 0.2% bis-acrylamide (w/v), 0.01% (w/v) ammonium persulfate and 0.07% (v/v) TEMED. Protein samples were solubilized in a medium containing 9.5 M urea, 1.25% SDS (w/v), 2% 2-mercaptoethanol (v/v), 2% ampholines (3-10), 6% Triton X-100 (w/v) and a trace amount of bromophenol blue. Aliquots (80 ug protein) were loaded at the cathodic end of the gels and overlaid with 6% Triton X-100 (w/v) and 2% ampholines (3-10). The upper (cathode) buffer was 50 mM NaOH, and the lower (anode) buffer was 25 mM H_3PO_4. Isoelectric focusing was conducted at 200 V for 30 min, 300 V for 30 min, and finally 500 V for 24 h at room temperature.

The second dimension slab gels (160 x 180 x 1.5 mm) consisted of an 8% acrylamide (w/v) resolving gel and a 5% acrylamide (w/v) stacking gel prepared according to Laemmli (1970). Focusing tube gels were equilibrated for 45 min in 2 x 10 ml of stacking gel buffer containing 0.1% SDS (w/v) and 2% 2-mercaptoethanol (v/v), and sealed on the slab gel apparatus with 0.8% (w/v) agarose in stacking buffer. Electrophoresis was performed at 15 mA per gel. Proteins were then

electrophoretically transferred to nitrocellulose filters (Hybond-C, Amersham, Arlington Heights, IL) (Towbin et al., 1979), and detected by autoradiography.

Isolation of chloroplast membranes

Chloroplast membranes were prepared by grinding leaves in cold 450 mM sorbitol, 20 mM Tricine-KOH (pH 8.4), 10 mM NaCl, 10 mM EDTA and 0.1% (w/v) BSA. The extract was filtered through Miracloth (Calbiochem, La Jolla, CA) and centrifuged at 3000 x g for 5 min. The pellet was washed with 10 mM Hepes (pH 7.9), 10 mM NaCl 5 mM EDTA and resuspended in a buffer containing 300 mM sorbitol, 20 mM Hepes (pH 7.9), 10 mM NaCl, 2 mM $MgCl_2$, 2.5 mM EDTA and 0.1% (w/v BSA). In the membrane preparations for SDS-PAGE the buffers lacked BSA, while $MgCl_2$ was omitted from the resuspension buffer in some Chl fluorescence measurements and fluorescence polarization measurements, as noted in the text.

Measurements of relative fluidity

The relative fluidity of thylakoid membranes was determined by steady state fluorescence polarization after the addition of the hydrophobic fluorophore DPH (Aldrich) (Barber et al., 1984). DPH (3mM stock in tetrahydrofuran) was mixed with membranes (50 ug Chl ml^{-1}) to a final concentration of 5 uM and incubated in the dark at room temperature for 40 min. The membranes were then pelleted at 3000 x g for 5 min and diluted in 100 mM sorbitol, 20 mM Hepes (pH 7.9), 10 mM NaCl to a final concentration of 10 ug ml^{-1} Chl and 1 uM DPH. The measurements were carried out on an SLM 4048 spectrofluorometer (SLM

Instruments, Urbana, IL) in a T-format. Excitation was provided by light at 360 nm with a half-bandwidth of 16 nm. Fluorescence was monitored at 460 nm with a half-bandwidth of 8 nm. The degree of polarization (P) was calculated by an on line Hewlett-Packard 9825 computer.

Photosynthetic electron transport measurements

Whole chain and PSI dependent electron transport activities were assayed at 25^{o}C in the presence of 0.1 mM NaN_3, using water and 0.5 mM N,N,N',N'-tetramethyl-p-phenylenediamine (reduced with 2.5 mM ascorbate) as donors, respectively, by monitoring the O_2 consumption by 0.1 mM methyl viologen in a Rank oxygen electrode. Chloroplast membranes (20-30 ug Chl) were added to the reaction mixture which contained 300 mM sorbitol, 20 mM Hepes (pH 7.9), 10 mM NaCl, 2 mM $MgCl_2$, 2.5 mM EDTA, 0.1 % w/v BSA, 0.1 uM gramicidin-D and 1 mM NH_4Cl. The PSI assay also contained 1 uM 3-(3,4-dichlorophenyl)-1,1-dimethylurea to inhibit PSII activity and 10 ug/ul superoxide dismutase. Saturating white light illumination (1200 uE m^{-2} s^{-1}) was provided by a high intensity microscope lamp. PSII mediated 2,6-dichlorophenolindophenol reduction was measured at 580 nm using a Hitachi 100-60 spectrophotometer as described (Steinback et al., 1979). PSII light response was determined in the oxygen electrode as O_2 evolution with 1.5 mM $K_3Fe(CN)_6$ as the electron acceptor. L,D-glyceraldehyde was also added (10 mM) to inhibit CO_2 dependent oxygen evolution.

Room temperature chlorophyll fluorescence

Room temperature fluorescence induction transients of isolated chloroplast membranes were measured in the presence of 10 uM DCMU (Paterson and Arntzen, 1982). Membranes were diluted in resuspension buffer to a final concentration of 5 ug/ml Chl and dark adapted for 5 min before use. The actinic light was provided by a microscope illuminator through a broadband blue optical filter (Corning 4-96), with onset of illumination controlled by an electronic shutter (Vincent Associates, Rochester, NY). Fluorescence was measured through a Corning 2-64 red filter by a photodiode placed 90^{o} to the incident light as described (Paterson at al., 1982). Transients were recorded on a Nicolet Explorer II digital oscilloscope.

Low temperature (77K) chlorophyll fluorescence

Aliquots of chloroplast membranes in resuspension buffer lacking $MgCl_2$ were diluted to a concentration of 10 ug/ml Chl in 60 % glycerol (v/v) and sodium fluorescein was added as an internal standard to a final concentration of 2 mM (Krause et al., 1983). Samples were then frozen in liquid N_2 in capillary tubes (0.5 mm i.d.). Fluorescence emission spectra were acquired using an SLM 4048 spectrofluorometer. Excitation was provided by light at 440 nm with a half-bandwidth of 4 nm. Fluorescence emission was scanned in 1 nm increments from 470 to 800 nm with a half-bandwidth of 1 nm. Storage and mathematical manipulations of spectra were performed by an on-line Hewlett-Packard 9825 computer.

Gas exchange

Short term gas exchange determination on single intact plants has been described (Somerville and Ogren, 1982). Plants were placed in a glass chamber with the roots submerged in a vial containing water. The chamber was immersed in a temperature controlled waterbath at 25^{o}C and connected by two ports to a source of gas of a desired composition, and an infrared gas analyzer (Analytical Development Company Series-225). The CO_2 concentration of the entering and exiting gas stream was continuously monitored with the gas analyzer in the differential mode. Measurements of dark respiration and photosynthesis at 100 and 300 uE m^{-2} s^{-1} were performed for each plant. After the completion of each experiment, fresh weight and Chl content (MacKinney, 1941) of the aerial portion of the plants were determined.

Electron microscopy

Rosette leaves of three week old plants were fixed in 2% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h, followed by an 1 h incubation in 1% (v/v) osmium tetroxide in the same buffer. The specimens were dehydrated in a graded ethanol series and embedded in Spurr's epoxy resin (1969). Both fixation steps and dehydration were done at 4^{o}C. Thin sections were stained with uranyl acetate and lead citrate and examined in a JEOL 100CX electron microscope. Quantitative data of chloroplast membrane profiles on electron micrographs were obtained using a map measurer from 20 chloroplast sections from two separate embeddings of wild type and mutant plant material grown at different times.

Chloroplast copy number

Chloroplast copy number per cell was determined in isolated protoplasts prepared as described (Kunst et al., 1988). Aliquots (10-20 ul) of protoplast suspension were pipetted on microscope slides and the protoplasts were flattened by the coverslip application, so that the chloroplasts formed a monolayer within cells and could be easily counted (McCourt et al., 1987).

Results

Effects of temperature on relative growth rate

In order to determine the effects of the altered leaf membrane lipid composition on growth of the *act1* mutant line JB25, we measured the rate of increase in fresh weight of mutant and wild type plants growing at different temperatures (10-34^{o}C). The optimal growth temperature for both the mutant and the wild type was approximately 27^{o}C, and their relative growth rates were very similar at temperatures ranging from 10-30^{o}C (Figure 4-1). At temperatures greater than 30^{o}C the mutant JB25 grew slightly more rapidly than the wild type. However, after about 6 days at high temperatures the growth slows down, and eventually both the mutant and wild-type plants turn chlorotic. In any case, this experiment indicates that there are no significant deleterious effects of the *act1* mutation, or other mutations in the background genotype, on the growth of line JB25. These observations also suggest that the altered lipid composition of the *act1* mutants improves the thermal tolerance of this race of *Arabidopsis*.

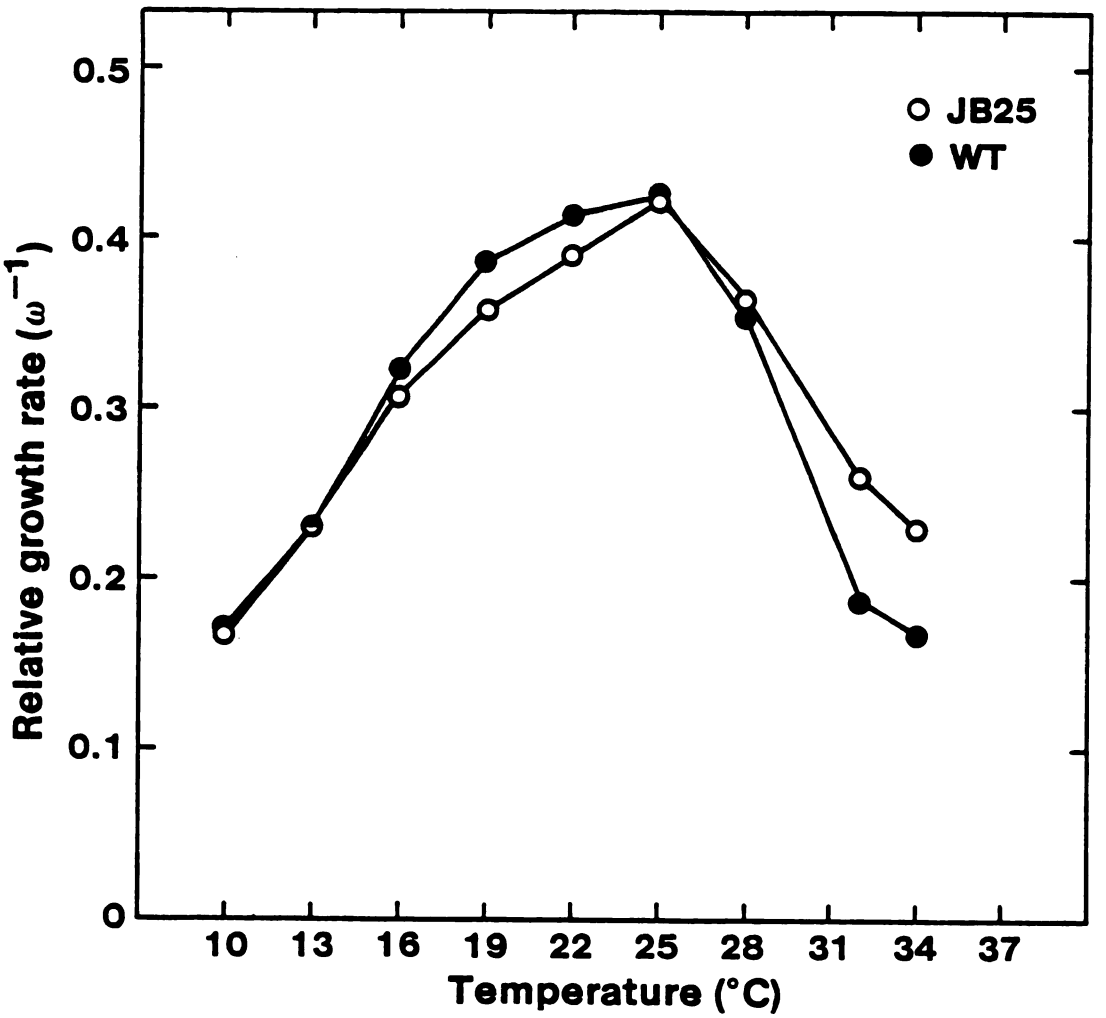

Figure 4-1. Effect of temperature on the relative growth rate of wild type and mutant Arabidopsis.

Effects of high temperature on stability of chloroplast membranes

To examine the thermal tolerance of the actl mutant further, the heat-induced changes in Chl fluorescence in mutant and wild-type leaves was measured (Figure 4-2). The rise in Chl fluorescence is thought to indicate an inhibition of excitation energy transfer from the LHCP antenna to PSII reaction centers, due to separation of LHCP from the PSII core (Armond et al., 1978). It is a sensitive indicator of the photosynthetic membrane stability, which is thought to depend on the membrane lipid composition (Berry and Bjorkman, 1980). The experiment was conducted by heating detached leaves from 25-57^{o}C at a rate of 1^{o}C min^{-1}, and measuring the fluorescence continuously (Schreiber and Berry, 1977). The fluorescence yield enhancement was observed at 43^{o}C in wild-type leaves (Figure 4-3), while the fluorescence did not rise in the mutant until 45^{o}C. This difference in the threshold temperature suggests greater thermal stability of the chloroplast membranes of the mutant.

In an effort to extend the fluorescence measurements, we compared the rates of photosynthetic electron transport by mutant and wild type chloroplast membranes incubated for 10 min at various temperatures ranging from 25-45^{o}C (Figure 4-3). The activities of both mutant and wild-type membranes rapidly declined following incubation at temperatures above 30^{o}C. Although the membranes of the mutant were more resistant than the wild type to thermal inactivation, the effect was subtle. The higher resistance of the mutant was also apparent when membranes were incubated for various times at 40^{o}C (Figure 4-4). Thus, these results suggest that the changes in composition of chloroplast membrane lipids in the mutant result in a slightly enhanced stability

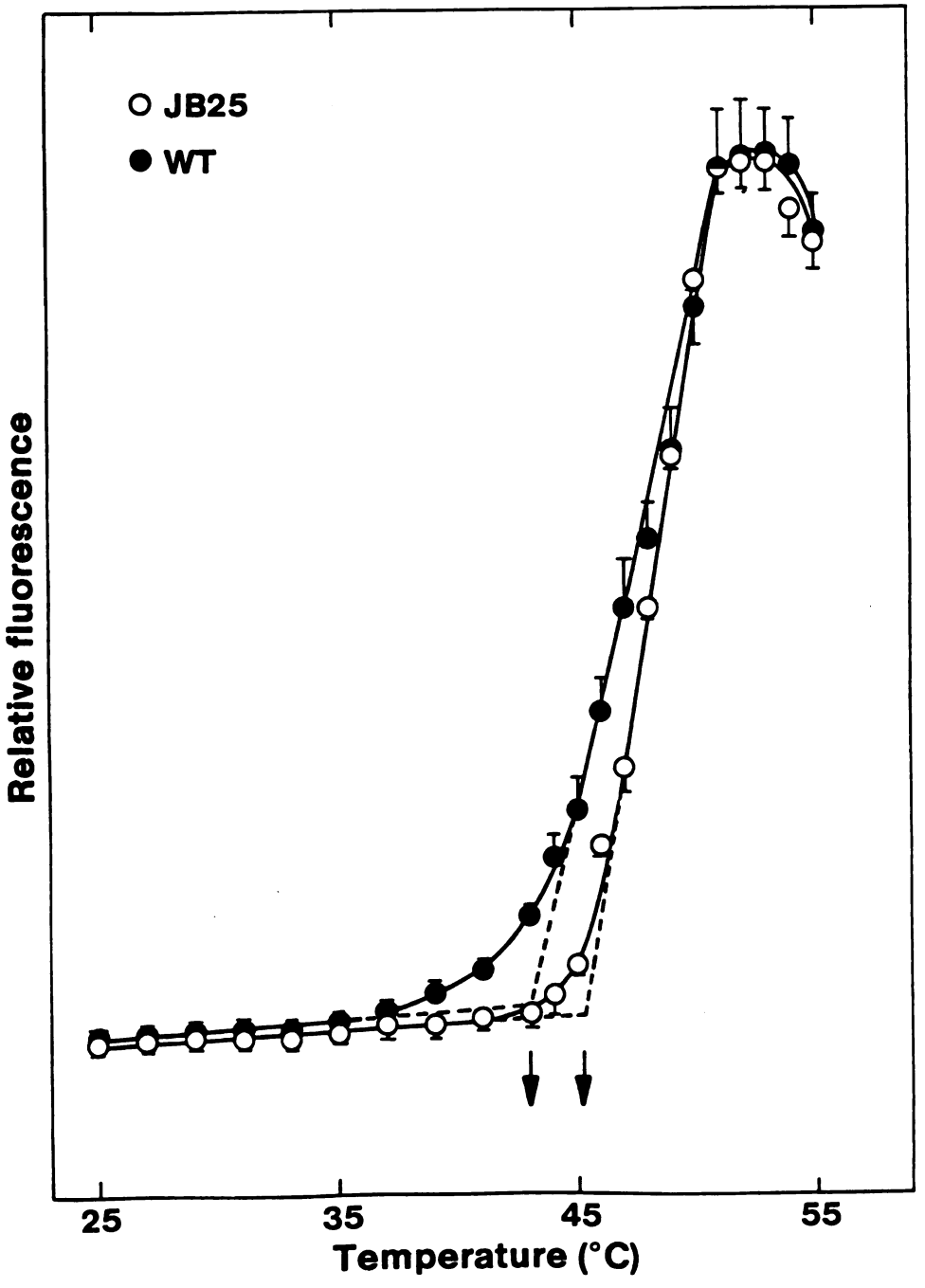

Figure 4-2. Temperature induced fluorescence enhancement yield of wild type and mutant leaves. Plants were grown at 22°C. The arrows indicate estimates of threshold temperatures at which fluorescence is enhanced. Each point represents the mean ± SD (n=3).

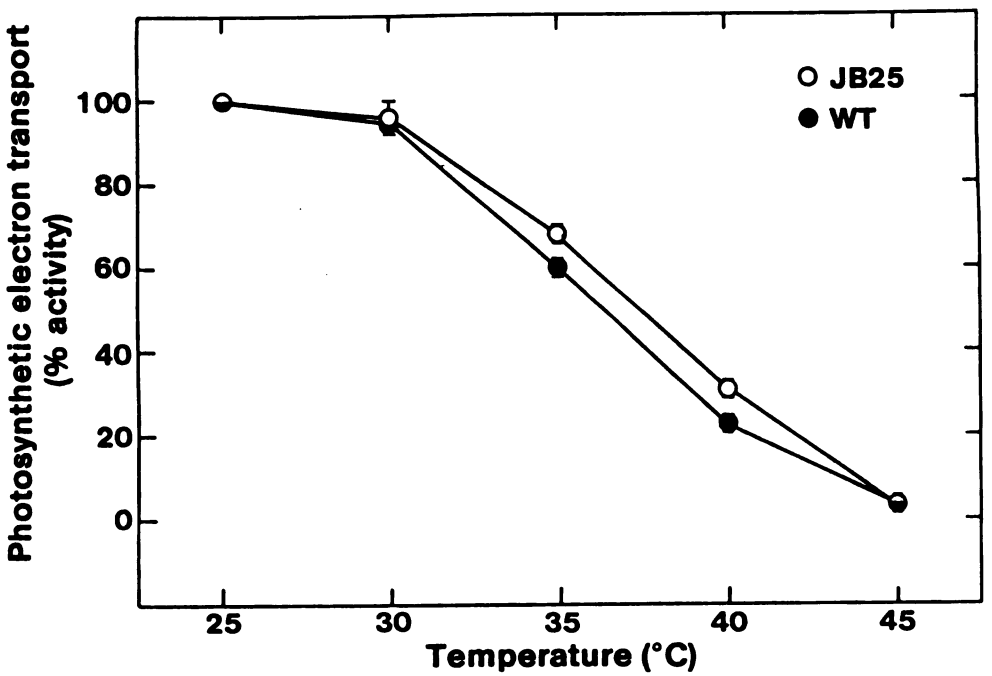

Figure 4-3. Effect of temperature on whole chain photosynthetic electron transport of thylakoid membranes from wild type and mutant Arabidopsis. Activity is expressed relative to that obtained with membranes preincubated in darkness at 4°C for 10 min. The maximal rates for the mutant and wild type were 169.4 umol O_2 mg Chl^{-1} h^{-1} and 156.7 umol O_2 $mgChl^{-1}$ h^{-1}, respectively. Each point represents the mean $\pm$ SD (n=4).

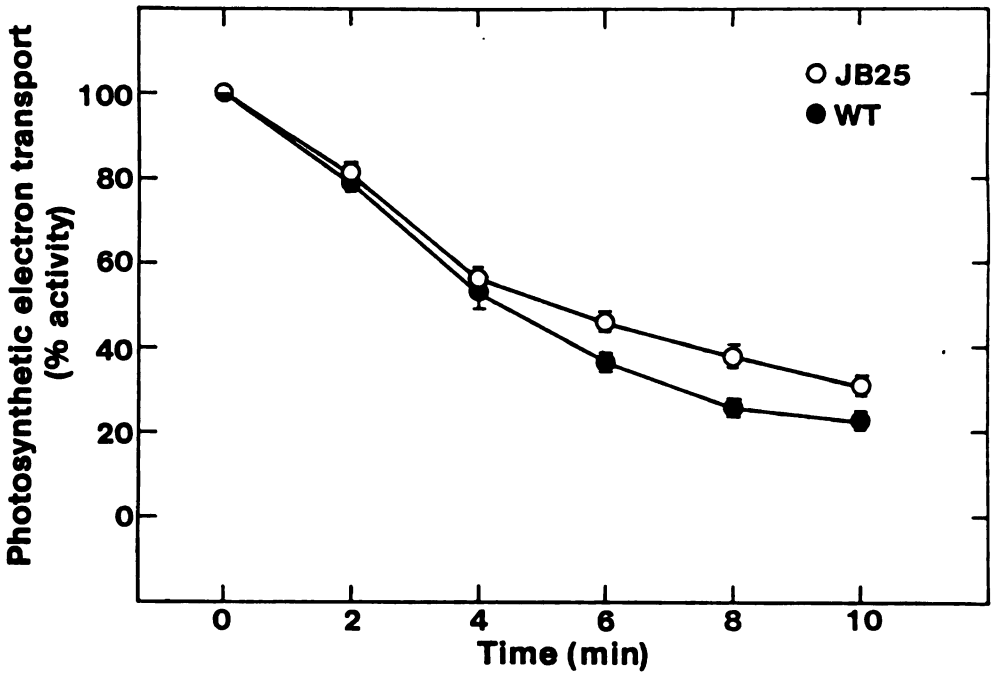

Figure 4-4. Decline in photosynthetic electron transport activity in chloroplast membranes from wild type and mutant Arabidopsis preincubated at 40°C. Activity is expressed relative to that obtained with membranes preincubated in darkness at 4°C for 10 min. The maximal rates for the mutant and wild type were 169.4 umol O_2 mg Chl^{-1} h^{-1} and 156.7 umol O_2 mg Chl^{-1} h^{-1}, respectively. Each point represents the mean ± SD (n=4).

of the membranes at high temperatures. However, because of the changes in ultrastructure, noted later, it is not possible to ascribe this effect specifically to the lipid composition.

Membrane fluidity

It has been suggested that the high proportion of polyunsaturated fatty acids in thylakoid membranes plays a major role in maintaining an extremely fluid matrix necessary for lateral movement of photosynthetic components (Quinn and Williams, 1983). The absence of 16:3 and concomitant changes in fatty acid composition due to the *act1* mutation might be expected to change thylakoid membrane fluidity. Therefore, fluorescence polarization (P) measurements were made on isolated membranes containing the hydrophobic fluorophore DPH, to determine the relative fluidity of thylakoids from mutant and wild type leaves. The relatively low P values obtained for wild-type *Arabidopsis* thylakoids were in agreement with those of other plant species (Barber et al., 1984), reflecting a highly fluid lipid bilayer (Figure 4-5). As with similar measurements made on the membranes from other mutants with altered lipid composition (McCourt et al., 1987, Chapter 6), the polarization values of the thylakoid membranes from mutant line JB25 were slightly higher at the majority of temperatures tested, but the difference was not statistically significant.

Effects of membrane lipid composition on Chl and protein content

Under all growth conditions examined i.e., growth at various temperatures in the range of 10-34^{o}C and 100-200 uE m^{-2} s^{-1}, leaves of the mutant JB25 were always slightly lighter green than those of the

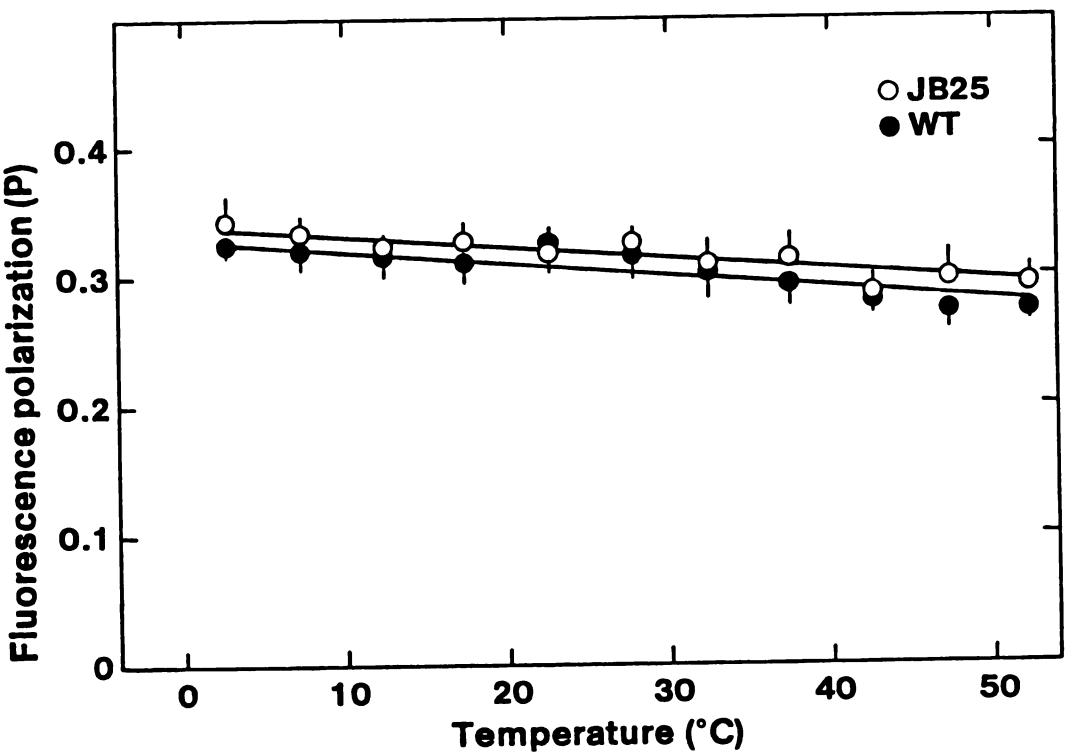

Figure 4-5. Effect of temperature on DPH fluorescence polarization by thylakoid membranes from wild type and mutant Arabidopsis. Each point represents the mean ± SD (n=10).

wild type, due to a 10% reduction in chlorophyll per unit fresh weight (Table 4-I). The analysis of 60 F_2 plants from a cross of WT x JB25 suggested that this phenotype is related to the actl mutation. All of the 16 plants with altered fatty acid composition exhibited the chlorotic phenotype, whereas all of the 44 plants with normal fatty acid composition had normal Chl content. Thus, the 16:3 deficiency in JB25 cosegregates with reduced Chl levels. The same conclusion was reached from a similar experiment with the independent allelic mutant LK8 (results not presented).

All the chlorophyll present in higher plants is thought to be bound to proteins of the thylakoid membrane (Markwell et al.,1979). Therefore, a decrease in chlorophyll content suggests a reduction in the amount of one or more Chl-protein complexes. Chloroplast membrane polypeptides of the mutant and wild type were separated by 2-dimensional SDS-polyacrylamide gel electrophoresis and examined in detail (Figure 4-6). The polypeptide pattern was very similar for both genotypes, but several differences were observed. No major polypeptides were absent from actl chloroplasts, although some were present in lower amounts than in the wild type. On the other hand, some other proteins were more abundant in the membranes of the mutant. There is also a small increase in Chl a/b ratio in the JB25 mutant, due to a preferential loss of Chl a. This result suggests that the stoichiometry of LHCP versus PSII and PSI might not be maintained. In order to determine whether this is the case, we compared the Chl-protein complexes from the mutant and the wild-type chloroplast membranes separated on SDS-polyacrylamide gels (Figure 4-7). Absorption spectra obtained for each of the Chl-protein complexes resolved in this manner

Table 4-I. Relative amounts of lipid, chl and protein in mutant and wild type Arabidopsis leaves and chloroplast membranes. Values are means ± SD (n=3).

	Wild type	JB25
LEAVES		
Chl/fwt (mg/g)	1.53 ± 0.08	1.40 ± 0.05
Chl a/b	2.93 ± 0.06	3.12 ± 0.08
Lipid/Chl (g/g)	2.48 ± 0.07	2.52 ± 0.01
Protein/Chl (g/g)	38.46 ± 0.15	38.38 ± 1.20
Protein/Lipid (g/g)	15.56 ± 0.90	15.24 ± 0.60
CHLOROPLAST MEMBRANES		
Lipid/Chl (g/g)	2.12 ± 0.08	2.45 ± 0.05
Protein/Chl (g/g)	12.27 ± 0.50	12.65 ± 0.18
Protein/Lipid (g/g)	5.79 ± 0.24	5.16 ± 0.07

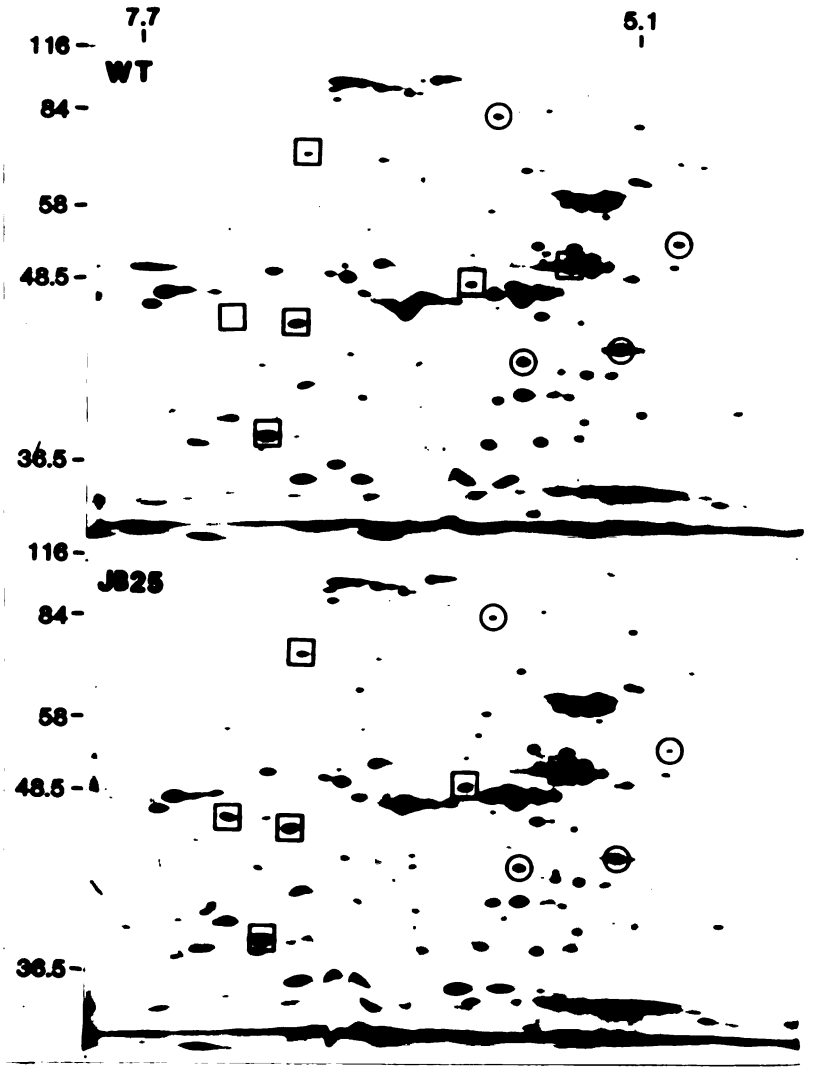

Figure 4-6. Autoradiographs of ^{35}S-Methionine labeled proteins of chloroplast membranes from wild type and mutant Arabidopsis separated by 2-dimensional SDS-polyacrylamide gel electrophoresis. The numbers at the top represent apparent pH, and those at the left apparent molecular mass. ◘ , proteins which are more abundant in the mutant than in the wild type; ⊙ , proteins which are more abundant in the wild type than in the mutant.

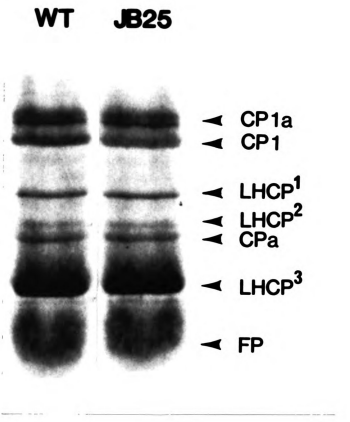

Figure 4-7. Chl-protein complexes of chloroplast membranes from wild type and mutant Arabidopsis. FP, free pigment. The nomenclature is from Anderson et al. (1978)

did not reveal any consistent changes in any of the complexes (Figure 4-8).

Photosynthetic characteristics

In an attempt to establish whether the wild-type lipid composition is specifically required to support maximal photosynthetic rates, we measured the rates of net photosynthetic CO_2 fixation in mutant and wild type plants under two different light intensities, and the rates of electron transport by isolated thylakoid membranes. CO_2 fixation rates of mutant JB25 and wild-type plants were not significantly different when expressed on either a Chl basis, or on a fresh weight basis (Table 4-II). Similarly, the rates of whole chain electron transport by thylakoid membranes were nearly identical in the mutant and wild type preparations at all light intensities (Table 4-II, Figure 4-9a), as were the PSI and PSII partial activities (Table 4-II, Figure 4-9b,c). The only difference between JB25 and wild type was a slight increase in JB25 PSI rate at higher irradiance levels.

Chlorophyll fluorescence measurements

The kinetics of induction of room temperature fluorescence is a measure of the rate of photoreduction of the primary electron acceptor Q_A of PSII. In the presence of DCMU, it depends only on the exciting light intensity and the effective cross-sectional area for light absorption by PSII (Melis and Harvey, 1981). The initial fluorescence level (F_o) is due to emission from the PSII antenna before the

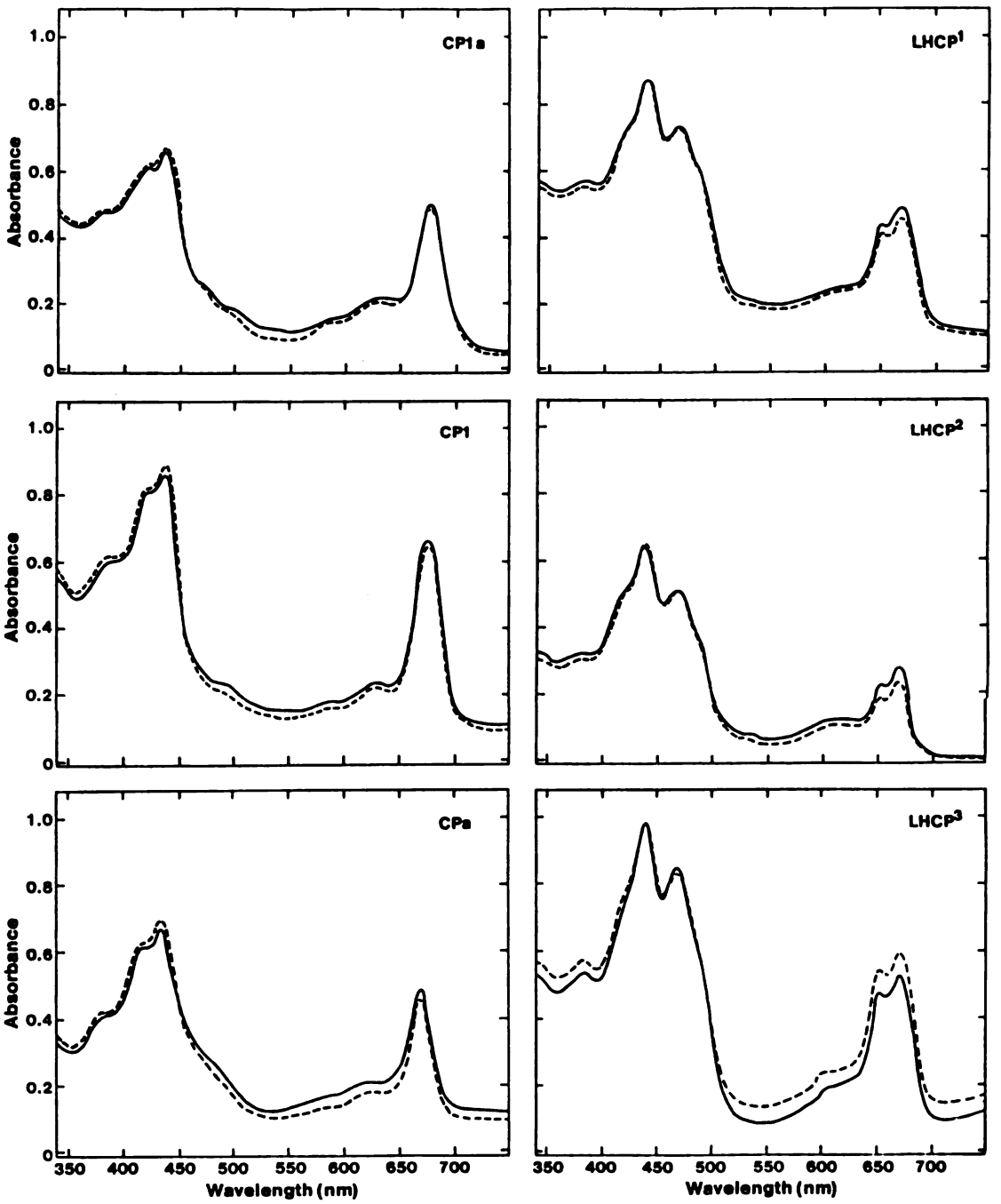

Figure 4-8. Absorption spectra of Chl-protein complexes of chloroplast membranes of the wild type (——) and mutant (---) Arabidopsis.

Table 4-II. Photosynthetic activities in mutant and wild type *Arabidopsis*.

	Wild type	JB25
NET CO_2 FIXATION		
	ug CO_2 mg Chl^{-1} h^{-1}	
Darkness	-[a]487.8 ± 13[2]	-450.2 ± 26
100 uE m^{-2} s^{-1}	2432.5 ± 100	2437.6 ± 61
300 uE m^{-2} s^{-1}	4766.9 ± 205	5086.0 ± 183
	ug CO_2 mg fwt^{-1} h^{-1}	
Darkness	-565.1 ± 23	-518.3 ± 30
100 uE m^{-2} s^{-1}	2802.2 ± 140	2798.0 ± 68
300 uE m^{-2} s^{-1}	5535.4 ± 285	5391.3 ± 169
ELECTRON TRANSPORT		
	umol O_2 mg Chl^{-1} h^{-1}	
Whole chain	145.9 ± 7	157.0 ± 7
PSI	383.2 ± 23	429.6 ± 20
PSII	410.4 ± 40	424.3 ± 26

[a] Net CO_2 evolution is indicated here as a negative value.

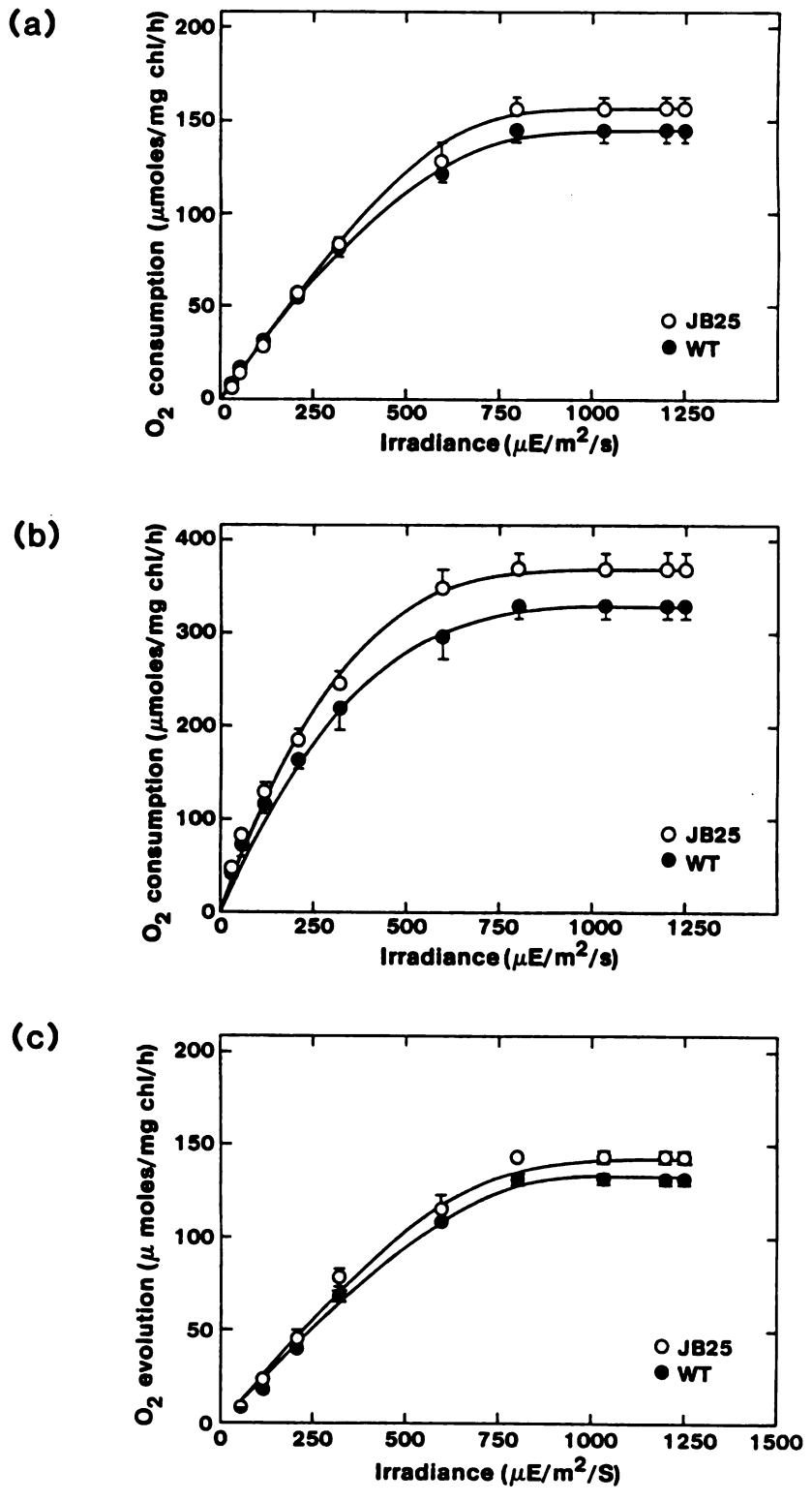

Figure 4-9. Light response curves for a) whole chain, b) PSI and c) PSII electron transport by wild type and mutant Arabidopsis. Each point represents the mean ± SD (n=3).

excitation energy is trapped by the reaction centers. Following onset of illumination, Chl fluorescence intensity rises to a maximal level F_m which is reached when all the primary acceptors of PSII are reduced. Variable fluorescence (F_v=F_m - F_o) is a measure of time required for the turnover of all the PSII reaction centers and it reflects the number of Chl molecules associated with these reaction centers.

The shape of the room temperature fluorescence transients obtained from JB25 and wild type thylakoid membranes were indistinguishable (Figure 4-10). There was also no difference in variable fluorescence values (expressed as F_v/F_o) (Table 4-III). These results considered in conjunction with almost identical PSII electron transport rates of mutant and wild-type plants suggest that their PSII antennas are structurally and functionally indistinguishable.

Low temperature (77K) Chl fluorescence emission spectra were used to compare the excitation energy distribution between Chl-containing components of thylakoid membranes isolated from mutant JB25 and wild type. In chloroplast membranes of higher plants, the component with an emission maximum at 685 nm is ascribed to LHCP of PSII, the 695 nm emission is attributed to PSII reaction center core complex, and the fluorescence emitted at 734 nm originates from PSI. The F_{685}/F_{734} ratio obtained with chloroplast membranes from the mutant was higher than the wild type (Table 4-III) in the presence or absence of $MgCl_2$. By normalizing to the magnitude of emission from the internal standard, fluorescein, it was apparent that the change in F_{685}/F_{734} ratio is caused by lower fluorescence emission at 734 nm, accompanied by increased Chl fluorescence at 685 nm. This result was confirmed using the independently isolated mutant line LK8 (Table 4-III). The results

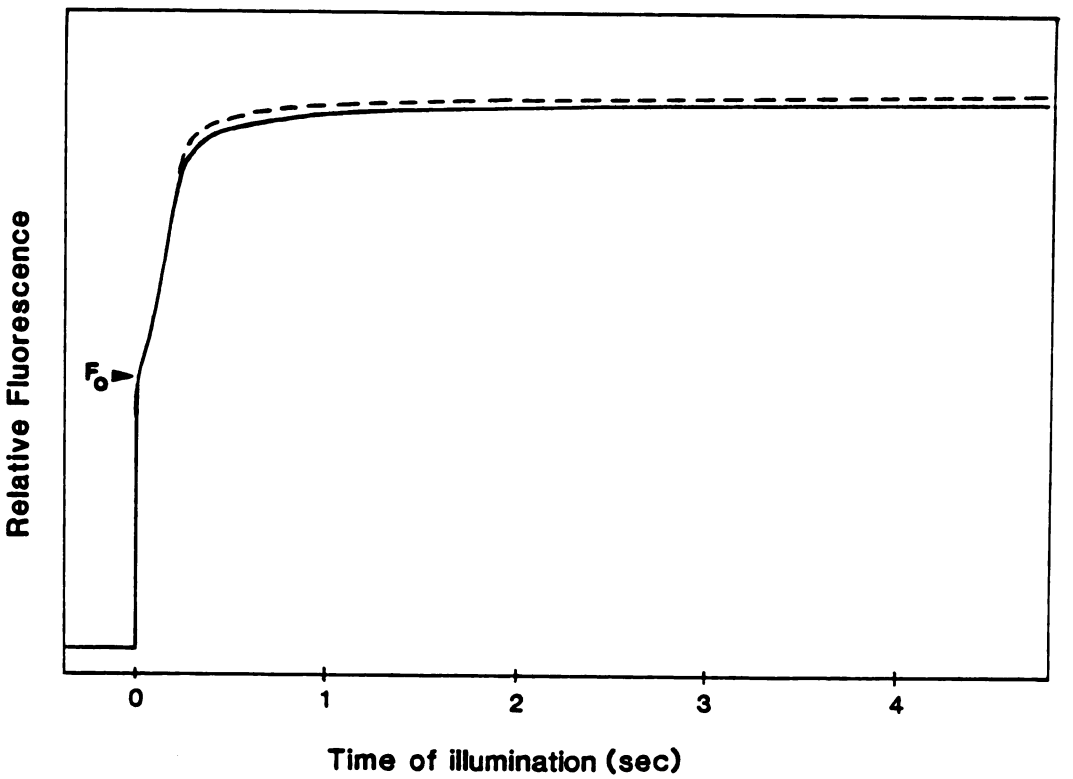

Figure 4-10. Room temperature fluorescence induction transients of isolated chloroplast membranes of the wild type (——) and mutant (---) Arabidopsis in the absence of DCMU.

Table 4-III. Room temperature fluorescence induction and low temperature (77K) fluorescence of isolated thylakoids.

		Wild type	JB25	LK8
ROOM TEMPERATURE FLUORESCENCE[a]				
F_o		1130 ± 33	1127 ± 11	-
F_m		3221 ± 132	3246 ± 65	-
F_v/F_o		1.85 ± 0.07	1.88 ± 0.02	-
77K FLUORESCENCE[b]				
F_{685}/F_{734}	$+Mg^{2+}$	0.78 ± 0.001	0.85 ± 0.002	0.84 ± 0.004
$F_{685/F734}$	$-Mg^{2+}$	0.65 ± 0.005	0.76 ± 0.006	0.78 ± 0.004

[a] Room temperature fluorescence was measured in the presence of 10 uM DCMU, n=10; Values are expressed in arbitrary units.

[b] 77K fluorescence was measured in the presence and absence of 5 mM $MgCl_2$, n=6.

of this experiment imply that the peripheral antenna of PSI in the mutant is structurally different, or that the efficiency of energy transfer between PSII and PSI is reduced in mutant chloroplasts.

Chloroplast ultrastructure and number

In order to examine the effects of changes in leaf lipid composition in the *act1* mutants on chloroplast structure, a morphometric analysis was compiled from electron micrographs of chloroplasts from the wild type and the allelic mutant lines JB25 and LK8. Even without a detailed analysis, a difference was apparent in the arrangement of chloroplast membranes between the two genotypes (Figure 4-11). The most striking was the alteration in the membrane appression in *act1* mutants. Quantitative analysis showed that the average number of thylakoid membranes per granum is decreased from 6.2 in the wild type to 3.8 in the mutant (Table 4-IV). This change is accompanied by a large increase in the number of grana per chloroplast, so that the total amount of appressed membranes in the mutant is close to the wild-type value. On the other hand, the total length of non-appressed membranes is increased in the mutants by 30%.

Discussion

Lipid composition and enhanced thermal tolerance

In spite of substantial changes in leaf lipid metabolism and membrane lipid composition the mutant line JB25 exhibits comparable or higher growth rates (at temperatures above $30^{o}C$) relative to the wild

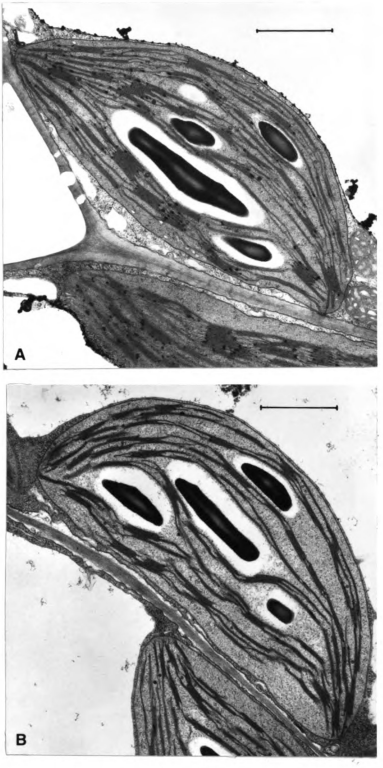

Figure 4-11. Transmission electron micrograph of chloroplasts from rosette leaves of (A) wild type and (B) mutant Arabidopsis. Bar = 1um

Table 4-IV. Morphometric analysis of chloroplasts from mutant lines and wild type Arabidopsis.*

	Wild type	JB25	LK8
Grana/plastid	54.4 ± 6.6	90.0 ± 7.2	87.6 ± 7.6
Thylakoids/granum	6.2 ± 3.7	3.7 ± 1.6	3.9 ± 1.7
Granal width (um)	0.4 ± 0.04	0.4 ± 0.03	0.4 ± 0.04
Stroma thylakoids/plastid	0.2 ± 0.01	0.2 ± 0.03	0.2 ± 0.02
Stroma thylakoid length (um)	103.9 ± 12	99.8 ± 10	96.1 ± 13
Appressed membrane/plastid (um)	114.1	97.0	104.2
Non-appressed membrane/plastid (um)	40.7	52.8	52.9
Total membrane (um)	154.8	149.8	157.1
Appressed/non-appressed membrane	2.8	1.8	2.0
Surface area (um^2/plastid)	9.9 ± 2	10.8 ± 1	10.7 ± 1

*Measurements were made on 20 chloroplasts from each line.

type. This observation is consistent with the results obtained for two other Arabidopsis mutants deficient in fatty acid unsaturation of chloroplast membranes (McCourt et al., 1987, Kunst et al., manuscript in preparation). In each of these cases, a reduction in the degree of lipid unsaturation is correlated with an enhanced growth rate at high temperatures. The acclimation to growth at high temperatures is usually accompanied by an increase in the threshold temperature at which Chl fluorescence enhancement occurs (Schreiber and Berry, 1977; Berry and Bjorkman, 1980). The mechanisms associated with this adaptive response are not known, but include substantial changes in membrane lipid unsaturation. A comparison of the actl mutant and wild type by this criterion also indicated increased thermal tolerance of the mutant. On the basis of these results, together with slower inactivation of photosynthetic electron transport in the mutant at elevated temperatures (Figures 4-3,4-4), we conclude that the fatty acid composition of chloroplast membranes may be an important component of the thermal adaptation response characterized in species such as Nerium oleander (Raison et al., 1982).

The fluorescence polarization measurements performed on JB25 indicate that the changes in lipid composition do not affect the fluidity of membranes from the mutant (Figure 4-5). This observation suggests that the superior acclimation to growth at elevated temperatures of JB25 is not due to changes in membrane fluidity. Thus, it is not apparent, at this time, why the actl mutant exhibits an enhanced thermal tolerance.

Chloroplast membrane function

When expressed on a Chl basis, the mutant had a higher rate of electron transport than the wild type. After correction for an 8.5% reduction in Chl content, the rates of whole chain electron transport were identical in the two genotypes. We could also not detect any changes in room temperature fluorescence between mutant and wild type thylakoid membranes (Table 4-III). These results suggest that the photosynthetic capacity of the mutant and the wild type is essentially equivalent.

One of the distinguishing characteristics of the mutant is an 8.5% reduction in Chl content (Table 4-I). This phenotype cosegregated with the altered lipid composition, indicating that the *act1* mutation causes both effects. The magnitude of the Chl deficiency parallels a 10% decrease in the protein/lipid ratio of chloroplast membranes. The mutant exhibits preferential loss of Chl a, but Chl b is also decreased by 4.5%. Since LHCP does not accumulate in the absence of chl b, a 4.5% decrease of Chl b in JB25 thylakoids suggests a corresponding decrease in LHCP content. However, a 4.5% reduction in the amount of LHCP cannot account for the 10% reduction in the protein/lipid ratio of mutant membranes. Thus, it is apparent that changes must have occurred in the amounts of other thylakoid polypeptides. A comparison of the polypeptide pattern of chloroplast membranes from the mutant and wild type indicated that several polypeptides are obviously reduced in amount in the mutant. However, the role of these polypeptides is not known, and it was not possible to determine by this criterion if the change was adequate to explain the reduced protein/lipid ratio.

Another change in JB25 with respect to the wild type concerns 77K fluorescence properties of chloroplast membranes. Measurements on isolated membranes from the mutant lines JB25 and LK8, show a decreased fluorescence emission at 734 nm, originating from PSI, and a concomitant increase in fluorescence yield at 685 nm, emitted by LHCP of PSII. This different distribution pattern of excitation energy in the mutant is reflected in a higher F_{685}/F_{734} ratio in both presence or absence of $MgCl_2$ (Table 4-III). Fluorescence emission at 734 nm arises from peripheral antennae of PSI (Mullet et al., 1980). Therefore, a reduced fluorescence yield at long wavelengths may be caused by changes within the PSI antennae pigment bed, or it may be attributed to a decrease in energy spillover from PSII to PSI. We favor the latter concept because we observe no significant changes in electron transport rates (after correction for the reduction in Chl content) or PSI light response.

A reduction in the amount of light energy transferred from PSII to PSI can, in principle, be explained in one of several ways. Since mobile LHCP is considered to play a major role in the regulation of excitation energy distribution between the photosystems, reduced LHCP content in mutant membranes would reduce the amount of energy reaching PSI. However, the relatively slight (4.5%) reduction in LHCP (expected on the basis of the reduced Chl b content) is not adequate to account for the fluorescence phenomena. It seems more likely that the structural organization of the chloroplast membranes (Figure 4-11) brought about by compositional changes of thylakoid membranes might impose limitations upon lateral migration of LHCP.

The alternate possibility, that a decrease in PSI fluorescence yield is affected by changes in the organization of PSI, cannot be completely ruled out, either. Chlorophyll fluorescence emission is considered a more sensitive monitor of changes in Chl-protein complexes than rates of PSI and PSII photochemistry (Burke et al., 1978). Therefore, our evidence (similar PSI electron transport rates and PSI light response of wild type and mutant thylakoids) might not be sufficient to support the conclusion that PSI antenna of the mutant is not changed.

Chloroplast ultrastructure

The most pronounced effects of the *act1* mutation are differences in the amount and arrangement of thylakoids within the chloroplasts. The number of apressed regions per chloroplast in mutant lines JB25 and LK8 is increased in comparison with the wild type, but the stacks contain fewer thylakoid membranes. The total length of non-appressed membrane is increased in both mutants. Similar changes in chloroplast ultrastructure in two independently isolated allelic mutants establish a causal relationship between the altered lipid composition and structural differentiation of thylakoid membranes. It has been suggested that membrane stacking in higher plant chloroplasts is mediated by LHCP present in the membrane (Staehelin and Arntzen, 1983). This model is based on observations that greening or mutant plastids which lack or are deficient in LHCP are correspondingly deficient in grana formation (Goodchild et al., 1966, Thornber and Highkin, 1974, Armond et al., 1976), and reconstitution experiments using liposomes and purified LHCP (Ryrie et al., 1980, McDonnel and Staehelin, 1980).

Results presented here indicate that the model for membrane appression based on LHCP is incorrect, or incomplete in some important way. Although a careful morphometric analysis does not appear to have been done on the barley chlorina mutant, or any of the other Chl b deficient mutants, it seems that the changes in ultrastructure in the actl mutant are at least as pronounced as in any of the mutant lines deficient in LHCP. Since no major difference in LHCP content between the wild type and mutant was observed, it is not possible to attribute the decrease in the degree of stacking to a reduction in the amount of LHCP. However, there are several quantitative differences between the mutant and wild type in the polypeptide pattern on 2-dimensional gels suggesting that an alteration in the amount of some other protein component of thylakoid membranes might be responsible for the changes in structural features of actl plastids. A thorough examination of 2-dimensional polypeptide pattern of chloroplast membranes from the barley chlorina mutant might, therefore, be informative in relating changes in specific polypeptides with the similar alterations in the degree of membrane appression observed in actl and chlorina mutants.

Ultrastructural changes in chloroplasts of the actl mutant may also be caused by changes in the organization of PSI antenna. Optimal efficiency of noncyclic photosynthetic electron flow depends on similar rates of charge separation at the two reaction centers. To ensure the balanced energy distribution between PSI and PSII, light harvesting pigments undergo changes referred to as "state I-state II" transitions. Preferential light absorption by PSII leads to a reversible phosphorylation of a population of the LHCP particles, and their subsequent migration to non-appressed membrane regions, so that more

excitation energy is distributed to PSI reaction centers (Staehelin and Arntzen, 1983). This adaptive response, known as state II, also results in a decrease of membrane stacking, due to incorporation of negatively charged phosphate groups into LHCP. Therefore, if there are changes in the structural organization of the PSI antenna of *act1* mutant, as suggested by a lower fluorescence emission at 734 nm and a decrease in the amount of Chl a, a reduced absorption by PSI would lead to state II, and a corresponding decrease in the extent of chloroplast membrane appression. Finally, we cannot rule out the possibility that the fatty acid composition of chloroplast lipids *per se* plays a role in the organelle biogenesis.

Why do 16:3 plants persist?

All described differences between mutant and wild-type plants are relatively minor, and are not deleterious to the mutants. The mutants are also not impaired in growth or development under a variety of environmental conditions. Therefore, we must conclude that under the conditions we have examined there are no major physiological advantages associated with the presence of the prokaryotic pathway.

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CHAPTER 5

A MUTANT OF ARABIDOPSIS THAT ACCUMULATES PALMITIC ACID IN LEAF LIPIDS

Abstract

Leaf membrane lipids of a mutant of Arabidopsis thaliana accumulate high amounts of palmitic acid (16:0) and show a corresponding decrease in unsaturated 16-carbon fatty acid levels as a consequence of a single nuclear mutation. Quantitative analysis of the fatty acid composition of individual lipids suggests that the mutant is deficient in the activity of the chloroplast n-9 fatty acid desaturase which normally introduces a double bond in 16-carbon acyl chains esterified to the sn-2 position of monogalactosyldiacylglycerol (MGD). Both chloroplast and extrachloroplast lipids are affected by the mutation. Thus, either there is substantial transfer of 16:0 acyl groups from MGD to all the leaf polar lipids, or 16:0 which would normally be used for MGD synthesis in the chloroplast is exported to the cytoplasm rather than being elongated to 18:0. Synthesis of MGD by the prokaryotic pathway is reduced 25-30%, but this deficiency is compensated for by the increased production of MGD through the eukaryotic pathway. This change in relative contribution of the two pathways of lipid biosynthesis in the mutant may be a regulated

response to the loss of chloroplast n-9 desaturase which reflects a requirement for polyunsaturated fatty acids for the assembly of chloroplast membranes.

Introduction

Trienoic fatty acids (18:3 and 16:3) are the predominant fatty acids of chloroplast membranes of higher plants. Typically they account for approximately two thirds of all the thylakoid fatty acids, and over 90% of the fatty acids of MGD, the most abundant chloroplast lipid (Gounaris and Barber, 1983). The reason for the high degree of fatty acid unsaturation is not known, but it is thought to be involved in providing an extremely fluid matrix for photosynthetic electron transport (Raison, 1980; Quinn and Williams, 1983). Since a relatively large decrease in trienoic fatty acid content in an Arabidopsis mutant had no effect on chloroplast function, but caused ultrastructural changes, it has also been suggested that lipid unsaturation may be primarily required for the formation of the characteristic ultrastructural features of chloroplasts (McCourt et al., 1987).

It is not known with certainty how many desaturase enzymes participate in the synthesis of trienoic acids in plant cells, and their compartmentation has not been precisely established. Isolated chloroplasts of '16:3' plants readily synthesize MGD in which 18:1 fatty acids at position sn-1 are converted to 18:2 and 18:3. Similarly, sequential desaturation of 16:0 to 16:3 takes place at position sn-2 of MGD (Roughan et al., 1979). However, desaturation of 18:1 and 18:2

fatty acids is not confined to MGD, because PG and SL have also been shown to convert these fatty acids to 18:3 (Roughan, 1985; Joyard et al., 1986). On the other hand, MGD seems to be the sole substrate for 16:0 desaturation, and 16-carbon fatty acids are not desaturated to any extent when esterified to other lipids. The main substrate for the desaturation of 18:1 to 18:2 outside the chloroplast is microsomal PC, on which some 18:3 synthesis also occurs (Slack et al. 1976; Roughan and Slack, 1982). These observations suggest the existence of a family of fatty acid desaturases located in the chloroplast or the endoplasmic reticulum, which use different glycerolipids for the desaturation reactions. The only exception is the soluble chloroplast 18:0-ACP desaturase, which inserts a double bond at the n-9 position of stearic acid while it is still bound to acyl carrier protein. This is also the only desaturase enzyme that has been partially purified (McKeon and Stumpf, 1982). All the other desaturases appear to be membrane bound enzymes that lose activity during membrane solubilization. This is particularly true for chloroplast desaturases which are inactivated by chloroplast rupture or exposure of intact chloroplasts to mild hypotonic conditions (Andrews and Heinz, 1987).

Difficulties associated with solubilization and reconstitution of desaturase acitivity in vitro have hindered traditional biochemical investigations of these enzymes. Therefore, we have initiated a genetic approach to study the desaturation process in plant membrane lipids by the isolation of a number of mutants with specific changes in unsaturation of their leaf fatty acids. We have previously characterized a mutant deficient in <u>trans</u>-16:1 synthesis (Browse et al., 1985a), and mutants that lack specific desaturases responsible

for the synthesis of n-3 (Browse et al. 1986a) and n-6 fatty acids (Browse et al., manuscript in preparation). Here we describe the biochemical characterization of a mutant deficient in conversion of 16:0 to cis-16:1.

Materials and methods

Plant material

The mutant line JB67 was isolated from the Columbia wild type of Arabidopsis thaliana (L.) Heynh. following mutagenesis with ethyl methane sulfonate, as previously described (Browse et al., 1985a). It was backcrossed to the wild type four times before being used for the experiments reported here. Plants were grown at 22°C with continuous fluorescent illumination (100-150 uE m^{-2} s^{-1}) on a perlite:vermiculite:sphagnum (1:1:1) mixture irrigated with a mineral nutrient solution (Haughn and Somerville, 1986).

Reagents

Sodium [^{14}C]-acetate (54 mCi $mmol^{-1}$) was obtained from Research Products International Corporation, Mt. Prospect, IL, and [^{14}C]-16:0-CoA (58 mCi $mmol^{-1}$) from DuPont, Wilmington, DE. Rhizopus arrhizus lipase suspension (50000 U ml^{-1}) was purchased from Boehringer Mannheim GmbH. Methanolic-HCl reagent was prepared by diluting a 3M solution (Supelco) to 1M with methanol.

Lipid analysis

Leaf material was frozen in liquid N_2 and lipids extracted with chloroform:methanol:formic acid (10:10:1 by vol.) as previously described (Browse et al, 1986b). Individual lipids were isolated by thin layer chromatography on silica gel G coated plates (Kunst et al., 1988), or $(NH_4)_2SO_4$-impregnated silica gel G plates (Khan and Williams, 1977), and transmethylated with methanolic-HCl after the addition of 14:0 methyl ester as internal standard. The resulting methyl esters were then quantified by gas chromatography (Browse et al, 1985b). Fatty acid positional distribution of MGD was established following degradation with Rhizopus arrhizus lipase (Boehringer Mannheim, triacylglycerol acylhydrolase EC 3.1.1.3) according to Fischer et. al. (1973). MGD (5 umol) was dissolved in chloroform:methanol (2:1 by vol.), and after the addition of Triton X-100 (4 mg in the same solvent), taken to dryness. The mixture was then dissolved in 1 ml 0.04M Tris-HCl buffer (pH 7.2), sonicated for 10 minutes, and the reaction was started by adding 2000 U of Rhizopus enzyme. The incubations were carried out at room temperature for 30 minutes with vigorous shaking and stopped by adjusting the pH to 4 with acetic acid. The reaction products were extracted and separated by thin layer chromatography on silica gel G plates using two consecutive solvent systems (Fischer et al., 1973).

Labeling of plants

The labeling of intact Arabidopsis plants with [^{14}C]-acetate and the determinations of distribution of radioactivity in the various lipids were done essentially as described (Browse et al., 1986b). Under

the conditions used, incorporation of [^{14}C]-acetate did not continue beyond 90 minutes after the label application.

Monoacyl-G3P acyltransferase assay

Chloroplast isolation and fractionation procedures have been described (Kunst et al., 1988). The enzyme activity was assayed at room temperature according to Frentzen et al. (1983). The 80 ul reaction mixture contained 250 mM Mops-NaOH (pH 7.4), 625 ug ml^{-1} BSA, 5uM [^{14}C]-16:0-CoA, 2 mM 1-oleoyl-G3P and 50 ug of chloroplast envelope protein.

Results

Genetic analysis

The isolation and genetic characterization of the mutant line JB67 (fadB) was described in Chapter 2.

Biochemical characterization

As shown in Table 2-IV, the increased levels of palmitic acid in the fadB mutant are accompanied by a decrease in 16:2 and 16:3 fatty acids. This phenotype could, in principle, be caused in one of several ways. First, a deficiency in the chloroplast enzyme monoacyl-G3P acyltransferase, that specifically esterifies the sn-2 position of G3P with a 16:0 acyl group, could lead to accumulation of palmitic acid within the chloroplast. Since fatty acids, with the exception of 18:0, are only desaturated after incorporation into glycerolipids (Roughan

and Slack, 1982), the inability of the mutant to synthesize lipids in the chloroplast would result in the absence of 16:2 and 16:3 acyl groups in its leaf lipids. To test this hypothesis we performed the monoacyl-G3P acyltransferase assay by measuring the incorporation of [^{14}C]-16:0-CoA by the envelopes (Figure 5-1). Envelopes from both lines synthesized similar levels of PA, PG and DAG indicating that the mutant has wild type levels of monoacyl-G3P acyltransferase activity.

The most likely explanation for the altered lipid composition of the mutant is that the fadB locus controls the activity of a fatty acid desaturase which is responsible for introducing the double bond at position n-9 of 16-carbon acyl groups in wild type plants. Since this desaturase activity has not yet been demonstrated by an in vitro assay, the precise enzymatic lesion in the mutant cannot be determined directly. However, it is well established that chloroplast MGD is the substrate for 16:3 synthesis. Therefore we analyzed the fatty acid composition of MGD in detail (Tables 5-I and 5-II). The data obtained show that 16:2 and 16:3 fatty acids are virtually absent from MGD in the mutant, while 16:0 fatty acid accumulates to more than 12-fold the levels of wild type. The degradation of MGD using Rhizopus lipase revealed that more than 94% of palmitic acid occurs at the sn-2 position of MGD in both mutant and wild type (Table 5-II). From these results we infer that the fadB mutation affects the activity of an n-9 desaturase which specifically desaturates 16-carbon acyl chains esterified to the sn-2 position of MGD. This headgroup specificity also implies that the desaturase is located in the chloroplast.

It is worth noting that the deficiency in n-9 desaturase results in the accumulation of palmitic acid at the sn-2 position of MGD,

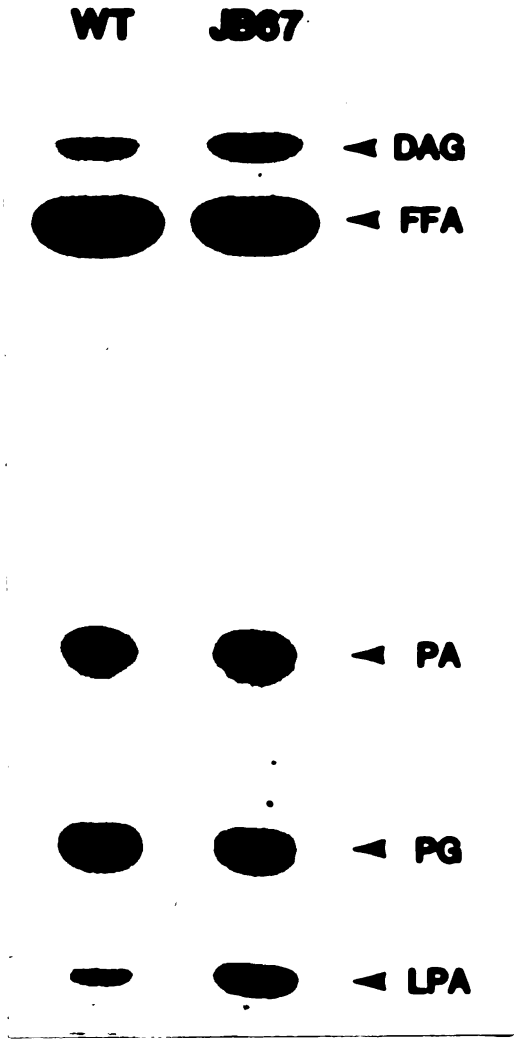

Figure 5-1. The distribution of radioactivity among the polar lipids following [^{14}C]-16:0-CoA labeling of isolated chloroplast envelopes of the mutant JB67 and wild type Arabidopsis. The same amount of radioactivity was applied to each lane.

Table 5-I. Fatty acid composition of leaf lipids from wild-type and mutant Arabidopsis grown at 22°C. Values presented are mol %. Dashes indicate that the acyl group was detected.

	MGD		DGD		SL		PG		PC		PE		PI	
	WT	JB67	WT	JB67	WT	JB67	WT	JB67	WT	JB67	WT	JB67	WT	JB67
16:0	1.1	12.6	12.7	28.3	40.2	44.2	29.1	30.8	22.8	36.8	30.0	38.0	43.5	52.4
16:1t	-	-	-	-	-	-	16.4	23.2	-	-	-	-	-	-
16:2	1.8	0.2	0.7	0.2	-	-	-	-	-	-	-	-	-	-
16:3	34.8	1.1	3.4	0.2	-	-	-	-	-	-	-	-	-	-
18:0	-	0.4	0.7	1.9	1.5	1.8	0.8	1.3	1.9	2.5	2.0	1.9	2.3	2.4
18:1	0.7	1.1	1.1	0.9	1.8	3.3	3.4	2.7	5.3	4.4	2.5	2.2	2.9	1.8
18:2	2.7	3.1	4.6	2.3	11.3	5.9	7.7	8.5	34.1	26.3	33.2	32.5	22.8	19.2
18:3	58.0	81.4	76.8	66.2	45.2	44.8	42.6	33.5	35.9	30.0	32.0	25.4	28.5	24.2
%	40.7	41.0	13.4	12.1	3.3	3.2	10.0	11.9	16.5	16.3	11.8	11.8	4.2	3.8

Table 5-II. Fatty acid distribution in MGD from wild type and mutant Arabidopsis established by degradation with Rhizopus arrhizus lipase. The lyso-compounds contain fatty acids only on the sn-2 position. The values are given as weight %.

	Untreated MGD		Lyso-compound	
Fatty acid	WT	JB67	WT	JB67
16:0	1.1	13.1	2.0	24.2
other 16C	35.7	0.4	69.0	0.8
18:0	tr.	1.2	-	-
18:1	0.6	0.6	0.2	0.2
18:2	2.4	3.5	1.2	3.2
18:3	60.1	81.2	27.6	71.6

rather than n-6 and n-3 isomers of 16:1, or n-6,n-3 isomers of 16:2. This observation suggests that the presence of a double bond at the n-9 position is required for the insertion of double bonds in n-6 and n-3 positions by other chloroplast desaturases.

Fatty acid composition of individual lipids

In order to investigate if the leaf polar lipids other than MGD are affected by the mutation at fadB locus, we determined the fatty acid composition of individual lipids from both mutant and wild type plants. The analysis showed that the proportions of various lipids are essentially the same in the mutant and wild type leaves (Table 5-I). On the other hand, there is a striking increase in the levels of palmitic acid in all the polar lipids of mutant, except for PG, which accumulates trans-16:1, and SL which seems relatively unaffected because the amount of 16:0 is already very high in the wild type. Thus, either there is a substantial transfer of acyl groups from MGD other chloroplast and extrachloroplast lipids (PE is entirely an extrachloroplast lipid), or 16:0 which would normally be utilized for MGD synthesis and desaturated to 16:3, becomes available for the synthesis of all the leaf polar lipids. Our results from a different lipid mutant of Arabidopsis deficient in G3P-acyltransferase (Kunst et al., 1988), indicate that 16:0 levels in leaf cells are highly regulated, and that greater than normal amounts of 16:0 are not utilized within the chloroplast, but get elongated to 18:0 and desaturated to 18:1 before being exported to the cytoplasm. Thus, the increased amount of 16:0 in the lipids of the fadB mutant are not readily explained.

Labeling of leaves

The relatively high levels of palmitic acid present in MGD in the mutant do not fully compensate for the reduction in unsaturated 16-carbon fatty acids. There is still an overall 63% decrease in the total amount of 16-carbon fatty acids in MGD in mutant plants, and therefore, a corresponding decrease in the amount of prokaryotic MGD. This may reflect either an alteration in fluxes through the two pathways of lipid synthesis, or an increased turnover of prokaryotic MGD containing high levels of saturated fatty acids. In an attempt to resolve this question and to determine the consequences of the enzyme deficiency on lipid biosynthesis in the mutant, we labeled the leaves of mutant and wild-type plants with [^{14}C]-acetate, and followed the distribution of radioactivity in polar lipids during the subsequent 142 hours (Figure 5-2). As we have shown previously (Browse et al., 1986b, Kunst et al., 1988), the labeling kinetics of wild-type plants demonstrate the parallel operation of the prokaryotic and eukaryotic pathways. Flux through the prokaryotic pathway leads to substantial labeling of MGD at the beginning of the experiment, while the increase in MGD label at longer times reflects the transfer of ^{14}C from PC made through the eukaryotic pathway. The distribution of label among various polar lipids in the fadB mutant is extremely similar to that of the wild type (Figure 5-2). However, the reduced rate of incorporation of label into MGD and, conversely, the relatively increased labeling of PC is consistent with a reduced synthesis of MGD by the prokaryotic pathway. The results also indicate that the eukaryotic pathway compensates for this reduction in prokaryotic MGD synthesis, since by the end of the experiment the amount of MGD in leaf tissue of the

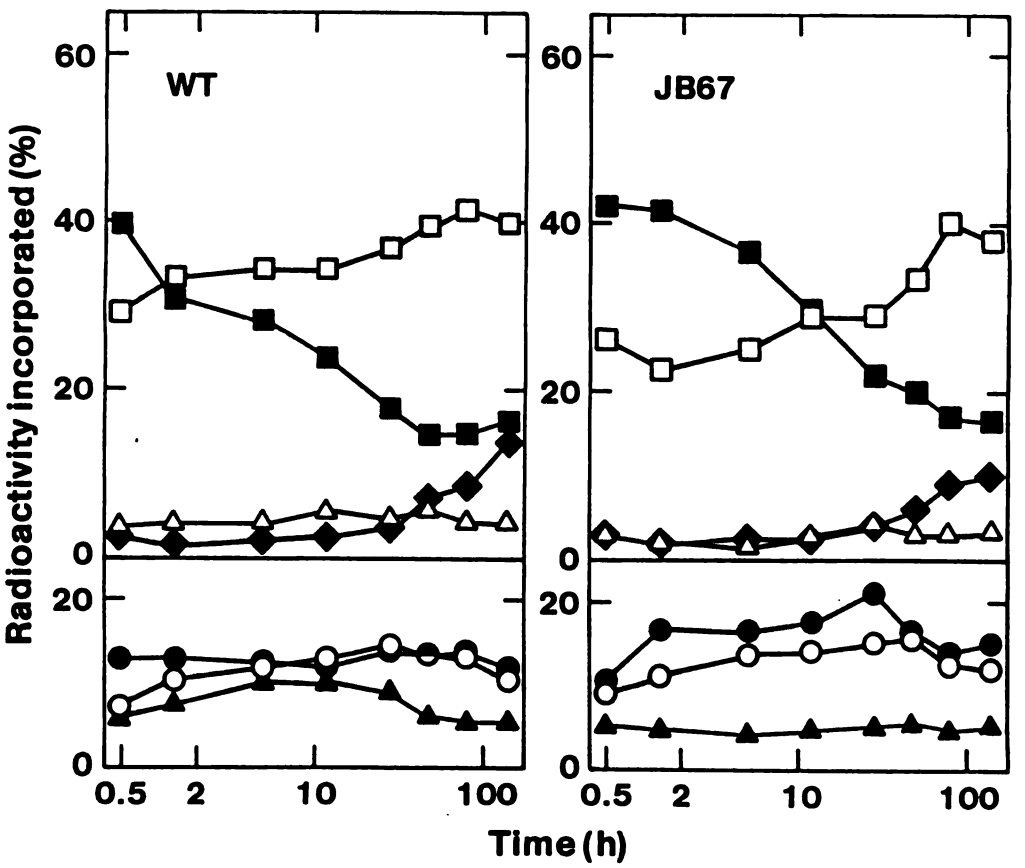

Figure 5-2. The distribution of radioactivity in leaf lipids of (A) wild type and (B) JB67 mutant of Arabidopsis after labeling with [^{14}C]-acetate. Symbols: ■, PC; □, MGD; ◆, DGD; ▲, SL; ○, PG; ●, PE; ▲, PI.

mutant reaches wild-type levels. The decline in total radioactivity per gram fresh weight during the course of the experiment was almost identical in the mutant and wild-type plants, suggesting that there is no major difference in their relative rates of lipid breakdown (data not shown). However, the pathway by which the components of MGD might be recycled are not known. Thus, it does not seem possible at this time to critically evaluate the concept that certain molecular species of MGD are turned over more quickly in the mutant.

Discussion

Because of the problems related to solubilization, purification and stability of the membrane-bound fatty acid desaturases of higher plants, very little information is available about these enzymes, and desaturation reactions _per se_. The subunit composition of the desaturases has not been elucidated, and the cofactors and electron transport components thought to be involved in the desaturation process have not been identified. Therefore, we could not determine the enzymatic lesion in _fadB_ mutant by direct enzyme assay. Nevertheless, normal monoacyl-G3P acyltransferase activity of the mutant (Figure 5-1), together with the analysis of fatty acid composition of MGD (Tables 5-I and 5-II) support the conclusion that the mutant line JB67 is able to synthesize MGD, but is deficient in a chloroplast n-9 desaturase due to a single nuclear mutation at the _fadB_ locus. This enzyme introduces the double bond only in 16-carbon acyl chains esterified to the _sn_-2 position of MGD synthesized in the chloroplast.

These features make the 16:0 desaturase unique in comparison with the n-6 (Browse et al.,manuscript in preparation) and n-3 enzymes (Browse et al., 1986a), which do not exhibit specificity with respect to acyl group chain length, its point of attachment to the glycerol backbone (sn-1 or sn-2), or the lipid head group. The observation that the mutant accumulates MGD containing 16:0 at position sn-2 indicates that introduction of the n-9 double bond is a prerequisite for further desaturation of 16-carbon acyl chains by the other chloroplast desaturases. Similar conclusions were reached for the desaturases of Chlorella presented with various monoenoic fatty acids as substrates (Howling et al., 1972).

The accumulation of 16:0 at position sn-2 of MGD is consistent with the expectation for a mutant unable to desaturate 16:0 to 16:1. However, the fadB mutation causes two other changes in lipid composition which are less readily explained. First, all the chloroplast and extrachloroplast polar lipids, except SL, show increased levels of palmitic acid or trans-hexadecenoic acid. In the case of DGD, this may be attributed to a greater proportion of this lipid being synthesized by the prokaryotic pathway. However, for PC, PE and PI, which are thought to be synthesized in the endoplasmic reticulum, the implication is that a greater amount of 16:0 must be available for lipid synthesis by microsomal membranes in the mutant. It is not obvious why this might be the case. On the contrary, we have recently shown that the amount of 16:0 exported from the chloroplast is not regulated simply by availability (Kunst et al., 1988). Thus, it seems necessary to propose that, in some way, the accumulation of MGD

with 16:0 at the sn-2 position stimulates transport of 16:0 from the chloroplast.

The other unusual effect of the fadB mutation is that the amount of prokaryotic MGD is decreased by more than 60%. The implication is that MGD containing 16:0 at sn-2 is either turned over rapidly and does not accumulate, or that this species inhibits the synthesis of MGD from DAG in the chloroplast. The latter explanation seems least likely since it is thought that DAG is normally converted to MGD before the desaturation at the sn-2 position occurs (Siebertz and Heinz, 1977, Heinz and Roughan, 1983). The reduced rate of accumulation of label in MGD at early times (Figure 5-2) is consistent with either possibility. Whatever the case, the decreased accumulation of prokaryotic MGD in the mutant does not lead to a decrease in the absolute amount of this lipid but, rather, is compensated by increased synthesis of eukaryotic MGD. Thus, by analogy with similar results obtained with a mutant deficient in plastid G3P-acyltransferase (Kunst et al., 1988) it is apparent that that the relative flux through the two pathways of lipid synthesis is tightly regulated in such a way that the physical properties of the lipid component of chloroplast membranes can be adjusted to offset the potentially deleterious effects of a major change in lipid unsaturation in the mutant. We anticipate that the mutant line described here will provide a useful tool for the elucidation of the mechanisms underlying the complex regulation of leaf lipid metabolism.

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CHAPTER 6

ENHANCED THERMAL TOLERANCE IN A MUTANT OF *ARABIDOPSIS* DEFICIENT IN PALMITIC ACID UNSATURATION

Abstract

A mutant of *Arabidopsis thaliana*, deficient in the activity of a chloroplast n-9 desaturase, accumulates high amounts of palmitic acid $(16:0)^4$, and exhibits an overall reduction in the levels of unsaturation of its leaf lipids. Under standard conditions (22^oC, 100-150 uE m^{-2} s^{-1} constant illumination) the altered membrane lipid composition has no effect on growth rate of the mutant, net photosynthetic CO_2 fixation, photosynthetic electron transport, or chloroplast ultrastructure. Similarly, fluorescence polarization measurements indicated that the fluidity of the membranes was not significantly different in the mutant and the wild type. However, at temperatures above 28^oC, the mutant grows more rapidly than the wild type. This observation suggests that the altered fatty acid composition results in increased thermal tolerance of the mutant. A comparison of the chloroplast membranes of the mutant and wild type by two additional criteria, temperature-induced fluorescence yield enhancement and whole chain electron transport of membranes preincubated at high

temperatures, confirmed the superior thermal properties of the mutant. Thus, it is apparent that the lipid composition plays a role in temperature adaptation of chloroplast membranes. Electrophoretic analysis of chlorophyll-protein complexes and Chl fluorescence measurements have shown that the oligomeric form of LHCP is slightly more labile in the mutant. On the other hand, the observed change in the efficiency of excitation energy distribution from LHCP to the reaction centers does not seem to affect the normal photosynthetic performance of mutant plants.

Introduction

The chloroplast membranes of higher plants have a distinct fatty acid composition characterized by an unusually high proportion of polyunsaturated acyl groups. Depending on the plant species, trienoic fatty acids (18:3 and 16:3) comprise up to 80% of total fatty acids in the membrane lipids of this organelle (Murphy, 1986). Furthermore, the atypical fatty acid Δ3, trans-16:1 is found esterified to the sn-2 position of the major chloroplast phospholipid, phosphatidylglycerol (PG). These features of chloroplast lipids are common and remarkably constant in a wide variety of species, suggesting that the fatty acid composition is important for maintaining photosynthetic function. In an attempt to elucidate the significance of fatty acid composition and unsaturation in photosynthesis, many different approaches have been used. They include reconstitution of photosynthetic components with lipid mixtures (Gounaris et al., 1983), alterations of lipids in situ

by heat (Gounaris et al., 1984), chemical inhibitors (Leech et al., 1985), lipase treatment (Rawyler and Siegenthaler, 1981), or hydrogenation of unsaturated fatty acids (Vigh et al., 1985, Quinn and Williams, 1983), as well as correlation of events during chloroplast development (Leech et al., 1973, Galey et al., 1980). However, these approaches have not proven successful in unequivocally establishing the specific role of acyl group composition in thylakoid membrane function.

As an alternative approach to this problem we have isolated a number of mutants with reduced levels of unsaturation of their leaf lipids (Browse et al., 1985a, Browse et al., 1986, Browse et al., manuscript in preparation, Chapter 5). Despite a detailed analysis of these mutants, we have not been able to detect any significant changes in photosynthetic properties of the mutants investigated. On the other hand, we have provided evidence that large decreases in the proportion of trienoic acids lead to changes in chloroplast ultrastructure (McCourt et al., 1987). Here I describe physiological studies of a mutant, designated *fadB*, deficient in the activity of the chloroplast n-9 desaturase, which specifically converts palmitic acid (16:0) at position *sn*-2 of MGD to *cis*-16:1 (Chapter 5). As a consequence, the mutant accumulates high levels of palmitic acid, and lacks polyunsaturated 16-carbon fatty acids. Therefore, it should be a useful tool in further evaluating the relationship between trienoic acid content and chloroplast structure and function.

Material and methods

Plant material and growth conditions

The mutant line JB67 was isolated from the Columbia wild type of Arabidopsis thaliana (L.) Heynh. as previously described (Browse et al., 1985a). The mutant carries a defective allele of a locus, designated fadB, required for the desaturation of palmitic acid at position sn-2 of MGD (Chapter 5). It was backcrossed to the wild type four times before being used for the physiological experiments described here. Unless otherwise indicated, plants were grown at 22°C under continuous fluorescent illumination (100-150 uE m^{-2} s^{-1}) on a perlite:vermiculite:sphagnum mixture (1:1:1) irrigated with mineral nutrients (Haughn and Somerville, 1986).

Measurements of growth rate

Plants were germinated at 22°C and grown under conditions described above. After seven days the temperature was adjusted as noted in the text. Samples of four plants were harvested at two day intervals, and the fresh weights of the aerial portions were measured. The relative growth rate (w^{-1}) was determined as the slope of the natural logarithm of the average fresh weight in mg plotted against time since the temperature adjustment. The increase in fresh weight was linear only during the first six days at all temperatures. The growth slowly ceased afterwards, especially at temperatures above 30°C. Therefore, only the values obtained during the initial 6 days after temperature adjustment were used for relative growth rate determinations.

Extraction and analysis of Chl, proteins, and lipids

Extracts were prepared by grinding leaves harvested at rosette stage (3 weeks) in cold 20 mM Tricine-KOH (pH 8.4), 5 mM $MgCl_2$ and 2.5 mM EDTA. Insoluble matter was removed by centrifugation at 100 x g for 10 min and Chl, proteins and lipids were assayed essentially as described (Chapter 4). Fatty acid composition of total leaf lipids was determined according to Browse et al. (1985b).

Isolation of chloroplast membranes

Chloroplast membranes were prepared by grinding washed leaves in cold 450 mM sorbitol, 20 mM Tricine-KOH (pH 8.4), 10 mM NaCl, 10 mM EDTA and 0.1% (w/v) BSA. The homogenate was filtered through Miracloth (Calbiochem, La Jolla, CA) and centrifuged at 3000 x g for 5 min. The pellet was washed with cold 10 mM Hepes (pH 7.9), 10 mM NaCl, 5 mM EDTA, and resuspended in 20 mM Hepes (pH 7.9), 10 mM NaCl, 2 mM $MgCl_2$, 2.5 mM EDTA and 0.1% (w/v) BSA. Thylakoid preparations for SDS-PAGE lacked BSA, while $MgCl_2$ was omitted in some fluorescence measurements, as described in the text. Chl was assayed by the method of MacKinney (1941).

Pigment-protein electrophoresis

Chloroplast membranes were isolated as described above. Pigment-protein electrophoresis was performed according to the method of Andersson et al. (1982), except that the sodium dodecyl sulfate (SDS, Sequanal Grade, Pierce, Rockford, IL) to Chl weight ratio was adjusted to 3.75 : 1.

L-[^{35}S]-Methionine labeling of thylakoid proteins, protein extraction and two-dimensional SDS-polyacrylamide gel electrophoresis

Labeling of chloroplast membrane proteins with [^{35}S]Methionine, protein extraction, and two-dimensional SDS-polyacrylamide gel electrophoresis were performed as described in Chapter 4.

Fluorescence polarization measurements

An estimation of the microviscosity of the thylakoid membranes was obtained by determining the steady state fluorescence polarization of the hydrophobic fluorophore DPH (Aldrich, Milwaukee, WI)(Barber et al., 1984). DPH (3 mM stock in tetrahydrofuran) was added directly to the thylakoid extract (50 ug Chl ml^{-1}) to a final concentration of 5 uM, and incubated for 40 min in the dark at room temperature. The membranes were then centrifuged at 3000 x g for 5 min and diluted with 100 mM sorbitol, 20 mM Hepes (ph 7.9), 10 mM NaCl to a final concentration of 10 ug ml^{-1} Chl and 1 uM DPH. Fluorescence polarization measurements were carried out as described by McCourt et al. (1987).

Photosynthetic electron transport measurements

Whole chain electron transport and PSI activity were measured in a Rank oxygen electrode with 1200 uE m^{-2} s^{-1} PAR, by adding an aliquot of thylakoid membrane extract to 1 ml of resuspension buffer to a final concentration of 20 ug Chl ml^{-1}, as previously described (Kunst et al., manuscript in preparation). PSII activity was assayed as DPIP reduction at 580 nm using a Hitachi 100-60 spectrophotometer according to Steinback et al., 1979). PSII light response was determined as described in Chapter 4. All assays were performed at 25^{o}C.

Chl fluorescence measurements

Room temperature fluorescence induction transients of isolated thylakoids were measured by the method of Paterson and Arntzen (1982). For low temperature (77K) fluorescence determinations, aliquots of thylakoid suspension were diluted to 10 ug ml^{-1} in 60% glycerol (v/v), and sodium fluorescein was added as an internal standard to a final concentration of 2 uM (Krause et al., 1983). Samples were then frozen in capillary tubes (0.5 mm inner diameter) in liquid N_2. Fluorescence was scanned from 470-800 nm at 77K on an SLM spectrofluorometer (McCourt et al., 1985).

Effects of temperature on Chl fluorescence

Temperature induced fluorescence yield enhancement was measured on dark adapted whole detached leaves by minor modifications of the method of Schreiber and Berry (1977). Weak (0.3 uE m^{-2} s^{-1}) monochromatic light at 480 nm with 4 nm half-bandwidth was directed at a 45^{o} angle to a leaf placed between two sheets of 0.1 mm thick mylar in a water filled cuvette in the SLM spectrofluorometer. Fluorescence emission from the leaf surface was monitored at 700 nm with 2 nm half-bandwidth. The temperature of the sample was increased at a rate of $1^{o}C$ min^{-1} and the fluorescence intensity was recorded simultaneously.

Gas exchange

Methods for short term gas exchange measurements on single intact Arabidopsis plants have been described (Somerville and Ogren, 1982). For each plant, measurements of dark respiration and photosynthesis at 100 and 300 uE m^{-2} s^{-1} were carried out, followed by fresh weight

determination and Chl assay (MacKinney, 1941) of the aerial portion of the plant.

Electron microscopy

Leaves from 3-week old plants were fixed in 2% glutaraldehyde (v/v) in 100 mM sodium cacodylate buffer (pH 7.2) for 2 h at 4°C, washed in the same buffer, and stained with 1% OsO_4 (w/v) for 1 h at 4°C. The samples were then rinsed with double distilled water and dehydrated in a graded ethanol series (70%, 80%, 95%, 100%). The infiltration with Spurr's epoxy resin was done in two steps at room temperature. The samples were first incubated with a mixture of 100% ethanol and Spurr's resin (1:1, v/v) for 1 h, followed by 100% Spurr's resin for 8 h. Thin sections were poststained with 5% (v/v) uranyl acetate and lead citrate (Reynolds, 1963), and examined in a JEOL 100CX electron microscope.

Chloroplast number determinations

Chloroplast number per cell was counted in isolated protoplasts prepared as described (Kunst et al., 1988). A 20 ul sample of protoplast suspension was pipetted onto a microscope slide and the protoplasts were flattened by application of a coverslip. The chloroplasts formed a monolayer within cells and could be easily counted (McCourt et al., 1987).

Results

Effects of temperature on growth

Several lines of evidence support the proposal that membrane lipid composition might play a role in thermal adaptation of plants. It was, therefore, of interest to examine the effects of fadB mutation on growth of the fadB mutant at different temperatures ranging from 10-34^{o}C (Figure 6-1). The rate of increase in fresh weight of mutant and wild type was very similar up to 28^{o}C. At temperatures greater than 28^{o}C the mutant plants grew more rapidly than the wild type (Figure 6-1). This observation suggests that the altered fatty acid composition results in increased thermal tolerance of the fadB mutant. However, this was apparent only during the first 6 days at temperatures above 30^{o}C, since the growth rate is greatly reduced after prolonged exposure to high temperatures, and both mutant and wild type plants eventually turn chlorotic.

Effects of high temperature on fluorescence

Since the fadB mutation primarily affects chloroplast lipids, the apparent effect of the mutation on thermal tolerance is most likely due to an effect on chloroplast membranes. Therefore, we examined the thermal stability of chloroplast membranes as measured by Chl fluorescence in intact leaves of mutant and wild type (Figure 6-2). A heat-induced increase in room temperature fluorescence has been attributed to the physical separation of the LHCP from the PSII core, which blocks the excitation energy transfer and leads to reemission of the energy as fluorescence. Therefore, Chl fluorescence is considered

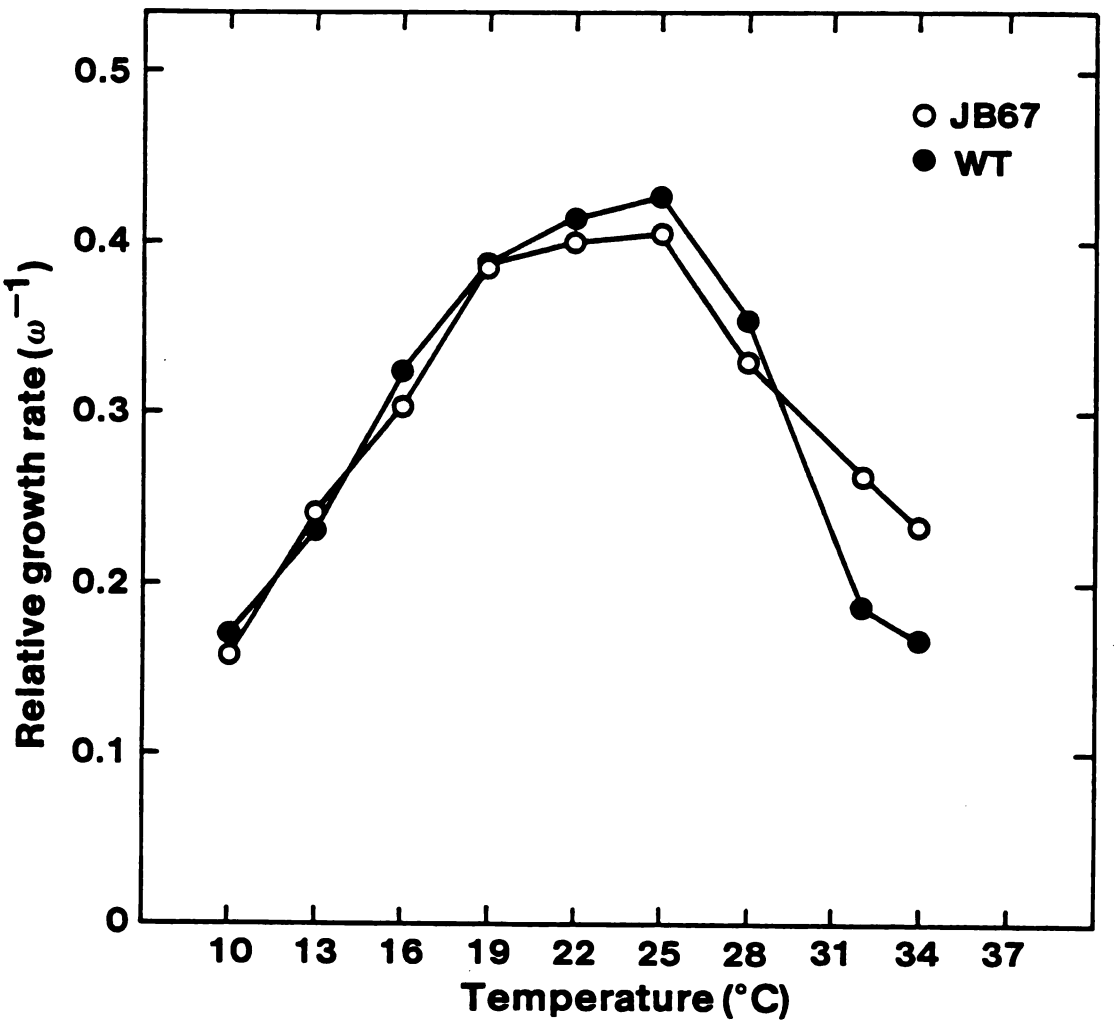

Figure 6-1. Effect of temperature on the relative growth rate of wild type and mutant Arabidopsis.

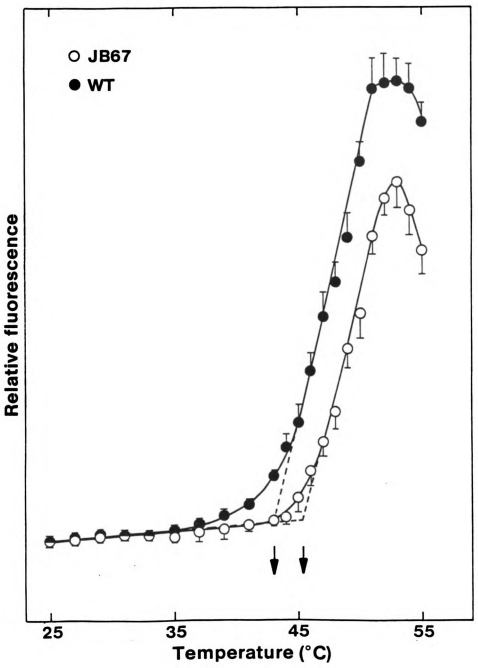

Figure 6-2. Temperature induced fluorescence enhancement yield of wild type and mutant leaves. Plants were grown at 22°C. The arrows indicate estimates of threshold temperatures at which fluorescence is enhanced. Each point represents the mean ± SD (n=3).

an intrinsic probe of lipid-protein interaction (Lynch and Thompson, 1984) and an indicator of photosynthetic membrane stability (Raison et al., 1982, Schreiber and Berry, 1977). The experiment was performed by slowly heating detached leaves at a rate of $1^{o}C\ min^{-1}$ up to $57^{o}C$ and continuously monitoring fluorescence levels. At approximately $43^{o}C$ the fluorescence started to rise rapidly in wild type leaves, while the transition in the level of fluorescence did not occur until $45^{o}C$ in the mutant. Thus, by this criterion, the fadB mutation appears to confer increased thermal stability upon chloroplast membranes.

Effects of temperature on photosynthetic electron transport

The effect of temperature on the stability of chloroplast membranes of the mutant line JB67 and wild type was also measured by incubating isolated membranes in darkness for 10 min at various temperatures from 25-$45^{o}C$, and then measuring whole-chain electron transport at $25^{o}C$. As shown in Figure 6-3, electron transport rates of both mutant and wild type declined steadily as the preincubation temperature was increased. However, there was a significant difference in the apparent stability of the mutant and wild-type membranes. In the wild type, electron transport activity drops to 60% after 10 min at $35^{o}C$, while the mutant exhibits a decrease of only 15% under the same conditions.

The kinetics of thermal inactivation of photosynthetic electron transport was examined by incubating isolated chloroplast membranes at $40^{o}C$ for various times, and then assaying the whole chain activity at $25^{o}C$. The results of this experiment (Figure 6-4) are consistant with a

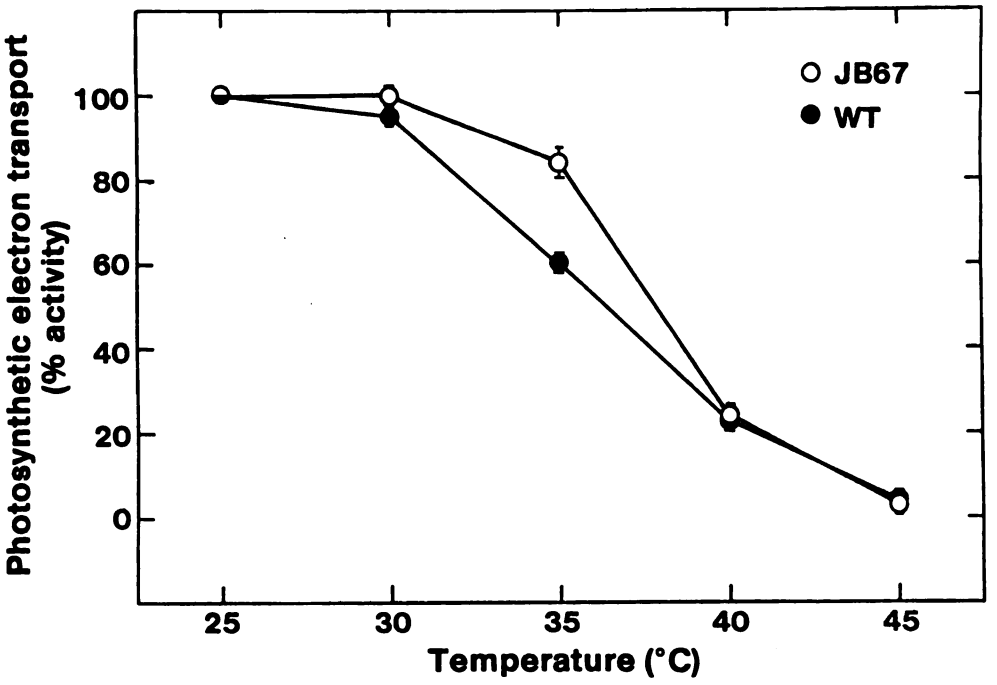

Figure 6-3. Effect of temperature on photosynthetic electron transport in chloroplast membranes from wild type and mutant Arabidopsis. Activity is expressed relative to that obtained with membranes preincubated in darkness at 4°C for 10 min. The maximal rates for the mutant and wild type were 161.2 umol O_2 mg Chl^{-1} h^{-1} and 146.9 umol O_2 mg Chl^{-1} h^{-1}, respectively. Each point represents the mean $\pm$ SD (n=4).

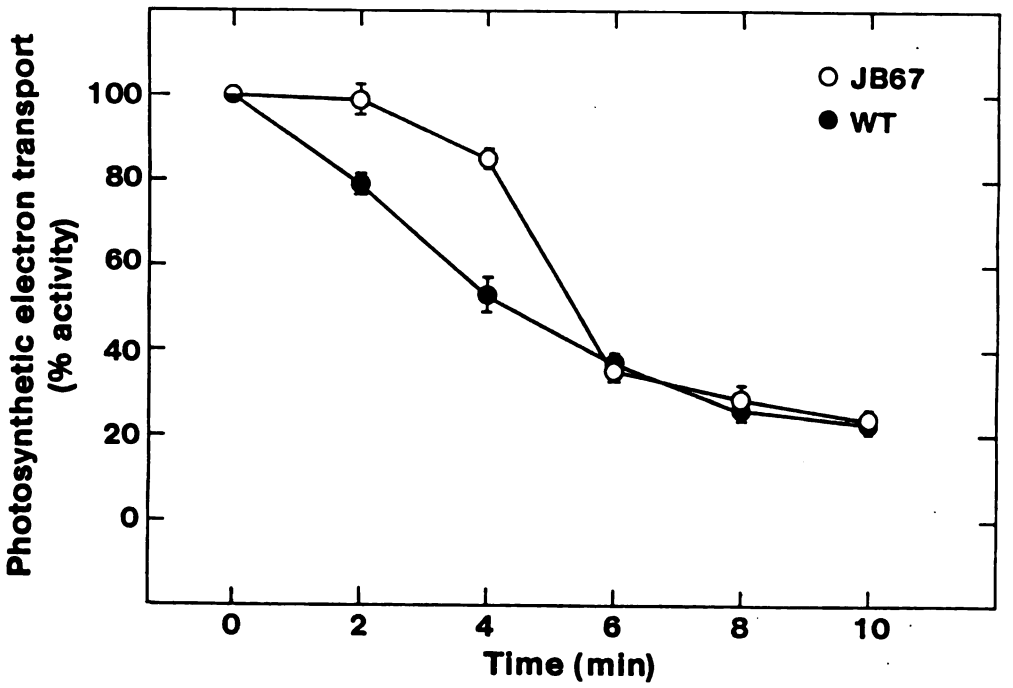

Figure 6-4. Photosynthetic electron transport activity in chloroplast membranes from wild type and mutant Arabidopsis preincubated at 40°C for various times indicated. Activity is expressed relative to that obtained with membranes preincubated in darkness at 4°C for 10 min. Maximal rates for the mutant and the wild type were 161.2 umol O_2 mg Chl^{-1} h^{-1} and 146.9 umol O_2 mg Chl^{-1} h^{-1}, respectively. Each point represents the mean ± SD (n=4).

delayed thermal inactivation of whole chain electron transport in chloroplast membranes of mutant plants. Therefore, it seems apparent that the composition of the lipid matrix plays a role in thermal tolerance of the photosynthetic membranes.

Membrane fluidity

To examine the effect of lipid composition on the fluidity of thylakoid membranes, we carried out fluorescence polarization measurements on isolated membranes from wild type and mutant Arabidopsis (Figure 6-5). Polarization (P) values of wild type membranes are similar to those reported for other plant species (Barber et al., 1984), and indicate a highly fluid lipid bilayer. The thylakoid membranes of the fadB mutant had slightly higher P values, suggesting a small decrease in membrane fluidity. A similar result was obtained for another Arabidopsis mutant with reduced amounts of polyunsturated fatty acids (McCourt et al., 1987). However, the difference in both mutants was below the limit of statistical significance.

Effect of membrane lipid composition on Chl and protein content

Under standard light conditions (100-150 uE m^{-2} s^{-1}) and various temperatures ranging from 10-34^{o}C, the mutant exhibits a slight chlorotic phenotype, due to a 15-20% reduction in Chl per unit fresh weight (Table 6-I). To determine if the reduction in Chl is related to the fadB mutation, 60 F_2 plants from a WT x JB67 cross were tested for cosegregation of the two phenotypes. Of 16 plants which showed increased levels of palmitic acid (16:0), all had reduced Chl levels on a fresh weight basis. All other plants had normal levels of Chl and

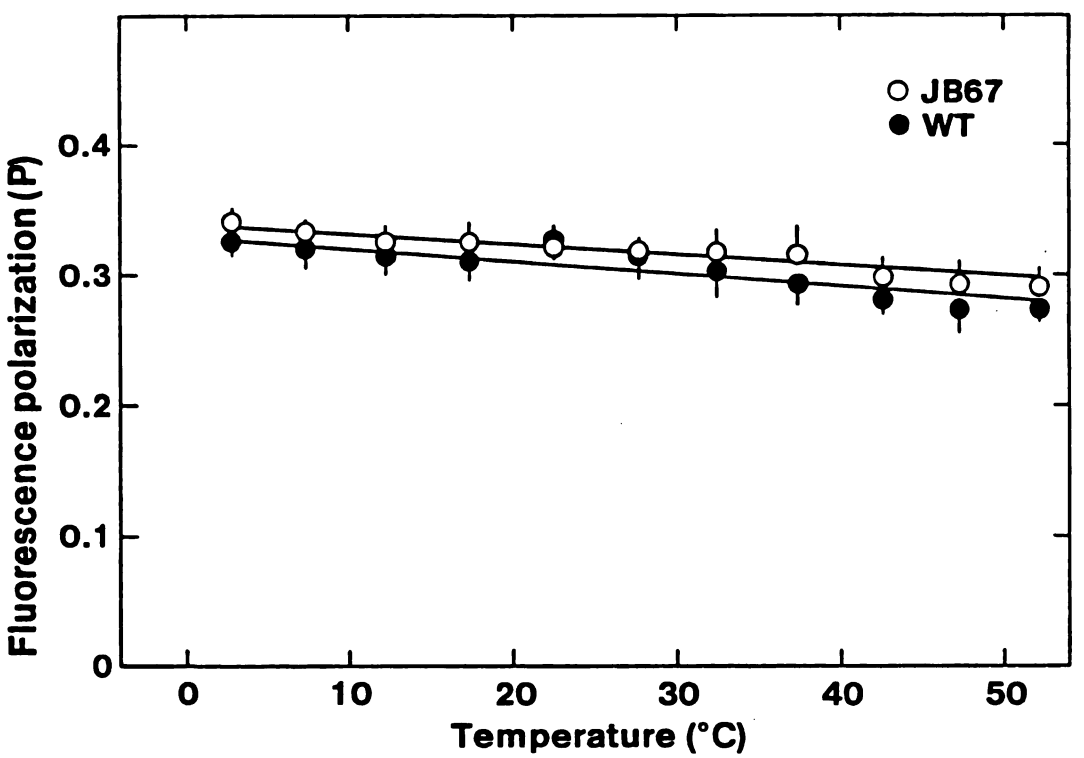

Figure 6-5. Effect of temperature on DPH fluorescence polarization of chloroplast membranes from wild type and mutant Arabidopsis. Each point represents the mean ± SD (n=10).

Table 6-I. Relative amounts of lipid, chl and protein in mutant and wild type Arabidopsis leaves and chloroplast membranes. Values are means ± SD (n=3).

	Wild type	JB67
LEAVES		
Chl/fwt (mg/g)	1.67 ± 0.01	1.39 ± 0.07
Chl a/b ratio	2.94 ± 0.10	3.21 ± 0.05
Lipid/Chl (g/g)	2.48 ± 0.07	2.58 ± 0.15
Protein/Chl (g/g)	38.46 ± 0.15	38.56 ± 1.60
Protein/Lipid (g/g)	15.56 ± 0.90	14.95 ± 0.30
CHLOROPLAST MEMBRANES		
Lipid/Chl (g/g)	2.12 ± 0.08	2.53 ± 0.09
Protein/Chl (g/g)	12.27 ± 0.50	16.66 ± 0.92
Protein/Lipid (g/g)	5.79 ± 0.24	6.79 ± 0.29

wild type levels of 16:0. Cosegregation of the two traits suggests that both phenotypes are caused by the same mutation.

Since all the Chl in higher plants is believed to be associated with proteins (Markwell et al., 1979), a decrease in Chl content suggests changes in one or more of the chl-protein complexes of thylakoid membranes in JB67 mutant. The higher a/b ratio of mutant leaves also suggests an alteration in the stoichiometry of LHCP to PSII and PSI. Therefore, we separated the chl-protein complexes of mutant and wild-type membranes by SDS-polyacrylamide gel electrophoresis in order to examine whether certain Chl-protien complexes are preferentially affected (Figure 6-6). The identity of the bands resolved by this method (CP1a, CP1, $LHCP^1$, $LHCP^2$, CPa, $LHCP^3$ and free pigments) was established by comparing the absorption spectra of individual bands (results not presented) with published values (Anderson et al., 1978). A comparison of the separation patterns of the chl-protein complexes from the wild type and mutant revealed that the mutant contains a slightly lower amount of $LHCP^1$, the presumed LHCP oligomer. A similar observation reported for another lipid mutant of Arabidopsis, which was deficient in the trans-16:1 acyl group (McCourt et al., 1985), was interpreted as indicating that an unsaturated fatty acid at sn-2 position of chloroplast PG might be important in stabilizing $LHCP^1$ against SDS mediated dissociation.

Photosynthetic characteristics

To estimate the effects of reduced levels of unsaturation on photosynthetic properties of the fadB mutant, we measured the rates of CO_2 fixation in mutant and wild-type plants (Table 6-II), as well as

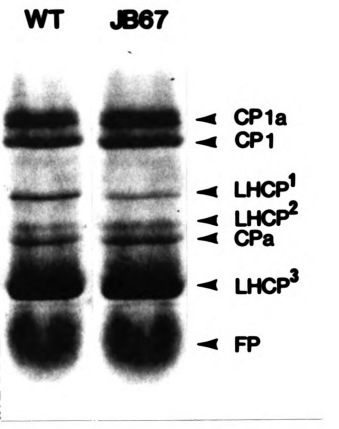

Figure 6-6. Chl-protein complexes of chloroplast membranes from wild type and mutant Arabidopsis. The nomenclature is from Anderson et al. (1978).

Table 6-II. Photosynthetic activities in mutant and wild type Arabidopsis.

	Wild type	JB67
NET CO_2 FIXATION		
	ug CO_2 mg Chl^{-1} h^{-1}	
Darkness	-[a]487.8 ± 13	-498.1 ± 34
100 uE m^{-2} s^{-1}	2432.5 ± 100	2761.5 ± 23
300 uE m^{-2} s^{-1}	4766.9 ± 205	5452.2 ± 122
	ug CO_2g fwt^{-1} h^{-1}	
Darkness	-565.1 ± 23	-563.8 ± 43
100 uE m^{-2} s^{-1}	2802.2 ± 140	3127.0 ± 56
300 uE m^{-2} s^{-1}	5535.4 ± 285	6015.0 ± 197
ELECTRON TRANSPORT		
	umol O_2 mg Chl^{-1} h^{-1}	
Whole chain	118.0 ± 2	119.0 ± 2
PSI	348.0 ± 8	351.1 ± 7
PSII	347.9 ± 35	360.4 ± 31

[a] Net CO_2 evolution is indicted here as a negative value.

electron transport activities in isolated chloroplast membranes. CO_2 fixation rates of both mutant and wild type were extremely similar when expressed on the basis of fresh weight. However, the mutant did exhibit 15% higher rates per unit Chl relative to the wild type (Table 6-II), probably due to a reduction of similar magnitude in leaf Chl content. There were also no major differences in the whole chain, or PSII and PSII partial electron transport activities between the mutant and wild type (Table 6-II, Figure 6-7). These data indicate that the changes in the fatty acid composition of chloroplast membranes from the mutant do not affect rates of photosynthetic electron transport.

Chl fluorescence measurements

Assuming that the LHCP oligomer is the native form in situ (Kuhlbrandt, 1984), it is possible that the reduced stability of LHCP[1] in the fadB mutant might be reflected in less efficient transfer of excitons from LHCP to the reaction centers. Low temperature fluorescence spectra are sensitive indicators of the efficiency of energy distribution between Chl-containing components of chloroplast membranes. Therefore, we examined 77K fluorescence spectra of the isolated chloroplast membranes of mutant and wild type. (Table 6-III, Figure 6-8). Reduced excitation energy transfer from LHCP to the photosystems would be expected to result in an increase of LHCP fluorescence (685nm) relative to PSII (695nm) and PSI (735nm) emission maxima. Indeed, the F_{685}/F_{734} ratio obtained for the mutant was higher than the wild type (Table 6-III) in both presence or absence of $MgCl_2$. By normalizing emission values of individual peaks at 685 nm and 734 nm to that of the internal standard, fluorescein, it became clear that the

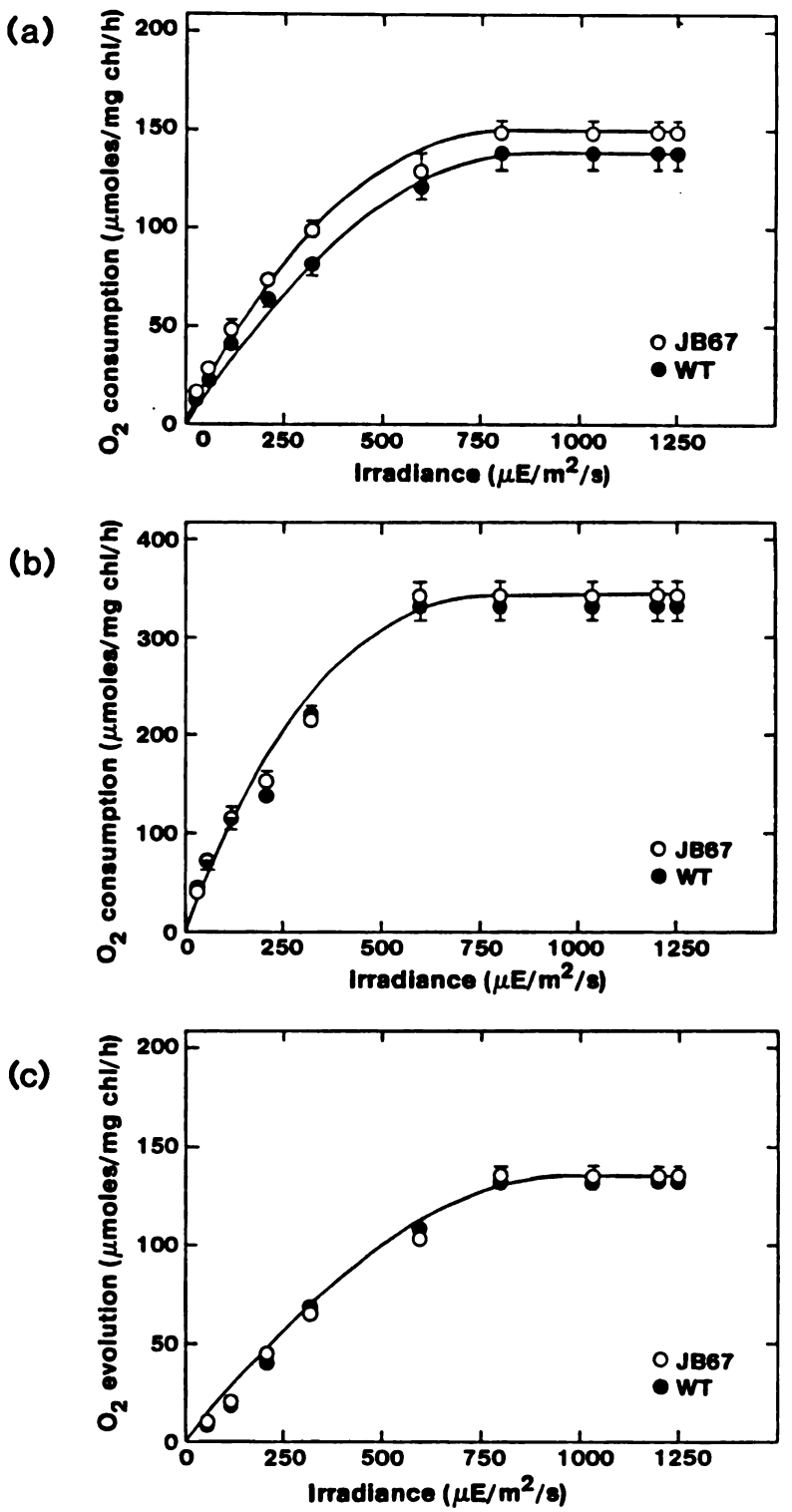

Figure 6-7. Light response curves for (A) whole chain, (B) PSI and (C) PSII electron transport by isolated chloroplast membranes from wild type and mutant Arabidopsis. Each point represents the mean ± SD (n=3).

Table 6-III. Room temperature fluorescence induction and low temperature (77K) fluorescence of isolated thylakoids.

	Wild type	JB67
ROOM TEMPERATURE FLUORESCENCE[a]		
Fo	1213 ± 19	1231 ± 25
Fm	3530 ± 90	3220 ± 67
Fv/Fo	1.91 ± 0.06	1.62 ± 0.07
77K FLUORESCENCE[b]		
	$+Mg^{2+}$	
F_{685}	3122 ± 66	3236 ± 68
F_{734}	4019 ± 84	3606 ± 98
F_{685}/F_{734}	0.78 ± 0.001	0.90 ± 0.01
	$-Mg^{2+}$	
F_{685}	3885 ± 96	4106 ± 93
F_{735}	5921 ± 85	4921 ± 117
F_{685}/F_{735}	0.66 ± 0.005	0.84 ± 0.005

[a] Room temperature fluorescence was measured in the presence of 10 uM DCMU, n=10; Values are expressed in arbitrary units.

[b] 77K fluorescence was measured in the presence and absence of 5 mM $MgCl_2$, n=6. Values are normalized to the emission peak of 2 uM fluorescein, which was assigned an arbitrary value of 9000.

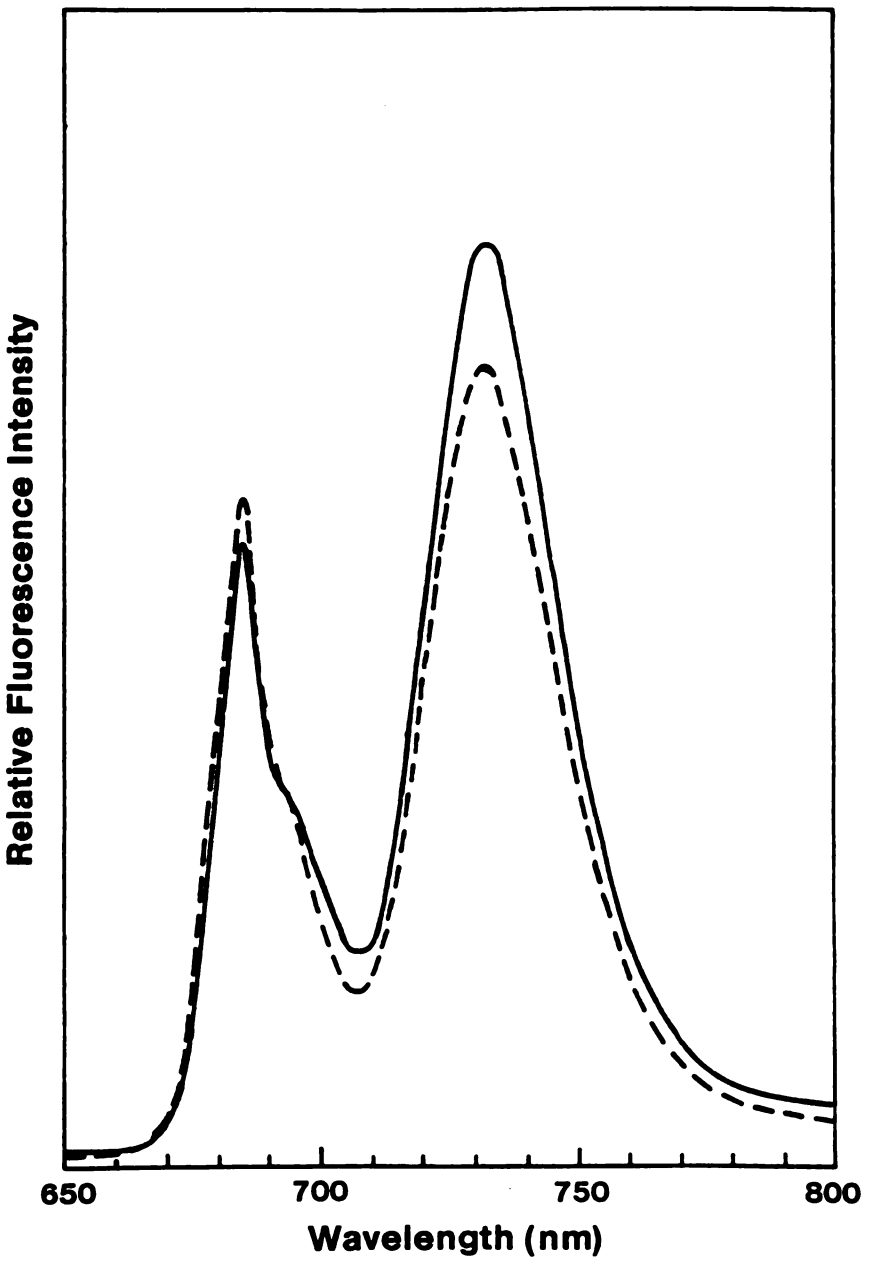

Figure 6-8. Chl fluorescence spectra of chloroplast membranes from wild type (——) and mutant (----) Arabidopsis in the absence of $MgCl_2$. The curves were normalized to the same value at 500 nm.

change in F_{685}/F_{734} ratio of the mutant is due to both a lower absolute fluorescence emission at 734 nm and a concomitant absolute increase in Chl fluorescence at 685 nm. This result is consistent with a reduction in the amount of light energy transferred from LHCP to the two photosystems.

In order to extend this observation the variable fluorescence of the mutant and wild type was measured at room temperature (Table 6-III). Room temperature Chl fluorescence is emitted from PSII and, in the presence of DCMU, depends only on the exciting light intensity, the number of Chl molecules active in transferring excitation energy to PSII reaction centers and the efficiency of transfer. Fo, the initial fluorescence level, is similar in the wild type and mutant membranes. On the other hand, maximal fluorescence (Fm), and the proportion of Chl active in photochemistry (Fv/Fo), are 15% lower in the fadB mutant (Table 6-III).

Chloroplast ultrastructure and number

We have recently provided evidence that fatty acid composition of chloroplast lipids may be an important factor regulating organelle biogenesis (McCourt et al., 1987). Thus, we examined the effect of the fadB mutation on chloroplast ultrastructure. The electron micrographs (Figure 6-9) of the mutant and wild type showed no obvious differences in chloroplast size, the arrangement of chloroplast membranes, the extent of stacking, or any other structural feature of the chloroplast. In addition, the number of chloroplasts per cell in the mutant (44.7 $\pm$ 15) and wild type (45.0 $\pm$ 13) were almost identical.

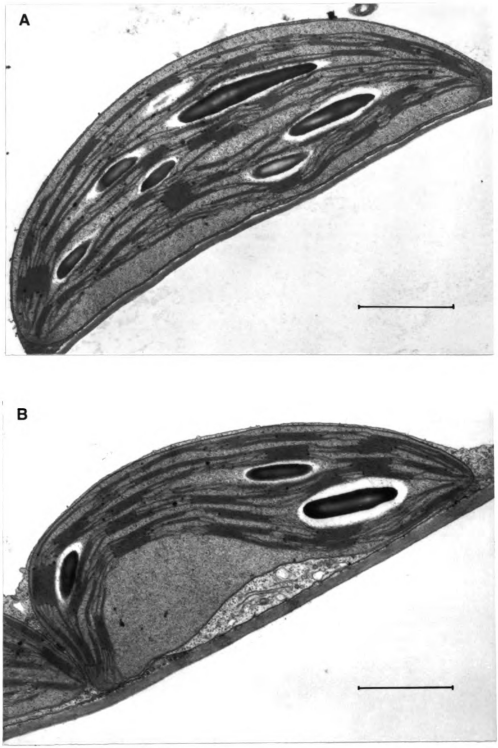

Figure 6-9. Transmission electron micrographs of chloroplasts from rosette leaves of (A) wild type and (B) mutant Arabidopsis. Bar = 1 um.

2-dimensional SDS-polyacrylamide gel electrophoresis

To examine the effect of the altered fatty acid composition on protein content of chloroplast membranes more thoroughly, we labeled the proteins with ^{35}S-Methionine and analyzed them by SDS-polyacrylamide gel electrophoresis. The polypeptide pattern of mutant and wild type plants (Figure 6-10) was almost identical, providing additional explanation as to why there were no apparent changes in chloroplast organization of the fadB mutant. However, there are subtle quantitative changes in several polypeptides associated with the fadB mutation (Figure 6-10). The amounts of several polypeptides are slightly reduced in comparison with the wild type, but there are also a few proteins that are more abundant in chloroplasts of the mutant. It would be interesting to know if any of these proteins play a role in the increased thermal stability of fadB membranes.

Discussion

A detailed comparison of the leaf lipid composition of the mutant line JB67 and wild type has indicated that the mutant is deficient in the activity of a chloroplast n-9 desaturase, due to a single nuclear mutation at fadB locus (Chapter 2). This lesion results in the accumulation of high amounts of 16:0 acyl group, and an overall reduction in the levels of membrane lipid unsaturation. Despite substantial changes in the membrane lipid composition of the fadB mutant, it exhibits normal growth, chloroplast ultrastructure and photosynthetic activity under standard conditions.

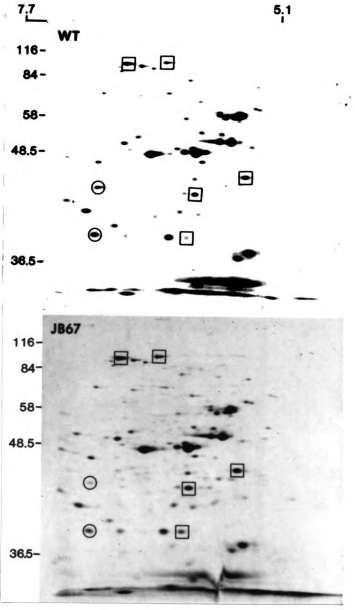

Figure 6-10. Autoradiographs of [^{35}S]-Methionine labeled proteins of chloroplast membranes from wild type and mutant Arabidopsis separated by two-dimensional SDS-polyacrylamide gel electrophoresis. The numbers at the top represent apparent pH, and those at the left apparent molecular mass. ◘ , proteins which are more abundant in the mutant than in the wild type; O , proteins which are more abundant in the wild type than in the mutant.

The growth rate of the mutant was higher than the wild type at elevated temperatures above 28°C (Figure 6-1). This effect was also observed in our analyses of the two other Arabidopsis mutants (McCourt et al., 1987, Chapter 4) with reduced amounts of polyunsaturated fatty acids. The factors involved in thermal stability of chloroplast membranes have not been elucidated. However, the results of Pearcy (1978), who reported the absence of 16:3 acyl group from MGD and a decrease in linolenic acid (18:3) with concomitant increases in more saturated fatty acids in membrane lipids of Atriplex lentiformis at high temperatures, suggest that changes in physical properites of the membranes might be the basis for thermal acclimation. Additional evidence was provided by Raison et al. (1982), whose work on Nerium oleander demonstrated that the decline in lipid unsaturation at high temperatures results in a less fluid lipid bilayer. The changes in fatty acid composition of leaf polar lipids of fadB mutant did not have a significant effect on chloroplast membrane fluidity. On the other hand, the mutant exhibited superior thermal stability by two criteria: a higher threshold temperature at which fluorescence yield was enhanced, and a slower rate of inactivation of whole chain electron transport at high temperatures. Therefore, it is concievable that the altered composition of the lipid matrix affects the stability of chloroplast membranes in the fadB mutant. It may well be that the changed physical properties of the lipid bilayer per se render the fadB membranes more stable. Alternatively, the changes in lipid-protein interactions, or changes in the amounts of specific proteins, due to alterations in leaf lipid composition, might confer increased thermal stability upon chloroplast membranes of the mutant. On the basis of the

results presented here, we cannot distinguish among these possibilities. However, there is evidence that the phase separation of non-bilayer lipids and the dissociation of light harvesting Chl-protein complexes of chloroplast membranes occur within the same temperature range (Gounaris et al., 1984). This correlation suggests that the stability of the protein-lipid association might be the most important factor determining the overall thermal stability of photosynthetic membranes.

An interesting feature of the fadB mutant is a 15% decrease in the amount of Chl per fresh leaf weight. Cosegregation analysis of altered fatty acid composition with reduced Chl content in the leaves of the mutant indicated that both these phenotypes are caused by the fadB mutation. An increase in Chl a/b ratio (Table 6-I) revealed that Chl b is lost from fadB chloroplasts. Since Chl b is associated with LHCP, a reduction in the amount of Chl b is indicative of a decrease in LHCP Chl-protein complex in chloroplast membranes. However, the loss of Chl b accounts for less than 25% of the total Chl absent from the leaves of the mutant, suggesting that other Chl-protein complexes are affected as well. Chl fluorescence measurements support this conclusion. The fluorescence emission at 695 nm and 734 nm, attributed to PSII snd PSI, respectively, is lower in fadB membranes, and there is a corresponding increase in LHCP fluorescence yield at 685 nm. Therefore, the excitation energy transfer from LHCP to PSII and PSI reaction centers is either less efficient, or there are structural changes within the PSII and PSI antennae of the mutant. Lower room temperature fluorescence emission of the mutant relative to the wild type indicates that the amount of Chl active in PSII photochemistry is

reduced. Therefore, it seems that fadB related changes in lipid composition result in a reduction of Chl associated with PSII and PSI reaction center complexes. However, it is apparent that this difference between the mutant and the wild type does not affect the overall photosynthetic performance of fadB mutant under the growth conditions examined.

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CHAPTER 7

CONCLUDING REMARKS

Summary

Each membrane in the cell has a distinct lipid composition with respect to both the head group and the acyl groups. However, the role of lipids and fatty acids in proper functioning of cell membranes remains uncertain. In order to directly address the question of how the lipid composition of thylakoid membranes affects chloroplast structure and function, we isolated a series of mutants of the crucifer Arabidopsis thaliana (L.) Heynh., with specific alterations in leaf lipid metabolism. Since relatively large changes in membrane lipids, like changes in the amount of various lipid headgroups could be lethal, we have concentrated on screening for mutants with altered leaf fatty acid composition by direct analysis using gas chromatography. In this dissertation I have described the isolation of two classes of mutants, act1 and fadB, deficient in the activity of chloroplast enzymes G3P acyltransferase and n-9 desaturase, respectively. I have characterized these mutants genetically and biochemically (Chapters 3 and 5), and examined the physiological consequences of the changes in their leaf membrane lipids (Chapters 4 and 6).

In the first mutant analyzed, a single recessive nuclear mutation at the *act1* locus causes a deficiency in the activity of the G3P acyltransferase, the first enzyme of the prokaryotic (plastid) pathway of glycerolipid synthesis. The principal results and conclusions of the work on *act1* mutant are:

(1) The lesion in the plastid pathway of lipid biosynthesis does not cause the accumulation of precursors within the organelle, but results in a redirection of fatty acids towards the cytoplasmic sites of lipid synthesis in the endoplasmic reticulum.

(2) The increased synthesis of glycerolipids by the eukaryotic pathway in the endoplasmic reticulum compensates for the loss of the prokaryotic pathway and provides, with the exception of the PG, almost normal amounts of all the lipids required for chloroplast membrane biogenesis.

(3) Differences in the distribution of radioactivity in leaf polar lipids between the wild type and the mutant after long term labeling provide definitive evidence for the parallel operation of prokaryotic and eukaryotic pathways in wild-type plants.

(4) The fatty acid composition of leaf lipids of the mutant is altered, since the acyltransferases of the two biosynthetic pathways exhibit different substrate specificities.

(5) *act1* mutation has a pronounced effect on structural features of chloroplasts. The number of appressed regions is increased in the mutant, but the number of membranes per stack is significantly reduced relative to the wild type. These changes were not associated with a major change in the amount of Chl a/b binding proteins, suggesting that

the model for membrane appression based on the properties of LHCP is incorrect or incomplete.

(6) Extensive changes in leaf lipid composition do not affect growth or development of the mutant under standard conditions. However, at temperatures above 28^{o}C the mutant grows slightly more rapidly. A comparison of the wild type and the mutant by two additional criteria: temperature-induced fluorescence yield enhancement and the rate of inactivation of whole chain electron transport activity in isolated chloroplast membranes preincubated at various increasing temperatures suggests an increased thermal stability of the photosynthetic membranes of the mutant.

The second mutant that I studied lacks polyunsaturated 16-carbon fatty acids and shows a corresponding increase in the amount of the 16:0 acyl group. These changes indicate that the mutant is deficient in the activity of a chloroplast n-9 fatty acid desaturase, due to a single nuclear mutation at a locus designated fadB. The major conclusions based on the analysis of the fadB mutant are:

(1) The mutation at fadB locus affects both chloroplast and extrachloroplast lipids. Thus, there is a substantial transfer of 16:0 acyl groups from MGD to all the leaf polar lipids, or 16:0 which would normally be used for MGD synthesis in the chloroplast is exported to the cytoplasm and utilized for the synthesis of lipids in this compartment.

(2) Synthesis of MGD by the prokaryotic pathway is reduced 25-30%, but this deficiency is compensated for by the increased production of this lipid in the endoplasmic reticulum.

(3) fadB related changes in leaf fatty acid composition result in increased thermal tolerance and enhanced stability of chloroplast membranes of the mutant at high temperatures.

(4) Under standard conditions the altered membrane lipid composition does not affect the vigor of the mutant, net photosynthetic CO_2 fixation, photosynthetic electron transport, chloroplast ultrastructure, or fluidity of chloroplast membranes.

Future directions

Additional mutants

The collection of acyl group mutants analyzed to date contributed considerably to our understanding of the desaturation process in the plant membranes and the control of cellular lipid metabolism. Furthermore, the mutants provided evidence for the possible functions of lipids in temperature responses of plants, and their involvement in the formation of chloroplast structure. However, because of the existence of the two pathways of lipid synthesis, the mutations in the prokaryotic pathway result in the increased compensatory synthesis of lipids through the eukaryotic pathway, so that the phenotypic effects produced by the mutations were not severe under standard growth conditions. Only one mutant has been isolated in the eukaryotic pathway of glycerolipid synthesis so far. It is not clear yet whether this lesion has any consequences on chloroplast lipid metabolism, or perhaps only affects the extrachloroplast lipids. In any case, isolation and characterization of more mutants in the eukaryotic pathway should be

useful in investigating the relevance of membrane fatty acid composition to a wide range of cellular processes including mitochondrial respiration, transport through the plasmalemma and tonoplast, or intracellular transport and secretion mediated by the Golgi apparatus. In addition, double mutants may be constructed with defects in both the prokaryotic and eukaryotic pathways. These mutants would probably have much more severe phenotypes and might be extremely valuable in testing how extensive the changes in lipid composition have to be before photosynthesis is affected, as well as which aspects of photosynthesis are most susceptible to alteration in fatty acid composition.

One of the major conclusions of our mutant analyses to date is that plants can withstand relatively large changes in membrane lipid composition without serious effects. Therefore, even the isolation of head group mutants is not inconceivable. It seems likely that the absence of a headgroup would be deleterious to the organism, but a leaky mutant, with a quantitative rather than qualitative variation in a certain headgroup, might provide answers to a lot of interesting questions concerning specific functions of lipids in different membranes.

Fatty acid desaturation

The fatty acid desaturase enzymes, with the exception of the soluble chloroplast desaturase (McKeon and Stumpf, 1982), have not been purified and characterized. The lack of information about plant lipid desaturases prevents the application of molecular genetic techniques in modifying the composition of storage lipids to suit industrial needs,

as well as the alteration of leaf lipids to possibly improve the acclimation of plants to different environmental conditions. The analysis of the fadB mutant, together with other mutants defective in the activities of chloroplast desaturases, provided important information about the desaturation of chloroplast lipids. The same mutants may be useful tools for cloning the desaturase genes. For example, if the defective gene product is missing or altered in the mutant, it may be possible to identify the wild-type polypeptide by comparing the polypeptide patterns of the mutant and wild type after separating them by 2-dimensional SDS-polyacrylamide gel electrophoresis. Another feasible approach in Arabidopsis is chromosome walking. Once the mutants are mapped, every desaturase gene within 250 kb of the restriction fragment length polymorphism (RFLP) marker on the Arabidopsis map can, in principle, be isolated. Alternatively, the fad mutants may be used for gene isolation by transposon tagging.

Chloroplast morphogenesis

Characteristic changes in chloroplast size and ultrastructure associated with changes in membrane fatty acid composition raise the possibility that high levels of acyl group unsaturation, characteristic of chloroplast membranes, might be an important factor involved in chloroplast structural differentiation and/or division. The polypeptide pattern of the act1 mutant resolved by 2-dimensional SDS-polyacrylamide gel electrophoresis revealed several quantitative differences with respect to the wild type. These differences might reflect an altered capacity of the lipid bilayer for incorporation of proteins. By comparing the protein pattern of another independent act1 mutant to the

wild type it might be possible to identify (a) specific protein(s) that show the same variation in both mutants and perhaps affect the chloroplast ultrastructure. The isolation of a gene that codes for this protein and its overexpression in transgenic plants may provide valuable information about the mechanisms involved in the assembly of chloroplast membranes, and/or the formation of appressed regions within chloroplasts.

Chilling sensitivity

Chilling sensitivity in plants has been correlated with a reversible phase separation of the lipids within cellular membranes, which effectively prevents the membranes and their constituent enzymes from functioning normally. Although plant membranes contain highly unsaturated lipids, which would not be expected to undergo phase transitions at physiological temperatures, the presence of as little as 1% of a disaturated phospholipid, such as PG, induces a thermal phase transition (Raison and Wright, 1983). In agreement with this, Murata et al. (1982) found a correlation between the high proportion of disaturated molecular species of PG and chilling sensitivity. Further surveys, involving a large number of species, generally support this correlation (Roughan et al., 1985, Bishop et al., 1986), although there were a few exceptions. Since the sn-2 position of PG is always esterified with 16:0 and trans-16:1 (which was also considered as a saturated fatty acid, by virtue of the position and isomerism of the double bond) acyl groups, the formation of disaturated PG depends on the specificity of the chloroplast G3P acyltransferase. Therefore, the ultimate test for the validity of Murata's hypothesis would be the

introduction of the G3P acyltransferase gene from a chilling sensitive species into a chilling resistant plant, to make it chilling sensitive, and subsequent analysis of PG acyl group composition of the transgenic plant. This approach depends upon first being able to eliminate the endogenous gene by the isolation of a null mutation. However, the actl mutant of Arabidopsis (a chilling resistant plant) described in this dissertation, with less than 4% of residual G3P activity, is also a good candidate for such an experiment.

Thermal tolerance

A detailed physiological characterization of actl and fadB mutants has shown that both mutants exhibit enhanced thermal tolerance relative to wild type plants. A similar result obtained for another mutant of Arabidopsis with altered leaf lipid composition (McCourt et al., 1987) suggests that membrane polar lipids might play a role in acclimation of plants to high temperatures. Thermal tolerance of plants is thought to be determined by the stability of their photosynthetic membranes. Gounaris et al. (1984) have shown that broad bean (Vicia faba) is adversely affected by temperatures above 35^{o}C. The ultrastructural changes involve: (1) a decrease in the amount of stacked membrane regions, (2) a decrease in size of EFs particles on freeze-fractured membranes, which was interpreted to indicate a dissociation of light harvesting units from the PSII core complex, and (3) the formation of phase separated aggregates of non-bilayer forming lipids. We have no information regarding the structural changes associated with heat-induced damage of chloroplast membranes in the lipid mutants of Arabidopsis. Freeze-fracture electron microscopic studies on actl and

fadB mutants might, therefore, be useful for obtaining additional evidence concerning the function of lipids, or lipid-protein association, in membrane stability at elevated temperatures.

An alternate approach to addressing the question of increased thermal stability of chloroplast membranes from act1 and fadB mutants would be to measure the physical properties of membrane lipids. In this context, it would be especially interesting to determine the threshold temperature for phase separation of membrane lipids using fluorescence polarization from the probe trans-parinaric acid, since differences in the transition temperature of polar lipids seem to correlate with different physiological performances at high temperatures (Raison et al., 1982).

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