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dissertation entitled

The Isolation and Characterization of Mutants of *Arabidopsis thaliana* (L.) Deficient in Fatty Acid Desaturation

presented by

Peter John McCourt

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Genetics and Botany

Major professor

Date September 29, 1986

THE ISOLATION AND CHARACTERIZATION OF MUTANTS OF *ARABIDOPSIS THALIANA* (L.) DEFICIENT IN FATTY ACID DESATURATION

BY

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A DISSERTATION

Submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

Genetics Program and Department of Botany

1986

ABSTRACT

THE ISOLATION AND CHARACTERIZATION OF MUTANTS OF ARABIDOPSIS THALIANA (L.) DEFICIENT IN FATTY ACID DESATURATION

BY

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In plant membranes each lipid class has a characteristic fatty acyl composition defined by chain length and degree, position, and stereochemistry of unsaturation. However, with few exceptions the functional significance of lipid acyl unsaturation remains uncertain. The major goal of this study was to isolate and characterize a series of mutants of the crucifer Arabidopsis thaliana (L.) Heynh. with specific alterations in leaf fatty acid composition. The mutants were isolated without selection by direct analysis of the leaf fatty acid composition of individual M2 plants using gas chromatography.

From approximately 2000 plants examined by this procedure, seven lines were isolated which showed stably inherited changes in fatty acid composition and of these, two were further analyzed at both the biochemical and physiological level.

The first mutant characterized was completely lacking the acyl group trans hexadecenoic acid due to a mutation at a single nuclear locus designated fadA. This fatty acid which is only found in chloroplast membranes in most higher plants was thought to play an

important role in various aspects of photosynthesis. However, detailed study of photosynthetic function in the fadA mutant suggests the role of trans hexadecenoic acid in chloroplast function is, at best, subtle.

The second mutant studied was characterized as deficient in both hexadecatrienoic (16:3) and linolenic (18:3) fatty acids. Both alterations were due to a single nuclear mutation at a locus designated fadD. This mutation affects the fatty acid composition both inside and outside the chloroplast and appears to be temperature sensitive. The multiple changes seen in the fadD mutant can be explained if the fadD gene product is found in different cellular compartments or the product of the reaction it governs is transported between compartments.

The fadD mutation appears to have little or no functional effect on photosynthesis but does alter chloroplast size, number and ultrastructure.

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LITERATURE REVIEW

Introduction

The most satisfactory explanation of how biological membranes can have enough stability to be an impermeabilty barrier between two compartments yet maintain the flexibility to selectively transport metabolites and macromolecules between such compartments has been the fluid-mosaic model of membrane structure proposed by Singer and Nicolson (1972). This model suggested that the lipids in biological membranes are organized as bilayers in which proteins needed for transporting ions, metabolites and macromolecules are embedded. While such a view implies heterogeneity at the protein level, it suggests that any lipid which can form a bilayer structure forming a barrier to polar or large nonpolar molecules under physiological conditions would be adequate for membrane function. Thus, for example, a phosphatidylcholine (PC) bilayer is not distinguished from a phosphatidylserine (PS) bilayer. However a survey of the lipid membrane composition of various cell types or even organelles within a particular cell type suggests that the situation is more complex. In

plants, many of the lipids found in the thylakoid and envelope membrane are not found elsewhere in the cell and vice versa (Harwood, 1980). Furthermore, each lipid class has a characteristic fatty acyl composition which is defined by chain length and degree, position and sterochemistry of unsaturation. However with a few exceptions the functional significance of these complex lipids and fatty acids remains uncertain in both animals (Stubbs and Smith, 1984) and plants (Quinn and Williams, 1983).

The major goal of this work was to use a genetic approach to understand the regulation and functional significance of lipid diversity. In principle, it should be possible to remove or to alter each individual class of lipid and fatty acid by mutation and then assay the physiological effect of this change on the organism. The use of such an approach would not only enable one to determine the role of specific lipids in membrane fuction but also, such mutants would be very useful in elucidating how these molecules are made. Because the enzymes that synthesize lipids and desaturate fatty acids are usually membrane-bound it has been difficult to characterize these proteins by conventional biochemical methods.

This study describes the isolation and preliminary characterization of a number of mutants in Arabidopsis thaliana (L.) Heyhn. with altered leaf lipid metabolism. These mutants which are the first reported in higher plants have been used to formulate models of lipid biosynthesis and to evaluate the physiological role of particular lipids. Because the only changes in lipid metabolism in these particular mutants are in the leaf tissue, the review of glycerolipid biosynthesis has been limited to photosynthetic tissue.

Lipid Structure

The major fatty acids of leaves are even numbered long chain hydrocarbons which have been classified into two distinct groups. The saturated fatty acids contain no double bonds in their carbon chains. Palmitate (hexadecanate) and stearate (octadecanoate) are the most prevalent members of this group making up roughly 10% and 2%, repectively of the total fatty acids of the leaf (Harwood, 1980). The other group, which are called unsaturated fatty acids, contain one or more double bonds in their acyl chains and are usually the most abundant fatty acids of the leaf. For example, linolenate (cis 9,12,15 octadeca-trienoate) which has double bonds between the 9^{th} and 10^{th}, 12^{th} and 13^{th}, and 15^{th} and 16^{th} carbons of the fatty acid from the carboxyl end, can represent up to 80% of the total leaf fatty acid in some plant species (Harwood, 1980). For the purpose of this study a shorthand nomenclature will be used to symbolize the various fatty acids (Table 1). For example, palmitate which is a 16 carbon saturated fatty acid is designated 16:0 whereas oleate, which contains 18 carbons with one double bond in the 9^{th} position, is designated 18:1.

Normally fatty acids are attached to a glycerol molecule in two of the three hydroxyl positions. The third hydroxyl group is usually esterified with a sugar residue (glycolipid) or a phosphatidic acid derivative (phospholipid). These sugar and phosphate moieties, termed the head group, represent the polar part of the lipid and face out into the aqueous solution when in a bilayer.

The presence of three esterified hydoxyl groups has allowed the naming of the two fatty acid esters by optical isomerism. In all cases studied in photosynthetic tissue the fatty acids are esterified to the

Table 1

Fatty acid nomenclature

	Symbol	Systematic Name	Common Name
Saturated			
	16:0	n - Hexadecanoic	Palmitic
	18:0	n - Octadecanoic	Stearic
Unsaturated			
trans-3	16:1	trans - Hexadecanoic	
cis 7	16:1	n - Hexadecamonoenoic	Palmitoleic
cis 7,10	16:2	n - Hexadecadienoic	
cis 7,10,13	16:3	n - Hexadecatrienoic	
cis 9	18:1	n - Octadecamonoenoic	Oleic
cis 9,12	18:2	n - Octadecadienoic	Linoleic
cis 9,12,15	18:3	n - Octadecatrienoic	Linolenic

sn-1 and sn-2 positions of the glycerol backbone and the head group is attached to the 3rd position. For example, monogalactosyldiacylglycerol (MGD) contains fatty acids in position sn-1 and sn-2 and a galactose moiety esterified to position sn-3.

A survey of the leaf lipid and fatty acid composition from one plant species to another shows a very similar distribution (Harwood, 1980). The most striking feature is the amount of glycolipid present in leaves. These lipids, which can be classified into three categories based on head group, are predominantly found in the chloroplast. MGD and digalactosyldiacylglycerol (DGD), which have one and two galactose residues respectively, represent roughly 40% and 25% of the total lipid of the cell and sulfoquinovosyldiacylglcerol (SL), which has 6-sulfo-deoxy-D-glucose as a head group, represents 5 to 10% of the total cellular lipid (Harwood, 1980; Barber and Gounaris, 1986). The fatty acid composition of these three glycolipids varies substantually. For instance MGD and DGD are almost completely composed of 18:3 or 16:3 acyl chains while a high proportion of the acyl chains of SL are saturated (Quinn and Williams, 1983). The high degree of unsaturation on MGD gives this lipid a cone shaped structure which prevents the formation of bilayers from pure preparations (Murphy, 1982). However, when mixed with proteins and other lipids normally found in the chloroplast, MGD spontaneously integrates into the bilayer (Quinn and Williams, 1983). This has led to the suggestion that this lipid may play a role in regulating insertion and packing density of proteins in the membrane (Murphy, 1982; Gounaris and Barber, 1983)

SL, which is a negatively charged lipid, has been proposed to associate closely with photosystem II and coupling factor, suggesting

it might be a boundary lipid (Barber and Gounaris, 1986). A boundary lipid is usually defined as a specific lipid which associates with a protein causing a stimulation of catalytic activity. Although hypothesized in plants, direct proof of boundary lipids has not been obtained.

The phospholipids in leaf tissue fall into five major groups, phosphatidylglycerol (PG), phosphatidylethanolamine (PE),phosphatidylserine (PS), phosphatidylcholine (PC), and phosphatidylinositol (PI). PG and PC which form 10 to 15% of the total lipid and PI which is roughly 3%, are found throughout the cell. However, PE and PS are not found in the chloroplast and are primarily located in the mitochondrion (Harwood, 1980). The PC and PI that are found in the chloroplast are located solely on the outer chloroplast membrane (Dorne et al, 1985). The PG is evenly distributed in both inner and outer envelopes and the thylakoids. Chloroplastic PG is of additional interest due to the occurance of trans-3-hexadecenoic acid (trans-$C_{16:1}$) at the second position of PG (Dubacq and Tremolieres, 1983). This fatty acid is unusual due to the position and conformation of the unsaturation (trans versus cis). In studies with phospholipases specific for the sn-2 position, it has been possible to show that trans-$C_{16:1}$ occurs only on the side of the thylakoid bilayer which faces the stroma (Unitt and Harwood, 1984). This specific location and the comigration of trans-$C_{16:1}$ with the light harvesting complex (LHCP) in SDS polyacrylamide gels under conditions which maintain the integrity of chlorophyll-protein complexes have led to the suggestion that this fatty acid is essential for efficient membrane insertion and

operation of this chlorophyll-protein complex in light capture (Dubacq and Tremolieres, 1983)

Lipid Biosynthesis

The observation that isolated chloroplasts readily synthesize 16:0, 18:0 and 18:1 from acetate in the light (Slack et al, 1977; Roughan and Slack, 1982) and that acyl carrier protein (ACP), a specific marker for fatty acid synthesis, is solely located in the chloroplast (Ohlrogge et al, 1979) has led to the conclusion that the chloroplast is the only site of de novo fatty acid biosynthesis in higher plant leaves. Therefore, it follows that all fatty acids found outside the chloroplastic compartment must be transported there from the chloroplast. Although the exact mechanisms that regulate the synthesis and distribution of fatty acids in plants are not clear, a number of studies have resulted in the formation of a rough outline of the overall process.

Stumpf and colleagues partially purified the fatty acid synthetase (FAS) from Spinacia oleracea (spinach) and have shown this enzyme complex is comprised of six loosely associated polypeptides which can catalyse the condensation of malonyl CoA to give 16:0-ACP and 18:0-ACP thioesters (Shimakata and Stumpf, 1982). The molecular organization of the FAS in plants appears to be quite similar to the Eschericha coli FAS, and E. coli ACP can be substituted for the spinach ACP in reconstitution studies even though the two forms show poor immunological crossreactivity (Ohlrogge et al, 1979; Simoni et al, 1967). Interestingly, the converse experiment in which E. coli ACP is replaced by spinach ACP in a bacterial synthetase reconstitution system

yields C_{12} through C_{18} fatty acids instead of the normal 16:0 and vaccinic (cis 11,18:1) bacterial fatty acids (Simoni et al, 1967). This suggests ACP may play an important role in chain termination reactions of the FAS.

Detailed studies of fatty acid synthesis in spinach have determined the existence of two B-keto-acyl-ACP synthases (Shimakata and Stumpf, 1982). One of these participates in the formation of 16:0 ACP from acetyl-ACP and the other, which can only use 14:0-ACP or 16:0-ACP as a substrate, produces only 18:0-ACP when added to a reconstitution system including both ACP synthases (Shimakata and Stumpf, 1982). The implication of such results is that regulation of these two B-keto-acyl-ACP synthases could control the ratio of 16 to 18 carbon fatty acids within the chloroplast.

Although roughly 80% of the 16:0-ACP synthesized in the chloroplast is converted to 18:0-ACP by the second B-keto-acyl-ACP synthase almost all the 18:0-ACP is metabolized to 18:1 ACP via a highly active NADPH dependent stromal desaturase (Nagai and Block, 1968). Hence, the major labeled products of intact chloroplasts supplied with radioactive [^{14}C]acetate are 16:0-ACP and 18:1-ACP.

16:3 and 18:3 Type Plants

The further metabolic fates of 16:0-ACP and 18:1-ACP made within the chloroplast appear to be dependent on the plant species. Following a detailed characterization of the leaf lipid composition of a variety of species, a pattern has emerged which has allowed the classification of plants into two groups. Those containing hexatrienoic acid (16:3-type plants) and those that do not (Roughan and Slack, 1984). Apiaceae,

Chenopodiaceae, Solanaceae and the Brassicaceae, which all belong to the former group, contain 50 to 60% 18:3 and 5 to 40% 16:3. The latter group, which include the families Fabaceae, Asteraceae, and Poaceae, have no 16:3 and very high levels of 18:3 (80% or more) hence they are given the name 18:3 type plants (Heinz and Roughan, 1983). The reason for these differences in acyl chain composition is directly related to the way these two groups of plants partition their newly synthesized fatty acids within the cell (Figures 1 and 2). In short term labelling studies of intact leaves with [^{14}C]acetate, 18:3 type plants (Figure 1) distribute the majority of the labeled fatty acids into phosphatidyl choline (PC) and a minor proportion into phosphatidyl glycerol (PG). On the other hand 16:3 type plants (Figure 2) produce approximately equal amounts of PC and the chloroplast-specific lipid monogalactosyldiacylglycerol (MGD) and a minor amount of PG under the same labelling conditions (Roughan and Slack, 1982). In leaf tissue, the endoplasmic reticulum is considered to be the major site of plant phospholipid metabolism and PC synthesis, in particular (Moore, 1984; Mudd, 1980). Hence, fatty acids made in the chloroplast appear to be exported to the ER where they become esterified to PC. In vitro labelling studies provide some insight as to how this transfer might occur. Incubation of isolated chloroplasts with CoA and ATP cause the accumulation of 18:1-CoA esters in the outer envelope (Roughan et al. 1976; Roughan et al. 1979). This and the recent report of the localization of an acyl CoA synthetase to the outer envelope (Andrews and Keegstra, 1983) have led to the suggestion that newly synthesized fatty acids are liberated from the ACP and diffuse into the lipid bilayer. Upon movement to the outer envelope the acyl CoA synthetase

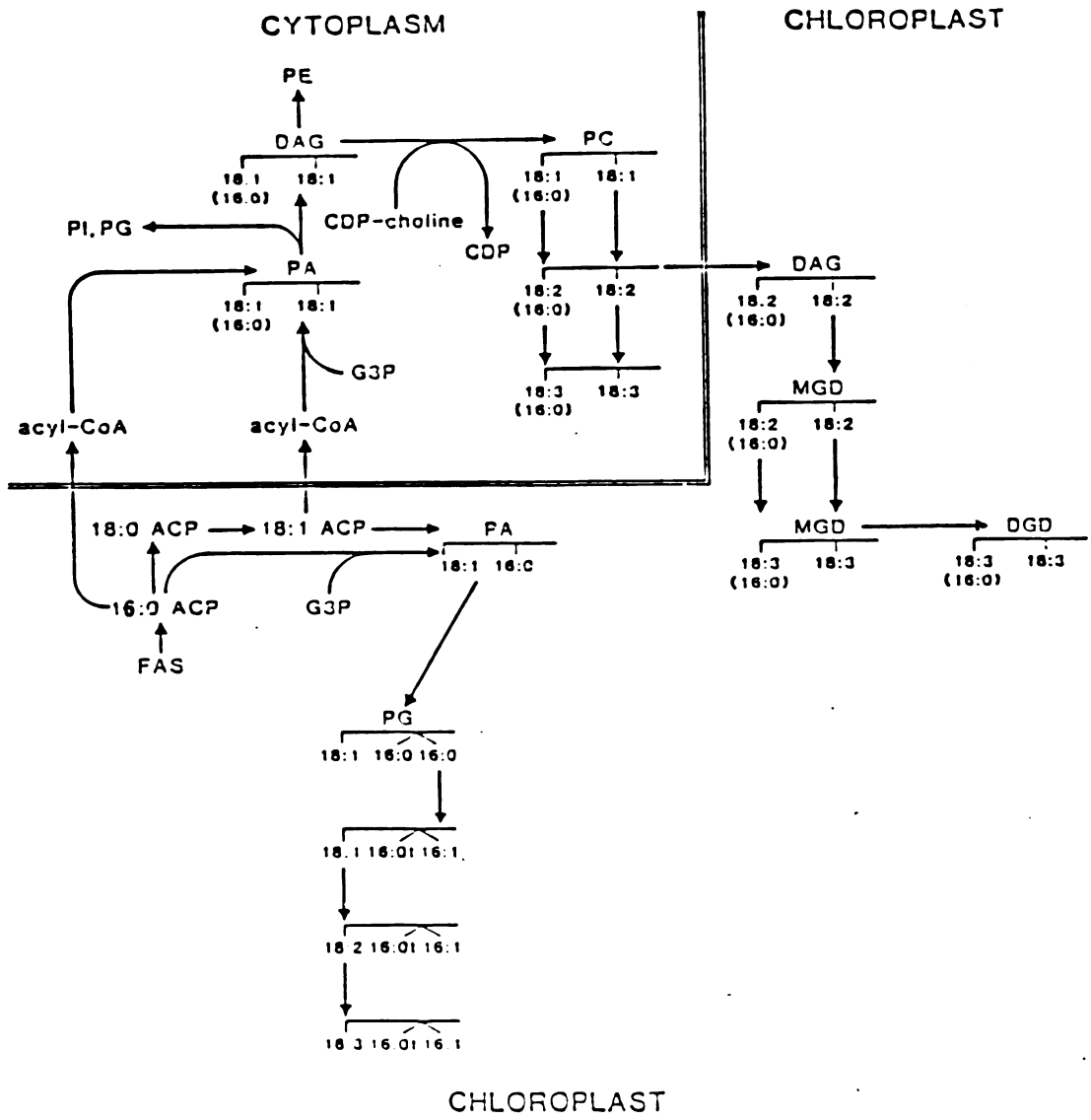

Figure 1. Lipid biosynthesis in 18:3-type plants

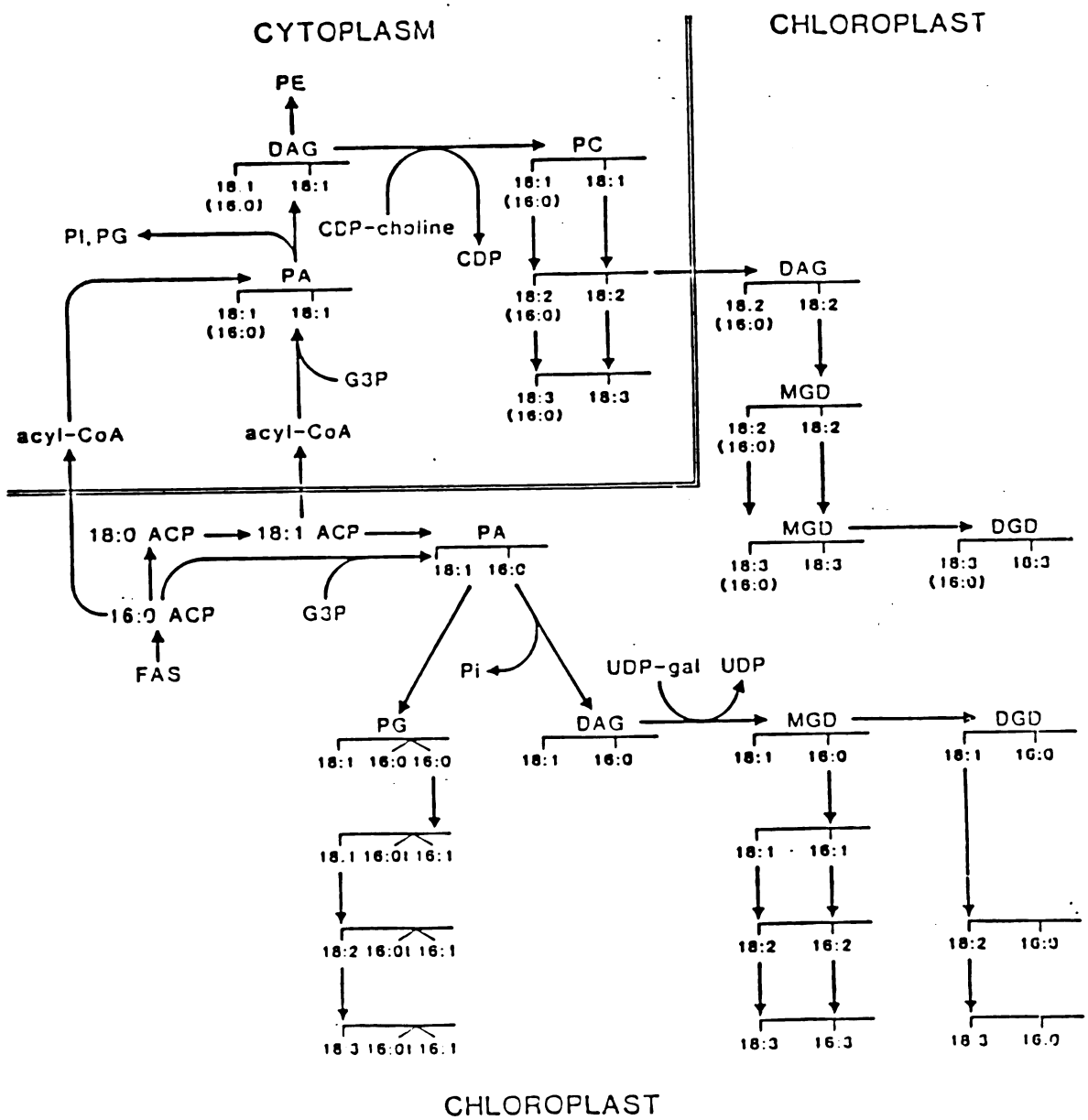

Figure 2. Lipid biosynthesis in 16:3-type plants

esterifies the fatty acid to CoA making it soluble for cytoplasmic transport to the ER. Although the mechanism of transfer of fatty acids across the envelope is unclear, the existence of a carnitine acyl transferase in the chloroplast suggests the mechanism may be similar to that characterized in animal mitochondria (McLaren et al, 1986). Until recently, the specificities of the enzymes which attach the exported 16:0 CoA and 18:1 CoA to glycerol-3-phosphate (G-3-P) in the cytoplasm could only be inferred from positional analysis of fatty acids attached to microsomal PC. However, purified microsomal fractions from Pisum sativum (pea) leaves show two acyl CoA G-3-P acyltransferases (Frentzen et al, 1984). One acylates either 16 or 18 carbons to the sn-1 position of G-3-P to form lysophosphatidic acid (LPA). The other acyl transferase, only esterifies 18 carbon fatty acids to the sn-2 position (Frentzen et al, 1984) giving phosphatidic acid (PA). Hence, PA formed via these cytoplasmic acyltransferases contains either 18 carbon fatty acids on the both sn-1 and sn-2 positions or 16:0 on the sn-1 position and an 18 carbon fatty acid on the sn-2 position (Figure 1). Both molecular species of PA can be used in the biosynthesis of PC and phosphatidylethanolamine (PE) but only sn-1 16:0 sn-2 18:1 PA is used for phosphatidylinositol (PI) synthesis (Roughan and SLack, 1984). These results indicate that the fatty acid composition of a particular lipid can play an important role in channelling lipids into various pathways.

The two molecular species of PC are also further channelled according to their fatty acid composition. In vivo pulse chase experiments using [^{14}C]acetate have demonstrated that radioactivity can be chased into chloroplastic MGD from PC in 18:3 plants (Roughan,

1975). Similar studies using double labelling in which fatty acids are labelled with ^{14}C and the glycerolipid backbone with ^{3}H have demonstrated that both labels are chased from PC into MGD at equal rates, suggesting that a complete lipid molecule is transferred in this process (Slack et al, 1977). However, 18:3 plants contain no 16:0 MGD indicating that (sn-1 16:0, sn-2 18:1) PC is excluded by this transfer mechanism.

The discovery of phospholipid exchange proteins (PLEP) in plants has suggested a possible mechanism of PC transport from the ER to the chloroplast (Ohnishi and Yamada, 1982). These proteins, are able to catalyse the exchange of phospholipids between microsomes and mitochondia and microsomes and chloroplasts (Kader et al. 1984) The evidence for PLEPs being involved in the transport of PC is that chloroplastic PC is only found in the outer envelope. By contrast, the apparent absence of phosphatidylcholine phosphatase activity and the inability of PLEPs to generate net membrane growth (since they can only exchange lipids), argues against such a mechanism. Results showing a rapid equilibrium between PC and diacylglycerol (DAG) in the ER of expanding maize leaves could mean that PC is converted to DAG in this compartment and that this species is transported to the chloroplast (Roughan and Slack, 1984). DAG would then be further metabolized to MGD via a well characterized chloroplast specific galactosyl transferase which uses cytoplasmic UDP-galactose as a second substrate (Block et al, 1983; Heemskerk et al, 1985). In principle, MGD can then be converted to digalactosyldiacylglycerol (DGD) by the addition of another galactose residue. However, this reaction has been difficult

to characterize in isolated chloroplasts and the exact nature of the galactose donor is not known.

Acyl analysis from [^{14}C]acetate in vivo labeling experiments has suggested that PC is the major site of 18:1 desaturation rather than oleoyl-CoA (Slack et al 1977). This observation was verified directly in purified microsomal fractions by showing [^{14}C]oleoyl-CoA was first esterified to lyso PC before it was desaturated (Murphy et al, 1983). Therefore in 18:3 plants it appears that 18:1 and 18:2 desaturations occur when the fatty acid is attached to the lipid.

In summary, 18:3 plants appear to export the majority of their newly synthesized fatty acids (16:0, 18:1) from the chloroplast to the ER where they are attached to phospholipids. The acyl transferases which carry out these esterifications attach 16:0 only to position 1 and 18 carbon fatty acids to either position. Cytoplasmic 16:0 is not desaturated futher via this pathway while 18:1 can be converted to 18:2 and 18:3. Microsomal PC, which appears to be a sink for fatty acid acylation is also the major site in 18:3 plants for 18:1 to 18:2 desaturation. Upon desaturation, some form of lipid (possibly DAG with 18:2, 18:2 acyl side chains) is transported back to the chloroplast where it is converted to MGD and further desaturated to 18:3. MGD can be further modified to DGD by addition of another galactose residue to the head group. Because a large proportion of this pathway is located in the cytoplasm, it has been termed the eukaryotic pathway of lipid synthesis.

In contrast to 18:3 type plants, 16:3 plants appear to have two ways of synthesizing galactolipids (Figure 2). The second pathway of synthesis is termed the prokaryotic pathway since it is limited to the

chloroplast (Roughan and Slack, 1982). After formation of 16:0 ACP, 18:0 ACP, and 18:1 ACP by the FAS, 16:3 plants can esterify the acyl chains directly to G-3-P within the chloroplast. Although these reactions also occur in 18:3 plants, the activities of the acyl transferases are relatively low compared to those of 16:3 species (Heinz and Roughan, 1983). The relatively low amount of PA that is made through this pathway in 18:3 plants is believed to be used only for chloroplastic PG synthesis (Andrews and Mudd, 1985). The acyl transferases that function in the prokaryotic pathway are quite different from the eukaryotic acyl transferases in terms of specificity. The prokaryotic enzyme is a stromal soluble protein which prefers 18:1-ACP to 16:0-ACP as a substrate (Gardiner et al, 1984). The product of this reaction, LPA, is soluble in the membrane and is converted to PA by an sn-2 specific membrane bound acyltransferase (Frentzen et al, 1983). Hence PA and subsequent lipids made from it via the prokaryotic pathway can be easily distinguished from lipids of eukaryotic origin by the position of the 16 carbon fatty acids. For example, 16:0 on the sn-1 position and an 18 carbon fatty acid on the sn-2 position of PA means it was synthesized by the eukaryotic pathway, while a lipid with the inverse arrangement of fatty acids would be synthesized via the prokaryotic pathway.

The fact that the two prokaryotic acyltransferases only use fatty acids attached to ACP implies that removal of the ACP could cause the fatty acid to enter the eukaryotic pathway. The localization of an acyl-CoA thioesterase, which converts acyl-ACP to acyl-CoA, in the inner envelope of the chloroplast may play a role in the partioning of fatty acids between the two pathways (Andrews and Keegstra, 1983; Block

et al, 1983). Although prokaryotic PA is also used to make chloroplastidic PG in 16:3 plants (as in 18:3 plants) the majority is hydrolyzed to form DAG via a phosphatidic acid phosphatase (Gardiner and Roughan, 1983). This DAG which can readily equilibrate with eukaryotic DAG (Figure 2) in the chloroplast membrane can be further metabolized to MGD and DGD by a similar mechanism as described for the eukaryotic pathway (Roughan and Slack, 1982). By determining the sn position of the 16 carbon fatty acids MGD and DGD, estimates suggest 50 to 60% of these lipids are made through the prokaryotic pathway in 16:3 type plants (Roughan and Slack, 1984). Furthermore, the 16:0 which is attached to position sn-2 of MGD is sequentially desaturated to 16:3. Although the 16:0 to cis 16:1 desaturation must be MGD specific, since it only occurs on that lipid, the subsequent 16:2 and 16:3 desaturations could be carried out by either 16 carbon specific desaturases or the desaturases which perform 18:1 and 18:2 desaturations.

As stated earlier, chloroplastic PG in both 18:3 and 16:3 plants is made through the prokaryotic pathway (Figures 1 and 2) and a number of studies have reported PG synthesis in isolated chloroplasts (Sparace and Mudd, 1982; Andrews and Mudd, 1985). Recently it has been shown that prokaryotic PA made in the envelope can be converted to CDP diacylglycerol using CTP and that this in turn reacts with G-3-P to form PG (Andrews and Mudd, 1985; Roughan, 1985).

The synthesis of SL, which is not shown in Figures 1 or 2, has attracted considerable attention, but the biosynthetic pathway remains uncertain (Barber and Gounaris, 1986). It has not yet been established whether or not the chloroplast is autonomous for SL synthesis, and the

results of positional analysis of fatty acids esterified to SL varies from species to species. Therefore, a general statement about the biosynthetic pathway of this lipid cannot be made (Harwood, 1980).

CHAPTER 2

MATERIALS AND METHODS

Growth Conditions

Arabidopsis thaliana (L.) Heynh., race Columbia, was used in this study. The seed source for the mutant search was an M2 population of ethyl methane sulfonate treated seed (Somerville and Ogren, 1979). A list of the plant lines used in this study which were either isolated from an M2 population or constructed from crosses is given in Table 2. M2D represents a particular batch of seed that was mutagenized.

Before physiological studies were performed, the mutants were backcrossed to wild type and mutant lines were reisolated in the F2 progeny. This procedure was repeated and the subsequent reisolated mutant lines were advanced to the F4 generation. All plants were grown under continuous fluorescent illumination (150 - 200 uEinsteins m^{-2} s^{-1}) at 19 C and 60% relative humidity in a mixture composed of equal parts of vermiculite, perlite and sphagnum irrigated with mineral nutrients (Somerville and Ogren, 1979)

Table 2

List of plant lines

Line	Genotype	Source / Derivation
JB1	fadD	M2D seed
JB25	pap	M2D seed
JB27	fadA	M2D seed
JB60	fadA	M2D seed
JB101	fadD gl-1	JB1 x CS2
JB601	fadA gl-1	JB60 x CS2
LIP1	pap fadD	JB1 x JB25
LIP2	fadA fadD	JB60 x JB1
MK1	an py ex gl-1 cer-2 Ms/ms	M. Koorneef

For measurements of growth rates, seeds were germinated at 19 C on 100 x 25 mm petri plates containing 25 ml of mineral nutrients solidified with 0.7% (w/v) agar at a density of 16 evenly spaced plants per dish. The light intensity was 120 uE m^{-2} s^{-1}. After seven days the temperature was adjusted as noted in the results. Because of the restricted air flow in the petri plates actual leaf temperature may have been slightly higher than the air temperature in the chamber. At three day intervals, for the next 21 days, plants were removed and the fresh weights of the aerial portion were determined. The relative growth rate was determined as the slope of the log of the fwt (mg) plotted against time since plating (days).

For tissue culture studies, callus was induced from leaves by placing sterile tissue on PG1 media of Negrutui et al (1975). Plates were incubated in the light at 23 C for about two weeks until green callus was visible on the leaves. This callus was removed and placed on fresh PG1 media and after one week the fatty acid composition was determined.

Gas Exchange

Measurements of CO_2 fixation and dark respiration were obtained on intact plants of Arabidopsis by gently removing soil from the roots and placing the plants in a glass cuvette which had two ports to allow gas flow into and out of the system. Dry gas of a desired composition (350 ul l^{-1} CO_2, 21% O_2, balance N_2) was passed through the reference cuvette of a Analytical Developement CO_2 Infrared Gas Analyser (IRGA) Type 225. After leaving this cuvette the gas was humidified to approximately 70% RH by bubbling it through water at 20 C. The gas then

passed through the plant chamber which was immersed in a circulating water bath so that the temperature at which gas exchange occurs could be controlled. The gas stream was then dehumidified before entry into the analytical cuvette of the IRGA by passage through a cold finger at 4 C. The error due to transpired water is negligible if the analyser is calibrated under the same conditions of humidity and flow rate as used during photosynthesis measurements. Flow rates for this open system were determined by a Matheson mass flow meter. The rates were usually between 50 - 150 ml min^{-1} depending on the size of the plant. The light intensity and temperature is given with each experiment.

Standardization of CO_2 fixation rates were determined in three ways: leaf area which was measured on a portable area meter (LI-COR 3000, Lamba Instruments Corp), by fresh weight, or by chlorophyll concentration.

Preparation Of Chloroplast Membranes

For the following procedures all operations were carried out at 4 C. Usually one pot of three week old plants (4-6 grams fwt) was homogenized in 100 ml of grinding buffer by two 5 second bursts at high speed in a Osterizer galaxie blender. The homogenate was passed through four layers of cheese cloth and centrifuged at 3000 g for 5 min. The pellet was resuspended in a small volume of washing buffer using a paint brush and about 30 ml of the same buffer was then added to ensure good chloroplast breakage. The suspension was centrifuged at 5000 g for 5 min. The supernatant was then discarded and the pellet was resuspended in about 1 - 2 ml of resuspension buffer. The compositions of the various buffer solutions are given in Table 3.

Table 3

Concentrations of solutions used to isolate thylakoid membranes

Final Concentration	Stock Conc.	Stock Volume/100 ml
Grinding Buffer		
50 mM Tricine-KOH (pH 8.4)	1.0 M	5.0 ml
10 mM NaCl	1.0 M	1.0 ml
400 mM Sorbitol	2.0 M	20.0 ml
10 mM EDTA (pH 8.0)	0.5 M	2.0 ml
Washing Buffer		
10 mM Hepes-KOH (pH 7.8)	1.0 M	1.0 ml
10 mM NaCl	1.0 M	1.0 ml
5 mM EDTA (pH 8.0)	0.5 M	1.0 ml
Resuspension Buffer		
20 mM Hepes-KOH (pH 7.8)	1.0 M	2.0 ml
10 mM NaCl	1.0 M	1.0 ml
300 mM Sorbitol	2.0 M	15.0 ml
5 mM $MgCl_2$	1.0 M	0.5 ml

For chlorophyll determinations, 100 ul of sample was removed and added to 5 ml of 80% (v/v) acetone. This solution was shaken and centrifuged for 1 min at 1000 g in a clinical centrifuge. The chlorophyll concentration was determined by measuring the absorbance of the supernatant at 645 nm and 663 nm and transformed according to the formula $(A_{645} * 20.2) + (A_{663} * 8.02) =$ mg Chl/ml (McKinney, 1941).

Electron Transport

Electron transport was measured in a Rank O_2 electrode by adding an aliquot of thylakoid membranes so that the final concentration was 20 ug chl ml^{-1} to one ml of resuspension buffer. Whole chain rates were measured by the reduction of methyl viologen (MeV). Because of MeV low redox potential MeV accepts electrons exclusively from photosystem I (PSI) and passes them to O_2 to make a superoxide radical which spontaneously dismutates to H_2O_2 and O_2. In the presence of NaN_3, a catalase inhibitor, the transport of electrons from water to PSI is measured as O_2 uptake (Mehler reaction). To uncouple electron transport from ATP synthesis NH_4Cl and Gramicidin-D were added to the reaction.

For photosystem II (PSII) measurements, 2,5-dimethyl-p-benzoquinone (DMQ) was added as an artifical electron acceptor and to eliminate electron transport reactions associated with PSI the plastoquinone antagonist 2,5-dibromo-3-methyl-6-isopropyl -p-benzoquinone (DBMIB) was added.

PSI electron transport rates were measured by inhibiting PSII activity with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). Electrons were supplied directly to PSI by reducing dichloroindophenyl (DCIP) to DCIPH with ascorbate (Asc). MeV was used as an electron acceptor from

PSI as described above. Because Asc can cause superoxide formation in the prescence of MeV, superoxide dimutase (SOD) was added. The solutions for the partial and whole chain assays are given in Table 4.

Room Temperature Fluorescence Measurements

Thylakoids were diluted in resuspension buffer to a final concentration of 5 ug Chl ml^{-1}. Illumination was by a Unitron microscope illuminator powered by a model C5-6, 6A stable output supply (Power One, Cornello CA). Chl fluorescence was measured with a photodiode positioned at a 90^{o} angle to the actinic light source. This source was filtered through a broadband blue optical filter (Corning 4-96). The onset of illumination was controlled by an electronic shutter (Vincent Associates, Rochester NY). The fluorescence was measured through a red cut off filter (Corning 2-64) to protect against scattered blue light from the actinic light souce. The voltage output of the photodiode was stored on a Nicollet model 206 digital recording oscilloscope.

Low Temperature Fluorescence

Isolated thylakoids were divided into two aliquots of resuspension buffer, one of which was supplemented with 5mM $MgCl_2$ and allowed to dark adapt for 30 min. The samples were then diluted to a concentration of 10 ug Chl ml^{-1} in a 60% glycerol (v/v) solution of the same buffers. Two hundred ul of this suspension was added to heat-sealed 11 inch pasteur pipets (5mm id), and kept frozen in liquid nitrogen in the dark until measurements were taken.

Table 4

Concentration of solutions used for electron transport assays

Final Concentration	Stock Solution	Stock Solution/100 ml
Whole Chain		
0.1 mM NaN_3	25 mM	4.0 ul
0.1 mM MeV	25 mM	4.0 ul
1.0 mM NH_4Cl	150 mM	6.7 ul
1.0 uM Gramiciden	1 mM	1.0 ul
PS II		
1.0 uM DBMIB	20 mM	5.0 ul
0.25 mM DMQ	100 mM (Methanol)	2.5 ul
PS I		
2.5 mM Asc	1 M	2.5 ul
1.0 ul DCMU	20 mM (Ethanol)	5.0 ul
0.5 DCIP	100 mM	5.0 ul
0.07 mg/ml SOD	7 mg/ml	10.0 ul
0.1 NaN_3	25 mM	4.0 ul
0.1 mM MeV	25 mM	4.0 ul

For whole-leaf measurements a wet leaf was placed on a metal spatula and held in place by water proof tape. The spatula was immersed in liquid N_2 to freeze the sample. For measurements, the spatula was rotated while in the fluorometer to an angle approximately 45^o to the excitation light. All fluorescence emission spectra were recorded using an SLM 4048 scanning spectrofluorometer (SLM Instruments, Urbana IL) operating in the ratiometric acquisition mode. Excitation was usually 480 or 440 nm light with a half band width of 8 nm. Fluorescence emission was scanned in 1.0 nm increments from 650 - 800 nm with a half band width of 2 nm. Acquisition, storage and mathematical manipulation of spectra were performed by an on line Hewlett Packard 9825 computer.

Movement Of LHCP

For phosphorylation experiments of LHCP, thylakoids were resuspended in resuspension buffer to a final concentration of 200 ug Chl ml^{-1}. To this, ATP at a concentration of 200 uM (10 ul from a 20 mM stock) and NaF to a concentration of 10 mM (20 ul of a 0.5 M stock) were added and the sample was kept in the dark for 30 min. The NaF was added to inhibit phosphatases. The samples were placed into a plexiglas waterbath maintained at 23 C and were continuously stirred on a magnetic stir plate. The assay was initiated by illumination from the side at a fluence of 300 uE m^{-2} s^{-1}. At specific times 20 ul was removed and quickly added to 1 ml of 60% (v/v) glycerol, 5 mM $MgCl_2$ resupension buffer giving a concentration of 10 ug chl ml^{-1}. These samples were frozen for low temperature fluorescence work and measured as described above. Movement of LHCP was determined by the change in F_{685} versus F_{734}. Fluorescence at 685nm is believed to represent LHCP

associated with PS II core particles (Staehelin and Arntzen, 1983). With phosphorylation of LHCP a decrease in the ratio of F_{685} to F_{734} due to movement of the antenna away from the PSII reaction centers is observed. Measurements were done on three independent thylakoid preparations.

Temperature Induced Fluorescence Yield Enhancement

The method used to monitor fluorescence yield (F_0) in whole detached leaves at various temperatures was a modification of the method used by Schreiber and Berry (1977). A leaf was positioned between two 45mm x 10mm strips of acetate and placed diagonally into a water filled cuvette. The cuvette was placed into the spectroflorometer so that the leaf was at a 45^o angle to the excitation light. A thermocouple was attached to the leaf to monitor the temperature. The excitation light which was at 480 nm with a 16 nm half band pass was filtered with neutral density filters so that the intensity was 0.3 uE m^{-2} s^{-1}. Fluorescence emission from the leaf surface was monitored at 700 nm with a 16 nm half band width. Sample temperature was increased at a rate of roughly 1.5 C min^{-1}. Measurements were made by simultaneously recording leaf temperature and fluorescence intensity.

Fluorescence Polarization Measurements

An estimation of the microviscosity of the thylakoid membranes was achieved by determining the steady state fluorescence polarization of the hydrophobic probe 1,6-diphenyl-1,3,5-hexatriene (DPH) inserted in this membrane. Because conditions which favour thylakoid stacking inhibit the uptake of DPH into the membrane (Barber et al, 1984)

thylakoids were isolated as usual but without $MgCl_2$ in the resuspension buffer. DPH was added from a 3 mM stock solution in tetrahydrofuran directly to a suspension of thylakoids (50 ug ml^{-1} chl) to a final concentration of 50 uM. After incubation in the dark for 40 min at room temperature, thylakoids were centrifuged (3000 g, 3 min), the supernatant was discarded and the pellet was respended in resuspension buffer containing no DPH to a final concentration of 10 ug chl ml^{-1}.

Fluorescence polarization was carried out on an SLM 4048 spectrofluorometer in the T format. In this conformation the intensities of the parallel and perpendicular components are measured simultaneously using two separate emission polarizers. The formula used to calculate fluorescence polarization (p) values was

$$p = \frac{Ivv - Ivh\ (Ihv/Ihh)}{Ivv + Ivh\ (Ihv/Ihh)}$$

where I is the intensity and v and h are the positions of the polarizers (Barber et al, 1984). For example Ivh corresponds to a vertical excitation polarizer and a horizontal emission polarizer. Excitation was provided by 360 nm light with a half band pass of 16 nm. the two emission polarizers were at 460 nm with a half band width of 8 nm.

Extraction and Analysis of Chl, Lipid and Proteins

Leaves were harvested at the rosette stage (3 weeks) and either fresh weight or leaf area were determined. Individual leaves were ground in 2 ml of 95% ethanol and centrifuged at 1000g for 1 min to remove the insoluble material. Chl concentrations were determined

according to the formula (A_{654} * 39.8)/1000 = mg chl/ml (Wintermans and Demots, 1965). To the Chl fraction 0.01 mg of 17:0 fatty acid methyl ester (FAME) in one ml of hexane was added and the suspension was dried down under nitrogen gas. Fatty acid methyl esters of the dried samples were prepared by transesterification in hot methanolic-HCl as described elsewhere. The known amount of 17:0 standard added was used as an internal standard to determine the amount of total fatty acid in the leaf.

For measurements of Chl, lipid and protein of fractionated samples, leaves from a pot of plants (4 to 6 g) were harvested and homogenized as was done for thylakoid preparations except the supernatants of both the grinding and washing steps were saved, combined and centrifuged at 80,000 g in an SW-40 rotor for 60 min at 4 C to sediment extrachloroplast membranes. The resulting pellet was termed the extrachloroplast or 80,000 g fraction.

Determinations of Chl and fatty acid amounts in the chloroplast fraction were done as described above. Quantities of fatty acids in the extrachloroplast fraction were corrected for the presence of chloroplast membranes by assuming that Chl in this fraction was associated with chloroplast lipid. This assumes the Chl in the 80000 g fraction was associated with the same specific lipids as Chl in the chloroplast fraction. The correction was made by subtracting the chloroplast contamination (mol chl in 80000 g fraction x mol fatty acid per mol chl in cloroplast fraction) from the total for the 80000 g fraction and adding that amount to the chloroplast fraction.

Protein concentrations of the extrachloroplast fraction were determined according to Markwell et al (1981).

Pigment-Protein Electrophoresis

Pigment-protein electrophoresis was performed by slight modifications of the method of Anderson et al (1978). Chloroplast membranes were isolated as described above except $MgCl_2$ and NaCl were omitted from all buffers. Membranes were solubilized before electrophoresis by incubation for 5 min at 22 C in a volume of solubilization buffer (300 mM Tris-Cl (pH 8.8), 10% (v/v) glycerol, and 5% (w/v) SDS) which gave an SDS : Chl ratio of 1 : 10. In some instances as noted in the results, NaCl was added to the solubilization buffer.

The acrylamide to N,N'-methylenebisacrylamide ratio was 30 : 0.8 for both the lower and stacking gels. The concentrations of the reagents and buffers used are given in Table 5.

After the gel had set (30 min), 10 - 20 ul of sample was loaded and run at 12 mA for 50-60 min at 4 C giving a migration distance of 4.5 cm for the free pigments. The absorbance of the pigment - protein containing bands in the gels was determined at 600 nm using a Gelman ACD-18 automatic computing densitometer.

LHC was isolated by density gradient ultracentrifugation by the method of Burke et al (1978). For rerunning LHCP oligomers and monomers by SDS - PAGE for investigations of protein composition,slices of the bands were removed from the gel with a razor blade and homogenized in a tenbrock. Two gel slices of the oligomeric band were reloaded for SDS-PAGE (Laemmli, 1970). For the monomeric band only one slice was used to prevent overloading.

Table 5

Concentration of solutions used in pigment-protein gel and buffer systems

Buffer System

	Buffer	Amount	Titrant
Upper Gel	0.56M Tris (pH 6.14)	6.78g/100ml	H_2SO_4
Lower Gel	1.68M Tris (pH 9.5)	20.34g/100ml	HCl
Upper Resevoir	0.04M Tris (pH 8.64)	4.84g/l	Boric Acid
Lower Resevoir	0.42M Tris (pH 9.5)	51.35g/l	HCl

Gel System

	Lower Gel (8%)	Stacking Gel (4%)
H_2O	9.2 ml	3.7 ml
Buffer	5.0 ml	0.5 ml
5% SDS	0.4 ml	0.1 ml
30% acry-0.8% Bis	5.3 ml	0.67 ml
APS (100mg/100ml)	100 ul	50 ul
Temed	10 ul	2.5 ul

Electron Microscopy

All stages of preparative electron microscopy were carried out at 1 C. The leaf tissue was fixed in 2% (v/v) glutaraldehyde in sodium cacodylate buffer (pH 7.2) for 1 hour and postfixed in 1% (w/v) osmium tetroxide in the same buffer. After dehydration in a graded ethanol series, the specimens were embedded in Spurr's Epoxy resin (Spurr, 1969). Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a JEOL 100 CX electon microscope. For obtaining quantitative measurements of the amount of membrane from electron micrographs a map wheel was used. Measurements were made on sections of 20 chloroplasts from both wild type and mutant lines.

Fatty Acid Methyl Ester Formation

Single Arabidopsis leaves (5-50 mg fwt) were placed in 13 x 100 mm screw capped glass tubes. Three molar methanolic-HCl (Sulpelco) was diluted to 1 M with reagent grade methanol, one ml was added to each sample and the tubes were sealed with Teflon lined caps and heated to 80 C for 1 h. After cooling, 0.3 to 1.0 ml of hexane and 1 ml of 0.9% NaCl were added and the fatty acid methyl esters (FAMES) were extracted into the hexane phase by vigorous shaking of the tubes. The tubes were centrifuged (1000g for 30s) to break any emulsion formed and to completely separate the phases. One or two ul of the hexane layer was injected directly onto the gas chromatograph (GC). GLC analysis was carried out on a Varian 3700 instrument equipped with a flame ionization detector. A six meter column (5% DEGS PS on 100/120 Supelcoport; Supelco, Bellefonte, PA.) with 30 ml min^{-1} N_2 as a carrier gas was used. Operating temperatures for the column, injection port,

and detector were 175 C, 210 C and 210 C respectively. Peaks were quantified by a Spectra Physics 2100 Auto lab integrator. Peaks were identified by comparing retention times with known standards.

Analysis Of Lipid Composition

Leaves from 4 to 6 g fwt of Arabidopsis plants were pulverized in liquid nitrogen with a mortar and pestle. The pulverized powder was transferred to a 50 ml polypropylene SS-34 centrifuge tube and 30 ml of chloroform-methanol (1:1 v/v) was added. This slurry was homogenized in a Polytron for approximately 30 sec and filtered through Whatman No.3 MM filter paper under vacuum. The funnel and paper were washed several times with chloroform- methanol giving a final volume of about 50 ml. To the filtered mixture 5 ml of 0.9% NaCl was added. The sample was vigorousrly shaken and separated into phases by centrifugation (1000g for 30s). The lower chloroform phase was removed for further analysis.

For complete separation of all the lipid species the chloroform extract was applied in 2 ml aliquots to a Biosil A (BIO-RAD) column (0.5 x 3.5 cm) in chloroform. The column was eluted with 10 ml chloroform (neutral fraction), 20 ml acetone (glycolipid fraction) and 10 ml methanol (phospholipid fraction). All three fractions were dried under nitrogen and respended in chloroform : methanol (1:1 v/v) for thin layer chromatography (TLC).

For TLC separations, Silica G TLC plates (Baker Si 250 PA, 200 microns) were activated by heating them to 100 C for 1 hour before spotting the sample. For lipid separations two solvent systems were employed. The first was acetone-benzene-water (91:30:8) (Khan and Williams, 1977). This system was excellent for separating PG from PE

but involved soaking the plates in 0.15M $(NH_4)_2SO_4$ for 15 min before activation. One problem with this system was the variabilty in Rf values from plate to plate.

The second system was chloroform-acetone-methanol-acetic acid-water (100:40:30:10:4) which was more reproducible but gave poor separation of SL, DDG and PC. However this was not a problem if the lipids were first separated into glycolipid and phospholipid fractions by silicic acid chromatography. After development, the plates were allowed to dry in the fume hood and standards were detected using an stream of air passed through a pasteur pipet containing iodine crystals. Usually one lane of sample was also exposed to the iodine vapours to allow detection of lipids for which standards were not availiable. Lipids were marked and the corresponding area on the plate which had not been exposed to iodine was scraped off into a 13x100 mm screw cap test tube. Three ml of chloroform : methanol (1:1 v/v) were added to each tube and then sealed with a teflon lined cap. The tubes were mixed and allowed to stand for a minimum of 15 min. One ml of 0.9% NaCl was added which upon gentle mixing caused a separation of chloroform from the methanol-water phase with the silica remaining at the interface. The chloroform layer was removed and the procedure was repeated with another ml of chloroform. The combined chloroform phases were dried under nitrogen and 1 ml of methanolic HCl was added to each tube. FAMEs of the lipids were made as described for fresh leaf samples. If quantification of the amount of lipid was desired an internal standard of 17:0 FAME (0.1 ug/ml) was added to the lipid sample before extraction into chloroform.

CHAPTER 3

MUTANT ISOLATION

Introduction

Although the use of mutational analysis to dissect the roles of lipids and fatty acids in microorganisms has led to considerable insight into the function of these macromolecules in membrane assembly and function, such an approach has not been considered widely applicable to higher plants. This generally reflects the inability to adapt microbial methodologies of mutant isolation to multicellular organisms. Bacterial and yeast mutants with altered lipid composition are usually isolated by two methods. One involves screening for mutants auxotrophic for lipid precursors by rescue with nutritional supplementation (Keith et al, 1969; Atkinson et al, 1980). However this method depends upon the ability to replica plate cells and the organisms ability to takes up the precursors readily. Neither of these criteria have been achieved in higher plants.

The alternative to supplementation is to screen a large population of individuals for temperature sensitive (ts) conditional mutants. However a ts mutation in any particular gene is generally much less

frequent than a null mutation. Therefore, some form of mutant enrichment, usually involving conditions which kill actively growing cells at nonpermissive temperatures, is required (Raetz, 1978). This in turn allows enough individuals to be screened to make the approach feasible. However adaption of such negative selection screens has met with limited success in plant tissue culture (Horsch and King, 1986) and is mechanistically difficult to do at the whole plant level.

A alternative approach for the isolation of well defined biochemical mutants at the whole plant stage has involved a detailed characterization of the regulation of the pathway of interest under a variety of experimental conditions. If conditions can be defined which appear to change the regulation of the pathway, mutants with altered growth response under these conditions might be defective in the characterized pathway. Such an approach has been used successfully to isolate mutants defective in photorespiratory metabolism (Somerville and Ogren, 1982). In the case of lipid biosynthesis, however, the results of biochemical and physiological studies on the regulation of these pathways have been conflicting (Kuiper, 1986). Early work on chilling sensitive and chilling resistant species of plants led to the proposal that the ratio of unsaturated to saturated fatty acids was instrumental in maintaining membrane integrity at various temperatures (Lyons and Raison, 1970). Although subsequent analysis of these ratios in a larger collection of species did not support this theory most studies did show that for any particular plant a change in unsaturation does occur upon shifting to different temperatures. For example, in Brassica napus upon transfer to 5 C an increase in 18:3 was detected in both roots and leaves (Smolenska and Kuiper, 1977). This increase is

most evident in phosphatidylcholine and phosphatidylethanolamine in the leaves and neutral lipids in the roots. Similar biochemical changes in 18:3 levels in Arabidopsis, which is also a member of the Brassicaceae, led to the suggestion that a class of mutants sensitive to chilling temperatures might also be disrupted in some aspect of fatty acid unsaturation. Based on the theory that the degree of lipid unsaturation affects the resistance of a plant to chilling, two chilling sensitive mutants of Arabidopsis were isolated; however, neither showed alterations in fatty acid metabolism (McCourt, 1983). The low number of mutants isolated did not allow evaluation of this method as a potential approach for the isolation of mutants with altered lipid membrane composition.

Because of the failure of this method to result in the isolation of plant lipid mutants, it was decided that a direct screen for changes in fatty acid composition under nonselective conditions should be attempted. The rationale for such a screen was as follows. All successful methods of lipid mutant isolation in microbial systems assume such defects are lethal to the organism. Hence, almost all of these mutants have a detectable phenotype. However, E. coli mutants completely defective in the synthesis of cyclopropane fatty acids show no impairment of growth under a variety of environmental conditions (Taylor and Cronan, 1976; Grogan and Cronan, 1986) suggesting changes can be made to the membrane lipid composition which are not essential for the life cycle of the organism. Therefore, an assay based on fatty acid methyl ester formation and subsequent gas chromatographic analysis was devised which permits rapid and quantitative analysis of fatty acids containing 16 or more carbons from a single leaf sample (see

Materials and Methods). An example of the results of a typical analysis of a leaf from the wild type is presented in Figure 3.

Results

Mutant Isolation

This single leaf assay was applied to a population of M2 Arabidopsis plants grown under normal laboratory growth conditions. From approximately 2000 plants examined in this way, 89 were retained for future analysis due to anomalies in fatty acid composition. Four to ten individual M3 progeny from each of the 89 lines were analysed for inheritance of the altered fatty acid composition. In those cases where all the progeny exhibited the mutant phenotype the most vigorous plant was saved and advanced by single seed descent. In instances where some of the plants appeared to be wild type more plants were analysed to see if the population was segregating for the original phenotype. This allowed the reselection of the mutation in the homozygous state. By repeating this process for several generations eight of the orginal 89 lines were found to have stably inherited changes in fatty acid composition and were given strain numbers beginning with JB1 (Table 2). These 8 lines were grouped according to phenotype based on whole leaf fatty acid composition. Although the exact reason for the low recovery of mutant phenotypes in the M3 generation is not known, in most cases the 81 lines which did not show a reproducible phenotype had very subtle changes in fatty acid composition and probably reflect physiological variation within a growing population.

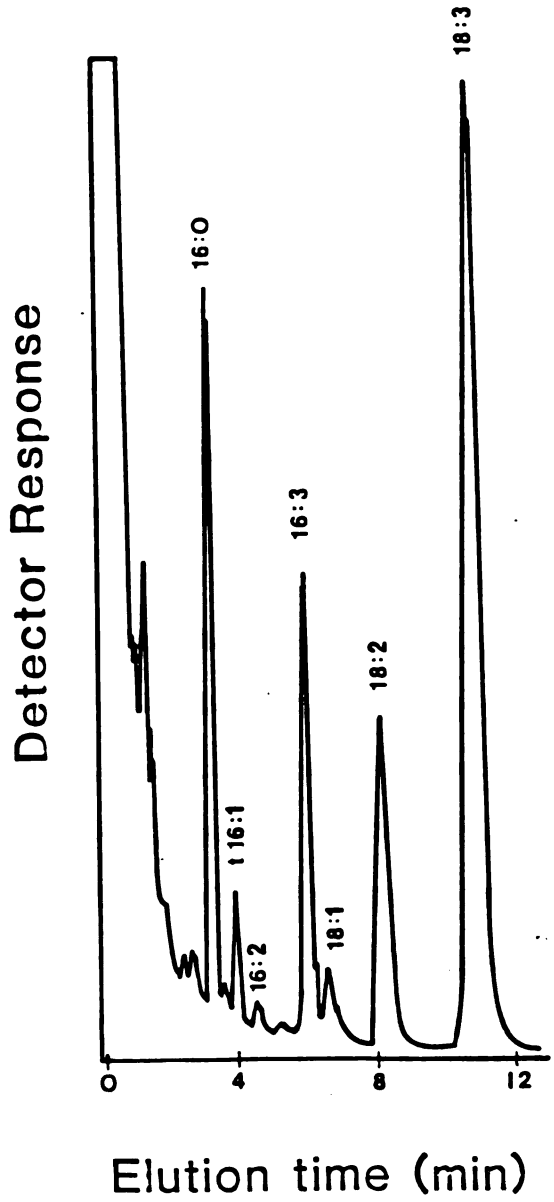

Figure 3. A typical gas chromatography tracing of fatty acids extracted from a single wild type leaf

Group I Mutants

Two mutants (JB27 and JB60) were isolated with nondetectable levels of the fatty acid 3-trans-hexadecenoic acid (trans-$C_{16:1}$) and corresponding increases in the amounts of 16:0. Normally less than 5% of the wild-type levels can be detected by this method. Otherwise the mutants were indistinguishable from wild-type in both fatty acid composition (Table 6) and viablity. The genetic basis of the phenotype in the line JB60, was determined by measuring the fatty acid composition of F1 and F2 progeny from crosses between JB60 and wild-type. The F1 progeny had approximately 50% as much trans-$C_{16:1}$ as wild-type (0.89 $\pm$ 0.25 mol % in the F1 progeny versus 1.74 $\pm$ 0.21 mol % in the wild-type, n=10 plants) suggesting a simply inherited codominant nuclear mutation. The frequency of the homozygous mutant phenotype in an F2 generation was determined by gas chromatographic analysis of the fatty acid composition of leaves from 57 F2 plants. Of these 13 completely lacked trans-$C_{16:1}$. This is a very good fit to the 3:1 hypothesis (X^2 (1)=0.14; P>0.9) indicating the deficiency is due to a single nuclear mutation at a locus that has been designated fadA (fatty acid desaturation gene A). The fadA mutation was mapped to chromosome 4 by F2 mapping (see Appendix B) from a cross of line MK1 by JB60.

Complementation analysis of JB60 with JB27 was carried out using a glabirous derivative of line JB60 (fadA, gl-1) as the female parent. The recessive leaf marker assured selfing had not occurred in the cross. The F1 progeny showed no detectable trans-$C_{16:1}$ indicating these two mutations are allelic.

Table 6

Percent of total fatty acids in Group I mutants (lacking trans-$C_{16:1}$). Each value represents the average of ten plants.

	Fatty Acid Composition (mol %)		
Fatty Acid	WT	JB27	JB60
16:0	15.8	17.0	18.3
t16:1	1.8	0.0	0.0
c16:1	0.2	0.3	0.2
16:2	0.7	0.8	0.8
16:3	12.6	12.5	11.6
18:0	1.0	0.9	1.0
18:1	2.7	2.8	2.7
18:2	18.9	19.3	18.9
18:3	47.5	47.5	47.2

Group II Mutants

One mutant (line JB1) was isolated with reduced levels of both 18:3 and 16:3 fatty acids and had corresponding increases in 18:2 and 16:2 (Table 7). On the basis of evidence presented later, this line is considered to carry a mutation at a single nuclear locus designated fadD. Upon subsequent analysis of this mutant it was observed that the fatty acid composition, although always different from wild-type, showed variability between plants grown within the same chamber. Subsequent experiments in which light intensity, light quality and temperature were varied, were carried out to determine the cause of this variation. No obvious difference could be detected in trienoic acid composition of plants grown for 3 weeks at 25 C under varying light intensities (Figure 4). By contrast, a marked effect was seen in the levels of both 18:3 and 16:3 at temperatures above about 23 C in the JB1 line, suggesting the fadD mutation confers temperature sensitivity upon dienoic acid desaturation (Table 7, Figure 5).

In order to determine the genetic basis for the reduced trienoic acid content, 317 F2 plants resulting from a cross of wild-type to JB1 were grown at 23 C and analysed for fatty acid composition. Of these, 244 F2 progeny resembled wild-type in fatty acid content and 73 had a fatty acid composition indistinguishable from the mutant parent grown at the same temperature. This segregation pattern is a good fit ($X^2(1)=0.66$; $P>0.9$) to the 3:1 hypothesis. The cosegregation of reduced 16:3 and 18:3 content strongly sugests both changes are caused by a single nuclear mutation. The fadD mutation was mapped to chromosome 3 by F2 mapping (see Appendix B) from a cross of line MK1 by JB1.

Table 7

Percent of total fatty acids in Group II mutants (deficient in 16:3 and 18:3) grown at 18 C and 27 C for three weeks. Each value represent average of ten plants.

	Fatty Acid Composition (mol %)			
	18 C		27 C	
Fatty Acid	WT	JB1	WT	JB1
16:0	13.3	13.4	15.8	17.4
t16:1	1.4	1.1	2.2	3.1
c16:1	0.3	0.3	0.2	2.7
16:2	0.2	3.4	0.3	6.0
16:3	13.8	6.9	10.7	2.0
18:0	0.4	0.4	1.3	1.4
18:1	1.1	2.4	3.7	9.2
18:2	12.7	18.1	17.0	39.3
18:3	55.6	52.0	48.7	18.6

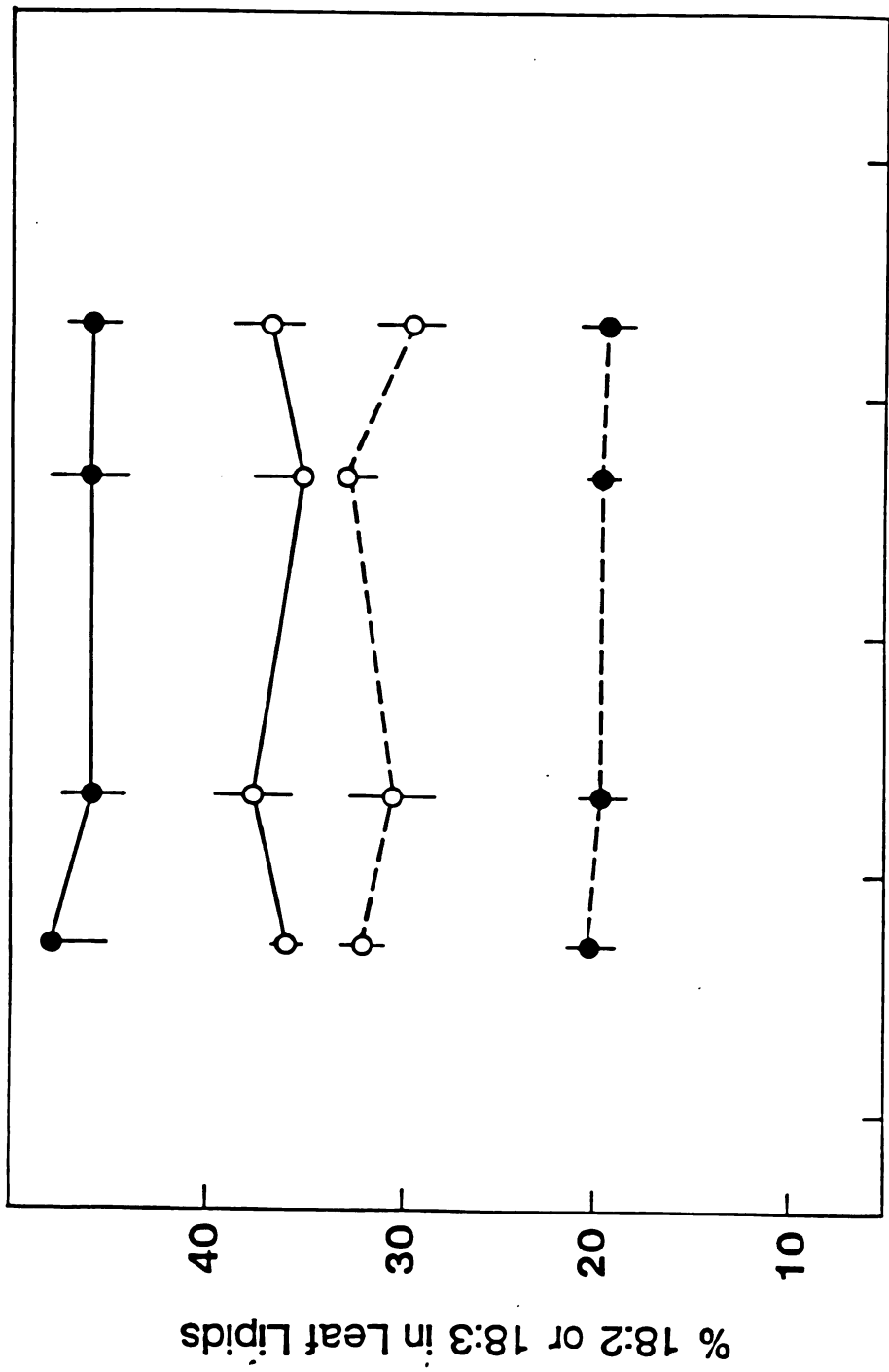

Figure 4. The effect of fluence rate on the proportion of 18:2 (---) and 18:3(—) in leaves of mutant JB1 (O) and wild type (O) Arabidopsis

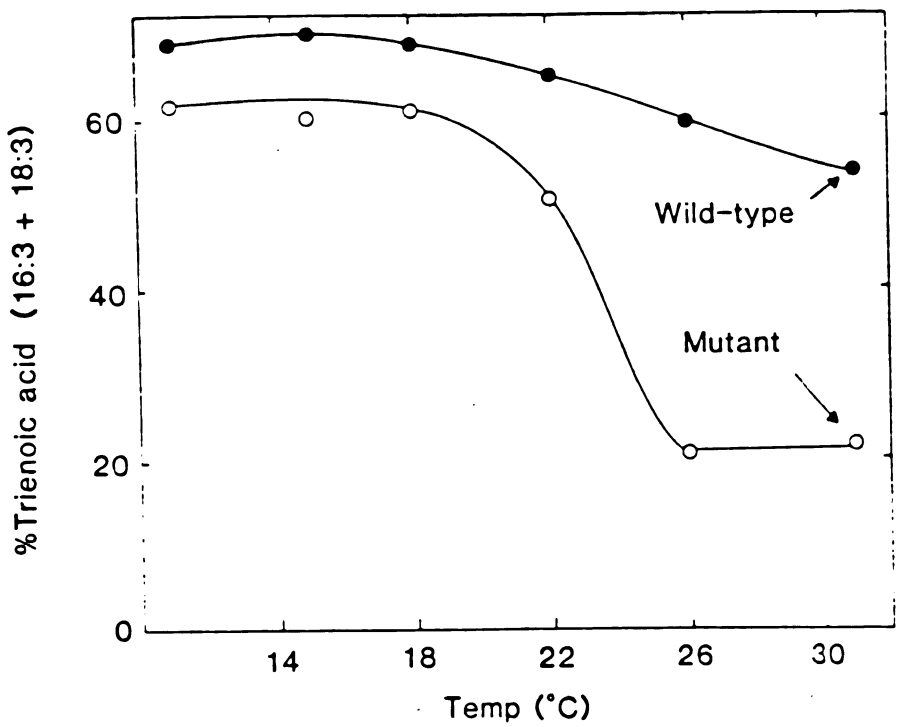

Figure 5. The effect of temperature on the proportion of trienoic fatty acids in leaves of mutant JB1 (O) and wild type (●) Arabidopsis

When F1 plants from a cross of JB1 by wild-type were grown at 23 C and the fatty acid composition of these plants was analysed, no distinguishable difference from wild-type could be detected. However F1 plants grown at 28 C had intermediate levels of 18:3 and 16:3 compared to wild-type (Table 8). Therefore, as with the fadA mutant, the level of 18:3 and 16:3 is probably regulated by the amount of active enzyme present.

Group III Mutants

Four mutants were isolated which showed similar whole leaf fatty acid alterations (Table 9). All had reduced amounts of 16:3 and no 16:2 or cis-16:1. These changes appeared to be compensated by increases in 18:1, 18:2, and 18:3 fatty acids. Although no further work was carried out on these mutants in this study, L. Kunst has shown that JB25 is also a simple nuclear mutation at a locus provisionally designated pap.

Double Mutants

Construction of double mutants for the testing of epistatic relationships between various mutations can help provide detailed information about the sequence of steps affected by these mutations in a biosynthetic pathway. For example, if one mutation occurs earlier in a linear pathway than another it should be epistatic to the second mutation. If the mutations fail to show epistasis then they probably do not affect the same pathway or there are alternate pathways. If double mutants are obtained which have a fatty acid composition different from both parents, although this makes the interpretation of the

Table 8

Percent of total fatty acids in wild type, JB1, and F1 progeny from a JB1 x wt cross grown at 28 C for three weeks. Each value represents the average of ten plants.

	Fatty Acid Composition (mol %)		
Fatty Acid	WT	JB1	F1 (WT x JB1)
16:0	17.4 ± 0.2	16.8 ± 0.1	17.6 ± 0.3
t16:1	2.0 ± 0.2	1.9 ± 0.3	1.8 ± 0.3
c16:1	0.5 ± 0.1	0.7 ± 0.1	0.9 ± 0.2
16:2	1.0 ± 0.1	7.6 ± 1.2	2.9 ± 0.9
16:3	10.0 ± 0.8	2.2 ± 1.4	7.7 ± 1.3
18:0	1.0 ± 0.1	1.4 ± 0.1	1.4 ± 0.1
18:1	4.4 ± 0.1	5.2 ± 0.5	4.6 ± 0.1
18:2	22.3 ± 0.3	39.8 ± 2.9	28.3 ± 2.6
18:3	39.1 ± 0.4	22.3 ± 2.8	33.7 ± 2.7

Table 9

Percent of total fatty acids in Group III mutants (deficient in 16:3 biosynthesis. Each value represents the average of ten plants.

	Fatty Acid Composition (mol %)				
Fatty Acid	WT	JB3	JB19	JB25	JB28
16:0	18.0	10.5	13.8	14.6	12.8
t16:1	1.7	1.4	1.0	1.1	0.9
c16:1	0.3	0.0	0.0	0.0	0.0
16:2	0.8	0.0	0.0	0.0	0.0
16:3	12.9	0.0	4.9	0.0	2.2
18:0	0.9	0.6	0.2	0.6	0.8
18:1	2.0	6.7	4.8	8.6	6.8
18:2	11.7	17.9	19.1	19.0	19.7
18:3	51.6	62.3	55.2	55.4	57.4

biosynthetic pathway more difficult it does provide completely new membrane compositions upon which to carry out physiological studies.

Because group-II (fadD) and group-III (pap) fatty acid phenotypes both appear to have altered 16:3 levels, it was of interest to determine their epistatic relationship. Of 49 F2 plants from a JB1 (fadD) by JB25 (pap) cross, one plant line showed a fatty acid composition similar to both parents. This phenotype was inherited stably for three generations, and the line was given the strain designation LIP1. As with JB25, the LIP1 line had no detectable 16:3, 16:2 or cis 16:1. However LIP1 also had reduced amounts of 18:3 and increased 18:2 in plants grown at 27 C characteristic of the JB1 (Table 10). The lack of 16 carbon unsaturated fatty acids was independent of temperature but the 18:3 levels were not (Table 11). The LIP1 line is of added interest because although it appears that the pap mutation is epistatic to the fadD mutation at the level of 16:3 synthesis the opposite is true for 18:3 synthesis. This suggests some features of the 16:3 and 18:3 biosynthetic pathway(s) are shared while others are not.

Of the F2 progeny that were screened from the JB1 by JB25 cross, the ratio of classes were 27 WT : 5 JB25 : 16 JB1 : 1 LIP1. This ratio is close to the 9:3:3:1 ratio expected for independent assortment ($X^2(3)=5.59$, $P>0.9$). This result in combination with normal 3:1 ratios for the single loci (FadD $X^2(1)=2.45$, pap $X^2(1)=4.25$, $P>0.9$) suggest these two loci are unlinked.

The F2 progeny from a JB60 (group I) by JB1 cross were also screened for double mutants. Eight of 120 plants lacked both trans-$C_{16:1}$ and had reduced levels of 16:3 and 18:3 (Table 10). All lines were tested for the inheritance of the phenotype and one strain was kept for further

Table 10

Percent of total fatty acids in double mutants LIP1 (JB1 x JB25) and LIP2 (JB60 x JB1) grown at 28 C. Each value represents the average of four plants.

	Fatty Acid Composition (mol %)				
Fatty Acid	WT	JB1	JB25	LIP1	LIP2
16:0	14.7	13.2	14.6	10.5	16.2
t16:1	2.7	2.7	1.1	2.0	--
c16:1	0.1	3.1	--	--	0.2
16:2	1.2	10.5	--	--	9.0
16:3	11.5	2.0	--	--	1.4
18:0	1.1	1.4	0.6	0.8	1.0
18:1	2.3	9.2	8.6	6.7	6.5
18:2	14.2	37.7	19.0	65.7	37.9
18:3	52.1	18.9	55.4	15.9	25.0

Table 11

Percent of total fatty acids in double mutants LIP1 (JB1 x JB25) and LIP2 (JB60 x JB1) grown at 19 C. Each value represents the average of four plants.

	Fatty Acid Composition (mol %)				
Fatty Acid	WT	JB1	JB25	LIP1	LIP2
16:0	15.0	13.4	14.6	11.7	17.8
t16:1	1.7	1.1	1.1	1.2	--
c16:1	0.3	0.3	--	--	0.4
16:2	0.8	3.4	--	--	5.0
16:3	12.9	6.7	--	--	6.6
18:0	0.9	0.4	0.6	0.6	0.4
18:1	2.0	2.4	8.6	9.0	2.7
18:2	11.7	18.1	19.0	29.6	21.6
18:3	53.6	52.0	55.4	45.5	48.7

analysis (LIP2). Again the 18:3 and 16:3 fatty acid compositions were affected by temperature as is expected of the JB1 phenotype but the trans-$C_{16:1}$ was absent at both temperatures (Table 11).

The ratio of classes from the screen were 73 WT : 19 JB1 : 20 JB60 : 8 LIP2. This excellent fit to the 9:3:3:1 hypothesis ($X^2(3)=1.30$, $p>0.9$) suggests that the two mutations are unlinked. This had also been established by F2 mapping of both fadA and fadD to known chromosomal markers (Appendix B). The lack of any epistasis between fadA and fadD was expected since trans-$C_{16:1}$ is only synthesized on PG and is not further metabolized (Dubacq and Tremolieres, 1983).

CHAPTER 4

PHYSIOLOGICAL AND BIOCHEMICAL STUDIES OF A MUTANT LACKING TRANS HEXADECENOIC ACID

Introduction

Aside from the family Orchidaceae (Roughan, 1986) the chloroplast membranes of all photosynthetic eukaryotes contain the unusual fatty acyl group 3-trans-$C_{16:1}$ which is always found esterified to the second position of phosphatidyl glycerol (Dubacq and Tremolieres, 1983). The fatty acid is atypical because of the trans configuration, and because of the position of the double bond near the carboxyl rather than the methyl end of the fatty acid. A specific role for the acyl group in photosynthesis has frequently been proposed because trans-$C_{16:1}$-PG occurs only in chloroplast membranes (Dubacq and Tremolieres, 1983), and is present in relatively low amounts in etioplasts but accumulates upon light-induced chloroplast development in parallel with the accumulation of the LHCP and the development of appressed membranes (Dubacq and Tremolieres, 1983; Galey et al, 1980; Mackender, 1979). Also, removal of trans-$C_{16:1}$ from PG by phospholipase-A2 treatment of isolated thylakoids was reported to alter the efficiency of light capture and to change the kinetics of

fluorescence induction (Duval et al, 1979). However, a specific role in photosynthesis has not been demonstrated (reviewed in Dubacq and Tremolieres, 1983).

Recently, evidence pertaining to a possible role for trans-$C_{16:1}$ was obtained from experiments in which the lipid content of isolated Chl-protein complexes was characterized. When thylakoid Chl-protein complexes are solubilized in low amounts of SDS and electrophoresed on polyacryamide gels which also contain low concentrations of SDS, the Chl-protein complexes separate into a characteristic pattern of about six major Chl-containing bands (Anderson et al, 1978). When these bands were extracted from the acrylamide gel and the lipid composition of each band measured, it was found that the LHCP band which is believed to correspond to an oligomeric form of LHCP, was significantly enriched with trans-$C_{16:1}$ (Tremolieres et al, 1981). The possible importance of the lipid was also suggested by experiments in which treatment of thylakoids with phospholipase-A2 before solubilization and electrophoresis caused the disappearance of the LHCP oligomer, which has been proposed to be the native form of the complex in vivo (Kuhlbrandt, 1984). Although it may be only coincidental, there is approximately enough trans-$C_{16:1}$ in the chloroplast membranes to satisfy a stoichiometry of one molecule per LHCP oligomer (Dubacq and Tremolieres, 1983). A role in LHCP oligomer formation or stabilization is also suggested by recent experiments showing that the rate of reconstitution of LHCP oligomer in liposomes is stimulated by the presence of trans-$C_{16:1}$ (Remy et al, 1984).

The mutant line of Arabidopsis thaliana (L.), JB60 which specifically lacks trans-$C_{16:1}$ has a compensating increase in palmitic

acid (16:0). The mutant is, therefore, believed to lack a specific desaturase which converts palmitic acid at the sn-2 position of PG to trans-$C_{16:1}$. As mentioned in a previous section the mutant, which has no obvious phenotype, was isolated by analyzing the fatty acid composition of several thousand randomly selected individuals from a mutagenized population. In a preliminary analysis of thylakoid ultrastructure and function we were unable to establish a difference between the mutant and the wild-type. Here, we describe the results of experiments designed to test the role(s) of trans-$C_{16:1}$ in formation of LHCP oligomer and in the functional association of LHCP and the photochemical reaction centers. Although fluorescence measurements suggest normal LHCP function, the LHCP oligomer appears less stable to dissociation by SDS in the mutant. A similar effect on the CP1a complex suggests that trans-$C_{16:1}$ also stabilizes the presumed oligomeric form of the PSI Chl-protein complex.

Results

Biosynthesis of Trans-$C_{16:1}$

The observation that 16:0 levels were higher in the fadA mutant than in wild-type plants suggests this fatty acid is a precursor of the trans isomer (Table 6). Support for this idea can also be inferred from radiotracer studies of trans-$C_{16:1}$ synthesis in Chlorella vulgaris (Nichols et al, 1964). Upon adding [^{14}C]palmitate to light grown cultures, radioactive trans-$C_{16:1}$ was exclusively found esterified to PG. Moreover the observation that [^{14}C]trans-$C_{16:1}$ when added to

cultures is randomly distributed among the lipids implies PG is the substrate for trans unsaturation. Thus, it was of interest to compare the fatty acid composition of PG from the mutant and the wild-type. The results of these measurements (Figure 6), which substantially increased the limit of detection, confirmed that the mutant completely lacked trans-$C_{16:1}$. The decrease in trans-$C_{16:1}$ was compensated by a proportional increase in the 16:0 content of PG. These observations suggest that the mutant is deficient in activity for a proposed desaturase which specifically converts 16:0 at position two of PG to trans-$C_{16:1}$ (Galey et al, 1980). If this interpretation is correct, the presence of trans-$C_{16:1}$ in the heterozygote would suggest that the amount of this fatty acid is regulated directly by the amount of enzyme activity rather than by some mechanism which senses and responds to the absolute concentration of this fatty acid in the membrane. Since this desaturase activity has not, as yet, been demonstrated by in vivo assay of cellular extracts or by tracer studies with intact chloroplasts (Guillot-Salomon et al, 1982) the precise enzymatic lesion in the mutant cannot be evaluated by these means at present.

The absence of trans-$C_{16:1}$ in a barley mutant deficient in chloroplast ribosomes (Haworth et al. 1983), and the inhibition of trans-$C_{16:1}$ synthesis by chloroplast protein synthesis inhibitors (Henry et al, 1983) have been interpreted as possible evidence that a gene for trans-$C_{16:1}$ synthesis is plastome-encoded. The Mendelian segregation of the fadA mutation does not support this concept. However, our results do not exclude the possibility that one or more proteins encoded by the chloroplast genome are also required for trans-$C_{16:1}$ synthesis.

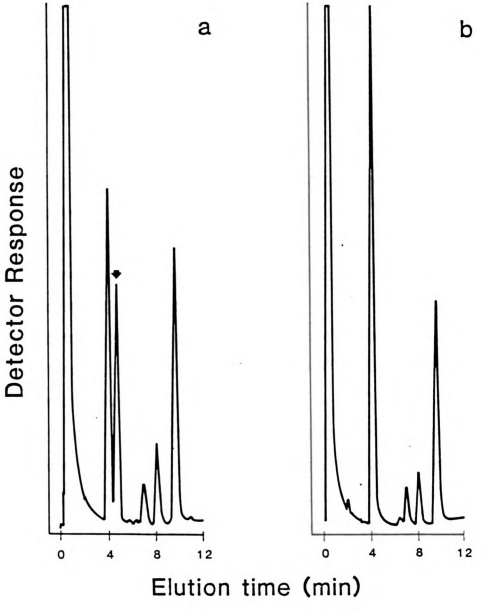

Figure 6. Fatty acid composition of PG from wild type (a) and mutant JB60 (b) Arabidopsis. The position of trans-C16:1 in the chromatogram from the wild-type is indicated by the arrow.

Cellular and Biochemical Studies

Because trans-$C_{16:1}$-PG is not present in etiolated tissue but accumulates during light-induced chloroplast development (MacKender, 1979) and occurs only in chloroplast membranes, it has been inferred that this lipid has a specific role associated with the light reactions of photosynthesis. Recently, attention has been focused on an apparent association of this lipid with the light harvesting chlorophyll a/b protein complex (LHCP) (Lam et al, 1984; Lynch and Thompson, 1984), which also accumulates in thylakoid membranes during light-induced chloroplast development and is thought to have an important role in the formation of the appressed membranes of the grana (Mackender, 1979). In order to critically evaluate the possibility that trans-$C_{16:1}$ is also involved in this process, thylakoid ultrastructure of mutant and wild-type chloroplasts was analyzed by electron microscopy of thin sections of whole leaves. The micrographs (Figure 7) showed no obvious differences in either the size or extent of granal development or any other major ultrastructural feature of the chloroplast, and are considered compelling evidence against the obligatory involvement of trans-$C_{16:1}$ in the development of thylakoid structure.

Recent models for the native structure of LHCP based on Fourier analysis of high resolution electron micrographs of two-dimensional LHCP crystals have indicated that LHCP is a trimer of three structurally equivalent subunits (Kuhlbrandt, 1984). It is believed that this oligomeric structure corresponds to a high molecular weight form of LHCP observed following electrophoresis of thylakoid proteins in acrylamide gels under conditions in which thylakoid proteins are solubilized with low amounts of SDS so that Chl-protein associations

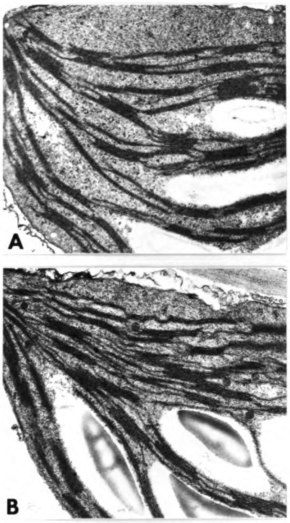

Figure 7. Transmission electron micrographs of ultrathin (80 to 100 nm) sections of whole leaves from wild-tupe (A) and mutant JB60 (B) Arabidopsis at x22,500 magnification.

remain intact (Lam et al, 1984; Lynch and Thompson, 1984). Furthermore, previous studies have shown that trans-$C_{16:1}$-PG comigrates with the LHCP oligomer in gels run under these conditions (Anderson et al, 1978). It was, therefore, of interest to examine the effect of the fadA mutation on the pattern of Chl-protein complexes resolved by this method. Separation of the Chl-protein complexes from wild-type Arabidopsis extracts revealed five major Chl-containing bands (Figure 8) CP1a, CP1, $LHCP^1$, $LHCP^3$ and free Chl.

Comparison of the electrophoretic separation patterns of the Chl-protein complexes from the wild-type and the mutant under standard conditions revealed that the mutant lacked the two Chl-containing bands designated CP1a and $LHCP^1$ (Figure 8). These bands are believed to represent the oligomeric forms of CP1 (the P700-Chl a-protein complex) and $LHCP^3$ (the presumed LHCP monomer), respectively (Anderson et al, 1978). The absence of the oligomeric form of LHCP in the mutant mimics similar results obtained following removal of the acyl group at position two of PG by phospholipase-A2 treatment of thylakoids (Remy et al, 1984).

The reduction of the amount of CP1a in the extracts of the mutant was unexpected since trans-$C_{16:1}$-PG has not previously been reported to be a component of this Chl-protein complex. However, a Chl a/b protein complex associated with the PSI complex has recently been reported (Haworth et al, 1983; Kyle et al, 1984; Lam et al, 1984). The results presented here raise the possibility that this complex has trans-$C_{16:1}$-PG specifically associated with it as a boundary lipid. Alternatively, the presence of trans-$C_{16:1}$-PG in the membrane may exert a nonspecific effect on both LHCP and CP1a stability.

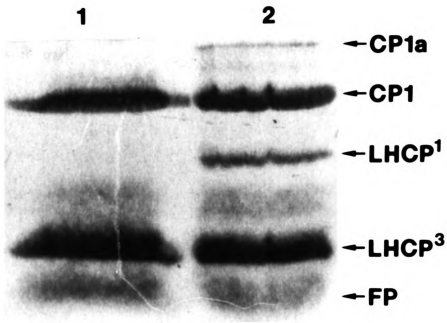

Figure 8. Pigment protein complexes of mutant JB60 (lane 1) and wild type (lane 2) thylakoids. FP denotes free pigment

Studies of cation effects on Chl-protein complexes have shown that removal of cations from solubilization buffers increased the proportion of Chl found in CPla and LHCP following electrophoresis in SDS polyacryiamide gels (Argyroudi-Akoyunoglou, 1981; Argyroudi-Akoyunoglou and Thomou, 1981) The addition of either $MgCl_2$ or NaCl converted an increased proportion of the oligomers into their respective monomers. Therefore, we examined the effect of cation concentration on the proportion of Chl associated with LHCP to determine if conditions could be found which would stabilize the oligomers of the mutant. The solubilization of thylakoid membranes in solutions of SDS containing very low concentrations of NaCl revealed that the thylakoids of the fadA mutant contained normal levels of the LHCP oligomer (Figure 9). As the NaCl concentration was increased from 0 to 100 mM, the amount of LHCP in both mutant and wild-type decreased from a maximum of about 7% to zero (Figure 9). However the membranes from the mutant were much more sensitive to salt-induced dissociation of LHCP oligomer than those of the wild-type. The concentration of NaCl which gave 50% reduction in LHCP concentration was about 13 mM in the mutant as compared to about 37 mM in the wild-type (Figure 9). Thus, it appears that trans-$C_{16:1}$ is not required for LHCP formation but in some way stabilizes the oligomer so that it is less susceptible to SDS-mediated dissociation.

Since it was now possible to visualize the four pigment protein complexes under low salt conditions in both mutant and wild-type, the bands were removed and analyzed by absorption spectroscopy (Figure 10). The absorption spectra of the gel slices derived from extracts of the two genotypes were very similar indicating that lack of trans-$C_{16:1}$ in

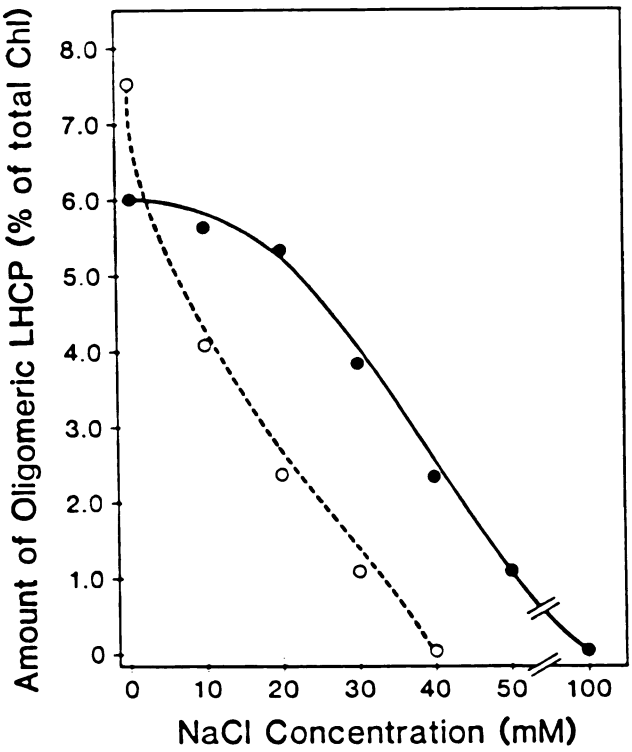

Figure 9. Effects of NaCl concentration on the proportion of Chl in LHCP oligomer in mutant JB60 (O) and wild type (●) Arabidopsis

the complex does not cause a major change in the orientation of the chlorophyll on the proteins under these conditions. CP1 and CP1a both showed a red maximum at 675 nm and a blue maximum at 438 nm which is characteristic of a P_{700} chlorophyll a-protein complex (Thornber and Highkins, 1974). $LHCP^1$ and $LHCP^3$ also showed similar spectra as would be expected for related complexes. The enhanced absorption at 672 nm and 472 nm is due to high levels of chl b relative to chl a which is commonly seen in chlorophyll a/b binding complexes (Anderson et al, 1978). If the oligomeric bands ($LHCP^1$) were rerun under SDS denaturing gel conditions (PAGE) no difference in protein composition of this complex could be detected (Figure 11, lane 6 wt, lane 3 JB60). To further characterize the protein composition of fadA thylakoids and LHCP, SDS PAGE was carried out on thylakoids and LHCP purified by density gradient centrifugation (Figure 11). The thylakoid protein composition of JB60 (lane 4) and wild-type (lane 5) were indistinguishable.

The density gradient purified LHCP monomers from wild-type (lane 7) and JB60 (lane 2) were also identical. The oligomeric LHCP from mutant could not be purified under these conditions but wild-type oligomers appear to have the same protein composition as monomers (lane 1). The major polypeptide for the Arabidopsis LHCP appears to have a molecular weight of 26 kd which is close to that published for the major LHCP polypeptides from other species (Darr, 1985). In summary, the lack of trans-$C_{16:1}$, although causing instability in the oligomeric form of LHCP under certain gel conditions, does not seem to effect polypeptide or chlorophyll composition of this complex under less harsh isolation conditions.

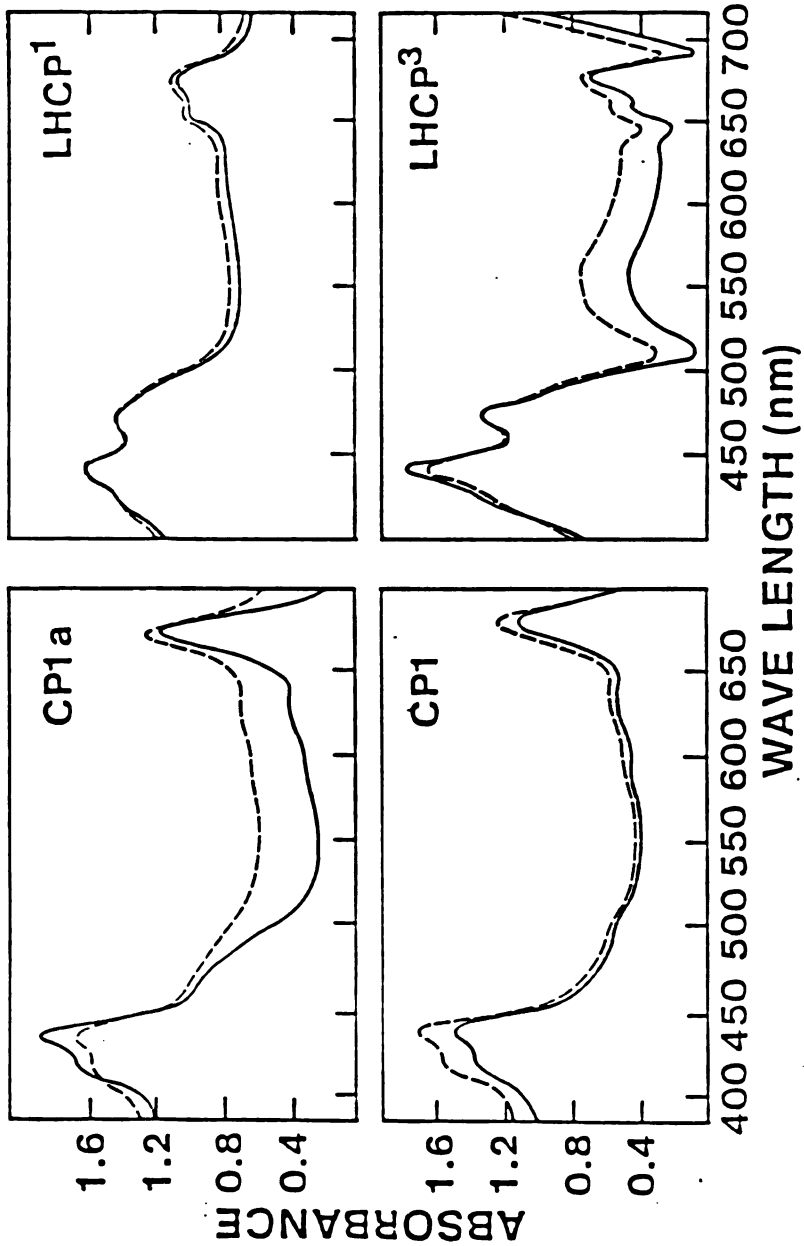

Figure 10. Absorption spectra of mutant JB60 (---) and wild type (—) chlorophyll-protein complexes resolved by SDS-PAGE

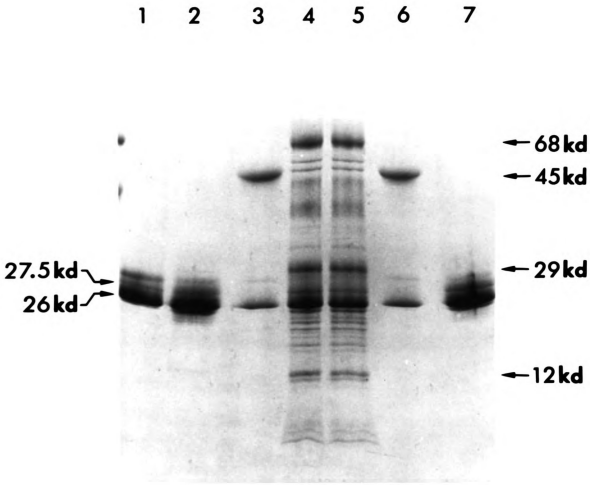

Figure 11. Protein composition of mutant JB60 (lane 4) and wild type (lane 5) thylakoids and purified LHCP1 and LHCP3 from JB60 and wild type separated by SDS-PAGE. Lane 1 is density gradient purified wild type LHCP1. Lane 2 and 7 are density gradient purified JB60 and wild type LHCP3 monomers. Lane 3 and 6 are gel purified wild type and JB60 LHCP1.

Photosynthetic Studies

Since the function of LHCP is to enhance the capture of light energy by PSII, we examined the effect of the fadA mutation on the irradiance response curve for the light reactions catalyzed by isolated thylakoids (Figure 12). A major role for trans-$C_{16:1}$ in LHCP function would be expected to result in a reduced rate of electron transport in the mutant lines at low irradiance. However, no difference was observed between the photosynthetic activities of mutant and wild-type.

Fluorescence Spectra

Chl fluorescence emission spectra are sensitive indicators of the efficiency of energy distribution between the Chl-containing components of the photosynthetic membranes. Preferential excitation of Chl b using 480 nm light results in most of the energy being distributed between the two photosystems. Detachment of LHCP from one or both of the reaction centers results in relatively increased fluorescence emission from LHCP and associated Chl-protein complexes. Thus, it is possible to monitor the relative extent of interactions between LHCP and the reaction centers in thylakoid membranes by comparing the fluorescence emissions at 685, 695, and 735 nm which have been attributed to LHCP, PSII, and PSI, respectively (Butler, 1978; Papageorgiou, 1975).

To test for the presence of an in vivo difference between LHCP function in mutant and wild-type we first compared the spectrum of Chl fluorescence from whole leaves at 77K (Figure 13). An alteration in the efficiency of exciton transfer from LHCP to the photosystems in the mutant would have been expected to result in a change in the ratio of

LHCP fluorescence (685 nm) relative to the other emission maxima. However, no significant difference was apparent between the mutant and the wild-type by this criterion (Figure 13).

In an attempt to relate the effects of cations on LHCP[1] stability to a functional property of LHCP, we examined the effect of cations on low temperature (77K) fluorescence emission spectra of isolated thylakoid membranes. Assuming that the LHCP oligomer is the native form in situ (Kuhlbrandt, 1984), it might be expected that if cations induced dissociation of the LHCP oligomer in intact membranes, this would be reflected in less efficient transfer of excitons from LHCP to the reaction centers. In this case one would expect more fluorescence at 685 nm and less at 734 nm. It should be noted, however, that a cation-induced change in the ratio of PSI to PSII fluorescence has previously been attributed to changes in the spatial organization of the Chl-proteins rather than to changes in the quaternary structure of individual proteins (Staehelin and Arntzen, 1983).

The Chl fluorescence spectrum of chloroplast membranes isolated in the absence of cations is presented in Figure 14. The spectrum, which is qualitatively very similar to that obtained with whole leaves, was not significantly different with respect to the wavelength of the emission maxima or the relative distribution of fluorescence between the two photosystems in mutant and wild-type. Addition of 5 mM $MgCl_2$ to the thylakoids caused an increase in the ratio of PSII to PSI fluorescence (Figure 14) as expected from previous studies concerning the effect of divalent cations on membrane appression and fluorescence (Butler, 1978; Staehelin and Arntzen, 1983). However, the ratio of PSI

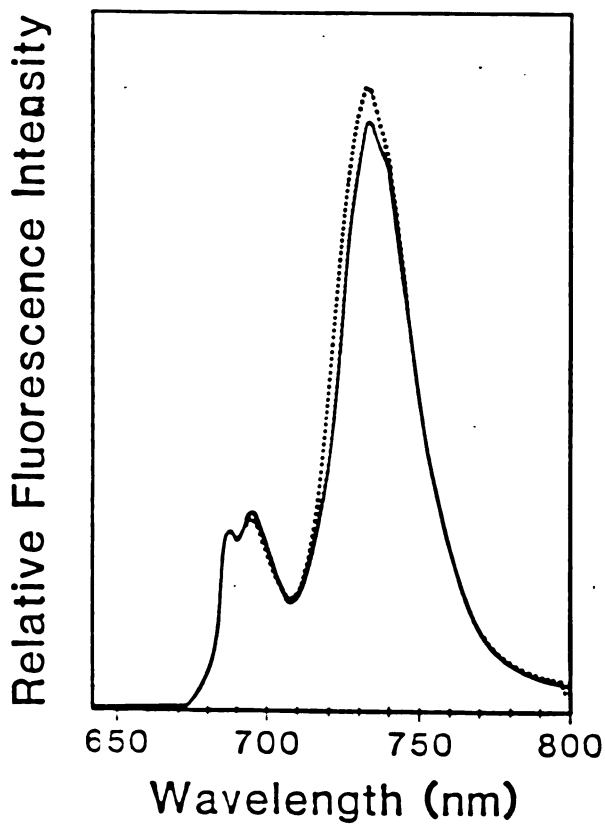

Figure 13. Chlorophyll fluorescence spectra of whole leaves from mutant (---) and wild type (—) Arabidopsis at 77K

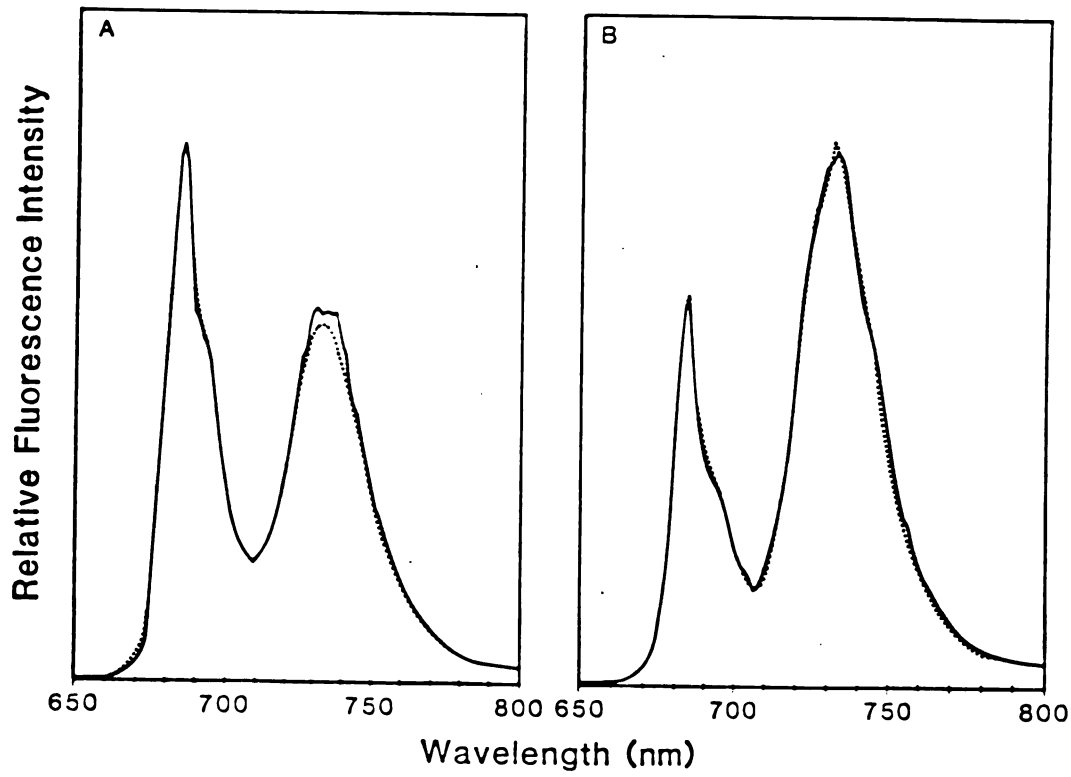

Figure 14. Chlorophyll fluorescence spectra of chloroplasts from mutant (---) and wild type (——) Arabidopsis in the presence (A) and absence (B) of $MgCl_2$ (5mM). The same preparation of chloroplasts were used for A and B.

to PSII fluorescence in the mutant was not significantly different from that of the wild-type.

A more extensive analysis of cation effects was performed by examining the effects of a range of NaCl concentrations on the photosynthetic lamellae. Incubation of thylakoids in increasing concentrations of NaCl in the range from 0 to 100 mM caused an essentially linear increase in the ratio of fluorescence at 685 nm to that at 734 nm (Figure 15). As noted above, a cation stimulated increase in PSII activity at the expense of PSI has previously been observed and is attributed to a decrease in energy spillover from PSII to PSI due to cation-induced changes in the spatial separation of PSI and PSII, which decreases the probability of exciton migration from PSII to PSI. In contrast to the results from the SDS-acrylamide gel experiments, no differential cation effect on the transfer of energy from LHCP to PSI in the mutant versus the wild-type was detected in the range of 0 to 100 mM NaCl (Figure 15). Energy transfer between LHCP and PSII and between the photosystems does not appear to be impaired as one might expect if LHCP structure was substantially altered (Staehelin and Arntzen, 1983)

Fluorescence Induction

In previous studies of the role of *trans*-$C_{16:1}$-PG, thylakoid membranes were depleted of *trans*-$C_{16:1}$ by treatment with phospholipase-A2 (Duval *et al*, 1979; Rawyler and Siegenthaler, 1981; Remy *et al*, 1984). Membranes treated in this way exhibited altered fluorescence induction kinetics which were interpreted as a reduction in the efficiency of light capture and the rate of plastoquinone

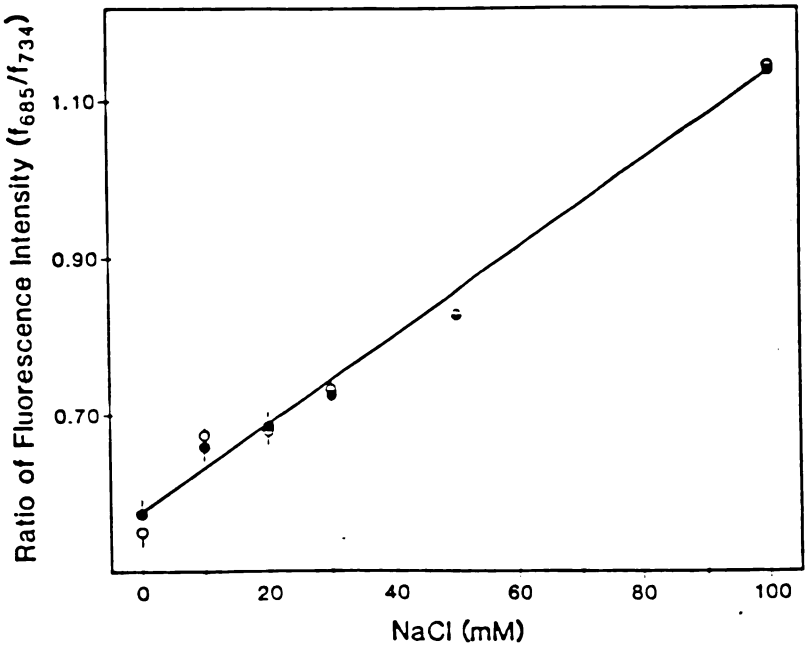

Figure 15. Ratio of fluorescence intensity from low temperature (77K) emission spectra of wild type (●) and mutant JB60 (○) thylakoids. Each point represents the mean ± SE (n=3).

reduction (Duval et al, 1979). To reexamine the relevance of these observations to understanding the role of trans-$C_{16:1}$-PG, the function of PSII and LHCP in wild-type and the mutant JB60 lacking trans-$C_{16:1}$-PG was compared by examining the kinetics of induction of room temperature fluorescence at 700 nm. Room temperature fluorescence primarily represents fluorescence emitted from PSII (Patterson and Arntzen, 1982). When electron transport is blocked with DCMU, the rise of the variable fluorescence (F_v) is a measure of the time required to close (cause a turnover of) all PSII reaction centers (thereby reaching maximal fluorescence, F_m). In this respect, the rise time of F_v is a relative measure of both the number of Chl active in transferring excitation energy to PSII reaction centers and of the efficiency of transfer. The minimum level of fluorescence, F_o, is due to emission from the antenna Chl of PSII which occurs before the excitation energy is trapped by the reaction centers (Butler, 1978).

F_o and the proportion of Chl active in photochemistry (F_v/F_o) appeared to be identical in the mutant and the wild-type in the absence of $MgCl_2$ (Table 12). Addition of 5 mM $MgCl_2$ to the membranes resulted in a dramatic increase in F_m due to Mg-induced changes in the spatial organization of the membranes (Staehelin and Arntzen, 1983) and a resulting decrease in the amount of spillover of excitation energy to PSI. The effect of cations on fluorescence characteristics of mutant and wild-type membranes was quantitatively and qualitatively indistinguishable under these conditions. These observations, in conjunction with previous studies showing that the mutant and the wild-type have indistinguishable rates of electron transport, suggest that the two genotypes have indistinguishable PSII photochemistry

Table 12

Room temperature fluorescence induction parameters of isolated thylakoids from mutant JB60 and wild type Arabidopsis in the presence and absence of 5mM $MgCl_2$.

Line	F_o	F_m	F_v/F_o
Wild Type (+Mg)	1164 ± 114	4746 ± 97	3.01 ± 0.14
JB60 (+Mg)	1122 ± 35	4735 ± 225	3.22 ± 0.07
Wild Type (-Mg)	1027 ± 35	2333 ± 71	1.27 ± 0.02
JB60 (-Mg)	1010 ± 13	2302 ± 89	1.28 ± 0.07

efficiency. This implies that the PSII antenna must be structurally similar. It is, therefore, apparent that the interpretation of previous studies employing lipase modification of membrane structure was confounded by the lack of specificity of the experimental approach (Duval et al, 1979; Rawler and Siegenthaler, 1981).

Effects of High Temperature on Fluorescence

Several authors have interpreted increases in Chl fluorescence which occur upon heating of leaves as an indicator of temperature-induced changes of photosynthetic membrane stability (Armond et al, 1978; Lynch and Thompson, 1984; Schrieber and Berry, 1977, see Appendix A). The heat-induced rise in F_0 which has been interpreted in terms of a breakdown in energy transfer from LHCP antenna pigments to PSII centers and related inhibition of photochemistry, has been taken as an indicator of the thermal stability of the PSII pigment system. More precisely, the fluorescence rise has been attributed to the physical separation of the LHCP from the PSII core, thereby blocking excitation energy transfer and leading to reemission of excitation energy from LHCP as fluorescence (Armond et al, 1978). The temperature at which enhanced fluorescence occurs may vary in response to environmental adaptation and appears to be affected by the lipid environment in which the proteins are embedded (Lynch and Thompson, 1984). In this respect, Chl fluorescence may be considered an intrinsic probe of lipid-protein interaction.

The effect of temperature on mutant and wild-type leaves was measured by appressing leaves to a temperature-controlled metal block

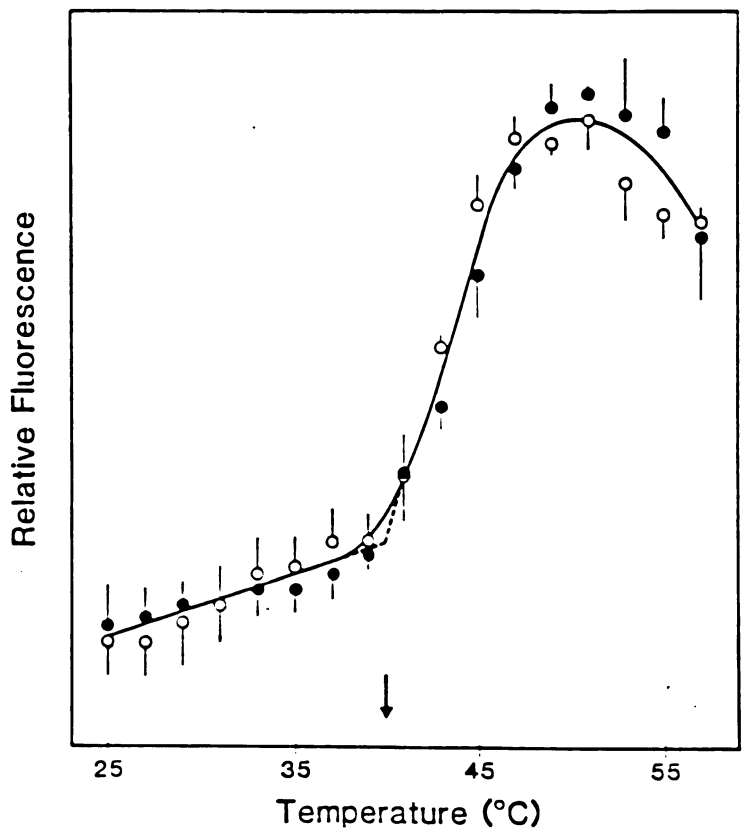

Figure 16. Temperature-induced fluorescence enhancement yield (F_o) of wild type (●) and mutant JB60 (○) leaves. Plants were grown at 21°C. The arrow indicates the threshold temperature at which fluorescence is enhanced. Each point represents the mean ±SE (n=4).

and measuring fluorescence continuously as the temperature of the heating block was increased from 25 to 56 C at a rate of about 1.5 C/min. At approximately 37 to 38 C a transition in the level of fluorescence was observed in both wild-type and mutants leaves (Figure 16). This response is similar to that observed by comparable experimental approaches with other species (Armond et al, 1978; Lynch and Thompson, 1984; Schrieber and Berry, 1977). There was no significant difference in the threshold temperature or magnitude of the fluorescence response of the mutant as compared to the wild-type. Thus, it does not appear that the absence of trans-$C_{16:1}$-PG has a significant effect on the thermal stability of the LHCP-PSII core association.

Discussion

The normal growth, chloroplast ultrastructure, and rate of photosynthesis in the fadA mutant grown under standard conditions indicate that the role of trans-$C_{16:1}$ is subtle. The most striking effect attributable to the mutation is the relatively reduced amount of LHCP oligomer which was recovered from mutant membranes following detergent-mediated thylakoid solubilization (Figures 8 and 9). This observation suggests a role for trans-$C_{16:1}$-PG in stabilizing the LHCP oligomer. The simplest hypothesis to explain the apparent instability of LHCP oligomer in the mutant would seem to be that PG containing trans-$C_{16:1}$ is more effective at preventing SDS from penetrating the subunit contact sites of the LHCP oligomer than PG alone . This is

consistent with the results from previous studies showing that phospholipase-A2 treatment of membranes leads to loss of the LHCP oligomer (Remy et al, 1982), and with studies showing that the LHCP oligomer extracted from SDS gels appears to be specifically enriched in PG containing trans-$C_{16:1}$ (Tremolieres et al, 1981). The results of experiments in which the presence of trans-$C_{16:1}$-PG in artificial liposomes enhanced the rate (but not the amount) of reconstitution of LHCP oligomer (Remy et al, 1984) also supports the concept that the lipid facilitates formation or stability of the LHCP oligomer. However, these observations are difficult to interpret since, for example, the addition of Triton X-100 to the SDS-solubilization buffer was also reported to increase the amount of $LHCP^1$ at the expense of $LHCP^3$ (Anderson et al, 1978). Similarly, the solubilization of thylakoids with the nonionic detergent octyl-B,D-glucoside rather than with SDS resulted in loss of the apparent trans-$C_{16:1}$-PG/$LHCP^1$ association (Henry et al, 1983). Also, analysis of the lipid composition of mechanically isolated stroma and grana lamellae revealed that the stroma lamellae, which contained low amounts of $LHCP^1$ had higher levels of trans-$C_{16:1}$-PG than PSII granal vesicles (Guillot-Salomon, 1982). Thus, it appears that the effect of the loss of trans-$C_{16:1}$-PG on $LHCP^1$ stability may reflect a nonspecific change in the overall properties of the photosynthetic lamellae rather than a specific effect on LHCP per se. The observation that the CP1a oligomer is also less stable in the mutant (Figure 9) lends credence to this view.

It is well established that cations stimulate thylakoid stacking (Izawa and Good, 1966), a process which involves LHCP (Staehelin and

Arntzen, 1983). Thus the possibility was considered that the differential cation enhancement of SDS-mediated dissociation of LHCP oligomer in the mutant was related to properties of the LHCP oligomer involved in bringing about membrane appression (Argyroudi-Akoyunoglou, 1981). We analyzed the effect of cations on LHCP function by measuring the effect of cation concentration on the efficiency of exciton transfer from LHCP to PSI (Figure 15). In principle, cation-induced dissociation of LHCP oligomers might be expected to lead to less efficient exciton transfer to PSI and, therefore, to increased fluorescence from LHCP at 685 nm. Although both mutant and wild-type showed a change in the ratio of PSI to PSII fluorescence, there was no significant difference between the two lines at any cation concentration. The absence of a differential effect of NaCl on the ratio of Chl fluorescence at 685 and 734 nm is considered evidence against an important role for trans-$C_{16:1}$ in conferring unique functional properties to the LHCP oligomer in vivo. Similarly, the absence of a differential effect of divalent cations on fluorescence induction kinetics in mutant versus wild-type (Table 12) indicated that the photochemical efficiency of PSII reaction centers are indistinguishable in the two lines. The effect of NaCl on the proportion of Chl found in $LHCP^1$ is, therefore, probably due to a stimulation of the activity of SDS rather than a specific effect on LHCP quaternary structure. We must conclude that the lipid has no significant in vivo effect on LHCP quaternary structure.

The apparent absence of an effect of the fadA mutation on PSI or PSII activity contrasts with the results of experiments involving lipase treatment of thylakoid membranes which were designed to examine

the role of trans-$C_{16:1}$. The lipase treatment was intended to exploit the head group and positional specificity of phospholipase-A2 to catalyze preferential removal of the acyl group at position two of PG and phosphatidyl choline (Rawler and Siegenthaler, 1981). In one study, phospholipase-A2 treatment increased the amount of light required to saturate the Hill reaction, decreased the variable fluorescence and increased the time required to reach maximal fluorescence (Duval et al, 1979). However, the implications of these observations were disputed by Rawyler and Siegenthaler (1981) who showed that both PG and phosphatidylcholine were affected by phospholipase-A2 to varying degrees, depending on the source of enzyme, and that PSII activity was severely depressed by phospholipase treatment. Whatever the reason for the effects of the lipase treatments the discrepancy between the functional properties of lipase-treated thylakoids and those of the fadA mutant illustrate the limited utility of lipolytic analysis in attempting to determine the functional significance of specific acyl groups.

The results of several studies have provided evidence that membrane lipid composition may exert an important influence on the stability of the association of LHCP with the PSII core (Armond et al, 1978;Lynch and Thompson, 1984; Schreiber and Berry, 1977). Indeed, on the basis of correlations between adaptive changes in lipid composition and the threshold for temperature-induced fluorescence, it has been suggested that trans-$C_{16:1}$-PG could play a role in mediating thermal stability of the LHCP-PSII complex (Lynch and Thompson, 1984). However, the absence of any differential effect of the fadA mutation on the threshold

temperature for temperature-induced fluorescence (Figure 16) renders a specific role for trans-$C_{16:1}$ in thermal adaptation untenable.

In conclusion, although we have independently reproduced the evidence for an effect of trans-$C_{16:1}$ on *in vitro* LHCP oligomer stability, we have not observed any functional significance associated with the absence of trans-$C_{16:1}$. On this basis we propose that trans-$C_{16:1}$-PG normally has no effect on the function of the photosynthetic lamellae. One possibility is the role is either restricted to an different environmental circumstance that we have not investigated, or to a specific phase of development. For example, since trans-$C_{16:1}$ accumulates concommitently with LHCP accumulation it seems possible that it facilitates insertion of proteins into the thylakoid membranes and thereby leads to more rapid membrane assembly.

The observation that the rate of $LHCP^1$ formation is enhanced in liposomes containing trans-$C_{16:1}$ (Remy *et al*, 1982) may be considered preliminary evidence in favour of this concept. Alternatively it is possible that this lipid is an element of the fine tuning mechanisms (Butler, 1978) which have evolved to optimize the efficiency of photosynthetic electron transport. This possibility would provide an example of the principle that many components of organisms may not be absolutly required, but serve very subtle functions which might only give a marginal selective advantage to the organism at best.

CHAPTER 5

A MUTANT DEFICIENT IN 18:3 AND 16:3 FATTY ACIDS

Introduction

The synthesis of -linolenate (18:3) in higher plants occurs by the sequential desaturation of stearate. The first double bond is inserted by a soluble chloroplast enzyme which utilizes stearoyl-ACP as its substrate and is closely associated with the fatty acid synthetase (Nagai and Bloch, 1968). The second and third double bonds are introduced only after the fatty acid has been incorporated into a glycerolipid molecule. As described in Chapter 1, glycerolipid synthesis is thought to involve two discrete pathways which have been designated the 'prokaryotic' and 'eukaryotic' pathways (Roughan and Slack, 1982). In this scheme, the 16:0 and 18:1 fatty acids synthesized de novo in the chloroplast may either enter the prokaryotic pathway in the chloroplast envelope or be exported as CoA esters to enter the eukaryotic pathway at extrachloroplast sites predominantly localized in the endoplasmic reticulum. In '16:3 species' such as Arabidopsis thaliana both pathways contribute to the production of chloroplast membrane lipids (Browse et al, 1986).

It is not yet apparent how many distinct desaturases are active in the leaves of 16:3 plants. Isolated intact chloroplasts of 16:3 plants are able to synthesize MGD by the prokaryotic pathway (Mckee and Hawke, 1978; Roughan et al, 1979), and 18:1 esterified to position sn-1 of this MGD is desaturated to 18:2 and 18:3. Similarly, 16:0 at position sn-2 of MGD is converted by sequential desaturations to 16:3 (Roughan et al, 1979). Desaturation of 18:1 and 18:2 on lipids synthesized by the prokaryotic pathway is not confined to MGD since [^{14}C]-18:1-PG synthesized by chloroplasts is sequentially converted to labelled 18:2- and 18:3-PG (Roughan, 1985). For lipids synthesized by the eukaryotic pathway, PC located in the endoplasmic reticulum is the predominant substrate for desaturation of 18:1 to 18:2 (Roughan and Slack, 1982; Slack et al, 1976). MGD of the eukaryotic pathway is thought to be the main substrate for the desaturation of 18:2 to 18:3 in leaves (Hawke and Stumpf, 1979; Roughan and Slack, 1982), although microsomal PC is probably the substrate for this reaction in developing seeds (Browse and Slack, 1981; Stymne and Appelquist, 1980). Unsaturated C-16 fatty acids are not produced to any extent by the eukaryotic pathway in leaves.

Although a broad outline of the pathways of lipid desaturation is available, many uncertainties remain. In particular, it is not yet established which lipids are substrates for desaturation, how many distinct desaturases exist, and whether 18:2 to 18:3 conversion occurs outside as well as inside the chloroplast. Given the predominance of trienoic fatty acids in leaf lipids and their suggested importance to photosynthesis and other plant functions (Gouraris and Barber, 1983; Quinn and Williams, 1983; Raison, 1980) it is important

to fully understand the operation and control of the fatty acid desaturases. However, each desaturation reaction is believed to involve the interaction of several membrane-bound components (Okayasu et al, 1981; Slack et al, 1976; Strittmatter et al, 1974) which have not yet been characterized.

This chapter describes the biochemical characterization of a group II mutant (fadD) which is deficient in 18:3 and 16:3 in the leaves and contains increased amounts of 18:2 and 16:2.

Results

Biochemical Characterization

The probable nature of the biochemical lesion in the fadD mutant is inferred from the observation that in leaves of plants grown at 26 C, the decreased amount of 16:3 and 18:3 fatty acids was accompanied by an increase of similar magnitude in the amounts of 16:2 and 18:2 (Table 7). Thus, although other possibilities are considered (see below and next chapter), the simplest hypothesis is that the mutant is deficient in a fatty acid desaturase which is normally responsible for introducing the double bond at position 15 of 18-carbon acyl groups and at position 13 of 16-carbon acyl groups. If this hypothesis is true the site for insertion of these double bonds is determined relative to the methyl end of the chain since it is n-3 in both cases. Furthermore, it seems likely that this desaturase is located in the chloroplast because chloroplast MGD is believed to be the substrate for 16:3 synthesis (Roughan et al, 1981).

As noted in Chapter 1 during the preliminary characterization of the mutant we observed some variability in the proportion of both 18:3 and 16:3 from one experiment to another. Analysis of the effects of various environmental influences led to the recognition that the amount of these fatty acids is dramatically affected by temperature in the mutant line but is much less affected in the wild-type (Figure 5). When grown at 18 C the fatty acid composition of the mutant is similar to that of the wild-type. By contrast, when grown at 26 C the mutant has only about 35% as much trienoic fatty acids (ie., 16:3 + 18:3) as the wild-type grown at the same temperature (Figure 5). As noted in a later section, the trienoic acid synthesis which occurs above 26 C may be due to the action of a second desaturase. The simplest explanation for this observation is that the genetic lesion in the fadD mutant renders a desaturase or a regulator of the desaturase(s) temperature sensitive so that it is almost normally functional at low temperatures but is largely inoperative at temperatures above about 26 C.

To further characterize the temperature effects on trienoic acid synthesis in the fadD mutant, temperature shift experiments were performed in which plants were grown at nonpermissive conditions for trienoic acid desaturation (28 C) for three weeks and then shifted to permissive conditions (19 C). The new synthesis of 16:3 can be detected within 48 hours after shifting and in the case of 18:3, changes in the total pools can be detected as early as 18 hours. (Figure 17) The difference in the rate of recovery of 16:3 versus 18:3 can not be explained at this time due to the lack of any knowledge of the product of the fadD gene or the kinetics of desaturation. However, it could

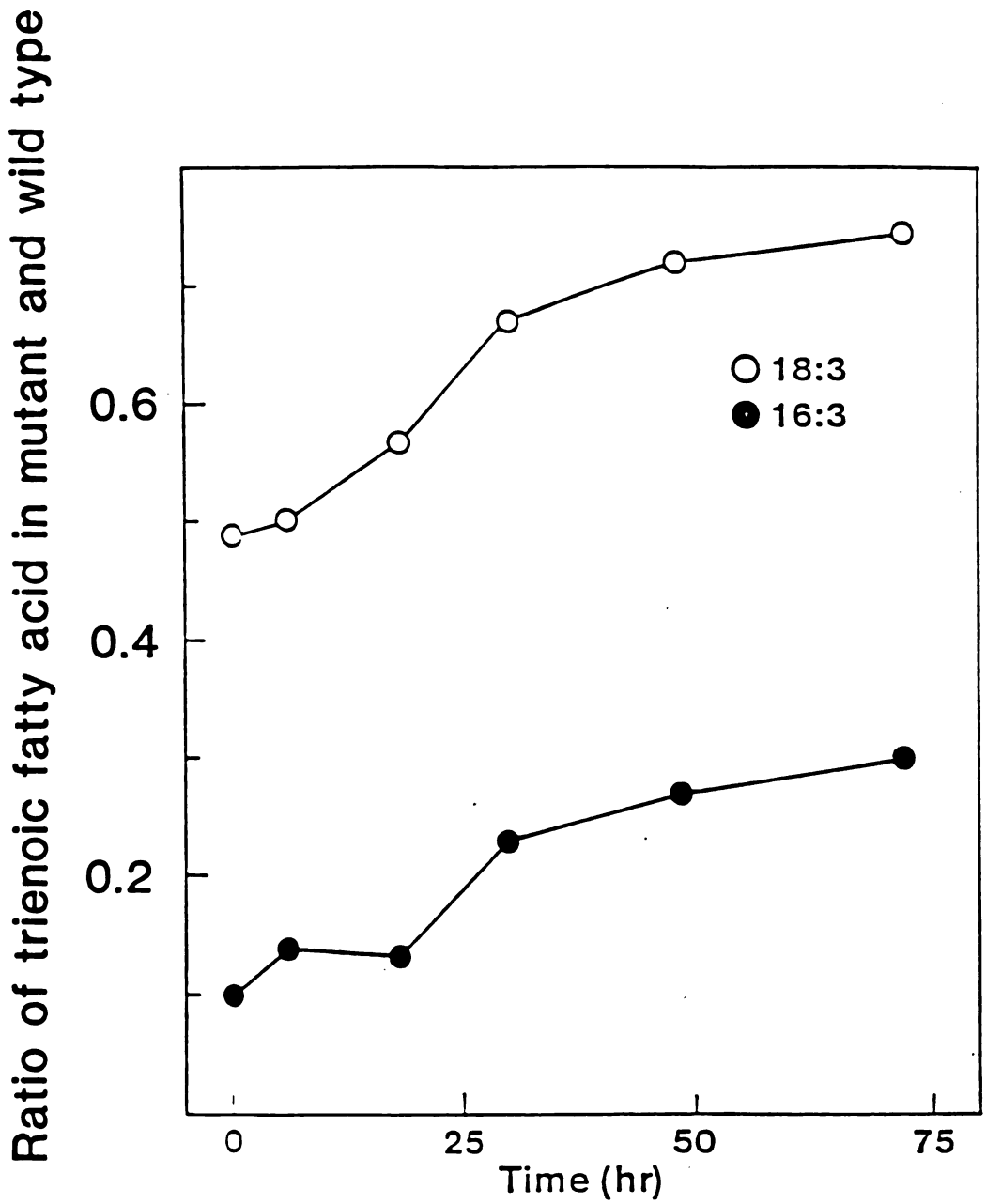

Figure 17. Ratio of the amount of trienoic fatty acids in mutant line JB1 and wild type after shifting plants from 27 C to 19 C

reflect different substrate specificities of the n-3 desaturase for 16:2 and 18:2.

The rapid increase in 18:3 synthesis upon shifting the mutant to 19 C is of interest with respect to questions concerning sites of synthesis and final location of trienoic acid. Although the thylakoid lamellae, which are highly enriched for 18:3 and 16:3 (Table 13), account for almost 80% of all membranes in higher plants (Harwood, 1980), it is believed that the major site of synthesis of these fatty acids is the chloroplast envelope (Douce and Joyard, 1980). If this is true, upon shifting the fadD mutant to permissive conditions the thylakoid membranes must import large amounts of 18:3 and 16:3 from the envelope. Unless there is an exchange of fatty acids out of the thylakoids for the incomming trienoic acids, this mechanism would demand massive membrane growth. Alternatively the thylakoid membrane may also contain n-3 desaturation activity which is affected by the fadD mutation and hence the desaturation of 18:2 may occur directly in the thylakoid without lipid transfer.

Fatty Acid Composition of Individual Lipids

Analysis of the fatty acyl composition of individual lipids from leaf tissue of mutant and wild-type plants grown at 28 C revealed that all the major polar lipids are affected by the fadD mutation (Table 14). However, the proportions of various polar lipids in the extracts were essentially the same for the mutant and the wild-type. The decreased levels of 18:3 and 16:3 and an increase in the corresponding dienoic fatty acids are the most striking differences between the wild-type and the fadD mutant. In addition, the amount of 18:1 appears

Table 13

Fatty acid composition of wild type and JB1 thylakoid membranes from plants grown at 28 C

	Fatty Acid Composition (mol %)	
Fatty Acid	WT	JB1
16:0	10.5	9.2
t16:1	4.8	4.4
c16:1	0.8	1.0
16:2	2.6	13.8
16:3	13.8	1.4
18:0	0.9	0.7
18:1	2.4	4.5
18:2	7.4	37.7
18:3	46.3	20.7

Table 14

Fatty Acid compositions of leaf lipids from wild-type and mutant JB1 *Arabidopsis*, grown at 28°C. Values presented are mol %. Dashes indicate that the acyl group was not detected.

Lipid	MGD		DGD		SL		PG		PC		PE		PI	
	WT	JB1	WT	JB1	WT	JB1	WT	JB1	WT	JB1	WT	JB1	WT	JB1
16:0	1.8	1.9	13.4	15.2	36.4	39.9	47.9 [a]	48.2 [a]	28.0	24.9	31.2	28.7	44.9	44.4
16:2	1.5	23.0	--	3.5	--	--	--	--	--	--	--	--	--	--
16:3	25.8	6.9	1.9	0.4	--	--	--	--	--	--	--	--	--	--
18:0	0.2	0.2	1.1	1.1	2.8	0.9	0.2	1.3	2.4	2.7	2.9	2.5	2.7	2.4
18:1	0.8	1.7	1.1	2.1	3.9	4.0	3.0	9.2	2.6	4.6	2.8	2.9	2.3	1.8
18:2	4.6	30.9	6.7	37.5	13.2	25.5	13.5	22.7	28.6	45.9	37.2	51.8	24.1	35.0
18:3	65.7	34.4	76.5	40.0	43.7	29.7	34.5	18.5	38.4	21.9	26.0	14.2	26.1	16.1
Proportion of leaf polar lipids (%)	32.7	35.6	18.5	18.9	4.0	3.5	8.8	7.5	19.2	18.2	9.9	10.3	6.9	6.2

a. Includes 16:1 *trans*

to be slightly increased in some lipids of the mutant raising the possibility that the increased amount of 18:2 partially inhibits the desaturation of 18:1 to 18:2.

These results suggest that a single enzyme may synthesize trienoic fatty acids for a variety of different lipids. The effect on those lipids which are thought to be desaturated in the chloroplast (ie., MGD, DGD, SL and PG) suggests that a chloroplast desaturase which does not show head group specificity is affected. However, PE and PI are largely or entirely extrachloroplast lipids (Mazliak, 1977) and these also show reduced 18:3 levels in the mutant (Table 14). Thus, either the fadD gene product regulates n-3 desaturase activities in more than one cellular compartment, or there is substantial movement of trienoic acyl groups between the chloroplast and extrachloroplast membranes. The striking thing about these results is that the ratio of 18:3 in the mutant to that in the wild-type is reduced to the same amount (0.57+0.06) in all of the lipid classes. This clearly indicates that the amount of 18:3 in all of the lipid classes is regulated by a common mechanism which is in some way controlled by the fadD gene. Similar results were obtained for plants grown at 23 C except that in this case the ratio of 18:3 in each lipid of the mutant to that in the wild-type was 0.71+0.02. It is noteworthy in this respect that the mutation resulted in a greater reduction in the amount of 16:3 than of 18:3. The proportion of 16:3 in MGD from the mutant grown at 28 C was only 27% of that found in MGD from wild-type plants (Table 14).

From the results in Table 14 it can be inferred that the fadD mutation reduces the level of desaturation of fatty acids at both the sn-1 and sn-2 position of glycerolipids. Position sn-2 is obviously

affected because 16:3 occurs only at this position on MGD (Browse et al, 1986). Similarly, chloroplast PG, which represents more than 85% of total leaf PG (Browse et al, 1986), contains 18:3 only at position sn-1. Since the amount of 18:3 on chloroplast PG is strongly reduced in the mutant it is apparent that the desaturation of acyl groups at the sn-1 position is affected by the mutation. Direct evidence that both the sn-1 and sn-2 positions of MGD and DDG are affected by the fadD mutation has been obtained by quantitative analysis of the acyl group composition of these lipids (Norman and St. John, 1986).

Lipid Composition Of Roots, Seeds And Callus Tissue

In order to determine if the fadD gene product is normally active in tissues other than leaves, the fatty acid composition of roots, mature seeds and callus induced from leaf tissue of the wild-type and the mutant grown at 23 C were compared. In these tissues, plastid membranes comprise a relatively small proportion of the total cellular lipids and a chloroplast n-3 desaturase would not be expected to be important in determining the fatty acid composition of these tissues. From the results of this experiment (Table 15) it appears that the fadD mutation is not expressed in these tissues since both roots, seeds and callus of the mutant have fatty acid compositions which are indistinguishable from the corresponding tissues of the wild-type. By contrast, the leaves of plants grown at this temperature show a pronounced decrease in the proportion of trienoic acids (Table 7).

Interestingly the callus which has no 16:3 fatty acids has roughly 55% 18:3 in both mutant and wild-type tissue (Table 15). The lack of 16:3 and the large amount of 18:3 impies that all linolenic acid

Table 15

Fatty acid composition of roots, seeds and callus tissue of wild type and JB1 Arabidopsis grown at 23 C

	Fatty Acid Composition (mol %)					
	Roots		Seeds		Callus	
Fatty Acid	WT	JB1	WT	JB1	WT	JB1
16:0	19.7	19.2	7.9	7.9	15.3	14.9
t16:1	-	-	-	-	-	-
c16:1	1.7	1.3	0.5	0.4	0.1	0.3
16:2	-	-	-	-	-	-
16:3	-	-	-	-	-	-
18:0	1.8	1.9	3.2	3.0	2.5	2.7
18:1	6.5	6.3	18.0	18.3	8.0	6.9
18:2	46.6	47.1	30.4	30.7	18.8	19.0
18:3	20.6	21.3	18.7	18.9	55.6	56.6
20:1	1.7	1.9	20.9	20.5	-	-

synthesis in callus is through the eukaryotic pathway. Alternatively some specific regulator of 16 carbon but not 18 carbon desaturation may be missing which is normally found in leaf tissue and both pathways of 18:3 synthesis are still operative. In this respect the callus tissue in both genotypes has a similar fatty acid composition to the Group 3 mutants (Chapter 3). These mutants lacked or had strongly reduced levels of 16:3 fatty acids in the leaves with proportional increases in the 18 carbon fatty acids. Whatever the reasons for this difference the fact that different tissue types express different desaturases will be of importance in understanding regulation of lipid synthesis in other plant tissues.

Discussion

The higher plant fatty acyl desaturases, with the exception of the stearoyl-ACP desaturase (Nagai and Bloch, 1968), are membrane-bound enzymes which are thought to accept glycerolipids as substrates (Browse and Slack, 1981; Roughan et al,1979; Slack et al, 1976; Stymne and Applquist, 1980). Although the subunit composition of these desaturases is not established, it seems probable that the activity of the desaturases may require the cooperation of electron transport components and associated reductases (Murphy et al,1983; Okayasu et al,1981; Strittmatter et al, 1974). Because the technical problems associated with solubilizing, purifying and reconstituting the plant enzymes have not yet been overcome, it was not possible to characterize the fadD mutant by direct enzyme assay. Nevertheless, the results presented here suggest that at temperatures above about 26 C the mutant line JB1 is deficient in activity of a desaturase(s) which is

responsible for inserting the final double bond during the synthesis of both 16:3 and 18:3 fatty acids.

It is possible that the primary effect of the fadD mutation is on some cellular component which is non-specifically required for the operation of one or more n-3 desaturases rather than affecting the primary structure of a desaturase per se. However, since it is probable that all the chloroplast glycerolipid desaturases make use of the same ancillary proteins, it seems unlikely that the defect is in one of these since the other desaturations are not affected.

An alternative hypothesis is that a specific structural or regulatory component of an n-3 desaturase has been made thermolabile by the mutation. Many examples of temperature-sensitive enzymes are known from similar genetic studies of microorganisms such as yeast and Escherichia coli. Interestingly in these systems mutations specifically affecting the structural gene of the desaturase also show no chain length specificity (Henry, 1982). An independent (nonconditional) mutation of the fadD locus is probably required to distinguish between this and other possibilities.

The observation that all the chloroplast polar lipids are affected in the mutant suggests that the desaturase controlled by the fadD gene acts on acyl chains with no apparent specificity for the point of attachment to the glycerol backbone (sn-1 or sn-2) or for the lipid head group. The results in Table 14 show that the ratio of 18:3 in the mutant to that in the wild-type is 0.57 $\pm$ 0.06 for all lipid classes. This striking similarity in the magnitude of the effect on all the lipid classes implies that the amount of 18:3 in all lipid classes is controlled in some way by the product of the fadD locus. This

observation would seem to have very important implications in understanding the regulation of membrane lipid desaturation. We have not observed any effect of the fadD mutation on the relative proportion of the various lipid classes in more than 10 independent batches of plants. However, in a complementary study in which different growth conditions were used (Norman and St John, 1986), the fadD mutant was observed to have reduced amounts of DDG. This potentially interesting observation raises the possibility that under some circumstances the amount of DDG is regulated by acyl group composition of MGD, the presumed precursor of DDG.

Since 16:3 is thought to be synthesized only in chloroplasts (Roughan and Slack, 1982), the desaturase affected by the fadD mutation must be present in the chloroplast. However, our results clearly indicate that both chloroplast and extrachloroplast lipids are affected by the fadD mutation. This is most clearly evident from the results of the membrane fractionation studies showing directly that extrachloroplast membranes have reduced amounts of 18:3. These findings may be explained in one of several ways. First, the mutation may affect the activity of a single desaturase which is active both inside and outside the chloroplast. This seems somewhat unlikely since nuclear encoded chloroplast proteins normally contain a leader sequence which results in transport into the chloroplast (Ellis, 1981). There are, however a number of cases where a single gene product is responsible for enzyme activity in two cellular compartments (Hopper et al, 1982; Natsoulis et al, 1986; Pratje and Guiard, 1986). It is also possible that the fadD gene controls two distinct isozymes of an n-3 desaturase which are located in the chloroplast and the

cytoplasm, respectively. This hypothesis can not be excluded at present.

Alternatively, it is possible that 18:3 (but not 16:3) fatty acids produced in the chloroplast are transferred to other cellular sites (Figure 18). The present formulation of the two pathway hypothesis for glycerolipid synthesis (Browse et al, 1986; Roughan and Slack, 1982) allows for the export of 18:1 and 16:0 fatty acids from the chloroplast as CoA esters, while mainly 18:2 is reimported in the form of glycerolipid, probably by means of a phospholipid transfer protein (Dubacq et al, 1984). If 18:3 formed by a chloroplast desaturase is normally exported to extrachloroplast membranes it might be mediated by reverse action of the phospholipid transfer protein or via hydrolysis of lipids followed by the formation of 18:3-CoA in the chloroplast envelope (Douce and Joyard, 1980). In either case 16:3, which is the second most abundant fatty acid in the chloroplast, must be specifically excluded since it is not normally found in the extrachloroplast lipids.

The level of 18:3 in the total leaf lipids of the mutant is not reduced as much as the level of 16:3 (Table 7). This might, in principle, be due to a differential effect of the mutation on the ability of the enzyme to catalyze 16:2 versus 18:2 desaturation. This idea is supported by temperature shift experiments which show a different rate of synthesis for 16:3 versus 18:3. Alternatively these observations may suggest that a second 18:2 desaturase is active in Arabidopsis leaves which either does not recognize 16:2 as a substrate or is not in the chloroplast. One possibility for this second enzyme is a microsomal 18:2-PC desaturase similar to the enzyme found in oilseeds

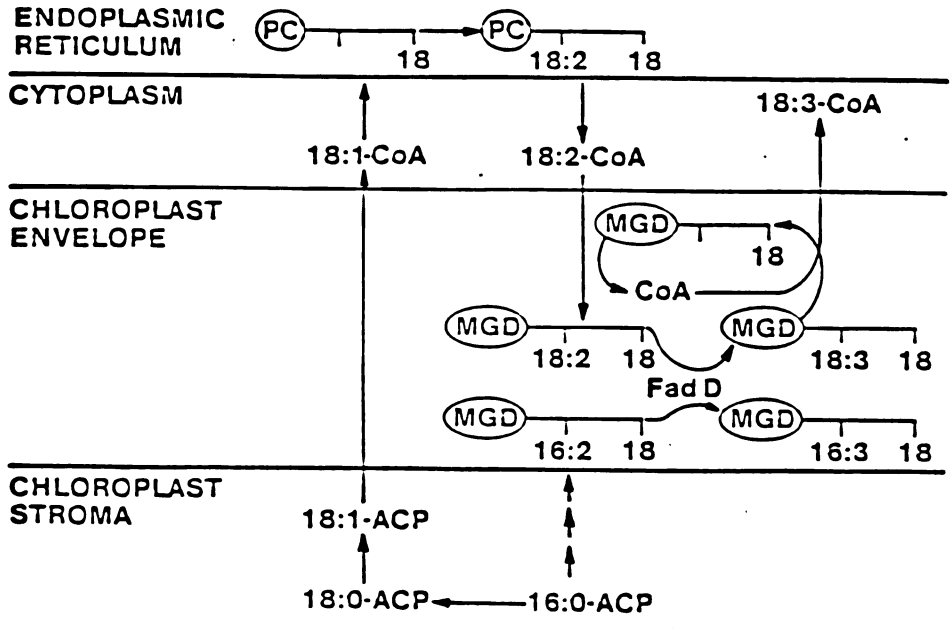

Figure 18. Possible mechanism of 18:3 exchange and transport from the chloroplast for acylation to cytoplasmic lipids. Upon return of 18:2 from the cytoplasm this species exchanges with an 18:3 fatty acid acylated to MDG. The 18:3-CoA is then free to go to the endoplasmic reticulum. The reduced amount of 18:3 and increased 18:2 due to the fadD mutation does not effect the rate of exchange.

(Browse and Slack, 1981). Indeed, such an enzyme appears to be active in non-photosynthetic tissues of Arabidopsis since the roots, seeds and callus of JB1 plants have fatty acid compositions which are indistinguishable from those of the same tissues of wild-type plants (Table 15). However, because there is still some 16:3 in leaves of the mutant at all temperatures, it appears that the fadD mutation described here is somewhat leaky. Thus, it is not possible to unequivocally confirm the existence of a second n-3 desaturase in Arabidopsis leaves or to deduce whether that enzyme has a different location or a different substrate specificity from the fadD gene product described here.

CHAPTER 6

PHYSIOLOGICAL CONSEQUENCES OF UNSATURATION

Introduction

Chloroplast membranes of all higher plants contain a high proportion and selective distribution of trienoic acids (Harwood, 1980). These observations in conjunction with the tight coupling of trienoic acid synthesis during light-induced chloroplast development has stimulated substantial interest in the role of these fatty acids in the chloroplast (Gounaris and Barber, 1983; Quinn and Williams, 1983; Raison, 1980). However with few exceptions this enthusiasm has not led to a clear understanding of the functional significance of these fatty acids in plants (Quinn and Williams, 1983).

The high level of unsaturation, as expected, provides an extremely fluid bilayer compared to other biological membranes and this observation has led to the suggestion that such an enviroment is essential for efficient electron transport and other diffusional processes. A number of studies have been attempted to test this hypothesis by artifically reducing thylakoid membrane unsaturation to various degrees using catalytic hydrogenation and then assaying

electron transport and light capturing ability (Vigh et al, 1985; Restall et al, 1979; Siefermann-Harms et al, 1982). The results, however, appear to be dependent on the method of hydrogenation and therefore inconclusive. For example, using a water soluble hydrogenating catalyst to reduce thylakoid membrane unsaturation, lowered the rates of whole chain electron transport (Vigh et al, 1985). However, similar experiments with a water-insoluble catalyst which gives the same overall reduction in unsaturation showed no effect on electron transport rates (Restall et al, 1979). These conflicting results and the fact that physical studies on artifical membranes have shown that the removal of the third double bond from the acyl side chain of a lipid does not dramatically affect the fluidity of the membrane further suggests that the major role of trienoic acids is not directly related to membrane fluidity.

Recent studies using hydrophobic fluorescence probes to estimate membrane fluidity have shown that the stromal lamellae are more fluid than the granal regions of the thylakoids (Ford et al, 1982). Since the fatty acid composition of the two lamella types are very similar, the difference has been attributed to the higher protein/lipid ratio of the grana versus the stroma (Ford et al, 1982). Moreover plants appear to alter their protein/lipid ratio rather than thylakoid fatty acid composition in response to temperature changes (Chapman et al, 1983). Although increasing the protein/lipid ratio brings about an ordering effect on membranes which results in a less fluid bilayer (Stubbs and Smith, 1983), the argument for altering protein/lipid ratios rather than unsaturation under various environmental conditions is based on the theoretical considerations of the molecular structure of MGD in

thylakoids. MGD, containing two trienoic fatty acids cannot form bilayers unless appropriately balanced with bilayer forming lipids such as DDG, PG, or PC. Although the role of nonbilayer forming lipids is not known, the observation that the inner surface of the thylakoid membrane has led to the proposal that these lipids help stabilize irregularly shaped regions of the membrane which have resulted from the insertion of protein - pigment complex (Murphy, 1983). This stabilization is essential for thylakoid integrity since roughly 80% of the surface area of this membrane is due to protein (Murphy, 1983). The observation that n-3 desaturation is correlated with chloroplast development and studies on the reconstitution of LHCP into saturated and unsaturated bilayers futher supports this view (Leech and Leech, 1976; Siefermann-Harms et al, 1982).

Other evidence pertaining to the function of trienoic acids has come from studies on the effects of sublethal doses of the herbicide SAN9785 applied to barley seedlings (Leech et al, 1985). This herbicide, which causes a reduction in the amount of 18:3 and a corresponding increase in the 18:2 content of treated plants, appears to cause both ultrastructural and photosynthetic changes (Leech et al, 1985). The ratio of granal to stromal lamellae increased and although primary photochemistry still occurred, both energy transfer between the photosystems and noncyclic electron transport were altered.

It is now widely accepted that the organization of the photosynthetic apparatus is intimately related to chloroplast ultrastructure (Anderson and Melis, 1983). It is thought PSI is located in the stromal or nonappressed region and PSII-LHCP are situated in the granal or appressed regions of the thylakoid. From this model of

chloroplast organization it was concluded that the altered energy transduction between the two photosystems was the result of the redistribution of the thylakoid lamellae which in turn was caused by the reduced 18:3 content.

In order to examine the the above models and other possible roles of trienoic acids in chloroplast structure and development a physiological and biochemical study of photosynthesis was carried out on the fadD mutant.

Results

Growth Rate of Mutant and Wild-Type.

Because of the widespread interest in the possible role of membrane lipid composition on thermal adaptation we examined the effect of temperature on growth of the fadD mutant line JB101 and the wild-type. Under the conditions of this experiment both the mutant and the wild-type had maximal growth rates at approximately 27 C and had very similar growth rates at all temperatures measured (Figure 19). Since the fadD mutation only affects lipid composition at temperatures of about 25 C or greater this observation does not permit an assessment of the role of trienoic acid composition at temperatures below this threshold.

Effects of Trienoic Acid Composition on Chl, Protein and Lipid Content.

When grown at temperatures above 25 C the leaves of the mutant line were chlorotic by comparison with the wild-type due to a 15% reduction in Chl g fwt^{-1} and 40% reduction in Chl per unit leaf area in the mutant (Table 16). Since all Chl is believed to be associated with

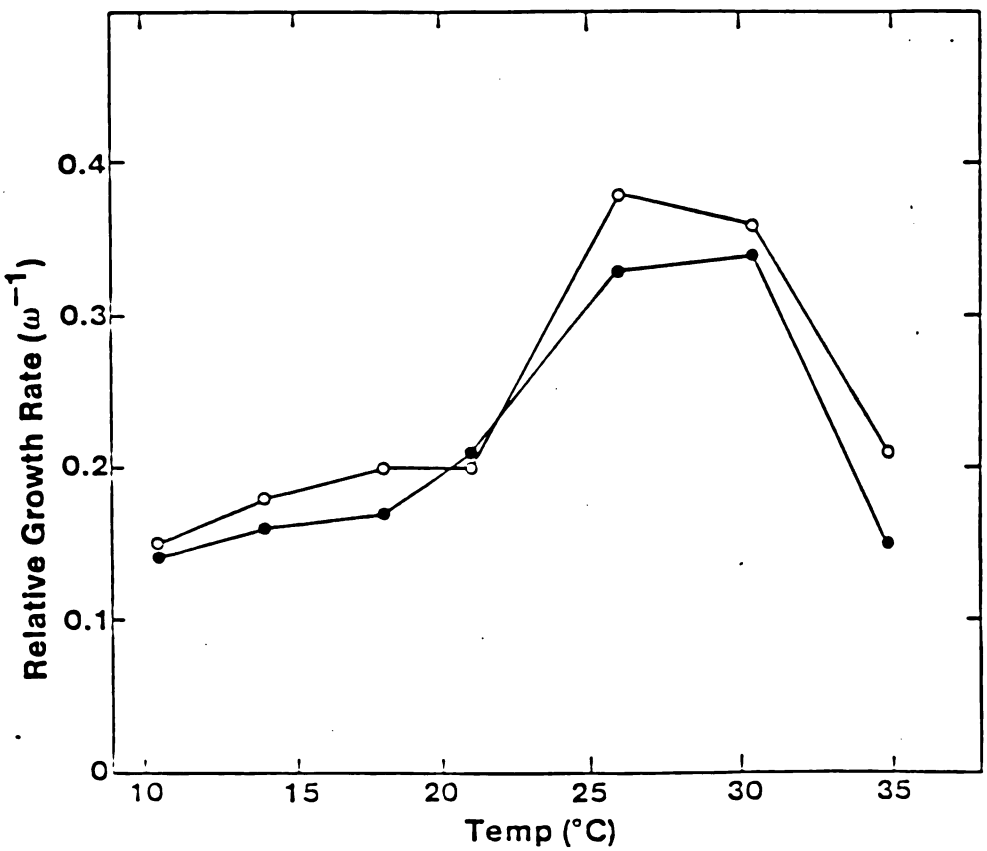

Figure 19. Effect of temperature on the relative growth rate of wild type (●) and mutant (○) Arabidopsis.

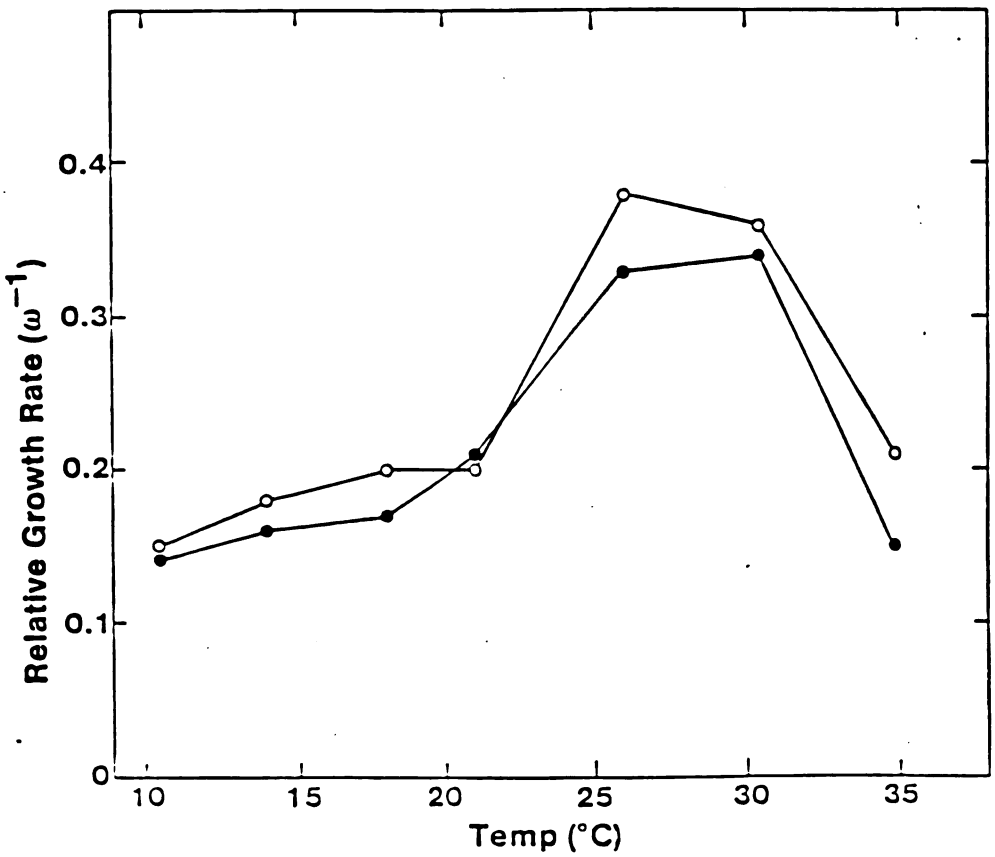

Figure 19. Effect of temperature on the relative growth rate of wild type (●) and mutant (○) Arabidopsis.

Table 16

Relative amounts of Lipid, Protein, and Chl in mutant JB1 and wild type *Arabidopsis* leaves and thylakoids

	WT	JB1
chl a/b ratio	3.42 ± 0.04	3.65 ± 0.12
chl/leaf area (ug/cm^2)	21.27 ± 2.21	13.74 ± 1.74
chl/fwt (ug/mg)	1.62 ± 0.10	1.39 ± 0.04
Total lipid/chl (ug/ug)	3.42 ± 0.08	3.47 ± 0.11
Total lipid/fwt (ug/mg)	3.84 ± 0.11	3.44 ± 0.13
Thylakoid Lipid/chl (ug/ug)	2.93 ± 0.08	2.73 ± 0.05
Extra chloroplast lipid/protein (ug/ug)	0.25 ± 0.03	0.26 ± 0.03
$\frac{\text{Chloroplast Lipid}}{\text{Extrachloroplast lipid}}$ (g/g)	2.53 ± 0.40	1.67 ± 0.05

thylakoid proteins (Markwell et al, 1979), the Chl content of higher plants is generally a good measure of Chl-binding protein complexes, such as the Chl a/b binding protein. Futhermore since each of these complexes has a distinct stoichiometry of Chl to protein, the observation that the Chl a/b ratio is not significantly different between the mutant and wild-type (Table 16) suggests that no one complex is affected.

Although the lipid/Chl ratio remained constant in the mutant compared to the wild-type a 15% reduction in total lipid was observed on a fwt basis (Table 16). These results suggest the mutant has less total membrane per cell. In order to examine whether both chloroplast and extra-chloroplast lipids were equally affected these two membrane fractions were separated by differential centrifugation and quantitated. Although the Chl/chloroplast lipid and protein/non chloroplast lipid ratios were similar between the two genotypes the chloroplast/nonchloroplast lipid ratio was reduced approximately 30% in the mutant (Table 16). The reason for the reduction of the overall amount of chloroplast membrane is not clear but the unaltered Chl/lipid ratio in the chloroplast suggests that the stoichiometry of these two components is maintained. Coupled regulation of Chl and glycerolipid synthesis has recently been observed in studies using Chl biosynthesis inhibitors (Kosmac and Feierabend, 1985).

To determine if the reduction in Chl was related to the fadD mutation, 44 F_2 plants from a wild-type X JB101 cross were tested for cosegregation of these phenotypes. Of the 10 plants which showed reductions in 18:3 and 16:3 levels all had a 10-15% reduction in Chl on a fwt basis. The 34 plants with normal or intermediate levels of

trienoic acids had normal levels of Chl. Furthermore all ten mutant lines gave a chlorotic phenotype in the next generation indicating that they were homozygous for the relevant mutation. The co-segregation of the alteration in fatty acid composition with reduced Chl levels suggests the two biochemical changes are due to the same mutation.

A further substantiation of the relationship between fatty acid composition and chlorosis was indicated by a pronounced temperature effect on Chl levels in the mutant. The fadD mutation affects fatty acid composition at temperatures above 25 C but has no major effect at temperatures below 18 C. Thus, the effect of temperature on Chl content was tested by growing plants at 27 C to allow full expression of the fadD phenotype. Plants were then shifted to 19 C to allow recovery of normal trienoic acid levels. During this permissive period the Chl/fwt ratio slowly returned to wild-type levels (Figure 20A) and the amount of trienoic acid increased to near wild-type levels (Figure 20C). By contrast, plants maintained at 27 C showed no change in fatty acid composition (Figure 20D) or Chl/fwt ratios (Figure 20B) over the same period. The observation that changes in both trienoic acid and Chl content are responsive to temperature suggests both are caused by the same mutation.

Chloroplast Ultrastructure.

The overall reduction in Chl and lipid levels in chloroplast membranes per cell in the mutant was unexpected since previous reports in which fatty acid compositions had been modified with SAN9785 did not show these effects (Leech at al, 1985). Morphometric analysis of electron micrographs of chloroplasts from JB1 and wild-type showed

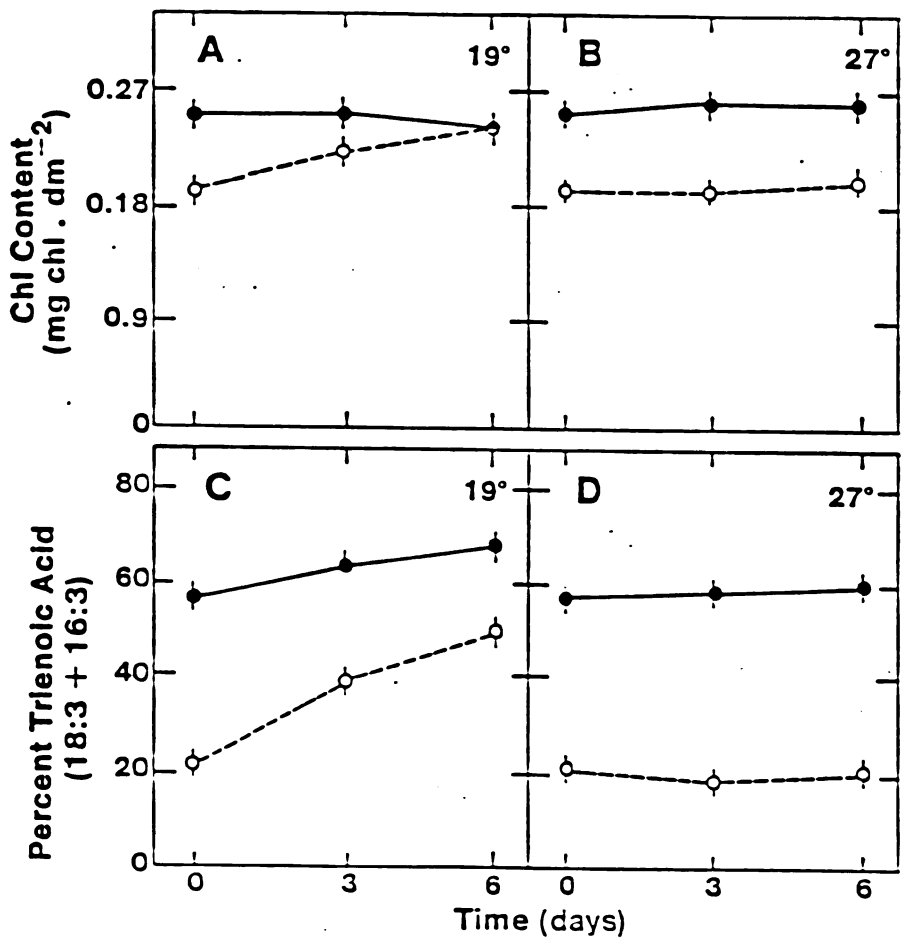

Figure 20. Chl and trienoic acid content following a shift of wild-type (●) and mutant (○) plants from 19 C to 27 C. Plants grown at 27 C were transferred to 19 C (A and C) or maintained at 27 C (B and D). Each point represents the mean ± SD (n=3).

similar but less dramatic changes in thylakoid ultrastructure to those observed in SAN9785 treated barley seedlings (Leech et al, 1985) (Table 17). The granal width was increased 20% with a similar reduction in stromal thylakoids and a slight reduction in grana/plastid. However the most striking feature of the morphometric analysis was the overall reduction in plastid size (Table 17). In the mutant, chloroplasts were roughly half the size of wild-type and both granal and stromal membranes were reduced to approximately 73% and 64% of wild-type amounts, respectively. This overall reduction in thylakoid and envelope membrane in the mutant is consistent with the lipid, Chl, and protein ratios shown in Table 16.

To determine if the smaller chloroplast size was compensated to any degree by increased chloroplast number, protoplasts were isolated from both wild-type and fadD leaves and the number of chloroplasts per protoplast were counted under a light microscope (Table 18). The mutant appears to have more chloroplasts per protoplast than wild-type raising the possibilty that smaller chloroplasts are the result of increased chloroplast divisions.

Photosynthetic Studies.

A preliminary test of the effects of the reduced trienoic acid content on photosynthesis was performed by measuring photosynthetic gas exchange in mutant and wild-type plants at various light intensities (Figure 21). Although CO_2-fixation rates of mutant and wild-type were indistinguishable when expressed on a Chl basis at all light intensities, the mutant showed a 20% reduction in CO_2-fixation rates when measured on the basis of fwt (Figure 21,Table 19). This is

Table 17

Morphometric analysis of chloroplasts from mutant JB1 and wild type Arabidopsis. Measurements were made on 20 chloroplasts from each line.

	WT	JB1
grana/plastid	54.1 ± 13.0	37.2 ± 8.2
thylakoids/granum	5.5 ± 3.3	4.8 ± 2.5
granal width (um)	0.40 ± 0.03	0.48 ± 0.03
stroma thylakoids/plastid (um)	67.0 ± 20.	55.7 ± 14.2
stroma thylakoid length (um)	0.26 ± 0.03	0.20 ± 0.03
total grana (um/plastid)	119.0	85.7
total stroma (um/plastid)	17.4	11.1
total thylakoids (um/plastid)	136.4	96.8
grana/stroma	6.8	7.7
surface area (um^2/plastid)	9.7 ± 2.0	5.3 ± 1.6

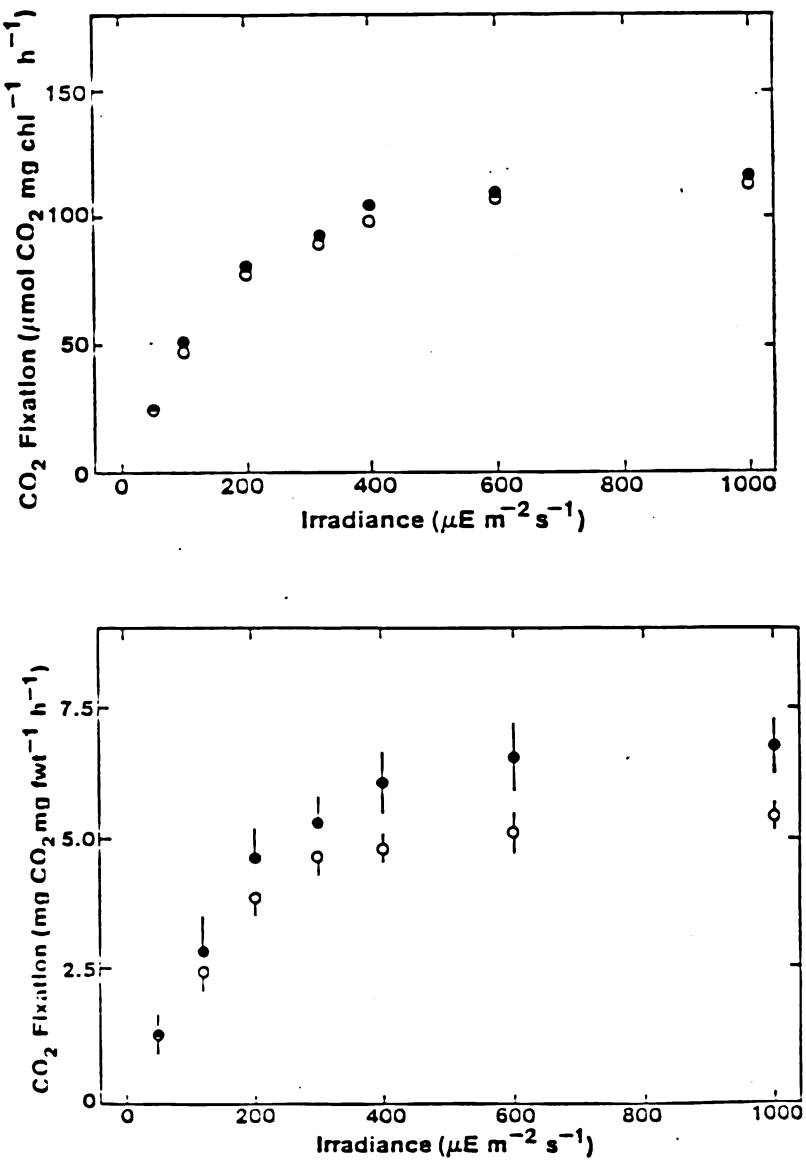

Figure 21. CO_2 fixation rates versus light intensity for mutant JB1 (O) and wild type (●) Arabidopsis on a chlorophlyll and fresh weight basis. Each point represents 3 plants.

Table 18

Number of chloproplast per protoplast in mutant JB1 and wild type *Arabidopsis* grown at 19 C and 27 C. (n = 50)

Temperature	WT	JB1
19 C	34.6 ± 11	32.4 ± 10
27 C	40.1 ± 15	57.9 ± 25

consistent with the overall reduction in the photosynthetic membranes in the mutant.

In order to measure the effect of lipid composition on the light reactions catalyzed by isolated thylakoids, plants were grown at 27 C to ensure maximum reduction of trienoic acid content and then assayed at various temperatures. Whole chain electron transport rates were very similar in mutant and wild-type at 6 C, 14 C and 25 C suggesting reduced unsaturation is not a rate limiting factor in plastoquinone diffusion (Table 19). Uncoupled PSI and PSII rates were also not dramatically different between mutant and wild-type, indicating that a high concentration of trienoic acid is not required to support these activities. These results contrast with studies in which thylakoids were exposed to hydrogenation in the presence of a water soluble paladium catalyst which reduced the level of unsaturated fatty acids (Vigh et al, 1985). Such exposure caused loss of whole chain but not the partial reactions of electron transport (Vigh et al, 1985). Our results suggest the loss of whole chain activity can not be attributed to trienoic acid content. It probably reflects the nonspecificity of the hydrogenation treatment which also reduces 18:2 to 18:1. Futhermore the observation that non water-soluble catalysts which cause near identical reductions in fatty acid unsaturation, do not affect electron transport rates also suggests that water soluble hydrogenation catalysts are nonspecific in action (Restall et al, 1979).

Table 19

Comparison of photosynthetic activities in mutant JB1 and wild type Arabidopsis

	WT	JB1
CO_2 fixation		
(umol CO_2 mg chl^{-1} h^{-1})	116.0 ± 2.5	114.1 ± 2.5
(mg CO_2 mg fwt h^{-1})	6.8 ± 0.6	5.5 ± 0.2
Electron transport rates (umol O_2 mg chl^{-1} h^{-1})		
Whole chain 25 C	261.4 ± 7.1	270.7 ± 19.1
14 C	202.3 ± 5.9	200.0 ± 9.3
6 C	132.9 ± 7.5	123.8 ± 11.3
PSII 25 C	261.8 ± 14.5	265.7 ± 4.3
PSI 25 C	375.4 ± 9.5	422.4 ± 16.7

Fluorescence Measurements.

As mentioned earlier, spectral analysis of fluorescence emitted from thylakoids excited with 440 nm light at 77 K can resolve three major peaks at 685, 695 and 734 nm. These peaks have been attributed to LHCP associated with PSII, PSII reaction centers, and PSI respectively (Bose, 1983; Butler, 1978). Interestingly SAN9785 treated plants show large enhancements of fluorescence at 685 and 695 nm relative to 734 nm at low temperature (Leech et al, 1985). The ratio of fluorescence at 685 nm/734 nm is considered a measure of PSII to PSI stoichiometry and in herbicide treated plants might reflect the altered ratio of appressed to non-appressed membranes seen in electron micrographs (Leech et al, 1985).

A comparison of the F_{685}/F_{734} ratio in mutant and wild-type thylakoids did not demonstrate any change in PSII/PSI organization in the absence or presence of $MgCl_2$ (Table 20). The fact that we did not observe any difference between the two genotypes under either condition implies that the Chl-protein complexes are structurally similar and the efficiency of energy transfer between the major complexes is normal.

This conclusion was substantiated by measuring variable fluorescence (F_0) at room temperature in the mutant and wild-type. Room temperature fluorescence is a kinetic measure of PSII activity and therefore it is a sensitive indicator of actual light capturing and PSII photochemistry. However, variable fluorescence was indistinguishable between mutant and wild-type in both high and low $MgCl_2$ implying normal PSII photochemistry efficiency in the mutant (Table 20).

Table 20

Room Temperature (F_v/F_o) and Low Temperature (F_{685}/F_{734}) fluorescence in isolated thylakoids from mutant JB1 and wild type *Arabidopsis*

	F_v/F_o	F_{685}/F_{734}
Wild Type (+Mg)	2.73 ± 0.11	1.49 ± 0.08
JB1 (+Mg)	2.61 ± 0.05	1.52 ± 0.08
Wild Type (-Mg)	1.03 ± 0.07	0.87 ± 0.01
JB1 (+Mg)	1.26 ± 0.16	0.88 ± 0.07

Membrane Fluidity

To estimate the effects of trienoic acid composition on the fluidity of thylakoid membranes fluorescence polarization measurements were carried out on freshly isolated thylakoids from mutant and wild-type. The principle of the measurement is that the fluorophor DPH readily partitions into thylakoid membranes (Barber et al, 1984), and upon excitation with polarized light emits polarized fluorescence. The orientation of the probe upon excitation and the plane at which the polarized fluorescence is re-emitted is directly dependent on the rotational diffusion of the probe during its excitation lifetime. This diffusive motion is in turn dependent on the viscosity of the membrane. The more fluid a membrane the faster the rotational diffusion of the probe during its excitation lifetime and hence the more depolarized the re-emitted fluorescence thus, high fluidity is reflected in low fluorescence polarization values (P).

The P values obtained for Arabidopsis thylakoids are very similar to those of other species (Barber et al, 1984) and in general indicate a very fluid membrane (Figure 22). Although the mutant shows higher polarization values at every temperature tested in comparison to wild-type samples the difference was just at the limit of statistical significance (Figure 22). These results suggest that considerable changes in the level of unsaturation by removal of the n-3 double bond caused only a small decrease in membrane fluidity. Physical studies on melting temperatures of 18:3 versus 18:2 and 18:1 are consistent with these findings and imply that the position and not the number of double bonds is important in maintaining fluidity (Stubb and Smith, 1984).

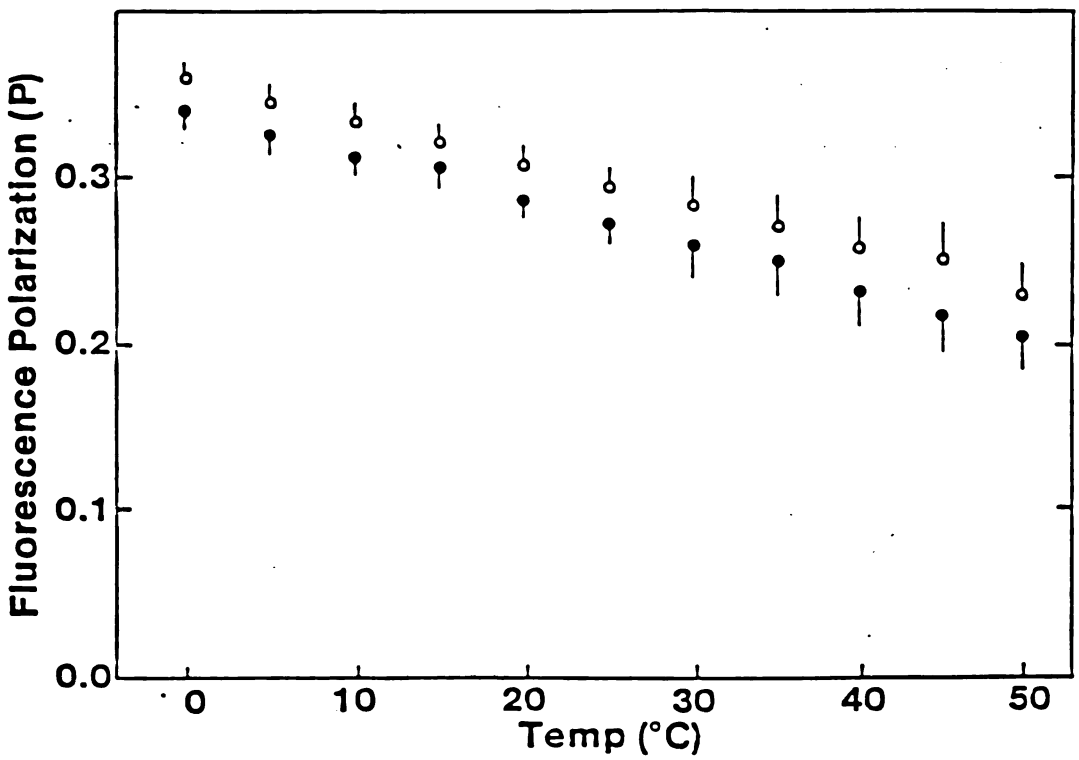

Figure 22. Effect of temperature on DPH fluorescence polarization by thylakoid membranes from wild-type (●) and mutant (○) JB1 Arabidopsis. Each point represents three independent replicates.

Considered together, the role of trienoic acids in membrane fluidity is relatively minor at this level of resolution.

Effects of Unsaturation on Protein Diffusion.

It has recently been recognized that light harvesting antenna complexes can undergo reversible lateral diffusion between appressed and non-appressed regions of thylakoid membranes under the appropriate environmental conditions (Staehelin and Arntzen, 1983). This phosphorylation-dependent process can be artificially regulated in isolated thylakoids by the addition of ATP and light and appears to be controlled by a protein kinase (Staehelin and Arntzen, 1983). The physical movement of LHCP in turn leads to a redistribution of energy between PSI and PSII. With the lateral movement of LHCP away from PSII in the grana towards PSI in the stromal lamellae, a greater portion of the incident radiation is partitioned to PSI thereby giving increased fluorescence at 734 nm. Because phosphorylation and subsequent movement of the complexes from PSII to PSI is a relatively slow process, the increased fluorescence from PSI (F_{734}) relative to PSII (F_{685}) can be monitored by 77K fluorescence emission spectra.

Analysis of low temperature fluorescence emission of PSII and PSI spectra indicate that LHCP phosphorylation does lead to a redistribution of absorbed energy between these photosystems in Arabidopsis and therefore LHCP diffusion but the kinetics of diffusion are nearly identical between mutant and wild-type samples (Figure 23). Therefore reduced unsaturation does not seem to hinder movement of

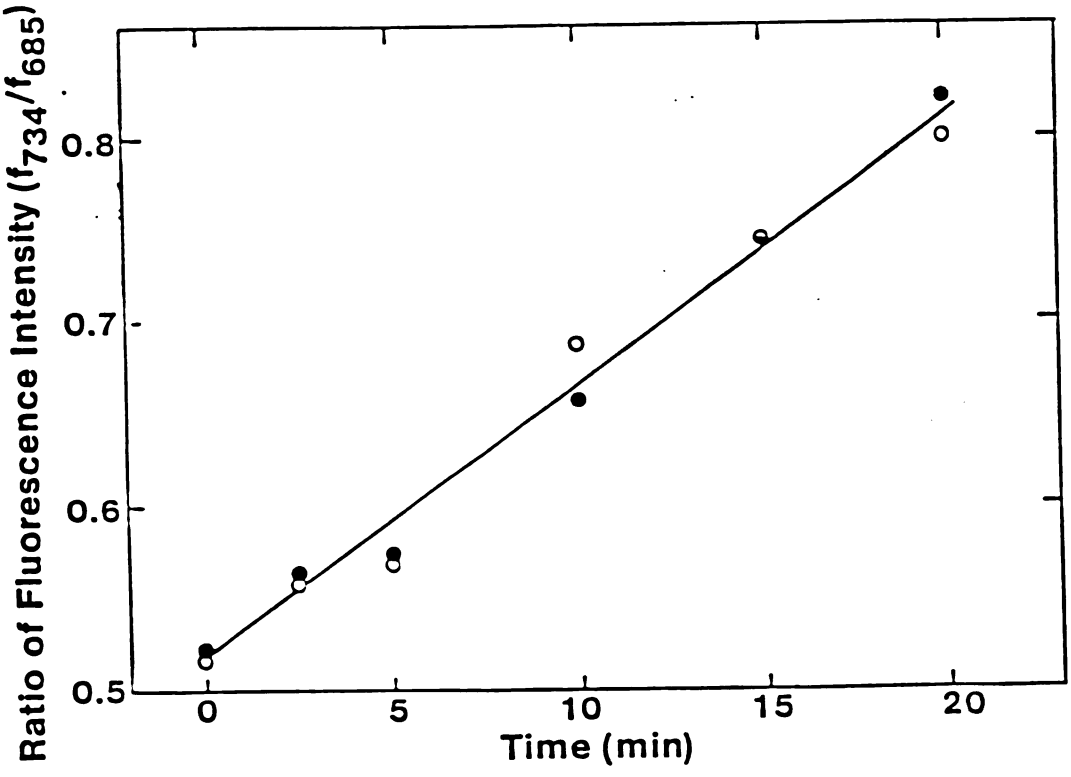

Figure 23. Rate of ATP-induced change in the ratio of 77K fluorescence (F734/F685) from thylakoid membranes of wild-type (●) and mutant JB1 (○) Arabidopsis. Each point represents the mean of three samples.

macromolecules such as proteins to any major extent in thylakoid membranes.

Effects of High Temperature on Fluorescence

As with the fadA mutant, the stability of pigment-protein complexes in the new lipid enviroment caused by the fadD mutation were assayed by heat induced fluorescence yield enhancement. However there was no significant difference in the temperature at which fluorescence started to increase in the mutant as compared to wild-type or in the magnitude of the responses (Figure 24). Thus the stability of the LHCP-PSII core association is not significantly affected by the reduced 18:3 and 16:3 composition of the membranes.

Discussion

Although the fadD mutation has no apparent effect on growth rate under controlled conditions the mutant is somewhat chlorotic by comparison with the wild-type due to a major reduction in the amount of chloroplast membranes per cell when grown at temperatures above 26 C. This alteration is expressed both as a reduction in the size of chloroplasts and in the amount and distribution of lammelar membranes within the chloroplasts (Table 17). Futhermore, although the chl/lipid and protein/lipid ratios are unchanged, the number of chloroplasts per cell appears to be substantially higher in the mutant (Table 14, Table 18).

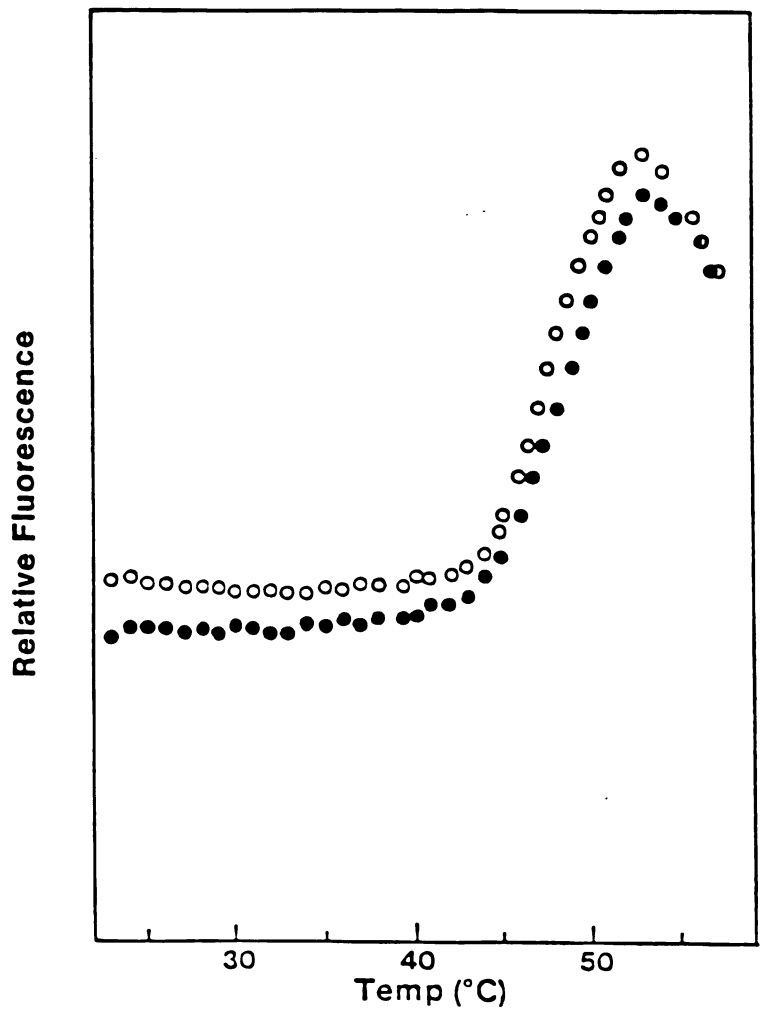

Figure 24. Temperature-induced fluorescence enhancement yield (fo) of wild-type (●) and mutant (0) Leaves. Plants were grown at 27 C. Each point represents the mean of 4 independent measurements.

The redistribution of the thylakoid lamellae, although similar to those reported in SAN9785 treated barley seedlings are less pronounced (Leech et at, 1985). Ultrastructural studies with 6 day old barley seedlings treated with sublethal doses of the herbicide SAN9785 reduced the amount of 18:3 from 76 mol % to 28% and increased the 18:2 levels from 7 mol % to 49%, in thylakoid membranes but caused no change in total lipid or chl levels/cell (Leech et al, 1985). In the treated plants, however, the grana were approximately 60% wider than the controls with fewer thylakoids/stack and less stromal lamellae (Leech et al, 1985). Although these structural changes were supported by lower chl a/b ratios and altered fluorescence emission, other chemicals which do not specifically effect 18:3 levels can cause similar ultrastructural and biochemical changes (Bose and Mannan, 1984; Festke et al, 1977). Therefore we believe that many treatments which cause perturbation of the photosynthetic apparatus during development can cause alterations in granal/stromal ratios irrespective of lipid composition.

By using chl/fwt ratios as a convenient indication of membrane growth it was possible to show that this parameter is affected by temperature in a similar way to that of trienoic acid synthesis (Figure 20). Along with the observation that these two traits co-segregate from an F_2 population derived from a wild-type x fadD cross we believe that the fadD gene product must somehow regulate both phenomena. There are two simple possibilities. 1) The fadD gene product is some cellular component which controls the assembly and growth of chloroplast membranes and the n-3 desaturase is one of the enzymes regulated by this gene product. 2) The fadD gene product is the structural gene for

the n-3 desaturase and the reduction in trienoic acids due to the mutation causes the reduction in chloroplast membrane. The former hypothesis would explain one of the discrepancies between this study and that in which 18:3 was reduced by treating seedlings with SAN9785. The changes in photosynthetic function attributed to decreased 18:3 content in the herbicide experiments are considered artifactual since we did not observe any similar change in the fadD mutant. Using the same reasoning it can be argued that reduced chloroplast membrane size observed in the mutant is not directly due to the trienoic acid levels since this does not occur in SAN9785 treated barley. Thus the effect measured is probably due to some other function affected by the altered fadD gene product. Moreover if the fadD gene product does regulate a number of functions the fact that the mutation is temperature sensitive and somewhat leaky might be because a null mutation in such a regulatory gene is lethal to the plant.

The direct testing of the above hypotheses of fadD action is at this time not possible but the fact that n-3 desaturation is closely regulated with membrane growth in the fadD mutant strongly suggests a role for 18:3 and 16:3 in membrane biogenesis. Support for this proposal can be inferred from developmental and greening studies of plants which have shown a direct correlation of trienoic acid synthesis and membrane biogenesis (Leech and Leech, 1976).

Although the fadD mutation translates into decreased unsaturation and structurally modified chloroplasts these changes have little effect on any functional aspect of this organelle. Photosynthesis as assayed by CO_2 fixation, electron transport (Table 19), and room temperature fluorescence (Table 20) showed no significant differences. Net CO_2

fixation was reduced on a fwt basis but this is only a reflection of the smaller photosynthetic apparatus since this difference was not detected on a chl basis. These results are in sharp contrast to studies carried out with chemically modified membranes (Leech et al, 1985; Bose and Mannan, 1984; Raison, 1980). We therefore suggest these methods induce non-specific changes and are not by themselves adequate to determine the functional significance of trienoic acids in vivo.

It has been suggested that the structural organization of the chloroplast involves lateral separation of the two photosystems (Anderson and Melis, 1983; Staehelin and Arntzen, 1983). Membrane fractionation studies suggest LHCP-PSII protein complexes are primarily located in granal or appressed regions of the thylakoid lamellae and PSI is associated with non-appressed or stromal membranes. The spatial separation of the photosynthetic light reactions necessitates the movement of plastoquinone and probably plastocyanin as long range carriers of electrons. Also, it is now well established that control of energy distribution between PSII and PSI under different light conditions involves the movement of LHCP between the reaction centers (Staehelin and Arntzen, 1983). Although each case involves very different diffusion constants, both movements are dependent on the membrane fluidity (Barber et al, 1984; Chapman et al, 1983). In a more general sense the lateral movement of all the membrane components is also determined by rates that are optimal for the overall system.

Because of the high proportion of polyunsaturated fatty acids in the thylakoids, and in particular trienoic acids, it has been suggested that these molecules play a major role in maintaining an extremely fluid matrix for lateral movement of photosynthetic components. The

introduction of double bonds into linear fatty acids causes kinking of the chains thereby disordering hydrophobic interactions between the fatty acids which in turn causes a more fluid bilayer. The relatively low fluorescence polarization values for thylakoid membranes of Arabidopsis (Figure 22) and other plant species (Barber et al, 1984) does reflect the very fluid environment of this compartment compared to other bilayers.

The reduced trienoic acid in the fadD mutant, however did not decrease the fluidity to any great extent (Figure 22). Rates of plastoquinone and plastocyanin diffusion as assayed by PSII and PSI partial reactions (Table 19) and protein diffusion as assayed by LHCP movement from PSII to PSI (Figure 23) support this view. Taken together with results obtained from model membrane systems (Stubbs and Smith, 1984) the conclusion drawn is that major changes in unsaturation may often result in only minor changes in fluidity.

The lack of any major effect of the fadD mutation on photosynthesis probably reflects the bias of a nonselective screen for fatty acid alterations. However the structural changes described above have defined a new class of mutants for chloroplast developmental studies. To date the majority of previously isolated mutants which affect chloroplast membrane development also disrupt photosynthetic function. This is largely a reflection of the screening procedure for isolation of such mutants which has relied on chlorosis or variegation as a phenotype. By contrast, mutant classes such as fadD were screened by looking for specific changes in a chloroplast component. By using such a criterion one can isolate mutations which alter chloroplast structure without disrupting function. In this sense, changes in fatty

acid metabolism may be an excellent method for the isolation of mutants with altered membrane growth and organization.

In conclusion it appears the high proportion of trienoic acid found in chloroplasts plays little if any role in the function of photosynthesis as assayed by a number of criteria. The observation that the growth of both chloroplast envelopes and thylakoids is inhibited in the fadD mutant however leads us to conclude that their is a function for trienoic acids in membrane biogenesis. The isolation of a collection of mutations which affect 18:3 and 16:3 levels would be very useful in further determining this relationship. Not only would such a collection allow the determination of the number of loci involved in the synthesis and regulation of these fatty acids but the recovery of a nontemperature sensitive null mutant which totally lacks 18:3 and 16:3 might shed some light on the nature of the fadD mutation. For example, if the fadD phenotype is due to change in a multifunctional regulator of chloroplast development it should be possible to isolate mutations in the structural gene(s) for trienoic acid synthesis which do not show these pleiotropic effects. Furthermore because the fadD mutation is somewhat leaky for trienoic acid synthesis it can be argued that many of the functions in which these fatty acids play a role can still occur.

APPENDIX A

Temperature Induced Fluorescence

As a method for estimating the stability of pigment-protein complexes in thylakoid membranes the technique of temperature induced fluorescence has become very popular (Schreiber and Berry, 1977; Armond et al, 1978). A number of studies have now been done to see if changes in fatty acid composition commonly seen in plants acclimating to various temperatures can be correlated with changes in fluorescence emmission (Raison et al, 1980; Lynch and Thompson, 1984). The general approach to using this technique is as follows. Detached leaves or isolated thylakoids are placed in a temperature controlled cuvette and excited with weak blue light (0.3uE m^{-2} s^{-1}, 480 nm) causing the sample to fluoresce at a low constant level (F_o). F_o, which represents photochemically inactive chlorophyll at room temperature, and is mostly PSII fluorescence from chlorophyll a (Armond et at, 1978). As the temperature is increased a sudden increase in F_o is observed at temperatures which appear to be dependent on the temperature at which the plant was grown (Armond et al, 1978). Plants grown at higher temperatures have fluorescence breaks at higher temperatures than plants grown at lower temperatures. From experiments using different

excitation wavelengths of light on plants grown at various temperatures it has been suggested that the increased fluorescence is due to an energy block between chlorophyll b (chl b) and chlorophyll a (chl a) (Armond et al, 1978). Normally the effiency of transfer between these two pigments is nearly 100%. Further studies involving low temperature fluorescence spectra of leaves heated above their break point showed a new 660 nm peak which probably represents free chl b. These results have led to a model suggesting that upon heating of the sample the chlorophyll a/b binding protein complex which contains all the chl b in a plant becomes dissociated from the PSII reaction centre thereby not allowing energy transfer to occur between chl b and chl a.

Because such a system could be useful as an assay of the effects of fatty acid alterations on membrane stability a more detailed characterization of heat induced damage on chloroplast function was carried out on wild-type Arabidopsis. As a first test of the use of this system, plants were grown at 12 C and 22 C until leaves were large enough to be assayed (2 to 3 weeks). Plants grown at cooler temperatures do show a lower break point in fluorescence emission than 22 C grown plants (Table 21). Although this change in fluorescence does correlate with changes in unsaturation ratios, the differences were not dramatic (Table 21).

Further analysis of the nature of the fluorescence rise was studied using low temperature fluorescence spectra of wild type leaves subjected to temperatures above the normal break point of 22 C grown plants (Figure 25). A typical spectrum shows increased 734 nm (F_{734}) and 695 nm (F_{695}) fluorescence peaks relative to 685 nm (F_{685}) fluorescence (Figure 25). It is generally accepted that F_{685} belongs to

Table 21

Temperature induced fluorescence breaks
and unsaturation ratios in wild type plants grown 12 and 22 C

	12 C	22 C
WT-1	40.4	45.2
WT-2	43.8	46.2
WT-3	42.9	45.0
AVERAGE	42.9 ± 1.8	45.5 ± 0.6
Unsaturation Ratio	5.47	4.99

$$\text{Unsaturation Ratio} = \frac{18:3+18:2+18:1+16:3+16:2+16:1}{16:0+18:0}$$

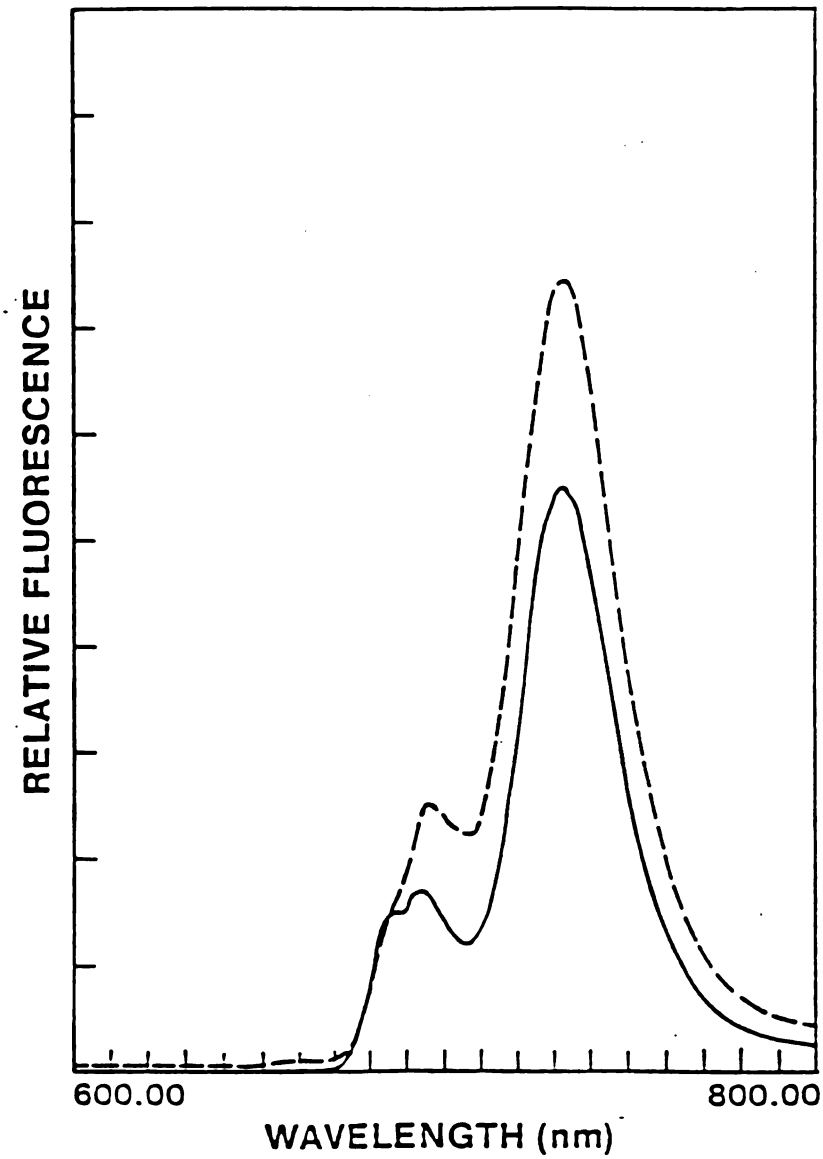

Figure 25. Low temperature fluorescence (77K) emission spectra of wild type leaf samples incubated at 22 C (—) or 53 C (---) for 5 min before freezing of the samples. Both spectra were normalized to 685 nm.

antenna of PSII and probabaly LHCP. That F_{695} is from the antenna of PSII and the core antenna of PSI and that F_{734} is fluorescence from the peripheral antenna of PSI (Butler, 1978; Bose, 1982). Because of the quenching of F_{685} and F_{695} by the high level of chlorophyll in the leaf and to determine which peaks are actually increasing or decreasing a similar experiment was carried out using thylakoids to which an internal standard was added (Carbofluor 1 ug ml^{-1}, F max = 500 nm). Comparisons of unheated and heated samples normalized to the internal stadard show that PSI external antenna are not dramatically altered by the heat treatment although the maxima was slightly blue shifted (Figure 26).

In contrast the PSII-LHCP associated fluorescence peaks are significantly affected, showing decreased fluorescence yield and shifts in peak maxima (Figure 26). This result supports the Armond hypothesis that PSII is preferentially affected by heating (Armond et al, 1978). The increased F_{695} and decreased F_{685} suggests that the antenna of PSII has somehow been affected. One possible cause for these changes could be the separation of LHCP from the rest of the PSII core particle. Recently emission spectra from purified LHCP have been shown to have an emission maximum of 680 nm (Bose, 1982). Therefore separation of LHCP from PSII should be reflected in a 5 nm shift in the 685 nm peak towards the blue. To see if such a shift was occurring in thylakoids, samples were heated for various times and frozen in liquid nitrogen for low temperature fluorescence analysis. The 680 to 690 nm region of the spectra was expanded for more detailed study (Figure 27). From the results it can be seen that as the sample is subjected to longer heating a shift in the F685 occurs towards 680 nm at the same time as

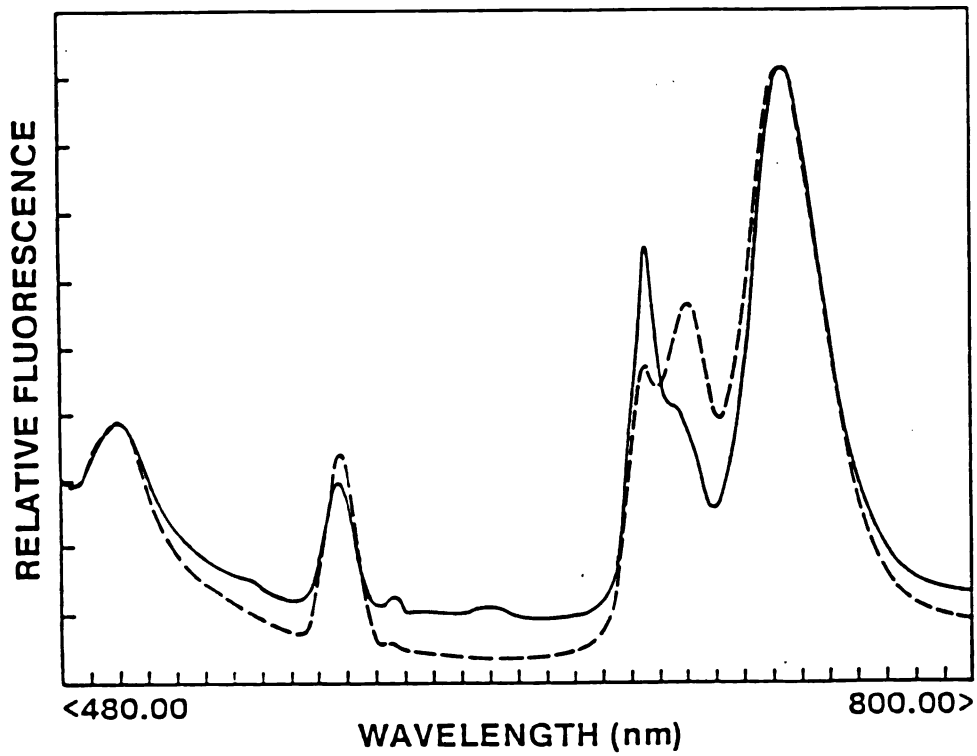

Figure 26. Low temperature fluorescence (77K) emission spectra of wild type thylakoids incubated at 22 C ( ) and 53 C (---) for 5 min before freezing of the samples. Carbofluor (Fmax 500 nm) was added as an internal standard to which both spectra were normalized.

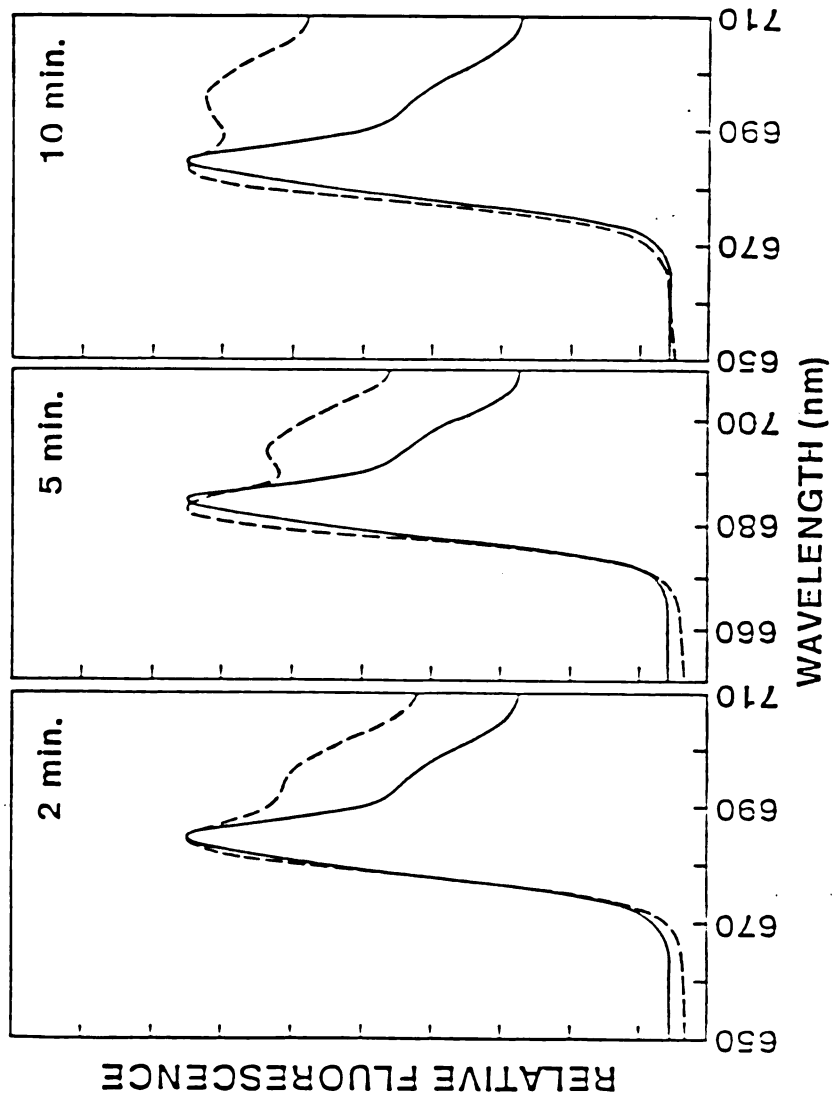

Figure 27. Low temperature fluorescence (77K) emission spectra of wild type thylakoids incubated at 22 C (—) or 2, 5, or 10 min at 53 C (---) before freezing the samples. Samples were normalized to the the most intense peak in the range of 680 nm to 690 nm.

an increase in F695 is happening. This indicates that indeed there is a separation of LHC from the PSII core and that this is a possible cause of the increased fluorescence emission upon heating leaves. Therefore this system for measuring protein stability in thylakoid membranes should be useful in analysis of Arabidopsis mutants with altered lipid membrane composition.

APPENDIX B

Chromosome Assignments

Because of the difficulty of constructing test crosses for mapping purposes in Arabidopsis, the fad mutants were assigned to chromosomes by scoring aberrant independent assortment of F_2 phenotypes from F_1 x F_1 crosses. The fad mutants were crossed to an Arabidopsis strain (MK1) which contains a visible marker for each chromosome. These mutations are listed in Table 2. For assignment to a chromosome each mutation was scored for 3:1 segregation and then 9:3:3:1 independent assortment. Any significant departure from these ratios not due to aberrant segregation was used to assign the fad mutations to chromosomes. The observed and expected frequencies and X^2 values are reported below for the fadA and fadD mutations.

JB60 (+ + + + + + fadA) x MK1 (an py gl cer ms +)

	+	an	+	py	+	gl	+	cer	+	ms	+	fadA
obs	120	35	119	39	117	38	126	28	108	35	116	39
exp	116	39	119	40	116	39	115	39	107	36	116	39
$X^2(1)$	0.52		0.08		0.02		3.81		0.05		0.02	

Independent assortment of fadA

Chromosome			+/+	+/-	fadA/+	fadA/-	$X^2(3)$
1	an	obs	91	29	25	10	
		exp	87	29	29	10	0.74
2	py	obs	87	31	29	8	
		exp	87	29	29	10	0.54
3	gl	obs	89	29	27	10	
		exp	87	29	29	10	0.18
4	cer	obs	89	37	26	2	
		exp	87	29	29	10	8.96 *
5	ms	obs	80	28	25	10	
		exp	80	27	27	9	0.30

JB1 (+ + + + + + fadD) x MK1 (an py gl cer ms +)

	+	an	+	py	+	gl	+	cer	+	ms	+	fadA
obs	62	24	75	11	64	21	67	14	58	19	65	16
exp	65	21	65	21	64	21	61	20	58	19	61	20
$X^2(1)$	0.39		6.84		0.03		2.56		0.004		0.19	

Independent assortment of fadD

		+/+	+/-	fadD/+	fadD/-	$X^2(3)$
an	obs	53	9	20	4	
	exp	48	16	16	6	4.87
py	obs	60	9	15	2	
	exp	48	16	16	6	8.10 *
gl	obs	49	15	21	0	
	exp	48	16	16	5	7.25 *
cer	obs	53	14	12	2	
	exp	46	15	15	5	3.76
ms	obs	49	9	14	5	
	exp	54	15	15	4	2.61

* signifies a significant difference

All the marker and fadA mutations segregated as expected for a simple mendelian trait ($X^2(1)<3.84$, $p<0.05$). This was also seen for independent assorment of the markers except cer and fadA which gave a high value. FadA was therefore assigned to chromosome 4. In the case of fadD two markers showed aberrant independent assortment. However the py marker also showed aberrant segregation from fadD, therefore, fadD was assigned to chromosome 3.

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