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Altered rates of protein transport in *Arabidopsis* mutants deficient in chloroplast membrane unsaturation

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Dedicated to Professor Rodney Croteau on the occasion of his 60th birthday.

Abstract

Protein transfer across membranes is mediated by protein machinery embedded in the membrane. The complement of different lipid classes within a membrane is known to influence the efficiency of some protein translocation processes, but very little is known about whether the fatty acid composition of the membrane bilayer also affects protein transport. We investigated this issue using three mutants of *Arabidopsis*, *fad6*, *fad5*, and *fad3 fad7 fad8*, that have reduced levels of fatty acid unsaturation in their thylakoid membranes. Interestingly, the effect of reduced unsaturation was different for three distinct pathways of protein transport. In thylakoids from all three mutants, transport of the OE17 protein on the ΔpH/Tat pathway was reduced by up to 50% relative to wild-type controls, when assays were run at 10, 20 or 30 °C. By contrast, transport of the OE33 protein on the Sec pathway was substantially increased in all the mutants at the three temperatures. Transport of the CF_OII protein (ATPg) on the 'spontaneous' pathway was largely unaffected by reduced unsaturation of the thylakoid membranes. Experiments with intact chloroplasts from wild-type *Arabidopsis* and the three mutants confirmed these changes in thylakoid transport reactions and also demonstrated an increased rate of protein import across the chloroplast envelope in each of the mutants. This increased capacity of chloroplast protein import may partially compensate for the reduced capacity of thylakoid transport by the ΔpH/Tat pathway. The *fad5*, *fad6* and *fad3 fad7 fad8* mutants used in this study grow normally at 15–20 °C, but exhibit reduced photosynthesis and growth, relative to wild-type controls, at low temperatures (4 °C). The results reported here indicate that protein transport and chloroplast biogenesis may well contribute to these low-temperature phenotypes.

Keywords: Arabidopsis; Fatty acid mutants; Chloroplast; Protein transport; Temperature responses; Thylakoid

1. Introduction

The biophysical reactions of light harvesting and electron transport during photosynthesis take place in the thylakoid membrane. During chloroplast biogenesis in higher plants, more than 100 different proteins must be efficiently transported into or through the thylakoid to ensure the correct assembly and functioning of the photosynthetic complexes (Peltier et al., 2002; Gomez et al., 2003). Some of these proteins are encoded by chloroplast genes. However, the majority are products of nuclear genes that are translated on free cytoplasmic ribosomes and posttranslationally imported through the chloroplast envelope and then into, or through, the thylakoid membrane.

A series of important discoveries over the last 10 years have revealed the complexity of this protein traffic. It has been known for some time that thylakoid proteins enter the chloroplast on the general import apparatus that also translocates stromal proteins across the two envelope

Abbreviations: *Arabidopsis thaliana*, Cruciferae; MGD, monogalactosyldiacylglycerol; PSI, PSII, photosystem I or II; SDS–PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; SSU, small subunit of ribulose bisphosphate carboxylase; X:Y, a fatty acid with X carbons and Y *cis* double bonds.

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membranes (Keegstra and Cline, 1999). Typically, proteins destined for the thylakoid lumen have a bipartite transit peptide at the amino terminus. One domain of this transit peptide facilitates interaction with the general import apparatus and transfer into the stromal compartment. This first domain is then removed by the stromal processing protease (VanderVere et al., 1995) to expose a second, luminal targeting domain that mediates transfer across the thylakoid and is then removed by the thylakoid processing protease (Chaal et al., 1998). Integral membrane proteins of the thylakoid follow a similar overall fate but some do not have a cleavable targeting domain controlling transfer from the chloroplast stroma to the thylakoid.

There are four distinct pathways for transfer of proteins into and through the thylakoid membrane. One of these is termed the chloroplast Sec pathway because it uses homologues of the bacterial SecA, SecY and SecE proteins that are involved in protein secretion in prokaryotes (Mori et al., 1999; Mori and Cline, 2001; Robinson et al., 2001). The chloroplast SRP (signal recognition particle) pathway is similar to both the endoplasmic reticulum SRP pathway and the more recently described prokaryotic SRP pathway, although it has some unique features (Schuenemann et al., 1998). Both the cpSec and cpSRP pathways require soluble protein and nucleotide cofactors (ATP or GTP, respectively) and share a common transport channel in the membrane which is analogous to the SecYEG translocation complex in bacteria (Schnell and Hebert, 2003; Van den Berg et al., 2004). These pathways typically translocate proteins in the unfolded state. By contrast, the ΔpH/Tat pathway requires no soluble cofactors but uses the transthylakoid pH gradient to facilitate the transfer of folded proteins across the bilayer. Identification of Hcf106 as an essential component of the ΔpH/Tat machinery (Settles et al., 1997) led to the recognition that eubacteria contain *hcf106* homologues (*TatA* and *TatE*) that are components of a third bacterial pathway for protein export, the Tat pathway (Berks et al., 2000). The ΔpH/Tat pathway is unusual in transporting fully folded proteins. Lastly, there are a number of proteins that insert into the thylakoid in the absence of an energy supply, soluble factors or known membrane components using the so-called Spontaneous pathway (Michl et al., 1994).

There is now considerable information about the biochemical requirements and proteins associated with each of these translocation mechanisms. However, much less is known about how the lipid composition of each membrane bilayer might influence protein transport. Some information is available for protein transfer through the chloroplast envelope via the general import apparatus. Studies of the chloroplast targeting peptides from the small subunit of ribulose bisphosphate carboxylase-oxygenase (van't Hof et al., 1991; Pinnaduwage and Bruce, 1996) and from ferredoxin (van't Hof et al., 1993) provided evidence that initial binding of the preproteins to the chloroplast envelope is mediated by lipids of the outer envelope. Using a vesicle-disruption assay, Pinnaduwage and Bruce (1996) demonstrated that a domain within the transit peptide interacts specifically with liposomes composed of lipids derived from the outer envelope. Interaction was dependent on the presence of monogalactosyldiacylglycerol and was substantially absent when this lipid was not a component of the liposomes. Monogalactosyldiacylglycerol is a class of glycerolipid molecules that forms inverted micelles (the HexII phase) in water (Sen et al., 1981). A second class of HexII-forming lipids, phosphatidylethanolamine, has been shown to be a critical membrane component to support protein transport across the plasma membrane in *E. coli* (Rietveld et al., 1993; Rietveld et al., 1995). Chloroplast protein import is also dependent of the presence of digalactosyldiacylglycerol, a bilayer-forming lipid that is characteristic of chloroplast membranes (Chen and Li, 1998). These perspectives on the roles of glycerolipids in membrane transport processes focus on the complement of different lipid classes, as defined by the headgroups of the glycerolipid molecules.

Very much less is known of the effects of changes in fatty acid composition of the membranes on protein transport processes. The fatty acid composition, and in particular the extent of fatty acid unsaturation, determines the fluidity of the membrane bilayer and also influences the polymorphic behavior of HexII-forming lipids (Marsh, 1990). These effects mean that it is likely that fatty acid composition influences membrane transport processes. There is substantial evidence that high levels of thylakoid unsaturation are required for efficient removal and replacement of the D1 protein of PSII (Zhang and Aro, 2002) and *Arabidopsis* mutants with reduced thylakoid unsaturation are susceptible to photoinhibition (Vijayan and Browse, 2002).

In the experiments reported here, we have investigated the functional significance of chloroplast fatty acid composition using a series of *Arabidopsis* mutants with specific defects in fatty acid desaturation. In the *fad5* mutant, 16:0 is increased at the expense of polyunsaturated fatty acids as a result of a mutation in the 16:0-MGD Δ7 desaturase (Kunst et al., 1989). In the *fad6* mutant, trienoic fatty acids are reduced by approximately 50% and replaced by monounsaturated fatty acids as a result of a mutation in the chloroplast 16:1/18:1 ω6 desaturase (Browse et al., 1989). Two isozymes of the chloroplast ω3 desaturase are encoded by *FAD7* and *FAD8*, while the endoplasmic reticulum ω3 desaturase, encoded by *FAD3*, also contributes to the production of 18:3 fatty acids found in chloroplast membranes. In *fad3 fad7 fad8* triple mutants (McConn and Browse, 1996) trienoic fatty acids are substantially replaced by the dienoic acids, 16:2 and 18:2.

Plants from these three mutant lines are substantially similar to wild type in vegetative growth and development, and in photosynthesis under standard culture conditions (22 °C and 100–150 μmol quanta m^{-2} s^{-1} illumination) indicating that a high degree of thylakoid unsaturation is not required to establish and maintain the photosynthetic machinery under these conditions. However, at 22 °C all the mutants contain slightly less chlorophyll than the wild

type show modest changes in chloroplast ultrastructure, and are more susceptible to photoinhibition at high light intensities (Hugly et al., 1989; Kunst et al., 1989; Vijayan and Browse, 2002). Furthermore, all the mutants are defective in chloroplast biogenesis at low temperature suggesting that assembly of photosynthetic complexes in the thylakoids is compromised (Hugly and Somerville, 1992; Routaboul et al., 2000).

Since a defect in membrane protein translocation provides one possible explanation for these observations, we have used in vitro assays of protein transport in thylakoids and intact chloroplasts from wild-type and mutant plants. Our results show that the Sec, ΔpH and Spontaneous pathways of thylakoid protein transport are altered in very different ways by the changes in fatty acid composition between wild-type and mutant membranes. Our results also provide evidence that disruption of one or more of the pathways may indeed contribute to the mutant phenotypes.

2. Results

For our investigations, we used proteins that are transported into or through the thylakoid by three different protein translocation pathways. The OE17 and OE33 (oxygen-evolution 17 and 33 kDa) proteins are both translated on cytoplasmic ribosomes. Each contains a bipartite targeting peptide that allows the preprotein (pOE17 and pOE33) to be transferred into the chloroplast stroma on the general import apparatus. Following processing to an intermediate form (iOE17 and iOE33), they are transported through the thylakoid via the ΔpH/Tat pathway (OE17) (Cline et al., 1993; Henry et al., 1997; Ma and Cline, 2000) or the Sec pathway (OE33) (Yuan and Cline, 1994). The pATPg protein (precursor of the CF_OII protein of the chloroplast ATP synthase) is also translated on cytoplasmic ribosomes but after chloroplast import it inserts spontaneously into the thylakoid before processing to the mature form (Michl et al., 1994). We used cDNA constructs encoding pATPg, iOE17 and iOE33 to assay the activities of protein transport pathways in the thylakoids from wild-type and mutant plants.

We began our studies with thylakoids prepared from *fad6* mutant plants and wild-type controls. The *fad6* thylakoids exhibited the greatest reduction in lipid unsaturation with an average of only 3.45 double bonds per glycerolipid molecule compared with 5.08 double bonds per molecule in wild-type thylakoids (Table 1). The triple mutant *fad3 fad7 fad8* and the *fad5* mutant had smaller reductions in overall thylakoid unsaturation. The *fad2* mutant, which is deficient in the Δ12 desaturase of the endoplasmic reticulum (Miquel and Browse, 1992), showed relatively small changes in thylakoid fatty acid composition with an overall level of glycerolipid unsaturation that was only 5% less than wild-type thylakoids.

The three protein precursors were incubated with wild type and *fad6* thylakoids using assays that were optimized for each transport activity (see Section 4). Assays were started by addition of ^{3}H-labeled preprotein and then sampled at different times up to 50 min (Fig. 1). This experiment demonstrated that protein transfer was linear with time up to at least 30 min in our assays, but also provided evidence that each pathway was affected differently by the alteration in lipid composition between wild-type and *fad6*. The iOE17 preprotein bound to a similar extent to wild-type and *fad6* thylakoids as indicated by the upper band on the gel (Fig. 1). However, the extent of transport of iOE17 through the membrane (as indicated by processing to the lower molecular weight mature OE17 protein) is greatly reduced in *fad6*, relative to wild type, throughout the entire time course of the experiment. A quite different

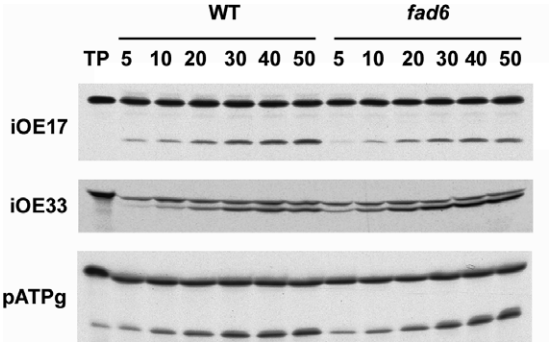

Fig. 1. Kinetics of protein transport into wild-type and *fad6* thylakoids. Thylakoids isolated from wild-type (WT) and *fad6* were incubated with ^{3}H-labeled thylakoid precursors (iOE17, iOE33 and pATPg). Samples were harvested at different times, with thylakoid membranes recovered by centrifugation and ^{3}H-proteins analyzed by SDS–PAGE and fluorography (see Section 4). Each lane contains thylakoid protein equivalent to 20% of that present in each assay. Lane TP contains 5% of the precursor added to the assays.

Table 1
Overall fatty acid composition of thylakoids from wild-type and mutant plants

Plant line	Fatty acid composition (percent of total)								Average double bonds per glycerolipid
	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3	
Wild type	7.9	5.6	2.3	19.6	0.6	1.4	4.4	58.2	5.08
fad6	8.4	17.9	1.3	1.2	0.4	31.0	2.2	37.6	3.45
fad5	19.1	5.3	0.6	1.1	1.1	1.1	3.4	68.5	4.46
fad3 fad7 fad8	6.8	6.3	16.4	1.3	0.4	3.5	61.1	4.3	3.63
fad2	7.2	6.2	1.6	18.2	1.3	8.1	4.0	53.5	4.81

Data are averages of three separate thylakoid preparations.

result is seen for iOE33, which is transported via the Sec pathway. Again, binding of the preprotein is similar for wild type and *fad6* but iOE33 is transported into the *fad6* thylakoids at a considerably higher rate than into wild-type membranes. The ATPg precursor also binds equally to wild-type and *fad6* thylakoids, but there is no detectable difference between wild type and mutant in the rate at which the protein is inserted and processed via the Spontaneous pathway. The differences evident on the autofluorographs were quantified by scintilation counting of protein bands eluted from the gels (Cline, 1986). Data for all the figures are included as Supplementary Data.

The results shown in Fig. 1 were somewhat unexpected, given that growth, development and photosynthesis of *fad6* and wild-type plants are very similar for plants grown at 15–25 °C. To find out how the three protein transport pathways are affected by temperature in wild-type and mutant thylakoids, we carried out assays at 10, 20, and 30 °C. The extent of precursor binding to thylakoids was largely unaffected by temperature or genotype, but the extent of protein transport showed characteristic differences (Fig. 2). For all the test proteins, transport declined progressively from 30 to 10 °C, except that iOE17 transport in wild-type thylakoids was slightly higher at 20 °C than 30 °C. At each temperature, the extent of ΔpH pathway transport in *fad6* thylakoids was reduced by 50–70% relative to the transport measured for wild-type thylakoids. Assays with the Sec pathway protein, iOE33 showed increased transport into *fad6* versus wild-type thylakoids at all three temperatures. The extent of transport into *fad6* thylakoids was 2–3-fold higher than transport into wild-type thylakoids. For the Spontaneous pathway, transport of pATPg was very nearly the same into wild-type and *fad6* thylakoids (Fig. 2).

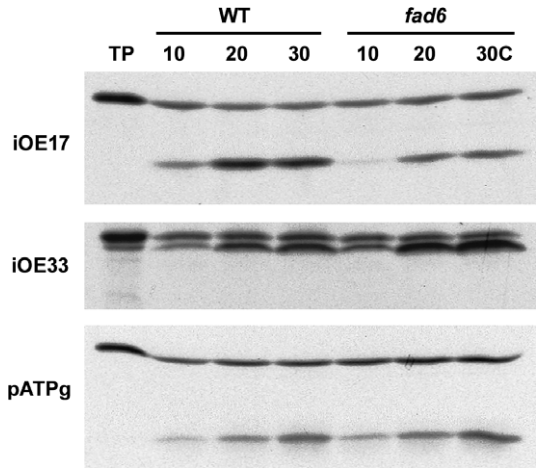

Fig. 2. Effects of temperature on protein transport into wild-type and *fad6* thylakoids. Thylakoids isolated from wild-type (WT) and *fad6* were incubated with ^{3}H-labeled thylakoid precursors (iOE17, iOE33 and pATPg) at 10, 20 or 30 °C for 20 min. At the end of the assay, thylakoid membranes were recovered by centrifugation and ^{3}H-proteins were analyzed by SDS–PAGE and fluorography (see Section 4). Each lane contains thylakoid protein equivalent to 20% of that present in each assay. Lane TP contains 5% of the precursor added to the assays.

The iOE17 and iOE33 proteins are derived from precursor proteins (pOE17 and pOE33, respectively) that are translated on cytoplasmic ribosomes and imported post-translationally through the chloroplast envelope via the general import apparatus (Keegstra and Cline, 1999). To extend our knowledge of transport processes for the OE17 and OE33 proteins, and to investigate the consequences of decreased thylakoid transport of iOE17 by the ΔpH pathway, we isolated intact wild-type and *fad6* chloroplasts and incubated them with ^{3}H-leucine labeled pOE17 and pOE33 proteins. As an additional control, we also included ^{3}H-labeled pSSU, the precursor to the small subunit of ribulose bisphosphate carboxylase-oxygenase, which is also imported on the general import apparatus.

The results of these chloroplast transport assays for *fad6* (Fig. 3) once again showed reduced accumulation (relative to wild type) of the mature OE17 protein at all assay temperatures. However, intact *fad6* chloroplasts accumulated substantial label in the iOE17 intermediate, even though this band is absent in wild-type chloroplasts. Interestingly, the sum of radioactivity in OE17 and iOE17 in the mutant averaged 35% higher than in the wild-type control. The OE33 protein showed increased accumulation in the mutant and there was no noticeable accumulation of iOE33 in either mutant or wild-type chloroplasts. These results indicate an increased capacity of the general import apparatus in *fad6* chloroplasts to transfer by pOE17 and pOE33 through the chloroplast envelope. For pOE33, all the imported protein appears to be efficiently processed

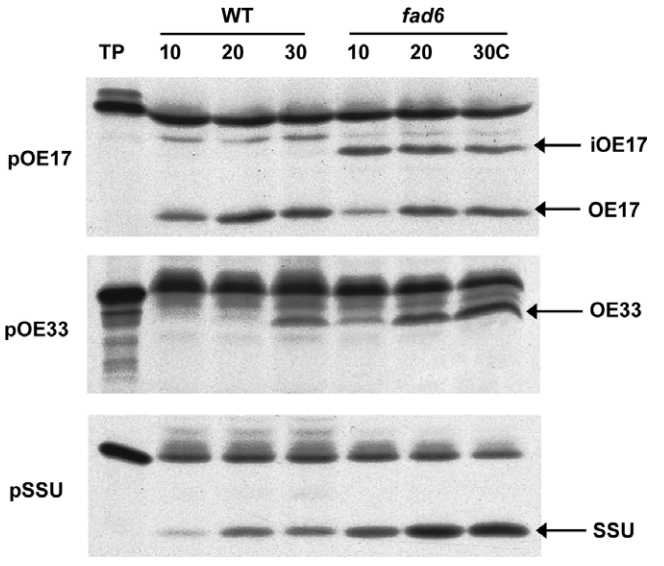

Fig. 3. Protein import into wild-type and *fad6* chloroplasts. ^{3}H-labeled precursors (pOE17, pOE33 and pSSU) were incubated with intact chloroplasts isolated from wild-type (WT) and *fad6* at 10, 20 or 30 °C for 20 min. At the end of the assays, chloroplasts were recovered by centrifugation and ^{3}H-proteins were analyzed by SDS–PAGE and fluorography. The fluorograms of the gels are shown. Each lane contains chloroplast protein equivalent to 20% of that present in each assay. Lane TP contains 5% of the precursor added to the assays. The positions of the intermediate precursor (iOE17) and the mature forms (OE17, OE33 and SSU) are marked.

to iOE33, transported through the thylakoid and matured to OE33. However, import and production of iOE17 in *fad6* chloroplasts greatly exceeds the capacity of the ΔpH machinery to transport the intermediate into the thylakoid. The increased capacity of *fad6* chloroplasts for protein import is markedly evident in the results obtained with pSSU (Fig. 3). At each assay temperature SSU accumulation in *fad6* chloroplasts is 3–4-fold higher than in the corresponding wild-type control.

The results in Figs. 1 and 2 indicate that *fad6* plants are compromised in thylakoid transport via the ΔpH pathway while the capacity for protein transport by the Spontaneous and Sec pathways was unaffected or increased in the mutant, relative to wild type. To find out if these changes in transport were a common characteristic of mutants with reduced levels of thylakoid lipid unsaturation, we investigated the three transport pathways in thylakoids isolated from *fad5* and *fad3 fad7 fad8* plants. In this series of experiments, we also included thylakoids from *fad2* mutant plants. The *fad2* mutation primarily reduces lipid unsaturation in the extrachloroplast membranes of leaf cells (Miquel and Browse, 1992) and thylakoid membranes from *fad2* plants show only a modest decrease in overall unsaturation relative to wild type (Table 1). Like the other mutants studied here, *fad2* is damaged at low temperature (Miquel et al., 1993). For *fad5* and *fad3 fad7 fad8* thylakoids the changes in transport processes, relative to wild-type controls, are strikingly similar to those seen in *fad6* thylakoids (Figs. 4a and b). Transport of iOE17 on the Δ pH pathway is substantially reduced at 10, 20 and 30 °C, while transport of iOE33 on the Sec pathway is increased and transport of pATPg via the Spontaneous pathway is similar in thylakoids from wild-type and mutant plants. By contrast, the experiment with wild-type and *fad2* thylakoids (Fig. 4c) revealed no detectable changes in transport for any of the three pathways at the three temperatures tested.

Intact chloroplasts isolated from *fad5* and *fad3 fad7 fad8* plants similarly showed import and transport capacities (for pOE17, pOE33 and pSSU) that were qualitatively similar to those exhibited by *fad6* chloroplasts and distinctively different from wild-type controls grown and assayed at the same time (Figs. 5a and b). However, the results obtained with chloroplasts from *fad2* plants were very similar to those observed in wild-type chloroplasts (Fig. 5c).

3. Discussion

Our analyses of three mutant lines deficient in chloroplast lipid unsaturation have uncovered distinct effects on different pathways of protein transport. The capacity of the general import apparatus, which moves proteins through the chloroplast envelope, and of the Sec pathway for protein transport through the thylakoid both increase in all three mutants relative to wild-type controls. Conversely, the rate of protein transport on the thylakoid

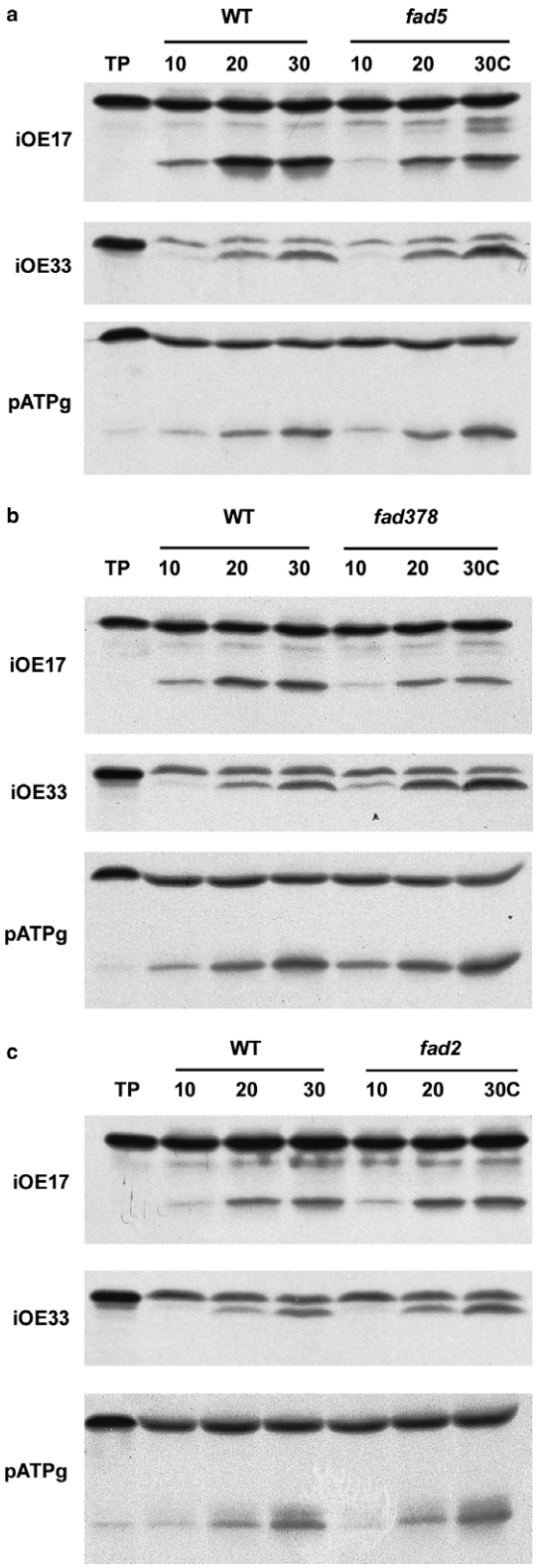

Fig. 4. Protein transport into wild-type, *fad5*, *fad3 fad7 fad8* and *fad2* thylakoids. Transport assays were conducted as described in Fig. 2. The assays for each mutant were carried out in parallel with wild-type controls that used plants that were grown and prepared alongside the mutants.

ΔpH pathway is markedly reduced in all the mutants, while the capacity of the Spontaneous pathway is similar in mutant and wild-type thylakoids.

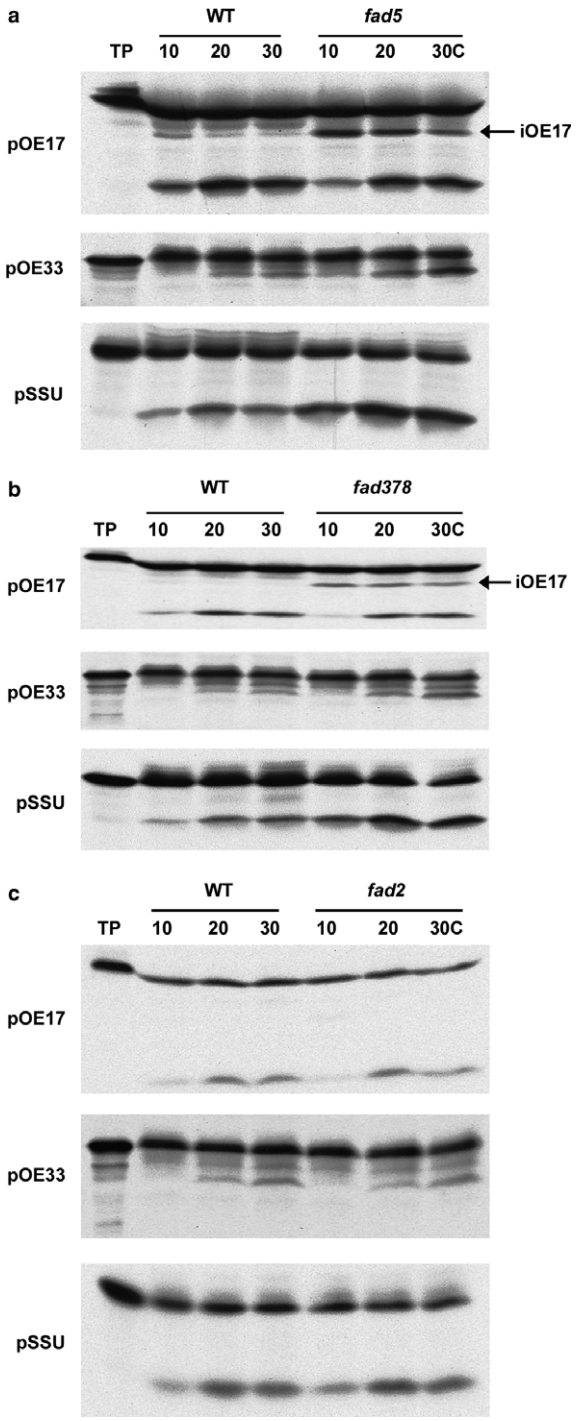

Fig. 5. Protein import into wild-type and *fad5*, *fad3 fad7 fad8* and *fad2* thylakoids. Import assays were conducted as described in Fig. 3. The assays for each mutant were carried out in parallel with wild-type controls that used plants that were grown and prepared alongside the mutant.

All our experiments used the standard convention of assembling assays and reporting results on a chlorophyll basis (Cline, 1986; Michl et al., 1994; Robinson et al., 2001). Because chlorophyll content is strongly correlated to the content of photosystems, it is the appropriate basis for assays measuring the transport of proteins that make up the photosynthetic machinery. The three lipid mutants investigated here, *fad6*, *fad5* and *fad3 fad7 fad8* all contain 5–20% less chlorophyll than wild type, when grown at 20–25 °C, either on the basis of leaf fresh weight or per gram of thylakoid glycerolipids (Hugly and Somerville, 1992; Hugly et al., 1989; Kunst et al., 1989; Routaboul et al., 2000). Data collected during the experiments reported here indicated a 10–12% reduction relative to wild-type. Any correction to the data on thylakoid protein transport (Figs. 1, 2 and 4) based on these differences in chlorophyll content would be relatively small, but it would make the differential in ΔpH pathway activity between mutant and wild type slightly larger. The increases in the Sec pathway (and in chloroplast protein import) between the mutants and wild type would be slightly reduced but remain substantial.

The reduced capacity of the ΔpH pathway in the mutants may reflect a direct effect of membrane unsaturation on the translocon (or associated components), and this is certainly consistent with other evidence that membrane translocation of proteins is sensitive to lipid composition (van't Hof et al., 1991, 1993; Rietveld et al., 1993, 1995; Pinnaduwage and Bruce, 1996; Bruce, 1998). Likewise, the altered fatty acid composition of *fad6*, *fad5* and *fad3 fad7 fad8* chloroplast membranes might stimulate activity of the Sec pathway and the general import apparatus. However, protein targeting and translocation are highly regulated processes and it is also entirely possible that altered transport capacities reflect regulated changes in the number of translocons in the chloroplast thylakoid and envelope. One possibility in this respect is that translocation capacities are upregulated in the mutants to compensate for deficiencies introduced by lowered membrane unsaturation, and that the phenotypes of the mutants reflect the extent to which such compensating mechanisms fail to restore optimal protein translocation capacity.

Irrespective of the exact mechanism by which the ΔpH pathway is reduced in the chloroplast lipid mutants, our results provide evidence that the deficiency in thylakoid protein transport on this pathway contributes to the defects in chloroplast biogenesis, and thus to reduced growth and photosynthesis of the mutants at low temperatures. We hypothesize that reduced protein transport on the ΔpH pathway is not substantially limiting at 22 °C, as evidenced by the normal growth of mutant plants at this temperature. As the temperature is lowered transport rates for all the translocation pathways decline; once the rate of the ΔpH pathway falls below a necessary threshold, the availability of ΔpH proteins inside the thylakoid becomes limiting. The defect in ΔpH pathway will reduce availability of only a subset of thylakoid proteins. However, available evidence indicates that this will prevent assembly of PS I, PS II and other protein complexes of the photosynthetic machinery, causing other protein components to be broken down (Saenger et al., 2002). Accumulation of chlorophyll–protein complexes will be reduced leading to chlorosis, which is characteristically found in leaves of the mutants developing at low temperature (Hugly and Somerville, 1992; Routaboul et al., 2000). The observation that mature leaves of *fad6* and *fad5* plants remain green after transfer to low temperature

(Hugly and Somerville, 1992) is consistent with the expectation that the extent of protein transport required for maintenance of mature chloroplasts is considerably less than that required during chloroplast biogenesis.

4. Experimental

4.1. Plant material

The fatty acid desaturation (*fad*) mutants of *Arabidopsis thaliana* have been reported previously (Kunst et al., 1989; Browse et al., 1989; Miquel and Browse, 1992; McConn and Browse, 1996). Note that *fad5* was originally named *fadB* and *fad6* was originally named *fadC*. Plants were grown on commercial potting mix in controlled environment chambers at 20–22 °C under a 8 h dark/16 h light (~100 μmol m^{-2} s^{-1}) cycle.

4.2. Chloroplast and thylakoid isolation

Intact chloroplasts were isolated from 19 to 21 day old *Arabidopsis* seedlings, following the method for pea chloroplast isolation (Cline, 1986) with small modifications. Briefly, ~30 g of fresh weight of the aerial portions of the plant was homogenized at 0 °C in 200 ml of grinding buffer, containing 50 mM Hepes–KOH, pH 8.0, 330 mM sorbitol, 10 mM EDTA, 5 mM sodium ascorbate, and 0.05% bovine serum albumin (Schuenemann et al., 1999) in a blender (Waring Commercial Blender, model 31 BL 92) for 3 × 1 s at low speed for chloroplast isolation or high speed for thylakoid preparations. The homogenate was filtered through two layers of cheese cloth and a single layer of Miracloth. After centrifugation of the filtrate (3 min at 3000*g*), the pellets were resuspended in grinding buffer (6 ml) and mixed with import buffer (35 mL), consisting of 50 mM Hepes–KOH, pH 8.0, and 330 mM sorbitol. After centrifuging again (3 min at 3200*g*), the pellet was resuspended twice in import buffer with subsequent centrifuging as above. Finally, the thylakoids were resuspended to a final concentration of 1 mg of chl/ml in import buffer. Chlorophyll determinations and other preparation methods were as described in Ma and Cline (2000); the thylakoid preparations contained very few intact chloroplasts but retained identical thylakoid transport capacity as that of intact chloroplasts.

4.3. Preparation of radiolabeled precursors

Protein substrates were produced with TNT Coupled Wheat Germ Extract System (Promega, Madison, WI, USA) in the presence of [^{3}H]-leucine [4TB_q/mmol] according to the manufacturer's specifications. In vitro transcription plasmids for iOE17 and pOE17 (Henry et al., 1997), iOE33 and pOE33 (Cline et al., 1993), pSSU (Anderson and Smith, 1986) and pATPg (Michl et al., 1994) have been described.

4.4. Protein transport and import assays

Thylakoid transport assays were conducted by incubating ^{3}H-labeled protein substrates (3 μl) with thylakoid preparations equivalent to chlorophyll (25 μg) at indicated temperatures. Transport assays with iOE17 were in a total volume of import buffer (75 μl) with the addition of 4 mM Mg-ATP. Assays with iOE33 were in a total volume of 90 μl buffer containing 24 mM Hepes/KOH (pH 8), 117 mM sorbitol, 4 mM $MgCl_2$ and 4 mM Mg-ATP. Transport assays for pATPg were in total volume of 53 μl containing 28 mM Hepes/KOH (pH 8), 156 mM sorbitol, and 5 mM $MgCl_2$. Chloroplast import assays were conducted by incubating ^{3}H-labeled protein substrate (2.5 μl) with the intact chloroplasts equivalent to chlorophyll (25 μg) at indicated temperature in a total volume of 75 μl import buffer containing 5 mM Mg-ATP. After incubation for 20 min, the thylakoids or chloroplasts were recovered by centrifugation, washed once with import buffer, analyzed by SDS–PAGE and fluorography (Bonner and Laskey, 1974), with the dried gels exposed to X-ray film. In general, experiments were repeated at least three times with similar results. Quantification of the extent of transport (and import) processes was accomplished by scintillation counting of radiolabeled proteins extracted from bands cut from the PAGE gels (Cline, 1986). The results of these analyses are included under Supplementary Data.

4.5. Measurement of fatty acid composition

A portion of the thylakoid pellet equivalent to 50 μg of chlorophyll was resuspended in 2.5% H_2SO_4 in MeOH (1 mL, v/v). After incubation for 1 h at 80 °C, the fatty acid methyl ester derivatives were extracted into hexane and analyzed by gas chromatography (Miquel and Browse, 1992).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2006.04.008.

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