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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