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Dynamin-related Proteins are Involved in Chloroplast Division and Morphogenesis

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Dynamin-related Proteins are Involved in Chloroplast Division and Morphogenesis

By

Hongbo Gao

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHOLOSOPHY

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Abstract

Dynamin-related Proteins are Involved in Chloroplast Division and Morphogenesis

By

Hongbo Gao

Chloroplasts are specialized organelles evolved from cyanobacteria as endosymbionts in plant cells. Homologs of the cyanobacterial cell division genes *FtsZ*, *MinD* and *MinE* have been identified previously in plants and shown to be important for chloroplast division. In this work, a new chloroplast division gene, *ARC5*, was identified by positional cloning in *Arabidopsis*. The *arc5* mutant has 1-15 enlarged dumbbell-shaped chloroplasts per cell, suggesting that ARC5 may be involved in the constriction of chloroplasts during division. A GFP-ARC5 fusion protein rescues the mutant phenotype of *arc5* and localizes to a ring at the chloroplast division site. Chloroplast import and protease protection assays indicate that the ARC5 ring is positioned on the outer surface of the chloroplast. Thus, ARC5 is the first cytosolic component of the chloroplast division complex to be identified. Phylogenetic analysis suggests that ARC5 is in the dynamin family of mechanochemical enzymes in eukaryotes, which are large GTPases involved in membrane fission and fusion in a variety of cellular processes. These results indicate that the chloroplast division machinery is of mixed evolutionary origin and that it shares structural and mechanistic similarities with both the cell division machinery of bacteria and the dynamin-mediated organellar fission machineries of eukaryotes. Additional experimental results demonstrate that a homolog of ARC5, ARC5H, is not involved in chloroplast division. Therefore, ARC5 is important but not essential for

chloroplast division and it probably evolved to facilitate the division of chloroplasts during endosymbiosis.

FZO is a more diverged dynamin-related protein involved in mitochondrial fusion in metazoa and fungi. However, an FZO-like protein in *Arabidopsis*, FZL, is targeted to chloroplasts. *fzl* knockout mutants show a chloroplast division defect with heterogeneity in chloroplast size and thinner thylakoid sacs with disorganized stacking. An *FZL-GFP* gene rescued the *fzl* mutant phenotype. Overexpression of FZL-GFP causes very long thylakoid sacs with considerably less stacking. FZL-GFP is a membrane protein present in both the thylakoids and chloroplast inner membrane. FZL-GFP is mainly localized to punctate or vesicle-like structures associated with chloroplasts and infrequently in the cytosol, but is not associated with mitochondria. The level of FZL-GFP expression correlates with the number of punctate and vesicle-like structures on the surface of the chloroplast, suggesting that these structures are coincident with FZL and that FZL may be involved in their formation. Mutation of a conserved lysine residue in the GTPase domain abolishes the punctate localization pattern and the ability of FZL-GFP to complement *fzl* mutant. FZL seems to be involved in the formation and trafficking of vesicles from the thylakoid to the chloroplast envelope and cytosol, and blocking of this process affects both thylakoid morphology and chloroplast division.

The finding that two dynamin-related proteins of eukaryotic origin, ARC5 and FZL, are required for chloroplast division and morphogenesis indicates that the chloroplast division machinery has been modified from the cyanobacterial cell division machinery during evolution of the organelle.

To my wife, Fuli, for her love and support.

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KEY TO ABBREVIATIONS

arc	accumulation and replication of chloroplasts
ATPase	adenosine triphosphatase
BAC	bacterial artificial chromosome
bar	BASTA resistant
BLAST	basic local alignment search tool
bp	base pair
C-terminus	carboxy terminus
CaMV	cauliflower mosaic virus
CAPS	cleaved-amplified polymorphic sequence
cDNA	complementary DNA
co-IP	co-immunoprecipitation
DAPI	4',6-diamidino-2-phenylindole, dihydrochloride
EST	expressed sequence tag
EMS	ethyl methanesulfonate
fts	filamentation temperature-sensitive
FZL	FZO-like
FZO	fuzzy onions
GFP	green fluorescence protein
GTPase	guanosine triphosphatase
INDEL	insertion-deletion
IPTG	isopropylthio-β-galactoside
kb	kilobase

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kD	kilodalton
LHC	light-harvesting complex
min	minicell
MS	Murashige and Skoog
N-terminus	amino terminus
OCS	octopine synthase
PAGE	polyacrylamide gel eletrophoresis
PCR	polymerase chain reaction
PD	plastid-dividing
Pfu	*Pyrococcus furiosus*
PS	photosystem
RT	reverse transcription
SDS	sodium dodecyl sulfate
T-DNA	transfer DNA
UTR	untranslated region

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Chapter 1

Evolution of the Chloroplast Division Machinery

Chloroplasts are specialized organelles that carry out photosynthesis and many other important processes such as fatty acid and amino acid biosynthesis in plant cells. Many of the biological pathways in chloroplasts are similar to those in cyanobacteria. Chloroplasts have a small circular genome of about 60-200 genes without associated histones (Gray, 1999; Howe et al., 2003; Raven and Allen, 2003). They have their own protein-synthesizing machinery, which more closely resembles that of prokaryotes than that found in the cytoplasm of eukaryotes (Harris et al., 1994). Molecular phylogenetic studies of the genomic sequences of chloroplasts in many plants and several species of cyanobacteria strongly support the idea that chloroplasts are derived from cyanobacteria (Chu et al., 2004). After the *Arabidopsis* and rice genomes were sequenced (Arabidopsis_Genome_Initiative, 2000; Yu et al., 2002), it was revealed that most of the cyanobacterial genes were transferred into the nuclear genome in the past, and that most of them have acquired sequences encoding chloroplast transit peptides. Many of the functional proteins of chloroplasts were also found to be homologous to cyanobacterial proteins (Douglas, 1998; Durnford et al., 1999; McFadden, 1999; Raven and Allen, 2003). Thus, it is now widely accepted that chloroplasts originated from cyanobacteria through endosymbiosis.

Higher plants have many chloroplasts per cell in green tissue. For example, the mature mesophyll cells of wild-type *Arabidopsis thaliana* contain about 80-110 chloroplasts per cell (Marrison et al., 1999). New chloroplasts are generated by binary division of existing chloroplasts (Leech et al. 1981). Chloroplasts divide during cell division and expansion (Possingh, 1969; Saurer, 1970; Dean, 1982; Ellis, 1985).

Dumbbell-shaped chloroplasts, which represent the division phase, can frequently be found in young leaf tissue. Chloroplast division has physiological importance for plant cells. Suppression of chloroplast division results in fewer and larger chloroplasts per cell (Osteryoung et al., 1998; Pyke, 1999; Stokes et al., 2000). A chloroplast division mutant was reported to have a defect in gravitropism (Yamamoto et al., 2002). Smaller and more numerous chloroplasts per cell provide an advantage compared to larger and fewer chloroplasts: smaller chloroplasts have more total surface area and may exchange materials with other parts of the cell more efficiently. They can also migrate faster in response to changes in light intensity and direction (Jeong et al., 2002). Multiple chloroplasts in one cell seems also very important in the history of chloroplast genome evolution: cells with multiple chloroplasts can withstand the breaking of some chloroplasts, leading to the gene transfer from the chloroplast genome to the nuclear genome (Timmis et al., 2004). This transfer is important for the establishment of the modern chloroplast and nuclear genomes (Martin, 2003).

Early studies of chloroplast division were limited to observation by either light or electron microscopy. More details of chloroplast division were obtained following improvement of electron microscopy techniques (Leech RM, 1981; Oross and Possingham, 1989; Robertson et al., 1996). Observation of proplastids, which are the progenitor plastid type, reveals a central constriction that has been interpreted to represent the division phase of proplastids. Similar to proplastid division, chloroplast division includes elongation of the chloroplasts to form a peanut shape, constriction of the division furrow, further narrowing of the division furrow to a thin isthmus that joins

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the two daughter chloroplasts, and finally pinching off of the narrow isthmus. The envelope membranes of daughter chloroplasts are then resealed (Leech RM, 1981; Oross and Possingham, 1989).

Electron-dense deposits are observed at the division site of plastids (Kuroiwa et al., 1998). They form ring-like structures on the stromal face of the inner plastid membrane and the cytosolic surface of the outer plastid membrane. They were named the inner plastid-dividing (PD) ring and outer PD ring. A ring-like structure called the middle PD ring is also found between the two envelope membranes of the plastid in some red algae, but not in higher plants (Kuroiwa et al., 1998). The inner PD ring forms first and is belt-like, while the outer PD ring is rope-like and narrower than the inner ring. In the process of constriction of the plastid division furrow, the width and thickness of the outer PD ring increase linearly (Miyagishima et al., 2001a; Miyagishima et al., 2001b). The width of the inner PD ring remains the same throughout the plastid division. It has been suggested that the inner PD ring decomposes during constriction, and the outer PD ring wraps around the chloroplast with a constant number of molecules during the constriction (Miyagishima et al., 2001a; Miyagishima et al., 2001b).

To document the division and development of chloroplasts, Leech's group isolated 11 chloroplast division mutants in *Arabidopsis*, and named them *arc*, for accumulation and replication of chloroplasts (Pyke and Leech, 1994; Marrison et al., 1999). In each of the mutants, the mutation is recessive and represent single nuclear locus. Among these mutants, *arc1* and *arc7* have smaller and more numerous

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chloroplasts while the other nine mutants show larger and fewer chloroplasts. *arc6* shows the most severe phenotype, with only 1-2 chloroplasts per cell (Robertson et al., 1995). *arc5* has about 10 large chloroplasts per cell, which are frequently dumbbell-shaped (Robertson et al., 1996; Gao et al., 2003). *arc3* and *arc11* have about 20 chloroplasts of various sizes per cell (Marrison et al., 1999). Double-mutants were made to elucidate the role of some *arc* genes in chloroplast development and division. Based on the phenotypes of single and double mutants, several hypotheses were developed (Pyke and Leech, 1994; Marrison et al., 1999). It was suggested that *ARC6* controlled the initiation of both proplastid and chloroplast division; *ARC3* had an important role in the initiation of chloroplast division and controlled the rate of chloroplast expansion; *ARC11* could function just before chloroplast division is initiated or at any time earlier than the chloroplast expansion phase, and controlled the central positioning of the final division plane; *ARC5* worked in the final stage of chloroplast division and facilitated the separation of the two daughter chloroplasts; *ARC1* was in a different pathway and down-regulated proplastid division. However, later cloning of several chloroplast division genes and their further characterization indicated that most of the hypotheses above were inaccurate (Itoh et al., 2001; Gao et al., 2003; Vitha et al., 2003; Fujiwara et al., 2004; Shimada et al., 2004).

With the rapid developments molecular biology and genomics, many bacterial cell division genes were cloned and the functions of their gene products have been extensively studied (Bramhill, 1997; Rothfield et al., 1999; Errington et al., 2003). Some of the chloroplast division genes have also been cloned recently (Osteryoung and Pyke,

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1998; Strepp et al., 1998; Colletti et al., 2000; Itoh et al., 2001; Gao et al., 2003; Vitha et al., 2003; Fujiwara et al., 2004; Maple et al., 2004; Raynaud et al., 2004; Shimada et al., 2004). The results indicated that the chloroplast division machinery has inherited some of the important features of the cyanobacterial cell division machinery and it also has acquired some eukaryotic features from the host cell (Osteryoung and Nunnari, 2003). This review illustrates the conservation and innovation of the chloroplast division machinery during evolution by comparing some of the key components of the division machineries between bacteria and chloroplasts.

I. Conservation between cyanobacterial cell division proteins and chloroplast division proteins

Since chloroplasts are derived from endosymbiotic cyanobacteria and many chloroplast-targeted proteins also originated from cyanobacteria, it is likely that most of the components of the chloroplast division machinery are derived from the cell division machinery of cyanobacteria (Osteryoung and Pyke, 1998; Osteryoung, 2000). Indeed, a search for homologs of bacterial cell division genes in the sequenced *Arabidopsis* genome led to the discovery of several chloroplast division genes: the *FtsZ* gene family, *MinD*, *MinE*, *ARC6* and *SulA* (Osteryoung and Vierling, 1995; Osteryoung et al., 1998; Colletti et al., 2000; Stokes et al., 2000; Itoh et al., 2001; Vitha et al., 2003; Maple et al., 2004; Raynaud et al., 2004).

FtsZ

FtsZ is a GTPase that was first found in *E. coli* and is conserved in almost all bacteria and archaea (Bramhill, 1997; Rothfield et al., 1999). FtsZ is related to tubulin, and is suggested to be the ancestor of tubulin (de Boer et al., 1992a; RayChaudhuri and Park, 1992; Lowe and Amos, 1998; Nogales et al., 1998b). The structures of FtsZ (Erickson, 1998) and the αβ-tubulin heterodimer (Nogales et al., 1998a) also show a high degree of similarity. FtsZ self-polymerizes, forms a ring at the equator of the bacterial cell, and determines the division site of the cell (Bi and Lutkenhaus, 1991). Blocking the function of FtsZ or changing the level of FtsZ blocks bacterial cell division and causes long filamentous cells (Dai and Lutkenhaus, 1992; Dewar et al., 1992). During different stages of the cell cycle FtsZ exhibits a series of different behaviors: assembly to form the Z ring at midcell, maintenance of the ring through continual subunit turnover, and constriction and disassembly of the ring (Romberg and Levin, 2003).

The polymerization mechanism of FtsZ is also similar to that of tubulin (Scheffers and Driessen, 2001; Scheffers et al., 2002; Scheffers and Driessen, 2002). *In vivo*, Z rings can be assembled within 1 to 3 min and disassembled within 1 min (Sun and Margolin, 1998; Sun et al., 1998). Fluorescence recovery after photobleaching showed that the Z ring is highly dynamic throughout its existence and constitutes about 30% of cellular FtsZ protein (Stricker et al., 2002). In addition, the FtsZ protein in the Z ring can exchange with FtsZ protein in the cytosol very quickly (Stricker et al., 2002). These characteristics are based on the GTP-dependent, reversible polymerization of FtsZ. GTP is hydrolyzed immediately after FtsZ polymerization, and the FtsZ polymer contains both GDP and

inorganic phosphate (Scheffers and Driessen, 2002). Preformed FtsZ polymers can be stabilized by addition of nonhydrolyzable GTP-γ -S with more than 95% of the nucleotide associated with the FtsZ polymer in the GDP form, and be rapidly destabilized by addition of GDP (Scheffers et al., 2000; Mukherjee et al., 2001; Scheffers and Driessen, 2001; Scheffers and Driessen, 2002). These data indicated that, similar to tubulin, phosphate release may also be important for FtsZ polymer dynamics.

In plants, there are at least two classes of FtsZ: FtsZ1 and FtsZ2 (Osteryoung and Vierling, 1995; Osteryoung et al., 1998; Strepp et al., 1998; Stokes et al., 2000; Stokes and Osteryoung, 2003). They are all targeted into chloroplasts by a cleavable transit peptide (McAndrew et al., 2001). The C-terminal core domain is conserved in FtsZ2 but not FtsZ1. Phylogenetic analysis indicates that they are all closely related to cyanobacterial FtsZ (Rensing et al., 2004). Both FtsZ1 and FtsZ2 were shown to be important for chloroplast division and they may have distinct roles in chloroplast division. Antisense suppression, knock-out and over-expression of FtsZ1 or FtsZ2 can cause enlarged and fewer chloroplasts in the cell (Osteryoung et al., 1998; Strepp et al., 1998; Stokes et al., 2000) (Deena Kadirjan-Kalbach, personal communication). In extreme cases, there is only one chloroplast per cell. FtsZ1 and FtsZ2 were shown to form a ring and colocalize at the division site of chloroplasts by immunofluorescence microscopy and expression of GFP fusion proteins in *Arabidopsis thaliana* (Mori et al., 2001; Vitha et al., 2001). Even in the antisense and overexpressing FtsZ plants, FtsZ1 and FtsZ2 are still colocalized (McAndrew et al., 2001) (Stan Vitha, personal communication). It was shown that in wild-type plants the molecular ratio between FtsZ1

and FtsZ2 is fixed at 1:2 (McAndrew, personal communication). Thus, it seems that the correct ratio between the two types of FtsZ is important for their proper function in chloroplast division.

Mitochondria are also derived from bacteria as endosymbionts but at a time earlier than chloroplasts (Dyall et al., 2004). The division machinery of mitochondria seems to be simpler than that of chloroplasts (Osteryoung and Nunnari, 2003). In animals, fungi and higher plants, there is no mitochondrial division protein with a prokaryotic origin (Osteryoung and Nunnari, 2003). However, FtsZ was found in some red algae and protists and shown to be localized to the middle of mitochondria and required for the normal morphology of mitochondria (Beech et al., 2000; Takahara et al., 2000; Gilson et al., 2003; Kiefel et al., 2004). In *Dictyostelium*, two mitochondrial FtsZs, FszA and FszB, were found (Gilson et al., 2003). FszA is localized to the future or recent division sites of mitochondria (Gilson et al., 2003). However, FszB was found in an electron-dense, submitochondrial body usually located at one end of the organelle (Gilson et al., 2003). The evolution of the mitochondrial division machinery seems to be an ongoing process. The mitochondrial FtsZ probably is the last bacteria-derived division protein to be left and it has been lost many times in different species (Kiefel et al., 2004).

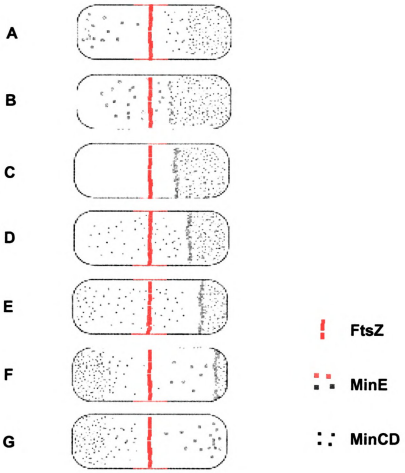
Min system

Proper cell division in bacteria requires accurate positioning of the division plane in the middle of the cell, which is determined by the placement of the FtsZ ring. The site of FtsZ ring assembly is in turn controlled by the *min* (*mini-cell*) operon, which

suppresses FtsZ polymerization at sites other than the middle of the cells (de Boer et al., 1989). MinC, MinD, and MinE are the proteins encoded by the *min* operon (de Boer et al., 1989), which is conserved in many bacteria including cyanobacteria. The Min proteins in *Escherichia coli*, when expressed at a certain ratio, undergo a highly dynamic localization cycle, during which they oscillate between the membrane of both cell halves, to ensure the proper placement of the cell division site (Figure 1) (de Boer et al., 1991; Mulder et al., 1992; Margolin, 2001; Meinhardt and de Boer, 2001).

The *min* system spatially regulates division through the topological regulation of MinCD, an inhibitor of cell division. MinCD inhibits division by preventing assembly of the Z ring (Bi and Lutkenhaus, 1993). A MalE-MinC fusion protein with full biological activity can interact with FtsZ and prevent polymerization without inhibiting FtsZ's GTPase activity (Hu et al., 1999). A functional GFP-MinC was shown to oscillate rapidly between the halves of the cell independently of FtsZ (Hu and Lutkenhaus, 1999; Raskin and de Boer, 1999b). However, GFP-MinC is a cytoplasmic protein with no oscillation in the absence of the other Min proteins (Hu and Lutkenhaus, 1999). The addition of MinD, which interacts with MinC, results in the localization of GFP-MinC on the membrane (Hu and Lutkenhaus, 1999). MinD is an ATPase located on the the inner membrane region of the cell envelope (de Boer et al., 1991). A functional GFP-MinD was also shown to have a rapid oscillation cycle which is imposed by MinE (Raskin and de Boer, 1999a). These results support a model in which MinD recruits MinC to inhibit FtsZ ring formation near the cell ends and force FtsZ assembly in the middle.

Figure 1 **A model for Min oscillation in *E. coli*** (adapted from Margolin, 2001). MinCD complexes first assemble on the membrane at one pole of the cell, while MinE is concentrated at the other pole (**A**). MinE diffuses towards MinCD (**B**), assembles into the MinE ring (**C**) and begins to disassemble the MinCD (**D**, **E** and **F**), causing MinCD to diffuse away from one pole (**D**, **E** and **F**). After the MinE ring has moved to one end of the cell, it begins to diffuse (**F** and **G**), lagging behind MinCD by a few seconds. In the meantime, MinCD is assembling in the other pole of the cell (**F** and **G**). The cycle then repeats.
Images in this figure are presented in color.


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In the absence of MinE, the coordinated expression of MinC and MinD leads to a general inhibition of cell division (de Boer et al., 1992b). In the yeast two-hybrid system, MinE reduces the interaction between MinC and MinD (Huang et al., 1996). MinE forms a dynamic ring that undergoes a repetitive cycle of movement first to one cell pole and then to the opposite pole (Fu et al., 2001). Taken together with studies of the dynamic behavior of MinCD, the MinE ring represents a cell structure that allows FtsZ ring formation at midcell by suppressing MinCD activity at this site.

To further investigate the molecular basis of the oscillation of the Min system, a series of *in vitro* experiments was done. MinD was shown to bind to phospholipid vesicles in the presence of ATP and to assemble into a well-ordered helical array that forms the vesicles into tubes in a cooperative fashion (Hu et al., 2002; Lackner et al., 2003). MinD can recruit either MinC or MinE in an ATP-dependent manner (Hu et al., 2003). MinE can promote bundling of MinD filaments as well as their disassembly by activating the ATPase activity of MinD (Suefuji et al., 2002). MinE stimulates dissociation of MinC from MinD:ATP-membrane complexes even without ATP hydrolysis (Hu et al., 2003). In contrast, MinC is unable to displace MinE bound to the MinD-bilayer complex (Hu et al., 2003; Lackner et al., 2003). These results suggest that MinE induces conformational changes in membrane-bound MinD, which result in the release of MinC and then the conversion of membrane-bound MinD (MinD:ATP) to cytoplasmic MinD (MinD:ADP).

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In some green algae, such as *Chlorella vulgaris*, *minD* and *minE* genes are present in the chloroplast genome and arranged in the same order as in *E. coli* (Wakasugi et al., 1997). In higher plants, these two genes are present in the nuclear genome and the gene products are targeted to chloroplasts by an N-terminal transit peptides (Colletti et al., 2000; Dinkins et al., 2001; Itoh et al., 2001; Maple et al., 2002; Reddy et al., 2002; Fujiwara et al., 2004). Transgenic *Arabidopsis* plants with reduced *MinD* expression exhibit variability in chloroplast size and number and asymmetrically constricted chloroplasts, suggesting that the chloroplast division machinery is misplaced (Colletti et al., 2000). Overexpression of *AtMinD* also inhibits chloroplast division in both *Arabidopsis* and tobacco (Colletti et al., 2000; Dinkins et al., 2001). Immuno-staining of FtsZ in plants over-expressing *MinD* shows random and short FtsZ filaments throughout the giant chloroplast, while parallel FtsZ rings are distributed ectopically at multiple sites along the enlarged chloroplast in the anti-sense *MinD* transgenic plants (Vitha et al., 2003). Giant chloroplasts were also observed in transgenic plants with higher or lower expression levels of *MinE* (Itoh et al., 2001; Maple et al., 2002; Reddy et al., 2002). Specifically, in the plants overexpressing *AtMinE*, the chloroplast division sites were misplaced, giving rise to either asymmetric or multiple constrictions along the length of chloroplasts (Maple et al., 2002). The phenotypes observed in *minD* and *minE* mutant plants resemble those of bacterial mutants with altered *minD* and *minE* expression levels, suggesting that their working mechanism may be similar.

Based on BLAST searches of the genome sequences of *Arabidopsis*, rice and *Chlamydomonas* and of the EST data from many plant species, *MinC* is missing in plants.

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According to the model of the Min system in bacteria, MinC is the protein that directly prevents FtsZ ring formation at non-mid- cell sites. Therefore, plants must have a protein with a role similar to that of MinC in order to allow the Min system to function in chloroplasts. One possibility is that the role of MinC is taken by another protein. In *Bacillus subtilis*, MinE is missing and its role probably is taken by DivIVA (Cha and Stewart, 1997; Edwards and Errington, 1997; Howard, 2004), supporting this hypothesis. But more likely, MinC is present in plants and the reason it has not been discovered is that its sequence is not well conserved. This is supported by the fact that all known cyanobacterial species have MinC, MinD and MinE but that protein sequences of MinC are much less conserved than those of MinD, MinE and many other cell division proteins. Still, the function of MinC may be well conserved. To find the MinC homolog in plants, approaches other than BLAST searches, such as searches based on protein structure, may be more useful.

FTN2 and ARC6

Ftn2 is a cell division gene conserved only in cyanobacteria but not in other bacteria (Koksharova and Wolk, 2002). A transposon insertion in the *Ftn2* gene in *Synechococcus sp. strain PCC 7942* blocks cell division. The mutant cells are up to 100-fold longer than wild-type cells and their colonies show an irregular spreading phenotype (Koksharova and Wolk, 2002). FTN2 has a domain of DnaJ cochaperones at its N-terminus and interacts with FtsZ in a bacterial two-hybrid system (Koksharova and Wolk, 2002; Mazouni et al., 2004). *In vitro* experiments with the purified proteins showed that

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FTN2 can also decorate the FtsZ filaments and that the DnaJ domain is critical for the decoration (Mazouni et al., 2004).

arc6 is a chloroplast division mutant in *Arabidopsis* with only one or two chloroplasts per cell (Pyke et al., 1994; Marrison et al., 1999). The *arc6* locus was mapped to a region containing the *Ftn2* homolog (Marrison et al., 1999). Sequencing of this gene revealed a nonsense mutation (Vitha et al., 2003). Complementation of the mutant phenotype by the wild-type gene confirmed that *ARC6* is the *Ftn2* homolog in plants (Vitha et al., 2003). Chloroplasts in the *arc6* mutant contain numerous short, disorganized FtsZ filament fragments (Vitha et al., 2003). *arc6* plants have a reduced level of FtsZ proteins but a normal level of *FtsZ* mRNA, indicating that their FtsZ proteins are less stable than that in wild type (Vitha et al., 2003). A functional ARC6-GFP was shown to be localized to a ring at the center of the chloroplasts (Vitha et al., 2003).

The data from Ftn2 in cyanobacteria and ARC6 in plants suggest that they may function similarly in stabilizing the constricting FtsZ ring.

SulA, Lon and HslVU

SulA, which is induced as part of the DNA-damage response (Huisman et al., 1980), inhibits bacterial cell division by interacting with FtsZ and preventing its polymerization (Mukherjee et al., 1998; Trusca et al., 1998). In *sulA* and *sulB* mutants, cell division is not blocked after DNA-damaging treatments as in wild type (Jones and

Holland, 1985). Induction of the expression of the wild-type *sulA* gene with the *lac* promoter by IPTG is sufficient to cause inhibition of cell division (Huisman et al., 1984). This inhibition can be suppressed by mutations in the *sulB* gene if *sulA* is not highly expressed (Huisman et al., 1984). Cloning of the *sulB* gene indicated that it encodes FtsZ (Jones and Holland, 1985). In the absence of FtsZ or in the *sulB114* mutant, SulA is extremely unstable with a half-life of only 3 min, in contrast to its normal half life of 12 min in the presence of FtsZ (Jones and Holland, 1985). These data suggest that SulA interacts directly with FtsZ *in vivo* to block cell division. In the presence of GTP and Mg^{2+}, SulA protein was shown to interact with FtsZ and form a stable complex in a molar ratio of approximately one to one (Higashitani et al., 1995). The role of GTP cannot be replaced by GDP or GTP-γ-S, suggesting that hydrolysis of GTP is required for the interaction between SulA and FtsZ (Higashitani et al., 1995). SulA inhibits the polymerization of FtsZ but not the polymerization of purified SulB mutant protein (Mukherjee et al., 1998; Trusca et al., 1998). By alanine scanning mutagenesis, the central region of SulA was shown to be essential for the FtsZ polymerization inhibition (Higashitani et al., 1997). By using deletion mutants, residues 3-27 and the 21 residues at the C-terminal end were shown not to be required for the inhibition, while the mutant protein lacking N-terminal residues 3-47 or 34 residues at the C-terminal end was inactive (Higashitani et al., 1997). SulA forms into a dimer either alone or in complex with FtsZ (Cordell et al., 2003). SulA blocks the polymerization of FtsZ and inhibits cell division by binding to the T7 loop surface of FtsZ (opposite to the nucleotide-binding site) without inducing conformational change (Cordell et al., 2003).

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SulA is induced by DNA damage and other stresses and has a very short half-life (Mizusawa and Gottesman, 1983). SulA is degraded by Lon protease and HslVU, an ATP-dependent protease (Mizusawa and Gottesman, 1983; Schoemaker et al., 1984; Kanemori et al., 1999; Seong et al., 1999). The cleavage is preferred at the central and C-terminal regions of SulA, which are important for the interaction with FtsZ and inhibition of cell division (Nishii and Takahashi, 2003). Either the deletion of the C-terminal region or a C-terminal addition of a protein tag can greatly increase the stability of SulA (Ishii and Amano, 2001).

Homologs of SulA are also found in plants and play a role in chloroplast division (Maple et al., 2004; Raynaud et al., 2004). The gene product of *AtSulA* is predicted to be targeted to the chloroplast. The expression pattern of *AtSulA* is similar to that of other chloroplast division genes, such as *FtsZ*s, *MinD* and *MinE*. In transgenic plants, AtSulA-GFP protein is imported into chloroplasts and the overexpression of *AtSulA* inhibits chloroplast division in various cell types. Overexpression of *AtSulA* can overcome the chloroplast division defect caused by overexpresion of *FtsZ*. Since the formation of too many FtsZ polymers can block chloroplast division, these data suggest that SulA in plants functions similarly to SulA in bacteria by preventing FtsZ polymerization.

Homologs of Lon and HslVU are also found in plants by BLAST search and are predicted with high scores to be chloroplast-targeted (Gao, unpublished). But their functions in chloroplast division have not been studied. Since chloroplasts have a non-dispensable genome, it is also important for chloroplasts to repair DNA damage before

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they divide. It is likely that the functions of the Lon and HslVU proteases are conserved in plants.

II. Innovations in the chloroplast division machinery during evolution

The endosymbiotic origin of chloroplasts occurred more than one billon years ago. Since then, chloroplasts have experienced great changes in the long history of evolution and acquired new protein components to function as an organelle in modern plants (Dyall et al., 2004). For example, chloroplasts have evolved protein import machinery complexes to import thousands of proteins encoded by the nuclear genome (Reumann et al., 1999; Reumann and Keegstra, 1999). Cyanobacteria have a cell wall between the inner and outer membrane, but there is no cell wall between the inner and outer envelope of chloroplasts and the outer envelope of chloroplasts has acquired many eukaryotic properties (Douce and Joyard, 1990; Dyall et al., 2004). Moreover, the division of chloroplasts must be under control of the host cells. Therefore, new components of the chloroplast division machinery must have evolved to adapt to the environment in host cells.

FtsZ1

In cyanobacteria, there is only one type of FtsZ. However, there are two types of FtsZ in plants: FtsZ1 and FtsZ2 (Osteryoung and McAndrew, 2001). FtsZ1 and FtsZ2 are closely related and very similar to the FtsZs in cyanobacteria (Osteryoung and McAndrew, 2001). FtsZs in most bacteria have a conserved N-terminal GTPase domain,

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C-terminal domain and the C-terminal core domain (Lowe and Amos, 1998; Mosyak et al., 2000). The C-terminal core domain at the extreme end contains a highly conserved sequence motif, D/E-I/V-P-X-F/Y-L, which is required for direct interactions between FtsZ and two other essential cell division proteins, ZipA and FtsA (Mosyak et al., 2000; Yan et al., 2000; Lowe and van den Ent, 2001). All three domains are conserved in FtsZ2 (Osteryoung and McAndrew, 2001). However, the C-terminal core domain is not present in FtsZ1 of plants (Osteryoung and McAndrew, 2001). Similar to FtsZ2, suppression of FtsZ1 expression or overexpression of FtsZ1 causes severe division phenotypes, suggesting that FtsZ1 and FtsZ2 are both essential for chloroplast division, but they may have different roles (Osteryoung et al., 1998; Stokes et al., 2000). Phylogenetic analysis indicates that FtsZ1 and FtsZ2 may have diverged at the very early stages of plant evolution, further supporting the distinct role of FtsZ1 in the evolution of the chloroplast division machinery (Stokes and Osteryoung, 2003; Rensing et al., 2004).

There are three possible explanations for the role of FtsZ1 in chloroplast division. First, the appearance of FtsZ1 during evolution may provide spacers for FtsZ proteins to form a ring suitable for chloroplasts. Since the diameter of bacterial cells is typically ~1 μm and the diameter of chloroplasts is typically 4-8 μm (Osteryoung et al., 1998; Koksharova and Wolk, 2002), one can envision that if there is only FtsZ2 in plants, there may be topological hindrances for FtsZ2 proteins to form a ring as large as required for chloroplast division. Second, the C-terminal end of FtsZ1 may have acquired new functions for the regulation of its activity. This is supported by the fact that the lack of the C-terminal end of FtsZ1 causes an intermediate division phenotype (Deena Kadirjan-

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Kolbach, personal communication). The third possibility is that the role of FtsZ1 is to provide enough proteins to be assembled into a ring together with FtsZ2 in chloroplasts. This seems to be less likely and can be tested by replacing *FtsZ1* with *FtsZ2*. If in the *FtsZ1* knockout plants a transgene of *FtsZ2* can rescue the mutant phenotype, the third hypothesis will be supported and the first and the second hypothesis will be invalidated.

ARC3

arc3 is a chloroplast division mutant in *Arabidopsis* with 10-20 chloroplasts per cell (Pyke and Leech, 1994; Marrison et al., 1999). Cloning of *ARC3* indicated that it encodes an FtsZ-like protein and that the *arc3* mutant is a null allele (Shimada et al., 2004). The N-terminus of ARC3 shares low similarity with the GTPase domains of FtsZ1 and FtsZ2. The C-terminus of ARC3 has a membrane-occupation-and-recognition-nexus (MORN) repeat motif similar to that of phosphatidylinositol-4-phosphate 5-kinases (PIP5K) (Shimada et al., 2004). Because many residues essential for the GTPase activity in FtsZ are not conserved in ARC3 (Osteryoung and McAndrew, 2001; Shimada et al., 2004), this protein may not have GTPase activity and its function might be quite different from that of FtsZ1 and FtsZ2. Since chloroplast division is still occuring in the null alleles of the *arc3* mutant (Pyke and Leech, 1994; Marrison et al., 1999; Shimada et al., 2004), ARC3 may have evolved to modify the chloroplast division machinery. Preliminary data show that ARC3 is a soluble protein located on the outer surface of the chloroplast in a ring-like structure at the early stage of chloroplast division (Shimada et al., 2004). These results are very surprising and it cannot yet be explained how ARC3 can work on the cytosolic side of the chloroplast envelope. However, the data on ARC3

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topology and localization were very preliminary (Shimada et al., 2004). Further experiments are needed to confirm these data before we can gain more insights about the role of ARC3 in chloroplast division.

ARC5

arc5 is a chloroplast division mutant in *Arabidopsis* with 1-15 enlarged dumbbell-shaped chloroplasts per cell (Pyke and Leech, 1994; Robertson et al., 1996; Marrison et al., 1999; Gao et al., 2003). The phenotype of *arc5* indicated that the *ARC5* gene product may be involved in the constriction of chloroplasts during division (Pyke and Leech, 1994; Robertson et al., 1996; Marrison et al., 1999; Gao et al., 2003). I cloned the *ARC5* gene by positional cloning (Gao et al., 2003). A *GFP-ARC5* fusion gene rescues the *arc5* mutant phenotype and the gene product is localized to a ring at the chloroplast division site (Gao et al., 2003). Chloroplast import and protease protection assays indicate that the ARC5 ring is positioned on the outer surface of the chloroplast (Gao et al., 2003). Similar results have also been observed for the homolog of ARC5 in a red alga (Miyagishima et al., 2003). Thus, ARC5 is the first cytosolic component of the chloroplast division complex to be identified. ARC5 is related to a group of dynamin-like proteins shown to be unique to plants by phylogenetic analysis (Gao et al., 2003; Miyagishima et al., 2003) (Figure 2). It has no obvious counterparts in prokaryotes, suggesting that it evolved from a dynamin-related protein present in the eukaryotic ancestor of plants.

Dynamin and its relatives are large GTPases that participate in a variety of organellar fission and fusion events in eukaryotes, including budding of endocytic and

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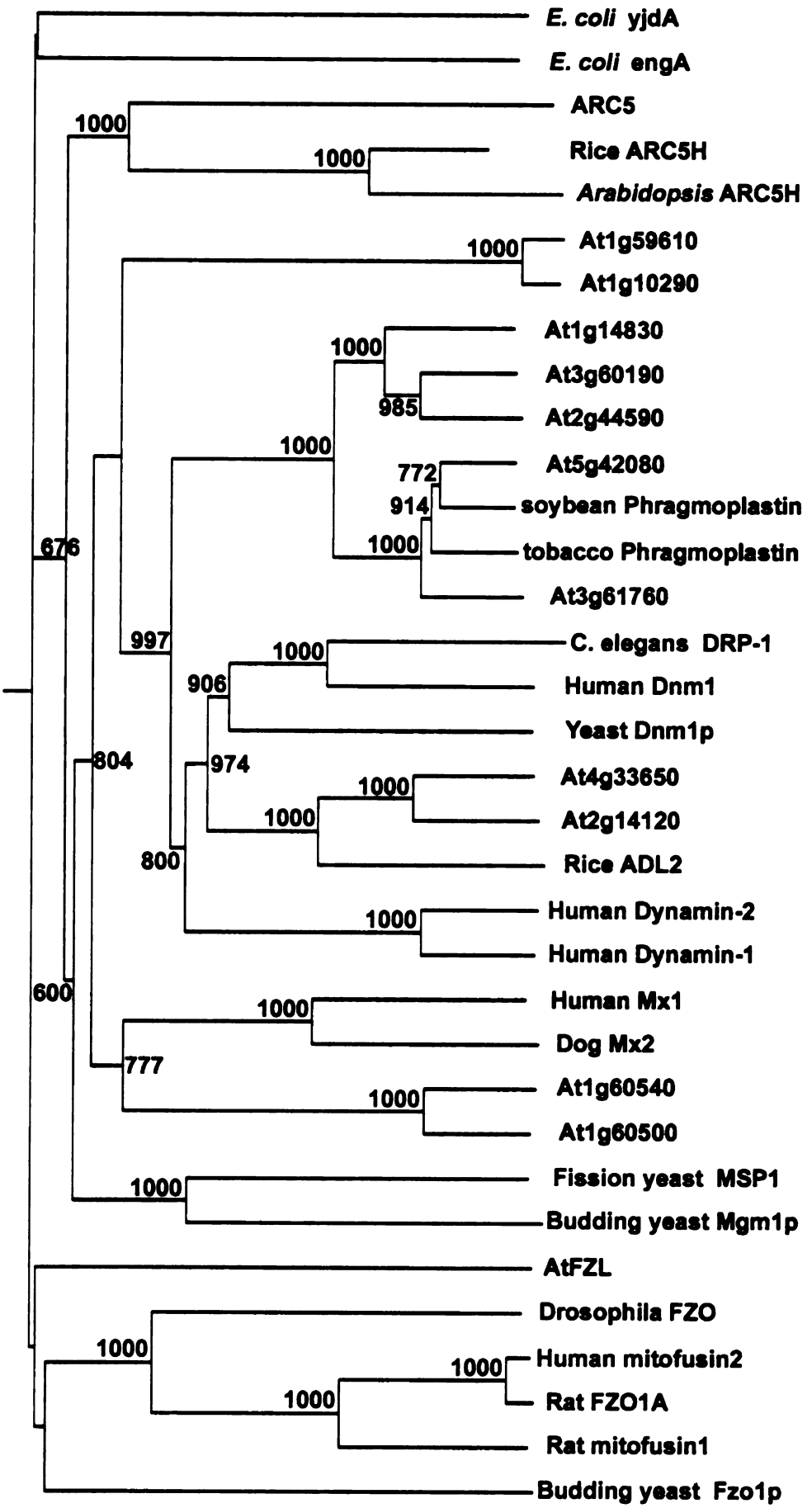
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Golgi-derived vesicles, mitochondrial fission, mitochondrial fusion, and plant cell plate formation (Chen et al., 1991; van der Bliek and Meyerowitz, 1991; Wilsbach and Payne, 1993; Gu and Verma, 1996; Pelloquin et al., 1998; Bleazard et al., 1999; Sesaki and Jensen, 1999; Hinshaw, 2000; Danino and Hinshaw, 2001). Structural analysis of dynamin indicates that it spontaneously self-assembles into rings and stacks of interconnected rings (Hinshaw and Schmid, 1995; Kelly, 1995; Carr and Hinshaw, 1997). Purified recombinant dynamin binds to a lipid bilayer in a regular pattern to form helical tubes that constrict and vesiculate upon GTP addition (Sweitzer and Hinshaw, 1998; Takei et al., 1998). In the *shibire* mutant of *Drosophila*, which has a mutation in the *dynamin-1* gene, many pit-like structures were observed to accumulate on the plasma membrane near presynaptic sites (Kosaka and Ikeda, 1983a, b). The neck portion of the pit-like structures was surrounded by cytoplasmic dense material, about 10 nm thick, reminiscent of a "collar" (Kosaka and Ikeda, 1983a). In GTP-γ-S-treated nerve terminals, tubular invaginations of the plasma membrane were surrounded by transverse electron-dense rings that were positive for dynamin immunoreactivity (Takei et al., 1995). These results, in addition to the finding that dynamin is capable of generating force, strongly support the hypothesis that dynamin is active in the fission reaction (Sweitzer and Hinshaw, 1998). Thus, it was suggested that dynamin is a mechanoenzyme directly involved in membrane remodeling when the vesicles are pinched off.

In contrast to most of the other members of the dynamin family, two dynamin-related proteins, FZO and Mgm1, are involved in membrane fusion in fungi and animals

Figure 2 **Phylogenetic analysis of dynamin-related proteins with an unrooted neighbor-joining tree.** Bootstrap values are shown at selected nodes with <50% bootstrap support. yjdA and engA in *E. coli* are GTPases involved in ribosome function and are used as the outgroup here. ARC5 is involved in chloroplast division. The function of ARC5H in rice and *Arabidopsis* is not clear. At1g59610 and At1g10290 are involved in the trafficking of Golgi-derived vesicles. At1g14830, At3g60190, At2g44590 and At3g61760 are also phragmoplastins and involved in cell plate formation in *Arabidopsis*. DRP-1, Dnm1, Dnm1p, At4g33650, AT2g14120 and rice ADL2 are involved in mitochondrial division. Dynamin-2 and dynamin-1 are involved in the budding of clathrin-coated endocytotic vesicles. Mx1 and Mx2 are Myxovirus resistance protein. The function of At1g60540 and At1g60500 is unknown. MSP1and Mgm1p mediates the fusion of the inner mitochondrial membrane during mitochondrial fusion. AtFZL is an FZO-like protein in *Arabidopsis* and is involved in the morphogenesis and division of chloroplasts. FZO, human mitofusin2, rat FZO1A, rat mitofusin1 and Fzo1p are involved in the fusion of the outer mitochondrial membrane during mitochondria fusion.


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At1g14830
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At2g44590
At5g42080
soybean Phragmoplastin
tobacco Phragmoplastin
At3g61760
C. elegans DRP-1
Human Dnm1
Yeast Dnm1p
At4g33650
At2g14120
Rice ADL2
Human Dynamin-2
Human Dynamin-1
Human Mx1
Dog Mx2
At1g60540
At1g60500
Fission yeast MSP1
Budding yeast Mgm1p
AtFZL
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Rat FZO1A
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(Hales and Fuller, 1997; Sesaki and Jensen, 1999; Shaw and Nunnari, 2002; Sesaki et al., 2003). Mutations in FZO and Mgm1 block mitochondrial fusion and result in a fragmented mitochondrial morphology and loss of mitochondrial DNA (Hales and Fuller, 1997; Sesaki and Jensen, 1999; Sesaki et al., 2003). FZO is directly inserted into the outer membrane of mitochondria by two transmembrane domains (Westermann, 2003). Mgm1, which is closely related to the mitochondrial fission protein Dnm1, is located in the mitochondrial intermembrane space and involved in the fusion of the mitochondrial inner membrane (Wong et al., 2000; Wong et al., 2003). The GTPase domain and two coiled-coil domains of FZO are exposed to the cytosol (Westermann, 2003). Interactions between the coiled-coil domains of FZO molecules was proposed to mediate the tethering between membranes from different mitochondria (Westermann, 2003; Koshiba et al., 2004). Hydrolysis of GTP may mediate the fusion of mitochondrial outer membranes by generating force for the movement of proteins and opening the attached membranes on the other side (Westermann, 2003; Meeusen et al., 2004).

Most of the dynamin-related proteins have four conserved domains: a GTPase domain, a middle-domain, a Pleckstrin homology (PH) domain and a GTPase effector domain (GED) (Hinshaw, 2000). Dynamin is proposed to be a mechano-enzyme and its GTPase activity may be involved in the generation of force (Warnock and Schmid, 1996; Sweitzer and Hinshaw, 1998). The middle-domain is involved in protein-protein interactions between dynamin molecules and is also essential for the function of dynamin (Smirnova et al., 1999). The PH domain is involved in the specific membrane binding of dynamin (Salim et al., 1996; Zheng et al., 1996). The GED domain interacts with the

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GTPase domain and modifies its activity (Fukushima et al., 2001). ARC5 is relatively more divergent from dynamin than other dynamin-related proteins (Gao et al., 2003). The GTPase domain and the middle domain of ARC5 can be aligned with other dynamin-related proteins better than can the PH and GED domains (Gao et al., 2003). ARC5 may therefore generate force to facilitate chloroplast division, but it functions at a site quite different from those of other dynamin-related proteins, and the diameter of the ARC5 ring is much larger than the diameter of the rings formed by other dynamin-related proteins. These results indicate that the chloroplast division machinery is of mixed evolutionary origin and that it shares structural and mechanistic similarities with both the cell division machinery of bacteria and the dynamin-mediated organellar fission machineries of eukaryotes.

III. Components that were probably lost or replaced by others during evolution.

Since chloroplasts lost their autonomy during their evolution, some of the bacterial functions are apparently no longer important for chloroplast function and the associated genes were lost. For example, nitrogen fixation is important for the survival of cyanobacteria but not important for the function of chloroplasts, so that many of the genes for nitrogen fixation were lost in evolution. This may also be partly true for chloroplast division genes. The morphology of plant chloroplasts is somewhat different from that of cyanobacteria. It is not surprising that some of the cell division proteins found in cyanobacteria are not conserved in plants. FtsA, Ftn6, FtsI and FtsW may be examples.

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FtsA belongs to a superfamily of ATPases that includes FtsA, DnaK, Hsp70, ParM, MreB, actin and hexokinase (Bork et al., 1992; Lowe et al., 2004). They all have similar structures and ParM and MreB have been shown to form filaments structurally similar to actin filments (van den Ent et al., 2002). FtsA, DnaK, Hsp70 and MreB are also very similar in sequence and may be homologs in different species with at least partially similar functions (Amos et al., 2004; Lowe et al., 2004). Sometimes, they may have the same function but have been annotated with different names in different species. This kind of confusion may be due to conservation and variations among these proteins but also to the fact that they were studied in different species from different perspectives. Here I will focus on FtsA and MreB, which function respectively in bacterial cell division and shape determination, and HSP70s, the homologs of cyanobacterial FtsA or MreB in plants.

FtsA was initially found by an *fts* screen (Wijsman and Koopman, 1976). FtsA is a bacterial cell division protein localized to a ring at the division site (Ma et al., 1996); Its localization depends on FtsZ, whereas FtsZ localization does not depend on FtsA (Addinall and Lutkenhaus, 1996). FtsA has no membrane-binding site and interacts with the C-terminal core domain of FtsZ (Ma and Margolin, 1999; Yan et al., 2000). In some *ftsZ* mutants with mutations in the coding region of the C-terminal core domain, FtsZ can form a ring but cannot recruit FstA (Ma and Margolin, 1999). In the absence of FtsA, several other cell division proteins cannot be localized to the division site (Errington et

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al., 2003; Rico et al., 2004). FtsA can be phosphorylated and bind ATP, but this seems to be not essential for its function (Sanchez et al., 1994). The molecular ratio between FtsA and FtsZ in E. coli is 1:100 (Dai and Lutkenhaus, 1992; Dewar et al., 1992). Alteration of this ratio will affect cell division.

MreB regulates the rod shape of bacterial cells in *Bacillus subtilis* and many other species and is believed to be the prokaryotic form actin (Jones et al., 2001; van den Ent et al., 2001a; Esue et al., 2005). MreB forms helical filamentous structures that surround the periphery of the cell, presumably just under the cytoplasmic membrane (Egelman, 2003). Knocking out of MreB in rod-shaped bacteria results in a spherical morphology (Jones et al., 2001). *In vitro*, MreB can assemble into two-stranded protofilaments with a subunit repeat similar to that of F-actin, except that the strands do not twist around each other (van den Ent et al., 2001a; van den Ent et al., 2001b). The crystal structure of MreB shows that its folding is also very similar to that of actin, except that there are insertions in actin (van den Ent et al., 2001a; van den Ent et al., 2001b). These insertions are important for the allosteric interactions within the actin subunit, subunit–subunit interactions in the filament, and interactions with other proteins.

Homologs of MreB or FtsA are also present in cyanobacteria and plants. The role of MreB or FtsA homologs in cyanobacteria is unknown. However, their homologs in plants are called HSP70. There are multiple copies of *HSP70* genes related to chloroplast function. In plant cells, when proteins are imported into chloroplasts, the HSP70s on the cytosolic side of the chloroplast import machinery may help to recognize the chloroplast

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transit peptide and unfold the proteins, HSP70s in the intermembrane space may be required for the translocation of chloroplast-targeted proteins, and the HSP70s on the stromal side may help the translocation and refolding of the proteins (Gray and Row, 1995; Jackson-Constan et al., 2001; Jarvis and Soll, 2002). Based on EST data, the mRNAs of these *HSP70* genes are much more abundant than those of FtsZ genes, in great contrast to the molecular ratio between FtsA and FtsZ in bacteria. Also, chloroplasts mostly have a spherical shape, similar to the bacteria that lack MreB. Thus, the homologs of FtsA or MreB in plants, chloroplast-targeted HSP70s, may not have a role in chloroplast division or morphology.

Ftn6

Ftn6 is a cyanobacteria-specific cell division protein. Knockout of *Ftn6* either by transposon insertion or homologous recombination affects cell division in *Synechococcus* sp. strain PCC 7942 and *Anabaena* sp. strain PCC 7120 (Koksharova and Wolk, 2002). The function of Ftn6 is unknown and there is no homolog of Ftn6 in other bacteria. Since not all the known cell division proteins in *E. coli* are found to have homologs in cyanobacteria and *vice versa*, Ftn6 may either have a role similar to some division proteins in *E. coli* or have a role unique to cyanobacterial cell division. The phenotype of *ftn6* mutants is not as severe as that of *ftn2* mutants (Koksharova and Wolk, 2002) and the protein sequences of Ftn6 are not well conserved in different cynaobacteria species. There is also no homolog of Ftn6 in plants, suggesting that the function of Ftn6 is not important for chloroplast division and that Ftn6 was lost during chloroplast evolution.

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FtsI

FtsI, also called penicillin-binding protein 3 (PBP3), is a transpeptidase required for cross-linking of the peptidoglycan cell wall at the bacteria cell division site (Nakamura et al., 1983; Weiss et al., 1997). FtsI is conserved in many bacteria including cyanobacteria (Margolin, 2000). It has a small cytoplasmic domain, a transmembrane domain, a domain of unknown function, and a transpeptidase domain (Wissel and Weiss, 2004). The last two domains reside in the periplasm (Wissel and Weiss, 2004). Immuno-staining and a functional GFP fusion protein indicated that FtsI is localized to the cell division site (Weiss et al., 1997; Weiss et al., 1999). Inhibition of the catalytic activity of FtsI blocks bacteria cell division but doesn't affect the localization of FtsI (Pogliano et al., 1997; Weiss et al., 1999). Localization of FtsI to the division site requires its membrane anchor, FtsZ, FtsW, FtsA, FtsQ, and FtsL (Weiss et al., 1999; Mercer and Weiss, 2002). It was suggested that the catalytic activity of PBP3 is stimulated by interaction(s) with other division proteins (Eberhardt et al., 2003). GFP-FtsI is localized to the division site during the later stages of cell growth and throughout the process of division (Weiss et al., 1999). Unconstricted FtsZ rings are stably trapped at the midpoint of the cell for several generations after inactivation of FtsI (Pogliano et al., 1997). These data suggest that FtsI functions in the late stage of cell division.

Chloroplasts do not have a peptidoglycan wall in the intermembrane space as do cyanobacteria. However, penicillin can inhibit chloroplast division in the moss *Physcomitrella patens* (Kasten, 1997; Katayama et al., 2003). This phenomenon is not observed in seed plants, such as tomato and *Arabidopsis* (Kasten, 1997). So far, it is

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unclear whether FtsI is conserved in moss or not. But in the sequenced rice and *Arabidopsis* genomes, no FtsI homologs are found. This indicates that, at least in higher land plants, FtsI probably was lost during evolution. If it is true that lower plants like moss require FtsI for chloroplast division, it will be interesting to learn how higher plants can overcome the loss of FtsI.

FtsW

FtsW is an essential bacterial cell division protein with 10 transmembrane domains and a large periplasmic loop (Ishino et al., 1989; Lara and Ayala, 2002). Both the N- and the C terminus of FtsW are located in the cytoplasm (Lara and Ayala, 2002). FtsW is also well conserved in cyanobacteria (Margolin, 2000). Although the sequence of FtsW is somewhat similar to that of the bacterial cell shape-determining protein RodA, mutations in FtsW only affect cell division and not cell shape (Khattar et al., 1994). FtsW is localized to the division site and interacts with FtsZ through its C-terminal tail (Datta et al., 2002). In the absence of FtsW, the formation of the FtsZ ring is affected, indicating that FtsW may have a role similar to that of another membrane protein, ZipA, in stabilizing FtsZ filaments. FtsW is also required for the localization of FstI to the cell division site (Mercer and Weiss, 2002). Thus, FtsW may link FtsZ ring formation in the cytoplasm to peptidoglycan synthesis in the periplasm at the bacteria cell division site.

Neither FtsW nor RodA was found to have a homolog in *Arabidopsis* or rice. Since there is no FtsI in higher plants, FtsW may not be required to be conserved for the localization FtsI. Moreover, the homolog of Ftn2, ARC6, is conserved in plants (Vitha

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et al., 2003). ARC6 has a transmembrane domain, its N-terminus is located in the stroma, and its C-terminus is located in the intermembrane space. ARC6 is also proposed to have a role in stabilizing FtsZ filaments at the chloroplast division site (Vitha et al., 2003) possibly replacing the function of FtsW.

IV. Perspective

The last decade has seen rapid growth of the knowledge in the areas of bacterial cell division and chloroplast division. The identification of many bacterial cell division proteins and some chloroplast division proteins indicates that the evolution of the chloroplast division machinery has involved both conservation and innovation.

FtsZ probably is the most important division protein for both bacteria and chloroplasts (Rothfield et al., 1999; Margolin, 2000; Osteryoung and McAndrew, 2001; Errington et al., 2003). FtsZ polymerizes and forms a ring at the division site. The FtsZ ring serves as a scaffold for the localization of many other division proteins. The Min system regulates the localization of the FtsZ ring to the mid-cell or mid-chloroplast (Colletti et al., 2000; Itoh et al., 2001; Lutkenhaus, 2002). SulA inhibits the assembly of FtsZ as part of the SOS system in bacteria (Lowe et al., 2004) and its homolog in plants also has a role in chloroplast division (Maple et al., 2004; Raynaud et al., 2004). Ftn2 is a cyanobacteria-specific cell division protein with a DnaJ domain (Koksharova and Wolk, 2002); it is also conserved in plants (Vitha et al., 2003). On the other hand, many bacteria cell division proteins may have been lost during evolution as chloroplasts

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evolved from bacteria into organelles. So far, only a few chloroplast division proteins are known. Most of these proteins are derived from the cynaobacterial cell division machinery.

When I started to clone *ARC5* in the fall of 2001, only FtsZ, MinD and MinE were known to be involved in chloroplast division and they had all been identified by a comparative genome approach (Osteryoung et al., 1998; Strepp et al., 1998; Colletti et al., 2000; Itoh et al., 2001). *arc5* is a chloroplast division mutant induced by EMS mutagenesis. Map-based cloning of *ARC5* indicated that it encodes a dynamin-related protein of eukaryotic origin (Gao et al., 2003) (see also chapter II). A homolog of ARC5, ARC5H, was shown not to have a function in chloroplast division (see chapter III). Therefore, ARC5 is important but not essential for chloroplast division. Since dynamin family-related proteins are proposed to be mechanoenzymes (Danino and Hinshaw, 2001), ARC5 may have evolved from the host to facilitate the division of chloroplasts by generating force from the outside of the organelles.

Bacterial cell division has been studied mostly in *E.. coli*. About 15 proteins are known to be involved in cell division in *E. coli* (Margolin, 2000). However, not all of these proteins are conserved in cyanobacteria (Margolin, 2000). Also, homologs of the cyanobacterial cell division proteins Ftn2 and Ftn6 are not found in other bacteria (Koksharova and Wolk, 2002). This indicates that different kinds of bacteria may have specific cell division proteins. Therefore, identification of more cynobacteria-specific cell division proteins may help to identify new chloroplast division proteins by comparative

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genomics. The previous mutant screening of chloroplast division mutants was far from saturation (Pyke and Leech, 1994; Marrison et al., 1999). A further mutant screening may also help to identify chloroplast division proteins that are not derived from cyanobacteria (Miyagishima et al., 2005). However, mutations in the genes with redundancy may not cause a phenotype and mutations in essential genes may be lethal. Identifying proteins that interact with the known chloroplast division proteins by biochemical methods and yeast two-hybrid assays can overcome this problem. With more chloroplast division proteins identified in the future, we can expect that more proteins of eukaryotic origin will be discovered.

In chapter IV, FZL, an FZO-like protein in *Arabidopsis* was shown to have a role in thylakoid morphogenesis and chloroplast division. FZO is a dynamin-like protein involved in mitochondrial fusion in fungi and animals (Sesaki and Jensen, 1999; Westermann, 2003; Meeusen et al., 2004). The domain structure of FZL is similar to that of FZO, suggesting that it may have similar biochemical function. FZL is a membrane protein localized to both the thylakoid and the chloroplast inner envelope, but not to mitochondria. Loss of function of *FZL* results in disorganization of thylakoid stacks and defects in chloroplast division. Overexpression of *FZL* causes very long thylakoid sacs with less stacking. Thus, the morphogenesis of thylakoids may also affect the division of chloroplasts.

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Chapter 2

ARC5, a Cytosolic Dynamin-like Protein from Plants, Is Part of the Chloroplast Division Machinery

Gao H, Kadirjan-Kalbach D, Froehlich JE, Osteryoung KW. Proceeding of the National Academy of Science of USA 2003 Apr 1; 100 (7):4328-33.

Dr. Deena Kadirjan-Kalbach did the cross between *arc5* and Col-0 wild type and generated an F_2 population for mapping the *ARC5* gene.

The work presented in Fig. 5 was done in collaboration with Dr. John E. Froehlich.

Abstract:

Chloroplast division in plant cells is orchestrated by a complex macromolecular machine with components positioned on both the inner and outer envelope surfaces. The only plastid division proteins identified to date are of endosymbiotic origin and are localized inside the organelle. Employing positional cloning methods in *Arabidopsis* in conjunction with a novel strategy for pinpointing the mutant locus, we have identified a gene encoding a new chloroplast division protein, ARC5. Mutants of *ARC5* exhibit defects in chloroplast constriction, have enlarged, dumbbell-shaped chloroplasts, and are rescued by a wild-type copy of *ARC5*. The *ARC5* gene product shares similarity with the dynamin family of GTPases, which mediate endocytosis, mitochondrial division, and other organellar fission and fusion events in eukaryotes. Phylogenetic analysis showed that ARC5 is related to a group of dynamin-like proteins unique to plants. A green fluorescent protein (GFP)-ARC5 fusion protein localizes to a ring at the chloroplast division site. Chloroplast import and protease protection assays indicate that the ARC5 ring is positioned on the outer surface of the chloroplast. Thus, ARC5 is the first cytosolic component of the chloroplast division complex to be identified. ARC5 has no obvious counterparts in prokaryotes, suggesting that it evolved from a dynamin-related protein present in the eukaryotic ancestor of plants. These results indicate that the chloroplast division apparatus is of mixed evolutionary origin and that it shares structural and mechanistic similarities with both the cell division machinery of bacteria and the dynamin-mediated organellar fission machineries of eukaryotes.

Introduction

The chloroplasts of plants and algae are widely believed to have evolved only once from a free-living cyanobacterial endosymbiont (1). Over evolutionary time, many of the genes once present in the endosymbiont have been transferred to the nuclear genome where they have acquired sequences encoding transit peptides that direct their gene products back to the chloroplast (1, 2). This scenario describes the origin of the five previously identified plastid division proteins in plants, all of which evolved from related cell division proteins in cyanobacteria, are encoded in the nucleus, and are localized inside the chloroplast. These include FtsZ1 and FtsZ2, tubulin-like proteins that localize to a ring at the site of plastid constriction (3-10), MinD and MinE, which regulate placement of the plastid division site (11-13), and ARTEMIS, which appears to mediate constriction of the envelope membranes (14).

Despite localization of the previously identified plastid division proteins inside the chloroplasts in plant cells, ultrastructural studies have shown that plastid division entails the coordinated activity of components localized outside as well as inside the organelle. In plants, the chloroplast division complex comprises electron-dense structures situated both on the stromal surface of the inner envelope membrane and on the cytosolic surface of the outer membrane (15). These structures have been termed the inner and outer plastid-dividing (PD) rings, respectively. A middle PD ring positioned in the intermembrane space has also been described in the red alga *Cyanidioschyzon merolae* (16), and the dynamics of assembly and disassembly of the three PD rings have been investigated in detail in this organism (17, 18). Although it was previously

hypothesized that the PD rings might contain FtsZ (4), recent evidence showing that the FtsZ ring assembles prior to and is separable from the PD rings in both *C. merolae* and plants (19, 20) indicate that this is not the case. Thus, while it is assumed that the PD rings represent multiprotein complexes, their compositions remain unknown.

The *Arabidopsis* mutant *arc5* contains an EMS-induced mutation conferring a chloroplast division defect in which chloroplasts initiate but rarely complete constriction (21, 22). As a result, *arc5* chloroplasts often exhibit a dumbbell shape (Fig. 1*B*). This phenotype suggested that the *ARC5* gene product might be a structural component of the chloroplast division complex. Here we show that *ARC5* is a member of the dynamin-family of GTPases, which have not been shown previously to participate in chloroplast division, and that it localizes to the chloroplast division site in plants. However, in contrast with other chloroplast division proteins, ARC5 is positioned on the cytosolic surface of the organelle and has no obvious homologues in prokaryotes. Our findings reveal that the chloroplast division machinery is an evolutionary hybrid, combining structural and mechanistic features acquired from both the prokaryotic ancestor of chloroplasts and its eukaryotic host.

Materials and Methods

Plant Material.

Arabidopsis thaliana strains Columbia (Col-0) and Landsberg *erecta* (L*er*) were used for all experiments as indicated. The *arc5* mutant was identified in the L*er* background by Pyke and Leech (21). Plants were grown as described previously (4).

Microscopy.

Phenotypes were analyzed as previously described (4), except that the images were recorded with a Coolpix 995 digital camera (Nikon Corporation, Tokyo, Japan). For *in vivo* detection of green fluorescent protein (GFP), fresh leaf tissue was mounted in water and viewed with an L5 filter set (excitation 455 nm to 495 nm, emission 512 to 575 nm) and a 100X oil immersion objective of a Leica DMR A2 microscope (Leica Microsystems, Wetzlar, Germany) equipped with epifluorescence illumination. Images were captured with a cooled CCD camera (Retiga 1350EX, Qimaging, Burnaby, British Columbia, Canada) and processed with Adobe Photoshop imaging software (Adobe Systems, San Jose, CA).

Fine-mapping of *ARC5*.

The *arc5* mutation was previously mapped between markers nga162 (20.6 cM) and AtDMC1 (32.6 cM) on chromosome 3 (23). To fine map *ARC5*, an F_2 population was generated from a cross between *arc5* and Col-0 wild type. Out of 7000 F_2 plants, 1720 mutants were identified by microscopy. Markers used for polymerase chain reaction (PCR)-based mapping (24) are provided as online supplemenatry material.

Identification of a Candidate *ARC5* Gene.

BAC clones MMB12 and MPN9 were double-digested with *Hinc*II and *Hind*III. The resulting fragments were inserted between the cauliflower mosaic virus (CaMV) 35S promoter and octopine synthase (OCS) terminator in the plasmid SN506 (4) that had been previously digested with *Sma*I and *Hind*III to remove the insert. Ligation products were amplified in *E. coli* strain DH5α, transferred to *Agrobacterium tumefaciens* GV3101, and introduced into wild-type Arabidopsis (Col-0) by floral dipping as described previously (25). T_1 transgenic plants were selected by growth on kanamycin and screened for an *arc5*-like phenotype by microscopy. The T-DNA inserts from the two *arc5*-like plants identified were amplified by PCR using vector primers flanking the inserts. The PCR products were sequenced to determine which fragments from the BAC clones were carried by these plants, as well as their orientation with respect to the promoter. Both plants carried the same *Hind*III-*Hinc*II fragment from At3g19730 (see Results). To confirm that this fragment was the source of the *arc5*-like phenotype in the T_1 transgenics, the fragment was subcloned into SN506 in the antisense orientation as described above and introduced into wild-type Col-0 plants. Following selection on kanamycin, the phenotypes of the resulting T_1 plants were determined by microscopy.

Amplification of *ARC5* cDNA.

Primers used for RT-PCR were 5'-GAAAAAGGAACGGCGACGAAAAC-3' and 5'-GCAAACATTGGACCAAAAAGCG-3'. Amplified cDNAs were subcloned into Bluescript KS+ (Stratagene) prior to sequencing.

Sequence Alignment and Phylogenetic Analysis.

The amino acid sequence of ARC5 was deduced from the cDNA sequence. The sequence alignment shown in Fig. 3*A* was performed with the CLUSTALW multiple alignment program (26) at the Biology Workbench 3.2 website (http://biowb.sdsc.edu/). Protein sequences used for the phylogenetic analysis shown in Fig. 3*B* were aligned with Clustal X (27) using default settings. The alignment is available upon request. Neighbor joining and maximum parsimony analyses were performed using PAUP version 4.0b10 (28) with default settings except for ties being randomly broken. Neighbor-joining and maximum parsimony analyses produced topologically identical trees. Bootstrap analyses were performed on the neighbor-joining and maximum parsimony trees with one thousand replications. GenBank accession numbers for proteins aligned with ARC5 (longer form, accession no. AY212885) are as follows: human Dynamin-1 (NP_004399), yeast Dnm1p (NP_013100), At1g53140 (NP_175722), rice dynamin like protein (BAB56031), ADL6 (AAF22291), At5g42080 (NP_568602), *Glycine* phragmoplastin (AAB05992), tobacco phragmoplastin (CAB56619), At2g44590 (NP_181987), human Dynamin II (NP_004936), ADL2a (NP_567931), ADL2b (NP_565362), rice ADL2-like protein (BAB86118), worm Drp-1 (AAL56621) and human Dnm1p/Vps1p-like protein (JC5695).

Complementation Analysis.

The genomic fragment corresponding to *ARC5* (At3g19730 and At3g19720 in the Arabidopsis database, see Results), including 1.9 kb and 1.1 kb of the 5' and 3' flanking

DNA, respectively, was amplified from MMB12 by PCR using the primers 5'-GGAATTCCGAGTCGAGTTGCTTTGTTG-3' and 5'-CGTCTAGAGCTTACCTCAAAGGTACATGGA-3'. The PCR product was digested with *Eco*RI and ligated into a derivative of the transformation vector pLH7000 (http://www.dainet.de/baz/jb2000/jb_2000direkt.htm) digested with *Eco*RI and *Sma*I. The construct was transferred to *A. tumefaciens* GV3101 and introduced into *arc5* plants by floral dipping. The phenotypes of the T_1 plants were determined by microscopy.

GFP-ARC5 Localization.

The GFP sequence was amplified from plasmid smRS-GFP (29) with the primers 5'-CGGGATCCATGAGTAAAGGAGAAGAACT-3' and 5'-GCTCTAGATAGTTCATCCATGCCATGT-3'. The PCR product was digested with *Bam*HI and *Xba*I. The *ARC5* coding region and 1.1 kb of the 3' flanking DNA were amplified from the MMB12 BAC clone with primers 5'-GGACTAGTACGATGGCGGAAGTATCAGC-3' and 5'-CGGGATCCGCACCGAAGGAGCCTTTAGATT-3'. The PCR product was digested with *Spe*I and *Eco*RI. cDNA fragments encoding GFP and ARC5 were subcloned into Bluescript KS+ (Stratagene) that had been digested with *Eco*RI and *Bam*HI to create a *GFP-ARC5* fusion construct. The *ARC5* promoter was amplified from MMB12 with primers 5'-GACTAGTTGGCTCAACGCTTACCTCAA-3' and 5'-CGGGATCCGCCATCGTCTCTTACGA-3', and cloned into Bluescript KS+ (Stratagene) between the *Spe*I and *Bam*HI sites. The promoter fragment was then subcloned into the plasmid containing the *GFP-ARC5* fusion construct at the 5' end of

the fusion. The resulting plasmid was digested with *Spe*I and *Eco*RI, and the promoter-*GFP-ARC5* cassette was subcloned into a derivative of the transformation vector pLH7000 (http://www.dainet.de/baz/jb2000/jb_2000direkt.htm). The plasmid was transferred to *A. tumefaciens* GV3101 and used to transform wild-type *A. thaliana* plants (Col-0) as described above. The GFP-ARC5 localization pattern was visualized by fluorescence microscopy in T_1 plants.

In Vitro Chloroplast Import and Protease Protection Assays.

Transcription/translation reactions, chloroplast isolation, *in vitro* import reactions, proteolytic treatments, and post-import fractionation and analysis were performed as described (7). The longer *ARC5* cDNA, after subcloning into Bluescript KS+ as described above, was used for these experiments.

Results

Phenotype of *arc5*.

Like other chloroplast division mutants, leaf mesophyll cells in *arc5* (L*er*) mutants contain fewer and larger chloroplasts than do wild type cells, which have about 120 chloroplasts at maturity (21, 22) (Fig. 1*A*). The *arc5* mutant has been reported to contain an average of 13 chloroplasts in fully expanded mesophyll cells (21, 22), though the phenotype appears somewhat more variable in our hands, with *arc5* cells typically containing between 3 and 15 chloroplasts (Fig. 1 *B* and *C*). This difference may reflect differences in growth conditions. Chloroplast number is fairly uniform within an

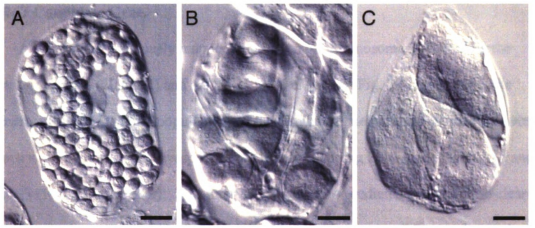


Fig. 1. Comparison of chloroplasts in *Arabidopsis* leaf mesophyll cells. (*A*) Wild-type (L*er*). (*B, C*) *arc5*. Cells are from fixed tissue. Bars: *A-C*, 10 µm.

individual *arc5* plant, and, as shown for other *arc* mutants (30), chloroplast size in *arc5* varies inversely with chloroplast number (not shown). As has been reported previously (21, 22), *arc5* chloroplasts frequently remain dumbbell shaped (Fig. 1*B*).

Fine Mapping of *ARC5* and a Novel Antisense Strategy for Identification of an *ARC5* Candidate Gene.

ARC5 was previously mapped to a region of chromosome 3 flanked by the markers nga162 (20.6 cM) and AtDMC1 (32.6 cM) (23) (Fig. 2*A*, upper bar). We generated a new mapping population and used standard methods to fine map the *arc5* locus to a 92-kb interval corresponding to two overlapping BAC clones, MMB12 and MPN9 (Fig. 2*A*, lower bar). This interval contained 24 predicted genes, but no clear *ARC5* candidate. To identify the gene most likely to represent *ARC5*, we digested MMB12 and MPN9 with restriction enzymes, and subcloned the resulting fragments between the CaMV 35S promoter and OCS terminator in a derivative of the plant transformation vector pART27 (31), creating a library of fragments spanning the *ARC5*-containing interval. The library was designed such that at least one fragment from each predicted gene in the interval would be ligated to the vector in the antisense orientation, with the expectation that an antisense fragment from *ARC5* would yield an *arc5*-like phenotype. The library was used to transform wild-type *Arabidopsis* plants (Col-0), and transgenic plants were screened for chloroplast division defects by microscopy.

Among 120 independent T_1 individuals examined, two had fewer and larger chloroplasts per cell than wild type (Fig. 2*E*), and resembled *arc5*. The inserts carried by

Fig. 2. Cloning of *ARC5*. (*A*) Fine mapping of *ARC5*. Triangle indicates the position of *ARC5*. (*B*) Open reading frames of *ARC5*. Black boxes represent exons; solid lines represent introns; gray box indicates the alternatively spliced intron. The mutation in *arc5* and its position are indicated. (*C*) Amplification of the transgene inserts in two *arc5*-like T1 plants produced by transforming wild type plants (Col-0) with a library of sense and antisense fragments derived from MMB12 and MPN9. 100 bp DNA ladder (New England Biolabs) is shown at left. Arrow indicates a *Hind*III-*Hinc*II fragment from At3g19730 common to both *arc5*-like transgenic lines. (*D-G)* Single leaf mesophyll cells from (*D*) Col-0 wild-type, (*E*) an *arc5*-like T_1 plant described in *C*, (*F*) the *arc5* (L*er*) parent line used for complementation by *ARC5*, and (*G*) an *arc5* plant transformed with the *ARC5* gene. Bars: *D-G*, 10 μm.

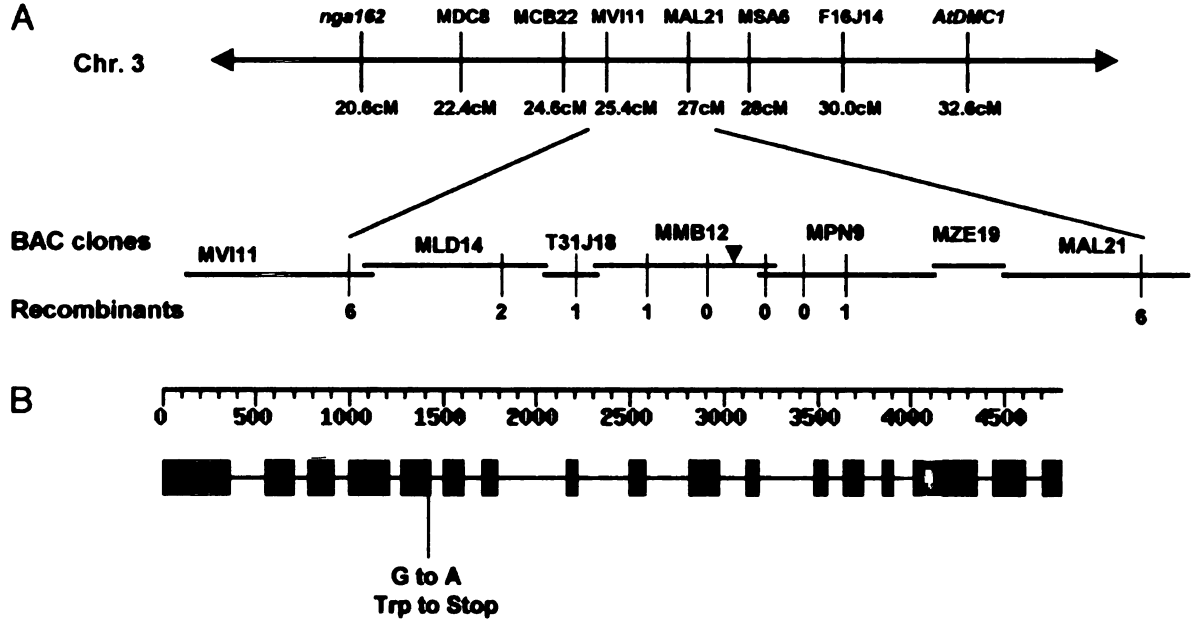
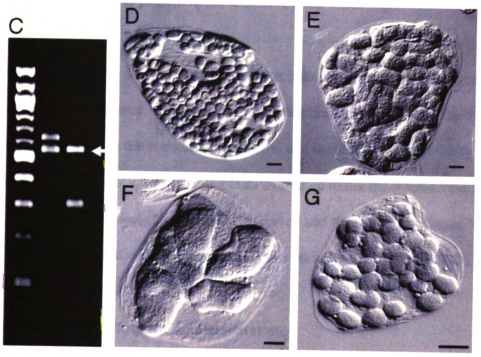

A
Chr. 3
nga162
MDC8
MCB22
MVI11
MAL21
MSA6
F16J14
AtDMC1
20.6cM
22.4cM
24.6cM
25.4cM
27cM
28cM
30.0cM
32.6cM
BAC clones
MVI11
MLD14
T31J18
MMB12
MPN9
MZE19
MAL21
Recombinants
6
2
1
1
0
0
0
1
6
B
0
500
1000
1500
2000
2500
3000
3500
4000
4500
G to A
Trp to Stop

Fig. 2 continued



the transgenes in these two transgenic lines were amplified by PCR and sequenced. Two different inserts were detected in both lines (Fig. 2*C*). One line carried a fragment derived from the BAC backbone DNA and a second fragment derived from At3g19730 on MMB12 (Fig. 2*C*, lane 2). The other line carried a fragment from At3g19760 on MMB12 as well as the same fragment from At3g19730 (Fig. 2*C*, arrow). In both plants, the At3g19730 fragment was present in the antisense orientation.

To confirm that the *arc5*-like plastid division phenotype in the two transgenic lines was caused by the shared At3g19730 fragment, we constructed a transgene containing this fragment in the antisense orientation, and introduced it into wild-type *Arabidopsis* (Col-0). Out of 80 transgenic plants examined under the microscope, 20% had chloroplast division defects similar to those of *arc5* and the transgenic line shown in Fig. 2*E*. These findings indicated that the *ARC5* gene corresponded to At3g19730.

Sequencing of *arc5* and Mutant Complementation.

At3g19730 has homology to the dynamin family of GTPases (32). In the *Arabidopsis* database, At3g19730 and the immediately adjacent sequence, At3g19720, were annotated as separate genes. However, sequence alignments to other dynamin-like proteins as well as EST data from *Arabidopsis* and other plants strongly suggested they were parts of a single gene, referred to hereafter as At3g19730/At3g19720, and that the annotated start codon for At3g19730 and stop codon for At3g19720 represented the true start and stop codons of this gene. This was confirmed by subsequent cDNA isolation and analysis (see below). To establish whether *arc5* contained a mutation in this region of

the genome, we sequenced the At3g19730/At3g19720 locus in both the *arc5* mutant and in wild-type Landsberg *erecta*. The data showed the presence in *arc5* of a G-to-A mutation, which altered a tryptophan codon (TGG) to a stop codon (TAG) (Fig. 2*B*). We then introduced a transgene containing the predicted At3g19730 /At3g19720 locus plus 1.9 kb and 1.1 kb of the 5' and 3' flanking DNA, respectively, into *arc5* mutants to determine whether the wild-type DNA could complement the *arc5* mutation. Microscopic analysis of T_1 transgenic plants indicated that the chloroplast division defect in the mutant was fully or partially rescued by the wild-type transgene (Fig. 2*G*). Differences in gene dosage resulting from position effects probably account for the partial complementation in some individuals (33). Taken together, the point mutation in At3g19730 /At3g19720 in *arc5*, complementation of the mutant phenotype by the wild-type gene, and ability of a fragment from At3g19730 /At3g19720 to confer an *arc5*-like phenotype in wild-type plants when expressed in the antisense orientation, indicate that the *ARC5* locus and At3g19730 /At3g19720 represent the same gene.

Analysis of *ARC5* cDNAs and Gene Products.

To gain more information on the *ARC5* transcription unit and encoded polypeptide, we used RT-PCR and primers spanning from 93 bp upstream to 152 bp downstream of the predicted At3g19730 /At3g19720 start and stop codons, respectively, to amplify the corresponding cDNA. Sequence analysis revealed two distinct cDNA species. The longer cDNA contained a sequence that was spliced out of the shorter cDNA as the 15th intron (Fig. 2*B*); however, its presence in the longer cDNA did not interrupt the reading frame. More than half the cDNA clones recovered were of this type. This

Fig. 3. ARC5 is a dynamin-like protein. (*A*) Alignment of ARC5 with Dynamin-1 from *Homo sapiens* and Dnm1p from *Saccharomyces cerevisiae*. Gray boxes indicate completely conserved residues; yellow boxes are identical residues; cyan boxes are similar residues; dashes indicate gaps. The domain structure is indicated by the lines above the alignment. Red, GTPase domain; green, middle domain; blue, PH domain; lavender, GED domain; black, PR domain. The dotted underline indicates the sequence encoded by the alternatively spliced intron in *ARC5*. The triangle indicates the position of the *arc5* mutation. (*B*) Phylogenetic analysis of ARC5 with an unrooted neighbor-joining tree. Bootstrap values are shown at selected nodes. The first and second bootstrap values are from the neighbor-joining and parsimony analyses, respectively. Nodes with less than 50% bootstrap support, or not present, are represented by a '-'. Accession numbers for the sequences aligned with ARC5 are listed in Materials and Methods.

Images in this figure are presented in color.

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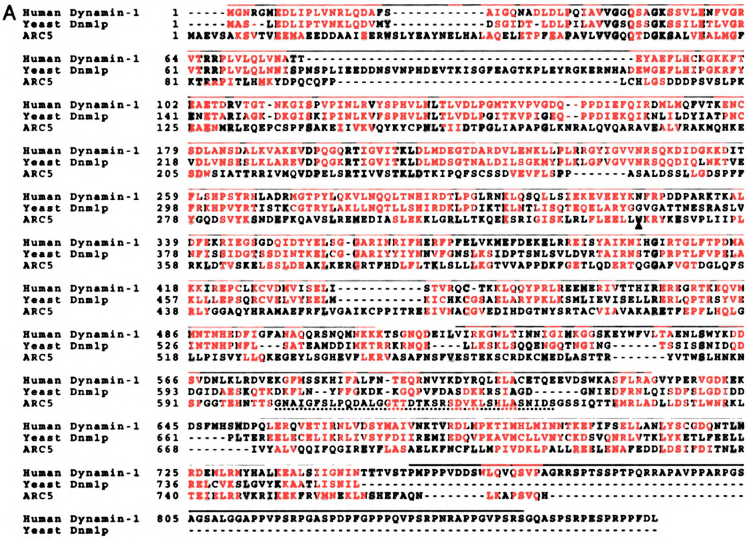
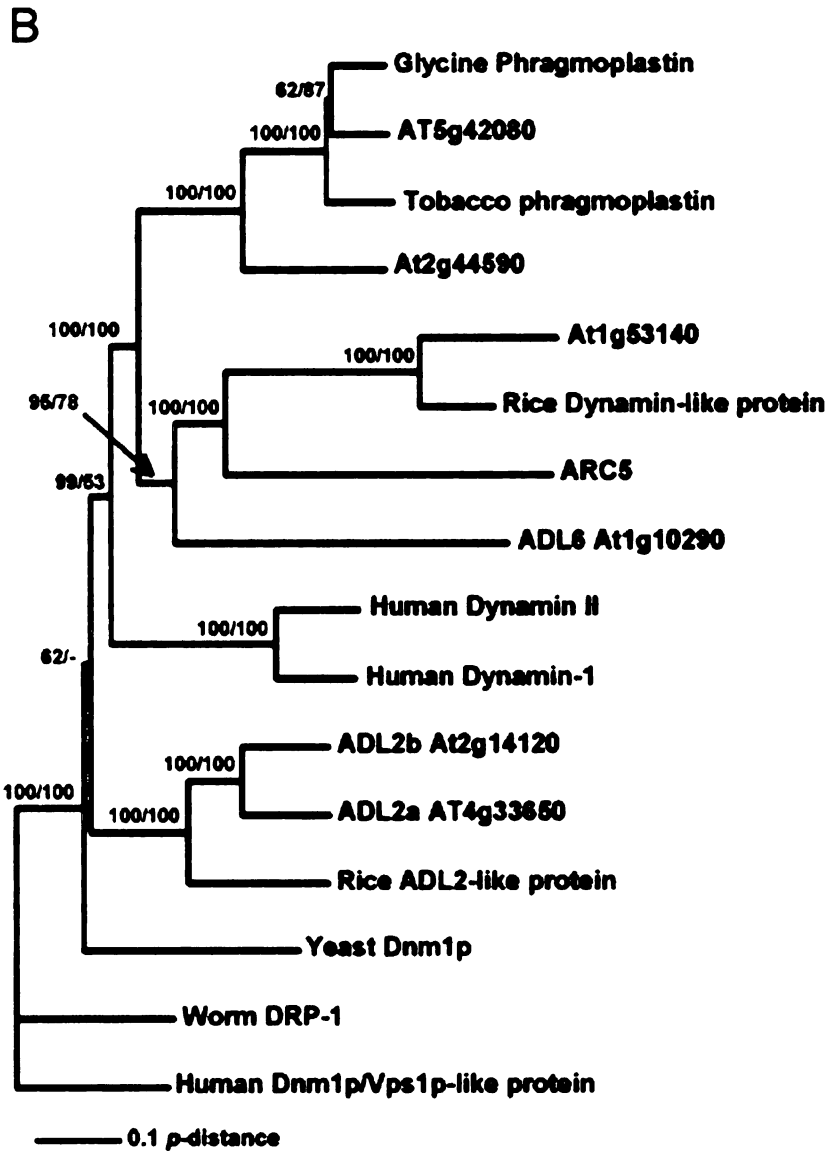

A
Human Dynamin-1
Yeast Dnm1p
ARC5

Fig. 3 continued



cDNA encoded a protein of 777 amino acids (Fig. 3*A*) and 87.2 kDa. The protein can be aligned over its entire length with numerous members of the dynamin family and contains three motifs found in other dynamin-like proteins: a conserved N-terminal GTPase domain, a pleckstrin homology (PH) domain shown in some proteins to mediate membrane association, and a C-terminal GTPase Effector Domain (GED) thought to interact directly with the GTPase domain and to mediate self-assembly (32, 34) (Fig. 3*A*). The shorter cDNA encoded a protein of 741 amino acids and 83.5 kDa identical to that of the larger gene product except for the absence of 36 amino acids encoded by the sequence of the 15th intron (Fig. 2*B* and 3*A*). These results suggest that the *ARC5* transcript is alternatively spliced. Alternative splicing of dynamin genes in several other organisms has also been documented (34). Because the shorter gene product lacks a portion of the PH domain (Fig. 3*A*), the proteins encoded by the two splice variants may have different activities or localization patterns.

Phylogenetic analysis was performed to investigate the relationship between ARC5 and other members of the dynamin family of proteins. Only full-length sequences were used, though EST data indicate that related proteins are present in many plants and in green algae (not shown). ARC5 clustered with a group of proteins found in plants, but was in a distinct clade from other dynamin-like proteins in Arabidopsis with functions in cell-plate formation and mitochondrial division (35, 36) (Fig. 3B). Surprisingly, the ARC5-like proteins clustered near ADL6, another Arabidopsis dynamin-like protein involved in vesicle trafficking from the trans-Golgi network to the vacuole in plants (37). This suggests that ARC5 and ADL6 could share a common dynamin-like ancestor.

Based on the similarity of ARC5 to dynamin and its relatives (Fig. 3*A*), we conclude that ARC5 represents a new class of a dynamin-like proteins that functions specifically in chloroplast division.

Localization of ARC5.

We expressed a GFP-ARC5 fusion protein in transgenic plants to investigate the subcellular localization of ARC5. Because overexpression of chloroplast FtsZ proteins can result in a dominant-negative phenotype (10), we used the native *ARC5* promoter to create the *GFP-ARC5* transgene for expression in wild-type plants (Col-0). Fluorescence microscopy showed that the fusion protein was localized in a ring-like pattern at the site of the chloroplast constriction (Fig. 4). This ring could be faintly detected in unconstricted chloroplasts, suggesting that ARC5 may act at an earlier stage of division than previously hypothesized (21, 22). However, ARC5 is not required for FtsZ ring formation, the earliest known event in the assembly of the chloroplast division apparatus (18, 19, 40), since the FtsZ ring can be detected in the *arc5* mutant (not shown). The GFP-ARC5 fusion protein was most obvious in visibly constricted chloroplasts, perhaps as a consequence of ring thickening during constriction. Similar localization patterns have been described for FtsZ1 and FtsZ2 (10). However, in contrast to the FtsZ rings, which are fairly uniform in fluorescence intensity, the GFP-ARC5 ring was more speckled in appearance (Fig. 4), suggesting that ARC5 localization at the division site may be discontinuous or that the ARC5-containing ring is not of uniform composition.

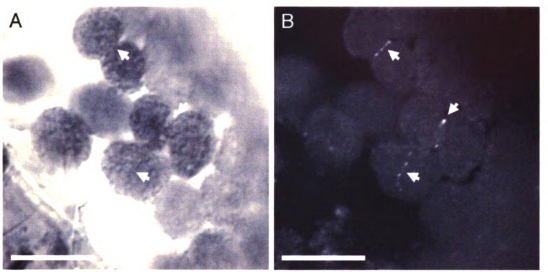
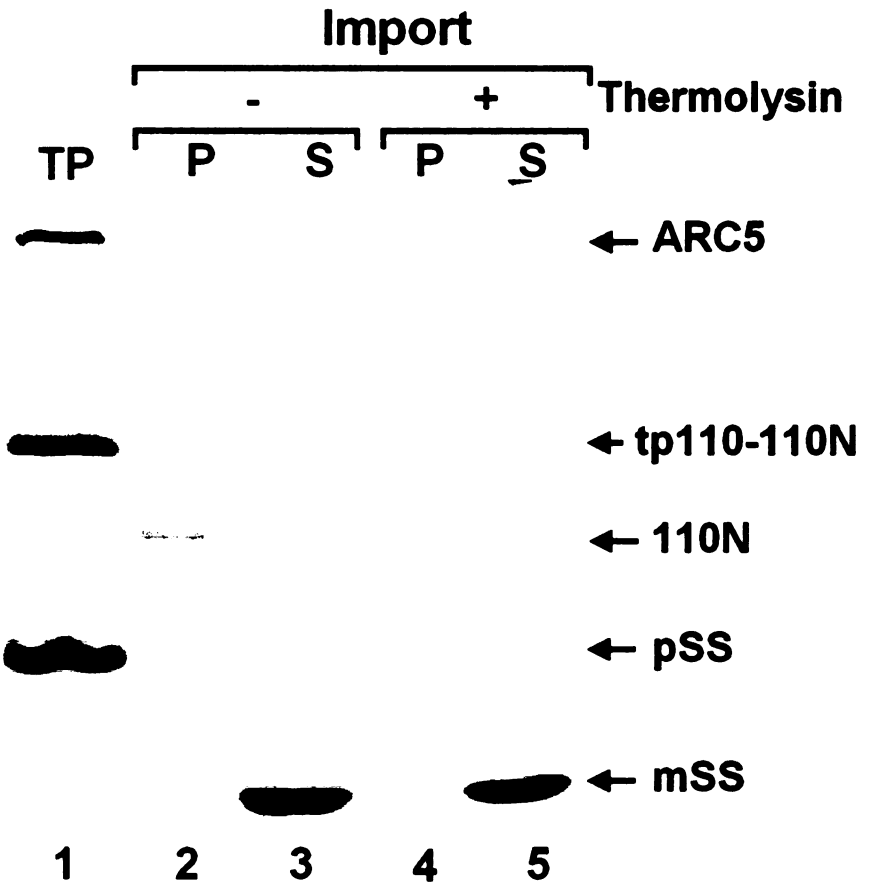


Fig. 4. GFP-ARC5 is localized to the constriction site of dividing chloroplasts. (*A*) Bright-field; (*B*) GFP fluorescence. Arrows indicate corresponding positions in the two images. Bars: *A-B*, 10 μm.

Fig. 5. ARC5 is on the outside surface of chloroplast. Radiolabeled ARC5 (upper panel), tp110-110N, the precursor of the inner envelope marker protein 110N (center panel), and pSS, the precursor of the stromal marker protein mSS (lower panel), were produced by coupled *in vitro* transcription/translation, and subsequently incubated with isolated pea chloroplasts (7). Chloroplasts were recovered by centrifugation and incubated with (+) or without (-) thermolysin. Intact chloroplasts were again recovered and fractionated into membrane (P) and soluble (S) fractions. TP, translation product.


Import
-
+
Thermolysin
TP
P
S
P
S
ARC5
tp110-110N
110N
pSS
mSS
1
2
3
4
5

Even though ARC5 mediates chloroplast division, it is not predicted by subcellular targeting prediction programs to be imported to the chloroplast (results not shown). To further define the topology of the ARC5-containing ring with respect to the chloroplast envelope membranes, we employed *in vitro* chloroplast import and protease protection assays. A radiolabeled translation product corresponding to the longer *ARC5* cDNA was generated by coupled transcription/translation (Fig. 5, top panel, lane 1), then incubated with isolated pea chloroplasts (Fig. 5, top panel, lanes 2-5). Subsequent fractionation of the chloroplasts indicated that the translation product was associated with the membrane fraction, but was not processed (Fig. 5, upper panel, lanes 2 and 3). The binding of the ARC5 translation product to isolated chloroplasts may be effected in part by the PH domain, which has been shown to mediate lipid binding of other dyanamin-like proteins (34, 38). In contrast, two chloroplast-targeted control proteins, one localized to the inner envelope (Fig. 5, center panel) and the other to the stroma (Fig. 5, lower panel), were processed upon import, consistent with the presence of N-terminal transit peptides, and associated with the membrane and soluble chloroplast fractions, respectively (Fig. 5, center and lower panels, lanes 2 and 3). In addition, the two control proteins were both protected from proteolysis by thermolysin, which does not penetrate the outer envelope (39) (Fig. 5, center and lower panels, lanes 4 and 5), whereas the ARC5 translation product was fully degraded by this protease (Fig 4*B*, upper panel, lanes 4 and 5). These data provide evidence that the ARC5-containing ring represented by the GFP-ARC5 fusion protein is situated on the cytosolic surface of the outer chloroplast envelope membrane. The position of ARC5 on the chloroplast surface is topologically

equivalent to that of Dnm1p, a dynamin-like protein that mediates mitochondrial division in yeast (40).

Discussion

Although the *arc5* mutation introduces a premature stop codon, the mutant phenotype probably results from a decreased rate of constriction rather than from a complete block in the process. This conjecture is based on the finding that *arc5/arc1* double mutants have more and smaller dumbbell-shaped chloroplasts than does *arc5*, indicating that *arc5* chloroplasts are capable of completing constriction (21, 23). The slow but continued chloroplast division in *arc5* may be due to the presence of a second *ARC5* homologue (At1g53140) in a duplicated region of the *Arabidopsis* genome (41) whose function might overlap to some extent with that of *ARC5*. Alternatively, it is conceivable that the *arc5* gene product, though severely truncated, could retain partial activity since the mutation occurs just downstream of the GTPase domain. In either case, because total chloroplast volume is maintained as a constant proportion of cell size both in wild-type *Arabidopsis* plants and in mutants with reduced chloroplast numbers (30), the enlarged size of *arc5* chloroplasts can probably be explained by sustained expansion of slowly constricting chloroplasts until the prescribed volume is reached.

Previous studies of the *arc5* mutant phenotype have suggested that the gene product functions in the constriction of chloroplasts, but not of undifferentiated proplastids (22). This could be the result of reduced gene dosage in the mutant; expression of the *ARC5* homologue in *arc5* mutants may be sufficient to maintain the

division of the few, small proplastids in the meristem, whereas higher levels of ARC5 and its homologue may be required for division of the much larger chloroplasts during leaf expansion. The observation that all the plastid division genes identified thus far are present in only one or two copies in the Arabidopsis genome suggests that the same gene products function in the division of all plastid types. Future analyses of protein levels and accumulation patterns for ARC5, its homologue, and other plastid division proteins will shed light on this issue.

Dynamin and its relatives are large GTPases that participate in a variety of organellar fission and fusion events in eukaryotes, including budding of endocytic and Golgi-derived vesicles, mitochondrial fission, mitochondrial fusion, and plant cell plate formation (reviewed in (32, 34)). Dynamin has also been shown to regulate actin assembly and organization at membranes (42). ARC5 defines a new class of dynamin-like proteins that function specifically in plastid division, and its identification extends the range of cellular processes in which dynamin-like proteins participate. The molecular mechanisms by which this large group of GTPases function is still uncertain. Recent structural data (43, 44), along with studies showing that dynamin self-assembles into rings (45) and can tubulate and vesiculate liposomes *in vitro* (46, 47), support proposals that dynamin forms a collar around the neck of budding vesicles during endocytosis and acts as a GTP-stimulated, force-generating "constrictase." However, other studies provide evidence that dynamin family members function as molecular switches, analogous to classical signaling GTPases (48). Functional investigations of dynamin and

its relatives will clearly be relevant to future studies aimed at determining the biochemical mechanism underlying ARC5 activity during chloroplast division.

The localization of ARC5 on the outer envelope surface at the site of chloroplast constriction parallels that of the yeast dynamin-related protein Dnm1p and its orthologues in animals and plants, which localize to the mitochondrial division site on the cytosolic side of the outer mitochondrial membrane (32, 34, 36, 40). This suggests that ARC5 is the chloroplastic counterpart of these mitochondrial division proteins. However, mitochondrial and chloroplast division are not equivalent processes. Chloroplast division requires the activity of several prokaryotically derived proteins in the chloroplast stroma, including FtsZ1, FtsZ2, MinD, MinE and ARTEMIS (6, 7, 11-13, 49), whereas related molecules are lacking in the mitochondria of animals, plants, and fungi, and no matrix-localized mitochondrial division proteins have been identified in these organisms.

Interestingly, the topology of the ARC5-containing ring is similar to that of the outer PD ring (15, 50). Like ARC5, which lacks homologues in cyanobacteria and was probably derived from the eukaryotic host cell, the outer PD ring has been proposed to be of eukaryotic origin (19). Thus, ARC5 could be an outer PD ring constituent. Further, the outer PD ring is composed of filaments about 5 nm in diameter (50), which is close to the diameter of previously observed dynamin strands (51). These similarities suggest that the outer PD ring filaments could be composed of ARC5. However, it has been suggested that the filament protein is an unidentified polypeptide of 56 kDa (50), which is considerably smaller than ARC5. Irrespective of the relationship between ARC5 and

the outer PD ring, our finding that a dynamin-related protein functions in chloroplast division indicates that the chloroplast division machinery is partly of prokaryotic and partly of eukaryotic origin, and shares at least one component in common with the machineries regulating fission of other eukaryotic organelles. Identification of this new chloroplast division protein will facilitate studies aimed both at further dissection of the plastid division machinery and at understanding how the activities of protein complexes separated by the two envelope membranes are coordinated to achieve constriction of the organelle.

Acknowledgement

We thank Kevin Pyke, University of Nottingham, and the Arabidopsis Biological Resource Center, Ohio State University, for *arc5* seeds, StanislavVitha for advice on microscopy, Kevin Stokes for help with phylogenetic analysis, Magali Tshiamala for technical assistance, and all the members of the laboratory for helpful comments. Supported by a grant from the Michigan State University Intramural Research Grants Program.

Online s

Table 1.

BAC clone	
MDC8	
MCB2	
MVI11	
MLD1	
T31J18	
MMB1	
F16J14	
MSA6	
MAL21	
MPN9	
MMB12	
MPN9	
MPN9	

Online supporting Information:

Table 1. PCR markers used for fine mapping *ARC5*

BAC clone	Primer sequences for PCR	Marker type, enzyme
MDC8	5'-GATTAATGAGACTATATATGAGAG-3' and 5'-ATCTGCATAACTTCAATTGAACTG-3'	INDEL*
MCB22	5'-GAACCCCCAGAATATCAACATC and 5'-GCTCTGATGGTGATTCTGGTAAC-3'	INDEL
MVI11	5'-GTAGCATTCTTTAGAGATTGATCTAG-3' and 5'-TATTCGAGTTTGAAATTATGATTTATGC-3'	INDEL
MLD14	5'-GCTACAGTTCTCAACCGGTAAATC-3' and 5'-CATAAGCTTTTATGCTCCAAAATAGTCTC-3'	INDEL
T31J18	5'-CTTGATCTTGTGTTCTGACATCTC-3' and 5'-CTAAACTATTCACAAATGCCATAGACG-3'	CAPS†, *Dra*I
MMB12	5'-AGCCGTCTTGTCCCATCATTAAAG-3' and 5'-GCACAAACAAACAGGGTCAATAGTTA-3'‡	CAPS, *Eco*RV
F16J14	5'-TTAAAGTGAAGCTTAAGCAGAGG-3' and 5'-CATTGTTAGAAAGTCAACACTTTG-3'	INDEL
MSA6	5'-GCAAGACATAACCAATGAACAAG-3' and 5'-GACACGTATGCGTTTCTAAGAG-3'	INDEL
MAL21	5'-CTCCAACTTCAAGCAAAACGGATG-3' and 5'-CTCTGTTTTTTGGGCTAGTGATGG-3'	INDEL
MPN9	5'-GCATACCCAATATCCTTTGTGC-3' and 5'-GATAGTATAACCAGAGGTTGGAG-3'‡	CAPS, *Tsp*509I
MMB12	5'-GAATCTTCTCAAACTGAAATCCACC-3' and 5'-TCGAAAGGAAGATCGGTGAACC-3'§	CAPS, *Taq*I
MPN9	5'-GATTGTGCTATGGTTCAGGAGTTC-3' and 5'-CATCAGCTATAACCTCCTCAGTG-3'§	CAPS, *Acc*I
MPN9	5'-ACTGACTATAAGGACCCCTCAAAC-3' and 5'-GTTGACCATAATTCATCCACCACTATTA-3'§	INDEL (cut by *Hind*III)

BAC, b

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§No reco

BAC, bacterial artificial chromosome.

*Insertion-deletion.

†Cleaved-amplified polymorphic sequence.

‡These two markers defined the final interval containing *arc5*.

§No recombination between these markers and *arc5* was observed.

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Chapter 3

ARC5H, a Homolog of ARC5, is not Involved in Chloroplast Division

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Abstract

arc5 is a chloroplast division mutant with 1-10 chloroplasts per cell. The mutation in the *arc5* mutant in *Arabidopsis* causes a stop codon in the middle of the gene. Nevertheless, chloroplasts divide in *arc5* albeit at a lower frequency than in wild type. One potential explanation for the chloroplast division defect in *arc5* is that an *Arabidopsis* homolog of *ARC5*, *ARC5H*, could be redundant to *ARC5*. However, an *arc5*/*arc5h* double mutant showed the same phenotype as *arc5*. Further, a *35S-ARC5H* transgene had no effect on chloroplast division either in *arc5* or in wild-type plants. ARC5H-GFP is localized to speckles in the nuclei and is not associated with chloroplasts. These data strongly suggest that ARC5H plays no role in chloroplast division. Therefore, ARC5 is important but not essential for chloroplast division, and it may have evolved from the eukaryotic host during evolution to generate force and facilitate the constriction of the chloroplast division furrow.

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Introduction

Chloroplasts carry out photosynthesis in plant cells. It is widely believed that chloroplasts originated from cyanobacteria as endosymbionts. In the mature mesophyll cell in wild-type *Arabidopsis* plants, there are about 100 chloroplasts, suggesting that chloroplast division occurs (Osteryoung and McAndrew, 2001). Known chloroplast division proteins include: FtsZ1, FtsZ2, ARC3, MinD and MinE, homologues of bacterial cell division proteins (Colletti et al., 2000; Itoh et al., 2001; Osteryoung and McAndrew, 2001; Shimada et al., 2004); ARC6, a homologue of the cyanobacteria-specific cell division protein Ftn2 (Vitha et al., 2003); and ARC5/AtDRP5B, a dynamin-related protein of apparent eukaryotic origin (Gao et al., 2003; Hong et al., 2003). FtsZ proteins are localized to and probably mark the division site in chloroplasts (Vitha et al., 2001) as do their homologues in bacteria (Errington et al., 2003). Improper localization of FtsZ interferes with normal chloroplast division (Vitha et al., 2003). FtsZ localization in chloroplasts is regulated by MinD and MinE, which, as in bacteria (de Boer et al., 1991; de Boer et al., 1992), probably prevent FtsZ ring formation at non-division sites (Colletti et al., 2000; Itoh et al., 2001; Margolin, 2001; Maple et al., 2002; Vitha et al., 2003). ARC6, a DNA-J-like protein localized to the chloroplast division site, probably is responsible for FtsZ ring stability or maintenance (Vitha et al., 2003). ARC5 is localized to the cytosolic surface of the chloroplast division site (Gao et al., 2003). The *arc5* mutant has 1-10 enlarged chloroplasts per cell that frequently have a dumbbell shape (Pyke and Leech, 1994; Robertson et al., 1996; Gao et al., 2003). The phenotype of *arc5* mutants and the localization of ARC5 indicate that ARC5 is involved in the constriction of chloroplasts during chloroplast division (Gao et al., 2003).

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The phenotype of *arc5* mutant also suggests that chloroplast division is not completely blocked. Since the *arc5* is a null allele, *ARC5* is either not essential for chloroplast division or is redundant to some other gene or genes. A homolog of ARC5, At1g53140 (hereafter ARC5H), was found in the *Arabidopsis* genome by a BLAST search of the protein database of *Arabidopsis* (http://www.arabidopsis.org/Blast/). Among all the dynamin-related proteins in *Arabidopsis*, ARC5H is not only closest to ARC5, but also can be aligned with ARC5 along its entire length (Figure 1A). This suggests that ARC5H may be redundant to ARC5 and responsible for the continued chloroplast division in the *arc5* mutant. If this is true, an *arc5/arc5h* double mutant should have a more severe chloroplast division phenotype than that of *arc5* and could even be lethal.

To test the above hypothesis, an *arc5/arc5h* double mutant was made. It showed a phenotype similar to that of *arc5*. Furthermore, an *ARC5H* gene driven by the 35S promoter could neither fully nor partially rescue the mutant phenotype of *arc5*. This transgene does not cause any chloroplast division phenotype in wild-type plants. Instead of being associated with chloroplasts, ARC5H-GFP is localized to a cluster of speckles outside the organelle. All these data strongly suggest that ARC5H is not redundant with ARC5 but has a function unrelated to chloroplast division.

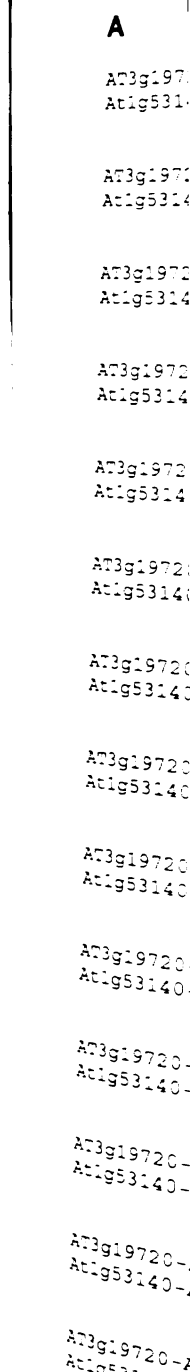


Figure 1 **ARC5H is a homolog of ARC5 and the closest relative of ARC5 in plants.** (**A**) Sequence alignment of ARC5 and ARC5H-AT1g53140 in *Arabidopsis*. The domain structure is indicated by the lines above the alignment. Red, GTPase domain; green, middle domain; blue, PH domain; lavender, GTPase effector domain. "*" indicates fully conserved residues; ":" indicates residues with strong conservation; "." indicates residues with weak conservation; "-" indicates gaps. (**B**) Phylogenetic analysis of dynamin-related proteins in *Arabidopsis* with a rooted neighbor-joining tree. Bootstrap values from the neighbor-joining analysis are shown at nodes with >50% bootstrap support. The functions of the proteins are indicted on the left side. (**C**) Phylogenetic analysis ARC5 and ARC5H in *Arabidopsis*, their homologs in rice and *Chlamydomonas*, and some other dynamin-related proteins with a rooted neighbor-joining tree. At4g33650, At2g14120, At1g59610 and At1g10290 have functions in mitochondrial division and trans-Golgi vesicle trafficking and are used as outgroups here. Bootstrap values from the neighbor-joining analysis are shown at nodes with >50% bootstrap support.

Images in this figure are presented in color.

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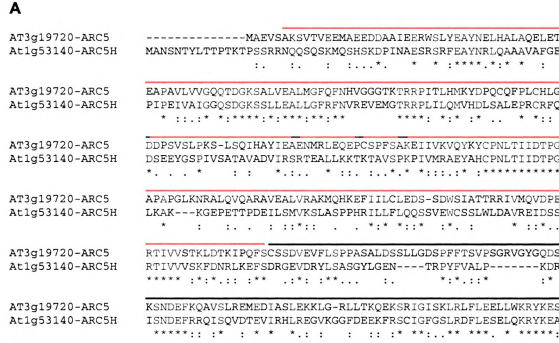

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Figure 1 continued

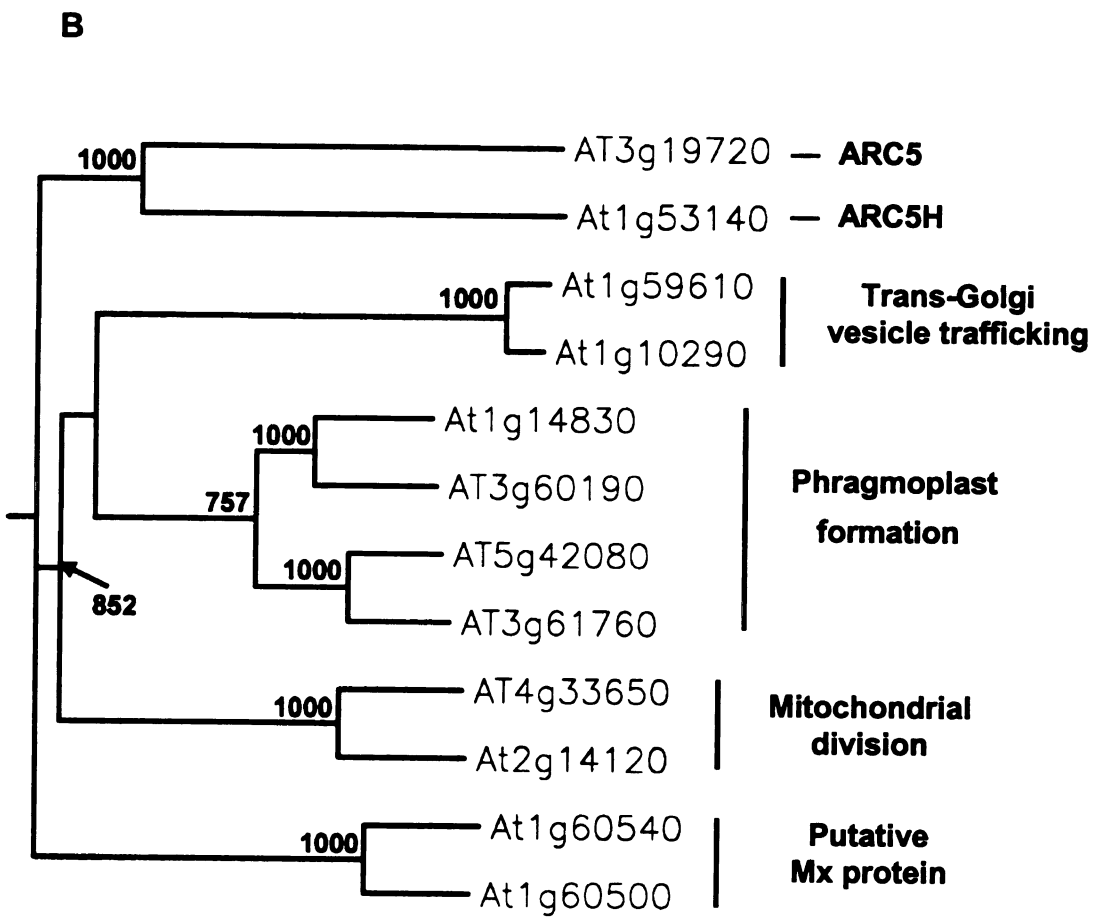
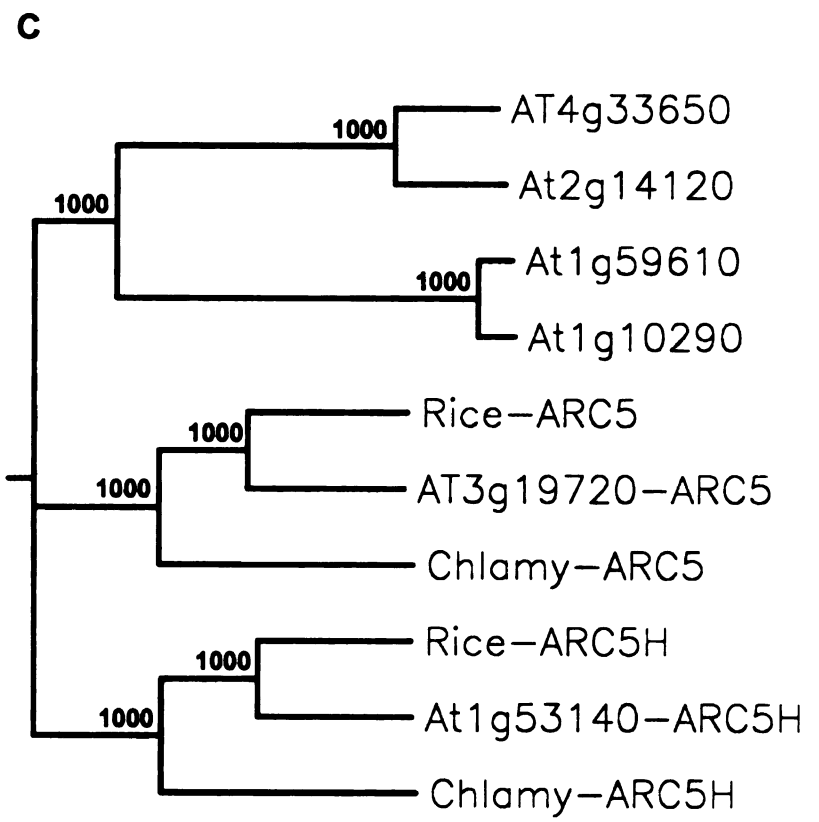


Figure 1 continued



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Results:

ARC5H is a homolog of ARC5 in *Arabidopsis*

To better understand the evolutionary relationship between ARC5 and ARC5H, a phylogenetic analysis of all the dynamin-related proteins in Arabidopsis was performed. As shown in Figure 1B, ARC5H is closely related to ARC5. The protein sequence of ARC5H can be aligned with that of ARC5 along its full length in a BLAST aligment, but all the other dynamin-related proteins can only be aligned with ARC5 in the regions of their GTPase and Conserved Middle domains (Figure 3 in Chapter 2). Although ARC5H was predicted to have a chloroplast transit peptide, the predicted transit peptide is only 36 amino acids and the score is 0.674 (http://mips.gsf.de/proj/thal/db/index.html), suggesting the reliability of this prediction is not high. The above data suggested that ARC5 and ARC5H have a close evolutionary relationship and may have similar function.

However, the sequences of ARC5 and ARC5H are not well conserved beyond the GTPase and Conserved Middle domains (Figure 1A). Further, separate orthologs of ARC5 and ARC5H exist not only in rice but also in the green alga *Chlamydomonas* (Figure 4). Phylogenetic analysis indicated that ARC5 and ARC5H are in different clades and that the two clades may have diverged before the split between plants and green algae. These data suggest that the function of ARC5 and ARC5H may not be identical.

Knock-out of *ARC5H* has no effect on chloroplast division

To test whether *ARC5H* is also involved in chloroplast division, the phenotypes of *ARC5H* knockout lines were checked. Two T-DNA insertion lines, Salk_065118 and

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Salk_066401, were ordered from ABRC. The homozygous knockout lines were confirmed by PCR (Figure 2B). Because the template DNA was from crude extracts, the yields of the PCR products varied in different samples. By sequencing the junction regions between the T-DNA left border sequences and the flanking genomic DNA, the insertion sites were confirmed to be the same as shown on the website http://signal.salk.edu/cgi-bin/tdnaexpress. The insertion sites in the two different lines are only 8 base pairs apart. In both lines, T-DNAs are inserted into the third intron (Figure 2A). The third and fourth exons are part of the coding regions for the GTPase domain in ARC5H. RT-PCR was done to detect *ARC5H* mRNA in Salk_065118 and no detectable *ARC5H* mRNA was found (Figure 2C). As controls, the cDNA of *ARC5H* was well amplified in wild-type plants and the cDNA of *ARC5* was well amplified in both Salk_065118 and wild type (Figure 2C). This means that the *ARC5H* gene is completely knocked out in Salk_065118 (hereafter named *arc5h*). The chloroplast sizes and numbers of *arc5h* plants were compared with that of wild-type plants and no obvious difference was found (Figure 2D and E). However, this may be due to redundancy between *ARC5* and *ARC5H*.

arc5/arc5h double mutant has a similar phenotype to _arc5_

If *ARC5H* is redundant with *ARC5*, we should observe a more severe chloroplast division phenotype in *arc5/arc5h* double mutants. To test this hypothesis, *arc5* was crossed with *arc5h* plants. In an F_2 segregation group of 70 plants, 16 plants with large chloroplasts were identified by phenotyping. The T-DNA insertions in *ARC5H* in 15 plants were checked by PCR and 5 plants were further identified to be homozygous

Figure 2 ***ARC5H* is not involved in chloroplast division.** (**A**) Gene structure of ARC5H. The T-DNA insertion sites in the two Salk lines are indicated by arrows. (**B**) PCR identification of the T-DNA insertion in Salk_065118. The first lane from the left is a 100bp DNA ladder; lane 1, homozygous insertion; lanes 2 and 4, wild type; lane 3, heterzygous insertion. (**C**) RT-PCR of *ARC5* and *ARC5H* in Salk_065118 and a wild-type plant. The first lane from the left is a 1Kb DNA ladder; lane1, RT-PCR of *ARC5H* in SALK_065118, *arc5h*; lane2, RT-PCR of *ARC5H* in a wild-type plant; lane3, RT-PCR of *ARC5* in SALK_065118, *arc5h*; lane4, RT-PCR of *ARC5* in a wild-type plant. (**D-G**) Comparison of the chloroplasts in *Arabidopsis* leaf mesophyll cells. Cells are from fixed 3-week-old leaf tissue. Scale bar represents 10 μm in **D—G**. (**D**) Wild type (Col). (**E**) Homozygous *arc5h* knockout line—SALK_065118. (**F**) *arc5*. (**G**) *arc5/arc5h* double mutant. (**H**) PCR identification of the T-DNA insertion in the F_2 group of the *arc5* X *arc5h* cross. Lanes with homozygous T-DNA insertions are indicated by arrows. (**I**) Verification of *arc5* mutation by PCR amplification and subsequent *Xba*I and *Hind*III digestion.

Images in this figure are presented in color.

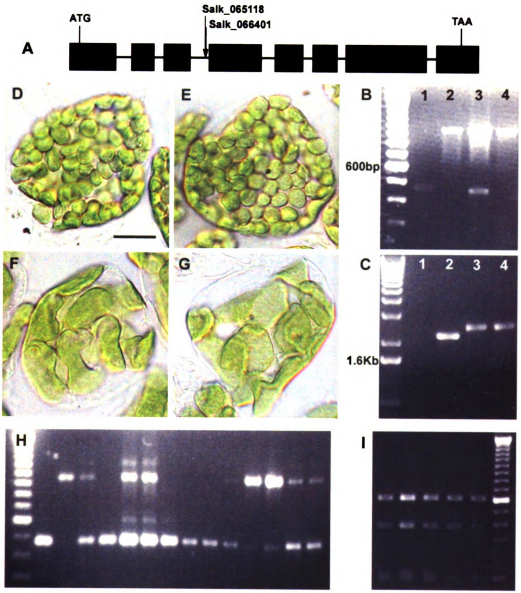
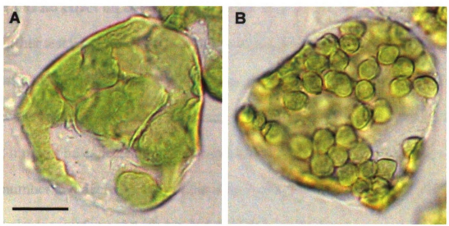

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G
H
I

Figure 3 ***35S-ARC5H* has no effect on chloroplast division.** (**A**) *35S-ARC5H* in *arc5*. (**B**) *35S-ARC5H* in wild type. Cells are from fixed 3-week-old leaf tissue. Scale bar represents 10 μm in **A** and **B**.

Images in this figure are presented in color.


A
B

knockout at the *arc5h* locus (Figure 2H). Those 5 plants are all homozygous *arc5* mutant (Figure 2I). The chloroplast sizes and numbers in the *arc5/arc5h* double mutants were compared with those in the *arc5* mutant and no obvious difference was found (Figure 2F and G). This suggests *ARC5H* is not involved in chloroplast division.

35S-ARC5H has no effect on chloroplast division in _arc5_ or wild-type plants

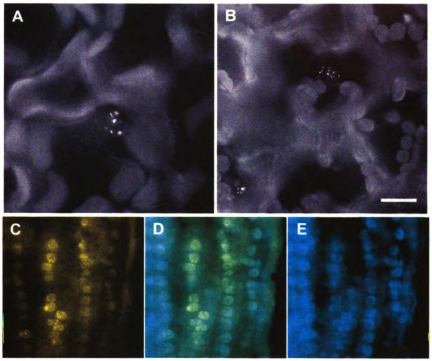
To further test whether *ARC5H* could be redundant with *ARC5*, a *35S-ARC5H* transgene was constructed and introduced into *arc5* to determine whether overexpression of *ARC5H* could complement the *arc5* mutant phenotype. Even in the case of partial redundancy, it might be expected that overexpression of *ARC5H* would increase chloroplast number and decrease chloroplast size in the *arc5* background. About 100 *arc5* mutant plants transformed with the *35S-ARC5H* transgene were checked by microscopy. Their phenotypes were found to be the same as that of *arc5* (Figure 3A and 2F). The *35S-ARC5H* transgene also caused no phenotype when expressed in wild-type plants (Figure 3B and 2D). These data further support the conclusion that *ARC5H* is not a chloroplast division gene.

ARC5-GFP is localized to speckles in the nucleus

To determine the localization of ARC5H, an *ARC5H-GFP* fusion gene with the native promoter was made and transformed into both the wild-type and *arc5h* plants. In the transgenic plants, ARC5H-GFP is localized to a cluster of speckles in the cell, instead of being localized to the division site of chloroplasts like ARC5 (Figure 4A and 4B).

Figure 4 **ARC5H-GFP is localized to a cluster of punctate structures in the nucleus.** (**A-B**) ARC5H-GFP in mesophyll cells of the cotyledons of 6-day-old plants. (**A**) *arc5*. (**B**) wild type. Arrows indicate GFP signals in the two images. Scale bar represents 10 μm in **A** and **B**. (**C-E**) ARC5H-GFP in root-tip cells of a wild-type plant. (**C**) ARC5H-GFP; (**E**) DAPI staining of DNA; (**D**) overlay of **C** and **E**.

Images in this figure are presented in color.


A
B
C
D
E

DAPI staining of the root tip cells indicated that ARC5H-GFP-labeled speckles are in the nucleus (Figure 4C, 4D and 4E). Like *35S-ARC5H*, *ARC5H-GFP* neither rescues the mutant phenotype of *arc5* nor affects chloroplast division in wild-type plant (Figure 4A and B). Therefore, the function of ARC5H is not related to chloroplast division.

Discussion:

Because the mutation in *arc5* creates a stop codon in the middle of the gene, the mRNA should be degraded by non-sense mediated decay and there should be no gene product (Baker and Parker, 2004; Maquat, 2004; Neu-Yilik et al., 2004). However, there are still 1-10 enlarged chloroplasts in the leaf cells of the *arc5* mutant (Figure 2F), indicating that plastid division still occurs to some degree in this null *arc5* background. It was thought that the homolog of *ARC5*, *ARC5H* might be responsible for chloroplast division in *arc5*. However, neither the *arc5/arc5h* double mutant phenotype is more severe than that of *arc5* (Figure 2G) nor can the overexpression of *ARC5H* rescue the phenotype of *arc5* (Figure 3A). Furthermore, the ARC5H-GFP fusion protein is not localized to chloroplasts (Figure 4). These data strongly suggest that *ARC5H* does not function in chloroplast division and consequently is not redundant with *ARC5*. Therefore, *ARC5* is not essential for chloroplast division. Possibly, the chloroplast division proteins with cyanobacterial origin are competent for the first several rounds of chloroplast division in the very young tissue in *arc5*.

One can image that in the very early stage of the endosybiosis, ARC5 was not involved in the division of the cyanobacterial cell, but that the cyanobacterium could still divide. During chloroplast evolution, the outer membrane of the chloroplast acquired some eukaryotic properties (Douce and Joyard, 1990; Dyall et al., 2004). The cyanobacterial cell division machinery must have been modified to adapt to this change during evolution. This may explain why some bacterial cell division genes were lost in plants and new chloroplast division genes, like ARC5, evolved. A similar scenario also happened in mitochondria. However, mitochondria are about one billion years older than chloroplasts and the *FtsZ* gene for mitochondrial division was lost in most eukaryotes (Beech et al., 2000), leaving the dynamin-related protein Dnm1 as the only possible force-generating protein for mitochondrial division (Otsuga et al., 1998; Sesaki and Jensen, 1999; Arimura and Tsutsumi, 2002). ARC5 may generate force to facilitate the division of chloroplasts or overcome the problems caused by the loss of some cyanobacterial cell division proteins.

It has been observed by electron microscopy that there are electron-dense materials on both the inner envelope and outer envelope at the chloroplast division site in the red alga *Cyanidioschyzon merolae* and higher plant *Pelargonium zonale*, which are named inner and outer plastid-dividing (PD) rings (Kuroiwa, 2000; Miyagishima et al., 2001; Kuroiwa et al., 2002). The PD rings are potential components of chloroplast division machinery and are proposed to be involved in chloroplast division (Kuroiwa, 2000). Especially, the outer PD ring may be responsible for constriction of the division furrow from the outside of chloroplast in the *arc5* mutant. However, there is no molecular

evidence to support the existence of the inner and outer PD rings. FtsZ was shown not to be in the inner PD ring (Kuroiwa et al., 2002) and the homolog of ARC5,CmDnm2 in *C. merolae*, was shown not to be in the outer PD ring (Miyagishima et al., 2003). ARC6 is also localized to the chloroplast division site and spans the inner membrane with its N-terminal region in the stroma and C-terminal region in the intermembrane space (Vitha et al., 2003). However, ARC6 is not found to have a homolog in *C. merolae* (Matsuzaki et al., 2004). So, it is unclear whether PD rings exist *in vivo* or not.

Our data suggest that ARC5 and ARC5H have different functions although they are closely related. A phylogenetic analysis indicated that ARC5 and ARC5H may have diverged earlier than the split between the plant and green algal lineages (Figure 1C). Analysis of the *Arabidospis* genome has shown that *ARC5* and *ARC5H* belong to two ancient duplicated regions that have some degree of synteny (Arabidopsis_Genome_Initiative, 2000). There were probably three large-scale genome duplications in *Arabidopsis* (Arabidopsis_Genome_Initiative, 2000; Ku et al., 2000; Simillion et al., 2002; Blanc et al., 2003; Ermolaeva et al., 2003): the recent one happened before the *Arabidopsis/Brassica* split and probably during the early emergence of the crucifer family; one happened after the monocot/dicot divergence; another one is even more ancient. It is generally accepted that genome duplication is an important driving force for evolution and that one of a pair of duplicated genes may be lost or acquire new function after the duplication (Blanc and Wolfe, 2004; Taylor and Raes, 2004). In the case of ARC5 and ARC5H, although it is unclear which has originated earlier, at least one of them has apparently acquired a new function after the duplication.

Material and methods

Plant material

Arabidopsis thaliana ecotype Columbia (Col-0) was used as wild type for all experiments. T-DNA insertion lines of *ARC5H* in the Col-0 background were ordered from the Arabidopsis Biological Resource Center (ABRC).The *arc5h* mutant was identified in the segregation groups from the Salk line: Salk_065118 and Salk_066401. Plants were grown as described (Osteryoung et al., 1998).

Sequence Alignment and Phylogenetic Analysis.

Full-length protein sequences were used for phylogenetic analysis and aligned with CLUSTALW (Thompson et al., 1994) using default settings. The phylogenetic tree was drawn by using the program DRAWTREE at the Biology Workbench 3.2 website (http://biowb.sdsc.edu/). Neighbor-joining analysis was performed by using the program CLUSTALTREE at the same website. Bootstrap analysis was performed on the neighbor-joining tree with one thousand replications.

The protein sequences of all the dynamin-like proteins in *Arabidopsis* were obtained from TAIR (http://www.arabidopsis.org). The sequences of the orthologs of ARC5 and ARC5H in *Chlamydomonas* were obtained from a BLAST search at the JGI website (http://genome.jgi-psf.org/chlre2/chlre2.home.html). The Genebank accession number for rice-ARC5H is BAD86966. The protein sequence of rice-ARC5 is derived from the cDNA sequence AK072318.

Microscopy

Phenotypes were analyzed as previously described (Gao et al., 2003), except that the images were recorded with a Coolpix 4500 digital camera (Nikon Corporation, Tokyo). For *in vivo* detection of GFP by conventional fluorescence microscopy, fresh leaf tissue or root tissue from young seedlings was mounted in water and viewed with an L5 filter set (excitation 460-500 nm, emission 512-542 nm) and 40× objective lense of a Leica DMR A2 microscope (Leica, Wetzlar, Germany) equipped with epifluorescence illumination. For DNA staining, roots were mounted in 1μg/ml DAPI solution and viewed with an A4 filter set (excitation 340-380 nm, emission 450-490 nm) and 40× objective lense. Images were captured with a cooled charge-coupled device camera (Retiga 1350EX, Qimaging, Burnaby, British Columbia, Canada). All images were processed with PHOTOSHOP imaging software (Adobe Systems, San Jose, CA).

PCR identification of the Salk lines and verification of the *arc5* mutation

Three primers 5'-CGGAGGACTATTGTTGTTGTGTCCA-3' (ARC5H-L), 5'-GGCACTCGATTGAGTAGGCTGC-3' (ARC5H-R) and 5'-GCGTGGACCGCTTGCTGCAACT-3' (LBb1) were used for verifying T-DNA insertion sites in the two Salk lines. ARC5H-L and ARC5H-R will give a band of 932 bp in wild-type plants. In T-DNA insertion lines, LBb1 and ARC5H-R will give a band of about 480 bp that corresponds to the junction region between the T-DNA left border and genomic DNA. Two bands will be amplified in heterozygous insertion lines. The mutation in *arc5* creates an *Xba*I cutting site. When the PCR products amplified by 5'-

CTGGAAAGAGAAGGGCATCAAG-3' and 5'-CTGGGAAGATTTTGACGAATGTTG-3' are cut by *Hind*III and *Xba*I, DNA from wild type will give two bands with the sizes of 480bp and 608bp, DNA from *arc5* will give three bands of 98bp, 382bp and 608bp. 100bp DNA ladder is from Invitrogen (Carlsbad, California).

RT-PCR

Primers used for RT-PCR of *ARC5H* were 5'-TGTCGATTCCAGGATGAAGAT TCTG-3' and 5'-TTGGGAATA AAACACCAGCCATG-3'. Primers used for RT-PCR of *ARC5* were 5'-GAAAAAGGAACGGCGACGAAAAC-3' and 5'-GCAAACATTGGACCAAAAAGCG-3'. RT-PCR was done with Qiagene OneStep RT-PCR kit. The 1Kb DNA ladder is from Invitrogen (Carlsbad, California).

Overexpression of ARC5H

The genomic DNA of *ARC5H* was amplified by Pfu DNA polymerase using the primers 5'-TCGTCAATGGCGAATTCAAACAC-3' and 5'-GCTAGGATCCATTATAGAGAACGAGCCACCGT-3'. The PCR product was digested by *Bam*HI and cloned between the *35S* promoter and *OCS* terminator in the transformation vector pFGC5941 (Genebank accession number AY310901), which was cut by *Swa*I and *BamH*I. The construct was transferred to *Agrobacterium tumefaciens* GV3101 and introduced into *Arabidopsis* plants by floral dipping.

ARC5H-GFP Localization

To make an *ARC5H-GFP* fusion gene, the genomic DNA of *ARC5H* with ~1.8 kb of the promoter region was amplified by Pfu DNA polymerase using the primers 5'-AGGTTGTCTTGGTTGTAAATCCTCTC-3' and 5'-GCATTGATCAGAACGAGCCACCGTTTTAAACA-3', digested by *Bgl*II, and then cloned into a derivative of the transformation vector pCAMBIA-1302 (CAMBIA, Canberra, Australia), pCAMBIA-1302-bar (the *hpt*II gene was replaced with the *bar* gene from pCAMBIA-3300), between the *Sma*I and *Bgl*II cutting sites. The construct was introduced into *Arabidopsis* plants as described above. Green fluorescence from GFP of the transgenic plants was detected by fluorescence microscopy.

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Chapter 4

FZL, an FZO-like Protein in Plants, Links Thylakoid Morphogenesis to Chloroplast Division

Abstract

FZO is a mitochondrial fusion protein in fungi and animals. We identified an FZO-like protein in plants, FZL, that was shown to have a chloroplast transit peptide. Knock-out of *FZL* caused a chloroplast division defect and disorganized stacking of thylakoids. The mutant phenotype was rescued by an *FZL-GFP* transgene. Overexpression of FZL-GFP caused very long thylakoid sacs with considerably less stacking than in wild type. These data indicate that FZL is involved in the morphogenesis of thylakoids. Fluorescence microscopy showed that FZL-GFP was localized to punctate structures associated with chloroplasts and in the cytosol. Chloroplast fractionation indicated that FZL-GFP is associated with both the thylakoid and inner envelope membranes. Immunogold labeling indicated FZL-GFP is localized to thylakoids, and to vesicle-like structures found underneath the inner envelope, protruding from chloroplasts, and in the cytosol. The level of FZL-GFP expression correlates with the number of punctate structures and vesicle-like structures on the surface of the chloroplast. These data suggest that the punctate structures and vesicle-like structures are coincident and that FZL may be involved in their formation. Mutation of a conserved lysine residue in the FZL GTPase domain abolished the punctate pattern of FZL-GFP localization and the ability to complement the knock-out mutant phenotype. Thus, FZL seems to be involved in vesicle formation and trafficking process from the thylakoid to the outside of chloroplasts, and blocking of this process affects both the thylakoid morphology and chloroplast division.

Introduction

Chloroplasts carry out photosynthesis and the biosynthesis of amino acids, lipids, starch and many other compounds in plant cells. The chloroplast envelope contains a permeable outer membrane and a less permeable inner membrane. Both membranes contain many proteins that regulate the transport of small molecules and proteins into and out of the chloroplast (Neuhaus and Wagner, 2000; Schleiff and Soll, 2000). Vesicle-like structures have been observed budding from or associated with the inner and outer membrane and may be involved in some transport processes into and (or) out of chloroplasts (Westphal et al., 2001a, 2003). Vesicle-like structures with double membranes were also observed containing stromal protein in the cytoplasm and in the vacuole (Chiba et al., 2003). The biological roles of these vesicles and the molecular mechanisms of their formation are unknown.

Localted within the chloroplast is a system of interconnecting, disk-shaped sacs called thylakoids (Trissl and Wilhelm, 1993). Stacked thylakoid sacs called grana are connected by non-stacked thylakoid sacs called lamellae. Generally, the sacs in all the grana of a chloroplast are similar in size and stacked in arrays. The thylakoid membrane is the location for light absorption and ATP synthesis. Photosystem (PS) II and LHC (light-harvesting complex) II are mainly located on the membrane of stacked thylakoids (Andersson and Anderson, 1980; Chow, 1984; Borodich et al., 2003). Most of the PS I complexes and the ATP synthase are located on the lamellae (Andersson and Anderson, 1980; Chow, 1984; Borodich et al., 2003). The formation of grana and the lateral heterogeneity of the protein complexes in the thylakoid membrane separate the slow (PS

II) and the fast (PS I) photosystems and makes photosynthesis more efficient (Trissl and Wilhelm, 1993; Westphal et al., 2003). The mechanisms controlling the organization of the thylakoid membranes and the differentiation of grana and lamellae are still unclear.

FZO and its homologues in fungi and metazoa are dynamin-like outer mitochondrial membrane proteins required for mitochondrial fusion (Hermann et al., 1998; Santel et al., 2003). FZO has a GTPase domain, two transmembrane domains and two coiled-coil domains (Hales and Fuller, 1997; Mozdy and Shaw, 2003; Westermann, 2003). The GTPase domain and the two coiled-coil domains of the FZO protein are exposed to the cytosol. Mutations in FZO block mitochondrial fusion and result in a fragmented mitochondrial morphology and loss of mitochondrial DNA (Hermann et al., 1998; Rapaport et al., 1998). Detailed mutational analysis has shown that the GTPase domain of FZO is important for its function, and that its transmembrane domains and coiled-coil domains are important for its localization to the outer membrane (Santel and Fuller, 2001; Rojo et al., 2002). Overexpression of mitofusin 1 (Mfn1), a human homologue of FZO, can increase mitochondrial length and interconnectivity (Legros et al., 2002). Interactions between the coiled-coil domains of FZO molecules were proposed to mediate the tethering between membranes from different mitochondria (Rojo et al., 2002; Westermann, 2003; Koshiba et al., 2004). Hydrolysis of GTP may mediate the fusion of mitochondrial outer membranes by generating force for the movement of proteins and opening the attached membranes on the other side (Westermann, 2003; Meeusen et al., 2004). The fusion of the mitochondrial inner membranes may be mediated by the Mgm1, which is located in the intermembrane space and resides on the

inner membrane of mitochondria (Wong et al., 2000; McQuibban et al., 2003; Wong et al., 2003). In fungi, Ugo1p links Fzo1p and Mgm1p for mitochondrial fusion (Sesaki and Jensen, 2001, 2004).

We identified an FZO-like protein in *Arabidopsis*, FZL, which is similar to FZO in both sequence and domain arrangement. Phylogenetic analysis indicated that FZL and FZO may have a common ancestor before the divergence of plants and animals; however, FZL is predicted to be targeted to chloroplasts with high probability. For this reason, we hypothesized that FZL might have a role in a chloroplast process requiring membrane fusion activity. To investigate the role of FZL, we cloned the *FZL* gene and characterized the function of its gene product. FZL was confirmed to be a chloroplast protein. Loss of function of *FZL* results in disorganized thylakoid stacks and heterogeneity of chloroplast size and number. Overexpression of FZL causes very long thylakoid sacs with much less stacking. Thus, FZL links the morphogenesis of thylakoids to the division of chloroplasts.

Results

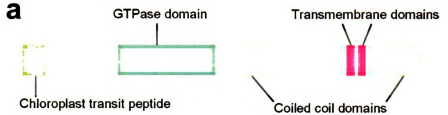
FZL is an FZO-like protein in plants

FZL (At1g03160) was identified by a BLAST search of the Arabidopsis protein database (http://www.arabidopsis.org/Blast/) using FZO as a query sequence. FZL is conserved in many photosynthetic eukaryotes, including tomato, rice and

Figure 1 **FZL is an FZO-like protein involved in chloroplast division.**

(**a**) Predicted domains of FZL. (**b**) Phylogenetic analysis of FZL based on aligment of the GTPase domains with an unrooted neighbor-joining tree. Bootstrap values from the neighbor-joining analysis are shown at nodes with >50% bootstrap support. "At" represents Arabidopsis thaliana. Accession numbers for the sequences aligned with FZL are listed in Materials and Methods. (**c**) Sequence alignment of the GTPase domains of FZL and FZO (GenBank accession number: AAF56110). Green boxes are identical residues; cyan boxes are similar residues; dashes indicate gaps. Arrow indicates K362, which is conserved in all the dynamin-related proteins and is changed to M in the *FZL(K362M)-GFP* mutant. (**d**) *fzl* mutant plants look pale and flower later than wild-type plants. Left, wild type plants; middle, *fzl* mutant plants; right, *fzl* mutant plants complemented by *FZL-GFP*. (**e**) Gene structure of *FZL*. Exons are represented by black boxes; introns are represented by grey boxes. The sites of the insertions in the two T-DNA insertion lines are indicated by arrows. (**f—i**): Chloroplast division phenotype and complementation from 4 week old fully expanded leaves. (**f**) Single cell from wild type plant. Cells in (**g** and **h**) are from *fzl* mutant plants. (**i**) Single cell from *fzl* mutant plants complemented by *FZL-GFP*. Scale bar represents 10 μm in **f—i**.

Images in this figure are presented in color.



c

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FZO AFF RTSN   A          HEIL PSA GH  SCF QQ QANGSNET  VTV
FZL VIV EFNS   T          GKEY KEG VP  NEI IF CYSDLESE  QRC

FZO EQED HMEL A SQLASAHSPGALK STL VVNMAKNRCSILDY VVLM
FZL QTHP GQY VQY -----------  APE ------------ TNIV

FZO   VD TA LDDC -SK CMD  FL  N S TVRRV RQ PE DVS L
FZL   TN IL RQQR TEE VPR  LF  S RRPLTES VA L- YIQW

FZO S PNL  NRWD AS
FZL K KFW  ---- SD
```

d

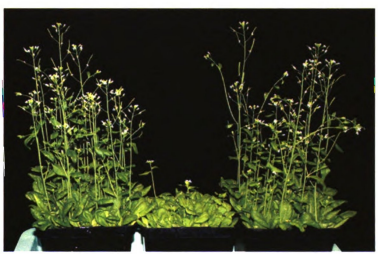


Figure 1 continued

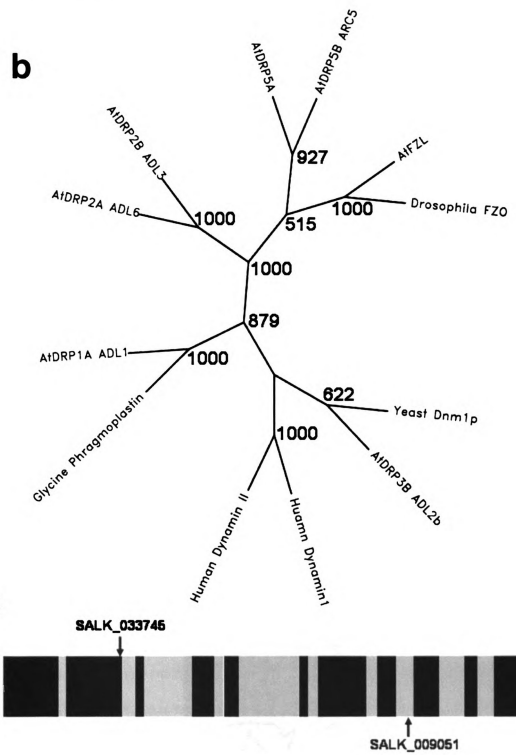
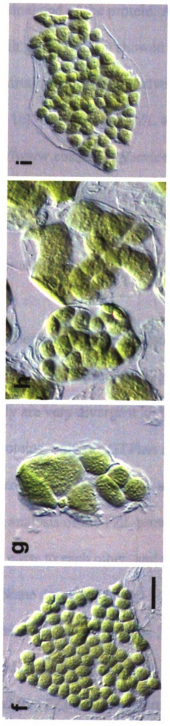


Figure 1 continued



Chlamydomonas (data not shown). The cDNA of *FZL* in *Arabidopsis* was amplified by RT-PCR and the resulting sequence showed that the annotation of the coding region lacked 270 residues at the C-terminal end of the protein. A BLAST search of the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) showed that FZL is similar to a cluster of proteins involved in mitochondrial fusion. The putative GTPase domain of FZL can be aligned with that of FZO (Figure 1c). Interestingly, FZL was predicted by TargetP (Emanuelsson et al., 2000) (http://www.cbs.dtu.dk/services/TargetP/) to have a chloroplast transit peptide (score, 0.871; reliability class, 2.) comprising the first 54 amino acid residues at the N-terminus (Figure 1a). This indicates that FZL is very likely to be targeted to the chloroplast *in vivo*.

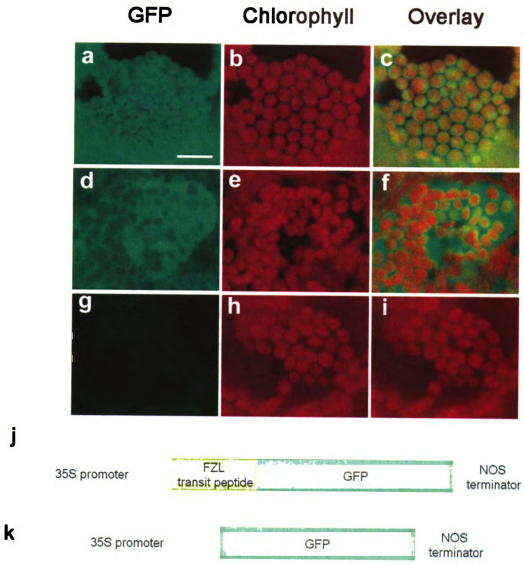
Phylogenetic analysis was performed to investigate the relationship between FZL and other members of the dynamin family of proteins (Figure 1b). Because the sequences of the members of dynamin family are very divergent and the GTPase domain is the most conserved region among these proteins, only the GTPase domains were used for the phylogenetic analysis. FZL clustered in the same branch as the mitochondrial fusion protein FZO (Figure 1b). Further analysis of the FZL protein sequence predicted two transmembrane domains situated close to each other, and two coiled-coil domains flanking the predicted transmembrane domains (Figure 1a). This domain arrangement is very similar to that of mitochondrial FZO (Westermann, 2003). These data suggest that *FZL* and *FZO* may have arisen and diverged from a same ancestral gene in the common ancestor of plants and animals and that FZL may play a role in a membrane remodeling process in the chloroplast similar to that of FZO in mitochondria.

FZL has a chloroplast transit peptide

To test the function of the predicted chloroplast transit peptide of FZL, the coding sequence from the third to the 67th amino acid residue was amplified by PCR and inserted into the plant transformation vector pCAMBIA-1302-BAR to make a 35S promoter-*FZL transit peptide-GFP*-NOS terminator construct (Figure 2j). This construct was transformed into wild type *Arabidopsis* plants by floral dipping (Clough and Bent, 1998). In mesophyll cells of transgenic plants, the GFP signal was coincident with chlorophyll autofluorescence, indicating its association with chloroplasts (Figure 2a-c). The GFP looks to be present in the stroma, not the chloroplast envelope (Figure 2a, c). In the GFP fusion genes, the second codon for methionine is located about one third of the way into the GFP coding sequence. If this codon is used as the start codon, the gene product will lack the predicted chloroplast transit peptide and the N-terminal one-third of the GFP and presumably will not fluoresce. As a control, the *GFP* gene without the coding sequence of the chloroplast transit peptide was transformed into wild type plants using pCAMBIA-1302-BAR (Figure 2k) and GFP remained in the cytosol of mesophyll cells (Figure 2d-f). In wild-type plants, no GFP signal could be detected and the very dim fluorescence was from chlorophyll autofluorescence (Figure 2g-h). These results confirm the prediction that FZL has a chloroplast transit peptide.

Figure 2 **FZL has a chloroplast targeted transit peptide.** (**a**—**c**) A single cell from a wild type plant transformed by CaMV35S promoter-*FZL transit peptide-GFP*-nopaline synthase (NOS) terminator. (**d**—**f**) A single cell from a wild type plant transformed by CaMV35S promoter-*GFP*-NOS terminator. (**g**—**i**) A single cell from a wild type plant. (**j**) The GFP construct in **a**—**c**. (**k**) The GFP construct in **d**—**f**. Scale bar represents 10 μm in **a**—**i**.

Images in this figure are presented in color.


GFP
Chlorophyll
Overlay
a
b
c
d
e
f
g
h
i
j
35S promoter
FZL
transit peptide
GFP
NOS
terminator
k
35S promoter
GFP
NOS
terminator

To test whether FZL also functions in mitochondria, we crossed *fzl* mutant plants with plants that have a mitochondria-targeted GFP (Kohler et al., 1997a). We compared the mitochondria in *fzl* and wild type plants by visual detection using fluorescence microscopy and found no obvious differences in size, morphology, motility, fluorescence intensity etc. (data not shown). This further suggests that FZL is only targeted to chloroplasts. This result is consistent with the observation that plants lack Mgm1p and Ugo1p (Wong et al., 2000; Sesaki and Jensen, 2001), which in yeast work in a complex with FZO during mitochondrial fusion (Sesaki and Jensen, 2004). These findings together suggest that FZL does not have the same function as mitochondrial FZO, but instead has a unique function in chloroplasts.

Loss of *FZL* function alters chloroplast size and morphology

To understand the function of FZL, T-DNA insertion lines of the *FZL* gene were obtained from the Arabidopsis Biological Resource Center (Columbus, Ohio, USA). The Salk_033745 line has an insertion in the region encoding the GTPase domain (Figure 1e). The Salk_009051 line has two tandem insertions: one in the intron between the two exons encoding the second transmembrane domain and a second in the 3'-UTR (Figure 1e). Both mutant lines had the same phenotype: leaves were visibly pale and flowering was delayed by several days compared with wild type (Figure 1d). Light microscopy showed that mature mesophyll cells in the *fzl* knockout lines contained only 1-20 chloroplasts per cell (Figure 1g, h), whereas wild-type plants had approximately 120 chloroplasts per cell (Figure 1f). Chloroplasts in the *fzl* mutants were heterogeneous in size. The heterogeneity was observed both within a single cell and among different cells (Figure 1g, h).

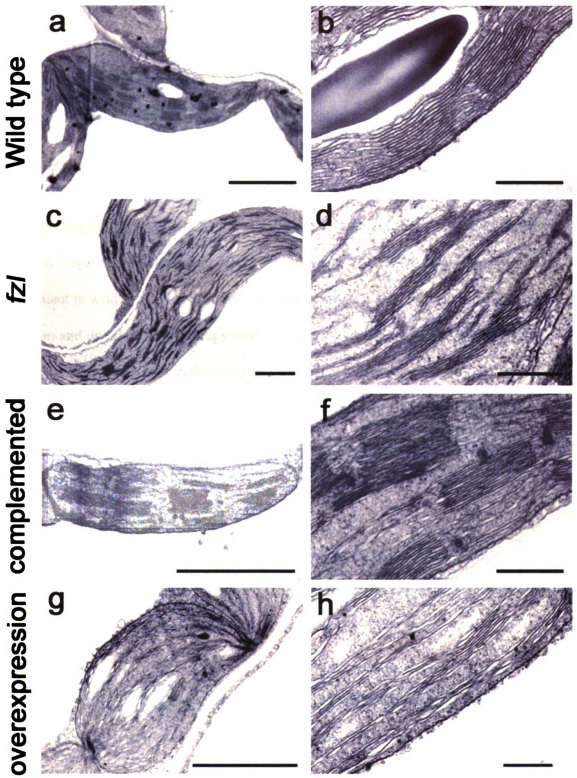
To confirm that the mutant phenotype resulted from the disruption of the *FZL* gene, the coding region of *FZL*, including ~0.8 kb upstream of the start codon, was amplified by PCR, inserted into the plant transformation vector pCAMBIA-1302-BAR with a *GFP* gene fused to the 3'-end, and transformed into *fzl* mutant plants by floral dipping (Clough and Bent, 1998). The mutant phenotypes, including enlarged chloroplasts with heterogeneous size, pale leaves and late flowering, were rescued by the transgene (Figure 1d, i). Expression levels higher than that needed to complement the mutant phenotype had no obvious effect on chloroplast number and size (Figure 3 and 7). This is in contrast to the overexpression of some other chloroplast division proteins in plants (Osteryoung et al., 1998; Colletti et al., 2000; Itoh et al., 2001) and to the overexpression of some bacterial cell division proteins in bacteria, which often have a dominant-negative effects (Dai and Lutkenhaus, 1992; de Boer et al., 1992).

FZL level affects the structure and the morphology of the thylakoid

To further investigate the effect of FZL on the ultrastructure of chloroplasts, *fzl* mutant, wild type and *FZL-GFP* over-expression plants were examined by transmission electron microscopy (TEM). The results showed that thylakoid structure was affected by both loss of function of FZL and overexpression of FZL-GFP.

In wild type plants, unstacked lamellae and stacked grana are well organized inside the chloroplast (Figure 3a, b) and the sacs in each granum are similar in size and connected by stroma lamellae. In *fzl* mutant plants, the thylakoid sacs appear thinner and

Figure 3 **FZL level is related to the structure of thylakoid.** TEM of the thylakoid structure in the plants with different levels of FZL. (**a** and **b**) Thylakoid structure of wild type plants. (**c** and **d**) Thylakoid structure of *fzl* mutant plants. (**e** and **f**) Thylakoid structure of *fzl* mutant plants complemented by normal levels of FZL-GFP. (**g** and **h**) Thylakoid structure of *fzl* mutant plants over-expressing FZL-GFP. Scale bar represents 2 μm in **a**, **c**, **e** and **g**. Scale bar represents 500 nm in **b**, **d**, **f** and **h**.

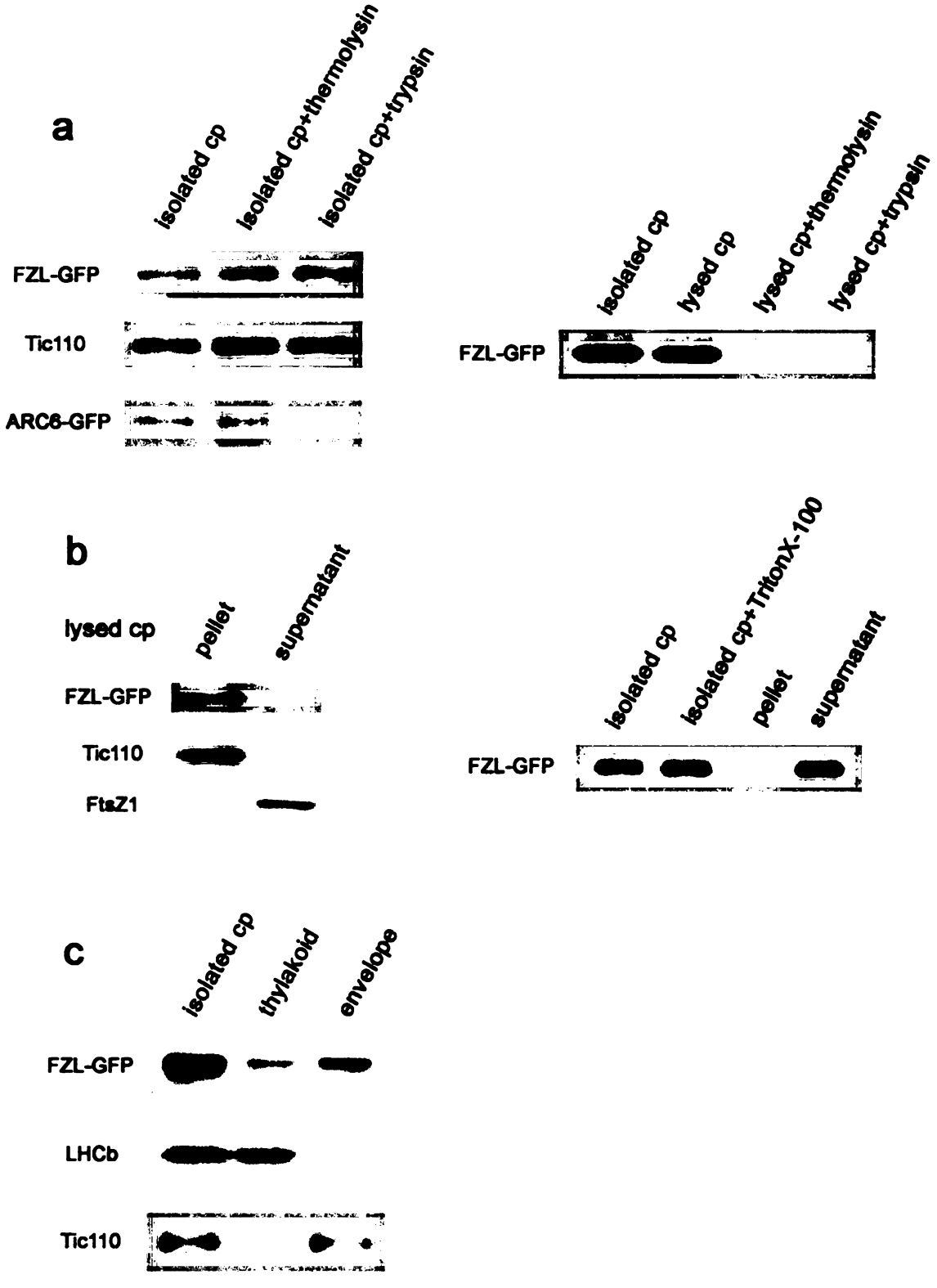

Wild type
fzl
complemented
overexpression
a
b
c
d
e
f
g
h

less organized and lack the lamellar thylakoid sacs seen in wildtype plants (Figure 3c, d). Furthermore, the thylakoid stacks are more compact and appear more variable in size (Figure 3d). In most of the *fzl* plants complemented by *FZL-GFP* with native promoter, each chloroplast has on average 1 or 2 GFP-labeled structures (Figure 5 a, b and e; Figure 7d) and the thylakoid structure looks similar to that in wild-type plants (Figure 3e, f; Figure 7f). In some of the *fzl* plants complemented by FZL-GFP expressed from the native promoter, FZL-GFP levels are much higher (Figure 7g-k) and there is considerably less stacking of thylakoid sacs and the unstacked stroma lamellae appear very long and swollen (Figure 3g and h; Figure 7i). However, unstacked sacs of stroma lamellae are infrequent in wild-type plants. The complementation of the defects of *fzl* in chloroplast division and thylakoid morphology further suggests that FZL-GFP is functional. The abnormal morphologies in both the *fzl* and the plants overexpressing FZL-GFP indicate that the level of FZL influences the morphology and structure of thylakoids. FZL seems to have a role in promoting the formation of stroma lamellae and (or) preventing the stacking of thylakoid sacs into grana.

Topology of FZL

To understand the function of FZL, we studied the topology of FZL by a protease protection assay with isolated chloroplasts from the *FZL-GFP* complemented mutant plants. Thermolysin, which cannot penetrate the outer chloroplast envelope membrane (McAndrew et al., 2001), and trypsin, which can penetrate the outer but not the inner membrane (McAndrew et al., 2001), were the proteases used for this assay. FZL-GFP was protected when the chloroplasts were treated with either thermolysin or trypsin

Figure 4 **FZL is a chloroplast-targeted membrane protein.** (**a**) FZL-GFP is protected from thermolysin and trypsin digestion. Isolated intact chloroplasts were incubated with thermolysin and trypsin (left). Lysed chloroplasts were incubated with thermolysin and trypsin. (**b**) FZL-GFP is a membrane protein. Isolated intact chloroplasts were lysed in hypotonic solution and separated into pellet fraction and supernant fraction (left). FZL-GFP is solubilized by 1% TritonX-100 (right). After centrifugation, FZL-GFP is present in soluble part. (**c**) FZL-GFP is present in both thylakoid and envelope of chloroplast. Isolated intact chloroplasts were lysed in hypotonic solution and separated into thylakoid and envelope fractions. Crude proteins representing approximately equivalent proportion of the chloroplast from each fraction were analyzed by SDS-PAGE and immunoblotting with antibodies against GFP, Tic110, FtsZ1 and LHCb. cp, chloroplast.


a
isolated cp
isolated cp+thermolysin
isolated cp+trypsin
FZL-GFP
Tic110
ARC6-GFP
isolated cp
lysed cp
lysed cp+thermolysin
lysed cp+trypsin
FZL-GFP
b
lysed cp
pellet
supernatant
FZL-GFP
Tic110
FtsZ1
isolated cp
isolated cp+TritonX-100
pellet
supernatant
FZL-GFP
c
isolated cp
thylakoid
envelope
FZL-GFP
LHCb
Tic110

unless the chloroplasts were osmotically lysed prior to protease treatment (Figure 4a). The control protein Tic110, an inner membrane protein facing the chloroplast stroma (Jackson et al., 1998), was also protected from digestion by thermolysin and trypsin. As a control for the intermembrane space, we used a C-terminal GFP fusion to ARC6, a protein whose C-terminus resides in the intermembrane space (Vitha et al., 2003). This ARC6-GFP was protected from digestion by thermolysin but not trypsin (Figure 4a). These data indicate that FZL is targeted to the stromal side of the chloroplast envelope.

The pellet and soluble fractions of the isolated chloroplasts were separated by centrifugation after chloroplast lysis by hypotonic solution. FZL-GFP and the inner envelope protein Tic110 (Jackson et al., 1998) were found in the pellet fraction, but not in the soluble fraction (Figure 4b). FtsZ1, a soluble stromal protein (McAndrew et al., 2001), was found mainly in the soluble fraction (Figure 4b). FZL-GFP was detected in the supernatant fraction after the chloroplasts were incubated in 1% TritonX-100 (Figure 4b). These data confirm that FZL is a membrane protein as predicted by sequence analysis.

To further determine the localization FZL-GFP, chloroplast membranes from hypotonically lysed chloroplasts were fractionated into thylakoid and envelope fractions by sucrose gradient centrifugation (Block et al., 2002). Immuno blot analysis indicated that FZL-GFP was present in both thylakoid and envelope fractions (Figure 4c). As controls, LHCb and Tic110 were detected only in the thylakoid and envelope fractions respectively (Figure 4c).

Localization of FZL by microscopy

In *fzl* plants complemented by *FZL-GFP*, FZL-GFP usually exhibited a punctate pattern of localization. Punctate structures were often associated with the periphery of the chloroplasts (Figure 5a, b). Less frequently, punctate structures were observed in the stroma (Figure 5c, d). When the expression levels were low, the GFP signal also appears to be present in the cytosol as well as the chloroplasts (Figure 5e, indicated by arrows). The same pattern of GFP localization is observed in wild type plants transformed with *FZL-GFP* (data not shown).

To further investigate the localization of FZL-GFP, we performed immunogold labeling with GFP antibodies in the *FZL-GFP* complemented plants. FZL-GFP was detected in thylakoids (Figure 6b), vesicle-like structures associated with the inner chloroplast envelope (Figure 6a), membrane protrusions from chloroplasts (Figure 6b, c), vesicle-like structures close to chloroplasts (Figure 6d) and vesicle-like structures in the cytosol (Figure 6e). These data are consistent with the observations made with fluorescence microscopy. The presence of FZL-GFP-labeled punctate or vesicle-like structures on the stromal and cytosolic sides of the envelope, and in the cytosol suggests that FZL-GFP or these structures are being trafficked.

Figure 5 **FZL-GFP is localized to punctate structures in *FZL-GFP* complemented *fzl* mutants.** (**a**, **b** and **e**) FZL-GFP-labeled punctate structures are associated with the chloroplast. (**a**) Extended focus confocal image. (**b**) Single section of the confocal images from **a**. (**c** and **d**) Single sections of confocal images show FZL-GFP labeled punctate structures in the stroma of chloroplast. (**e**) Conventional fluorescence image. Arrows indicate FZL-GFP labled punctate structures. Image depth is 0.5 μm in **b**, **c** and **d**. Scale bar represents 5 μm in **a**—**d**. Scale bar represents 10 μm in **e**.
Images in this figure are presented in color.

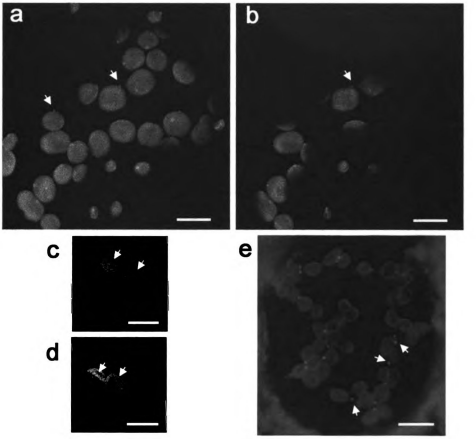
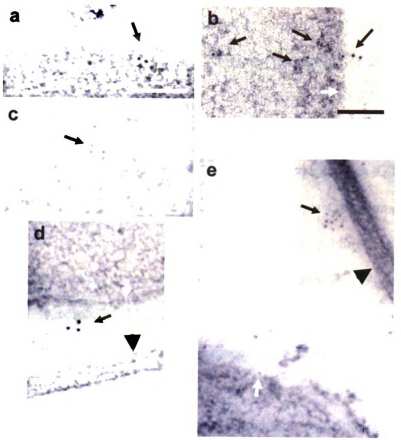

a
b
c
d
e

Figure 6 **FZL-GFP is localized to vesicle-like structure in *FZL-GFP* complemented *fzl* mutant with immuno gold labeling.** (**a**) FZL-GFP is localized to vesicle-like structures underneath the chloroplast envelope. (**b**) FZL-GFP is localized to stroma and protrusion-like structure on chloroplast envelope. (**c**) FZL-GFP is localized to protrusion-like structure on chloroplast envelope. (**d** and **e**) FZL-GFP is localized to vesicle-like structures in cytosol. Black arrows indicate gold particles. White arrows indicate chloroplast envelope; adjacent dark areas are stroma. Triangles indicate cell wall. Scale bar represents 200 nm in **a**, **c** with the same magnification. Scale bar represents 200 nm in **b**, **d** and **e** with the same magnification.

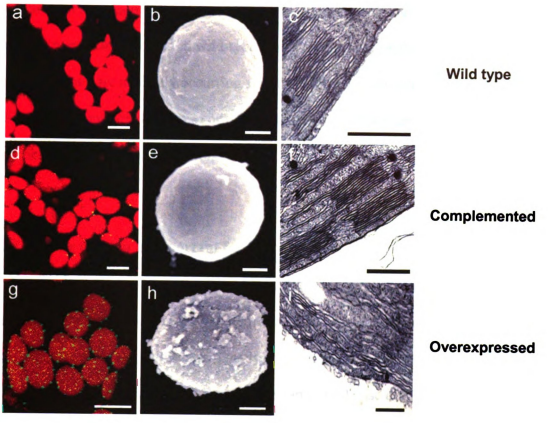

a
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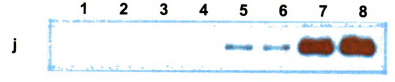
Increased levels of FZL result in more punctate and vesicle-like structures on the surface of chloroplasts

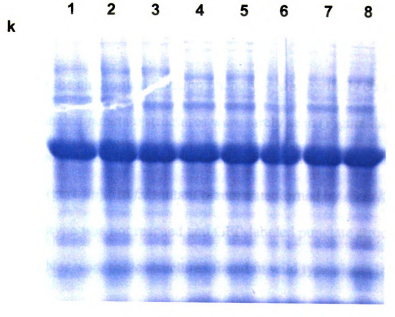
To gain further insight into the function of FZL, we analyzed the effect of FZL-GFP expression level in the *fzl* mutant background. In the *FZL-GFP* complemented mutant plants, probably because of positional effect and the number of the inserted transgenes, the level of FZL-GFP was variable. The relative levels of FZL-GFP were estimated by visual estimation of the strength of the GFP fluorescence signal or by immuno-blot detection (Figure 7d, g, j and k; Figure 8a, b and e). When FZL-GFP is expressed at a level to be able to fully complement the chloroplast division phenotype with normal thylakoid morphology, the GFP-labeled punctate structures are often seen associated with the chloroplast envelope or in the cytosol (Figure 7d-f; Figure 5 a, b and e). When *FZL-GFP* is expressed at higher levels, the chloroplast division defect is still rescued but more punctate structures are found associated with the chloroplast (Figure 7g). Smaller GFP-labeled structures can also be seen in the stroma in these plants (Figure 7g). The number and size of GFP-labeled punctate structures inside the chloroplast increased with increasing FZL-GFP expression level (Figure 5c and d; Figure 7g). This probably means that, when these punctate structures are in excess, they accumulate and fuse together inside chloroplasts. Scanning electron microscopy (SEM) of isolated chloroplasts from plants expressing different levels of FZL-GFP showed that higher FZL-GFP levels are associated with an increased number of structures resembling vesicles protruding from the outer envelope (Figure 7b, e and h). TEM of leaf sections further indicated a positive correlation between FZL-GFP levels and the number of vesicle-like

Figure 7 **Correlation between number of punctate and vesicle-like structures and the level of FZL-GFP.** (**a**, **b** and **c**) Chloroplasts of wild type plants. (**d**, **e** and **f**) Chloroplasts of *fzl* mutant fully complemented by FZL-GFP. (**g**, **h** and **i**) Chloroplasts of *fzl* mutant with higher FZL-GFP levels. (**a**, **d** and **g**) Confocal Laser Scanning Microscopy (CLSM) image. (**b**, **e** and **h**) Scanning Electron Microscopy (SEM) image. (**c**, **f** and **i**) Transmission Electron Microscopy (TEM) image. Scale bars, 5 μm in **a**, **d** and **g**, 1 μm in **b**, **e** and **h**, 500 nm in **c**, **f** and **i**. (**j** and **k**) Relative levels of FZL-GFP in different *fzl* transgenic lines expressing FZL-GFP. Shown in **j** is the immunoblot with GFP antibodies. The loading of the proteins was measured by Coomassie blue staining and shown in **k**. Lane 1 and 2, *fzl*; lane 3 and 4, *fzl* partially complemented by FZL-GFP; lane 5 and 6, *fzl* fully complemented by FZL-GFP; lane 7 and 8, *fzl* plants with higher FZL-GFP levels.

Images in this figure are presented in color.


a
b
c
Wild type
d
e
f
Complemented
g
h
i
Overexpressed


1 2 3 4 5 6 7 8
j


1 2 3 4 5 6 7 8
k

structures on the chloroplast surface (Figure 7c, f and i). Although infrequent, these structure were also observed in wild-type plants (Figure 7b) (Robertson et al., 1996), suggesting they are naturally occurring structures. These data further suggest that the GFP-labeled punctate structures correspond to the vesicle-like structures and that FZL may be involved in their formation.

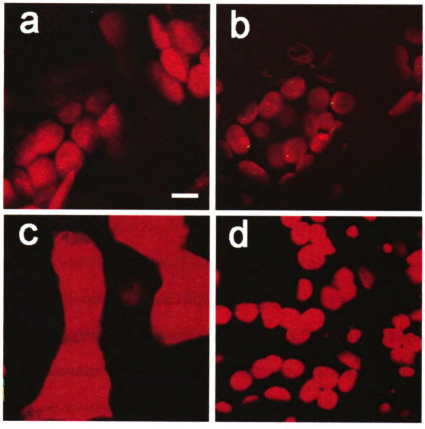
The GTPase domain is important for FZL function

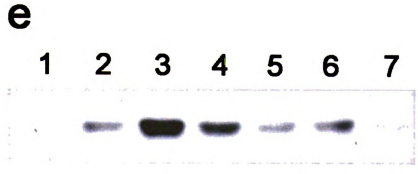
To further investigate the role of FZL in the chloroplast, a mutant of *FZL*, *FZL (K362M)-GFP* was made. In the gene product of this mutant, lysine 362 (Figure 1c) of FZL was changed to a methionine. This lysine is conserved not only in the GTPase domains of dynamin family members, but also in the GTPase domains of many other proteins. Mutating the equivalent residue has been shown to abolish the function of FZO and other dynamin-related proteins (Hermann et al., 1998; Arimura and Tsutsumi, 2002; Orth et al., 2002). Mutation of residue 362 from lysine to methionine was predicted to reduce the GTPase activity of FZL without altering its structure.

When the chloroplast division phenotype was partially complemented by *FZL-GFP*, GFP-labeled punctate structures were rarely seen (Figure 8a). In fully complemented plants, GFP-labeled punctate-like structures were observed more frequently (Figure 8b). In *fzl* mutant plants transformed by *FZL(K362M)-GFP*, the mutant phenotype was not rescued and GFP-labeled punctate structures were absent (Figure 8c). Nor were these structures found in wild type plants transformed with

Figure 8 **Mutations in the GTPsae domain affect the localization and function of mutant FZL-GFP.** (**a** and **b**) FZL-GFP in *fzl* plants (**a**) *fzl* mutant is partially complemented by low levels of FZL-GFP. (**b**) fzl mutant is complemented by normal levels of FZL-GFP. FZL-GFP is localized to vesicle-like structures. (**c** and **d**) K362M mutation blocks the formation of GFP labeled vesicle-like structures. (**c**) FZL(K362M)-GFP cannot complement the mutant phenotype of *fzl*. (**d**) FZL(K362M)-GFP in wild type plant. The GFP labeled vesicle-like structure is not seen as in **b**. Images are of same magnification in (**a-d**) and scale bar represents 5 μm. (**e**) Immuno blot of FZL-GFP and FZL(K362M)-GFP. Approximately 2 mg of fresh weight was loaded in each lane. Lane 1 and (**a**), *fzl* mutant partially complemented by low level of FZL-GFP; Lane 2 and (**b**), *fzl* mutant fully complemented by low level of FZL-GFP; Lanes 3-5, *fzl* mutant plants expressing FZL(K362M)-GFP; Lane 6-7, wild type plants expressing FZL(K362M)-GFP. Lane 3 and 6 are corresponding to (**c**) and (**d**) respectively.

Images in this figure are presented in color.


a
b
c
d


e
1 2 3 4 5 6 7

FZL(K362M)-GFP (Figure 8d). Immuno blots showed that the mutant proteins were expressed at levels comparable to those in mutant plants complemented by *FZL-GFP* (Figure 8e). These data indicate that the GTPase activity of FZL is required for the localization of FZL in a punctate pattern, and also suggest that this punctate localization pattern is important for FZL function.

Discussion:

In this paper, we identified FZL, an FZO-like protein in plants, and studied its function. Mutation of *FZL* causes abnormal thylakoid morphology and a chloroplast division defect with heterogeneity in chloroplast size. FZL-GFP was shown to be present in both the inner envelope membrane and thylakoids of the chloroplast. FZL-GFP was mainly localized to punctate or vesicle-like structures associated with chloroplasts and infrequently with the cytosol. These data indicate that FZL is not an orthorolog of FZO and mitofusins, mitochondrial fusion proteins in fungi and metazoa, but has a unique function in chloroplasts.

FZL is very similar to two proteins found in *Chloroflexus aurantiacus* (Genebank accession number: ZP_00021046) and *Deinococcus radiodurans* R1 (Genebank accession number: NP_294959). Because the GC content of these two genes is very different from that of the flanking sequences and the whole genomes, the two genes seem to be the result of horizontal gene transfer (Makarova et al., 2001; Da Lage et al., 2004). The GTPase domains of FZL and FZO proteins can be aligned with those of dynamin and

dynamin-related proteins, but they lack the other domains of the dynamin family and are only more similar to dynamin-related proteins when compared with other GTPases. The fact that FZO is involved in mitochondrial fusion and FZL is apparently involved in chloroplast function implies that they diverged very early. However, FZL and FZO are similar in both sequence and domain arrangement and a phylogenetic analysis indicated that they are more related to each other than other dynamin related proteins, suggesting that FZL may be mechanistically similar to FZO, mediating membrane fusion through the interaction of its coiled-coil domains and GTP hydrolysis.

FZL may have membrane fusion activity as does FZO. We speculate that FZL is involved in a vesicle-trafficking process in the chloroplast and blocking of this process affects the properties of the thylakoid and chloroplast envelope membranes, and results in a change in thylakoid morphology and dysfunction of the chloroplast division machinery.

This is based on the following evidence: First, FZL has a chloroplast transit peptide and FZL is a membrane protein found in both the thylakoid and the envelope fraction of chloroplasts (Figure 4). Second, FZL-GFP was localized to thylakoids, vesicle-like structures associated with the inner chloroplast envelope, membrane protrusions from the chloroplast and vesicle-like structures in the cytosol based on both fluorescence microscopy and immuno-gold labeling (Figure 5 and Figure 6), suggesting that FZL and these structures undergo trafficking. Third, the number of GFP-labeled punctate structures and vesicle-like structures on the surface of chloroplasts was positively correlated with the level of FZL-GFP, further supporting the hypothesis that

the punctate structures correspond to vesicle-like structures and that FZL is involved in the formation and (or) the trafficking of these structures. Fourth, in *fzl* mutant plants partially complemented with low levels of FZL-GFP, the punctate pattern of GFP signal can rarely be seen; in *fzl* mutant plants fully complemented with FZL-GFP and with normal thylakoid morphology, many FZL-GFP-labeled punctate structures were visible on the cytosolic side of the chloroplast envelope; in plants with high levels of FZL-GFP, some of the FZL-GFP-labeled punctate structures were found in the stroma but were smaller in size (Figure 5c, d). If the function of FZL is membrane fusion as is true for FZO in fungi and metazoa, it is likely that the vesicles are originally very small and then aggregate and fuse to form larger vesicles as they are transferred across the chloroplast envelope. Finally, a mutation, K362M, completely abolished the function of FZL-GFP and its punctate localization pattern. This further supports the notion that FZL mediates the fusion of small vesicles into larger vesicles in the stroma and their subsequent trafficking to the envelope and cytosol, and this dynamic vesicle-trafficking process is required for normal chloroplast thylakoid morphology.

In most eukaryotic cells, the Golgi apparatus is an array of stacked membrane sacs. Vesicles traffic from one sac to another by fusion and regeneration (Mayer, 2002). The proper functioning of the Golgi apparatus requires many GTPases of the dynamin (Henley and McNiven, 1996; Jones et al., 1998) and Rab GTPase families and other proteins such as golgins with coiled-coil domains (Barr, 1999; Short et al., 2001; Barr and Short, 2003; Diao et al., 2003; Jackson, 2003; Satoh et al., 2003). GTPase activity and the interaction between the coiled-coil domains of these proteins are important for

the maintenance of the dynamic structure of the Golgi apparatus and for the fusion and trafficking of vesicles for secretion. In spite of their many differences, the structure of the Golgi apparatus and chloroplast thylakoid are somewhat similar as they are both made up of well-organized, stacked membrane sacs. Also similarly, a dynamin-like GTPase with two coiled-coil domains and two trans-membrane domains, FZL, is involved in the maintenance of the structure of thylakoid stacks, suggesting that thylakoid and Golgi may share similarities in their biogenesis. Although it has been shown that the vesicle-transport system seen in the Golgi is related to Golgi structure (Short et al., 2001; Panic et al., 2003), no such relationship has been shown to exist for thylakoids and vesicle-transport. If, as proposed here, FZL is involved in both the structural maintenance of the thylakoids and chloroplast vesicle-trafficking, the Golgi apparatus and thylakoid may also have in common the feature of vesicle-trafficking as a means of maintaining structure.

In the vesicle secretion system of yeast and animal cells, vesicles originate from the Golgi and are transported to and then fuse with the plasma membrane (Tooze et al., 2001; Mayer, 2002). If the FZL-GFP-labeled vesicles are transported out of chloroplasts, this process should involve transport across both the inner membrane and outer chloroplast envelope. This is in contrast to the single membrane involved in Golgi vesicles-trafficking and these two processes may have many mechanistic differences. However, as with the chloroplast vesicle fusion proposed here, mitochondrial fusion requires the fusion of double membranes (Mozdy and Shaw, 2003). Recent work has shown that three mitochondrial fusion proteins in *S. cerevisiae*, FZO1p, Mgm1p and

Ugo1p, form a complex spanning the outer and inner membranes of the mitochondria and that their interactions are important for mitochondrial fusion (McQuibban et al., 2003; Sesaki et al., 2003; Wong et al., 2003). Although the mechanism of fusion is thus far not very clear, a similar mechanism may be responsible for vesicle trafficking out of chloroplasts and include a protein complex spanning the inner and the outer membranes of the chloroplast.

Vesicle-like structures on the outer envelope or in the stroma and close to the inner envelope have been observed either by chance or under specific experimental conditions (Robertson et al., 1996; Kroll et al., 2001; Westphal et al., 2001b; Westphal et al., 2001a; Chiba et al., 2003; Westphal et al., 2003). VIPP1 is proposed to be a protein involved in thylakoid biogenesis and maintenance via the budding of vesicles from the inner envelope membrane (Kroll et al., 2001; Westphal et al., 2001b). The vesicle transport of FZL seems to be a distinct pathway for two reasons: first, for FZL, the direction is from stroma to cytosol, but for VIPP1, the direction is likely from inner envelope to thylakoid. Second, the thylakoid membrane system is degraded in *vipp1* mutants but not in *fzl* mutants, suggesting that FZL influence the morphology of the thylakoids whereas VIPP1 is involved in biogenesis of the thylakoids. Vesicles close to the inner envelope of the chloroplast were also observed to accumulate at 4°C and their formation and fusion were affected by specific inhibitors(Westphal et al., 2001a, 2003); however, the direction of the movement of these vesicles is unclear. Rubisco-containing vesicles with double membranes have been found in the cytoplasm in naturally senescing

leaves of wheat (Chiba et al., 2003). This supports the proposal that there is a vesicle-trafficking process from the chloroplast to the cytosol.

Stromules are described as stroma-filled tubular structures extending from the plastid envelope membrane (Kohler et al., 1997b) and are clearly shown to be tubules dynamically protruding outward from chloroplasts and connected to each other (Kohler et al., 2000; Arimura et al., 2001). Vesicle-like structures filled with GFP have been observed either associated with stromules or detached from the plastid body (Kohler et al., 1997b; Pyke and Howells, 2002). It was proposed that the vesicle-like structures are cleaved from the plastids and represent a plastid export mechanism (Pyke and Howells, 2002). We studied *fzl* mutant plants with a chloroplast-targeted GFP as a marker for the stroma and found no defects in stromule formation (data not shown). Additionally, GFP-filled vesicles were shown to be bead-like or located at the enlarged end of stromules (Kohler et al., 1997b). Therefore, FZL does not appear to be involved in stromule formation. However, stromules may provide tracts for the trafficking of FZL-GFP-labeled vesicles or some of the FZL-GFP-labeled vesicles may make up a portion of the stromules.

Bacteria have been known to secrete vesicles from the outer membrane. Recently, it was shown that in *Escherichia coli* these vesicles have an important role in the secretion and delivery of bacterial protein toxins to mammalian cells (Beveridge, 1999; Miller et al., 2003; Wai et al., 2003). Since chloroplasts originated from cyanobacteria and many bacterial cellular processes are still preserved within the organelle, chloroplasts

may have a vesicle secretion system similar to that in bacteria. To date, the cargo of FZL-GFP-labeled vesicles is unknown, but the fact that vesicle formation is involved in the maintenance of thylakoid structure and chloroplast division implies that blocking of the dynamic vesicle-trafficking process may affect the underlying composition of the thylakoid and inner chloroplast membranes and consequently the function of chloroplasts. Thin layer chromatograph (TLC) analysis of total lipids and gas chromatograph (GC) analysis of total fatty acids showed no difference between wild type and *fzl* plants (data not shown); however, the mutant phenotype may be due to abnormal distribution of lipids or (and) fatty acids. Alternatively, FZL may be involved in some unknown signaling pathway that controls the development and division of chloroplasts. Further dissection and discovery of more components involved in this chloroplast vesicle-trafficking process will provide more details of its role.

If FZL is involved in the dynamic process of vesicle trafficking from the thylakoid to the cytosol, as proposed, loss of FZL function may severely affect the properties of the inner membrane. Subsequently, the function and (or) the localization of some chloroplast division proteins may be affected, resulting in enlarged chloroplasts in *fzl* mutants. The lack of visible GFP-labeled vesicle-like structures and the lack of complementation in the *FZL(K362M)-GFP* transformed *fzl* plants further indicates that the vesicles formed in the chloroplast and (or) their trafficking are important for chloroplast division. Alternatively, the abnormal thylakoids in *fzl* mutants may not be separated well during chloroplast division, affecting the constriction of chloroplasts and resulting in enlarged chloroplasts with heterogeneity.

Materials and Methods

Plant material

Arabidopsis thaliana ecotype Columbia (Col-0) was used for all experiments as wild type. T-DNA insertion lines of *FZL* in the Col-0 background were ordered from Arabidopsis Biological Resource Center (ABRC) (Alonso et al., 2003).The *fzl* mutant was identified in the segregation group from Salk lines: Salk_033745 and Salk_009051 (Alonso et al., 2003). Plants were grown as described (Gao et al., 2003).

Microscopy

Phenotypes were analyzed as previously described (Gao et al., 2003), except that the images were recorded with a Coolpix 4500 digital camera (Nikon Corporation, Tokyo). For *in vivo* detection of GFP with conventional fluorescence microscope, fresh leaf tissue was mounted in water and viewed with an L5 filter set (excitation 460-500 nm, emission 512-542 nm) and 40× objective lenses of a Leica DMR A2 microscope (Leica, Wetzlar, Germany) equipped with epifluorescence illumination. Images were captured with a cooled charge-coupled device camera (Retiga 1350EX, Qimaging, Burnaby, British Columbia, Canada). Chlorophyll was detected by the same method but with the TX2 filter set (excitation 540-580 nm, emission 608-683 nm). Confocal laser scanning microscopy (CLSM) was done with a Zeiss Pascal LSM 5.0 (Carl Zeiss, Germany). A 488nm argon ion laser line was used for excitation of GFP and chlorophyll flourescence. A BP 505-515 nm emission filter was used for GFP and a LP 650 nm emission filter was

used for chlorophyll. Images were viewed with a 40× oil immersion objective and processed with the Zeiss Pascal software (version 3.2 SP2).

For scanning electron microscopy (SEM) of isolated chloroplasts, chloroplasts from 3-week-old plants were isolated as in (Fitzpatrick and Keegstra, 2001), fixed with 4% glutaraldehyde for 2 hours and dehydrated in a ethanol sereies (Klomparens, 1986).The samples were sputter coated with gold, and imaged using a JEOL JSM 6400V SEM (JEOL USA, Inc., Peabody, MA, USA) at 7-10 kV of accelerating voltage.

For transmission electron microscopy (TEM) of chloroplasts, 3-week-old leaf tissue was cut and fixed in 2.5% glutaraldehyde and 2.0% paraformaldehyde in phosphate buffer 0.1 M, pH7.4 for approximately 24 hours at 4 °C. Postfixation was done in 1% osmium tetroxide. Samples were embedded in silicone molds and the polymerization was done for 24 hours at 60 °C. Thin sections were stained with 2% uranyl acetate and lead citrate before viewing with a JEOL 100CX transmission electron microscope (JEOL Inc., Peabody, MA, USA).

Immuno-gold labeling was done as in (Vandenbosch, 1991). The 3-week-old leaf tissue was cut and fixed in 0.2% glutaraldehyde and 3.0% paraformaldehyde in phosphate buffer 0.1 M, pH7.4, for approximately 24 hours at 4 °C. The primary antibodies against GFP (Clontech, Palo Alto, CA, USA) were diluted 30 times and the secondary antibodies conjugated with 10nm colloidal gold (Sigma, Saint Louis, MO, USA) were diluted 20 times. The samples were viewed with TEM as above.

All images were processed with PHOTOSHOP imaging software (Adobe Systems, San Jose, CA).

Sequence analysis of FZL

Primers used for RT-PCR amplification of *FZL* cDNA were 5'-CACAGACGAAGGTATCTCACTCTC -3' and 5'-TGTCAAGAAGGGAAAACCCGAC -3'. Amplified cDNAs were sub-cloned into Bluescript KS+ (Stratagene, La Jolla, CA, USA) before sequencing. The amino acid sequence of FZL was deduced from the cDNA sequence. TargetP (Emanuelsson et al., 2000) (http://www.cbs.dtu.dk/services/TargetP/) was used for predicting the subcellular location the FZL. The transmembrane domains of FZL were predicted with TMHMM program (http://biowb.sdsc.edu/) and TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html). The coiled-coil domains were predicted with the Paircoil program (Berger et al., 1995).
The sequence alignment shown in Figure 1c was obtained using the program CLUSTALW (Thompson et al., 1994) and Boxshade at the Biology Workbench 3.2 website (http://biowb.sdsc.edu/). The GenBank accession number for FZO from *Drosophila melanogaster* is AAF56110.

Protein sequences of the GTPase domains were used for the phylogenetic analysis in Figure 1b and aligned with CLUSTALW (Thompson et al., 1994) using default settings. The alignment is available on request. The phylogenetic tree was drawn by using the

program DRAWTREE at the Biology Workbench 3.2 website (http://biowb.sdsc.edu/). Neighbor joining analysis was performed using the program PROTPARS at the same website. Bootstrap analysis was performed on the neighbor-joining tree with one thousand replications. GenBank accession numbers for proteins aligned with FZL are as follows: human Dynamin1 (NP_004399), human Dynamin II (NP_004936), yeast Dnm1p (NP_013100), AtDRP3B (NP_565362), AtDRP1A (NP_851120), *Glycine* phragmoplastin (AAB05992), AtDRP2A (AAF22291), AtDRP2B (NP_176170), AtDRP5A (NP_175722), AtDRP5B (AY212885), *Drosophila* FZO (AAF56110).

Test of the transit peptide of FZL

A derivative of the transformation vector pCAMBIA-1302 (CAMBIA, Canberra, Australia), pCAMBIA-1302-bar (the *hpt*II gene was replaced with the bar gene from pCAMBIA-3300), which has a 35S CaMV promoter-*GFP*-NOS terminator cassette, was used as a negative control (Figure2 d-f). The corresponding coding sequence of the predicted transit peptide was amplified by PCR using the primers 5'-GCTTCCATGGGAACTCTAATCTCTCACCGGC -3' and 5'-GCATAGATCTGGACGCTTGTAACCACCAG -3'. The PCR product was digested with *Nco*I and *Bgl*II and ligated into pCAMBIA-1302-bar. The start codon is in the NcoI cutting site. This constructs were transferred to *Agrobacterium tumefaciens* GV3101 and introduced into *Arabidopsis* plants by floral dipping. Green fluorescence from GFP and red fluorescence from chlorophyll in the transgenic (Figure2 a-c) and wild type plants (Figure2 g-h) were detected by fluorescence microscopy.

Complementation analysis and FZL-GFP localization

The genomic fragment corresponding to *FZL*, including ~740bp of the 5' flanking DNA, was amplified from genomic DNA by PCR using the primers 5'-GACACACATATTAGGATTGGTTGCAC -3' and 5'-TACCACTAGTTCTCATCTCGTC TCGTGATACATG -3'. The PCR product was digested with *Spe*I and ligated into the transformation vector pCAMBIA-1302-bar, which was digested with *Sma*I and *Spe*I. The construct was transferred to *Agrobacterium tumefaciens* GV3101 and introduced into *fzl* plants by the floral dipping method. The phenotypes of the T_1 plants were determined by microscopy. In addition to fluorescence microscopy, FZL-GFP levels were measured by immunoblot. Monoclonal GFP antibodies were purchased from Clontech (Palo Alto, CA, USA).

Protease protection assay, fractionation of chloroplasts and immunoblotting

Chloroplast isolation, protease protection assays and immunoblotting were done as described (Bruce et al., 1994; McAndrew et al., 2001). Crude proteins representing approximately 4 μg chlorophyll content in each lane were analyzed by SDS-PAGE and immunoblotting in Figure 4a. Chlorophyll was quantified by adding 10 μl chloroplast suspensions to 990 μl 80% acetone and measuring the absorbance of the supernatant at 645 and 663 nm (Bruce et al., 1994).

Fractionation of chloroplasts was done as in (Block et al., 2002). To fractionate chloroplasts into envelope and thylakoid, chloroplasts were broken in 10 mM MOPS pH

7.6, 4 mM $MgCl_2$, 1 mM phenylmethanesulfonyl fluoride and 1 mM caproic acid, and then separated on a step gradient of 0.93 M to 0.6 M sucrose in 10 mM MOPS pH 7.6, 1 mM $MgCl_2$ by centrifugation at 70 000 g for 1 h. The thylakoid and envelope fractions were precipitated by acetone and suspended in equal amounts of SDS loading buffer so that equal sample volumes represent an equivalent proportion of the chloroplast. Crude proteins of different fractions in each lane of Figure 4b and 4c were from chloroplasts with 4 μg chlorophyll content. For immunoblottings, GFP antibodies were the same as above with a 1:10,000 dilution. Tic110 and FtsZ1 antibodies antibodies were diluted as described (Jackson et al., 1998; Stokes et al., 2000). LHCb antibodies used at 1: 20,000 were kindly provided by Kenneth Cline.

Mutagenesis of FZL

To create FZLK362M mutation, the 5' part of the genomic DNA of FZL was amplified by the PCR using the primers 5'- GACACACATATTAGGATTGGTTGCAC -3' and 5'- Phos-TTCCAGAGTTAAATTCCCCCAC -3'. The 3' end of the genomic DNA of *FZL* was amplified by PCR using the primers 5'- TGTCAACGGTTATCAATGCACTTCT -3' and 5'- TACCACTAGTTCTCATCTCGTCTCGTGATACATG -3' and digested with *Spe*I. The two PCR products were ligated into transformation vector pCAMBIA-1302-bar as above. The constructs were introduced into plants as above.

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Chapter 5

Conclusion and Future Directions

The genomes of many bacteria and eukaryotes, including several species of cyanobacteria and the model plant *Arabidopsis thaliana*, were sequenced in the last decade. This makes it much easier to identify the genes involved in many biological processes, including chloroplast division. Bacterial cell division is best studied in *E. coli* and many of the cell division proteins in *E. coli* are well conserved in cyanobacteria (Margolin, 2000; Errington et al., 2003). Several chloroplast division proteins, such as FtsZ1, FtsZ2, MinD, MinE, Ftn2 and SulA, were identified by searching for homologs of cyanobacterial cell division proteins in the *Arabidopsis* genome (Osteryoung and Vierling, 1995; Osteryoung et al., 1998; Strepp et al., 1998; Colletti et al., 2000; Stokes et al., 2000; Itoh et al., 2001; Vitha et al., 2003; Maple et al., 2004; Raynaud et al., 2004). This indicated that chloroplasts inherited some of the important components of the cyanobacterial cell division machinery during endosymbiosis.

However, not all the choloroplast division genes are derived from cyanobacteria; *ARC5*, a eukaryotic-derived chloroplast division gene, was identified by positional cloning in *Arabidopsis* (Gao et al., 2003). Phylogenetic analysis indicated that ARC5 is related to a group of dynamin-related proteins unique to plants (Gao et al., 2003; Miyagishima et al., 2003). Dynamin and its relatives are large GTPases that participate in a variety of organellar fission and fusion events in eukaryotes, including the budding of endocytotic and Golgi-derived vesicles, mitochondrial fission, mitochondrial fusion, and plant cell plate formation, etc. (Chen et al., 1991; van der Bliek and Meyerowitz, 1991; Wilsbach and Payne, 1993; Gu and Verma, 1996; Pelloquin et al., 1998; Bleazard et al., 1999; Sesaki and Jensen, 1999; Hinshaw, 2000; Danino and Hinshaw, 2001). Dynamin

was shown to constrict lipid tubes *in vitro* (Sweitzer and Hinshaw, 1998; Danino et al., 2004) and this family of proteins is proposed to be mechanochemical enzymes (Danino and Hinshaw, 2001). ARC5 was localized to the chloroplast division furrow on the cytosolic surface (Gao et al., 2003). The phenotype of the large dumbbell-shaped chloroplasts in the *arc5* mutant suggests that ARC5 may generate force during the constriction of the division furrow (Pyke and Leech, 1994; Robertson et al., 1996; Gao et al., 2003). Thus, the chloroplast division apparatus is of mixed evolutionary origin and shares structural and mechanistic similarities with both the cell division machinery of bacteria and the dynamin-mediated organellar fission machineries of eukaryotes.

The phenotype of the *arc5* mutant also suggests that chloroplast division is not completely blocked (Pyke and Leech, 1994; Robertson et al., 1996; Gao et al., 2003). Since *arc5* is a null allele, *ARC5* is either not essential for chloroplast division or is redundant to some other gene. A homolog of *ARC5*, *ARC5H*, was found in the *Arabidopsis* genome, suggesting it might be redundant to *ARC5*. However, an *arc5/arc5h* double mutant showed the same phenotype as *arc5* and a *35S-ARC5H* transgene showed no effect on chloroplast division in either *arc5* or wild type plants. Moreover, ARC5H-GFP was localized to the nucleus and not chloroplasts. These data strongly suggest that ARC5H does not function in chloroplast division. Therefore, although ARC5 is important for chloroplast division, it is not an essential chloroplast division protein.

Cyanobacteria are Gram-negative bacteria with a cell wall between the inner and the outer cell membranes. FtsI, a transpeptidase conserved in many bacteria, is involved

in the synthesis of the new cell wall during cell division (Nakamura et al., 1983; Weiss et al., 1997; Wissel and Weiss, 2004). As there is no cell wall between the inner and outer membranes of chloroplasts and no homolog of FtsI is found in the sequenced genomes of *Arabidopsis* and rice, the inner and outer membranes of chloroplasts may be connected by some protein complex, such as the chloroplast import machinery. In the *arc5* mutant, although the constriction of the chloroplast outer membrane is defective, components of the chloroplast division machinery that are derived from cyanobacteria may be sufficient for the first several rounds of chloroplast division in the cells of very young tissues. The total volume of chloroplasts in one cell relative to the volume of the cell in leaf tissue is believed to be kept at a certain ratio (Pyke, 1999). When leaf cells start to quickly expand but the chloroplasts cannot divide efficiently, chloroplast size will increase. With the increase of chloroplast size, the chloroplast division machinery may work even less efficiently.

When chloroplast envelopes were changed during evolution, some chloroplast division proteins may be lost and chloroplasts may divide inefficiently. However, there are may be some disadvantages to have less chloroplasts with relatively larger size (Jeong et al., 2002). The chloroplast division machinery must be further modified to overcome this problem. Probably in this kind of situation, ARC5 evolved from the host cell to facilitate the division of chloroplasts from the cytosolic side. A similar scenario may have occurred in the evolution of Dnm1 for mitochondria division. However, mitochondria are more ancient than chloroplasts and mito-FtsZ, which was found only in some lower

eukaryotes (Beech et al., 2000; Gilson et al., 2003; Kiefel et al., 2004), may be the last relic of the bacterial cell division machinery in mitochondria.

ARC5 is relatively divergent from other dynamin-related proteins (Gao et al., 2003). The GTPase and middle domains of ARC5 can be aligned with those in other dynamin-related proteins better than can the PH and GED domains (Gao et al., 2003). The PH domains of dynamin family proteins determine the specificity of membrane binding (Salim et al., 1996; Zheng et al., 1996). The lower conservation in the PH domains between ARC5 and other members of dynamin family may underlay their different locations in the cell. ARC5 may generate force to facilitate chloroplast division like dynamin. However, ARC5 functions at a different site and forms a ring with a much lager diameter than do other dynamin-related proteins.

FZO is an even more diverged dynamin-related protein involved in the fusion of the mitochondrial outer membrane in metazoa and fungi (Hales and Fuller, 1997; Sesaki and Jensen, 1999). It has a GTPase domain, two transmembrane domains and two coiled-coil domains (Shaw and Nunnari, 2002). I found an FZO-like protein in plants, FZL, with a similar domain arrangement. However, FZL was shown to be targeted to the chloroplast. The *fzl* knockout mutant has disorganized stacking of thylakoid grana and a chloroplast division defect with heterogeneity in chloroplast size. Overexpression of FZL-GFP resulted in very long thylakoid membrane sacs with considerably less stacking than in wild type. FZL-GFP is a membrane protein localized to both the thylakoid and the inner chloroplast envelope membrane.

Thylakoids in cyanobacteria are simple parallel membrane sacs without stacking or visible differentiation in the membrane structure (Vothknecht and Westhoff, 2001; Westphal et al., 2003). In contrast, thylakoids in plants are organized stacks of membrane sacs (grana) connected by fewer unstacked sacs (lamellae) (Dekker and Boekema, 2005). Grana and lamellae have different roles in photosynthesis and their differentiation makes photosynthesis more efficient (Trissl and Wilhelm, 1993). FZL is in the same plylogenetic branch as FZO in the dynamin family and has a domain arrangement similar to that of FZO, suggesting that they may share similarity in the mechanisms of their functions. It is interesting that FZL evolved to regulate the morphology of thylakoid. Although it is not clear how the thylakoids are separated during chloroplast division and cyanobacterial cell division, analysis of FZL does indicate that the morphogenesis of thylakoids affects chloroplast division.

ARC5 is the first cytosolic component of the chloroplast division machinery to be identified. FZL is the first known protein that dramatically affects both thylakoid morphology and chloroplast division. In budding yeast, Dnm1 is a dynamin-related protein localized to the division site of mitochondria. It interacts with other proteins, such as Mdv1, Net2 and Fis1 (Cerveny et al., 2001; Tieu et al., 2002; Cerveny and Jensen, 2003), on the cytosolic side to control mitochondria division. In fungi and metazoa, FZO, Ugo1 and Mgm1 are in a protein complex involved in the fusion of mitochondrial outer membranes (Wong et al., 2003). So, ARC5 and FZL may interact with other proteins during chloroplast division and thyalkoid morphogenesis. In *E. coli*, there are about ten

proteins localized to the cell division site and interacting with each other. In chloroplasts, it is possible that there are additional proteins that interact with FtsZ and ARC6 at the division site either on the stromal side or in the intermembrane space. Therefore, more genes need to be identified for a better understanding of the mechanism of chloroplast division.

Homologs of cyanobacterial cell division proteins in *Arabidopsis* were shown to be involved in chloroplast division (Osteryoung and Vierling, 1995; Osteryoung et al., 1998; Colletti et al., 2000; Stokes et al., 2000; Itoh et al., 2001; Vitha et al., 2003; Maple et al., 2004; Raynaud et al., 2004). Most of the cyanobacterial cell division proteins were found based on homology to known cell division proteins in *E. coli*. However, some cell division proteins in *E. coli* have no homologs in cyanobacteria. In contrast, Ftn2 and Ftn6 are cell division proteins unique to cyanobacteria (Koksharova and Wolk, 2002). Since *Ftn2* and *Ftn6* were identified by transposon mutagenesis in a non-saturating screen, it is possible that there are still some cyanobacteria-specific cell division proteins have not been identified. Further genetic screening of cyanobacterial cell division mutants by transposon and chemical mutagenesis with high saturation may identify new cyanobacteria-specific cell division proteins and help to identify new chloroplast division proteins. Indeed, some additional cyanobacterial cell division mutants have recently been obtained by transposon mutagenesis (Miyagishima et al., 2005).

Genetic screening by Leech's group and others has identified 12 *arc* loci in *Arabidopsis* (Pyke and Leech, 1994; Marrison et al., 1999). ARC6, ARC10, ARC11 and

ARC12 correspond to homologs of the cyanobacteria cell division proteins Ftn2, FtsZ1, MinD and MinE, respectively (Colletti et al., 2000; Vitha et al., 2003; Fujiwara et al., 2004) (and Miyagishima, personal communication). The N-terminal half of ARC3 has some similarity to the GTPase domain of FtsZ and the C-terminal half of ARC3 has a membrane-occupation-and-recognition-nexus (MORN) repeat motif (Shimada et al., 2004). ARC5 is a plant-specific dynamin-related protein of eukaryotic origin (Gao et al., 2003). Thus, mutant screening is another very useful approach for identifying chloroplast division genes.

However, the genetic screening of the chloroplast division mutants is far from saturation. A further mutant screening in *Arabidopsis* and the subsequent gene cloning may identify new chloroplast division genes, especially genes of eukaryotic origin. Most of the *arc* mutants with an apparent chloroplast division phenotype have a non-sense mutation in the corresponding genes. It is not clear that whether mutations in the unknown chloroplast division genes cause gametophyte lethality or not. EMS causes alkylation of guanine and almost all the mutations are G/C-to-A/T transitions in EMS mutagenesis (Greene et al., 2003). Since the *Arabidopsis* genome has a ~40% GC content in the genic regions (Yu et al., 2002), there may be some limitations to use of EMS mutagenesis in *Arabidopsis*, although it has been successfully used. Other chemical mutagens can be applied as a complement to EMS in mutant screening.

In *Arabidopsis*, there are many gene families (Arabidopsis_Genome_Initiative, 2000) and different members in the same gene family may be redundant. Mutations and

even the loss of function of these genes may not cause any phenotype. Therefore, other approaches are needed to overcome the shortcomings of mutant screening. Techniques to detect protein-protein interaction, such as yeast-two-hybrid (Y2H) screening and co-immunoprecipitation, are powerful and routine approaches for identifying new components in protein complexes and may be used for identifying unknown components in the chloroplast division machinery.

Y2H screening has been used in many cases either for finding new proteins or for a better understanding of the relationship between interacting proteins in a biological process. A genome-wide Y2H screen identified 957 putative protein interactions in yeast (Uetz et al., 2000). Y2H screening can be easily carried out. So, all the known proteins involved in chloroplast division can be used as baits for identifying the proteins with which they interact. This will not only identify new components of the chloroplast division machinery but also reveal the protein interactions in this complex.

Another approach, co-immunoprecipitation (co-IP) is also very useful in the analysis of protein complexes. For example, the chloroplast protein import machineries are membrane protein complexes and they can be isolated in protein extracts by co-IP (Schnell et al., 1994; Akita et al., 1997). New components were found by analyzing all the proteins in the complex. Another example is the COP9 signalosome, a regulatory protein complex conserved from plants to humans (Wei and Deng, 2003). Some of the components in this complex were initially identified by genetic methods. Later on, all the components in this protein complex were identified by co-IP. This method may also be

applied to the chloroplast division machinery for discovering new chloroplast division proteins.

Finally, the mechanism of chloroplast division at the biochemical and biophysical level is still largely unknown. Cyanobacteria have only one FtsZ, while plants have FtsZ1, FtsZ2 and ARC3 (Osteryoung et al., 1998; Shimada et al., 2004). These three types of FtsZ proteins have distinct functions in chloroplast division (Osteryoung et al., 1998; Shimada et al., 2004). Why are three types of FtsZ proteins needed in plants for chloroplast division and how do they interact with each other? MinD and MinE are also present in the chloroplast (Colletti et al., 2000; Itoh et al., 2001), but MinC is missing. How are the MinD and MinE in plants different from those in cyanobacteria? What is the role of ARC6 in the chloroplast division? How does it regulate the activity of FtsZ at the chloroplast division site? How is ARC5 involved in chloroplast division? Is it also a mechanoenzyme like dynamin? Study of the crystal structures of the individual proteins or the proteins interacting with each other in the chloroplast division machinery will help us to answer the above questions and understand the molecular mechanism of chloroplast division.

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