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KEVIN D. STOKES

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By

Kevin David Stokes

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ABSTRACT

AN ANALYSIS OF THE EXPRESSION, FUNCTION, AND EVOLUTION OF THE FTSZ PLASTID DIVISION GENES

By

Kevin David Stokes

The photosynthetic capabilities of plants cells are dependent on the presence and maintenance of chloroplasts. The chloroplast complement of higher plant mesophyll cells, often very numerous, is maintained by division as cells differentiate and expand. Chloroplasts are evolutionarily derived from cyanobacteria through an endosymbiotic event and continue to bear several ancestral characteristics. The prokaryotic origins became even more evident when a chloroplast-targeted homologue of the bacterial cell division protein FtsZ was identified in plants. In bacteria, FtsZ is the first known protein to assemble at the division site. FtsZ has GTPase activity and polymerizes into a ring that encircles the cell at the cyoplasmic membrane surface. Following assembly of the FtsZ ring at the division site, several other proteins are recruited for assembly of a functional cell division apparatus.

In contrast to most bacteria that encode a single *FtsZ* gene, plants have multiple nuclear encoded FtsZ proteins that are localized to the chloroplast. The plant homologues have been grouped into two families, FtsZ1 and FtsZ2, based on sequence comparisons. Arabidopsis plants express three different FtsZ homologues: one FtsZ1 family member, AtFtsZ1-1, and two FtsZ2 family members, AtFtsZ2-1 and AtFtsZ2-2. Antisense repression experiments indicate members of both families are required for

chloroplast division, suggesting that chloroplasts have evolved a more complex division apparatus than is present in bacteria.

In an effort to better understand the role of the FtsZ1 and FtsZ2 proteins in chloroplast division, experiments were designed to investigate their function, expression, and evolution. To investigate some of the FtsZ functions in plastid division, the FtsZ proteins were overexpressed in Arabidopsis and the effects on chloroplast division were observed. The results indicate a stoichiometric balance is required for division and that disruption of that balance inhibits chloroplast division. Experiments with *promoter-GUS* fusion constructs were used to determine where and when FtsZ is expressed in Arabidopsis. Some of the FtsZ expression patterns were confirmed by measuring the cDNA distribution patterns. The results indicate the three *FtsZ* homologues are coordinately expressed in several plant tissues including roots, meristems, and young leaves. *FtsZ* expression occurs in tissue regions with rapidly dividing chloroplast populations and is consistent with the role of FtsZ in chloroplast division.

In an effort to understand when and why chloroplasts evolved two FtsZ families, we performed phylogenetic analyses, compared genetic structures, and compared conserved protein sequences. Phylogenetic analyses indicate the *FtsZ1* and *FtsZ2* sequences diverged before the split between the chlorophycean and charophycean green algal lineages and possibly earlier. Genetic structure comparisons reveal intron positions are conserved within the *FtsZ1* and *FtsZ2* family members but differ between them, also supporting an early divergence. Comparison of conserved protein sequences indicated several regions in which the FtsZ1 and FtsZ2 family members differ. These conserved differences may define functional differences between the FtsZ1 and FtsZ2 proteins.

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Ala

arc

ATP

ATPase

BAC

BLAST

bp

C-termin

cDNA

cpm

CTP

d

dATP

dGTP

DNA

DTT

EDTA

EST

EMS

FAM

fs

KEY TO ABBREVIATIONS

Ala	Alanine
arc	Accumulation and replication of chloroplasts
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphotase
BAC	Bacterial artificial chromosome
BLAST	Basic local alignment search tool
bp	Base pair
C-terminus	Carboxy terminus
cDNA	Completmentary DNA
cpm	Counts per minute
CTP	Cytidine tripohosphate
d	Day
dATP	Deoxyadenosine triphosphate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EST	Expressed sequence tag
EMS	Ethyl methanesulfonate
FAM	6-carboxyfluorescein
fts	Filamentation temperature-sensitive

g

GDP

GFP

GTP

GTPase

GUS

HPLC

hr

IPTG

Kan^r

kD or kDa

L

M

mg

min

mM

mRNA

MS

N-terminus

ng

nm

PAGE

PCR

g	Gram
GDP	Guanosine diphosphate
GFP	Green fluorescence protein
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
GUS	β-glucuronidase
HPLC	High-performance liquid chromatography
hr	Hour
IPTG	Isopropylthio-β-galactoside
Kan^r	Kanamycin resistant
kD or kDa	Kilodalton
L	Liter
M	Molar
mg	Milligram
min	Minute
mM	Millimolar
mRNA	Messenger RNA
MS	Murashige and Skoog
N-terminus	Amino terminus
ng	Nanogram
nm	Nanometer
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction

PD

Phe

RACE

RT

RNA

s

SEDS

SDS

Suc

T-DNA

TBR

TAMRA

UTP

UTR

X-gluc

PD	Plastid dividing
Phe	Phenylalanine
RACE	Rapid amplification of cDNA ends
RT	Reverse transcription
RNA	Ribonucleic acid
s	Second
SEDS	Shape, elongation, division, and sporulation
SDS	Sodium dodecyl sulfate
Suc	Sucrose
T-DNA	Transfer DNA
TBR	Tree bisection-reconnection
TAMRA	6-carboxy-N,N,N',N'-tetramethylrhodamine
UTP	Uridine triphosphate
UTR	Untranslated region
X-gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronid

CHAPTER 1

Introduction

Cell division is a critical process for the proliferation of any organism. In 1968 Hirota et al. (1968) reported an experiment designed to decipher the molecular basis of bacterial cell division by isolation of bacterial mutants that were unable to divide at elevated temperatures. Due to their filamentous morphology, the mutants were called *fts* for *f*ilamentation *t*emperature-*s*ensitive. One of the proteins identified from the screen, FtsZ, has been shown to be a key component of the bacterial cell division machinery (Lutkenhaus et al., 1980). FtsZ proteins are structurally homologous to tubulins, have GTPase activity, and polymerize (Lutkenhaus and Addinall, 1997). FtsZ forms a ring at the cell division site and is an early step in the assembly of the cell division apparatus, which includes at least ten other components: ZapA, FtsA, ZipA, FtsK, FtsQ, FtsL, FtsB, FtsW, FtsI, and FtsN (Errington et al., 2003; Margolin, 2003). To date, only homologues of FtsZ have been identified in higher plants, where they have been shown to function in the division of chloroplasts.

FtsZ Ring Formation in Bacteria

Although the filamentous phenotype observed in bacterial mutant screens indicated FtsZ had a role in cell division, it was a report by Bi and Lutkenhaus (1991) that suggested FtsZ formed a ring at the division site. Immunoelectron microscopy experiments localized FtsZ protein to a region at the midcell of *Escherichia coli*, a position that correlated to the division site. Cross-sectional micrographs of bacterial cells indicated the protein formed a ring at the inner surface of the outer membrane, which remained at the leading edge of the septum throughout constriction. FtsZ rings seemed to form just prior to septal constriction, since localization was only observed in longer cells

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and not in shorter cells that had recently divided (Bi and Lutkenhaus, 1991). Quantification by immunoblotting indicated there are about 5,000-20,000 FtsZ molecules in each *E. coli* cell, which is enough protein to encircle the bacterium about 20 times at the division site (Pla et al., 1991; Dai and Lutkenhaus, 1992; Lu et al., 1998). Similar observations of the FtsZ protein localizing to the division septum have also been made in *Bacillus subtilis* (Wang and Lutkenhaus, 1993). Mutations in other downstream division genes, like *ftsQ* and *ftsI*, produced cells with slightly constricted phenotypes and indicated FtsZ ring formation is one of the first steps that regulates the assembly of the division apparatus (Begg and Donachie, 1985).

Bacterial FtsZ Binds and Hydrolyzes GTP

Sequence comparisons identified motifs within the FtsZ protein that are similar to other GTP binding proteins. In eukaryotic tubulins the tubulin signature motif is proposed to be involved in GTP binding (Bourne et al., 1991) and this motif is conserved in the FtsZ protein (Erickson, 1995). Several groups used nucleotide-binding experiments to determine whether, like the tubulins, FtsZ also bound and hydrolyzed GTP (de Boer et al., 1992; RayChaudhuri and Park, 1992; Mukherjee et al., 1993). A single mutation in the tubulin signature motif significantly reduced GTP binding and cells expressing this mutant FtsZ protein failed to initiate division (de Boer et al., 1992). These findings indicated that GTP binding and hydrolysis are important for FtsZ function in cell division. Interestingly, this single mutation changed FtsZ from a GTPase to an ATPase, at least *in vitro* (RayChaudhuri and Park, 1994).

Bacterial FtsZ Proteins Form Polymers

In addition to displaying GTPase activity, tubulins polymerize into multimeric structures (Weisenberg, 1972). Since FtsZ has protein sequences and GTPase activity that are similar to tubulins, the polymerization capabilities of bacterial FtsZ were investigated. After incubating purified FtsZ protein with Mg^{2+} and GTP, protein filaments were observed by electron microscopy (Mukherjee and Lutkenhaus, 1994). This filamentation was not observed when GTP was absent or when mutant protein that was unable to bind GTP was used in the assay. However, filaments were observed when mutant protein was used that could bind but not hydrolyze GTP. These results indicate that FtsZ polymerization is dependent on nucleotide binding but not hydrolysis (Mukherjee and Lutkenhaus, 1994). Similar conclusions were reported by Bramhill and Thompson (1994) who observed GTP-dependent polymerization of FtsZ protein filaments by electron microscopy.

Large, polymerized FtsZ protein structures will sediment by centrifugation whereas free protein will not. Using the amount of protein that sediments as a measure of polymerization, various nucleotide cofactors such as GTP, GDP, GTPγS, dGTP, and ATP were tested for their ability to promote polymerization of FtsZ into large structures (Bramhill and Thompson, 1994). Only when both Mg^{2+} and GTP were incubated with FtsZ protein did significant sedimentation occur. These results indicated hydrolysis is important for polymerization into large structures (Bramhill and Thompson, 1994), but polymerization was later determined not to require hydrolysis (Scheffers and Driessen, 2002). It was reported that polymerization into these larger structures was enhanced by a

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drop in the pH during the polymerization reaction (Erickson et al., 1996). Addition of GTP in the Bramhill and Thompson (1994) experiments actually lowered the pH and unknowingly enhanced formation of these large FtsZ polymer structures (Erickson et al., 1996).

Detailed analysis of these large FtsZ polymer structures was done with high-resolution electron microscopy (Erickson et al., 1996). Although formation was enhanced at lower pH, large complexes were observed to form in the pH range of 5.5 to 7.0, in the presence of either GTP or GDP. In these polymerization experiments several large structures were reported. One of the simplest structures was a long, straight filament. More complex structures consisted of two-dimensional sheets made of two or more of these filaments. Even more complex tubular polymers that are composed of several filaments were observed. Filaments were also observed that formed minirings. The miniring curvature was also manifested as filaments spiraling away from polymer sheets or tubular structures. All these structures, including the long straight filaments, curved filaments and minirings, and filament sheets, are similar to structures formed by tubulin. In addition, optical diffraction and computer image reconstruction of FtsZ and tubulin sheets indicated the lattice spacing is very similar between the two proteins. These results supported the similarity between the tubulin and FtsZ structures (Erickson et al., 1996).

FtsZ and Tubulin Proteins are Structurally Similar

Additional support for tubulin and FtsZ functional and structural similarity was reported by de Pereda et al. (1996). The structures of α, β, and γ tubulin as well as FtsZ

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were predicted using alignments of several sequences of each protein from various organisms. Although tubulin and FtsZ protein sequences overall are only slightly similar, regions of the predicted secondary structures were very similar, including the region containing the tubulin signature motif. To support these structural predictions, limited proteolysis experiments confirmed that regions of the tubulin proteins predicted to be exposed could be cleaved. Although proteolysis experiments were not done with the FtsZ protein, the predictions supported similar protein structures for both tubulin and FtsZ (de Pereda et al., 1996).

The similarity between FtsZ and tubulin was graphically demonstrated by the determination of their respective crystal structures. Nogales et al. (1998) determined the crystal structure of the αβ tubulin heterodimers that had been polymerized into sheets. The FtsZ crystal structure was determined by Löwe and Amos (1998) using protein from *Methanococcus jannaschii*. Both the tubulin and FtsZ structures are very similar, with each protein consisting of two domains that are connected by a linker (Löwe, 1998; Löwe and Amos, 1998; Nogales et al., 1998; Nogales et al., 1998). The N-terminal portion of FtsZ is called the GTPase domain (Löwe, 1998; Löwe and Amos, 1998) and consists of residues 38-227 of the *M. jannaschii* protein that form a six-stranded β-sheet with two and three helices on either side. The arrangement of the β-sheet and helices is consistent with the Rossman-fold topology (Rossmann et al., 1974). The GTP nucleotide is bound to one side of the β-sheet and makes contact with six loops. Because the structure of this domain in FtsZ and tubulin were distinct from that in other classical GTPases, it was proposed that FtsZ and tubulin form a new family of GTP hydrolyzing enzymes (Löwe, 1998; Nogales et al., 1998). The extreme N-terminal residues of tubulin protrude from

the molecule and make important crystal contacts (Löwe and Amos, 1998). However, because this extension is highly variable among FtsZ proteins, being very short in *E. coli* FtsZ, it is unlikely to have a significant role in FtsZ polymerization.

The C-terminal domain of the FtsZ protein consists of residues 228-356 and is connected to the N-terminal domain by a long helix. This region has a parallel four-stranded central β-sheet with two helices supporting it on one side (Löwe, 1998; Löwe and Amos, 1998). This domain has sequence similarity to calmodulins that bind calcium, and make binding of calcium feasible in the FtsZ protein (Löwe and Amos, 1998). The residues at the extreme C-terminus protrude from the molecule and are disordered. Comparisons among the FtsZ proteins indicate the C-termini are very divergent among different organisms. In tubulin, the corresponding region forms two helices that sit on the surface of the molecule (Nogales et al., 1998) and are located outside of the microtubule (Wolf et al., 1996). It was proposed that this C-terminal region of tubulin contacted microtubule-associated proteins or motor proteins (Nogales et al., 1998). By analogy, the C-terminus of FtsZ is likely to be important for interactions with other proteins. The divergence of the C-terminal region in both tubulin and FtsZ may reflect differences in the contacts that are required in different organisms.

A region within the N-terminal domain of α-tubulin, called the T3 loop, makes important contacts with GTP. Specifically, the loop contacts the γ-phosphate of the GTP molecule (Nogales et al., 1998). Analysis of a molecular model of the microtubule, based on the crystal structure of the α,β-tubulin dimer (Nogales et al., 1998), indicates that a conformational change in the T3 loop might cause hydrolysis of the γ-phosphate (Nogales et al., 1999). A similar conformational change was predicted from molecular

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models of the FtsZ T3 loop (Diaz et al., 2001). To test this prediction Diaz et al. (2001) mutated a threonine residue in the T3 loop to a typtophan, making an FtsZ protein that had different spectral characteristics when GTP or GDP were bound. Analysis of this mutated FtsZ protein indicate there is a change in the position of the T3 loop when GTP or GDP is bound (Diaz et al., 2001).

Analysis of the crystal structure of α,β-tubulin (Nogales et al., 1998) and microtubule modeling (Nogales et al., 1999) revealed a region that includes the T7 loop that contacted the GTP nucleotide bound to an adjacent monomer. The T7 loop region of FtsZ is also determined to be near the GTP nucleotide of an adjacent monomer by three-dimensional reconstruction of FtsZ polymerized into sheets (Löwe and Amos, 1999). Furthermore, mutation of residues within the T7 loop region severely reduces both polymerization and GTP hydrolysis (Lu et al., 2001; Scheffers and Driessen, 2001; Scheffers et al., 2002). These results indicate that GTP hydrolysis occurs in an active site that is composed of two FtsZ monomers.

Dynamics of Bacterial FtsZ Polymer Formation

Evidence that FtsZ dimers are present *in vivo* was reported by Di Lallo et al. (1999). They constructed a chimeric gene that consisted of *FtsZ* and a portion of the λ repressor that is active only as a dimer. The repressor fragments alone were unable to dimerize, but functional repressor was present when the *FtsZ-repressor* chimera was expressed in *E. coli*. This indicated FtsZ formed dimers that allowed the repressor to become active. Using this as an assay, *FtsZ* mutants were isolated that could no longer dimerize. Mapping of the mutations provided insights into regions that were involved in

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FtsZ dimerization (Di Lallo et al., 1999). In another study, point mutations within *FtsZ* genes were isolated that either affected GTP binding or polymerization of the FtsZ protein (Lu et al., 2001). The polymerization mutants had normal, or near normal, GTPase activity and filament assembly *in vitro,* but were unable to complement an *FtsZ* with a different temperature sensitive mutation. These results indicated mutations located on the lateral surface of FtsZ are not required for filament polymerization but are important for filament-to-filament interactions that make up the higher-order structures (Lu et al., 2001).

Several reports have investigated the dynamics of FtsZ polymer formation and GTP hydrolysis. One question has been whether FtsZ monomers are added to a growing polymer in a cooperative or isodesmic manner (Romberg et al., 2001). Assembly of cooperative polymers requires a nucleation event followed by rapid growth of a multi-stranded complex; the monomer concentration is critical in this type of assembly. The polymers also tend to be very long whereas isodesmic polymers are short. Isodesmic polymers are single-stranded, require no nucleation event, and require no critical monomer concentration. Using electron microscopy and light scattering measurements (the longer the polymer the more light is scattered) to analyze the kinetics of polymer formation, Romberg et al. (2001) suggested that FtsZ polymer formation is isodesmic. Similar conclusions were reached by Rivas et al. (2000) using sedimentation analysis to measure the kinectics of polymer formation.

In a recent report however, Caplan and Erickson (2003) suggest that FtsZ polymer assembly is cooperative while disassembly is isodesmic. Caplan and Erickson (2003) used isothermal titration calorimetry to measure the heat of FtsZ self-association to

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investigate the thermodynamics of FtsZ polymerization. When proteins interact, heat is released. By measuring the amount of heat generated in reactions with different FtsZ concentrations the total number of protein interfaces could be inferred and these measurements were not biased toward longer molecules, as were the light scattering and sedimentation experiments. When FtsZ and GDP are injected into the calorimeter, the change in heat followed an isodesmic model. Under the conditions that were used almost all of the GDP-bound FtsZ protein was in a polymerized form. When the polymerized FtsZ was injected into the calorimeter, the change in heat was due to disassembly. Therefore, the authors suggest disassembly of FtsZ polymers is isodesmic. However, when FtsZ and GTP were injected into the calorimeter, a critical FtsZ concentration was required for polymerization and an isodesmic process could not model the thermodynamics of polymer formation. Therefore, the authors concluded assembly of FtsZ polymers is apparently cooperative (Caplan and Erickson, 2003).

Understanding the exact role that GTP hydrolysis plays in FtsZ polymer formation has been difficult to determine, although progress has been made. Mingorance et al. (2001) incubated radiolabeled GTP with FtsZ protein, filtered the reaction to remove free nucleotide and protein, and collected polymerized protein. By analyzing the amount of bound radiolabel, they determined that most of the nucleotide in the FtsZ polymers was GTP. When labeled GTP was added to preformed polymers, they measured a rapid exchange of bound nucleotide. From these results it was suggested that hydrolysis of GTP destabilizes the polymers, but by quickly exchanging GDP with fresh GTP the polymers were restabilized (Mingorance et al., 2001). However, their method was flawed as it assumed the phosphate released by hydrolysis was rapidly removed from

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the proteins (Scheffers and Driessen, 2002). Scheffers et al. (2000) performed similar nucleotide binding assays as Mingorance et al. (2001), but they extracted the bound nucleotide from the protein and analyzed the nucleotides by thin layer chromatography. They found that almost all of the bound nucleotide is hydrolyzed to GDP. In addition, they also found the phosphate released by the hydrolysis remains present in the FtsZ proteins. This indicated hydrolysis of GTP is very rapid and, by itself, does not destabilize polymers. In addition, Scheffers et al. (2000) found that GDP-containing polymers are stabilized by non-hydrolyzable GTP analogues in polymerization reactions. This result indicated that the FtsZ polymers, like tubulin, might have a GTP cap that stabilizes the polymerized structure.

Although several properties of FtsZ have been described, many of the functions and structures that are physiologically relevant are still unclear. FtsZ proteins bind GTP and hydrolysis occurs very rapidly upon dimerization or polymerization (Löwe and Amos, 1998; Scheffers and Driessen, 2002). Polymerization of FtsZ into filaments is likely an important step in the formation of the division ring. However, it is unclear whether the filaments form *de novo* at the site or whether short oligomers form in the cytosol and then aggregate at the division site (Stricker et al., 2002). The structure of the FtsZ ring is also unclear, but probably consists of linear filaments bundled together, since tubular structures have never been observed in bacteria by electron microscopy (Lu et al., 2000).

Although the structure of the FtsZ ring is still unresolved, it seems to be very dynamic with subunits being exchanged throughout its existence (Stricker et al., 2002). Using green fluorescent protein (GFP)-labeled FtsZ and fluorescence recovery after

photobleaching, Stricker et al. (2002) observed that bleached signal in FtsZ ring structures recovers rather rapidly, even in constricting cells. This indicates that the ring structure, even during constriction, is dynamic with its components rapidly exchanging with cytoplasmic pools of FtsZ protein.

The force-generating component of the ring structure is unknown. However, the FtsZ ring itself has been implicated based on observations that GTP hydrolysis causes FtsZ filaments to favor a curved conformation (Lu et al., 2000). Whether FtsZ is the force-generating component or not, one important function of the FtsZ ring seems to be the recruitment or localization of other protein components to the cell division apparatus (Errington et al., 2003).

Other Bacterial Cell Division Proteins

MinC, D, and E

The positioning of the FtsZ ring at the division site involves the functions of three proteins that make up the *min* system, MinC, MinD, and MinE. In *E coli*, these three Min proteins are encoded by and expressed from a single operon (de Boer et al., 1989). When *MinC* or *MinD* is mutated, FtsZ ring formation occurs not only at midcell but also at the poles, but no ring forms when MinE is mutated (de Boer et al., 1989, 1992). These and other results (Rothfield et al., 1999) indicate that MinC and MinD act in a complex as inhibitors of FtsZ ring formation, while MinE acts as a topological inhibitor of MinCD. A specific mutation in MinC reduces the ability of the protein to inhibit division, reduces its affinity for FtsZ, and reduces the inhibition of FtsZ polymerization (Hu et al., 1999). These observations indicate MinC interacts directly with FtsZ to prevent polymerization.

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In fact, fusion experiments with domains of MinC indicated the N-terminus interacts with FtsZ while the C-terminus was involved in dimerization with MinD (Hu and Lutkenhaus, 2000). Interactions between MinC and MinD have also been observed with the yeast two-hybrid system (Huang et al., 1996) and by proteolysis experiments (Szeto et al., 2001).

The process by which the Min system regulates placement of the FtsZ ring is a very dynamic. Localization studies of GFP-labeled MinC indicate the protein oscillates from pole to pole (Hu and Lutkenhaus, 1999). The oscillation is dependent on MinD since its removal results in MinC becoming diffuse throughout the cytoplasm. MinD, therefore, seems to be the oscillating component that binds MinC to the membrane at the cell poles (Raskin and de Boer, 1999; Raskin and de Boer, 1999). The oscillation of MinC from pole to pole likely inhibits FtsZ ring formation. MinE, which functions to keep the MinCD protein complex from the mid-cell, is also localized to a ring (Raskin and de Boer, 1997). Careful analysis of GFP-tagged MinE indicates the ring is not stationary but oscillates from one side of mid-cell to the other. The oscillation seems to define a boundary beyond which MinCD is excluded. These results indicate there is a complex oscillating system that is important to properly place the FtsZ ring at mid-cell for cell division and keep FtsZ rings from forming at cell poles.

Following the placement and assembly of the FtsZ ring, at least ten other components of the cell division apparatus localize to the division site (Chen and Beckwith, 2001; Errington et al., 2003). Nine of the components assemble in a specific order that starts with ZipA and FtsA and is followed by FtsK, FtsQ, FtsL, FtsB, FtsW, FtsI, and FtsN. The tenth component, the recently discovered ZapA, is likely one of the

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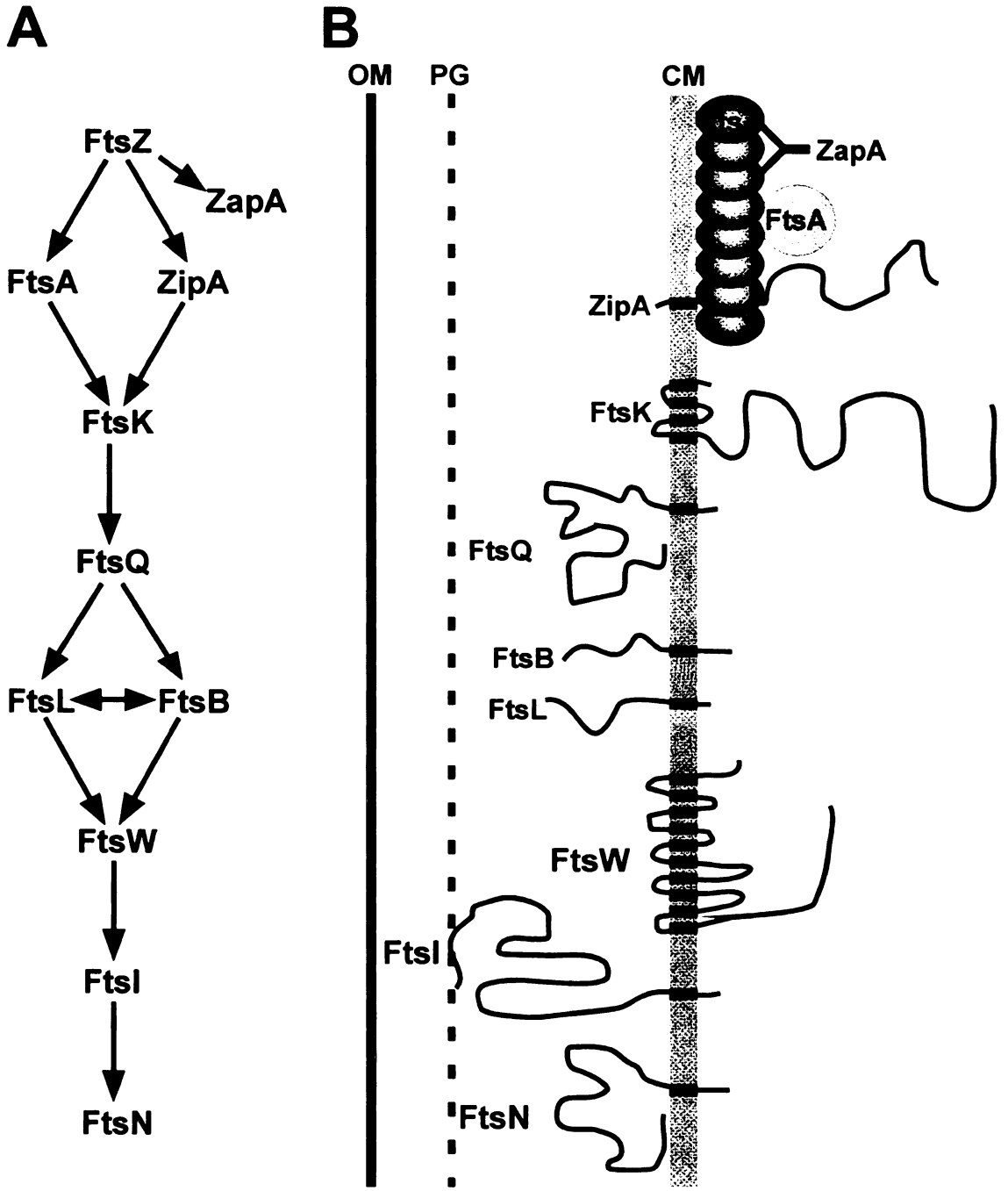
first components to assemble following FtsZ ring formation, although the exact timing is unclear. The order of assembly of the cell division components and a schematic representation and their predicted membrane topology are diagrammed in Figure 1. The functions of each component of this intricately orchestrated assembly will be discussed in the following sections.

ZapA

ZapA was recently identified in a screen for genes whose overexpression could antagonize a MinD overexpression block on cell division (Gueiros-Filho and Losick, 2002). ZapA is not essential for division since knocking out the gene resulted in no apparent defect in septum formation. However, when FtsZ levels are reduced, cell division requires ZapA. Fluorescence microscopy of a GFP-tagged ZapA protein showed it is localized to the division site and affinity chromatography indicated it interacts with FtsZ. Structural predictions suggest ZapA is a cytoplasmic protein with a coiled-coil domain, which indicates it may dimerize. Polymerization studies showed that ZapA promotes assembly of FtsZ into bundled filaments *in vitro*, which indicated ZapA might function in binding filaments together in the FtsZ division ring. One mechanism proposed for inhibition of cell division by MinD is that MinD inhibits the association of FtsZ filaments. ZapA, however, induces association of FtsZ filaments, which may be the reason overexpression of ZapA antagonizes the effects of MinD overexpression. Although ZapA was originally identified in *Bacillus subtilis*, orthologues are present in many bacterial species, including one in *E. coli* that also localizes to the division site (Gueiros-Filho and Losick, 2002).

Figure 1. The sequential assembly pathway of the cell division components at mid cell of *E. coli*.

A, The dependency pathway for protein localization to the division site. Arrows point from proteins that are required before the protein is localized to the division site. B, Schematic representation of the 11 known *E. coli* cell division proteins and their predicted topology in the cell membrane. Components that have been shown to interact with FtsZ are diagramed in contact with the FtsZ ring. OM, outer memberane; PG, peptidoglycan; CM, cell memberane.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FtsA

FtsA was identified in the initial screen for filamenting mutants (Hirota et al., 1968). Immunofluorescence microscopy showed that FtsA localizes to the division site only if FtsZ is located at the division site (Addinall and Lutkenhaus, 1996). FtsA does not have a membrane-spanning domain, but is associated with the cytoplasmic membrane (Pla et al., 1990). Mutations in the C-terminus of the FtsZ protein prevent localization of FtsA to the division site and indicate there is a direct interaction between the two proteins (Ma and Margolin, 1999). The crystal structure of FtsA from *Thermotoga maritima* has been determined and its structure resembles that of actin (van den Ent and Lowe, 2000). Although the function of FtsA is unknown, it is required for the recruitment of other downstream components of the division apparatus. Analysis of the crystal structure led Löwe and van den Ent (2001) to suggest a peptide located at the C-terminus of FtsA may interact with and be involved in the recruitment of the other cell division components.

ZipA

ZipA was identified in an affinity-blotting screen for proteins that bound FtsZ (Hale and de Boer, 1997). GFP-tagged ZipA localizes to the ring structure at the division site and is required for septum formation. Additional localization studies indicated FtsZ polymerization does not require ZipA, but without ZipA the other components of the division apparatus are not recruited to the septum (Hale and de Boer, 1999; Liu et al., 1999; Pichoff and Lutkenhaus, 2002). The localization of ZipA and FtsA to the FtsZ ring is independent, but recruitment of the other division components requires the presence of both. FtsZ polymerization studies indicated that ZipA stabilizes FtsZ filaments and even

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promotes bundling (RayChaudhuri, 1999). Furthermore, 143 residues at the C-terminus of ZipA are sufficient to induce bundling of FtsZ filaments (Hale et al., 2000). The ZipA protein has a flexible tether between a membrane-spanning domain and an FtsZ binding domain (Hale and de Boer, 1997; Ohashi et al., 2002), suggesting that ZipA anchors FtsZ to the cell membrane. Sequence comparisons indicate ZipA has microtubule binding domains (RayChaudhuri, 1999) and X-ray crystallography shows that ZipA interacts with a small fragment of FtsZ (Mosyak et al., 2000). Specifically, ZipA interacts with a 17 amino acid peptide that is located at the C-terminus of FtsZ. This C-terminal peptide is in the same region where FtsA binds to FtsZ. Although ZipA has a membrane-spanning domain but FtsA does not, a single amino acid change in FtsA bypasses the requirement for ZipA in cell division (Geissler et al., 2003). These results indicate both ZipA and FtsA have functional overlap although there are significant structural differences between them.

FtsK

By mapping a temperature-sensitive cell division mutant Begg et al. (1995) identified the gene *ftsK*. FtsK is localized to the division septum (Wang and Lutkenhaus, 1998; Yu et al., 1998) and its localization requires the prior localization of FtsZ, ZipA, and FtsA (Hale and de Boer, 2002; Pichoff and Lutkenhaus, 2002). FtsK has a domain that is predicted to span the membrane several times and a cytosolic domain that has homology to other nucleotide-binding domains (Begg et al., 1995). Several studies have indicated FtsK interacts with chromosomes and that this interaction is important for cell division (Liu et al., 1998; Yu et al., 1998; Steiner et al., 1999; Barre et al., 2000; Boyle et

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al., 2000). A recent report showed by analytical gel filtration and electron microscopy that FtsK proteins form multimers (Aussel et al., 2002). In addition, the FtsK multimers can translocate along DNA duplexes *in vitro* (Aussel et al., 2002) and indicate FtsK may be a motor protein that moves replicated chromosomes through the division septum. Another function of the FtsK-chromosome interaction may be to keep the division apparatus between the replicated chromosomes.

FtsQ

FtsQ was described by Begg et al. (1985) in a screen for cell division mutants. FtsQ localizes at the division septum in an FtsK-dependent manner (Chen and Beckwith, 2001; Hale and de Boer, 2002; Geissler et al., 2003) and immunofluorescence microscopy and GFP-tagging experiments confirmed FtsK is localized to a ring at the cell division site (Buddelmeijer et al., 1998; Ghigo et al., 1999). Although essential for division, the specific function of FtsQ is unclear (Chen et al., 1999). Various observations of the *B. subtilis* FtsQ homologue, DivIB, led Errington et al. (2003) to postulate DivIB recruites FtsL to the division site. In support of this hypothesis, when FtsQ proteins with site-specific mutations were expressed in cells, FtsL failed to localize to the division septum (Chen et al., 2002).

FtsL

FtsL was identified in a screen for envelope proteins that are required for cell division (Guzman et al., 1992). The protein has been localized to the septum by GFP-tagging experiments in an FtsQ-, but not FtsI-, dependent manner (Ghigo et al., 1999).

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The FtsL protein has a domain that is located in the cytosol, a domain located in the periplasm, and a membrane-spanning domain that separates the other two domains (Guzman et al., 1992). The periplasmic domain has a coiled-coil motif that indicates the protein may multimerize. Mutagenesis of the coiled-coil motif makes FtsL dimers less stable in SDS treatment (Ghigo and Beckwith, 2000). Experiments where the domains of FtsL were swapped with similar domains from other proteins (for example, swapping the coiled-coil domain of FtsL with a coiled-coil domain from another protein) indicated all three of the FtsL protein domains are required for function, even though the cytosolic and transmembrane domains are not required for localization or dimerization (Ghigo and Beckwith, 2000). However, the role of FtsL in septum formation is still unclear.

FtsB (YgbQ)

YgbQ was identified in two different laboratories at about the same time, one working in *Vibrio cholerae* and the other in *E. coli* (Buddelmeijer et al., 2002). The Beckwith laboratory predicted FtsL would not dimerize *in vivo* very well and did computer-based searches for a structurally similar protein as a possible dimerization partner. YgbQ was the most promising protein identified from the search (Buddelmeijer et al., 2002). The protein was renamed FtsB in a recent review (Buddelmeijer and Beckwith, 2002). GFP-tagging of FtsB showed the protein was localized to the division septum in an FtsQ-dependent manner, whereas mutations in downstream components, such as FtsW, did not disrupt FtsB localization (Buddelmeijer et al., 2002). When cells were depleted of FtsB, FtsL becomes unstable, which indicates that FtsB stabilizes FtsL and suggests that both assemble at the division site at about the same time.

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FtsW

FtsW was identified in a mutagenesis screen for cell division mutants and is localized to the *mra* region of the *E. coli* chromosome (Ishino et al., 1989). FtsW is required for cell division and is localized to the division septum (Boyle et al., 1997; Wang et al., 1998). Although Khattar et al. (1997) observed the presence of FtsZ rings when one mutant FtsW protein was expressed, the FtsZ ring was not always observed with all the different FtsW mutants (Boyle et al., 1997; Khattar et al., 1997; Wang et al., 1998). Therefore, the exact point at which FtsW localized to the septum was unclear, but the results suggested that it might be quite early in the assembly of the division apparatus. Khattar et al. (1997) also identified two different polypeptide products of the *ftsW* gene. It was suggested that one of these products might be involved in the early steps of assembling the division apparatus and that it stabilizes the FtsZ ring. However, Mercer and Wiess (2002) disputed this finding because they found that FtsW mutantions did not destabilize the FtsZ ring, at least not significantly. Furthermore, GFP-tagging indicated that FtsW is recruited to the septum after FtsL and FtsB, but before FtsI (Buddelmeijer and Beckwith, 2002; Mercer and Weiss, 2002). Structural analysis of the *Streptococcus pneumoniae* FtsW protein indicates it has 10 membrane-spanning segments and a large extracytoplasmic domain (Gerard et al., 2002). FtsW, along with RodA and SpoVE, are founding members of the SEDS (shape, elongation, division, and sporulation) protein family (Henriques et al., 1998) and each SEDS protein seems to work in conjunction with a transpeptidase (Matsuhashi et al., 1990). Since FtsW and FtsI, a transpeptidase, are

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expressed from the same operon (Ishino et al., 1989; Boyle et al., 1997) it has been suggested that FtsW may function in conjunction with FtsI (Mercer and Weiss, 2002).

FtsI

FtsI was purified based on its ability to bind Cephalexin, a penicillin-type antibiotic (Tamura et al., 1980). Because it bound penicillin and was the third protein to be identified with this property, FtsI was originally called PBP-3. Proteolyic digestion of inverted membranes suggested FtsI is anchored to the cell membrane at the amino terminus with the bulk of the protein located in the periplasmic space (Bowler and Spratt, 1989). Wang et al. (1998) showed that FtsI localizes to the division septum and that localization is dependent on the previous localization of FtsL to the division site (Weiss et al., 1999). In addition to septum localization, FtsI was also observed to localize at the cell poles by immunofluorescence microscopy, although the significance of this was unknown (Weiss et al., 1997). The PBP proteins, to which FtsI belongs, are peptidoglycan transpeptidases and synthesize murein (Spratt, 1977; Goffin and Ghuysen, 1998). Specifically, synthesis of septal peptidoglycan by FtsI is required during cell septation but not during cell elongation (Botta and Park, 1981).

FtsN

FtsN was identified in a screen for suppressors of an *ftsA* temperature-sensitive mutation (Dai et al., 1993). A late recruit to the division site, FtsN requires FtsK, FtsQ, FtsL, and FtsI for localization to the septum (Chen and Beckwith, 2001). The protein has a noncleavable hydrophobic sequence that binds it to the membrane (Dai et al., 1993).

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The function of FtsN is unclear, but it does have sequence similarity to cell wall amidases. Because of this similarity, Errington et al. (2003) suggested that FtsN allows septum constriction by hydrolyzing certain bonds in the cell wall.

Stiochiometric Ratios are Important Among Bacterial Cell Division Components

One important factor in the functionality of the cell division machinery is the maintanance of stoichiometric ratios among the different protein components. Inhibition of cell division in the FtsZ mutant (Lutkenhaus et al., 1980) is due to decreased amounts of functional protein, which causes an imbalance in the ratio of the division components. An imbalance can also occur when protein expression is increased. When FtsZ protein levels were slightly increased, the number of initiated division events increased (Ward Jr and Lutkenhaus, 1985). This increase was manifested by constriction and separation of cells at both the cell center and at abnormal positions near the cell poles that resulted in non-viable minicells. However, dramatically increasing FtsZ protein levels inhibited all cell division and resulted in a filamentous phenotype similar to that observed when FtsZ levels were reduced. The inhibition of cell division observed with high FtsZ expression could be rescued by simultaneous overexpression of FtsA protein (Dai and Lutkenhaus, 1992). Overexpression of ZipA also inhibits cell division, but division can be rescued by concomitant overexpression of FtsZ (Hale and de Boer, 1997). These results suggest that the components of the bacterial cell division machinery are in a strict stoichiometric balance and that disruption of that balance is detrimental to cell division.

Developmental Patterns of Chloroplasts

The chloroplast complement of a mature mesophyll cell results from both proplastid (undifferentiated plastids) and chloroplast division. Proplastids are located in the root and shoot meristems. Proplastids that have constricted centers have been observed in shoots of spinach as well as in the roots of spinach, pea, barley, tomato, maize, lettuce, pine, watermelon, and wheat (Chaly and Possingham, 1981). The constrictions are likely an indication that the proplastids they are undergoing division. Further support for proplastid division comes from microscopic observations of Arabidopsis *arc6* mutant plants (Robertson et al., 1995). The chloroplasts in the *arc*6 mutants are very large, and only one or two are present in each mesophyll cell. Electron micrographs of the shoot apex from the *arc*6 mutant plants reveal very few and large proplastids in each cell, whereas proplastids in the shoot apex of wild-type Arabidopsis plants are smaller and more numerous. This indicates proplastid division is severely reduced in the *arc6* mutant plants.

During leaf development, mesophyll cells differentiate and expand. While the cells expand, the chloroplasts (differentiated proplastids) divide. Possingham and Lawrence (1983) summarized several studies that reported the number of chloroplasts in cells at different developmental stages. In spinach, beet, pea, bean, and wheat as mesophyll cells expand there is an increase in the number of chloroplasts in each cell (Possingham and Smith, 1972; Boffey et al., 1979; Lamppa et al., 1980; Scott and Possingham, 1980; Whatley, 1980; Tymms et al., 1983). In addition, measurements of cell size and chloroplast number in Arabidopsis and tobacco mesophyll cells indicate that the bigger the cell, the more chloroplasts it contains (Boasson et al., 1972; Kameya, 1972;

Pyke and Leech, 1992; Pyke et al., 1994; Robertson et al., 1996). When cultured spinach or tobacco leaf disks are treated with cytokinin the size of the mesophyll cells increases, as does the number of chloroplasts in each cell (Boasson et al., 1972; Possingham and Smith, 1972; Possingham et al., 1988). This relationship between cell size and chloroplast numbers has also been observed in *Polytrichum* moss cells (Paolillo and Kass, 1977). This increase in chloroplast numbers indicates the chloroplasts are dividing while the mesophyll cells expand and suggest there is a close relationship between cell expansion and chloroplast division.

In the monocot wheat, chloroplast division occurs in a relatively small region of the leaves where cell expansion is greatest (Pyke, 1997). Microscopic examination of the leaves of Arabidopsis, a dicot, indicates chloroplast division occurs over a longer period of cell development and expansion than in wheat (Pyke and Leech, 1992; Pyke, 1997). Therefore, the region where most chloroplast division occurs is less pronounced in Arabidopsis leaves, and other dicot leaves, than it is in wheat leaves. However, a chloroplast division gradient is still observed in dicots from leaf base to tip, with most of the division occurring in the basal portion of the Arabidopsis leaf (Possingham and Smith, 1972; Possingham, 1973; Leech et al., 1981; Pyke et al., 1991). Besides young leaves, cells in the rapidly expanding cotyledons and the basal stock of developing petals also have numerous chloroplasts undergoing division (Pyke, 1997; Pyke and Page, 1998).

Dividing chloroplasts with several different shapes have been observed in electron micrographs (Leech et al., 1981; Leech, 1986; Leech and Pyke, 1988). From these observations, predictions have been made regarding the morphological changes that occur to the chloroplasts during division. The first proposed morphological change is a

constriction at the center of the plastid, followed by twisting around this constriction that ends with separation of the two daughter chloroplasts (Chaly et al., 1980; Leech et al., 1981). The constriction suggests chloroplasts divide by binary fission, similar to bacteria. The significance of the chloroplast twisting is unknown, but may be a result of external forces encountered by the chloroplast near the end of division. Ultrastructural studies in ferns identified the presence of electron-dense rings at the constriction of dividing chloroplasts (Duckett and Ligrone, 1993). These rings, called plastid dividing (PD) rings, have also been observed in red algae, green algae, and angiosperms (Hashimoto, 1986; Kuroiwa, 1989; Oross and Possingham, 1989; Ogawa et al., 1994), which indicates they may be ubiquitous in all plants.

Although most of the observed PD rings in the various plants appear to be singlets, a double-ring structure was observed in *Avena sativa* (barley) (Hashimoto, 1986) and in the red alga *Cyanidioschyzon merolae* (Miyagishima et al., 1998), with one PD ring located on the cytosolic surface and a second on the stromal surface of the chloroplast membrane. Careful studies of the unicellular red alga *Cyanidium caldarium* revealed not only the double PD ring structure, but the presence of a third PD ring (the middle PD ring) located in the intermembrane space (Miyagishima et al., 1998).

Although the PD rings have been identified in many plants, following their presence throughout chloroplast division has been difficult. Kuroiwa et al. (1998) were able to observe PD ring formation, contraction, and dispersion throughout the entire division process using synchronized cultures of the red alga *C. caldarium*. Formation of the stromal PD ring occurs first, followed by formation of the middle and outer PD rings (Miyagishima et al., 1998, 1998; Miyagishima et al., 1999; Miyagishima et al., 2001;

Miyagishima et al., 2001). Disassembly of the three rings is just the reverse of their formation. Throughout chloroplast division, the PD rings remain associated with the leading edge of the constricting plastid until dispersal upon chloroplast separation (Kuroiwa et al., 1998; Kuroiwa et al., 2002). It should be noted that the presence of a middle PD ring has been questioned, due to difficulty in resolving the inner and outer leaflets of the membrane (Hashimoto, 2003). Even so, the presence of an inner and outer PD ring suggests at least two complexes are involved in chloroplast division.

It had been hypothesized that the stromal PD ring could be at least partly composed of FtsZ protein, because both the PD and FtsZ rings form at the stromal surface of the inner chloroplast envelope membrane (Miyagishima et al., 1998; McAndrew et al., 2001). However, careful examination of the FtsZ and PD rings in *C. merolae* by Miyagishima et al. (2001) determined they are separate and distinct structures. Furthermore, analysis indicates the FtsZ ring forms first and is followed by the formation of the stromal and cytoplasmic PD rings. In the late stages of chloroplast division, just prior to separation, the FtsZ ring disappears before the two PD rings (Miyagishima et al., 2001). Electron microscopic observations in *Pelargonium zonale* (geranium) also indicate the FtsZ ring forms before the stromal or cytoplasmic PD rings and disappear in the late stages of chloroplast division in higher plants (Kuroiwa et al., 2002). Measurements of the diameter of the rings indicate the thickness of the outer PD ring increases while the FtsZ and inner PD ring diameters remain constant throughout division (Kuroiwa et al., 2002). From these observations the authors suggested that the FtsZ and inner PD rings decompose (lose components) throughout division while the

outer PD ring does not. They also suggest that the thickening of the outer PD ring might indicate it generates the force for chloroplast constriction (Kuroiwa et al., 2002).

Although the composition of the cytoplasmic PD ring is unknown, isolation of intact outer PD rings from *C. molerae* has allowed for some protein characterization. Immunoblot analysis failed to detect FtsZ protein in samples enriched in the isolated outer PD rings, providing evidence that the outer PD ring is not composed of FtsZ protein (Miyagishima et al., 2001; Kuroiwa et al., 2002). Furthermore, immuno-electron microscopy with FtsZ antibodies determined the FtsZ ring is located in the chloroplast stroma and not the cytoplasm. Negative staining of isolated outer rings revealed the structure is a bundle of 5-nm filaments and SDS-PAGE analysis indicated a 56-kDa protein is a major component (Miyagishima et al., 2001).

The *Arc* Mutants and Chloroplast Division

The first evidence for a genetic control of chloroplast division was the isolation of a set of *arc* (*a*ccumulation and *r*eplication of *c*hloroplasts) mutants in Arabidopsis. These mutants were isolated through a screen of T-DNA- and EMS-mutagenized plants (Pyke and Leech, 1992; Pyke et al., 1994; Robertson et al., 1995; Robertson et al., 1996; Marrison et al., 1999) and show a range of defects in chloroplast division. Plants with the *arc1* mutation have an increase in the number of chloroplasts, while the size of those chloroplasts are reduced (Pyke and Leech, 1992). In contrast, the *arc2*, *arc3*, and *arc6* mutant plants have increased chloroplast size but decreased chloroplast number (Pyke and Leech, 1992; Pyke et al., 1994). The most severe chloroplast division mutant is the *arc6* mutant, which has one or two greatly enlarged chloroplasts in each mesophyll cell.

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Another interesting mutant is *arc*5, which also has reduced numbers of enlarged chloroplasts that are constricted in the center and seem to be arrested in the final stage of chloroplast division (Robertson et al., 1996).

Several reports describe a strong correlation in the *arc* mutants between the size of the mesophyll cell and the number of the chloroplasts they contain (Pyke and Leech, 1992; Pyke et al., 1994; Robertson et al., 1996; Marrison et al., 1999). As the number of chloroplasts in a cell decreases, the chloroplast size increases. This keeps the total chloroplast area within the cells constant. Compared to the 80 chloroplasts in a mature mesophyll cell of wild-type Arabidopsis plants, for instance, plants with the *arc*6 mutation have one greatly enlarged chloroplast, while plants with the *arc1* mutation have increased numbers of smaller chloroplasts. These results suggest the *arc* gene products are not involved in maintaining the chloroplast volume during cell expansion (Pyke and Leech, 1992; Pyke et al., 1994; Robertson et al., 1996; Marrison et al., 1999).

Studies with the *arc* mutants have provided insights into the process of plastid division. Double mutants of *arc1* with *arc3*, *arc5*, *arc6*, or *arc11* have phenotypes that are intermediate between the two parental mutant plants, suggesting that the *ARC1* gene acts on a pathway independent of the other genes (Marrison et al., 1999). The *ARC6* gene seems to function upstream of *ARC3, ARC5,* and *ARC11* since the double mutants all have one or two enlarged chloroplasts in the mesophyll cells, similar to the parental *arc6* phenotype. The ARC3 protein is predicted to have a role in chloroplast division, but not proplastid division, since *arc3* mutant plants have about 16 chloroplasts, which is the estimated number of proplastids that differentiate into chloroplasts. Most of the *arc*5 double mutant crosses resulted in chloroplasts that have a constricted phenotype and

provided further support that the *ARC5* gene affects the final stages of chloroplast division.

The gene encoding *ARC5* was identified and reported by Gao et al. (2003). Sequence analysis indicated the gene codes for a dynamin-like protein of eukaryotic origin, since no prokaryotic homologues were identified. These results indicate the chloroplast divison apparatus is composed of proteins of both prokaryotic and eukaryotic desent. Chloroplast import assays indicate ARC5 is located on the cytoplasmic surface of the outer chloroplast envelope, and studies with GFP-tagged ARC5 localized it to a ring at the division site (Gao et al., 2003). Although the exact function of ARC5 is unclear, based on studies with other dynamin proteins it may function in constriction of the chloroplast, possibly as a force generating protein (Sweitzer and Hinshaw, 1998), or it may be a molecular switch (Sever et al., 2000; Gao et al., 2003; Miyagishima et al., 2003).

Another recently identified protein that is required for chloroplast division was ARTEMIS (Fulgosi et al., 2002). In contrast to ARC5, ARTEMIS was of prokaryotic ancestry. When the cyanobacterial homologue is mutated, bacterial division is inhibited. Similarly, in Arabidopsis *ARTEMIS* mutants, chloroplast division is inhibited. Immunoelectron analysis, immunoblot analysis of isolated envelopes and thylakoid membranes, and envelope extraction experiments indicated ARTEMIS is an integral inner envelope membrane protein. ARTEMIS has some sequence similarity to translocases, which led the authors to suggest ARTEMIS may not have a direct role in chloroplast division but may influence the translocation of other chloroplast division proteins (Fulgosi et al., 2002).

The gene encoding ARC6 has also been recently identified (Vitha et al., in press). ARC6 is located in the inner envelope membrane and has a large domain that faces the chloroplast stroma. In addition, an ARC6-GFP fusion protein localizes to the division ring. Although ARC6 homologues are present in cyanobacteria, they have not been identified in other bacteria (Vitha et al., in press). This indicates ARC6 and its orthologues may be unique to photosynthetic organisms. Chloroplast division is inhibited in *arc6* mutants and in plants overexpressing an *ARC6* transgene, resulting in plants with one or two chloroplasts per mesophyll cell. Immunofluorescent microscopy of chloroplasts from the *arc6* plants shows that FtsZ1 and FtsZ2 proteins form short filaments, while plants overexpressing wild-type ARC6 have long FtsZ1 and FtsZ2 protein filaments. Also, sequence comparisons identified a J-domain in ARC6 that is typical of DnaJ co-chaperones. The microscopy results along with sequence similarity to co-chaperones indicates ARC6 might have a role, either directly or indirectly, in stabilizing FtsZ rings in chloroplasts (Vitha et al., in press).

FtsZ Function in Chloroplast Division

Identification of an FtsZ homologue in plants indicated the proteins involved in chloroplast division have a prokaryotic origin (Osteryoung and Vierling, 1995). The first plant FtsZ homologue was identified in Arabidopsis through a homology search of the Expressed Sequence Tag database dbEST (Newman et al., 1994) using the *E. coli* FtsZ protein as a query sequence. Of the known prokaryotic FtsZ proteins, sequence comparisons indicated the plant homologue is most closely related to cyanobacterial FtsZ proteins, which is consistent with chloroplasts being ancestors of a cyanobacterial

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endosymbiont (McFadden, 2001). The *FtsZ* gene is located in the eukaryotic nucleus, which suggests it was transferred from the cyanobacterial endosymbiont during chloroplast evolution (Osteryoung and Vierling, 1995). Chloroplast import assays followed by protease digestion demonstrated that the plant FtsZ protein is localized to the chloroplast stroma, where it is processed by removal of the transit peptide (Osteryoung and Vierling, 1995).

Proof that plant FtsZ proteins function in chloroplast division came from studies in moss and Arabidopsis. Strepp et al. (1998) used homologous recombination to knock out an *FtsZ* gene in the moss *Physcomitrella patens*, which resulted in cells with large, undivided chloroplasts. The large chloroplasts have diameters similar to wild-type chloroplasts, but their lengths were much longer. This lengthened chloroplast phenotype is similar to the filamentous phenotype observed in bacterial *FtsZ* mutants (Strepp et al., 1998). In Arabidopsis, antisense repression of either of two nuclear-encoded *FtsZ* genes reduces chloroplast numbers from 100 to as few as one greatly enlarged chloroplast in each cell (see Chapter 2; Osteryoung et al., 1998). Sequence comparisons of the two Arabidopsis FtsZ proteins with other plant FtsZ homologues indicate they belong to two different families, called FtsZ1 and FtsZ2. These results established that chloroplast division requires members of two FtsZ families.

To further investigate the functional properties of the FtsZ proteins, transgenic plants were constructed with transgenes that overexpress either *AtFtsZ1-1* or *AtFtsZ2-1* (see Chapter 3; Stokes et al., 2000). Immunoblot analyses and microscopic examination of the chloroplasts indicate increased AtFtsZ1-1 levels inhibits chloroplast division, and that AtFtsZ1-1 levels are directly correlated with the severity of the division defect.

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Slight increases in AtFtsZ2-1 levels do not inhibit chloroplast division (Stokes et al., 2000). However, analysis of the genomic *AtFtsZ2-1* sequence, and comparisons with other plant *FtsZ* sequences that had become available, indicated the *AtFtsZ2-1* cDNA used in the overexpression study was truncated, missing the amino terminus containing the chloroplast import signal. Therefore, I isolated a full-length cDNA clone for *AtFtsZ2-1* as well as for a second Arabidopsis *FtsZ2* homologue, *AtFtsZ2-2* (McAndrew et al., 2001). Import assays using the full-length Arabidopsis FtsZ proteins indicate that all three are localized to the chloroplast stroma (McAndrew et al., 2001). Fujiwara and Yoshida (2001) independently isolated cDNA clones for *AtFtsZ2-1* and *AtFtsZ2-2* and determined that both proteins, tagged with GFP, are targeted to tobacco chloroplasts. In addition to localization studies, McAndrew et al. (2001) observed that overexpressing high levels of full-length AtFtsZ2-1 protein inhibits chloroplast division, whereas slight increases do not inhibit division. These results indicate that both AtFtsZ1-1 and AtFtsZ2-1 proteins have similar properties when their expression is increased. Overexpression of FtsZ1 or FtsZ2 proteins probably inhibits chloroplast division by disrupting a stoichiometric balance that is required among the division proteins.

FtsZ Rings in Chloroplasts

Using an FtsZ1 protein from *Pisum sativum* (pea), Gaikwad et al. (2000) showed that the plant FtsZ protein can polymerize, *in vitro,* to form multimers. The pea FtsZ protein can also complement a mutant *E. coli* FtsZ protein (Gaikwad et al., 2000). Mori et al. (Mori et al., 2001) used fluorescence microcopy and immunogold localization to show that FtsZ1 protein assembles into a ring structure in *Lilium longiflorum*

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chloroplasts. In Arabidopsis plants, Vitha et al. (2001) observed both FtsZ1 and FtsZ2 rings that colocalize at the chloroplast midpoint. Colocalization is also observed in plants where chloroplast division is inhibited due to overexpression of *AtFtsZ1-1*, overexpression of *AtFtsZ2-1*, or mutations in *arc6*. In their studies on the PD rings in geranium, Kuroiwa et al. (2002) not only determined that the FtsZ ring is distinct from the inner and outer PD rings but that antibodies against FtsZ1 and FtsZ2 proteins react to the FtsZ ring, providing further evidence that the ring was composed of both proteins. These studies indicate that plant FtsZ proteins can polymerize, like the bacterial protein, but that chloroplast division involves a complex ring structure that is composed of at least two different FtsZ proteins.

Other Potential FtsZ Functions

Besides rings and filaments, FtsZ protein has also been observed in other complex formations in plant cells. One structure is a cytoskeleton-like network of FtsZ filaments in chloroplasts of *P. patens* overexpressing an FtsZ2 protein (Kiessling et al., 2000). Other researchers (Vitha et al., 2001) have also observed these networks of FtsZ filaments in Arabidopsis. However, the network is likely an artifact of FtsZ overexpression since it is only observed in plants overexpressing FtsZ protein (Kiessling et al., 2000; Fujiwara and Yoshida, 2001; Vitha et al., 2001).

During analysis of FtsZ-GFP fusion experiments, Vitha et al. (2001) noticed that AtFtsZ1-1 could be detected in "stromules", which are thin connections between two chloroplasts (Köhler et al., 1997). Plant FtsZ proteins, therefore, could have a structural role in the formation of these thin chloroplast-to-chloroplast connections. However,

AtFtsZ1-1 was only detected in the stromules of plants overexpressing the AtFtsZ1-1 protein and not in wild-type plants. Therefore, the localization of FtsZ to the stromules may also be an artifact of high AtFtsZ1-1 levels.

Another piece of evidence that FtsZ may function outside of plastid division comes from the isolation of a *L. longiflorum* cDNA from generative cells (Mori and Tanaka, 2000). Generative cells have no plastids and isolation of *FtsZ* cDNA from these cells indicated to the authors that the *FtsZ* genes might have functions that are not associated with plastid division. Since the experiments only detected *FtsZ* cDNA and not protein, the transcript may be produced but not translated. Further experiments will be required to determine if FtsZ protein is present in the generative cells that lack plastids.

FtsZ1 and FtsZ2 rings have also been observed in chloroplasts that do not seem to be undergoing division in mature leaf mesophyll cells (Vitha et al., 2001). If the chloroplasts are not dividing, do the FtsZ proteins have other, as yet unidentified function? In addition, the amount of FtsZ1 transcript increased in abundance during petal development and was higher in yellow and orange varieties of *Tegetes erecta* L. (marigold) than in white varieties (Moehs et al., 2000). Although the presence of chloroplasts in the petals, especially at the base, cannot be ruled out, the increase in FtsZ transcript as petals develop and in the yellow and orange marigold varieties suggests FtsZ proteins may have a function in chromoplasts.

Expression of *FtsZ* in Cyanobacteria

FtsZ expression in *Prochlorococcus*, a cyanobacterium, has been shown to be coordinated with cell division (Holtzendorff et al., 2001; Holtzendorff et al., 2002). Cell

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division of *Prochlorococcus sp.* strain PCC 9511 cultures becomes synchronized when they are grown in a turbidostat with 12 hour day and night cycles. When the cultures are synchronized under these light conditions DNA replication occurs in the late afternoon and is followed by cell division at night (Holtzendorff et al., 2001). Immunoblot analysis of samples removed from the turbidostat over a three-day period indicates *FtsZ* expression is at a minimum during the day and maximum at night. Therefore, FtsZ expression seems to be correlated with the cell cycle. These results are supported by studies of naturally occurring synchronous *Prochlorococcus* populations in the Red Sea (Holtzendorff et al., 2002). In these natural populations, the amount of expressed *FtsZ* mRNA was measured by real-time reverse transcriptase-polymerase chain reaction. As in the previous experiment, *FtsZ* expression is maximal at night when the cells are dividing, and at a minimum during the day. Differential expression of FtsZ has also been observed in *Anabaena*, where vegetative cells express FtsZ, but terminally differentiated heterocysts do not (Kuhn et al., 2000).

Expression of *FtsZ* in Plants

A few studies have investigated the regulation of *FtsZ* gene expression in plants. El-Shami et al. (2002) used a synchronized culture of *Nicotiana tabacum* cells to study FtsZ expression during the cell cycle. They observed oscillations of *FtsZ* expression that coincide with the cell cycle. In another set of experiments, Gaikwad et al. (2000) observed that the highest levels of a *P. sativum FtsZ* gene is in young leaves while old leaves have significantly less *FtsZ* expression. These expression results, along with the

cyanobacterial studies, suggest *FtsZ* expression in plants correlates with the cell cycle, but there may also be other factors that affect expression.

Light and the growth hormone cytokinin are two other factors that may have a role in regulating FtsZ expression. When cultured spinach leaf disks were incubated with cytokinin not only was cell expansion induced, but chloroplast division was also induced (Possingham et al., 1988). Measurements of transcript amount indicate *FtsZ* expression is increased in excised cytokinin treated cucumber cotyledons, but not auxin treated cotyledons (Ullanat and Jayabaskaran, 2002). Also, Ullanat and Jayabaskaran (2002) observed increased *FtsZ* expression in light-treated cucumber cotyledons. Similarly, Gaikwad et al. (2000) noted that *FtsZ* expression increases when pea seedlings are exposed to light. Although informative, the plant studies only investigated the expression of a subset of the *FtsZ* genes encoded in these plants. In fact, only one *FtsZ1* gene was analyzed in the *P. sativum* study (Gaikwad et al., 2000) even though FtsZ2 protein has been detected in peas (McAndrew et al., 2001). The *N. tabacum* study only investigated one of at least four *FtsZ1* and one of at least two *FtsZ2* genes (El-Shami et al., 2002). And the expression of only one of at least three *FtsZ2* genes was analyzed in the cucumber study (Ullanat and Jayabaskaran, 2002). Analysis of the expression of all FtsZ genes in a plant is required before it can be concluded that all *FtsZ* genes are regulated in the same manner. The expression pattern of the three Arabidopsis *FtsZ* genes is the subject of chapter 4.

Mitochondrial Division and FtsZ

Mitochondria are thought to have originated from an α-proteobacterial endosymbiont, rather than a cyanobacterium as has been proposed for chloroplasts (Gray et al., 2001). Two FtsZ proteins have been described in the golden-brown alga *Mallomonas splendens* and the red alga *Cyanidioschyzon merolae*: one localizing to the chloroplast and the other localize to the mitochondria (Beech et al., 2000; Takahara et al., 2000). Sequence comparisons indicate that the mitrochondrial FtsZ proteins are more closely related to α-proteobacterial FtsZ proteins than to the cyanobacterial proteins, which is in accordance with the presumed mitochondrial endosymbiotic origin (Gray et al., 2001). Although FtsZ proteins that localized to the mitochondria have been identified in these algae and protists, they are the exception rather than the rule among eukaryotes (Beech and Gilson, 2000; Beech et al., 2000; Gilson and Beech, 2001). In fact, few mitochondria actually use FtsZ for organellar division. For example, the yeast *Saccharomyces cerevisiae*, the nematode *Caenorhabditis elegans*, and plants like *Arabidopsis thaliana* do not encode an FtsZ protein in their nuclear or mitochondrial genomes (Goffeau et al., 1996; Osteryoung, 2000). These results indicate that FtsZ did function in mitochondrial division anciently, but was lost during the evolution of the organelle.

Instead of an FtsZ-based apparatus, mitochondrial division in plants, animals, and yeast involves systems where dynamins perform critical roles. In yeast, Dnm1 is a dynamin-like protein that is required for mitochondrial division (Bleazard et al., 1999; Sesaki and Jensen, 1999). In mutant *dnm1* cells, mitochondria aggregate into networks or highly branched structures. Dnm1 is localized to the cytoplasmic surface of the

mitochondria, either at the site of constriction in dividing mitochondria or at the ends, if the mitochondria have recently divided. These results indicate Dnm1 functions in mitochondrial division. In addition to Dnm1, several other proteins required for mitochondrial division have now been identified, including Fzo1, Mdv1p, and Fis1p (Bleazard et al., 1999; Sesaki and Jensen, 1999; Fekkes et al., 2000; Mozdy et al., 2000; Tieu and Nunnari, 2000; Cerveny et al., 2001; Tieu et al., 2002). Besides yeast, dynamin-like proteins involved in mitochonrial division have been identified in the protozoan *Dictyostelium discoideum* (Wienke et al., 1999), *Caenorhabditis elegans* (Labrousse et al., 1999), humans (Smirnova et al., 1998; Pitts et al., 1999), and Arabidopsis (Arimura and Tsutsumi, 2002). These results indicate that in fungi, animals, and plants dynamin-like proteins, and not FtsZ, are involved in mitochondrial division.

FtsZ Studies Reported Hereafter

The primary aim of the work described in this thesis is to better understand the function, expression, and phylogenetic relationships of the plant FtsZ proteins. Work by Osteryoung and Vierling (1995) reported an FtsZ homologue in Arabidopsis is imported into the chloroplast. From this result it was hypothesized that the Arabidopsis FtsZ protein is involved in chloroplast division. Chapter 2 describes the isolation of a second Arabidopsis FtsZ homologue. Sequence comparisons indicate that the two Arabidopsis FtsZ proteins are grouped into different families, FtsZ1 and FtsZ2. To investigate whether the two *FtsZ* genes are required for chloroplast division, plants expressing antisense constructs of each gene were analyzed. Microscopic examination of the mesophyll cells from these transgenic plants revealed that chloroplast numbers were

greatly reduced and enlarged. The results from this chapter indicate that members of both the FtsZ1 and FtsZ2 family are required for chloroplast division.

Further investigation into the functions and relationships of the FtsZ1 and FtsZ2 proteins are described in chapter 3. In bacteria, increasing FtsZ protein levels inhibited cell division (Bi and Lutkenhaus, 1990). We postulated that increased FtsZ1 or FtsZ2 protein levels in Arabidopsis plants would inhibit chloroplast division. To test this hypothesis, transgenic plants were prepared with constructs that overexpress the Arabidopsis *FtsZ* genes. Microscopic examinations in combination with immunoblot analysis indicated greatly increased AtFtsZ1-1 levels inhibit chloroplast division. In plants with the *AtFtsZ2-1* overexpressing transgene, only slight increases of AtFtsZ2-1 expression were observed and chloroplast division did not seem to be affected in these plants. Inhibition of chloroplast division was observed in some AtFtsZ2-1 transgenic plants, but immunoblot analysis indicated expression of the *AtFtsZ2-1* gene was reduced, not increased.

Transgenic plants with constructs designed to overexpress the *AtFtsZ2-2* gene have now been prepared and are described in the final section of chapter 3. However, the transgenic plants did not overexpress *AtFtsZ2-2*. Most transgenic plants had a wild-type-like chloroplast phenotype and immunoblot analysis indicated FtsZ protein levels were similar to those in wild-type plants. A few transgenic plants did have severely reduced chloroplast division, but instead of overexpressing the protein, the level of both AtFtsZ2-1 and AtFtsZ2-2 protein were reduced. This *AtFtsZ2-2* overexpression data were not available at the time the *AtFtsZ1-1* and *AtFtsZ2-1* overexpression studies were published, but are now included at the end of chapter three.

The work in chapter 4 investigates the expression of the three Arabidopsis *FtsZ* genes. Because FtsZ1 and FtsZ2 proteins are both required for chloroplast division, we postulated that the three Arabidopsis *FtsZ* genes are expressed in tissues where chloroplasts are dividing. To investigate expression of the *FtsZ* genes in Arabidopsis, transgenic plants with the *GUS* reporter gene fused to the promoters for the Arabidopsis *FtsZ* genes were prepared. Histochemical staining of these plants indicated that both *FtsZ2* genes are coordinately expressed in many tissues, especially in tissues where chloroplast division is common. Very little staining was observed in the plants with the *AtFtsZ1-1 promoter-GUS* fusion construct and evidence is discussed that there are sequences after the start codon that are required for *AtFtsZ1-1* expression. However, measurements of RNA indicated all three Arabidopsis *FtsZ* genes are coordinately expressed. Measurements of transcript amounts also indicate the Arabidopsis *FtsZ* genes are expressed at a constant stoichiometric ratio throughout leaf development, and this ratio may be important for chloroplast division.

Most bacteria encode a single *FtsZ* gene, while plants encode multiple homologues that belong to two families. In an effort to better define the differences between the FtsZ1 and FtsZ2 family members, phylogenetic and sequence comparisons were performed and the results are reported in chapter 5. Phylogenetic analysis of FtsZ protein or cDNA sequences supports the grouping of the plant proteins into the FtsZ1 and FtsZ2 families and indicates the two families diverged before the chlorophycean and charophycean green algae lineages split. Genetic structure comparison indicated that the intron positions are conserved within, but not between, the FtsZ1 and FtsZ2 family members. These results further support an early divergence of the two FtsZ families.

Finally, we determined the amino acid residues that are conserved among the members of the FtsZ1 and FtsZ2 families. The residues that are conserved in each family, but that differ between them, may define their functional differences and will guide future work to determine why plants have two FtsZ families.

CHAPTER 2

Osteryoung KW, Stokes KD, Rutherford SM, Percival AL, Lee WY (1998)

Chloroplast division in higher plants requires members of two functionally divergent gene families with homology to bacterial *ftsZ*. Plant Cell **10:** 1991-2004

Abstract

The division of plastids is critical for viability in photosynthetic eukaryotes, but the mechanisms associated with this process are still poorly understood. We previously identified a nuclear gene from Arabidopsis encoding a chloroplastlocalized homolog of the bacterial cell division protein FtsZ, an essential cytoskeletal component of the prokaryotic cell division apparatus. Here, we report the identification of a second nuclear-encoded FtsZ-type protein from Arabidopsis that does not contain a chloroplast targeting sequence or other obvious sorting signals and is not imported into isolated chloroplasts, which strongly suggests that it is localized in the cytosol. We further demonstrate using antisense technology that inhibiting expression of either Arabidopsis *FtsZ* gene (*AtFtsZ1-1* or *AtFtsZ2-1*) in transgenic plants reduces the number of chloroplasts in mature leaf cells from 100 to one, indicating that both genes are essential for division of higher plant chloroplasts but that each plays a distinct role in the process. Analysis of currently available plant FtsZ sequences further suggests that two functionally divergent *FtsZ* gene families encoding differentially localized products participate in chloroplast division. Our results provide evidence that both chloroplastic and cytosolic forms of FtsZ are involved in chloroplast division in higher plants and imply that important differences exist between chloroplasts and prokaryotes with regard to the roles played by FtsZ proteins in the division process.

Introduction

A number of metabolic pathways crucial for plant growth and development are housed in plastids. Among the various types of plastids present in plants, chloroplasts have been studied most extensively because of their role in photosynthesis. However, plastids also synthesize various amino acids, lipids, and plant growth regulators and so are assumed to be essential for viability of all plant cells (Mullet, 1988). For plastid continuity to be maintained during cell division and for photosynthetic tissues to accumulate the high numbers of chloroplasts required for maximum photosynthetic productivity, plastids must divide. Most of the available information concerning the process of plastid division is based on morphological and ultrastructural observations of dividing chloroplasts. During division, chloroplasts exhibit a dumbbell-shaped appearance in which the division furrow becomes progressively narrower. It is therefore generally agreed that chloroplast division occurs by a binary fission mechanism involving constriction of the envelope membranes (Leech, 1976; Possingham et al., 1988; Whatley, 1988).

In plastids from a variety of higher plant and algal species, an electron-dense "plastid dividing ring" of unknown composition has been described in association with the zone of constriction. The electron-dense material often can be resolved into two concentric rings, one on the stromal face of the inner envelope and one on the cytosolic face of the outer envelope (Hashimoto, 1986; Oross and Possingham, 1989; Duckett and Ligrone, 1993; Kuroiwa et al., 1998). Little else is known regarding the structure of the division apparatus. Valuable information on the plasticity and genetic complexity of plastid division has been obtained from study of the *arc* mutations (for accumulation and

replication of chloroplasts) in the model plant system Arabidopsis (Pyke and Leech, 1992, 1994; Robertson et al., 1996; Pyke, 1997). These mutations define at least seven nuclear genes important in the control of plastid number in higher plants. However, none of the *arc* loci has as yet been isolated, and until recently, the molecular events underlying chloroplast division in plants have remained entirely undefined.

A significant development in understanding the mechanistic basis for chloroplast division was the discovery that the nuclear genome of Arabidopsis encodes a eukaryotic homolog of the bacterial cell division protein FtsZ (Osteryoung and Vierling, 1995). The *ftsZ* gene was originally identified in a temperature-sensitive mutant of *Escherichia coli* that formed bacterial filaments at the restrictive temperature due to incomplete septum formation (Lutkenhaus et al., 1980), hence the designation fts (for filamenting temperature-sensitive). FtsZ is a rate-limiting cytoskeletal component of the cell division apparatus in prokaryotes (Ward Jr and Lutkenhaus, 1985; Baumann and Jackson, 1996; Margolin et al., 1996; Wang and Lutkenhaus, 1996), assembling at the nascent division site into a contractile ring just inside the cytoplasmic membrane (Bi and Lutkenhaus, 1991; Lutkenhaus and Addinall, 1997).

Recent studies have revealed that FtsZ is a structural homolog and possibly the evolutionary progenitor of the eukaryotic tubulins (Erickson, 1995; de Pereda et al., 1996; Erickson et al., 1996; Erickson, 1998; Löwe and Amos, 1998), and it can undergo dynamic GTP-dependent assembly into long polymers in vitro (de Boer et al., 1992; RayChaudhuri and Park, 1992; Mukherjee et al., 1993; Bramhill and Thompson, 1994; Mukherjee and Lutkenhaus, 1994; Bramhill, 1997; Yu and Margolin, 1997; Mukherjee and Lutkenhaus, 1998). In a previous study (Osteryoung and Vierling, 1995), we

identified an *FtsZ* gene (*AtFtsZ1-1*) from Arabidopsis whose putative product exhibited between 40 and 50% amino acid identity with most of its prokaryotic counterparts. We further demonstrated that the gene product is synthesized as a precursor in the cytosol and post-translationally targeted to the chloroplast by virtue of an N-terminal chloroplast transit peptide. These findings provided direct evidence that the chloroplast division machinery in photosynthetic eukaryotes evolved from the endosymbiotic ancestor of chloroplasts and led us to hypothesize that this chloroplast-localized FtsZ has a function analogous to that of FtsZ in prokaryotes, that is, that it is a key structural component of the chloroplast division apparatus in plants (Osteryoung and Vierling, 1995).

A role for a plant FtsZ in the division of chloroplasts was recently confirmed by Strepp et al. (1998), who reported that a targeted knockout of *PpFtsZ* , an *FtsZ* homolog from the nonvascular plant *Physcomitrella patens* , resulted in severe disruption of chloroplast division in that organism. The localization of the *PpFtsZ* gene product was not investigated; however, the finding that it differs from the previously published Arabidopsis FtsZ in that it lacks an N-terminal extension that could function as a chloroplast transit peptide is an interesting observation. In this report, we describe the isolation of an additional cDNA from Arabidopsis encoding an FtsZ protein that also lacks a potential chloroplast targeting sequence. In addition, we provide experimental evidence that this protein is not localized in the chloroplast and demonstrate that both the chloroplast-targeted and nontargeted forms of Arabidopsis FtsZ are critical for the division of higher plant chloroplasts. Finally, we propose that plant *FtsZ* genes can be grouped into two families whose products are localized in different subcelluar

Figure 1. Alignment Showing Homology of AtFtsZ2-1 to AtFtsZ1-1 and Several Prokaryotic FtsZ Proteins.

Identical amino acids are boxed. Asterisks indicate residues conserved among tubulins and FtsZ proteins (Erickson, 1998); double underlining indicates the tubulin signature motif (de Boer et al., 1992); dots indicate gaps in the alignment. The alignment was performed using CLUSTAL W 1.7 (Thompson et al., 1994). GenBank accession numbers for the proteins in the alignment are as follows: *Bacillus subtilis*, M22630; *Staphylococcus aureus*, U06462; *Anabaena* sp, U14408; AtFtsZ1-1, U39877; AtFtsZ2-1, AF089738; *E. coli*, AE000119; and *Rhizobium meliloti*, L25440.

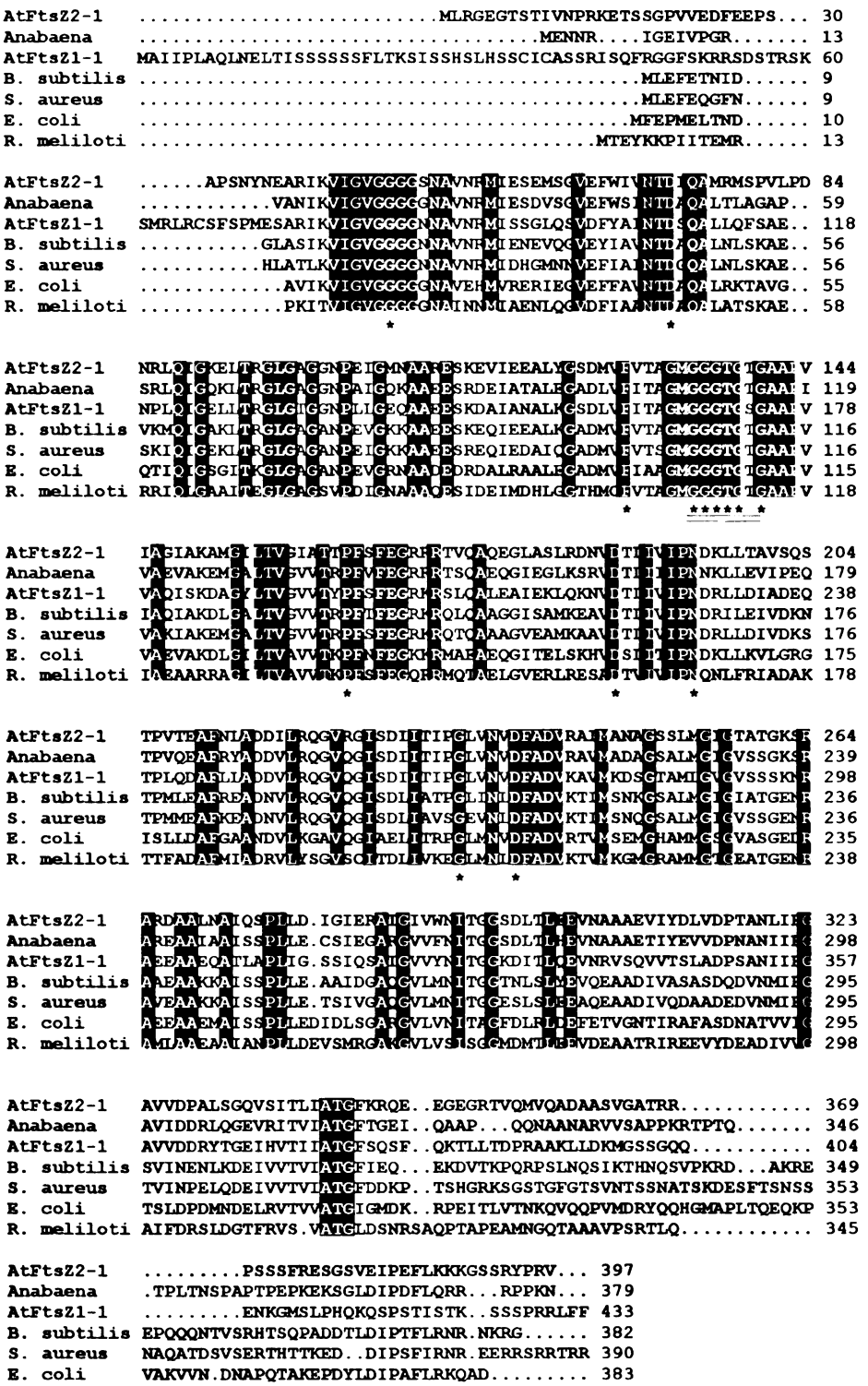
AtFtsZ2-1 ..... MLRGEGTSTIVNPRKETSSGPVVEDFEEPS... 30
Anabaena ..... MENNR....IGEIVPGR..... 13
AtFtsZ1-1 MAIIPLAQLNELTISSSSSSFLTKSISSHSLHSSCICASSRISQFRGGFSKRRSDSTRSK 60
B. subtilis ..... MLEFETNID..... 9
S. aureus ..... MLEFEQGFN..... 9
E. coli ..... MFEPMELTND..... 10
R. meliloti ..... MTEYKKPIITEMR..... 13
AtFtsZ2-1 .....APSNYNEARIKVIGVGGGGSNAVNRMIESEMSGVEFWIVNTDIQALMRMSPVLPD 84
Anabaena .....VANIKVIGVGGGGNNAVNRMIESDVSGVEFWSINTDAQALTLAGAP.. 59
AtFtsZ1-1 SMRLRCSFSPMESARIKVIGVGGGGNNAVNRMISSGLQSVDFYAINTDSQALLQFSAE.. 118
B. subtilis .....GLASIKVIGVGGGGNNAVNRMIENEVQGVEYIAVNTDAQALNLSKAE.. 56
S. aureus .....HLATLKVIGVGGGGNNAVNRMIDHGMNNVEFIAINTDGQALNLSKAE.. 56
E. coli .....AVIKVIGVGGGGNNAVEHMVRERIEGVEFFAVNTDAQALRKTAVG.. 55
R. meliloti .....PKITVIGVGGGGGNAINNMIAENLQGVDFIAANTDAQALATSKAE.. 58
AtFtsZ2-1 NRLQIGKELTRGLGAGGNPEIGMNAAEESKEVIEEALYGSDMVFVTAGMGGGTGTGAAPV 144
Anabaena SRLQIGQKLTRGLGAGGNPAIGQKAAEESRDEIATALEGADLVFITAGMGGGTGTGAAPI 119
AtFtsZ1-1 NPLQIGELLTRGLGIGGNPLIGEQAAEESKDAIANALKGSDLVFITAGMGGGTGSGAAPV 178
B. subtilis VKMQIGAKLTRGLGAGANPEVGKKAAEESKEQIEEALKGADMVFVTAGMGGGTGTGAAPV 116
S. aureus SKIQIGEKLTRGLGAGANPEIGKKAAEESREQIEDAIQGADMVFVTSGMGGGTGTGAAPV 116
E. coli QTIQIGSGITKGLGAGANPEVGRNAAEDRDALRAALEGADMVFIAAGMGGGTGTGAAPV 115
R. meliloti RRIQLGAAIVEGLGAGSVPDIGNAAAQESIDEIMDHLGGTHMCFVTAGMGGGTGTGAAPV 118
AtFtsZ2-1 IAGIAKAMGILTVGIATTPFSFEGRRFTVQAQEGLASLRDNVDTLIVIPNDKLLTAVSQS 204
Anabaena VAEVAKEMGALTVGVVTRPFVFEGRRFTSQAEQGIEGLKSRVDTLIIIPNNKLLEVIPEQ 179
AtFtsZ1-1 VAQISKDAGALTVGVVTYPFSFEGRRFSLQALEAIEKLQKNVDTLIVIPNDRLLDIADEQ 238
B. subtilis IAQIAKDLGALTVGVVTRPFIFEGRRFQLQAAGGISAMKEAVDTLIVIPNDRILEIVDKN 176
S. aureus VAKIAKEMGALTVGVVTRPFSFEGRRFQTQAAAGVEAMKAAVDTLIVIPNDRLLDIVDKS 176
E. coli VAEVAKDLGILTVAVVTKPFNFEGKKRMAEAEQGITELSKHVDSLITIPNDKLLKVLGRG 175
R. meliloti IAEAARRAGILTVAVVTKPFSFEGQRRMQTAELGVERLRESADTVIVIPNQNLFRIADAK 178
AtFtsZ2-1 TPVTEAFNLADDILRQGVRGISDLITIPGLVNVDFADVRAIMANAGSSLMGIGTATGKSR 264
Anabaena TPVQEAFRYADDVLRQGVQGISDLITIPGLVNVDFADVRAVMADAGSALMGIGVSSGKSR 239
AtFtsZ1-1 TPLQDAFLLADDVLRQGVQGISDLITIPGLVNVDFADVKAVMKDSGTAMIGVGVSSSKNR 298
B. subtilis TPMLEAFREADNVLRQGVQGISDLIATPGLINLDFADVKTIMSNKGSALMGIGIATGENR 236
S. aureus TPMMEAFKEADNVLRQGVQGISDLIAVSGFVNLDFADVKTIMSNQGSALMGIGVSSGENR 236
E. coli ISLLDAFGAANDVLKGAVQGIAELITRPGLMNVDFADVRTVMSEMGHAMMGSGVASGEDR 235
R. meliloti TTFADAFMIADRVLYSGVSGITDLIVKPGLMNLDFADVKTVMKGMGRAMMGTGEATGEDR 238
AtFtsZ2-1 ARDAALNAIQSPLLD.IGIEPATGIVWNITGGSDLTLDEVNAAAEVIYDLVDPTANLIFG 323
Anabaena AREAATAAISSPLLE.CSIEGARGVVFNITGGSDLTLHEVNAAAETIYEVVDPNANIIFG 298
AtFtsZ1-1 AEEAAEQATLAPLIG.SSIQSATGVVYNITGGKDITLQEVNRVSQVVTSLADPSANIIFG 357
B. subtilis AAEAAKKAISSPLLE.AAIDGAQGVLMNITGGTNLSLMEVQEAADIVASASDQDVNMIFG 295
S. aureus AVEAAKKAISSPLLE.TSIVGAQGVLMNITGGESLSLEEAQEAADIVQDAADEDVNMIFG 295
E. coli AEEAAEMAISSPLLEDIDLSGARGVLVNITAGFDLRLDEFETVGNTIRAFASDNATVVIG 295
R. meliloti AMLAAEAAIANPLLDEVSMRGAKGVLVSISGGMDMTLFEVDEAATRIREEVYDEADIVVG 298
AtFtsZ2-1 AVVDPALSGQVSITLIATGFKRQE..EGEGRTVQMVQADAASVGATRR..... 369
Anabaena AVIDDRLQGEVRITVIATGFTGEI..QAAP...QQNAANARVVSAPPKRTPTQ..... 346
AtFtsZ1-1 AVVDDRYTGEIHVTIIATGFSQSF..QKTLLTDPRAAKLLDKMGSSGQQ..... 404
B. subtilis SVINENLKDEIVVTVIATGFIEQ...EKDVTKPQRPSLNQSIKTHNQSVPKRD...AKRE 349
S. aureus TVINPELQDEIVVTVIATGFDDKP..TSHGRKSGSTGFGTSVNTSSNATSKDESFTSNSS 353
E. coli TSLDPDMNDELRVTVVATGIGMDK..RPEITLVTNKQVQQPVMDRYQQHGMAPLTQEQKP 353
R. meliloti AIFDRSLDGTFRVS.VATGLDSNRSAQPTAPEAMNGQTAAAVPSRTLQ..... 345
AtFtsZ2-1 .....PSSSFRESGSVEIPEFLKKKGSSRYPRV... 397
Anabaena .TPLTNSPAPTPEPKEKSGLDIPDFLQRR...RPPKN... 379
AtFtsZ1-1 .....ENKGMSLPHQKQSPSTISTK..SSSPRRLFF 433
B. subtilis EPQQQNTVSRHTSQPADDTLDIPTFLRNR.NKRG..... 382
S. aureus NAQATDSVSERTHTTKED..DIPSFIRNR.EERRSRRTRR 390
E. coli VAKVVN.DNAPQTAKEPDYLDIPAFLRKQAD..... 383

Figure 1

compartments and suggest a model for the functional role of plant FtsZ proteins in the division of chloroplasts that takes all of these findings into account.

Results

FtsZ Proteins are Encoded by a Small Gene Family in Arabidopsis

We identified the first eukaryotic *FtsZ* gene from Arabiodpsis in the expressed sequence tag (EST) database (Newman et al., 1994) on the basis of its high degree of conservation with bacterial *ftsZ*. *B*ecause the gene product was imported into chloroplasts, we originally referred to it as cpFtsZ. However, in keeping with guidelines for a standard nomenclature for plant genes (Lonsdale and Price, 1997), we have now renamed this gene *ARATH;FtsZ1-1* , which we henceforth designate as *AtFtsZ1-1*.

The existence of at least one additional nuclear *FtsZ* gene in Arabidopsis was subsequently indicated by the appearance in the database of a second EST with partial homology to both *AtFtsZ1-1* and prokaryotic *FtsZ* genes. Because the sequence of this EST suggested that the cDNA from which it was derived was rearranged, a polymerase chain reaction fragment containing the region of homology with *FtsZ* was amplified from Arabidopsis genomic DNA and used to screen an Arabidopsis cDNA library in an effort to isolate a nonrearranged clone. Three identical full-length cDNAs encoding a second homolog of Arabidopsis FtsZ were isolated. Comparisons between the encoded polypeptide, designated AtFtsZ2-1, and several other FtsZ proteins are shown in Figure 1 and Table 1. The amino acid sequence exhibits ~50% identity to both AtFtsZ1-1 and to bacterial FtsZ proteins and contains all of the features conserved among FtsZ proteins. These include the tubulin signature motif involved in GTP binding (de Boer et al., 1992;

Table 1. Percentage of Identity[a] between the Arabidopsis FtsZ Proteins and Those of Several Prokaryotes

Species	AtFtsZ1-1[b]	AtFtsZ2-1[b]
Anabaena sp	64.5	62.3
Bacillus subtilis	54.1	60.5
Staphylococcus aureus	59.9	58.4
E. coli	51.0	51.0
Rhizobium meliloti	49.5	45.9

[a]Calculated in pairwise comparisons by using the SIM local alignment algorithm (Huang and Miller, 1991) with the default parameters specified on the ExPASy Molecular Biology Server, Swiss Institute of Bioinformatics (http://expasy.hcuge.ch/sprot/sim-prot.html). Accession numbers are provided in the legend to Figure 1.
[b]AtFtsZ1-1 and AtFtsZ2-1 share 59.4% identity.127

RayChaudhuri and Park, 1992; Mukherjee et al., 1993), other residues conserved among tubulins and FtsZ proteins, and a stretch near the N terminus that is highly conserved among all FtsZ proteins (Figure 1). Among the prokaryotic FtsZ sequences, AtFtsZ2-1 is most closely related to that of the cyanobacterial species *Anabaena* (Table 1). This suggests that like *AtFtsZ1-1*, *AtFtsZ2-1* probably had an endosymbiotic origin. However, a notable difference between the two Arabidopsis FtsZ proteins is that AtFtsZ1-1 contains a long extension at its N terminus relative to most prokaryotic FtsZ proteins that was shown previously to function as a chloroplast transit peptide (Osteryoung and Vierling, 1995). AtFtsZ2-1 lacks an N-terminal extension.

To investigate whether additional *FtsZ* genes exist in Arabidopsis, we probed DNA and RNA gel blots at moderate stringency with either the *AtFtsZ1-1* or *AtFtsZ2-1* cDNA. On both DNA and RNA gel blots, *AtFtsZ1-1* hybridized with a single band, as shown in Figures 2A and 2B (lanes 1), indicating that AtFtsZ1-1 in Arabidopsis is likely encoded by a single gene. In contrast, *AtFtsZ2-1* hybridized with two bands, which were distinct from those recognized by *AtFtsZ1-1* (lanes 2). These results indicate the existence of at least three genes encoding FtsZ proteins in Arabidopsis: one encoding AtFtsZ1-1, one encoding AtFtsZ2-1, and one encoding a protein that is closely related to AtFtsZ2-1. Recent sequence data from the Arabidopsis genome project have confirmed this conclusion (described below).

AtFtsZ2-1 Is Not Imported into Isolated Chloroplasts

To test whether AtFtsZ2-1 could be targeted to the chloroplast, we performed an in vitro chloroplast import experiment identical to that conducted previously for AtFtsZ1-

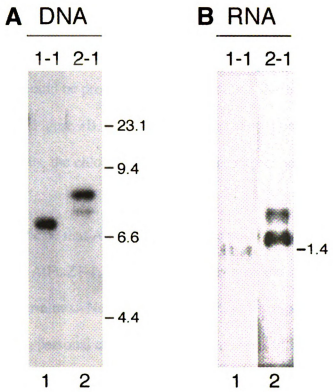

Figure 2. Hybridization Analysis of *AtFtsZ1-1* and *AtFtsZ2-1* in Arabidopsis.

Hybridizations with either the *AtFtsZ1-1* (1-1) cDNA (lanes 1) or the *AtFtsZ2-1* (2-1) cDNA (lanes 2) were conducted at moderate stringency. Length markers in kilobases are indicated at right.

(A) DNA gel blot. Genomic DNA (1.5 µg) was digested with BamHI, separated on a 0.7% agarose gel, and transferred to a nylon membrane for hybridization. The signal shown for the faster migrating fragment in lane 2 disappeared when the blot was washed at high stringency (data not shown).

(B) RNA gel blot. Poly(A)$^+$ RNA was isolated from leaf tissue, and 1.5 µg was separated on a 1.5% agarose gel containing formaldehyde and transferred to a nylon membrane for hybridization.

1 (Osteryoung and Vierling, 1995). The results are shown in Figure 3. A full-length radiolabeled AtFtsZ2-1 translation product was synthesized in vitro (Figure 3A, lane 1). The addition of isolated pea chloroplasts failed to effect import or proteolytic processing of the translation product (Figure 3A, lane 2). In a control reaction run at the same time, the AtFtsZ1-1 translation product was imported and processed (Figure 3B, lanes 1 and 2), and the import product could be protected from degradation during a postimport treatment with protease (Figure 3B, lane 3), as has been shown previously (Osteryoung and Vierling, 1995). Thus, the chloroplasts used for the experiment were import competent and intact.

These results indicate that AtFtsZ2-1 cannot be posttranslationally targeted to the chloroplast in vitro as is AtFtsZ1-1, which is consistent with the apparent absence of a transit peptide at its N terminus. Neither could AtFtsZ2-1 be imported into isolated yeast mitochondria (R. Jensen, personal communication). Analysis of the amino acid sequence by PSORT, a computer algorithm designed to identify potential intracellular sorting signals (Nakai and Kanehisa, 1992), did not predict other targeting sequences. We conclude from these findings that AtFtsZ2-1 is probably not localized in chloroplasts or mitochondria in vivo but is likely a cytosolic protein.

Expression of *AtFtsZ1-1* or *AtFtsZ2-1* Antisense Constructs Disrupts Chloroplast Division in Transgenic Arabidopsis

We previously hypothesized a role for AtFtsZ1-1 in chloroplast division based on its high degree of conservation with the bacterial FtsZs and the demonstration that it is localized in the chloroplast (Osteryoung and Vierling, 1995). To test whether AtFtsZ1-1

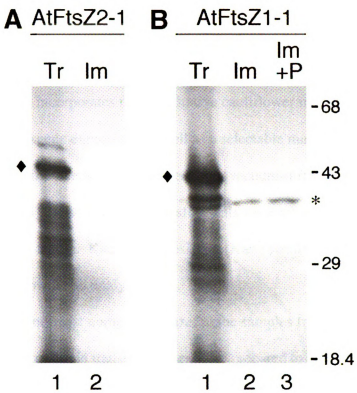

Figure 3. In Vitro Assay for Post-Translational Import of AtFtsZ2-1 to the Chloroplast.

In vitro transcription and translation reactions were performed to obtain full-length, radiolabeled translation products (lanes 1; full-length products indicated by diamonds). Chloroplasts isolated from pea seedlings were incubated with the translation products for 30 min and then reisolated by centrifugation through Percoll to remove unimported radioactivity (lanes 2). Reisolated chloroplasts were dissolved in SDS sample buffer, and import products were analyzed by SDS-PAGE and fluorography. Equal amounts of chloroplast extract, as determined by chlorophyll measurements, were loaded on the gel (Chen et al., 1994). Molecular mass standards are indicated at right in kilodaltons. Tr indicates translation products; Im indicates import products.
(A) Import assay for AtFtsZ2-1. No radioactivity was recovered with the chloroplasts after reisolation, indicating that none of the radiolabeled translation product was imported.
(B) Control import assay for AtFtsZ1-1. As shown previously (Osteryoung and Vierling, 1995), the radiolabeled precursor (lane 1) was processed upon import (lane 2), and the import product (asterisk) was protected from proteolysis by thermolysin (lane 3). Im+P indicates import product protected from proteolysis.

and AtFtsZ2-1 function as chloroplast division proteins, we conducted experiments to determine whether expression of antisense versions of either gene in transgenic Arabidopsis plants would inhibit chloroplast division, yielding plants with reduced numbers of chloroplasts. Antisense genes were constructed in the binary vector pART27 (Gleave, 1992), which incorporates the constitutive cauliflower mosaic virus 35S promoter to drive transgene expression as well as a selectable marker conferring plant resistance to kanamycin. Seeds were collected from vacuum-infiltrated (Bechtold et al., 1993; Bent et al., 1994) individuals, and transformants were selected by germination on kanamycin-containing media. Kanamycin-resistant (Kan^r) seedlings were transferred to soil for subsequent growth and analysis.

To analyze chloroplast number and size, tissue samples from the first fully expanded leaves from multiple transgenic lines were prepared to allow visualization of individual leaf mesophyll cells under the microscope (Pyke and Leech, 1991). In both the *AtFtsZ1-1* and *AtFtsZ2-1* antisense plants, a significant proportion of the Kan^r T_1 individuals exhibited drastically reduced numbers of chloroplasts. The phenotypes of these plants fell into two distinct classes, shown in Figure 4. The most commonly observed phenotypic class consisted of plants in which the mesophyll cells contained between one and three greatly enlarged chloroplasts, indicating that chloroplast division was severely disrupted. Indeed, the cells in most of these plants appeared to contain only a single enormous chloroplast (Figures 4A and 4C), compared with ~100 smaller chloroplasts in mature mesophyll cells from wild-type plants (Figures 4E and 4F). The other phenotypic class consisted of plants having between 10 and 30 chloroplasts of intermediate size in mature mesophyll cells (Figures 4B and 4D). Within an individual

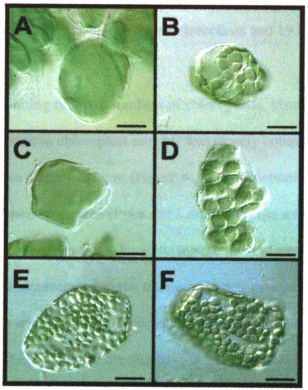

Figure 4. Phenotypes of Transgenic Plants Expressing Antisense Constructs of *AtFtsZ1-1* or *AtFtsZ2-1*.

Tissue from the first leaves of 23-day-old plants was prepared for visualization of individual mesophyll cells by using Nomarski interference contrast optics, as described by (Pyke and Leech, 1991).
(A) and **(B)** Cells from transgenic plants expressing the *AtFtsZ1-1* antisense gene.
(C) and **(D)** Cells from transgenic plants expressing the *AtFtsZ2-1* antisense gene.
(E) and **(F)** Cells from wild-type Arabidopsis.
Bars in **(A)** to **(F)** = 25 μm.
Images in this dissertation are presented in color.

transformant, most of the mesophyll cells appeared to be affected to the same extent. Interestingly, a continuous series in numbers of chloroplasts was not observed among the antisense plants, as might be expected from variations in transgene expression (Hooykaas and Schilperoort, 1992). Of 184 T_1 plants showing reduced chloroplast numbers in the two antisense experiments, 165 had one to three chloroplasts and 19 had 10 to 30 chloroplasts.

In all plants exhibiting reduced numbers of chloroplasts, visual inspection suggested that the reduction in chloroplast number was closely compensated for by a corresponding increase in chloroplast size (Figure 4). This observation was confirmed by measurements of chloroplast plan area (Pyke and Leech, 1992) as a function of mesophyll cell size in several transgenic lines having the most severe phenotypes. The data shown in Figure 5 demonstrate that the relationship between total chloroplast plan area and cell size over a wide range of cell sizes was almost the same in the transgenic and wild-type plants. These data indicate that despite the presence of only a single chloroplast in most of the mesophyll cells, the total chloroplast compartment volume was conserved. Similar results have been reported for several of the *arc* mutants (Pyke and Leech, 1992).

DNA gel blot analysis on a subset of plants from both antisense experiments yielded distinct hybridization patterns, confirming that the reduced chloroplast numbers in different transgenic lines were the result of independent T-DNA insertion events (results not shown). Most of the transgenes segregated as single loci based on analysis of segregation for Kan^r in the T_2 and T_3 generations. The phenotypes have remained heritable through the T_4 generation, although in some lines a proportion of the Kan^r

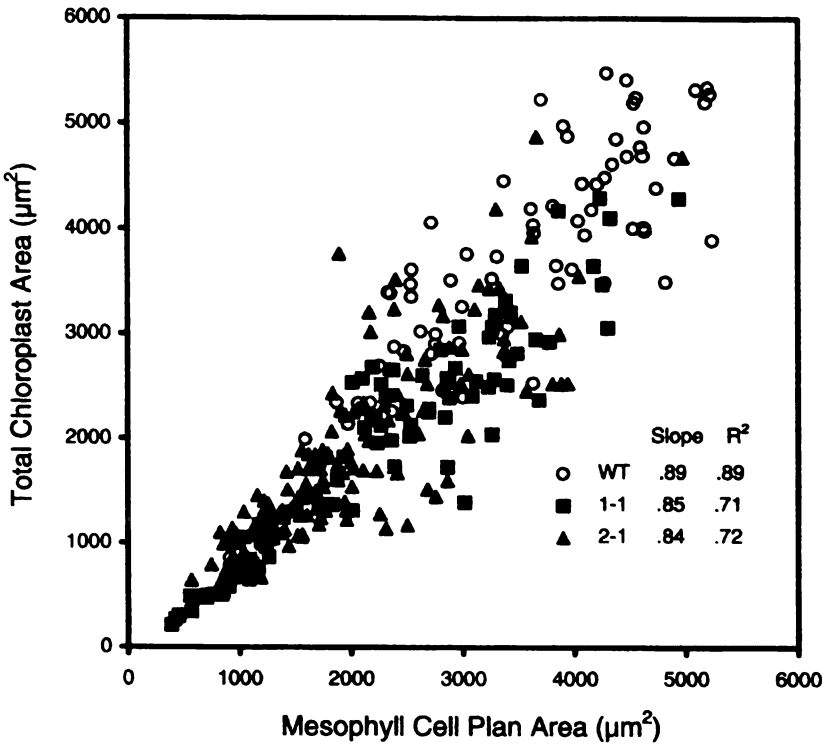

Figure 5. Relationship between Mesophyll Cell Plan Area and Total Chloroplast Plan Area in Transgenic and Wild-Type Plants.

Chloroplast plan areas were measured over a wide range of cell sizes in several *AtFtsZ1-1* (1-1, filled boxes) and *AtFtsZ2-1* (2-1, filled triangles) antisense and wild-type (WT, open circles) plants. The slopes and R^2 values calculated from linear regressions are shown.

progeny in each generation have reverted to wild type with regard to chloroplast number, suggesting the possibility of transgene silencing in those individuals (Matzke and Matzke, 1998). Despite the greatly reduced numbers and enlarged sizes of the chloroplasts in the antisense plants, their outward appearance under the conditions used in this study did not differ noticeably from the wild type. Flowering, seed production, and seed viability appeared normal for all transgenic plants, although subsequent analysis may reveal small variations, as has been observed for *arc6*, which grows somewhat more slowly than the wild type (Pyke et al., 1994).

AtFtsZ1-1 and AtFtsZ2-1 Have Distinct Functions in Chloroplast Division

A significant result of the transgenic plant experiments was that chloroplast numbers were reduced in plants expressing antisense copies of either *AtFtsZ1-1* or *AtFtsZ2-1*. An important question arising from these findings was whether in each experiment this phenotype was due to downregulation of only the gene targeted for antisense suppression or whether expression of both genes was inhibited. To address this issue, we performed ribonuclease protection assays to investigate levels of *AtFtsZ1-1* and *AtFtsZ2-1* RNA in several transgenic lines showing the most severe phenotypes. In the *AtFtsZ1-1* antisense plants, the levels of *AtFtsZ1-1* RNA were reduced significantly below those present in the wild type, as shown in Figure 6A (lanes 1 to 3). However, *AtFtsZ2-1* RNA levels in these same plants were unaffected (Figure 6B, lanes 1 to 3). Similarly, in the *AtFtsZ2-1* antisense lines, only *AtFtsZ2-1* RNA levels were reduced (Figure 6B, lanes 4 and 5); *AtFtsZ1-1* RNA remained at wild-type levels (Figure 6A,

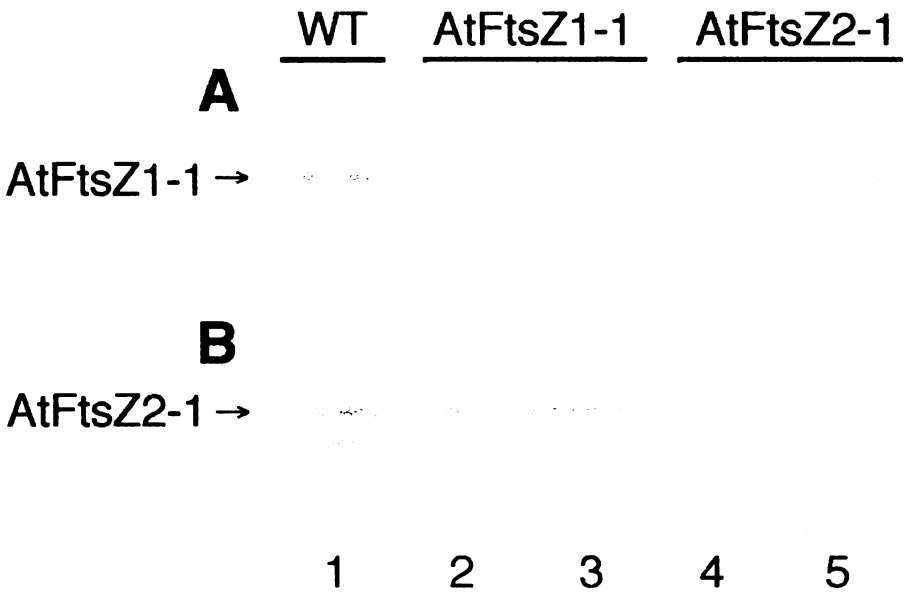

Figure 6. RNase Protection Assays of *AtFtsZ1-1* and *AtFtsZ2-1* Expression Levels in Independent Transgenic Antisense Lines.

Total RNA was isolated from 18-day-old plants exhibiting one to three large chloroplasts in mesophyll cells and hybridized with a radiolabeled RNA probe specific for either *AtFtsZ1-1* **(A)** or *AtFtsZ2-1* **(B)** RNA. After treatment with RNase, the protected fragments were separated on a sequencing gel and detected by autoradiography. Lanes 1, wild type (WT); lanes 2 and 3, two independent *AtFtsZ1-1* antisense lines; and lanes 4 and 5, two independent *AtFtsZ2-1* antisense lines. Arrows indicate positions of fully protected fragments.
(A) RNase protection of *AtFtsZ1-1* RNA.
(B) RNase protection of *AtFtsZ2-1* RNA.

lanes 4 and 5). These results confirm that chloroplast division could be inhibited in the transgenic plants by downregulation of either *AtFtsZ1-1* or *AtFtsZ2-1*.

Taken together, the results from the antisense experiments establish that *AtFtsZ1-1* and *AtFtsZ2-1* are not functionally redundant with regard to their roles in chloroplast division. Expression of both genes is necessary to achieve wild-type numbers of mesophyll cell chloroplasts in Arabidopsis.

Two Nuclear *FtsZ* Gene Families in Plants

Recently, a candidate for the second *AtFtsZ2* gene whose existence was predicted from hybridization studies (Figure 2) was revealed as a consequence of the Arabidopsis genome sequencing program. In a BLAST sequence similarity search (Altschul et al., 1990), we identified two independent genomic clones derived from different bacterial artificial chromosome (BAC) libraries that had overlapping end sequences with significant homology to *AtFtsZ2-1*. Further sequence analysis of these BAC clones yielded a partial amino acid sequence sharing 83% identity with AtFtsZ2-1 in the region of overlap, confirming the existence of a second gene closely related to *AtFtsZ2-1* in Arabidopsis. We designate this newly identified gene as *AtFtsZ2-2*.

Six nuclear *FtsZ* genes from four plant species representing dicots, monocots, and lower plants, including the three Arabidopsis sequences described above, are currently documented in the database. Their deduced amino acid sequences are aligned in Figure 7A, and the percentages of amino acid and DNA identity in pairwise comparisons among them are presented in Figure 7B. Both AtFtsZ2-1 and AtFtsZ2-2 share >75% amino acid identity with PpFtsZ from the moss *P. patens*. In contrast, these three proteins share

Figure 7. Plant *FtsZ* Genes Can Be Grouped into Two Families on the Basis of Their Deduced Amino Acid Sequences.

GenBank accession numbers for the proteins used in these analyses are as follows: AtFtsZ2-1, AF089738; AtFtsZ2-2, B25544+B96663 (overlapping BAC clones); PpFtsZ, AJ001586; AtFtsZ1-1, U39877; PsFtsZ, Y15383; and rice EST, C27863.
(A) Alignment of the deduced amino acid sequences of six plant *FtsZ* genes currently represented in the databases, performed using CLUSTAL W 1.7 (Thompson et al., 1994). Identical residues within the FtsZ1 family are boxed in gray. Identical residues within the FtsZ2 family are boxed in black. AtFtsZ2-2 and the rice EST are partial sequences. Asterisks indicate identity among all plant FtsZ proteins shown at that position in the alignment. Dots represent gaps in the alignment.
(B) Amino acid and DNA sequence identities in pairwise comparisons among six plant *FtsZ* genes. Percentages of identity were calculated as described in Table 1. Darker shading indicates amino acid sequence comparisons. Lighter shading indicates DNA sequence comparisons. Boxed groupings at left and right, respectively, show identities within the FtsZ2 and FtsZ1 families. Asterisks indicate partial sequences. Dashes indicate partial sequences that could not be compared because they do not overlap.

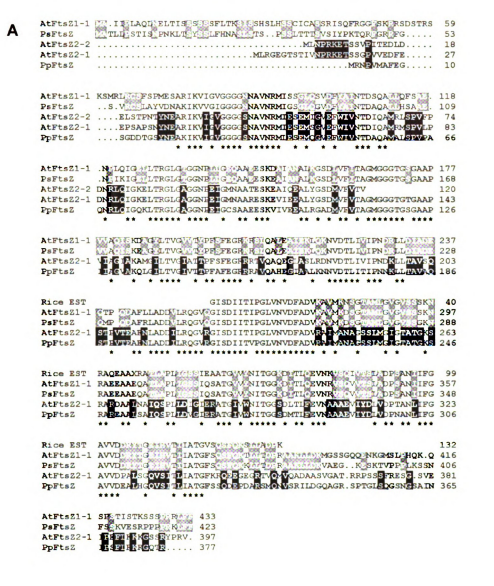
A
AtFtsZ1-1
AtFtsZ2-1
AtFtsZ2-2
PsFtsZ
PpFtsZ
Rice EST
B
AtFtsZ2-1
AtFtsZ2-2*
PpFtsZ
AtFtsZ1-1
PsFtsZ
Rice EST*
82 68 60 61 61
83 68 58 62 -
77 76 59 59 58
59 61 57 78 79
59 57 57 86 80
57 - 57 91 90

Figure 7

≤61% identity with AtFtsZ1-1. However, the proteins encoded by *PsFtsZ*, a full-length *FtsZ* cDNA from pea (*Pisum sativum*), and by a partial rice cDNA identified in the EST database (rice EST) share >85% identity with AtFtsZ1-1 (Figure 7B). Furthermore, like AtFtsZ1-1, PsFtsZ has an obvious N-terminal extension (Figure 7A), with all of the hallmarks of a chloroplast targeting sequence (Von Heijne et al., 1991), but neither PsFtsZ nor the partial rice protein exhibit >61% identity with either AtFtsZ2-1 or AtFtsZ2-2. Comparisons of DNA sequences among the six plant FtsZ sequences show similar trends (Figure 7B).

The comparative data shown in Figures 7A and 7B provide evidence that the existing plant FtsZ sequences can be classified into two distinct families on the basis of their overall amino acid sequence similarities. This proposal is strongly supported by the results of parsimony analysis for phylogenetic relatedness, shown in Figure 8. One FtsZ family appears to comprise precursor proteins with N-terminal extensions (Figure 7A) that are synthesized in the cytosol and post-translationally delivered to the chloroplasts where the transit peptide is processed. Based on sequence comparisons (Figures 7A and 7B) and parsimony analysis (Figure 8), AtFtsZ1-1 and PsFtsZ are both members of this family, as is the partial protein defined by the rice EST. We designate this group the FtsZ1 family. The other family, which we designate FtsZ2, includes AtFtsZ2-1, AtFtsZ2-2, and PpFtsZ. None of the FtsZ2 family members contains an obvious extension at the N terminus (Figure 7A) that would suggest localization in the chloroplasts, which is consistent with the inability of AtFtsZ2-1 to be imported into isolated chloroplasts in vitro (Figure 3). These data suggest that the FtsZ2 proteins so far identified are localized in the cytosol.

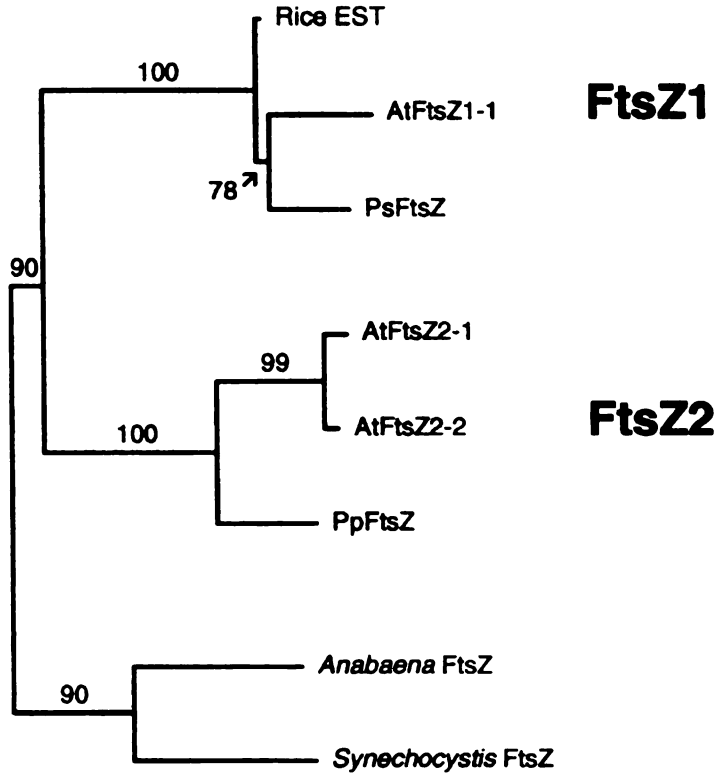

Figure 8. Phylogenetic Relationships among Plant FtsZ Proteins.

Weighted maximum parsimony analysis was performed with PAUP 3.1.1 (Swofford, 1998), using the exhaustive search option. Gaps were treated as missing. A single most parsimonious tree was produced. Horizontal branch lengths are proportional to amino acid step differences. Numbers above the horizontal branches indicate bootstrap confidence limits (>50%) supporting each clade of 1000 branch-and-bound replicates. The *Anabaena* sp (GenBank accession number Z31371) and *Synechocystis* sp (GenBank accession number D90906) sequences were defined as the outgroup.

Despite the apparent localization of the FtsZ1 and FtsZ2 family members in different subcellular compartments, our antisense experiments with transgenic plants clearly establish that members of both families, AtFtsZ1-1 and AtFtsZ2-1, play critical roles in the division of chloroplasts in Arabidopsis. Based on these findings, in conjunction with previous ultrastructural observations of plastid dividing rings both inside and outside the chloroplast (Kuroiwa et al., 1998), we propose that chloroplast division in higher plants is mediated by at least two functionally distinct forms of FtsZ: one represented by AtFtsZ1-1, which is localized inside the chloroplast and functions on the stromal surface of the inner envelope, and one represented by AtFtsZ2-1, which is localized in the cytosol and functions on the cytosolic surface of the outer envelope.

Discussion

Functional Divergence of *FtsZ* Genes in Plants

Our discovery that multiple *FtsZ* genes are present in the Arabidopsis nuclear genome provided the first clue that the FtsZ family in plants is functionally more complex than in prokaryotes. In prokaryotes, FtsZ is almost always encoded by a single gene (Lutkenhaus and Addinall, 1997). The amino acid sequence data derived from the four full-length cDNAs available from Arabidopsis (AtFtsZ1-1 and AtFtsZ2-1), pea (PsFtsZ), and moss (PpFtsZ) and the two partial sequences available from Arabidopsis (AtFtsZ2-2) and rice (rice EST) provide evidence that plant FtsZ proteins from evolutionarily divergent species can be grouped into two families—FtsZ1 and FtsZ2. Within each family, the proteins analyzed thus far share amino acid sequence identities ranging from 76 to 91%, whereas between families, the amino acid identities drop to

≤61%. The antisense experiments demonstrating similar phenotypes in plants that underexpress members of either family reveal that both FtsZ families contribute to and are essential for chloroplast division in higher plants but indicate that they have unique functions in that process.

Further analysis suggests that the functional distinction between the two FtsZ families in plants is at least partially a consequence of their differential subcellular localizations. At least two members of the FtsZ1 family, AtFtsZ1-1 and PsFtsZ, possess extensions at their N termini when compared with most prokaryotic FtsZ proteins, whereas at least three members of the FtsZ2 family, AtFtsZ2-1, AtFtsZ2-2, and PpFtsZ, have no such extensions. This correlation is evident even though the parsimony analysis producing the two clades was based only on regions of overlap and therefore excluded the poorly conserved N termini. Consistent with the presence of N-terminal extensions in FtsZ1 proteins, our in vitro chloroplast import results indicated that AtFtsZ1-1 can be imported into isolated chloroplasts (Osteryoung and Vierling, 1995). In addition, the database entry for PsFtsZ (GenBank accession number Y15383) indicates that this protein also is localized in the chloroplast. In contrast, AtFtsZ2-1 lacks an N-terminal extension and cannot be imported into isolated chloroplasts, despite its demonstrated role in chloroplast division. PpFtsZ, which we have assigned to the FtsZ2 family, also lacks an N-terminal extension but has been shown to function in chloroplast division (Strepp et al., 1998). We have been unable to find convincing evidence in the literature of nuclear-encoded chloroplast proteins that lack N-terminal targeting sequences. Therefore, although definitive localization of the plant FtsZ proteins awaits further experimentation, it seems probable that the FtsZ2 family members, which clearly function in chloroplast

division, are localized in the cytosol, whereas the FtsZ1 family members are localized in the chloroplast.

Concordance between Functional and Structural Studies

Many ultrastructural studies of chloroplast division have reported the appearance of an electron-dense ring, termed the plastid dividing ring, in the zone of constriction before separation of the daughter plastids (reviewed in Kuroiwa et al., 1998). A similar electron-dense ring in dividing bacteria has been shown to contain FtsZ (Bi and Lutkenhaus, 1991). In algae and land plants, the plastid dividing ring can be resolved into two concentric rings that appear to reside on opposite sides of the chloroplast envelope (Hashimoto, 1986; Oross and Possingham, 1989; Duckett and Ligrone, 1993; Kuroiwa et al., 1998). A simple model incorporating these cytological observations with our findings on the role of FtsZ proteins in the division of chloroplasts is depicted in Figure 9. The model predicts that chloroplast-localized FtsZ1 is a component of the inner plastid dividing ring, which is localized in a position analogous to that of the FtsZ ring in dividing bacteria. Although our in vitro import data indicated that newly imported AtFtsZ1-1 protein was in the soluble chloroplast fraction (Osteryoung and Vierling, 1995), we expect to find that during plastid division in vivo at least a portion of the protein is localized to the inner envelope surface. This is consistent with the finding that the FtsZ ring in bacteria is able to undergo assembly and disassembly in vivo (Addinall et al., 1997; Pogliano et al., 1997). With regard to the composition of the outer plastid dividing ring, several reports have described the appearance of fine filaments around the isthmus of dividing chloroplasts (Chida and Ueda, 1991; Ogawa et al., 1994; Kuroiwa et

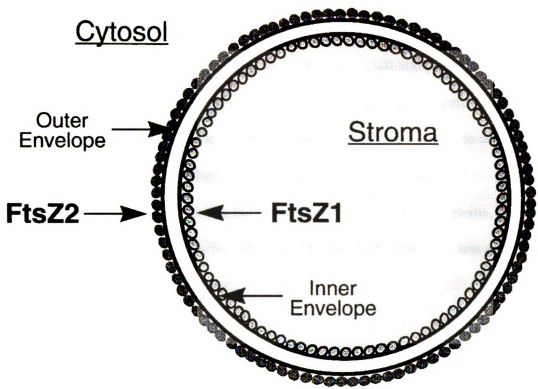

Figure 9. A Tentative Model for FtsZ Localization and Function in Division of Higher Plant Chloroplasts.

The model predicts that (1) FtsZ1 family members are post-translationally targeted to the stroma, where they assemble at the surface of the inner chloroplast envelope to become a component of the inner plastid dividing ring; (2) FtsZ2 family members remain in the cytosol and assemble at the surface of the outer envelope to become a component of the outer plastid dividing ring; and (3) the two FtsZ containing rings function together on opposite sides of the chloroplast envelope to effect constriction of the organelle. Other molecules, such as actin, may also participate.

al., 1998), supporting the idea that cytoskeletal elements participate in chloroplast division at the cytosolic surface.

There has been some speculation that actin may be a component of the outer ring, although the evidence is inconclusive (Kuroiwa et al., 1998). Our model predicts that the outer ring is composed at least partially of FtsZ2, although actin could also be a component or may interact with it. The fact that both chloroplast and putative cytosolic forms of FtsZ are required for chloroplast division suggests that the two FtsZ-containing plastid dividing rings proposed in the model function together in some way to accomplish constriction of the chloroplast. Whether FtsZ proteins in plants or bacteria are themselves force generating or whether other molecules, such as motor proteins, provide the motive force necessary for division remains unknown. Experiments to test and further refine this model are under way.

Implications of Transgenic Plant Phenotypes for Developmental Patterns of Plastid Division

An interesting observation from our antisense experiments is that reduced expression of the *FtsZ* genes does not yield a continuum in the number of mesophyll cell chloroplasts present in different transgenic lines, as might be expected from variations in transgene expression (Hooykaas and Schilperoort, 1992). Rather, the phenotypes fall into two discrete classes in which the cells contain either one to three large chloroplasts or 10 to 30 intermediate-sized chloroplasts. Analysis of plastid division patterns in several species indicates that division of proplastids in the shoot apical meristem is sufficeint to maintain the proplastid population at 10 to 20 per cell, whereas increased division of

developing or fully differentiated chloroplasts results in the proliferation of mesophyll cell chloroplasts that normally accompanies leaf maturation (Pyke, 1999). Consequently, we infer from the phenotypes of the transgenic plants that both proplastid and chloroplast division are inhibited in plants with the most severe phenotypes, whereas plants with intermediate phenotypes are apparently inhibited primarily in division of differentiated chloroplasts. Because similar phenotypes are observed among *AtFtsZ1-1* and *AtFtsZ2-1* antisense lines, these findings further imply that both of these genes function in proplastid as well as in chloroplast division. In addition, the lack of a continuum among the observed phenotypic classes suggests that threshold levels of FtsZ expression may be necessary for plastid division, possibly for complete formation of the plastid dividing rings, and that constriction of a few small plastids in the meristem and young leaf cells may require lower levels of FtsZ than does constriction of larger chloroplasts later in leaf development. These conjectures can be investigated by further analysis of the transgenic plants for FtsZ expression levels and plastid numbers in different cell types.

Different Division Mechanisms in Chloroplasts and Mitochondria?

It seems reasonable to suppose that chloroplasts and mitochondria, both of which presumably evolved from eubacterialike endosymbionts (Gray, 1989, 1993), would divide by similar mechanisms. Therefore, it is surprising that BLAST searches to date have failed to identify FtsZ homologs in the recently completed *Saccharomyces cerevisiae* nuclear or mitchondrial genomes or in the genomes of any other nonphotosynthetic eukaryote (K.W. Osteryoung, unpublished results). These findings suggest that the mechanism of mitochondrial division differs fundamentally from that of

chloroplasts and bacteria. Because *FtsZ* is an ancient gene family, predating the split between the eubacteria and archaebacteria (Margolin et al., 1996; Wang and Lutkenhaus, 1996), this observation further implies that an FtsZ-based division apparatus may have been present early in the evolution of mitochondria but was supplanted over evolutionary time by a different system. Perhaps studies of primitive eukaryotes will eventually reveal remnants of a prokaryotically derived mitochondrial division apparatus.

Conclusion

The data presented here confirm that the mechanism of chloroplast division in higher plants has its evolutionary origins in the mechanism of prokaryotic cell division, as was first suggested by the discovery of a chloroplast-localized form of FtsZ. Evidence already exists that some additional components of the chloroplast division machinery are also of prokaryotic origin. For example, homologs of *MinD*, a gene involved in the positioning of the FtsZ ring in bacteria (de Boer et al., 1991; de Boer et al., 1992), are present in the plastid genome of the unicellular green alga *Chlorella vulgaris* (Wakasugi et al., 1997) and the nuclear genome of Arabidopsis (K.W. Osteryoung and K.A. Pyke, unpublished data). However, the demonstration that multiple forms of FtsZ are involved in chloroplast division suggests that the prokaryotic division apparatus has been elaborated during the evolution of photosynthetic eukaryotes, resulting in a more complex machinery for chloroplast division in which constituents of both the chloroplast and cytosol cooperate to achieve constriction of the organelle. Further studies to firmly establish the subcellular localization of different FtsZ proteins in plants and define other components of the plastid division apparatus will no doubt reveal additional similarities

and differences between the mechanisms of chloroplast and prokaryotic cell division.

Methods

Plant Material

Arabidopsis thaliana ecotype Columbia was used for all experiments. Seeds were sown on Supersoil (Rod McLellan Co., South San Francisco, CA) potting mix and vermiculite (4:1 mix) and stratified at 4°C for 48 hr in the dark before germination. Plants were grown in controlled environment chambers at a relative humidity of 40% and provided daily with 16 hr of light (125 μmol m^{-2} sec^{-1}) at 20°C and 8 hr of dark at 18°C. The age of the plants was taken from the date the seeds were placed in the light.

cDNA Library Screening

Primers flanking the region of homology to *FtsZ* present in an expressed sequence tag (EST) (accession number Z48464) were used to amplify the corresponding region of Arabidopsis genomic DNA. The primers had the following sequences: foward primer, 5'-CCAGGCTATGAGAATGTCT- 3'; and reverse primer, 5'-CTGTGACAAAGACCATATCTGAGC- 3'. The amplified fragment was gel purified, radiolabeled with ^{32}P-dATP by the random primer method (Feinberg and Vogelstein, 1983), and used as a probe to screen the λPRL2 cDNA library (Newman et al., 1994) at high stringency (65°C, 0.2 × SSC [1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate]). The library, obtained from the Arabidopsis Biological Resource Center (Columbus, OH; stock number CD4-7), was constructed in the λZipLox vector (Gibco BRL). Three strongly hybridizing clones were obtained. The pZL1 plasmids containing the

hybridizing cDNAs were excised from the phage DNA according to the manufacturer's instructions. End sequencing of the inserts indicated that all three clones were identical. One clone was sequenced completely on both strands and designated *AtFtsZ2-1*.

Hybridization Analysis

The methods used for DNA extraction, RNA extraction, poly(A)$^+$ RNA isolation, and RNA and DNA gel blot analyses were performed as described previously (Osteryoung et al., 1993). For DNA gel blot analysis, 1.5 μg of genomic DNA was digested with BamHI and electrophoresed through a 0.7% agarose gel. Blots were washed in 0.2 × SSC at 46°C before being exposed to film at −80°C with an intensifying screen. For RNA gel blot analysis, 1.5 μg of poly(A)$^+$ RNA isolated from rosette leaves of 3- to 4-week-old plants was separated on a 1.5% agarose gel. Blots were washed in 0.1 × SSC at 60°C and exposed to film for 1 week.

Chloroplast Import Assays

In vitro chloroplast import assays were performed for AtFtsZ1-1 and AtFtsZ2-1 as described by (Osteryoung and Vierling, 1995) and (Chen et al., 1994).

Construction of Antisense Genes and Plant Transformation

The plasmid SN506 (Norris et al., 1998), a derivative of the binary plant transformation vector pART27 (Gleave, 1992), was digested with XhoI and XbaI to remove the insert. The gel-purified vector fragment was ligated directionally to a 743-bp XbaI-AvaI fragment spanning nucleotides 132 to 875 of the *AtFtsZ1-1* cDNA or to a

1164-bp SpeI-AvaI fragment spanning nucleotides 126 to 1290 of the *AtFtsZ2-1* cDNA. The ligation products were amplified in *Escherichia coli*, and the resulting plasmids were purified and transferred to *Agrobacterium tumefaciens* GV3101 (Koncz and Schell, 1986). Transformation of Arabidopsis was done using the vacuum infiltration method (Bechtold et al., 1993; Bent et al., 1994).

Selection of Transgenic Plants

T_1 seeds collected from vacuum-infiltrated plants were sown on Rockwool (GrodanHP; Agro Dynamics, East Brunswick, NJ) (Gibeaut et al., 1997), saturated with nutrient solution containing 100 mg/L kanamycin (Fisher Scientific, Hampton, NH), covered with a clear plastic lid, and stratified and grown as described above. Kanamycin-resistant (Kan^r) seedlings were transplanted to soil at 14 days by cutting the surrounding Rockwool and placing both Rockwool and seedlings in soil. T_2 seed were collected from individual T_1 plants at maturity.

For analysis of transgene segregation ratios, T_2 or T_3 seed was surface sterilized, sown on plates containing 100 mg/L kanamycin in nutrient medium (4.3 g/L Murashige and Skoog salts [Gibco BRL], 1% sucrose, B5 vitamins [Gibco BRL], and 0.8% Phytagar [Gibco BRL]), incubated at 4°C for 2 days, moved to the light for germination, and grown as described above; resistance or sensitivity to kanamycin was scored 10 days later. Lines segregating as homozygous were used for subsequent studies.

Analysis of Transgenic Phenotypes by Microscopy

Analyses of the transgenic phenotypes were performed initially with 14-day-old

Kan^r T_1 seedlings and later with established homozygous lines at 14 and 23 days. The first leaf was removed with a razor blade and prepared for microscopy as described by Pyke and Leech (1991). A longitudinal strip was cut along the center of the leaf to ensure a representative sample of cell sizes. Fixed tissue samples were macerated on a microscope slide by using the blunt end of a scalpel, then suspended in a drop of 0.1 M Na_2–EDTA, pH 9, and covered with a coverslip. Samples were analyzed with Nomarski (Olympus Optical, Tokyo, Japan) differential interference contrast optics, using an Olympus (Olympus America, Melville, NY) BH-2 microscope. Images for analysis and publication were captured by computer using an Optronics (Goleta, CA) DEI-750 digital CCD camera and Adobe Premiere (Adobe Systems, San Jose, CA) imaging software.

Chloroplast numbers were counted by eye under the microscope. Mesophyll cell and chloroplast plan areas were analyzed from captured CCD images on a Macintosh-type (Power Computing Corporation, Round Rock, TX) computer using the NIH-Image public domain software (http://rsb.info.nih.gov/nih-image/). Total chloroplast plan area was taken as the product of chloroplast number and mean chloroplast size per cell (Pyke and Leech, 1994).

RNase Protection Assays

Total RNA was isolated from 18-day-old plants, as described previously (Logemann et al., 1987), using 1 g of leaf tissue from independent transgenic lines (T_3) or from the wild type. Only transgenic individuals exhibiting severely reduced numbers of chloroplasts were used for RNA isolation. After precipitation, the RNA pellet was resuspended in buffer (50 mM Mes, pH 7.0, and 2.5 mM magnesium acetate) and treated

for 15 min with 4 units of RNase-free DNase (Promega), then extracted with phenol–chloroform, and precipitated with sodium acetate and isopropanol. The final RNA pellet was washed with 70% ethanol and resuspended in 50 µL of sterile H_2O.

Plasmids used for probe synthesis were constructed as follows. For the *AtFtsZ1-1* probe, the pZL1 plasmid (Gibco BRL) containing the *AtFtsZ1-1* cDNA (GenBank accession number U39877), which was provided by the Arabidopsis Biological Resource Center (stock number 105K17T7), was digested with ClaI and XbaI to remove from the insert all but the 88 nucleotides at the 5' end of the cDNA. The overhangs were filled in with the Klenow fragment of DNA polymerase I (Promega) and ligated back together. For the *AtFtsZ2-1* probe, the pZL1 plasmid containing the *AtFtsZ2-1* cDNA, obtained from the library screen described above, was digested with SpeI and XbaI to remove from the insert all but 129 nucleotides at the 5' end of the cDNA, and the ends were ligated back together.

For probe synthesis, the plasmids were linearized with SmaI upstream of the inserts. Radiolabeled antisense RNA was generated by in vitro transcription. The reactions contained 2 µg of linearized plasmid, 500 2 µM each of ATP, GTP, and CTP, 1 mM DTT, 20 units of RNasin (Promega), 2 µL of 5 X transcription buffer (Promega), 2.5 µL of ^{32}P-UTP (800 Ci/mmol; ICN, Costa Mesa, CA), and 18 units of SP6 RNA polymerase (Promega). After a 1-hr incubation at 37°C, the reactions were treated with 4 units of DNase (Promega) for 15 min. The probes were purified by electrophoresis through a 6% polyacrylamide gel. The full-length probes were excised from the gel and eluted by incubation in 50 µL of elution buffer (Ambion Inc., Austin, TX) overnight at 37°C.

RNase protection assays were performed using the RPA II ribonuclease protection assay kit (Ambion, Inc.), as described in the Streamlined Procedure in the manual supplied by the manufacturer. Hybridizations were conducted overnight at 42°C by using 30 μg of total RNA and ~5×10^4 cpm of probe. The final RNA pellet was resuspended in 3 μL of loading buffer and heated at 95°C for 2 min before being electrophoresed through a 6% polyacrylamide sequencing gel. The gel was transferred to filter paper and dried. The protected RNA fragments were detected by autoradiography on XAR-5 (Eastman Kodak Co., Rochester, NY) or Bio-Max (Eastman Kodak Co.) film for 2 days at −80°C by using an intensifying screen.

Identification of Other Plant *FtsZ* Genes and DNA Sequence Analysis

The additional plant FtsZ sequences used in Figure 7 were initially identified by using the BLAST sequence similarity search (Altschul et al., 1990). The partial sequence shown for AtFtsZ2-2 represents a contig formed by two bacterial artificial chromosome (BAC) end sequences (GenBank accession numbers B25544 and B96663) that share an overlap of ~85 nucleotides on opposite strands. Pairwise comparisons, multiple sequence alignments, and parsimony analysis (Swofford, 1998) were performed as indicated in the legends for Figures 1, 7, and 8.

Acknowledgments

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Note Added In Proof

A recent update of the *FtsZ* sequence from *Physcomitrella patens* (Strepp et al., 1998; GenBank accession number AJ001586) indicates that the encoded protein contains an N-terminal extension. This additional sequence information does not influence the assignment of *PpFtsZ* to the *FtsZ2* gene family. However, the conclusion that N-terminal extensions are attributes of FtsZ1, but not FtsZ2, family members may be premature, or may apply only to higher plant FtsZ proteins.

CHAPTER 3

Stokes KD, McAndrew RS, Figueroa R, Vitha S, Osteryoung KW (2000) Chloroplast division and morphology are differentially affected by overexpression of *FtsZ1* and *FtsZ2* genes in *Arabidopsis*. Plant Physiol **124:** 1668-1677

Abstract

In higher plants, two nuclear gene families, *FtsZ1* and *FtsZ2*, encode homologues of the bacterial protein FtsZ, a key component of the prokaryotic cell division machinery. We previously demonstrated that members of both gene families are essential for plastid division, but are functionally distinct. To further explore differences between FtsZ1 and FtsZ2 proteins we investigated the phenotypes of transgenic plants overexpressing *AtFtsZ1-1* or *AtFtsZ2-1*, Arabidopsis members of the *FtsZ1* and *FtsZ2* families, respectively. Increasing the level of AtFtsZ1-1 protein as little as three-fold inhibited chloroplast division. Plants with the most severe plastid division defects had 13- to 26-fold increases in AtFtsZ1-1 levels over wild type, and some of these also exhibited a novel chloroplast morphology. Quantitative immunoblotting revealed a correlation between the degree of plastid division inhibition and the extent to which the AtFtsZ1-1 protein level was elevated. In contrast, expression of an *AtFtsZ2-1* sense transgene had no obvious effect on plastid division or morphology, though AtFtsZ2-1 protein levels were elevated only slightly over wild-type levels. This may indicate that AtFtsZ2-1 accumulation is more tightly regulated than that of AtFtsZ1-1. Plants expressing the *AtFtsZ2-1* transgene did accumulate a form of the protein smaller than those detected in wild-type plants. AtFtsZ2-1 levels were unaffected by increased or decreased accumulation of AtFtsZ1-1 and vice versa, suggesting that the levels of these two plastid division proteins are regulated independently. Taken together, our results provide additional evidence for the functional divergence of the *FtsZ1* and *FtsZ2* plant gene families.

Introduction

The first identified proteins of the chloroplast division machinery were homologues of the essential bacterial cell division protein FtsZ (Osteryoung and Vierling, 1995; Osteryoung et al., 1998; Strepp et al., 1998). In contrast with most bacterial genomes that contain only a single gene encoding FtsZ, the nuclear genome of Arabidopsis contains at least three *FtsZ* genes encoding members of two distinct protein families, FtsZ1 and FtsZ2. *AtFtsZ1-1* and *AtFtsZ2-1*, members of the *FtsZ1* and *FtsZ2* families, respectively, have been shown to be essential for chloroplast division. When the level of either gene is diminished, chloroplast division is inhibited, yielding cells with as few as one very large chloroplast. One important difference between AtFtsZ1-1 and AtFtsZ2-1 is their predicted localization. AtFtsZ1-1 is targeted to the chloroplast, as is a closely related FtsZ protein from pea (Gaikwad et al., 2000), and is thought to be a component of a division ring that forms on the stromal side of the inner envelope membrane. AtFtsZ2-1, in contrast, lacks a chloroplast transit peptide, is not targeted to the chloroplast in vitro, and is hypothesized to be a constituent of a division ring that assembles on the cytoplasmic surface of the outer envelope membrane. The two rings together are postulated to coordinate chloroplast division (Osteryoung et al., 1998).

Bacterial FtsZ is a self-associating cytoskeletal GTPase evolutionarily and structurally related to the eukaryotic tubulins (de Boer et al., 1992; RayChaudhuri and Park, 1992; Mukherjee and Lutkenhaus, 1994; Erickson, 1997; Yu and Margolin, 1997; Löwe and Amos, 1998; Nogales et al., 1998; Nogales et al., 1998). Early in the bacterial division cycle, prior to the onset of cytokinesis, FtsZ assembles into a ring at the division plane that encircles the cell on the inner surface of the cytoplasmic membrane. At least

eight other essential proteins are then recruited to the division site and function to complete cytokinesis. Throughout this process, the FtsZ ring remains localized at the leading edge of the division septum (Bi and Lutkenhaus, 1991; Bramhill, 1997; Lutkenhaus and Addinall, 1997; Pogliano et al., 1997; Rothfield and Justice, 1997; Nanninga, 1998; Rothfield et al., 1999).

Overexpression of FtsZ in bacteria has yielded important insights into the properties of this protein. First, FtsZ appears to be rate limiting in the division process since slight overproduction of FtsZ increases cell division (Ward Jr and Lutkenhaus, 1985). This increased division results in the formation of small, inviable "minicells" that lack chromosomes due to the occurrence of divisions not only at midcell, but also near the cell poles. Second, high overexpression of FtsZ inhibits cell division and results in the formation of bacterial filaments (Ward Jr and Lutkenhaus, 1985). This appears to be due in part to a stoichiometric imbalance between FtsZ and other division proteins because the filamentation phenotype can be relieved by simultaneous overexpression of FtsA or ZipA, two other bacterial cell division proteins that interact with FtsZ directly and colocalize with FtsZ to the midcell (Ward Jr and Lutkenhaus, 1985; Wang and Gayda, 1990; Dai and Lutkenhaus, 1992; Hale and de Boer, 1997; Wang et al., 1997; Hale and de Boer, 1999; Mosyak et al., 2000).

Although chloroplast division involves some proteins homologous to components of the bacterial division machinery, division of higher plant chloroplasts differs from bacterial cell division since it requires the coordinated activities of at least two FtsZ proteins and does not involve cell wall ingrowth at the division site (Osteryoung and Pyke, 1998; Osteryoung et al., 1998). In the studies described here we sought to further

explore the functional differences between FtsZ1 and FtsZ2 proteins by analyzing the phenotypes of Arabidopsis plants overexpressing *AtFtsZ1-1* or *AtFtsZ2-1*. The results reveal additional parallels between chloroplast and bacterial cell division with regard to the behavior of FtsZ, but support a difference in the roles played by FtsZ1 and FtsZ2 in chloroplast division. The data also suggest that the levels of AtFtsZ1-1 and AtFtsZ2-1 are regulated independently in Arabidopsis, and that FtsZ1 may have an additional function inside the organelle in regulating chloroplast morphology.

Results

Production of Antibodies Specific for Recognition of AtFtsZ1-1 or AtFtsZ2-1

In preparation for investigating AtFtsZ1-1 and AtFtsZ2-1 protein levels in this and other studies we produced antipeptide antibodies specific for detection of these two polypeptides on immunoblots. The specificities and reactivities of the affinity-purified antibodies, designated 1-1A and 2-1A, respectively, were analyzed in a series of immunoblotting and competition binding assays (Figure 1). Each antibody was highly selective for its target protein. In Arabidopsis leaf extracts the 1-1A antibodies reacted with a single polypeptide of 40 kD (Figure 1, A, lanes 1 and 3 and B, lanes 1 and 5), whereas the 2-1A antibodies reacted primarily with a polypeptide of 46 kD, though one of 45 kD was also detected (Figure 1, A, lanes 4 and 5 and B, lanes 2 and 4). In competition binding assays, immunoreactivity of the 1-1A antibody with the 40-kD protein was prevented when the antibodies were preincubated with recombinant AtFtsZ1-1 protein (Figure 1A, lane 2), but not with recombinant AtFtsZ2-1 protein (Figure 1A, lane 3). Likewise, immunoreactivity of the 2-1A antibodies with the 46- and 45- kD

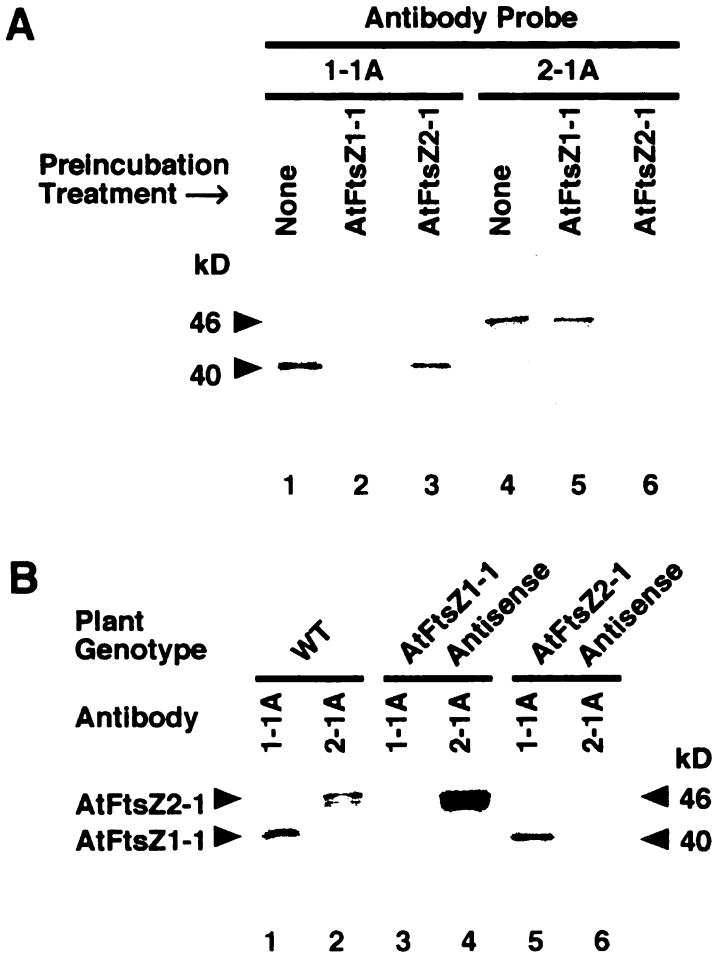

Figure 1. Specificity of AtFtsZ antipeptide antibodies.

A, Immunoblots of proteins isolated from wild-type Arabidopsis leaf extracts were probed with 1-1A (lanes 1-3) or 2-1A (lanes 4-6) antibodies raised against peptide from AtFtsZ1-1 or AtFtsZ2-1, respectively. Antibodies were preincubated with purified, recombinant AtFtsZ1-1 protein (lanes 2 and 5) or AtFtsZ2-1 protein (lanes 3 and 6). B, Immunoblot of proteins isolated from leaf extracts of wild-type (lanes 1 and 2), *AtFtsZ1-1* antisense (lanes 3 and 4), or *AtFtsZ2-1* antisense (lanes 5 and 6) plants. Blots were probed with either 1-1A (lanes 1, 3, and 5) or 2-1A (lanes 2, 4, and 6) antibodies. The 46- and 40-kD polypeptides are indicated by markers. Equivalent volumes of plant extracts were loaded in each lane.

polypeptides was blocked when the antibodies were preincubated with recombinant AtFtsZ2-1 protein (Figure 1A, lane 6), but not with AtFtsZ1-1 protein (Figure 1A, lane 5). Furthermore, the 1-1A antibodies detected the 40-kD polypeptide in extracts from wild-type plants and transgenic plants expressing an *AtFtsZ2-1* antisense construct (Figure 1B, lanes 1 and 5), but not in plants expressing an antisense *AtFtsZ1-1* construct (Figure 1B, lane 3). Conversely, the 2-1A antibodies detected the 46- and 45-kD polypeptides in extracts from wild-type plants and transgenic plants expressing an *AtFtsZ1-1* antisense construct (Figure 1B, lanes 2 and 4), but not in plants expressing an antisense *AtFtsZ2-1* construct (Figure 1B, lane 6). Neither antibody cross-reacted with prokaryotic FtsZ proteins in bacterial extracts (not shown). From these results, we conclude that the 1-1A antibodies specifically recognize AtFtsZ1-1, which migrates at 40 kD, whereas the 2-1A antibodies specifically recognize either two distinct forms of AtFtsZ2-1, which migrate at 46 and 45 kD, or AtFtsZ2-1 and a closely related polypeptide. The absence of both bands in the AtFtsZ2-1 antisense lines (Figure 1B, lane 6) is most consistent with the former possibility.

Overproduction of AtFtsZ1-1 Inhibits Chloroplast Division in Transgenic Arabidopsis

AtFtsZ1-1 was overexpressed in Arabidopsis under the control of the cauliflower mosaic virus 35S (35S) promoter in the vector pART27 (Gleave, 1992). The kanamycin-resistant (kan^r) transgenic plant lines used in this report were confirmed to be independent transformants by Southern-blot analysis (data not shown). With the exception of a slight twist in some of the leaves, all kan^r plants exhibited normal growth

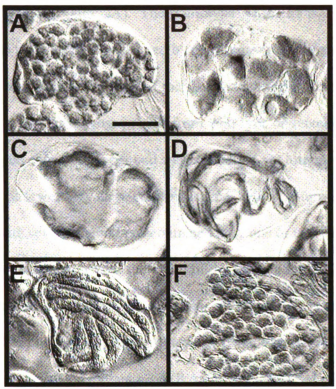

Figure 2. Phenotypes of transgenic plants overexpressing *AtFtsZ1-1* or *AtFtsZ2-1*.

Mesophyll cells are shown from the first leaves of 23-d-old plants transformed with the empty pART27 vector (A), the *AtFtsZ1-1* sense transgene (B-E), or the *AtFtsZ2-1* sense transgene (F). Tissue was prepared for imaging with differential interference contrast optics using methods described previously (Pyke and Leech, 1991). Bar=25 μm in all figures. A three-dimensional rotating reconstruction of cells with phenotypes similar to those shown in C and D can be found in a video supplement at www.plantphysiol.org.

and development. However, microscopic examination of mesophyll cells revealed distinct phenotypes in plants overexpressing *AtFtsZ1-1* when compared with wild-type plants of the Columbia ecotype, which typically contain 80-100 chloroplasts in fully expanded mesophyll cells (Osteryoung et al., 1998), or to control plants transformed with the empty pART27 vector (Figure 2A). The phenotypes of the transgenic plants consistently fell into three categories defined by chloroplast number and size. Transgenic plants were classified as "wild-type-like" if the size and number of chloroplasts in mesophyll cells were similar to those in cells from wild-type or vector controls. Plants with 15 to 30 enlarged chloroplasts per cell were classified as "intermediate" (Figure 2B), and those with five or fewer very large chloroplasts per cell were termed "severe" (Figure 2C). Most plants with severe phenotypes contained only one or two chloroplasts per cell and these organelles usually appeared flattened, filling the thin layer of cytoplasm surrounding the vacuole. Despite the enlarged size, chloroplast ultrastructure consistently appeared normal (data not shown). Similar "severe" phenotypes have been described for the Arabidopsis plastid division mutant *arc6* (Pyke and Leech, 1994), for plants transformed with antisense *AtFtsZ1-1* or *AtFtsZ2-1* transgenes (Osteryoung et al., 1998), and for *FtsZ* knockout mutants in the moss *Physcomitrella patens* (Strepp et al., 1998). The proportion of T_1 individuals exhibiting wild-type-like, intermediate, and severe phenotypes were 19%, 39%, and 42%, respectively (Table 1). Therefore, over 80% of the kan^r plants expressing the *AtFtsZ1-1* sense transgene displayed defects in chloroplast division.

Table 1. Phenotypic distribution of transgenic T_1 plants.

Phenotype	Percentage of Transgenic Plants[a]	
	AtFtsZ1-1	*AtFtsZ2-1*
Wild-type-like	19	72
Intermediate	39	14
Severe	42	14

[a]Total number of plants analyzed: *AtFtsZ1-1*, 129; *AtFtsZ2-1*,

The Severity of Chloroplast Division Inhibition Is Proportional to AtFtsZ1-1 Protein Level

AtFtsZ1-1 protein levels in T_3 kan^r plants representing wild-type-like, intermediate, and severe phenotypes were investigated by immunoblot analysis. Proteins in leaf homogenates were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the affinity-purified AtFtsZ1-1 antipeptide antibodies. An immunoreactive 40-kD polypeptide was detected that varied in amount among different transgenic lines (Figure 3A, lanes 5-10), but comigrated with authentic AtFtsZ1-1 from wild-type and empty-vector control extracts (Figure 3A, lanes 1-4). These results indicate that most of the AtFtsZ1-1 protein in the overexpression lines was properly targeted to the chloroplast and processed. However, a slower-migrating immunoreactive polypeptide of 72 kD was also detected in plants with increased levels of the 40-kD polypeptide (Figure 3A, lanes 7-10). Competition binding assays have shown that this polypeptide does contain AtFtsZ1-1 (not shown), and may represent a non-dissociated form of the protein, possibly a dimer. A similarly migrating polypeptide is occasionally observed in wild-type plants as well (data not shown). The increased levels of the 72-kD polypeptide in overexpression lines with high AtFtsZ1-1 levels may be related to the ability of FtsZ proteins to form dimers and multimers in a concentration-dependent fashion (Di Lallo et al., 1999; Sossong et al., 1999; Rivas et al., 2000; White et al., 2000). An FtsZ1 homologue from pea has also been shown to form multimers in vitro (Gaikwad et al., 2000).

Visual inspection of immunoblots from the AtFtsZ1-1 overexpression lines suggested a correlation between the level of AtFtsZ1-1 accumulation and the severity of

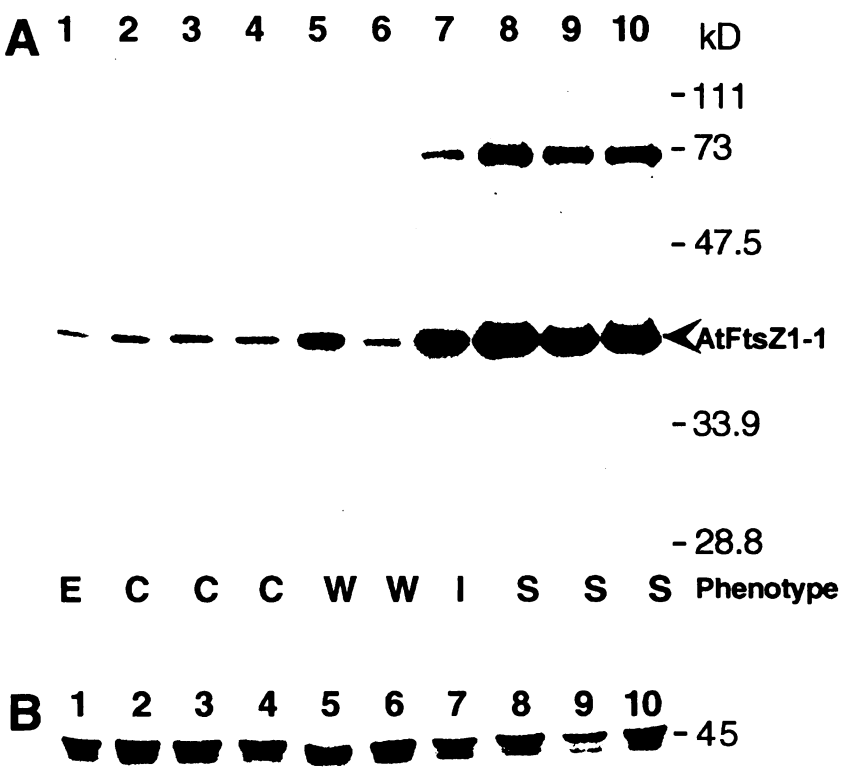

Figure 3. Immunoblot analysis of plant extracts overexpressing *AtFtsZ1-1*.

Proteins in extracts from 23-d-old transgenic plants were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antipeptide antibodies raised against AtFtsZ1-1 (A) or AtFtsZ2-1 (B). Lane 1, Empty vector control (E); lanes 2 through 4, wild type (C); lanes 5 through 10, transgenic plants with wild-type-like (W, lanes 5 and 6), intermediate (I, lane 7), or severe (S, lanes 8-10) phenotypes. Equal loading of all samples was confirmed by staining the membranes with ponceau S (data not shown).

chloroplast division defect. In extracts from plants with wild-type-like (Figure 3A, lane 5 and 6) and intermediate (Figure 3A, lane 7) phenotypes, the levels of the 40-kD AtFtsZ1-1 polypeptide were similar to, or slightly higher than, those seen in extracts from the vector controls (Figure 3A, lane 1) and non-transformed wild-type plants (Figure 3A, lanes 2-4), whereas in plants with severe phenotypes (Figure 3A, lanes 8-10), AtFtsZ1-1 protein levels were noticeably elevated. To further analyze this relationship, the level of AtFtsZ1-1 in the transgenic plants relative to that in control plants was quantified by immunoblotting (Figure 4). For this purpose, a calibration curve was constructed from densitometric analysis of the 40-kD polypeptide in four lanes loaded with increasing volumes of whole leaf extract from control plants transformed with the empty vector (Figure 4, lanes 1-4). For transgenic extracts, the volumes analyzed were adjusted to maintain protein levels within the linear range of the standard curve (Figure 4, lanes 5-13). The relative level of the 40-kD AtFtsZ1-1 polypeptide in each sample was then calculated from the standard curve based on the densitometry readings and sample volume. The calculated protein levels in extracts from three separate non-transformed wild-type plants (Figure 4, lanes 5-7) were comparable with those from empty vector control plants, indicating that the increased protein levels observed in the transgenic plants were due to expression of the AtFtsZ1-1 transgene and not to the vector. In transgenic plants classified as wild-type-like, the relative level of AtFtsZ1-1 protein was similar to the control level or elevated no more than two-fold (Figure 4, lanes 8 and 9). Protein levels between three-fold (Figure 4, lane 10) and 6-fold (not shown) above control levels were measured in plants with an intermediate chloroplast phenotype. Although data from only a single intermediate line are shown in Figures 3 and 4, two

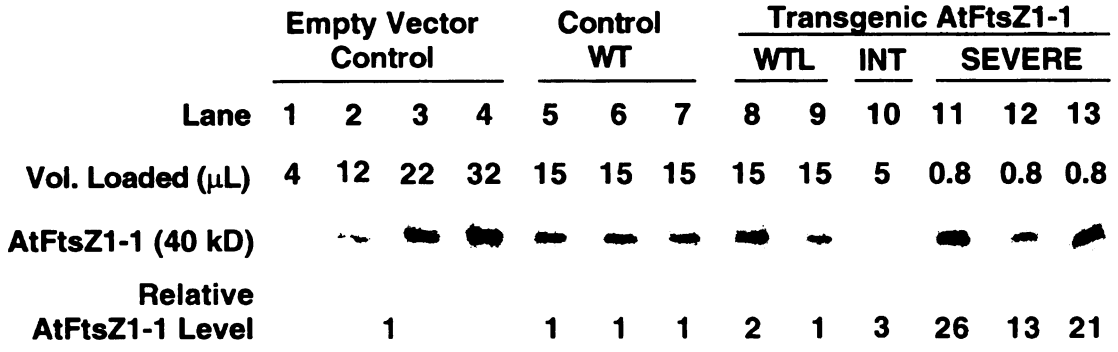

Figure 4. Relative levels of the 40-kD AtFtsZ1-1 polypeptide in plants expressing the *AtFtsZ1-1* transgene.

Densitometry readings from an immunoblot loaded with increasing amounts of extract from an empty vector control plant (lanes 1-4) were used to construct a standard concentration curve for AtFtsZ1-1. AtFtsZ1-1 levels in the other plant extracts (lanes 5-13), all loaded so that the densitometry reading produced by the 40-kD AtFtsZ1-1 polypeptide on immunoblots fell within the linear range of the standard curve, were then calculated, taking into account the volume loaded. The volume loaded, signal produced on immunoblots, and calculated level of AtFtsZ1-1 relative to that in the empty-vector controls are shown for three Columbia wild type plants (lanes 5-7), and transgenic plants with wild-type-like (WTL, lanes 8 and 9), intermediate (INT, lane 10), or severe (SEVERE, lanes 11-13) phenotypes.

additional lines with intermediate phenotypes also had approximately 3-fold more AtFtsZ1-1 than controls, whereas one line had 6-fold more (not shown). Plants exhibiting the most severe phenotypes had AtFtsZ1-1 levels ranging from 13- to 26-fold over control levels (Figures 3A, lanes 8-10, and 4, lanes 11-13). Our data indicate a correlation between the level of AtFtsZ1-1 and the severity of chloroplast division inhibition. However, we cannot rule out the possibility that the observed division defects resulted from accumulation of the 72-kD form of AtFtsZ1-1 rather than from overproduction of the protein per se. This 72-kD band was not quantified, but its levels in the transgenic lines appeared to correlate with those of the 40-kD polypeptide (Figure 3, lanes7-10, and data not shown).

AtFtsZ1-1 Overexpression Produces a Novel Chloroplast Morphology in Some Transgenic Plants

In addition to the phenotypes described above, an interesting and unusual phenotype was encountered in three independent AtFtsZ1-1 overexpression lines with severe phenotypes. A small number of cells in these plants contained chloroplasts that appeared long and narrow, giving the impression of worms or noodles (Figure 2, D and E). Two of these plant lines had only a few noodle-like chloroplasts per cell (Figure 2D), whereas one line had about 15 (Figure 2E). The diameter of these chloroplasts varied somewhat, but was comparable to that of wild-type chloroplasts. The length, however, was many times longer than in wild type, and the chloroplasts meandered in unique patterns around the cytoplasm of the cell. Only a small proportion of the cells in these plants displayed the noodle-like phenotype; the vast majority of cells exhibited a typically

severe chloroplast morphology. The noodle-shaped chloroplasts were only observed in transgenic plants with high levels of AtFtsZ1-1 protein, including those represented in lanes 8 through 10 of Figure 3, but have not been found in all such lines.

Slight Overexpression of AtFtsZ2-1 Does Not Disrupt Chloroplast Division

The effect of *AtFtsZ2-1* overexpression in transgenic plants was also investigated. The T_1 generation of kan^r plants transformed with the *AtFtsZ2-1* transgene contained cells with wild-type-like, intermediate, and severe chloroplast phenotypes, similar to those described for the *AtFtsZ1-1* transgenic plants, and had no obvious abnormalities in growth or development. However, in contrast to the *AtFtsZ1-1* overexpression lines, 72% of the *AtFtsZ2-1* kan^r T_1 individuals exhibited a wild-type-like phenotype (Figure 2F), whereas plants with intermediate and severe phenotypes each constituted only 14% of the total (Table 1). Furthermore, in subsequent generations the lines with intermediate and severe phenotypes reverted to the wild-type-like phenotype. The extent of this reversion was such that only one transgenic line retained reduced chloroplast numbers by the T_3 generation. Because of this trend, T_1 and T_3 plants were studied further.

AtFtsZ2-1 protein levels were analyzed in whole leaf extracts from T_1 and T_3 plants by immunoblotting. In extracts from transgenic plants exhibiting wild-type-like phenotypes (Figure 5A, lanes 2, 3, 6-9, 11, 13, and 16-18), the same 46- and 45-kD polypeptides present in wild-type and empty-vector control plants (Figure 5A, lanes 1 and 2) were detected. The levels of the 46-kD polypeptide in these lines were comparable with control levels, although some extracts exhibited a slight increase (Figure 5A, lanes 4 and 8). We have observed that the intensity of the 45-kD band varies

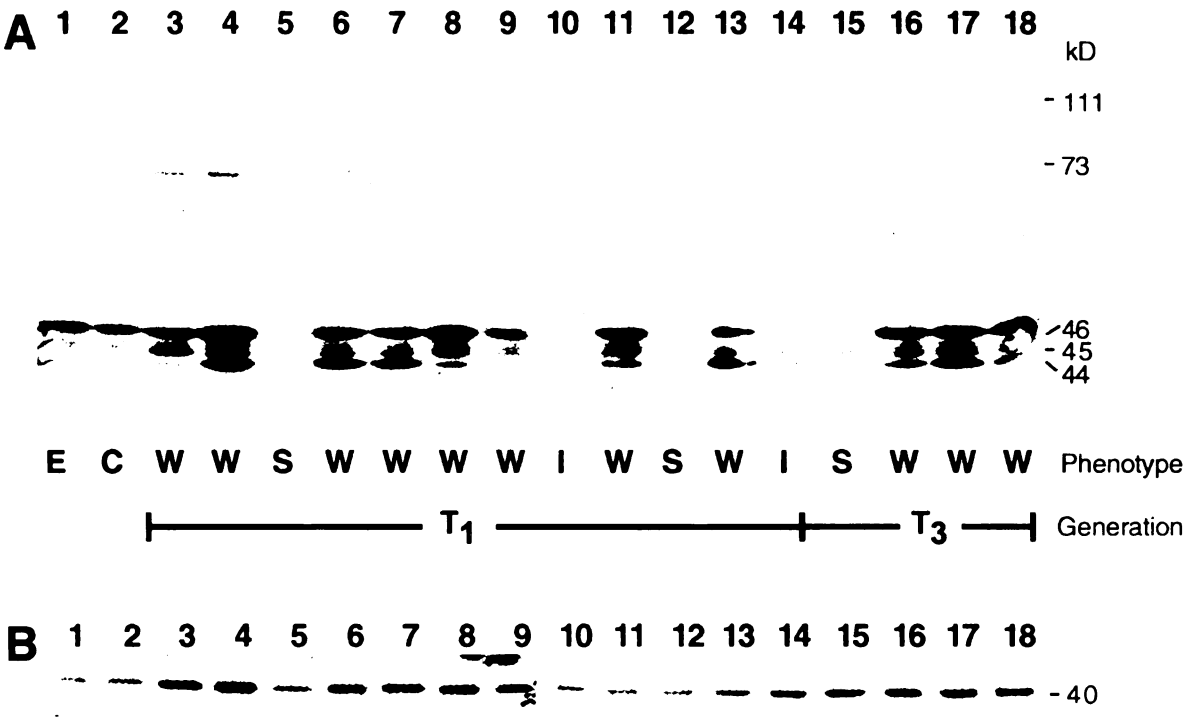

Figure 5. Immunoblot analysis of transgenic plant extracts expressing the *AtFtsZ2-1* transgene.

Proteins in extracts from 23-d-old transgenic plants were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antipeptide antibodies raised against AtFtsZ2-1 (A) or AtFtsZ1-1 (B). Lane 1, Empty vector control (E); lane 2, wild type (C); lanes 3 through 18, T_1 or T_3 transgenic plants with wild-type-like (W, lanes, 3, 4, 6-9, 11, 13, 16-18), intermediate (I, lanes 10 and 14), or severe (S, lanes 5 and 15) phenotypes. Equal loading of all samples was confirmed by staining the membranes with ponceau S (data not shown).

considerably among individuals in wild type (not shown), and the levels of the 45-kD protein in the transgenic plants did not appear to vary outside this range. The more obvious result of *AtFtsZ2-1* overexpression was the accumulation of a 44-kD polypeptide, detected in all kan^r plants with a wild-type-like phenotype, but not detected in any of the controls. Accumulation of this polypeptide was not correlated with any noticeable plastid division defect. In contrast, all transgenic lines with intermediate or severe phenotypes (Figure 5A, lanes 5, 10, 12, and 14), including the line retaining a severe phenotype into the T_3 generation (lane 15), had reduced levels of the 46- and 45-kD species present in controls, and did not accumulate the 44-kD polypeptide present in lines with the wild-type-like phenotypes. These data suggest that inhibition of chloroplast division in the *AtFtsZ2-1* transgenic plants resulted from cosuppression rather than overexpression of *AtFtsZ2-1*.

AtFtsZ1-1 and AtFtsZ2-1 Accumulation Are Regulated Independently of One Another

To learn whether overproduction of AtFtsZ1-1 was accompanied by a change in AtFtsZ2-1 levels or vice versa, the levels of both proteins in extracts from each set of transgenic plants were analyzed on duplicate immunoblots. In all *AtFtsZ1-1* overexpression lines analyzed, AtFtsZ2-1 protein remained at wild-type levels (Figure 3B). In a converse manner, the level of AtFtsZ1-1 protein in the *AtFtsZ2-1* transgenic lines was unaffected by the level of AtFtsZ2-1 protein (Figure 5B). Further, antisense repression of *AtFtsZ1-1*, though reducing AtFtsZ1-1 protein to nearly undetectable levels (Figure 1B, lane 3) and severely inhibiting chloroplast division (Osteryoung et al., 1998),

had no affect on accumulation of AtFtsZ2-1 (Figure 1B, lane 4), or vice versa (Figure 1B, lanes 5 and 6). Therefore, we conclude that the phenotypes associated with manipulation of *AtFtsZ1-1* or *AtFtsZ2-1* expression levels, whether from an increase or decrease, result from altered accumulation of only one and not both proteins. In addition, although *AtFtsZ1-1* and *AtFtsZ2-1* are co-expressed in wild-type plants (Figure 1B, lanes 1 and 2; Osteryoung et al., 1998), the collective results of overexpression and antisense experiments suggest that FtsZ1 and FtsZ2 protein levels are regulated independently in Arabidopsis.

Discussion

Correlation between AtFtsZ1-1 Accumulation and Plastid Number

Plants producing AtFtsZ1-1 at levels ranging from 13- to as high as 26-fold over wild-type levels exhibited drastically reduced numbers of enlarged chloroplasts, indicating a severe inhibition of chloroplast division. This phenotype is comparable with the filamentation phenotype observed in *E. coli* cells expressing *FtsZ* at high levels. However, when bacterial FtsZ levels were only slightly elevated, extra divisions were induced near the cell poles, resulting in the formation of minicells. Furthermore, when levels of overexpression were below those resulting in filamentation, the minicell phenotype was proportional to the degree of FtsZ overexpression (Ward Jr and Lutkenhaus, 1985). Based on these data we anticipated that slightly elevated AtFtsZ1-1 protein levels might increase the frequency of chloroplast division, yielding plants with cells containing smaller, more numerous chloroplasts. Instead, AtFtsZ1-1 levels as little as three-fold over wild-type levels inhibited, rather than increased, chloroplast division.

However, we have observed a few transgenic lines with more than 150 tiny chloroplasts in mesophyll cells (data not shown), but these plants were chlorotic and died as seedlings, preventing further analysis of their phenotypes or *AtFtsZ1-1* expression levels. Nevertheless, these observations suggest that elevated AtFtsZ1-1 levels could, under a limited set of circumstances, increase the frequency of chloroplast division. Based on the observation that plants with two-fold more AtFtsZ1-1 than wild type had wild-type numbers of chloroplasts, whereas plants with 3-fold more protein had intermediate numbers, indicating partial inhibition of division, we would predict that AtFtsZ1-1 accumulation only within this narrow range would lead to increased numbers of chloroplasts, and that levels above this are inhibitory for plastid division. This idea is consistent with the behavior of FtsZ in bacteria and with the possibility that the plastid division defect resulting from AtFtsZ1-1 overproduction reflects a stoichiometric imbalance in plastid division components. It is possible that the use of a weak promoter instead of the strong 35S promoter might allow identification of more plants with only slightly elevated levels of AtFtsZ1-1, and perhaps increased chloroplast numbers, for further study. It is also possible that simultaneous overexpression of additional chloroplast division proteins (including AtFtsZ2-1) might be required to permit an increase in the plastid division frequency. This is suggested by the finding that the increased frequency of cell divisions observed when *FtsZ* is overexpressed in *E. coli* only occurs when *FtsA* is also overexpressed at similar levels (Begg et al., 1998). Plant survival alternatively may be compromised by increased chloroplast numbers, which could account for the small number of plants identified with this phenotype. This conjecture is supported in part by the phenotype of the Arabidopsis *arc*1 chloroplast

division mutant, which is characterized by slightly increased numbers of small chloroplasts (Pyke and Leech, 1992; Marrison et al., 1999). *arc*1 grows more slowly and is pale early in its development compared with either wild type or other *arc* mutants that have reduced numbers of enlarged chloroplasts.

Expression of the *AtFtsZ2-1* Sense Transgene Does Not Produce a Plastid Division Phenotype

In contrast to the dramatic phenotypes associated with *AtFtsZ1-1* overexpression, more than 70% of the plants transformed with the *AtFtsZ2-1* sense transgene displayed a wild-type-like phenotype. In fact, immunoblotting results (Figure 5A, lanes 5, 10, 12, 14, and 15) indicated that all plastid division defects observed among these transgenic lines were due to cosuppression of endogenous *AtFtsZ2-1* expression rather than to overexpression, similar to the plastid division defects observed in plants expressing an *AtFtsZ2-1* antisense transgene (Figure 1B, lane 6; Osteryoung et al., 1998). However, authentic AtFtsZ2-1 protein did not accumulate substantially over wild-type levels in the overexpression experiments (Figure 5A), which may indicate that higher levels are lethal or that AtFtsZ2-1 accumulation is more tightly regulated than that of AtFtsZ1-1. The only phenotype associated with *AtFtsZ2-1* transgene expression (when it did not result in cosuppression) was the presence of a 44-kD form of AtFtsZ2-1 not detected in wild-type plants. Because this polypeptide was smaller than the one produced by in vitro translation of the predicted *AtFtsZ2-1* open reading frame (Osteryoung et al., 1998; R.S. McAndrew, S. Vitha and K.W. Osteryoung, unpublished data), it may represent a degradation product or an aberrantly processed form of AtFtsZ2-1. Accumulation of this

polypeptide at the observed levels had no effect on plastid division, however. Overall, the differences in the phenotypes of plants expressing *AtFtsZ1-1* and *AtFtsZ2-1* transgenes further support a difference in the functions of FtsZ1 and FtsZ2 proteins.

Aberrant Chloroplast Morphology Associated with High Levels of AtFtsZ1-1 Protein

The long, narrow chloroplasts observed in some of the *AtFtsZ1-1* overexpression lines occurred only in transgenic lines exhibiting severe plastid division defects and high AtFtsZ1-1 protein levels. Although at present we cannot be certain of the biological relevance of this unique phenotype, it could suggest an additional role for chloroplast-localized FtsZ1 proteins in the control of chloroplast shape. Immunofluorescence data indicating the presence of longitudinally oriented AtFtsZ1-1-containing filaments in the noodle-shaped chloroplasts (S. Vitha and R. McAndrew, unpublished observations) are consistent with this idea. The assembly of many such filaments in plastids with high AtFtsZ1-1 levels could restrict their ability to expand laterally, allowing plastid expansion to occur only longitudinally to produce narrow, elongated chloroplasts. The noodle phenotype alternatively could reflect an abnormality in the formation of stromules, narrow tubular connections between plastids through which protein molecules can pass (Köhler et al., 1997). At present there is no direct evidence that plant FtsZ proteins participate in chloroplast shape determination or stromule biogenesis, but the notion that FtsZs function in multiple processes, like their tubulin structural homologues, is not incompatible with their cytoskeletal properties.

Materials and Methods

Construction of Sense Transgenes and Plant Transformation

Full-length cDNAs for *AtFtsZ1-1* (accession no. U39877) and *AtFtsZ2-1* (accession no. AF089738) in the plasmid vector pZL1 (Gibco BRL) were obtained as described previously (Osteryoung et al., 1998). A gel-purified *Sma*I-*Cla*I fragment containing the complete *AtFtsZ1-1* cDNA sequence was ligated directionally behind the 35S promoter in pART7 (Gleave, 1992) digested with the same enzymes. The resulting plasmid was digested with *Not*I and the fragment containing the *AtFtsZ1-1* insert was ligated into *Not*I-digested pART27 (Gleave, 1992) to create the plasmid pAP202 containing the *AtFtsZ1-1* transgene. The *AtFtsZ2-1* transgene was constructed by digesting the pART27 derivative pSN506 (Norris et al., 1998) with *Eco*RI and *Hin*dIII, and replacing the insert with an *Eco*RI-*Hin*dIII fragment containing the complete *AtFtsZ2-1* cDNA to create pRF501. pAP202 and pRF501 were purified from *Escherichia coli* and transferred to *Agrobacterium tumefaciens* GV3101 (Koncz and Schell, 1986). Restriction analysis confirmed that no rearrangements occurred in the transfer to *Agrobacterium*. Arabidopsis ecotype Columbia was transformed by vacuum infiltration (Bechtold et al., 1993; Bent et al., 1994) with pAP202, and by floral dip (Clough and Bent, 1998) with pRF501 or the pART27 empty vector.

Selection and Propagation of Transgenic Plants

T_1 seed from the inoculated plants were collected, sown on plates containing nutrient medium (4.3 g/L Murashige and Skoog salts, 1% [w/v] Suc, B5 vitamins, and 0.8% [w/v] Phytagar [Gibco-BRL]) and 100 mg/L kanamycin (Fisher Scientific,

Hampton, NH), incubated at 4°C for 2 d, and moved to controlled environment chambers for germination. Chambers were set at a relative humidity of 40%, with 16 h of light daily (125 μmol m^{-2} s^{-1}) at 20°C, and 8 h of darkness at 18°C. The age of the plants was taken from the date of seed transfer to the growth chamber following cold treatment. After 10 d in the growth chamber, kan^r plants were transferred to a mixture of Supersoil potting mix (Rod McLellan Co., San Francisco) and vermiculite (4:1).

T_2 and T_3 seeds were sown on Rockwool (GrodanHP; Agro Dynamics, East Brunswick, NJ) saturated with Hoagland nutrient solution containing 100 mg/L kanamycin (Gibeaut et al., 1997). Seeds were covered with plastic and incubated at 4°C for 2 d, then transferred to growth chambers for germination. After 14 d, kan^r plants were transferred to soil and grown as described above.

Microscopic Analysis

When plants were 18 d post-germination, the first leaf was removed with a razor blade and prepared for microscopic analysis as described (Pyke and Leech, 1991). Samples were then viewed with differential interference contrast optics using a BH-2 microscope (Olympus America, Melville, NY). Images were captured by computer using a DEI-750 digital charged-coupled device camera (Optronics, Goleta, CA) and Adobe Photoshop (Adobe Systems, San Jose, CA) software.

Generation of Antipeptide Antibodies

Peptides corresponding to regions of AtFtsZ1-1 and AtFtsZ2-1 predicted to constitute highly accessible and immunogenic epitopes were designed using the crystal

structure of *Methanococcus janaschii* (Löwe and Amos, 1998), epitope mapping data for monoclonal antibodies against *E. coli* FtsZ (Voskuil et al., 1994), and molecular modeling programs. Peptides corresponding to residues 201 through 215 in AtFtsZ1-1 (EGRKRSLZALEAIEK) and residues 168 through 184 in AtFtsZ2-1 (RRRTVQAQEGLASLRD) were synthesized, purified by HPLC, and coupled to keyhole limpet hemocyanin (Pierce, Rockford, IL). These peptides, designated 1-1A and 2-1A, respectively, were injected into rabbits (nos. 4164 and 4166, respectively) for the production of polyclonal antibodies (Alpha Diagnostics, San Antonio, TX). The antisera obtained were partially purified by ammonium sulfate precipitation (50% final saturation) followed by dialysis against phosphate-buffered saline (140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), as described (Harlow and Lane, 1988). Antibodies were further purified on affinity columns prepared by immobilizing the peptide antigens to SulfoLink Coupling Gel (Pierce) according to the manufacturer's standard protocol, yielding final protein concentrations of 0.7 and 0.9 mg ml^{-1} for antibodies 1-1A and 2-1A, respectively, determined using the Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA). These preparations were diluted for immunoblotting as described below.

Immunoblotting Procedures

Tissue for immunoblot analysis was collected from leaves of 21-d-old plants with microscopically verified mesophyll cell phenotypes. Tissue was homogenized in a microcentrifuge tube with 10 μL of grinding buffer {60 mM Tris[tris(hydroxymethyl)-aminomethane]-HCl, pH 8.0, 100 mM dithiothreitol, 2% [w/v] SDS, 15% [w/v] Suc, 5

mM ε-amino-*N*-caproic acid, 1 mM benzamidine HCl, and 0.01% [w/v] bromophenol blue} per milligram of leaf tissue plus a few grains of sterilized sand. Homogenized tissue was heated for 15 min at 70°C and stored at -20°C until use. Prior to electrophoresis, samples were reheated to 70°C for 5 min and centrifuged (3 min, 14,000*g*) to remove particulates. Proteins were separated by standard SDS-PAGE on 11% (w/v) polyacrylamide gels (Bio-Rad, Richmond, CA) and transferred to nitrocellulose membranes (0.45 µm, Micron Separations, Westborough, MA). Except where indicated, sample volumes loaded on gels were equivalent. Membranes were blocked for 30 min in TBST (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, and 0.2% [v/v] Tween 20) containing 2% (w/v) Carnation non-fat dry milk (Nestle Food Company, Glendale, CA), then incubated in TBST plus 2% (w/v) nonfat dry milk (TBST-milk) containing affinity-purified AtFtsZ1-1 or AtFtsZ2-1 antibodies at dilutions of 1:1,500 and 1:3,000, respectively. Incubations with primary antibody were carried out in Seal-a-Meal bags (Dazey Corp., Century, KS) shaken vigorously overnight at room temperature. After four 10-min washes in TBST, membranes were incubated with horseradish peroxidase-conjugated goat-anti-rabbit secondary antibodies (Fisher, Pittsburgh) for 15 min at 1:4,000 dilution in TBST-milk. Following four 10-min washes in TBST, membranes were developed using Renaissance Western Blot Chemiluminescence Reagent (NEN Life Science Products, Boston) and the signal was recorded on X-OMAT 1s film (Kodak, Rochester, NY).

Competition Binding Assays

To determine antibody specificity, immunoblotting experiments were performed as described above, except that prior to probing membranes, each diluted antibody was preincubated for 2 to 4 h in TBST-milk with an equimolar amount of purified, recombinant AtFtsZ1-1 (residues 41-269) or AtFtsZ2-1 (residues 92-282) on a rocking platform. These truncated versions of AtFtsZ1-1 and AtFtsZ2-1 were obtained by expressing the corresponding cDNA fragments in the expression vector pJC40 (Clos and Brandau, 1994) as 10-histidine-tagged FtsZ fusion proteins in *E. coli* BL21(λDE3)/plysS cells, following induction with isopropylthio-β-galactoside (1 mM) at 37°C. The recombinant proteins were purified from inclusion bodies in cell lysates using metal chelation chromatography (His-Bind Resin, Novagen, Madison, WI) according to the manufacturer's protocol for denaturing conditions. Protein concentrations of truncated AtFtsZ1-1 or AtFtsZ2-1 following chromatography, determined using the Bio-Rad Protein Assay reagent (Bio-Rad), were 1.75 and 1.24 mg ml^{-1}, respectively.

AtFtsZ1-1 Quantification

Quantification of FtsZ protein levels was performed by scanning the film used for chemiluminescent detection of signals on immunoblots into the computer using a Mirage IIse imager (UMAX Technologies, Fremont, CA) and Binuscan software (Binuscan, New York). Densitometry measurements were used to quantify the levels of AtFtsZ1-1 from a standard curve prepared by evaluating the intensities of the 40-kD polypeptide in four lanes loaded with increasing amounts of an AtFtsZ1-1-containing plant extract prepared from a control plant transformed with the empty pART27 vector. A best-fit curve

calculated from the data had an R^2 value of 0.98. This curve was used to calculate the relative amount of protein in the other samples, which were loaded so that the signal generated on the immunoblot was in the linear range of the standard curve. The level of AtFtsZ1-1 protein in each extract was calculated relative to the control sample.

Acknowledgements

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Chapter 3 - Supplemental Data

AtFtsZ2-2 Cosuppression Inhibits Chloroplast Division

Background

Wild-type Arabidopsis plants express three different FtsZ proteins, AtFtsZ1-1, AtFtsZ2-1 and AtFtsZ2-2, which are localized to the chloroplast stroma (McAndrew et al., 2001). Previous research indicated overexpression of *AtFtsZ1-1* inhibited chloroplast division (Stokes et al., 2000). Attempts to overexpress *AtFtsZ2-1* did not result in any inhibition of chloroplast division. However, immunoblots indicate that only slight increases in protein expression were obtained with the transgenic plants. In the *AtFtsZ2-1* transgenic plants in which chloroplast division was inhibited, the amount of AtFtsZ2-1 protein was reduced due to cosuppression. At the time the overexpression experiments were published by Stokes et al. (2000) the *AtFtsZ2-2* gene had not been isolated and characterized. Since that report the *AtFtsZ2-2* transcript has been isolated and reported (McAndrew et al., 2001). In an effort to determine the effect of increased AtFtsZ2-2 protein on chloroplast division, plants were prepared with an *AtFtsZ2-2* overexpressing transgene. Most of the transgenic plants had a normal chloroplast phenotype, but FtsZ protein levels were similar to those in wild-type plants. Severe inhibition of chloroplast division was only observed in transgenic plants where AtFtsZ2-1 and AtFtsZ2-2 levels were both reduced.

Material and Methods

Preparation of *AtFtsZ2-2* Overexpressing Construct

Total RNA was isolated from 19-day-old soil grown plants as described by McAndrew et al. (2001). Reverse transcription (RT) reactions were performed using the Superscript II (Gibco-BRL) enzyme as instructed by the manufacturer, with 10 µg of total RNA in a 30 µL reaction volume and the reverse primer 5'-AGTGGGTCTAGAGGCGAGGA. PCR amplification of the *AtFtsZ2-2* gene was performed on 600 ng of RNA from the above RT reaction using the forward primer 5'-CAGAATGGCAGCTTATGTTTCTCC and the reverse primer used in the RT reactions. The amplified *AtFtsZ2-2* gene was cloned into pBuescript digested with *Sma*I. The resulting plasmid, called pKS135, was digested with *Xba*I and the fragment containing the *AtFtsZ2-2* gene was purified and cloned into the shuttle vector pART7 (Gleave, 1992) that had been digested with the same enzyme. This plasmid, called pKS168, was digested with *Not*I and the fragment with the *AtFtsZ2-2* insert cloned into pART27 (Gleave, 1992) that had been digested with the same enzyme. The final plasmid was called pKS170.

Plant Transformation and Screening for Kanamycin Resistant Plants

The pKS170 plasmid was purified from *Escherichia coli*, transferred to *Agrobacterium tumefaciens* GV3101 (Koncz and Schell, 1986), and then transformed into *Arabidopsis thaliana* ecotype Columbia (Col-0) plants by floral dip (Clough and Bent, 1998). T_1 and T_2 seed were sown on MS plates as described by Stokes et al. (2000), except 50 mg/L kanamycin was used for selection of transgenic plants. After the

transfer of transgenic plants to soil, the growth conditions, microscopic analysis, and immunoblotting procedure were as described by Stokes et al. (2000).

Results

Transgenic plants were generated with a construct designed to overexpress *AtFtsZ2-2* in an effort to determine the effect of increased AtFtsZ2-2 on chloroplast division. Numerous T_2 plants from three independently transformed lines (Figure 6; transgenic lines A, B, C) were analyzed for both their chloroplast phenotypes in mesophyll cells and FtsZ protein levels. When the transgenic plants are compared to wild-type plants (Fig 6; Wt), the transgenic plants grouped into three different FtsZ protein profiles and chloroplast phenotype combinations. One group of transgenic plants had a normal chloroplast phenotype and normal levels of AtFtsZ1-1, AtFtsZ2-1, and AtFtsZ2-2 protein (Figure 6, lanes 2-5). Although the four plants in this group are from the same transgenic line, this FtsZ protein profile and chloroplast phenotype was the most commonly observed among all the transformed lines that were tested (data not shown). A second group of plants had decreased levels of AtFtsZ2-2, but near-normal levels of AtFtsZ1-1 and AtFtsZ2-1 (Figure 6, lanes 8-10). These plants, from two independent lines, also had normal chloroplast phenotypes. The third group had a severe chloroplast phenotype, which was characterized by one very enlarged chloroplast per cell. FtsZ protein profiles for these plants had reduced AtFtsZ2-2 and AtFtsZ2-1 protein levels, but near normal AtFtsZ1-1 levels (Figure 6, lanes 6 and 7). Plants with this severe chloroplast phenotype were only observed in one transgenic line (data not shown).

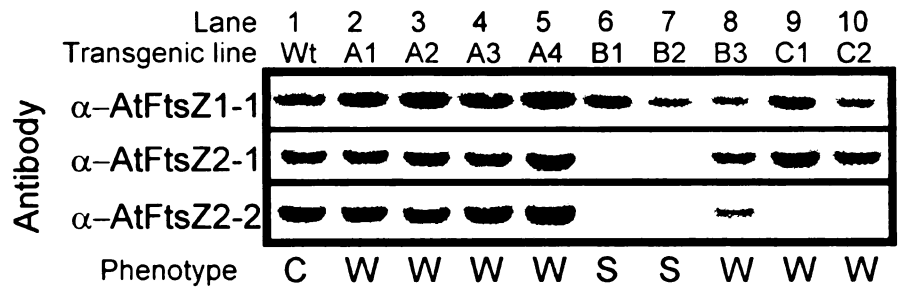

Figure 6. Immunoblot analysis of transgenic plant extracts expressing the *AtFtsZ2-2* transgene.

Protein extracts from T_2 transgenic plants were separated by SDS-PAGE, transferred to PVDF, and probed with antipeptide antibodies raised against AtFtsZ1-1, AtFtsZ2-1, or AtFtsZ2-2. Samples are from three independent transformed lines (A, lanes 2-5; B, lanes 6 and 7; C, lanes 8-10): lane 1, Wild-type control (C); lanes 2-10, transgenic plants with wild-type-like (W, lanes 2-5 and 8-10) or severe (S, lanes 6 and 7) phenotypes. Equal amounts of extract were loaded on four gels and confirmed by Coomassie staining one of those gels (data not shown).

Discussion

Instead of increased amounts of AtFtsZ2-2 protein, transgenic plants with the overexpressing *AtFtsZ2-2* construct had near normal or reduced FtsZ protein levels. The only lines in which chloroplast division was severely inhibited had undetectable levels of AtFtsZ2-1 and AtFtsZ2-2, indicating the chloroplast phenotype is a result of cosuppression. A few plants had reduced AtFtsZ2-2 levels and near-normal levels of AtFtsZ1-1 and AtFtsZ2-1, but chloroplast division did not seem to be affected. This result indicates that a reduction in AtFtsZ2-2 protein levels by itself does not inhibit chloroplast division. A recently isolated *AtFtsZ2-2* knockout plant that has no detectable AtFtsZ2-2 protein but normal levels of AtFtsZ2-1 protein supports this result (unpublished results). The chloroplast phenotype in the mesophyll cells of this AtFtsZ2-2 knockout plant is intermediate, with about 15 enlarged chloroplasts. The intermediate phenotype indicates there is some chloroplast division, but the frequency of chloroplast division has been reduced. The most common phenotype among the transgenic lines was normal FtsZ protein levels and normal chloroplast size and number. Although the exact reason for this result is unclear, it may be that expressing *AtFtsZ2-2* with a strong promoter is detrimental to plant viability. Cosuppression that resulted in inhibition of chloroplast division was also observed when *AtFtsZ2-1* was overexpressed with a similar construct (Stokes et al., 2000). However, overexpression of *AtFtsZ2-1* was achieved using the native promoter (McAndrew et al., 2001). Therefore, overexpression of AtFtsZ2-2 may also require a construct that uses the native *AtFtsZ2-2* promoter.

CHAPTER 4

Coordinate expression of the *FtsZ1* and *FtsZ2* genes in *Arabidopsis thaliana* plants

Abstract

The Arabidopsis genome encodes three chloroplast-localized FtsZ proteins that belong to two different families, FtsZ1 and FtsZ2. Members of both families perform roles in chloroplast division but little is known about their developmental patterns of expression. We used *β-glucuronidase* (GUS) reporter gene assays, real-time reverse transcriptase-polymerase chain reaction, and immunoblot assays to investigate RNA and protein expression patterns for all three Arabidopsis *FtsZ* family members. Analysis of GUS reporter staining patterns as well as the FtsZ transcript distribution indicates that the *FtsZ1* and *FtsZ2* genes are coordinately expressed throughout the plant. A high level of *FtsZ* gene expression occurs in the young expanding leaves but expression is reduced in the older, fully expanded leaves. These expression patterns correspond to reported patterns of plastid division (Pyke and Leech, 1992; Pyke, 1997), and are consistent with the role of the FtsZ's in plastid division. Measurements of the *FtsZ* transcript amounts indicate *AtFtsZ2-1* is the most abundant, followed by *AtFtsZ1-1*, and *AtFtsZ2-2*. In addition, the ratio of *FtsZ1* to *FtsZ2* transcript is constant throughout leaf development.

Introduction

Plastid division is important for the maintenance of plant photosynthetic competence. The chloroplast complement of a cell results from division of undifferentiated proplastids and green chloroplasts (Chaly and Possingham, 1981; Whatley, 1993; Robertson et al., 1995; Pyke, 1997). Proplastid division in root and shoot meristems is likely important for maintaining proplastid numbers in these rapidly dividing cell populations (Possingham and Lawrence, 1983), while chloroplast division in monocot and dicot leaves is correlated with cell expansion (Boasson et al., 1972; Possingham and Smith, 1972; Possingham and Lawrence, 1983; Pyke et al., 1994; Robertson et al., 1996). However, the region of the leaf where chloroplast division occurs differs between monocots and dicots. In the monocot wheat, chloroplast division occurs in a relatively small region of the leaf where cell expansion is greatest (Leech and Pyke, 1988; Pyke, 1997). In contrast, chloroplast division in Arabidopsis, a dicot, occurs over a longer period of cell development and expansion (Pyke and Leech, 1992; Pyke, 1997). This makes the region where chloroplast division occurs less pronounced in Arabidopsis than in wheat leaves. However, most chloroplast division still occurs in cells in the basal portion of both monocot and dicot leaves (Possingham and Smith, 1972; Possingham, 1973; Leech et al., 1981; Pyke et al., 1991). In Arabidopsis dividing chloroplasts have also been observed at the base of developing petals and in the rapidly expanding cotyledons (Pyke, 1997; Pyke and Page, 1998). It is in the tissues where plastid division is actively occurring that we anticipate chloroplast division genes, like *FtsZ*, to be expressed.

In bacteria, FtsZ is a cytoskeletal GTPase protein with structural homology to the eukaryotic tubulins that localizes to a ring at the cell division site (Bi and Lutkenhaus, 1991; Bramhill, 1997; Lutkenhaus and Addinall, 1997; Löwe and Amos, 1998). Most bacteria encode a single FtsZ protein, whereas plants contain multiple nuclear encoded copies of the FtsZ proteins. The plant FtsZ proteins have been grouped into two families, FtsZ1 and FtsZ2, based on conserved molecular features (Osteryoung et al., 1998). Members of both families are localized to rings at the chloroplast division site inside the stroma (Osteryoung et al., 1998; Gaikwad et al., 2000; Fujiwara and Yoshida, 2001; McAndrew et al., 2001), and are required for chloroplast division (Osteryoung et al., 1998; Strepp et al., 1998). In Arabidopsis, there are three *FtsZ* genes, one *FtsZ1* gene, *AtFtsZ1-1*, and two *FtsZ2* genes, *AtFtsZ2-1* and *AtFtsZ2-2*. Expression of *AtFtsZ1-1* and *AtFtsZ2-1* has been reported in Arabidopsis leaves by detecting the proteins by immunofluorescence microscopy as well as immunoblot analysis (Stokes et al., 2000; McAndrew et al., 2001; Vitha et al., 2001). No information regarding the expression of the third Arabidopsis FtsZ gene *AtFtsZ2-2* has been reported except that the gene product is imported into the chloroplast (McAndrew et al., 2001). Very little has been reported about patterns of *FtsZ* expression in Arabidopsis.

Although roles for both *FtsZ1* and *FtsZ2* genes in chloroplast division have been established (Osteryoung and Vierling, 1995; Osteryoung et al., 1998; Strepp et al., 1998), the relationship of their expression patterns to the patterns of plastid division during development has not been determined. In marigold (*Tagetes erecta* L.) an *FtsZ1* transcript was isolated from petals and was expressed at a higher level in the petals than in leaves (Moehs et al., 2000). There is some evidence indicating that *FtsZ* expression

responds to light in cucumbers and peas (Gaikwad et al., 2000; Ullanat and Jayabaskaran, 2002), cytokinin treatment in cucumbers (Ullanat and Jayabaskaran, 2002) and to the cell cycle in tobacco (El-Shami et al., 2002). However, the studies with cucumber investigated the expression of only one, of several, *FtsZ2* genes (Ullanat and Jayabaskaran, 2002), while the study in peas involved only an *FtsZ1* gene (Gaikwad et al., 2000). The tobacco study investigated one, of several, *FtsZ1* genes and one, of two, *FtsZ2* genes (El-Shami et al., 2002). Furthermore, the significance of cell cycle-dependent FtsZ expression is unclear since the study used a non-photosynthetic tobacco cell culture (El-Shami et al., 2002). None of these studies investigated the expression patterns of both *FtsZ1* and *FtsZ2* or their relationship to FtsZ protein levels and patterns of plastid division in the whole plants.

In an effort to better understand the expression of the *FtsZ* genes in Arabidopsis, experiments were performed that included promoter fusions, transcript quantification, and immunoblot analysis. To understand the spatial and temporal expression of the three Arabidopsis *FtsZ* genes, we used the promoters from each to drive expression of the *β-glucuronidase* (*GUS*) reporter gene in transgenic Arabidopsis plants. We report here that the GUS staining pattern, in conjunction with transcript quantification, indicates the three Arabidopsis *FtsZ* genes are coordinately expressed in roots, stems, shoot apex, and expanding leaves. Measurements of the relative levels of the three *FtsZ* gene transcripts indicate *AtFtsZ2-1* is the most abundant, followed by *AtFtsZ1-1*, and *AtFtsZ2-2*. Furthermore, the ratio of *FtsZ1* to *FtsZ2* transcripts remains constant throughout leaf development. These results indicate that expression of the *FtsZ* genes is coordinated and that their relative stoichiometric ratio may be important for chloroplast division.

Materials and Methods

Determination of the FtsZ's 5'-UTR Using 5'-RACE

Amplification of the 5'-UTR for the *FtsZ* genes was performed using the FirstChoice RLM-RACE kit (Ambion, Austin, TX) as described by the manufacturer using 10 ug of total RNA that had been isolated from 19-day-old *Arabidopsis thaliana* ecotype Columbia (Col-0) plants. Two serial PCR amplification reactions utilized nested forward primers provided in the kit with sequence-specific nested reverse primers. The reverse primers for the *AtFtsZ1-1* gene were, in order of use, 5'-CGCATAGAAATCAACACTCTG and 5'-AACGGCATTGTTACCACCAC. The 5'-UTR of the *AtFtsZ2-1* gene was amplified with the reverse primers 5'-ACCACCTCCCACACCAATAACC and 5'-AGTCCCTTCACCTCTAAGCAT. The reverse primers for the *AtFtsZ2-2* gene were 5'-TGAACCACCACCTCCAACGCC and 5'-AGTAGATAACTCATCCAAATCCTC. The amplified products were gel-purified and ligated into pBluescript II KS (Stratagene) for sequencing.

Construction of the *FtsZ Promoter-GUS* Constructs

All promoter constructs were made using pKS1207, a gift from Dr. Dean Dellapenna (Michigan State University). pKS1207 is a derivative of pART27 (Gleave, 1992) in which the pBI101 *GUS* gene has been insterted into the *Not*I restriction site. The *GUS* gene was amplified from pBI101 with the forward primer 5'-AGTCGGCCGAAGCTTGCATGCCTGCAGGTC and the reverse primer 5'-TGCCGGCCGGAATTCCCGATCTAGTAACAT, each primer has an *Eag*I restriction

site engineered at the end. The PCR-amplified *GUS* gene was digested with the enzyme *Eag*I and ligated into pART27 that had been digested with the *Not*I enzyme. The final pKS1207 plasmid was sequenced for accuracy.

Plants transformed with pKS1207 served as a promoterless control. The promoter for *AtFtsZ1-1* was amplified from genomic Arabidopsis DNA using the primers 5'-GAAGGATCCCAGACACTTTCTC (forward) and 5'-GTTTACTTCCTCTGCTTTCAGAGAAG (reverse). The amplified product was ligated into a *Sma*I-digested pBluescript II KS (Stratagene, La Jolla, CA) vector. The promoter was isolated from the resulting plasmid by digestion with *Eco*RI, the overhangs were then filled in with Klenow, and then digested with *Xba*I. The isolated *AtFtsZ1-1* promoter fragment was then directionally ligated into pKS1207 that had been digested with *Hin*dIII, the overhangs filled in, and then digested with *Xba*I. The resulting plasmid, pKS174, contains 1679 bp of *AtFtsZ1-1* promoter sequence, but sequencing indicated one base pair immediately upstream of the *AtFtsZ1-1* start codon (position –1 of the promoter) was incorrect. The reporter gene for *AtFtsZ2-1* was constructed similarly, except that the primers 5'-TTCTCTGCTCTCTTGATGATCA (forward) and 5'-AATGAGACCAATCACTGCAGG (reverse) were used for promoter amplification. The final construct, pKS154, contains 1775 bp of the *AtFtsZ2-1* promoter sequence. For the *AtFtsZ2-2* reporter gene, the primers 5'-TCTCGAAACGTTTATGCCAT (forward) and 5'-TCTGAGACTACAGAAGCAACCAAA (reverse) were used to amplify the promoter region, which was cloned into pBluescript as described above. The fragment with the *AtFtsZ2-2* promoter was excised from the resulting plasmid by digestion with *Hin*dIII and *Xba*I and then directionally cloned into pKS1207 that had been digested with the same

enzymes. The final plasmid, pKS156, contains 1311 bp of the *AtFtsZtsZ2-2* promoter sequence. The promoters in pKS154 (*AtFtsZ2-1*) and pKS156 (*AtFtsZ2-2*) were sequenced to confirm sequence integrity.

Plant Transformation and Growth

The plasmids pKS1207, pKS174, pKS154, and pKS156 were purified from *Escherichia coli*, transferred to *Agrobacterium tumefaciens* GV3101 (Koncz and Schell, 1986), and then transformed into *Arabidopsis thaliana* ecotype Columbia (Col-0) plants by floral dip (Clough and Bent, 1998). T_1 and T_2 seed were sown on MS plates as described by Stokes et al. (2000), except 50 mg/L kanamycin was used for selection of transgenic plants. After transferring the transgenic plants to soil, growth conditions were as described by Stokes et al. (2000). For etiolated seedlings, seeds were grown on plates that were treated identically to the light-grown seedlings, except the plates were covered with aluminum foil. The age of the plants was taken from the date of plate transfer to the growth chamber, following 2 days of cold treatment. After 8 days in the growth chamber, etiolated and light-grown seedlings were harvested for GUS staining and kan^r plants were transferred to soil. Whole plants and inflorescence tips were harvested for histochemical staining after growing for 19 or 32 days, respectively.

GUS Staining

The GUS staining proceedure is adapted from protocols by Jefferson et al. (1987) and Rodrigues-Pousada et al. (1993). Harvested tissue was immediately fixed in 90% acetone for 15 minutes, then rinsed in wash solution (50 mM $NaPO_4$ pH 7.2, 0.5 mM

$K_3Fe(CN)_6$, and 0.5 mM $K_4Fe(CN)_6$) five times, 15 minutes each wash. The wash solution was then replaced with stain solution (wash solution, 0.1% Triton X-100, and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide [X-Gluc]). Before addition to the stain solution the X-gluc (ANGUS Buffers and Biochemicals, Niagara Falls, NY) was dissolved in N-N-dimethylformamide to a concentration of 100 mM. After the stain solution was added to the tissue, the samples were briefly placed under vacuum then incubated overnight at 37°C. Tissue was then cleared with several changes of 70% ethanol and stored in 50 mM $NaPO_4$ pH 7.2 at room temperature. GUS expression analysis was performed on three independently transformed lines for each construct. Only one representative line for each construct is reported, since the staining pattern for each of the three tested lines was the same.

Detecting *GUS* Transcript by RT-PCR

Seeds were grown on MS plates with 50 mg/L kanamycin, or without kanamycin for wild-type seed, as described above. RNA was isolated from the shoots of 10-day-old plate-grown seedlings using the RNeasy plant mini kit (Qiagen, Valencia, CA) as directed, except about 400 mg of tissue was used and volumes of the kit solutions RLT, RPE, and ethanol were doubled from that described in the first five steps of the manual. Contaminating genomic DNA was removed by treating the RNA samples with DNase, then re-isolating the RNA with the kit described above. Reverse transcription (RT) reactions were performed using the Superscript II (Gibco-BRL) enzyme as instructed by the manufacturer, with 15 µg of total RNA in a 60 µL reaction volume and the reverse primers 5'- TGATCCCATTTTGTCAAGGAGTT (*AtFtsZ1-1*) and 5'-

TTCGTTGGCAATACTCCACA (GUS). PCR amplification of a 382 bp *AtFtsZ1-1* gene fragment used the *AtFtsZ1-1* reverse primer indicated above with the forward primer 5'-TTGCAGATGTGAAGGCAGTC. PCR amplification of a 467 bp *GUS* gene fragment used the *GUS* reverse primer indicated above with forward primer 5'-TGCAACTGGACAAGGCACTA.

Since the *GUS* gene used in our constructs does not have any introns, the amplified fragments from RNA or contaminating genomic DNA would yield the same size product. Therefore, to test for contamination by genomic DNA, we also amplified a fragment of the *AtFtsZ1-1* gene, which has introns that are removed from the sequence. Another purpose of the *AtFtsZ1-1* control was to confirm whether endogenous *AtFtsZ1-1* transcript levels could be detected in the samples. Isolation of genomic DNA for use in the PCR reactions was done as described by Neff et al. (1998).

Immunoblot Analysis of the FtsZ Proteins in Roots

Protein extracts were prepared from 19-day-old Arabidopsis ecotype Columbia (Col-0) plants as described previsously (Stokes et al., 2000). Total protein in each sample was measured using the RC DC Protein Assay kit (Bio-Rad, Hercules, CA) as directed by the manufacturer. Four polyacrylamide gels were loaded with equal amounts of total protein and the extracts separated by SDS-PAGE. Comparison of the loaded protein samples was visually determined by staining one of the gels with Coomassie Brilliant Blue R250. Immunoblot analysis was performed according to procedures described by Vitha et al. (2001).

Plant Material for Real-Time RT-PCR Analysis

Wild-type Arabidopsis ecotype Columbia (Col-0) seed was sown in soil, vernalized 2 days at 4°C, and then grown in growth chambers under the same conditions as above. After 19 days, the plants were harvested and tissue was separated into three fractions consisting of the first and second pair of leaves, the third leaf pair, and the remaining tissue (which included the fourth leaf pair, shoot apex, stems, and some root fragments). The separated tissue was immediately frozen in liquid nitrogen.

Nucleic Acid Isolation for Real-Time RT-PCR Analysis

The separated and frozen tissue, described above, was ground in a mortar and pestle that had been pre-chilled with liquid nitrogen. Total RNA was isolated from the ground tissue as described by McAndrew et al. (2001). Isolated RNA was treated with RNase-free DNase (Promega, Madison, WI) and then extracted with phenol/chloroform to remove proteins. Isolated RNA was again treated with DNase and then purified on RNeasy plant mini kit (Qiagen, Valencia, CA) as directed. These RNA samples were used as the template for the real-time RT-PCR analysis of *AtFtsZ1-1*, *AtFtsZ2-1* and *AtFtsZ2-2* transcript levels.

Real-Time RT-PCR Analysis

Reverse transcription (RT) of 5 μg total RNA was performed using the Taqman® RT-PCR Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's directions. Amplification and detection of *AtFtsZ1-1* transcript during the Taqman®

assay involved the primers 5'-CCACAGGCTTCTCTCAGTCATTC (forward) and 5'-TGATCCCATTTTGTCAAGGAGTT (reverse) along with the Taqman® probe 5'-AGAAGACACTTCTGACTGATCCAAGAGCAGCT, at concentrations of 300 nM, 300nM, and 150 nM respectively. The *AtFtsZ2-1* transcripts were detected with the primers 5'-GCTACGGGTTTCAAACGACAA (forward) and 5'-AGCTCCAACTGACGCAGCAT (reverse) with the Taqman® probe 5'-AGAAGGACGAACAGTTCAGATGGTACAAGCA, at concentrations of 900 nM, 300 nM, and 150 nM respectively. For detection of *AtFtsZ2-2* transcripts, primers 5'-GAAGGAGAAGGGAGGCCACT (forward) and 5'- GACGTCTTGTGGCTCCCATT (reverse) were used with the Taqman® probe 5'- CAGGCGACACAAGCGGATGCAT at concentrations of 900 nM, 300 nM, and 150 nM respectively. The three Taqman® probes were synthesized and labeled with the 5' fluorescent reporter dye FAM (6-carboxyfluorescein) and the 3' quencher dye TAMRA (6-carboxy-N,N,N',N'-tetramethylrhodamine) by the manufacturer (Applied Biosystems). Primers, probe, and optimized conditions for EF1α detection were as described by Tian and DellaPenna (2001). Real-time RT-PCR reactions used 200 ng of total RNA from the RT reactions, gene-specific primers and probe, and Taqman® Universal PCR Master Mix (Applied Biosystems) as described in the kit's manual. Optimization of primer and probe concentrations was done as directed by the manufacturer. Quantification of transcripts for each gene in the samples was based on a comparison to a calibration curve consisting of a dilution series of a plasmid containing the cDNA for the gene of interest. Measurements were repeated on plant samples taken from four biological replications.

Transcript amounts were either compared based on total RNA or on the *EF1α* transcript amount for each sample in each biological replication.

Results

The Structures of the 5'-UTR Differ Between the *FtsZ* Genes in Arabidopsis

Before the promoter expression experiments were initiated, there was some question as to the sequence of the *FtsZ* genes in Arabidopsis. Specifically, the start codon for the *AtFtsZ2-1* gene was in question. To identify the correct start codon of each *FtsZ* gene we determined the sequence of each 5'-UTR (untranslated region) by 5'-RACE (rapid amplification of cDNA ends). The 5'-RACE experiments confirmed the start codon for each Arabidopsis *FtsZ* gene and determined the *AtFtsZ2-1* cDNA used in the overexpression construct described in chapter 3 (Stokes et al., 2000) was truncated. Subsequently, a full length *AtFtsZ2-1* cDNA was isolated and characterized (McAndrew et al., 2001). Although the 5'-RACE experiments were important in determining the full-length transcripts (McAndrew et al., 2001), the sequence of the 5'-UTR's were not reported.

Figure 1 diagrams the structure the *AtFtsZ1-1*, *AtFtsZ2-1*, and *AtFtsZ2-2* primary transcripts upstream of the start codon. The 5'-UTR of *AtFtsZ1-1* extends 89 bp upstream from the translation start site, and does not contain any introns. The *AtFtsZ2-1* gene has a 452 bp intron that is removed, to produce a final 5'-UTR of 164 bp. The 5'-UTR of the *AtFtsZ2-1* gene has been determined by Fujiwara and Yoshida (2001) who reported a similar length and the removal of same 5'-UTR intron. Introns were also detected in the 5'-UTR region of the *AtFtsZ2-2* gene, but in this case three differentially

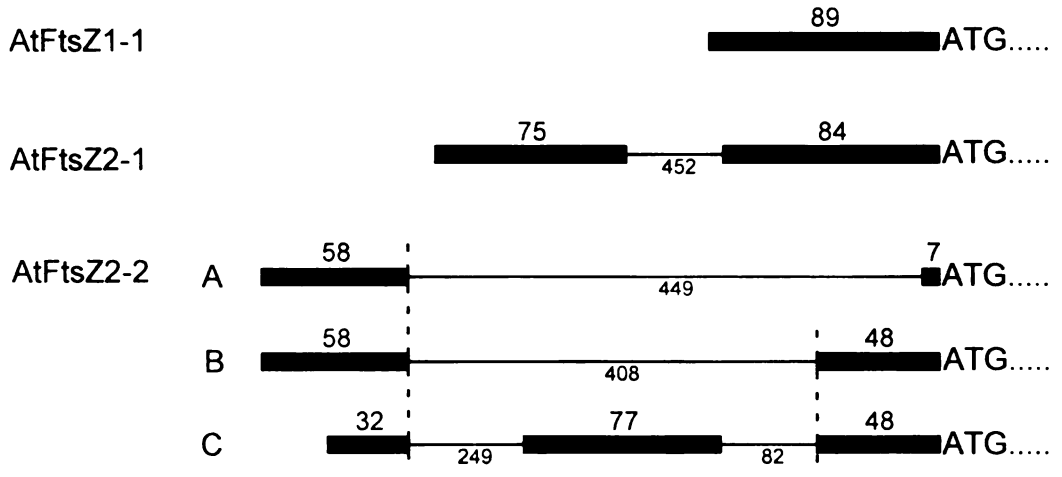

Figure 1. Genetic structure of the 5'-UTR region of the three *FtsZ* genes.

The exon and intron structure of the 5'-UTR region for each *FtsZ* gene up to the translation start site (ATG) are diagramed. Exons are represented as bars and are drawn to scale with the size (bp) above, while introns are shown as lines with their size (bp) below, but are not drawn to scale. Three *AtFtsZ2-2* transcripts that have differentially spliced 5'-UTR regions (AtFtsZ2-2 A, B, and C) were isolated. Dashed lines indicate junctions common among the *AtFtsZ2-2* species.

spliced species were isolated (Figure 1; AtFtsZ2-2A, B, and C). One of these (A) has a 449 bp intron that is removed, while another species (B) has a 408 bp intron that is removed. In contrast to the first two, species (C) has two removed introns. The intron junctions nearest the 5' end of the UTR's are identical in all three *AtFtsZ2-2* species, as well as the intron junction nearest the start codon of species B and C (Figure 1, dashed lines). The results indicate that both *AtFtsZ2-1* and *AtFtsZ2-2* transcripts have introns that are removed from the 5'-UTR region whereas the *AtFtsZ1-1* does not.

GUS Reporter Gene Expression Patterns

To study the expression patterns of the three Arabidopsis *FtsZ* genes, we generated transgenic plants expressing a *GUS* reporter gene under control of the *AtFtsZ1-1* (pKS174), *AtFtsZ2-1* (pKS154), or *AtFtsZ2-2* (pKS156) promoter. The promoter regions were chosen based on the results of the 5'-UTR analysis, which confirmed the position of the start codon for each *FtsZ* gene. To analyze the developmental and tissue-specific expression patterns of the different *FtsZ* genes, transgenic plants at various stages of development were histochemically stained for GUS expression (Figure 2). Both light-grown and etiolated whole seedlings were stained at 8 days, whole plants at 19 days, and floral shoots at 32 days. No GUS staining was detected in transgenic plants with a promoterless *GUS* construct (pKS1207), which served as a negative staining control (Figure 2, A0-H0).

Transgenic plants expressing the *AtFtsZ2-1* and *AtFtsZ2-2* reporter construct exhibited identical patterns of GUS staining (Figure 2, A2-I2 and A3-I3 respectively). Expression was observed in the roots, shoot apex, and cotyledons of etiolated seedlings

Figure 2. Histochemical localization of FtsZ-promoter activity during development of transgenic Arabidopsis.

Histochemical GUS staining in Arabidopsis plants transformed with constructs containing no promoter (A0-H0), the *AtFtsZ1-1* promoter (A1-H1), the *AtFtsZ2-1* promoter (A2-H2), or the *AtFtsZ2-2* promoter (A3-H3) fused to the *GUS* reporter gene. Histochemically stained tissue from (A0-A3) 8-day etiolated seedlings; (B0-B3) 8-day light-grown seedlings; (C0-C3) 19-day 1st leaf pair; (D0-D3) 19-day 2nd leaf pair; (E0-E3) 19-day 3rd leaf pair; (F0-F3) 19-day 4th leaf pair; (G0-G3) 19-day root, stem, meristem, and immature leaves; and (H0-H3) 32-day floral shoots with inserts showing an opened floral bud. Note that pollen grains are stained in H1 but not H2 or H3. Bars = 5 mm.
Images in this dissertation are presented in color.

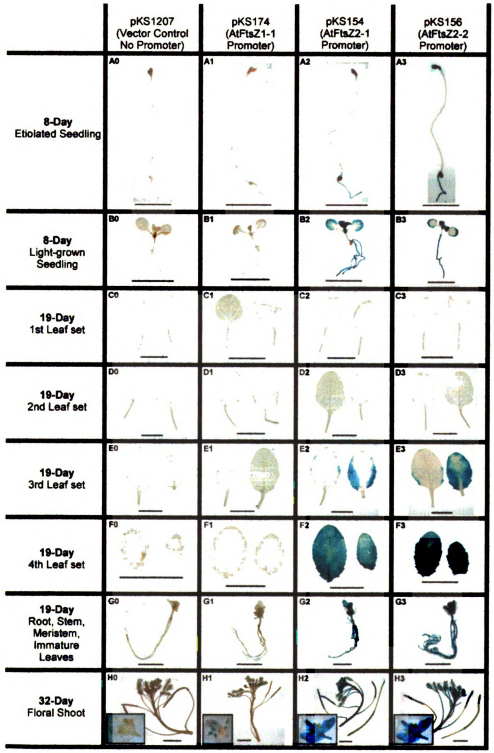
pKS1207 (Vector Control No Promoter)
pKS174 (AtFtsZ1-1 Promoter)
pKS154 (AtFtsZ2-1 Promoter)
pKS156 (AtFtsZ2-2 Promoter)
8-Day Etiolated Seedling
8-Day Light-grown Seedling
19-Day 1st Leaf set
19-Day 2nd Leaf set
19-Day 3rd Leaf set
19-Day 4th Leaf set
19-Day Root, Stem, Meristem, Immature Leaves
32-Day Floral Shoot
A0
A1
A2
A3
B0
B1
B2
B3
C0
C1
C2
C3
D0
D1
D2
D3
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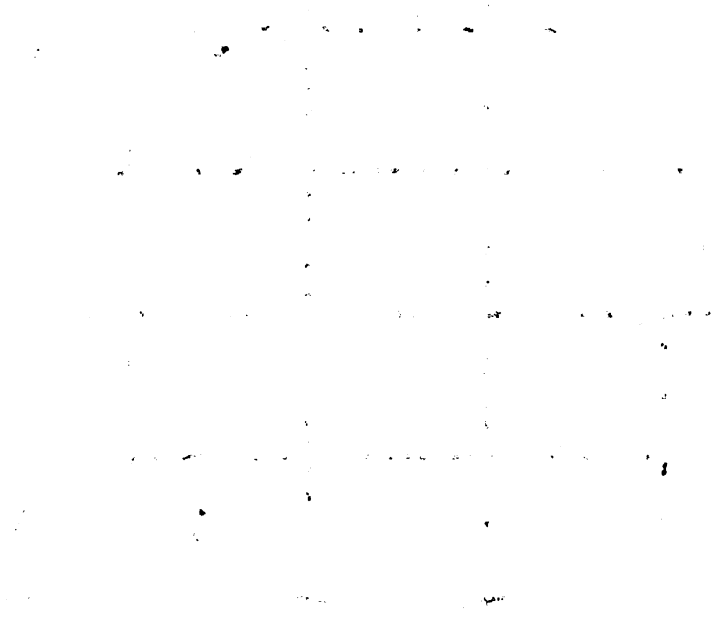
Figure 2



(Fig 2; A2, B2, A3, B3), suggesting light stimulation is not required for the *FtsZ* promoters to drive expression. Intense staining of cotyledons from etiolated seedlings compared to the staining at the margins of light-grown seedling cotyledons indicates the *FtsZ2* promoters drive reporter gene expression prior to or during cotyledon expansion, when there is significant chloroplast division (Pyke, 1997) but expression is reduced when the cotyledons have reached full expansion. The roots, shoot apex, and first leaf pair of light-grown seedlings also have an intense GUS staining pattern, consistent with *FtsZ* expression in tissues with actively dividing proplastids and chloroplasts (Pyke, 1997).

In the 19-day-old plants with the *FtsZ2* promoter constructs, the GUS staining intensity was strongest in the roots, stems, shoot apex, and fourth (youngest) leaf pair (Figure 2; F2, G2, F3, G3). Portions of the third leaf pair were also stained, but the staining was generally restricted to the outside edges of the leaf base (Figure 2, E2 and E3). In the older, fully expanded leaves, very little GUS staining was observed (Figure 2; C2, D2, C3, D3). Therefore, the reporter gene assays suggest that *FtsZ2* genes are expressed at higher levels in young, expanding leaves than in mature leaf tissue.

Much of the floral shoot tissue in plants with the *FtsZ2* promoter constructs (Fig 2, H2 and H3) showed intense staining, including the floral buds. Staining of the petal base indicates *AtFtsZ2-1* and *AtFtsZ2-2* are expressed in this tissue, which has a significant population of cells with constricted chloroplasts (Pyke and Leech, 1992; Pyke, 1997; Pyke and Page, 1998). No staining was observed in the pollen sac or pollen grains of plants with either *FtsZ2 promoter-GUS* construct.

In contrast, transgenic plants with the *AtFtsZ1-1* reporter gene had staining in only the pollen grains (Figure 2, H1 and inset). GUS staining was not observed in the other floral tissues, in the 19-day-old plants, or in either of the seedlings (Figure 2, A1-H1). These results are inconsistent with those of Vitha et al. (2001), who used the same promoter fragment to expression a GFP-tagged AtFtsZ1-1 protein in leaves of transgenic Arabidopsis plants. Furthermore, *AtFtsZ1-1* RNA was also detected in vegetative tissue by real-time reverse transcriptase-polymerase chain reaction (RT-PCR, described below) and AtFtsZ1-1 protein has been detected in leaf extracts (Stokes et al., 2000; McAndrew et al., 2001; Vitha et al., 2001). These data indicate that the staining pattern observed with the GUS reporter construct does not accurately represent expression of the endogenous *AtFtsZ1-1* gene.

The *GUS* Reporter Gene Is Not Transcribed Under Control of the *AtFtsZ1-1* Promoter

One possible explaination for the lack of GUS staining in the transgenic plants with the *AtFtsZ1-1 promoter-GUS* construct could be that expression is below the level required for histochemical detection. Therefore, we performed RT-PCR to determine whether *GUS* transcript could be detected in transgenic seedlings with the *AtFtsZ1-1 promoter-GUS* construct, which would indicate whether the *GUS* reporter gene is being transcribed. Total RNA was isolated for this experiment from 10-day-old plate-grown wild-type Arabidopsis plants, transgenic plants with the *AtFtsZ2-1 promoter-GUS* construct, and two independently transformed lines with the *AtFtsZ1-1 promoter-GUS* construct. Figure 3 shows the RT-PCR-amplified *AtFtsZ1-1* and *GUS* products that were

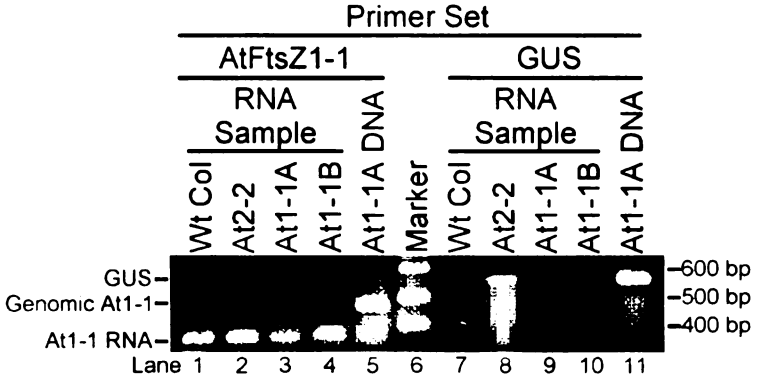


Figure 3. Detection of GUS expression in transgenic Arabidopsis seedlings with the *AtFtsZ1-1 promoter-GUS* fusions.

An agarose gel with the RT-PCR amplified *AtFtsZ1-1* (At1-1, Lanes 1-5) or *GUS* (GUS, lanes 7-11) gene fragments from RNA isolated from 10-day-old seedling shoots of wild-type Arabidopsis (Wt Col, lanes 1 and 7), a transgenic line with the *AtFtsZ2-2 promoter-GUS* construct (At2-2, lanes 2 and 8), and two independently transformed lines with the *AtFtsZ1-1 promoter-GUS* construct (At1-1A, lanes 3 and 9; At1-1B, lanes 4 and 10). PCR amplification of the *AtFtsZ1-1* and *GUS* fragments from genomic DNA from one of the AtFtsZ1-1 promoter lines (At1-1A, lanes 5 and 11) served as a control.

separated on an agarose gel from these plant samples. When primers for *AtFtsZ1-1* are used, PCR amplification of *AtFtsZ1-1* from the genomic DNA results in a product that is 96 bp longer, due to the presence of an intron, than the corresponding fragment amplified from RNA by RT-PCR. Comparison of the fragment amplified from genomic DNA (Figure 3, lane 5) to that from the RNA samples (Figure 3, lanes 1-4) indicates there is no detectable genomic DNA contamination in the RNA preparations. Since the endogenous *AtFtsZ1-1* transcript is detected in all the RNA samples, even those from wild-type Arabidopsis plants, the *GUS* transcript should be detectable if it is present at similar levels.

Samples from wild-type plants were used as a negative control for *GUS* expression, since no *GUS* gene is present in these plants. Expression of *GUS* transcript is not detected in this negative control sample (Figure 3, lane 7). As a positive control, a transgenic plant line with the *AtFtsZ2-1 promoter-GUS* construct was used because expression of GUS in this plant line was detected histochemically. As expected, *GUS* transcript was detected in this positive control sample (Figure 3, lane 8). When two independently transformed *AtFtsZ1-1 promoter-GUS* lines were tested, no *GUS* transcript was detected (Figure 3, lanes 9 and 10), although the *GUS* gene fragment was amplified from genomic DNA (Figure 3, lane 11). These results indicate that the *GUS* gene, although present in the plants, is either not transcribed under the control of the *AtFtsZ1-1* promoter that was used in the *GUS* reporter experiments or is very unstable. Because the construct used by Vitha et al. (2001) utilized the identical promoter to express the GFP-tagged AtFtsZ1-1 protein but included the entire genomic sequence of *AtFtsZ1-1*, these

results suggest that expression of *AtFtsZ1-1* requires regulatory elements located downstream of the 5'-UTR.

Arabidopsis Roots Express FtsZ Proteins

The staining patterns in the *promoter-GUS* experiments indicated the *FtsZ2* genes are expressed in the roots, stems, shoot apex and young leaves of 19-day-old plants. In pea plants, Gaikwad et al. (2000) reported that expression of an *FtsZ1* gene in pea was highest in the leaves but very low in roots and stems. In order to determine if all three Arabidopsis *FtsZ* genes are expressed in roots we performed immunoblot analysis on extracts from roots, shoot apex and stems, and young leaves of wild-type Arabidopsis plants (Figure 4A). For these immunoblot analyses, tissue similar to that shown in Figure 2G but from wild-type plants was divided into two fractions: a root fraction and a shoot fraction containing the stem, shoot apex, and 4th leaf pair. In addition to these two fractions, tissue from the 3rd leaf pair was also analyzed. All three Arabidopsis FtsZ proteins are detected in each of the three tissue fractions. Immunoblot signals for all three FtsZ proteins are strong in the shoot apex and young leaf extracts but the AtFtsZ1-1 and AtFtsZ2-1 protein signals in the roots are significantly less. In fact, the signal for the AtFtsZ2-1 protein in the root extracts was only observed with very long exposure times and cannot be seen in Figure 4.

Samples for the immunoblot analysis were loaded according to equal total protein, but Coomassie Brilliant Blue G250 staining of one gel indicated the amount of total protein in the root extracts was lower than the amount of protein in the extracts from the shoot apex and young leaves (Figure 4B). This loading error is likely due to soil that

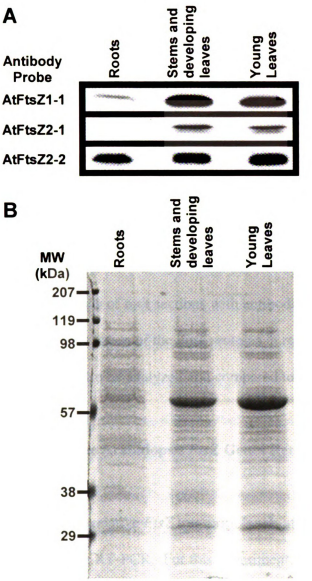


Figure 4. Immunoblot detection of FtsZ protein in root, stem, shoot apex, and immature leaf extracts.

Immunoblot analysis of AtFtsZ1-1, AtFtsZ2-1, or AtFtsZ2-2 proteins was performed on extracts from roots, shoots, and young leaves of 19-day wild-type Arabidopsis plants. Samples were loaded with equal total protein onto four different gels and separated by SDS-PAGE. A, Three of the gels were transferred to PVDF membrane and probed with antibodies specific for AtFtsZ1-1, AtFtsZ2-1, or AtFtsZ2-2. B, the fourth gel was stained with Coomasie Brilliant Blue G250 for visual comparison of the separated proteins.

remained on roots following harvest that caused inaccurate determinations of total protein. Because of this loading error, the amount of FtsZ protein signal in the root extracts cannot be accurately compared to the amount of protein in the other two extracts. This is especially significant for the AtFtsZ2-1 protein in root extracts and may be the reason the signal was so weak.

To obtain a better comparison of the amount of FtsZ protein in root extracts may require the Arabidopsis plants to be grown hydroponically rather than in soil. Hydroponically grown plants are free of the soil that seemed to affect protein assays and may result in more root tissue per plant. An alternative experiment for this section may be immunofluorescence microscopy of root sections with antibodies for each of the FtsZ proteins. Not only would the expression of the FtsZ proteins in roots be determined but their localization patterns could also be analyzed and compared to those in chloroplasts.

Relative Expression Levels of the Arabidopsis *FtsZ* Genes Remain Constant Throughout Leaf Development

We sought to compare the relative *FtsZ* transcript levels at various developmental points using quantitative real-time RT-PCR. For this experiment, tissue from 19-day soil-grown wild-type plants was separated into three pools based on the *FtsZ2 promoter-GUS* experiments representing tissue with high, intermediate, or low GUS staining. The shoot consisted of the fourth leaf pair, shoot apex, stems, and roots that comprised the high GUS-staining tissue (see Figure2, F and G). The young leaves consisted of the third leaf pair and comprised the intermediate GUS staining tissue (see Figure2, E). The old leaves consisted of the first and second leaf pairs and comprised the low GUS staining

tissue (see Figure2, C and D). Total RNA was isolated from each sample and the amount of each *FtsZ* transcript as well as the *EF1α* transcript was determined. The entire experiment was repeated four times. The amount of transcript reported in Figure 5 is an average from all four biological replicates.

The amount of *FtsZ* transcript based on equal amounts of total RNA is diagramed in Figure 5A. The amount of *FtsZ* transcript relative to that of *EF1α* is shown in Figure 5B. In Figure 5A, the amount of *AtFtsZ1-1*, *AtFtsZ2-1*, and *AtFtsZ2-2* transcript in one intermediate GUS-staining sample was more than two standard deviations away from the average of those in the other three biological replicates and was removed from the analysis. However, this sample was included in Figure 5B since the amount of each transcript was not more than two standard deviations from the average of the other three when compared relative to the amount of *EF1α* transcript.

Comparison of transcript levels in each of the tissues indicates the *FtsZ* genes are expressed at different levels, with *AtFtsZ2-1* being the most abundant, followed by *AtFtsZ1-1* and *AtFtsZ2-2* (Fig 5; grey, white, and black bars respectively). Furthermore, the ratio of *FtsZ1* to *FtsZ2* (combining the amounts of *AtFtsZ2-1* and *AtFtsZ2-2*) transcript is 1 to 2.4, 1 to 2.8, and 1 to 3.4 in the high, intermediate, and low GUS-staining tissue pools, respectively. These data suggest that a constant ratio of about one *FtsZ1* transcript to three *FtsZ2* transcripts is present throughout leaf development.

When the concentrations of the *AtFtsZ1-1*, *AtFtsZ2-1*, and *AtFtsZ2-2* transcript were compared among the three tissue pools, each was most abundant in the high GUS-staining pool. Lower concentrations of the three transcripts were observed in the other

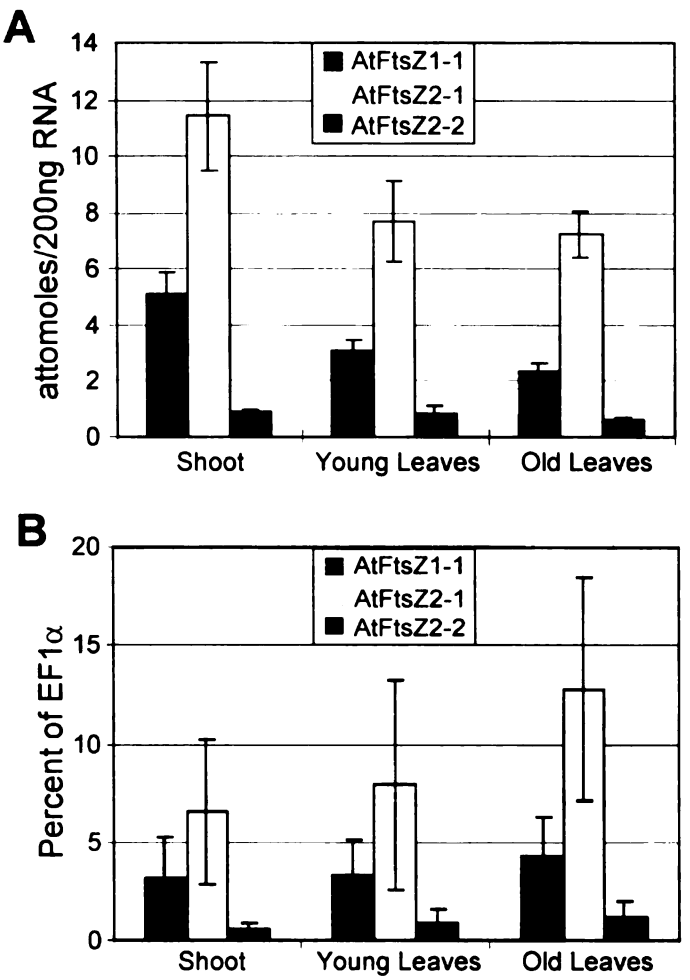


Figure 5. Quantification of *FtsZ* mRNA in various Arabidopsis tissues.

The concentration of *AtFtsZ1-1*, *AtFtsZ2-1*, and *AtFtsZ2-2* transcript was determined by real time RT-PCR in tissue from old leaves (gray bars), young leaves (white bars), and the shoot of the Arabidopsis plant (root, stem, and immature leaves; black bars). These three tissue pools represent plant tissues with low, intermediate, or high GUS staining. A, The reported amount is attomoles of *FtsZ* transcript per 200 ng of total RNA analyzed. B, The amount of *FtsZ* transcript is reported as a percentage of the *EF1α* transcript amount in each sample.

pools. The differences between the high and low GUS-staining pools for each transcript are statistically significant. The distribution of the *AtFtsZ1-1*, *AtFtsZ2-1*, and *AtFtsZ2-2* transcripts correlates with the patterns of GUS expression observed in the transgenic plants with the *FtsZ2 promoter-GUS* constructs.

The amount of *EF1α* transcript was also measured in each of the samples and was to be used for a loading control. The amount of each *FtsZ* transcript was compared as a percentage of the *EF1α* transcript (Figure 5B). Relative to *EF1α*, the *AtFtsZ2-1* transcript is still the most abundant followed by *AtFtsZ1-1* and *AtFtsZ2-2*. However, expressed in this way, the data show greater variation between the biological replications, and the differences between transcript amounts are not statistically significant. Only in the old leaf samples is there any significant difference between the amount of *AtFtsZ2-1*, *AtFtsZ1-1*, and *AtFtsZ2-2* transcript. When the amount of each *FtsZ* transcript is compared among the three pools, they are each more abundant in the old leaf extracts than they are in the extract from the shoot apex pool, contrary to the patterns of GUS expression in the *FtsZ* promoter experiments and to the patterns of cell expansion and chloroplast division (Pyke and Leech, 1987; Pyke et al., 1991; Pyke, 1997). However, because of variations between the biological replications, any trends of *FtsZ* expression between the different tissues are not significant.

Expression of *EF1α* in the three tissue pools, and between the biological replications, seems to vary much more than that of the three *FtsZ* genes. Therefore, *EF1α* seems to constitute a worse internal control than does basing the comparisons on total RNA. There are reasons why *EF1α* may not be a good control. One reason may be that *EF1α* expression may change differently during development than does the

expression of the *FtsZ* genes. Another reason may be the difference in transcript abundance, where *EF1α* is between 10- and 100-fold more abundant than any of the *FtsZ* transcripts. Instead of *EF1α*, the amount of *Geranylgeranyl Diphosphate Synthase 1* (*GGPS1*) or either *β-hydroxylase* 1 or 2 transcript may be a better internal control because their abundance is more similar to that of the *FtsZ* transcripts (Eva Collakova, personal communication), assuming they are constitutively expressed throughout development.

Discussion

Arabidopsis plants express three *FtsZ* genes that belong to two different families and members of both families are required for chloroplast division (Osteryoung et al., 1998; McAndrew et al., 2001). To better understand the functional relationship among the three *FtsZ* genes, we investigated their expression patterns. In the three tissue pools tested, the *AtFtsZ2-1* transcript was most abundant, followed by the *AtFtsZ1-1* and *AtFtsZ2-2* transcripts. Furthermore, for every *FtsZ1* transcript, there were approximately three *FtsZ2* transcripts. These results indicate that the three *FtsZ* genes are expressed at a constant ratio throughout leaf development, and are in reasonable agreement with other analyses in our laboratory showing that FtsZ1 and FtsZ2 protein levels are maintained at a constant 1:2 ratio throughout leaf development (Chi-Ham et al., 2003; McAndrew et al., 2003). The difference between the transcript and protein ratios may be due to differences in translational regulation or transcript and protein turnover rates. Both transcript and protein distribution patterns suggest that the *FtsZ* genes are expressed at a constant stoichiometric ratio, which may be important for chloroplast division.

Microscopic analyses indicate that FtsZ1 and FtsZ2 proteins co-localize to rings at the chloroplast division site (McAndrew et al., 2001; Vitha et al., 2001; Kuroiwa et al., 2002). It is unclear whether these rings are composed of separate FtsZ1 and FtsZ2 homopolymers or if they represent a heteropolymeric structure (McAndrew et al., 2001; Vitha et al., 2001). Although there seems to be a stoichiometric balance among the three *FtsZ* transcripts, the ratio of *FtsZ1* to total *FtsZ2* may be all that is important for chloroplast division (i.e., AtFtsZ2-2 may be functionally redundant). Also, the inhibition of chloroplast division does not seem to be a gene dosage effect since neither the transcripts or the proteins are present in a 1:1:1 ratio. Disruption of the stoichiometric ratio among the FtsZ proteins, and possibly other components of the chloroplast division apparatus, seems to be detrimental to chloroplast division (Osteryoung et al., 1998; Stokes et al., 2000; Vitha et al., 2001).

In leaves, the staining patterns in transgenic plants with either of the *FtsZ2 promoter-GUS* constructs were strongest in young leaves but diminished as the leaves aged. Consistent with this staining pattern, analyses of transcript distribution indicated that *FtsZ2* as well as *FtsZ1* levels are highest in young leaves but are reduced in older leaves. This expression pattern correlates with reported patterns of cell expansion and chloroplast division that are reported for Arabidopsis leaves (Pyke et al., 1991; Pyke and Leech, 1992; Pyke, 1997) and is consistent with the role of FtsZ proteins in chloroplast division.

Transgenic plants expressing either of the *FtsZ2 promoter-GUS* constructs exhibited GUS staining in roots. This was true in light-grown and etiolated seedlings as well as in the 19-day-old plants. In addition, immunoblot analysis detected each of the

FtsZ proteins in root extracts. Although chloroplasts are not present in roots, non-green plastids are present. Therefore, the expression of the *FtsZ* genes in root tissue indicates that FtsZ proteins may also have a function in the division of non-green plastids and in the division of all plastids. However, additional experiments are required to determine if the ratio of the FtsZ proteins and their ring structures are identical in all dividing plastids.

Staining of transgenic plants expressing the *AtFtsZ1-1 promoter-GUS* construct indicated the *GUS* transgene was not transcribed in young seedlings (Figure 3). However, RNA and protein analyses indicate the endogenous *AtFtsZ1-1* gene is expressed in seedlings and throughout the plant (Figure 2, and 3). Vitha et al. (2001) expressed a GFP-tagged version of *AtFtsZ1-1* bearing the same *AtFtsZ1-1* promoter that was used in our study but containing the entire genomic *AtFtsZ1-1* sequence. Fluorescence microscopy detected the GFP-tagged protein in leaves as well as in the root and shoot apices of transgenic Arabidopsis plants (Vitha et al., 2001), personal communication). Together, the data described here and those of Vitha et al. (2001) suggest that sequences downstream of the 5`-UTR are required for proper expression of *AtFtsZ1-1* since expression is not observed with the promoter alone, but is when the full genomic *AtFtsZ1-1* sequence is used. There are several reports of other genes that require sequences downstream of the 5`-UTR for proper expression (Luehrsen and Walbot, 1991; Curie et al., 1993; Donath et al., 1995; Sieburth and Meyerowitz, 1997; Silverstone et al., 1997; Itoh et al., 1999; Kim and Guiltinan, 1999; Kloti et al., 1999). For example, proper expression of the *GA1* gene requires sequences through the first two introns of the coding region (Silverstone et al., 1997).

CHAPTER 5

Stokes KD, Osteryoung KW (2003) Early divergence of the *FtsZ1* and *FtsZ2* plastid division gene families in photosynthetic eukaryotes. Gene, **In Press**

Abstract

Homologues of the bacterial cell division protein FtsZ are found in higher plants where they function as key components of the chloroplast division complex. In contrast to most bacteria that encode a single FtsZ protein, plants encode multiple proteins that group into two families, FtsZ1 and FtsZ2. Using new sequence data from a broad range photosynthetic organisms, we performed a series of analyses to better understand the evolutionary history of the plant FtsZ families. Multiple phylogenetic analyses strongly support the grouping of the plant *FtsZ* genes and proteins into distinct *FtsZ1* and *FtsZ2* clades. Protein features representing potentially significant functional differences between FtsZ1 and FtsZ2 are identified. Genomic structure comparisons show that exon length and intron position are conserved within each clade, but differ between the clades except at one position. Our data indicate that the divergence of the FtsZ1 and FtsZ2 families occurred long before the evolution of land plants, preceding the emergence of the green algae. The results are consistent with proposals that the two FtsZ families evolved distinct functions during evolution of the chloroplast division apparatus, and indicate that genetic and functional differentiation occurred much earlier than previously hypothesized.

Introduction

Cell division in bacteria requires the interaction of multiple proteins at the division site, and the most extensively studied among them is FtsZ (Lutkenhaus and Addinall, 1997). FtsZ is a structural homologue of the eukaryotic tubulins, and is probably their evolutionary predecessor (Erickson, 1998). Like tubulin, FtsZ is a GTPase that polymerizes into filaments *in vitro* (Mukherjee and Lutkenhaus, 1998). *In vivo*, formation of FtsZ into a cytokinetic ring at the midcell division site is the first step in assembly of the bacterial cell division machinery (Lutkenhaus and Addinall, 1997). Several key residues important for FtsZ enzyme activity, polymerization, and interactions with other cell division proteins have been identified (Lutkenhaus and Addinall, 1997; Löwe, 1998; Lu et al., 2001). Of considerable interest are the interactions between a short, conserved stretch of amino acids in FtsZ called the C-terminal domain, and the two division regulators FtsA and ZipA (Ma and Margolin, 1999; Mosyak et al., 2000). This interaction is essential for cell division in *Escherichia coli* (Ma and Margolin, 1999), though ZipA and FtsA are less widespread in bacteria than is FtsZ (Rothfield et al., 1999). In most prokaryotes, a single gene encodes *FtsZ*, but exceptions exist (Margolin and Long, 1994).

Chloroplasts are descendants of an ancient cyanobacterial endosymbiont acquired by a eukaryotic host (Martin and Herrmann, 1998; Gray, 1999; McFadden, 1999), and, like prokaryotes, divide by binary fission. The discovery of a nuclear gene in plants encoding a chloroplast–targeted form of FtsZ that is closely related to the FtsZ sequences in cyanobacteria provided compelling evidence for the prokaryotic ancestry of the chloroplast division machinery (Osteryoung and Vierling, 1995). Subsequently, plant

FtsZ proteins were shown to be essential for chloroplast division (Osteryoung et al., 1998; Strepp et al., 1998), indicating that chloroplasts have retained functional components of the prokaryotic cell division machinery during their evolution. In contrast, FtsZ no longer functions in the division of fungal, plant, or animal mitochondria, which descended from an ancient α-proteobacterium (Gray, 1999). However, the recent discovery of α-proteobacterial-like, mitochondrial-targeted FtsZ proteins in several primitive unicellular eukaryotes strongly suggests their presence in the endosymbiotic ancestor of mitochondria (Beech et al., 2000; Takahara et al., 2000; Gilson and Beech, 2001). Phylogenetic analysis has shown that mitochondrial and chloroplastic FtsZ proteins in extant eukaryotes have distinct evolutionary origins that parallel the unique endosymbiotic histories of the two organelles (Beech et al., 2000; Takahara et al., 2000; Gilson and Beech, 2001).

The chloroplastic FtsZ proteins currently identified, all of which are encoded by nuclear genes, group into three major families: red/brown algae, FtsZ1, and FtsZ2 (Beech et al., 2000; Gilson and Beech, 2001; El-Shami et al., 2002; Wang et al., 2003). The former are found only in red and brown algae, whereas the latter two have been reported in higher plants and in *Chamydomonas reinhardti*. Consistent with the presumed monophyletic origin of chloroplasts (Gray, 1999), all three FtsZ groups probably arose from a single *FtsZ* gene present in the cyanobacterial ancestor of chloroplasts, and members of each group have been shown to assemble into mid-plastid rings (Vitha et al., 2001).

Genome data suggest that *FtsZ1* and *FtsZ2* sequences are represented in many higher plants. Functional analysis in *Arabidopsis* has shown that members of both

families are required for chloroplast division in this organism (Osteryoung et al., 1998). FtsZ1 and FtsZ2 proteins are targeted to the chloroplast stroma by cleavable transit peptides (Osteryoung and Vierling, 1995; Gaikwad et al., 2000; Fujiwara and Yoshida, 2001; McAndrew et al., 2001) and are tightly colocalized (Vitha et al., 2001), suggesting they may be components of the same ring structure. FtsZ1 and FtsZ2 family members are distinguished by conserved differences in their amino acid sequences (Osteryoung and McAndrew, 2001; El-Shami et al., 2002). Especially noteworthy are the presence in FtsZ2, but not FtsZ1, of a short carboxy-terminal domain important for FtsZ function in bacteria (Osteryoung and McAndrew, 2001), and a single amino acid difference in the highly conserved "tubulin signature motif" (El-Shami et al., 2002). Because FtsZ2 proteins share slightly higher similarity with the cyanobacterial FtsZs than do FtsZ1 proteins, it has been suggested that FtsZ2 may be the more ancestral form of FtsZ in higher plants (El-Shami et al., 2002). In addition, because both forms of FtsZ had until recently only been identified in vascular plants, it had been postulated that the emergence of the two families may have been coincident with the emergence of this group of land plants (Osteryoung and McAndrew, 2001). However, a recent report showing that FtsZ1 and FtsZ2 homologues are present in the green alga *C. reinhardtii* indicates that the two families emerged as distinct clades before the evolution of land plants (Wang et al., 2003). The functional significance of this evolutionary divergence with regard to chloroplast division is not yet understood.

In the course of our studies on chloroplast division in higher plants, we have become particularly interested in understanding when during the evolution of chloroplasts the FtsZ1 and FtsZ2 families diverged from one another. Towards this end, we initiated a

series of sequence and phylogenetic analyses to reconstruct the evolutionary history of the chloroplast division FtsZs. These studies differ from those previously reported (Beech et al., 2000; Kiessling et al., 2000; Gilson and Beech, 2001; El-Shami et al., 2002; Wang et al., 2003) in four respects. First, we have focused solely on the plastid FtsZ lineages rather than on bacterial, mitochondrial and plastid lineages, providing a more focused picture of plastid FtsZ evolution. Second, we have incorporated a larger number of plastid FtsZ sequences from a wider range of photosynthetic eukaryotes, including *Oryza sativa* and the green alga *C. reinhardtii*, providing a more informative evolutionary perspective than earlier studies. Third, we have examined both protein and cDNA sequences to provide a more thorough analysis of the sequence relationships. Fourth, we have investigated intron positioning, as well as sequence comparisons, as an additional indicator of relatedness among *FtsZ* genes within and between organisms. The detailed analyses reported here strongly support the emergence of FtsZ1 and FtsZ2 as distinct clades before the split between the charophycean green algae, from which land plants evolved (Graham and Wilcox, 2000), and the chlorophycean green algae. Further, our results, which include analysis of evolutionary rates and testing of constraint trees, provide additional support for the peculiar FtsZ branching order observed previously (Beech and Gilson, 2000; Kiessling et al., 2000; Wang et al., 2003). We discuss possible explanations for this unexpected tree topology. In addition, we have identified conserved differences between the FtsZ1 and FtsZ2 protein families whose further study may help to elucidate why two forms of FtsZ evolved to function in chloroplast division.

Materials and Methods

Sequences and Their Alignment

Accession numbers for the protein and cDNA sequences used in this study are listed in Table 1 and most are available from GenBank (http://www.ncbi.nlm.nih.gov). The *Synechococcus* WH8102, *Trichodesmium erythraeum*, *Nostoc punctiforme*, *Prochlorococcus marinus* MED4 and *Prochlorococcus marinus* MIT9313 *FtsZ* sequences were obtained from the DOE Joint Genome Institute (http://www.jgi.doe.gov/JGI_microbial/html/). The *Anabaena sp.* PCC7120 and *Thermosynechococcus elongatus* BP-1 sequences are available at the Kazusa DNA Research Institute (http://www.kazusa.or.jp/cyano/cyano.html). Protein sequences were initially aligned with Clustal X (Thompson et al., 1997), but final adjustments were done manually and unaligned regions at the N- and C- termini were removed. The cDNA alignment was done similarly, except the final adjustments were based on the protein alignment. Both alignments are available upon request.

Phylogenetic Analyses

Neighbor joining, maximum parsimony, quartet puzzling and maximum likelihood analyses were all performed using PAUP 4.0v10b (Swofford, 1998). Ties were randomly broken and, for the cDNA analyses, the 3rd codon position was removed. Parsimony analyses were performed using heuristic searches and tree bisection-reconnection (TBR) swapping. All minimal trees were saved. The "COLLAPSE if maximum length is zero" option was in effect during the searches, and character changes were interpreted under ACCTRAN optimization. Characters were unweighted and

Table 1. Accession numbers for FtsZ protein and cDNA sequences

Letter[a]	Organism Name	Accession Number	
		Protein	cDNA
A	*Nicotiana tabacum* 2-1	CAB89288	AJ271750
B	*Nicotiana tabacum* 2-2	CAC44257	AJ311847
C	*Oryza sativa*	CLB17724_5[b]	CLB17724_5[b]
D	*Gentiana lutea*	AAF23771	AF205859
E	*Lilium longiflorum*	BAA96782	AB042101
F	*Arabidopsis thaliana* 2-1	AAC35987	AF089738
G	*Arabidopsis thaliana* 2-2	AAK63846	AF384167
H	*Oryza sativa*	CL005296_338[b]	CL005296_338[b]
I	*Physcomitrella patens* 2	CAB76386	AJ249139
J	*Physcomitrella patens* 1	CAB54558	AJ249138
K	*Chlamydomonas reinhardtii*	AAM22891	AF449446
L	*Cyanidioschyzon merolae*	BAA85116	AB032072
M	*Cyanidium caldarium* RK-1	BAA82871	AB023962
N	*Guillardia theta* (nucleomorph)	CAA07676	AJ007748
O	*Galdieria sulphuraria*	BAA82090	AB022594
P	*Mallomonas splendens*	AAF35433	AF120117
Q	*Galdieria sulphuraria*	BAA82091	AB022595
R	*Trichodesmium erythraeum*	Gene1949[c]	Gene1949[c]
S	*Synechocystis sp.* PCC6803	NP_440816	NC_000911
T	*Anabaena sp.* PCC7120	CAA83241[c]	Z31371[c]
U	*Nostoc punctiforme*	Contig 502 Gene 61[c]	Contig 502 Gene 61[c]
V	*Thermosynechococcus elongatus* BP-1	Gene tll2382[c]	Gene tll2382[c]
W	*Synechococcus* PCC7942	AAC26227	AF076530
X	*Prochlorococcus sp.*	CAB56201	AJ011025
Y	*Prochlorococcus marinus* MED4	Gene1658[c]	Gene1658[c]
Z	*Prochlorococcus marinus* ddlb	CAB95028	AJ237851
AA	*Synechococcus* WH8102	Gene 549[c]	Gene 549[c]
BB	*Prochlorococcus marinus* MIT9313	Gene1268[c]	Gene1268[c]
CC	*Nicotiana tabacum* 1-2	CAB41987	AJ133453
DD	*Nicotiana tabacum* 1-3	CAB89287	AJ271749
EE	*Tagetes erecta*	AAF81220	AF251346
FF	*Arabidopsis thaliana* 1-1	AAA82068	U39877
GG	*Pisum sativum*	CAA75603	Y15383
HH	*Nicotiana tabacum* 1-4	AAF23770	AF205858
II	*Nicotiana tabacum* 1-1	CAB89286	AJ271748
JJ	*Oryza sativa* 1-1	AAK64282	AF383876
KK	*Chlamydomonas reinhardtii*	BAB91150	AB084236
LL	*Clostridium propionicum*	AAC32266	AF067823
MM	*Escherichia coli* O157:H7	P06138	X55034
NN	*Bacillus subtilis*	AAA22457	M22630

[a]Letters to the left correspond to sequences represented in Figures. 1 and 2 and Table 2.
[b]Sequences derived from genomic sequence.
[c]Sequence source given in Materials and Methods

unordered, and gaps were treated as missing data. Bootstrapping was performed on trees obtained from each technique. One thousand replications were used for neighbor joining and maximum parsimony bootstrap analyses, as well as for the quartet puzzling frequency. Values from the different analyses indicate that the same sequences were present at similar nodes. When multiple trees were obtained, bootstrapping analysis was performed on the first tree in the list. For the maximum likelihood analysis, the Hierarchical Likelihood Ratio Tests and Akaike Information Criterion models were selected using Modeltest 3.06 (Posada and Crandall, 1998), which also provided the appropriate settings for analysis in PAUP 4.0v10b (Swofford, 1998) [PAUP command lines for each criterion were, respectively: Lset Base=(0.2705 0.2473 0.2146) Nst=6 Rmat=(1.0000 2.9264 1.0000 1.0000 3.4941) Rates=gamma Shape=0.7144 Pinvar=0.2225; and Lset Base=(0.2629 0.2330 0.2251) Nst=6 Rmat=(2.6121 4.5906 1.3185 1.6823 6.0001) Rates=gamma Shape=0.7111 Pinvar=0.2231]. Bayesian Inference was performed using the MRBAYES program (Huelsenbeck and Ronquist, 2001) with parameters set for four chains, 1.5 X 10^6 generations, and trees saved every 100 generations. The first 3000 trees were not included in the frequency analysis. Bayesian Inference analyses using the Hierarchical Likelihood Ratio Tests and Akaike Information Criterion produced identical trees with nearly identical frequency values. Values from the Hierarchical Likelihood Ratio Test criterion are reported in Figure 2.

Testing Constraint Trees

Constraint trees were produced using the program MacClade 4.0 (Maddison and Maddison, 2000). Trees were tested against the original maximum likelihood tree using

PAUP 4.0v10b (Swofford, 1998) and the Kishino-Hasegawa two-tailed test with a normal test distribution. Results are given as the *p*-value scores reported by the test.

Relative Rate Test

The relative rate test was performed using sequences taken from the alignments for the phylogenetic analyses. The analysis was performed using the program Phyltest version 2.0 (ftp://ftp.bio.indiana.edu/molbio/ibmpc). All protein comparisons were done using the proportion-of-differences and poisson correction distance estimation methods. Each cDNA comparison was performed with the proportion-of-differences, Jukes-Cantor, and Kimura 2-parameter distance estimation methods. Settings for all distance estimation methods were provided in the program.

Genetic Structure Comparisons

Regions of the *O. sativa* genome containing possible *FtsZ* genes were identified in the Torrey Mesa Research Institute rice database (http://portal.tmri.org/rice/). Accession numbers for the clones containing *FtsZ* genes are CL005296_338, CLB17724_5, and CL000716_185. The cDNA and protein sequences of the *O. sativa* *FtsZ* genes were determined using Netstart 1.0 at http://www.cbs.dtu.dk/services/NetStart/, Netgene2 at http://www.cbs.dtu.dk/services/NetGene2/, Genescan at http://genes.mit.edu/GENSCAN.html, and by comparison to known plant *FtsZ* sequences. Genomic sequences for the *Arabidopsis thaliana FtsZ* genes were obtained from The *Arabidopsis* Information Resource database (Huala et al., 2001). The accession

numbers for the *Physcomitrella patens* genomic sequences are AJ249138 and AJ249139. The *C. reinhardtii* genomic *FtsZ* sequences are located on scaffold sequences 85 and 262, which were obtained from the DOE Joint Genome Institute (http://genome.jgi-psf.org/chlre1/chlre1.home.html). Comparisons between the genomic and cDNA sequences were used to determine the intron and exon structures.

Protein Comparisons

The full-length protein sequences reported in Table 1 were aligned using the program Clustal X (Thompson et al., 1997) with default settings. The alignment was adjusted manually to mirror the protein alignment described for the phylogenic analyses, and to align the plant and algal C-terminal peptide sequences with that in *E. coli*. Regions at the amino termini containing the chloroplast transit peptides, and highly divergent regions near the carboxy termini, were removed. The entire *E. coli* FtsZ protein sequence was included in the alignment. The final alignment is available upon request. From the final alignment, residues that are identical in all available sequences of each clade were determined. Similar residues were determined using default settings in the Boxshade program at the Biology Workbench 3.2 website (http://workbench.sdsc.edu/).

Results

Phylogenetic Analysis of FtsZ Proteins from Photosynthetic Eukaryotes

Two recent events have provided critical insights into the plant FtsZ evolutionary origins. First, two *FtsZ* sequences from a green alga, *C. reinhardtii*, became available in

the database. Second, analysis of the newly sequenced genome of *O. sativa* (rice) (Goff et al., 2002) provided a full complement of monocot *FtsZ* homologues. Focusing on the FtsZ sequences from photosynthetic organisms (Table 1), including the *O. sativa* and *C. reinhardtii* sequences, we investigated their evolutionary relationships by phylogenetic analysis. Only available sequences that were full-length or near full-length were used to most accurately represent the evolutionary relationships. The outgroup was chosen based on a preliminary phylogenetic analysis by our group and on previous studies (Beech et al., 2000; Gilson and Beech, 2001), and consists of bacterial FtsZ sequences from *E. coli*, *Bacillus subtilis*, and *Clostridium propionicum*. BLAST searches identified several *Bacillus* FtsZ sequences as the closest relatives of the cyanobacterial FtsZs. The *B. subtilis FtsZ* gene, one of these close relatives, was chosen for the outgroup. The extreme amino and carboxy termini of the protein sequences used in this study are highly divergent and could not be aligned; therefore, they were omitted from the alignment. The trimmed protein sequences are about 322 amino acids long and correspond to residues 12 through 304 (Ala to Phe) of the *B. subtilis* sequence.

The protein alignment was analyzed using neighbor joining and maximum parsimony comparative techniques. The neighbor joining tree is shown in Figure 1, along with bootstrap support values for the neighbor joining and maximum parsimony analyses (first two numbers at nodes) and quartet puzzling frequencies (third number). Although six trees were obtained with maximum parsimony (not shown), the strict consensus indicated the differences were within, rather than between, clades. Both neighbor joining and maximum parsimony analyses separate the proteins into four clades: the cyanobacteria, red/brown algae, FtsZ1, and FtsZ2 families. Strong support is seen

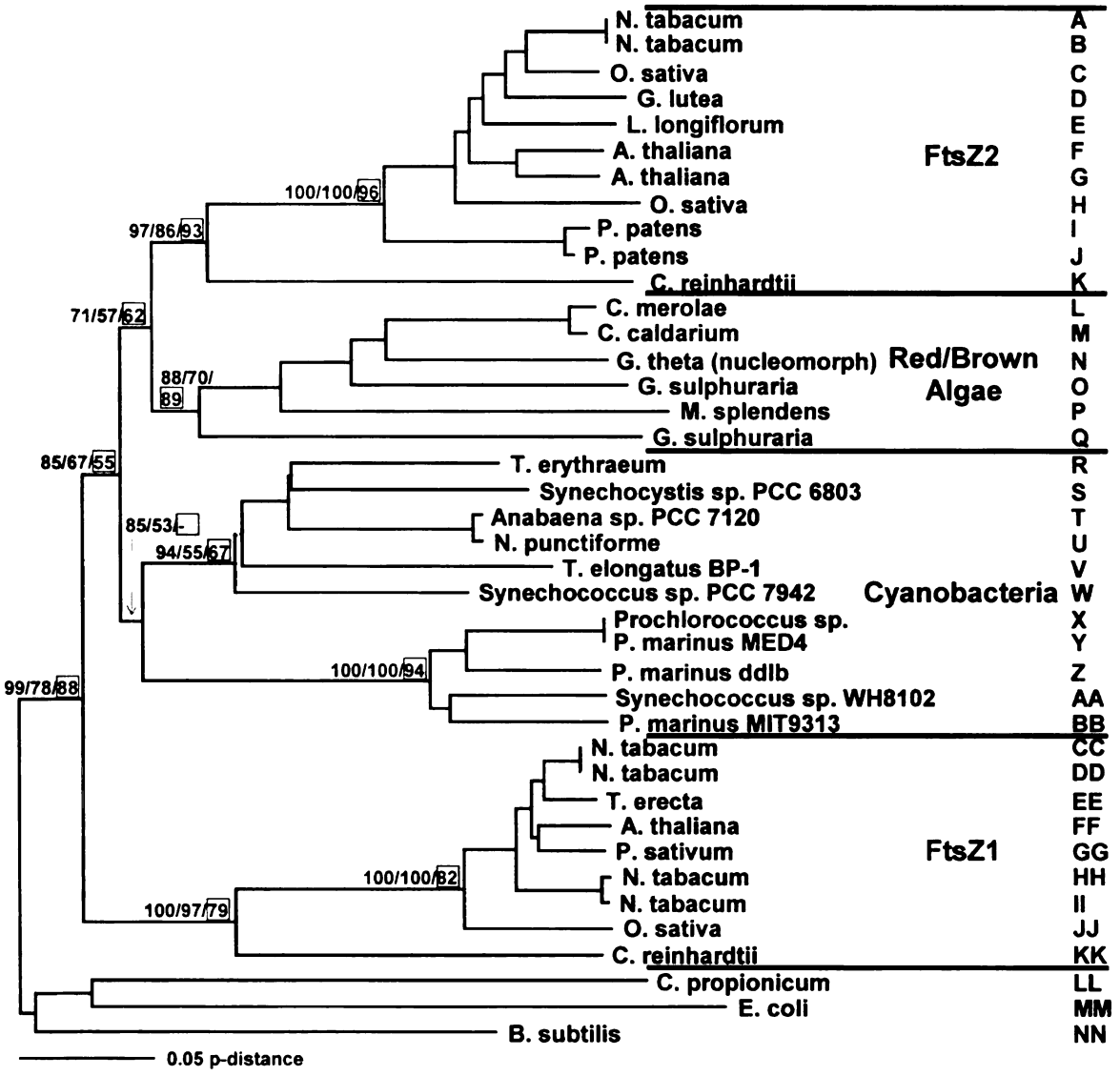


Figure 1. Phylogenetic analysis of *FtsZ* proteins.

A neighbor joining tree from analysis of FtsZ protein sequences is shown. Bootstrap support values from the neighbor joining (first number) and maximum parsimony (second number) analyses are shown at selected nodes. Boxed numbers are quartet puzzle frequencies from analysis of the parsimony tree. Nodes with less than 50% support, or not present, are represented by a dash. Letters to the left refer to the sequences listed in Table 1. Alignment is available upon request.

for the FtsZ1 (100/97/79) and FtsZ2 (97/86/93) clades, with slightly reduced support for the red/brown algae (88/70/89). The cyanobacteria have the least support (85/53/-) as a single clade; the analyses suggest there may actually be two clades (100/100/94 and 94/55/67). Although resolution of the cyanobacterial proteins into a single clade is weak, both neighbor joining and parsimony trees indicate that all the cyanobacterial sequences are sister to the red/brown algae and FtsZ2 clades. As indicated in previous reports (Osteryoung and Vierling 1995; Osteryoung et al., 1998; Beech et al., 2000), the plant FtsZ protein sequences are more closely related to those in cyanobacteria than in other prokaryotes (not shown).

An interesting result of the phylogenetic analyses is that the FtsZ1 clade branches prior to the other three clades rather than after the cyanobacterial clade (Figure 1). This branching order is supported by intermediate to weak bootstrap and frequency scores (85/67/55). The early branching of the FtsZ1 proteins before the cyanobacterial proteins seems inconsistent with the evolution of chloroplasts, as discussed below. Support for branching of the cyanobacterial (71/57/62) from the red/brown and FtsZ2 (71/57/62) clades is less strong, suggesting the differences between these clades are not as pronounced.

Significantly, our analyses place all the chloroplastic FtsZ proteins from red/brown algae into a single clade, whereas the sequences from the chlorophycean green alga *C. reinhardtii* are split between the FtsZ1 and FtsZ2 clades. Wang et al. (2003) recently reported a similar result. These findings indicate that the divergence of the FtsZ1 and FtsZ2 families found in plants may have occurred between the divergence of red and green algae.

Phylogenetic Analysis of FtsZ cDNA Sequences from Photosynthetic Eukaryotes

The phylogenetic relationships among the *FtsZ* cDNA sequences were analyzed using neighbor joining, maximum parsimony, and maximum likelihood algorithms (Swofford, 1998). The cDNA alignment used in this set of experiments was based on that of the proteins. The third codon position was removed for these analyses due to its variability. The maximum parsimony analysis produced seventeen different trees, but the strict consensus tree indicated that the differences in branching patterns occurred only within clades. Figure 2 shows the maximum likelihood tree with bootstrap support from the neighbor joining and maximum parsimony analyses (first two numbers) and likelihood frequencies from a Bayesian Inference analysis (Huelsenbeck and Ronquist, 2001) (third number) indicated at selected nodes.

The cDNA experiments group the sequences into the same four clades as the protein analyses, namely FtsZ1, FtsZ2, red/brown algae, and cyanobacteria (Figure 2). Support for each clade is generally strong (100/99/100, 99/97/100, 96/85/100, and 96/-/97, respectively) and the branching order was the same as that obtained for the protein analyses. There is reasonable support in the cDNA analyses for the early branching of the FtsZ1 clade before the cyanobacterial, red/brown algal, and FtsZ2 clades (72/72/94). Like the protein analyses (Figure 1; Wang et al., 2003), the cDNA analyses place distinct *C. reinhardtii* sequences in the FtsZ1 and FtsZ2 clades. The potential grouping of the cyanobacterial sequences into two clades is supported by bootstrap and frequency values of 93/100/100 and 100/65/100. These two cyanobacterial clades are almost identical to those observed in the protein analyses; the only difference is in the position of the

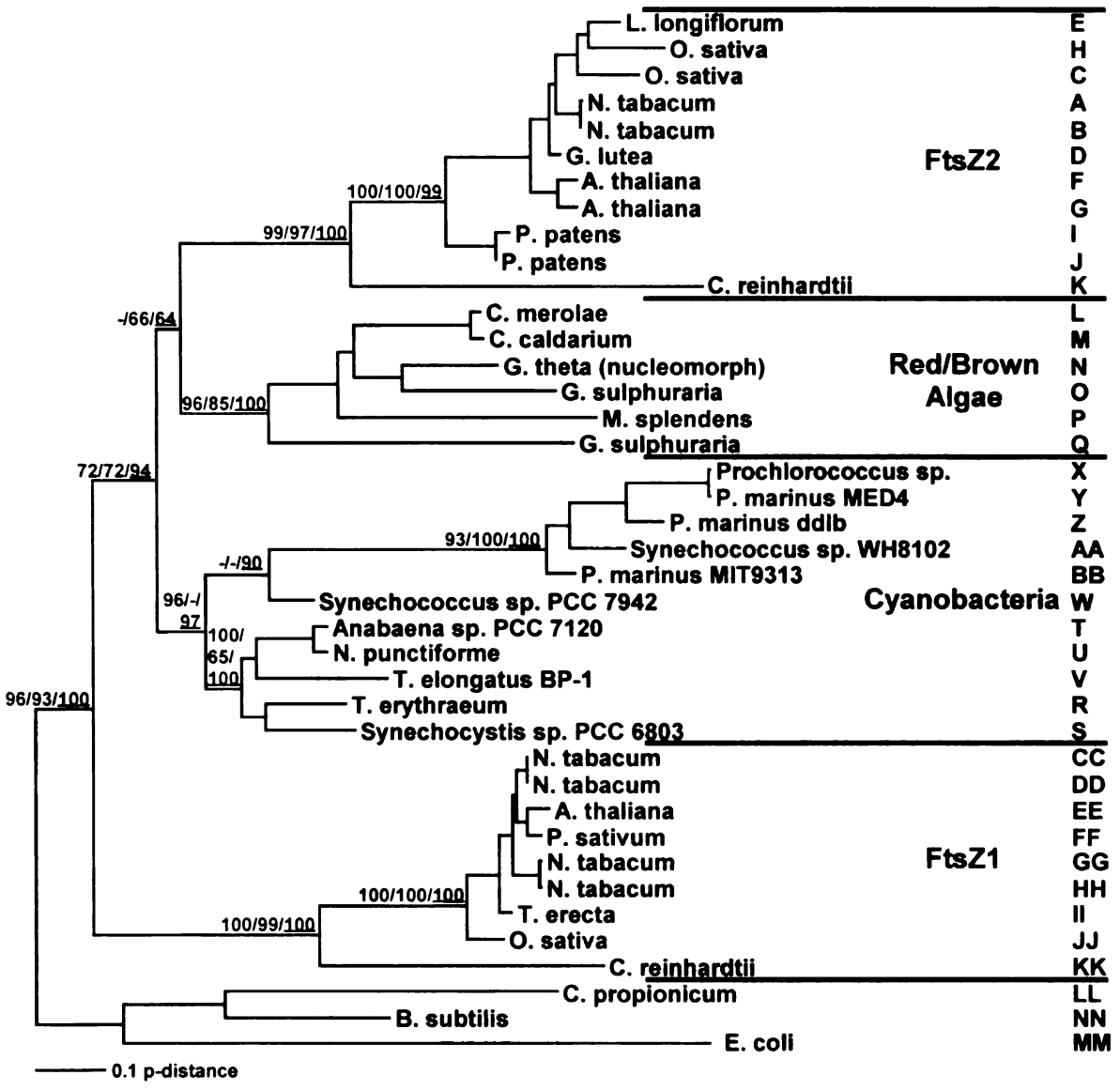


Figure 2. Phylogenetic analysis of *FtsZ* cDNA sequences.

A Maximum likelihood tree using the first two codon positions of the cDNA sequences is shown. Bootstrap support values from neighbor joining (first number) and maximum parsimony (second number) analyses are shown at selected nodes. Bayesian Inference frequencies are underlined. Nodes with less than 50% support, or not present, are represented by a dash. Letters to the left refer to the sequences listed in Table 1. Alignment is available upon request.

Synechococcus sp. PCC7942 sequence. Except for the node at which the red/brown algae and FtsZ2 clades branch, all Bayesian Inference frequencies shown (Figure 2) are greater than 90% and strongly support this as the most likely tree under the selected criteria.

We next tested the significance of the maximum likelihood tree from Figure 2 (shown in simplified form in Figure 3A) against two constraint trees in which the branching order among the four major clades was rearranged (Figure 3, B and C; branching within clades was not altered). In one constraint tree (Figure 3B), the positions of the cyanobacterial and FtsZ1 clades have been switched from that shown in Figure 3A, such that the cyanobacterial clade branches first, followed by the FtsZ1 and subsequently the FtsZ2 and red/brown algae clades. In the second constraint tree (Figure 3C) the FtsZ1 clade has been placed as sister to the FtsZ2 clade, with the cyanobacterial clade branching first. These two constraint trees better represent the relationships we expected based on chloroplast evolution theories (Gray, 1999) and on the strong similarity of both the FtsZ1 and FtsZ2 proteins to the cyanobacterial FtsZs (Osteryoung et al., 1998; Osteryoung and McAndrew, 2001). When the significance of either constraint tree was tested against the maximum likelihood tree, the *p*-value was 0.000. This means that, at greater than 99% confidence, the data do not support a branching order that differs from that shown in the maximum likelihood tree (Figure 3A). Therefore, phylogenetic analyses based on both cDNA and protein data support the grouping of FtsZ sequences into four clades, with the FtsZ1 sequences branching first, followed by the cyanobacteria, then the red/brown algae and FtsZ2 sequences.

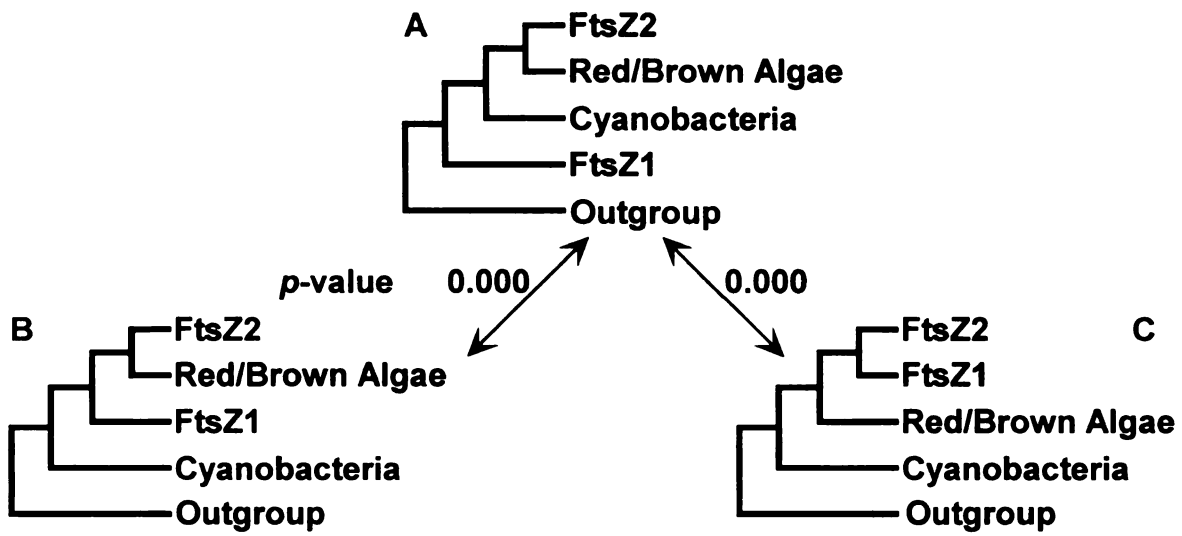


Figure 3. Testing the maximum likelihood tree against two constraint trees.

All trees have been simplified in this diagram. The maximum likelihood tree, A, from Figure 2 is compared to two different constraint trees, B & C, in which the relationships between the clades have been altered. The low *p*-values indicate the relationships in the two constraint trees are not supported by the data.

Testing for Differences in The FtsZ1 and FtsZ2 Evolutionary Rates

One possible explanation for the branching order determined from the phylogenetic analyses is that the *FtsZ1* sequences evolved more rapidly than the *FtsZ2* sequences. To test this possibility, we performed a relative rate test on the *FtsZ1* and *FtsZ2* sequences. Only sequences from the same organism were compared in order to minimize the effect of different selective pressures encountered by different organisms. Because of this restriction, only the *C. reinhardtii*, *O. sativa*, *A. thaliana*, and *Nicotiana tabacum* sequences could be tested. Every *FtsZ1* sequence was tested against every *FtsZ2* sequence from the same organism, using both protein and cDNA sequences. All FtsZ1 and FtsZ2 sequences were compared against each of three outgroups: (1) *B. subtilis* only, (2) *C. propionicum* only, and (3) *C. propionicum*, *E. coli*, and *B. subtilis* combined. At a 95% confidence limit, no significant difference in the evolutionary rate was detected for any of the sequence combinations tested. Thus, the relative rate test did not detect a significant difference in the rates at which the FtsZ1 and FtsZ2 sequences have evolved.

Genetic Structure Is Conserved Within the Plant FtsZ Clades

The relationship between the *FtsZ1* and *FtsZ2* sequences was further investigated using genetic structure (intron position/exon length) comparisons. Fujiwara and Yoshida (2001) reported that the intron positions in the two *A. thaliana FtsZ2* sequences are identical. We analyzed all three *A. thaliana FtsZ* genes, as well as genes from *O. sativa*, *P. patens*, and *C. reinhardtii* (Figure 4). Although relatively few genomic sequences are available for this analysis, especially among members of the FtsZ1 clade, those available represent very diverse groups. Due to high sequence variability in the 5' and 3' ends of

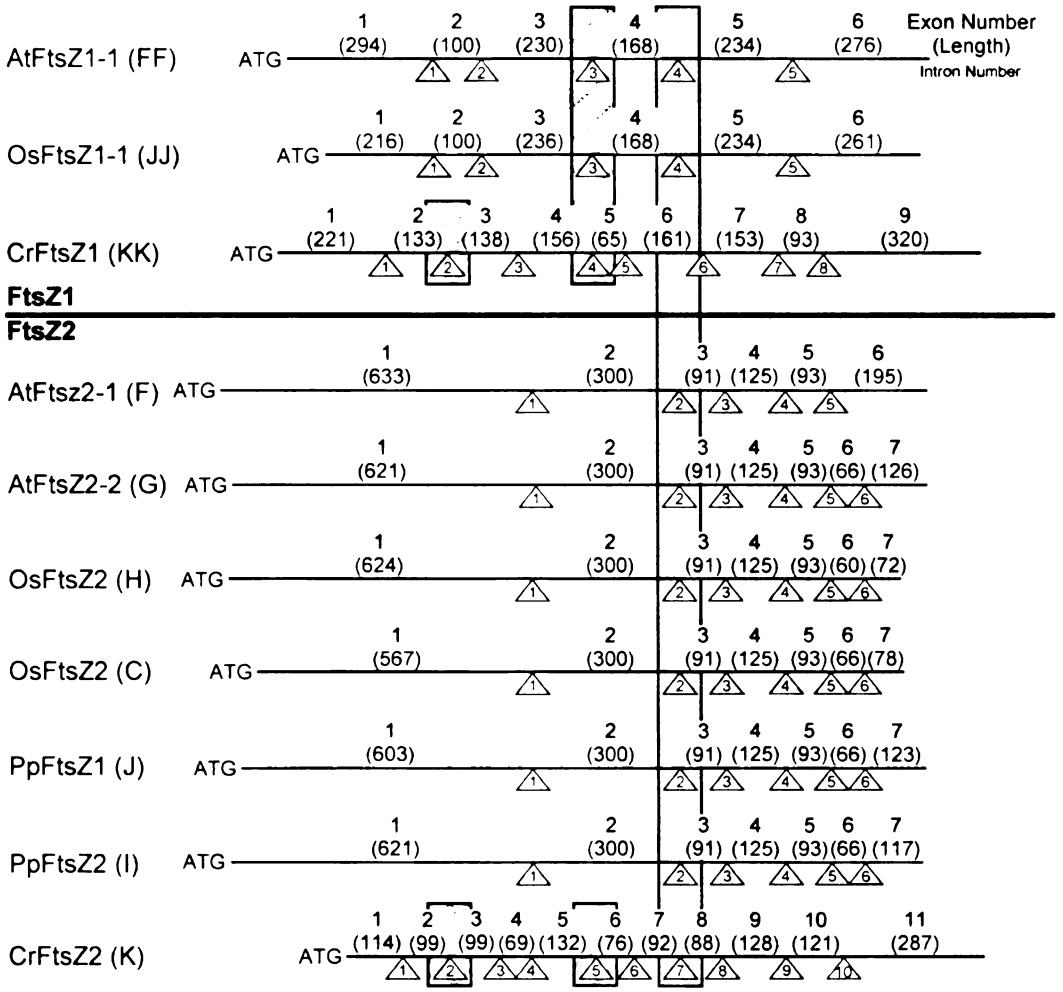


Figure 4. Comparison of *FtsZ1* and *FtsZ2* genetic structures.

Aligned *A. thaliana*, *P. patens*, *O. sativa*, and *C. reinhardtii FtsZ* cDNA sequences are represented by solid horizontal lines, and drawn to scale. Letters in parentheses correspond to sequences listed in Table 1. Intron positions are indicated by numbered triangles. Exon numbers are indicated above the horizontal line; exon lengths in bp are in parentheses. The shaded box indicates an intron positioned similarly in the *FtsZ1* and *FtsZ2* genes. Hatched boxes indicate *C. reinhardtii* and plant *FtsZ1* introns located at equivalent positions. The crosshatched boxes represent intron positions shared only in the two *C. reinhardtii* sequences. The *CrFtsZ1* sequence corresponds to the *CrFtsZ3* sequence reported by Wang et al. (2003).

the coding regions, a cDNA sequence alignment based on that used for the phylogenetic analysis shown in Figure 2 was used for genetic structure comparisons.

In the *O. sativa* and *A. thaliana FtsZ1* genes, all five introns (1, 2, 3, 4 and 5) are located at identical positions (Figure 4). The introns vary considerably in sequence and length (Table 2), but the exon lengths are identical, the only exception being a 6 bp insertion in the third exon of the *O. sativa FtsZ1* gene. These results indicate that intron locations in the *FtsZ1* sequences from angiosperms are conserved.

The conservation of intron locations among the *FtsZ2* genes is even more striking (Figure 4). The six introns in the *A. thaliana*, *O. sativa*, and *P. patens FtsZ2* genes, though variable in sequence and length (Table 2), are in identical positions, with two exceptions. The first is in the *AtFtsZ2-1* sequence, which lacks the sixth intron present in the other *FtsZ2* genes. The second is a six bp shift in the position of the sixth intron of one *O. sativa* sequence, possibly due to a deletion, that reduces the length of the sixth exon from 66 to 60 bp. Otherwise, monocot, dicot, and moss *FtsZ2* sequences have a conserved genetic structure.

Although intron locations within the plant *FtsZ1* and *FtsZ2* genes are conserved, the intron locations between them are significantly different. Only the fourth intron in the *FtsZ1* genes and the second intron in the *FtsZ2* genes are located at equivalent positions (shaded box in Figure 4). The presence of similarly positioned introns in the *FtsZ1* and *FtsZ2* genes from diverse land plants suggests that both clades originated from a common ancestral gene bearing a corresponding intron. Ancient duplication of this ancestral gene presumably allowed the two clades to diverge early and extensively from each other.

Table 2. Comparison of *FtsZ* intron lengths

Gene Family	Sequence[a]	Intron Number[b] 1	2	3	4	5	6	7	8	9	10
	AtFtsZ1-1 (FF)	102	93	89	106	96	NA	NA	NA	NA	NA
FtsZ1	*OsFtsZ1* (JJ)	357	266	468	68	192	NA	NA	NA	NA	NA
	CrFtsZ1 (KK)	126	165	198	122	163	173	133	186	NA	NA
	AtFtsZ2-1 (F)	165	85	301	202	82	NA	NA	NA	NA	NA
	AtFtsZ2-2 (G)	367	90	377	148	85	106	NA	NA	NA	NA
	OsFtsZ2 (H)	287	474	343	258	143	155	NA	NA	NA	NA
FtsZ2	*OsFtsZ2* (C)	90	132	500	1006	278	636	NA	NA	NA	NA
	PpFtsZ1 (J)	462	312	204	79	217	276	NA	NA	NA	NA
	PpFtsZ2 (I)	460	338	187	84	225	455	NA	NA	NA	NA
	CrFtsZ2 (K)	74	86	100	371	150	107	119	114	154	181

[a]Letters in parentheses correspond to sequences listed in Table 1.

[b]The intron number corresponds to labeled triangles in Figure 4.

NA, not applicable.

The genetic structures of the *C. reinhardtii FtsZ* genes are partially conserved with those of the plant genes. The *C. reinhardtii FtsZ1* gene shares one intron position with the plant *FtsZ1* genes (Figure 4, introns 4 and 3, respectively). Three *C. reinhardtii FtsZ2* introns are positioned similarly to those in the plant *FtsZ2* genes (introns 4, 7, and 9 versus 1, 2, and 4, respectively). Intron 7 in *C. reinhardtii FtsZ2* corresponds to the intron that is common to both *FtsZ1* and *FtsZ2* in plants (shaded box in Figure 4), though this intron is lacking in the *C. reinhardtii FtsZ1* gene. Intron 5 in *C. reinhardtii FtsZ2* corresponds to introns 4 and 3 in the *C. reinhardtii* and plant *FtsZ1* genes, respectively (hatched boxes in Figure 4). Finally, intron 2 is shared by both *C. reinhardtii* genes (crosshatched boxes in Figure 4), but is absent from the plant genes. The positions of the other introns in the two *C. reinhardtii FtsZ* genes are unique to each gene.

FtsZ1 and FtsZ2 Protein Comparisons

The early divergence of the FtsZ1 and FtsZ2 families and their conservation in both plants and *C. reinhardti* is consistent with the hypothesis that the two proteins evolved to have distinct functions. Because protein function is ultimately defined by amino acid composition, we compared the FtsZ protein sequences in an effort to identify regions that may represent functionally important differences between FtsZ1 and FtsZ2 (Figure 5). Consensus sequences for each clade defined in Figure 1 were aligned, except for the highly divergent chloroplast transit peptides. The alignment included the divergent carboxy termini omitted from the phylogenetic analyses because it contains a region of potential functional significance as described below. A full-length *E. coli*

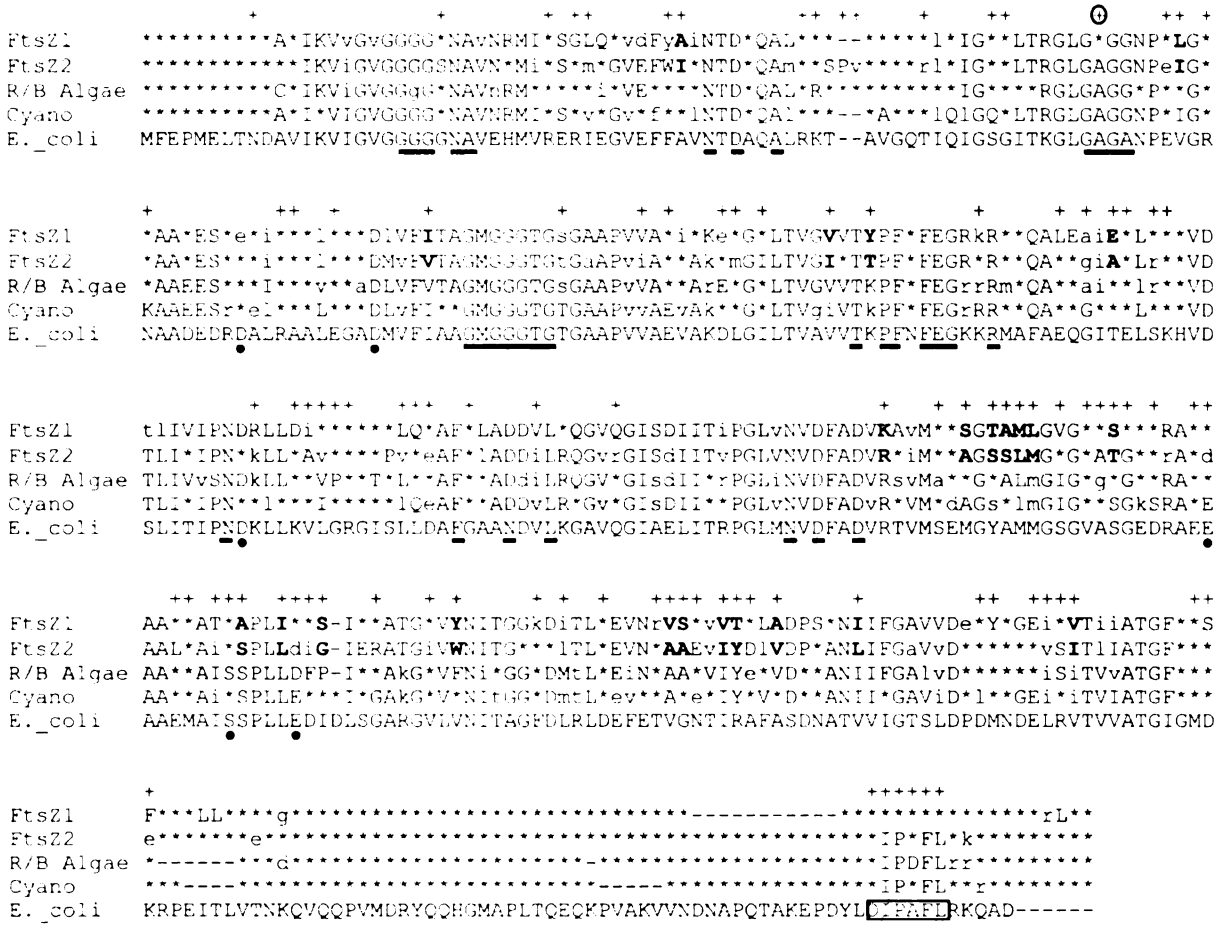


Figure 5. Comparison of FtsZ proteins from photosynthetic organisms.

Consensus sequences are shown for the FtsZ1, FtsZ2, Red/Brown algae (R/B algae), and cyanobacterial (cyano) clades. For all sequences within a clade, capital letters and small letters indicate identical and similar residues, respectively. Positions where amino acids are not conserved are indicated by asterisks. Gaps in all members of a clade are represented by dashes. Residues shown by structural analysis to either contact GTP (Löwe, 1998) or to be required for regulation of GTPase activity (Erickson, 1998) are underlined in the *E. coli* protein. Residues located on the side of the *E. coli* protein that are required for FtsZ function but not for GTPase activity (Lu et al., 2001) are labeled with dots. Residues where the FtsZ1 and FtsZ2 differ are marked at the top with a '+'; bold letters indicate residues that are completely conserved within, but that differ between, the FtsZ1 and FtsZ2 clades. The circled '+' is a nucleotide binding residue that was previously recognized to differ between the FtsZ1 and FtsZ2 clades (Osteryoung and McAndrew, 2001).

protein is included in the alignment as a reference because it is functionally the best characterized of the bacterial FtsZ proteins.

FtsZ sequences in all four clades share significant similarity with the *E. coli* protein, particularly in regions previously shown to contact GTP (Löwe, 1998), to be required for GTP activity (Erickson, 1998), or to be important for other aspects of *E. coli* FtsZ function (Lu et al., 2001) (underscores and dots in Figure 5). With one exception (circled plus), all the residues important for GTPase activity (underscores) are conserved in all the FtsZ proteins analyzed in this study. However, because we are most interested in the features that may functionally distinguish the FtsZ1 and FtsZ2 proteins, we used the alignment to highlight the differences between these two clades. The residues indicated in bold represent amino acids that are invariant within the two clades, but that differ between them. None of these conserved differences occur at positions corresponding to the functionally characterized residues in *E. coli* FtsZ, and most occur downstream of the regions in the *E. coli* protein associated with GTPase activity (underscores). In addition to these differences, the FtsZ1 and FtsZ2 proteins differ in the presence of a C-terminal peptide similar to a region in *E. coli* FtsZ (boxed residues in Figure 5) shown to be critical for its interaction with two other bacterial cell division proteins, FtsA and ZipA (Ma and Margolin, 1999; Mosyak et al., 2000). This peptide is identifiable in the FtsZ2 proteins as well as in the cyanobacterial and most of the red/brown algae proteins, but is missing in the FtsZ1 proteins. The absence of this peptide in FtsZ1 and its presence in FtsZ2 suggests the two proteins families may interact with different sets of proteins during chloroplast division, though obvious homologues of ZipA and FtsA are lacking in both plants and cyanobacteria.

Discussion

Early Divergence of the FtsZ1 and FtsZ2 Families

Phylogenetic analyses have defined two major groups of green algae, one containing the charophycean green algae, which are sister to the land plants, and the other containing the chlorophycean green algae, which include *C. reinhardtii* (Graham and Wilcox, 2000). Previously, we hypothesized that the emergence of FtsZ1 and FtsZ2 as separate clades might have accompanied the evolution of vascular plants (Osteryoung et al., 1998). This conjecture was based primarily on the lack of FtsZ1-like sequences from other organisms in the public databases. However, the grouping of the *C. reinhardtii FtsZ* genes into the *FtsZ1* and *FtsZ2* clades is well supported by phylogenetic analyses based on both amino acid (Figure 1; Wang et al., 2003) and cDNA alignments (Figure 2). This strongly suggests that the emergence of FtsZ1 and FtsZ2 as separate clades predated not only the split between the charophycean green algae and land plants, but also the split between the two major green algal lineages. To date, FtsZ1 sequences have only been reported in angiosperms and *C. reinhardtii*, but if the two FtsZ families originated as early as the data suggest, then members of both families should eventually be discovered in mosses and other land plants, in charophycean green algae, and in other chlorophycean green algae.

Relationship of the FtsZ1 Clade to the Other Three Clades

The phylogenetic analyses consistently yielded trees in which the FtsZ1 clade forms a sister group to the clade containing the FtsZ2, red/brown algal, and

cyanobacterial *FtsZ* sequences (Figs. 1 and 2). Although this branching order has also been observed (though not discussed) in previous reports (Beech et al., 2000; Kiessling et al., 2000; Wang et al., 2003), it is not consistent with the relationships predicted based on the presence of only a single *FtsZ* gene in all cyanobacterial genomes sequenced to date and on the presumed monophyletic origin of chloroplasts (Gray, 1999). If the FtsZ1 and FtsZ2 sequences shared a common cyanobacterial ancestor, one would expect them to be more closely related to each other than to the cyanobacterial FtsZ sequences. We tested the possibility that the observed branching order might be an artifact of a difference in the rates of FtsZ1 and FtsZ2 evolution, but were unable to detect such a difference. This could be because the portions of the protein sequences aligned in our analysis are functionally constrained, and do not reflect the true evolutionary rates. In fact, the *E. coli* and *Arabidopsis* FtsZ protein sequences are about 50% identical in this region, indicating a high degree of conservation among FtsZ proteins from widely divergent organisms.

Another possibility for the failure to detect a difference in evolutionary rate could be that the two clades diverged at equal rates. If so, this would suggest that the *FtsZ2* sequences evolved similarly to their cyanobacterial counterparts, perhaps due to specific functional constraints, while the *FtsZ1* sequences evolved to fulfill a new function. In this case, although evolutionary rates would be equal for both clades, the sequence changes would be in different directions. Another potential explanation is that the branching of the *FtsZ1* and *FtsZ2* clades is too deep for the relative rate test to detect a significant difference (Avise, 1994). A related possibility is that the outgroup is too distantly related to the sequences analyzed. However, the *B. subtilis* FtsZ used in the outgroups for the relative rate test is one of the closest currently available relatives of the

cyanobacterial FtsZ proteins based on sequence homology. Regardless of whether the branching order is an artifact of the analyses or reflects the true evolutionary history of the *FtsZ* gene families, the deep branching of the FtsZ1 and FtsZ2 clades supports their early divergence.

Comparisons of exon length and intron distribution among the *FtsZ* genes also support the early divergence of *FtsZ1* and *FtsZ2*. Although the intron positions in the angiosperm *FtsZ1* genes are conserved, they differ from the conserved intron positions in the *FtsZ2* genes, except at a single location. Assuming that both clades originated from a single ancestral *FtsZ* gene, these data indicate that a majority of the introns were inserted into the sequences following an ancestral duplication. The fact that the *C. reinhardtii* and plant *FtsZ* genes share partial similarity in their genetic structures is also consistent with the divergence of the FtsZ1 and FtsZ2 families prior to the split between the two green algal lineages. However, additional genomic *FtsZ* sequences from other green algae are needed for a more complete understanding of the relationship between *FtsZ* evolution and gene structure.

Evolutionary Origins of the Chloroplastic FtsZ1 and FtsZ2 Sequences

Exactly when the duplication and divergence of the ancestral *FtsZ* gene occurred remains unknown, but two general scenarios can be envisioned (Figure 6). In the first, the duplication and divergence might have occurred after a single *FtsZ* gene was transferred from the cyanobacterial endosymbiont to the eukaryotic nucleus, but before separation of the two major green algal lineages (Figure 6A). This scenario would

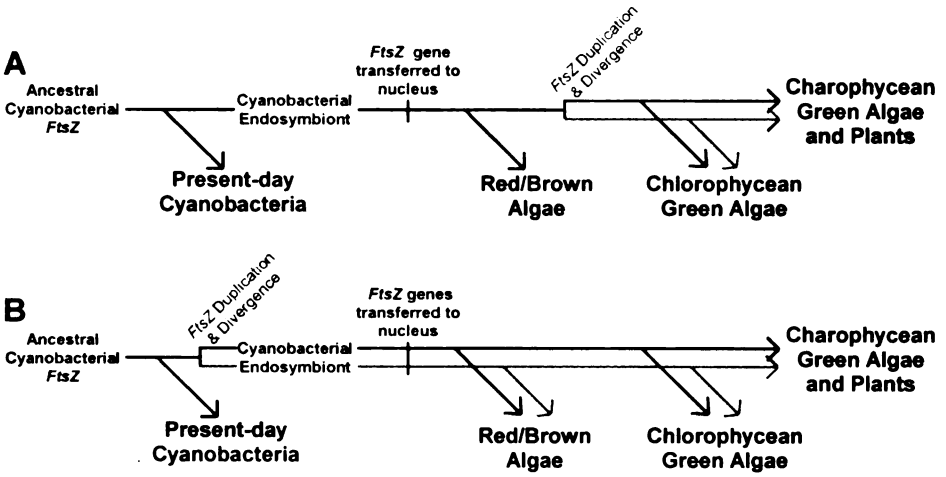


Figure 6. Two scenarios for the evolutionary origins of the plant *FtsZ* gene families.

Transfer of the *FtsZ* gene(s) from the cyanobacterial endosymbiont to the eukaryotic nucleus is marked with a vertical line. The evolutionary timeline of the *FtsZ* gene is represented as a horizontal line. Gray and black horizontal lines represent divergence in the *FtsZ* genes. A, a single *FtsZ* gene is transferred from the cyanobacterial endosymbiont to the eukaryotic nucleus. Duplication and divergence of the *FtsZ* gene occurs before divergence of the two major green algal lineages. B, Duplication and divergence of the *FtsZ* genes occur in the cyanobacterial ancestor of chloroplasts. Both genes would be transferred to the eukaryotic nucleus, giving rise to separate *FtsZ1* and *FtsZ2* clades.

explain why two FtsZ families are present in both chlorophycean green algae (*C. reinhardtii*) and plants. In a variation, divergence could also have occurred earlier, prior to emergence of the red and brown algae, though at present there are no data to suggest this is the case. Additional genome data from more basal organisms will shed light on the timing of *FtsZ* duplication and divergence. However, this first scenario (Figure 6A) does not explain the branching of the *FtsZ1* clade before the cyanobacterial sequences (Figs. 1 and 2). In the second scenario, duplication of the *FtsZ* gene might have occurred in the cyanobacterial progenitor of chloroplasts (Figure 6B). In this case, the extant cyanobacteria, which so far bear only a single *FtsZ* gene, would either have lost one of the duplicated genes or would have descended from the chloroplast progenitor before the duplication occurred. This scenario would explain the relationships we observe in our phylogenetic analyses, but would suggest that, in the red and brown algal lineages, one of the FtsZ families was lost or that a second FtsZ family has yet to be discovered. This scenario would also suggest that a cyanobacterium with both an FtsZ1-like and an FtsZ2-like gene, more closely related to the original endosymbiont, existed in the past and could still be discovered.

Although not a widely accepted idea (Palmer, 2003), it is also conceivable that chloroplasts may be polyphyletic in origin (Stiller et al., 2003), and that the two *FtsZ* gene families originated from different endosymbiotic events. At present we cannot rule out any of the above scenarios with the currently available information. Addition to the public databases of new *FtsZ* sequences and genome data from evolutionarily relevant taxa will help to elucidate the history of the chloroplast *FtsZ* genes, and may shed light on why two forms of FtsZ evolved to function in chloroplast division.

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CHAPTER 6

Summaries and Future Directions

Summary

The major thrust of the work presented in this thesis has been to better understand the function of FtsZ proteins in chloroplast division. The functional, expressional, and evolutionary FtsZ comparisons that have been discussed in this work provide a better understanding of the role FtsZ proteins play in chloroplast division. First, my work has helped us recognize that chloroplast division requires members of two FtsZ families, FtsZ1 and FtsZ2. Second, my studies have shown that chloroplast division seems to involve a stoichiometric balance of FtsZ1 and FtsZ2 proteins. Third, my expression studies have shown that the *FtsZ* genes are coordinately expressed in many tissues, including tissues in which chloroplast division occurs in a majority of the cells. Also, the *FtsZ* genes are expressed at different levels, consistent with the hypothesis that a stoichiometeric balance between the FtsZ proteins is required for chloroplast division. Finally, my analyses have shown that sequence and structural characteristics among the FtsZ1 and FtsZ2 families are very conserved, especially in higher plants. This conservation suggests there are differences in functions between FtsZ1 and FtsZ2 proteins. The results discussed in this thesis have laid the foundation for future work on the functions and interactions of the FtsZ proteins, which is central to understanding the complex and important process whereby plastids divide, a process that has only now begun to be explored.

Chapter Summaries and Future Directions

Function of FtsZ1 and FtsZ2 Proteins in Chloroplast Division

Isolation of two Arabidopsis *FtsZ* genes that belong to different families, *FtsZ1* and *FtsZ2*, indicated there is a significant difference between chloroplast and bacterial division, since the latter requires a single *FtsZ* gene in most cases. To determine the functions of the FtsZ1 and FtsZ2 proteins, plants expressing antisense *FtsZ* transgenes were generated. When FtsZ1 or FtsZ2 expression is reduced in the transgenic plants, chloroplast division is inhibited, which reduces the number of chloroplasts from about 100 in a normal mesophyll cell to as few as one very large chloroplast. These results indicate that both FtsZ1 and FtsZ2 proteins function in chloroplast division.

Although *FtsZ1* and *FtsZ2* family members are both functional in chloroplast division, Arabidopsis plants actually express two *FtsZ2* family members, *AtFtsZ2-1* and *AtFtsZ2-2*. Antisense repression experiments could not differentiate between the functions of the two FtsZ2 proteins since expression of both *AtFtsZ2-1* and *AtFtsZ2-2* is repressed in the *AtFtsZ2-1* antisense lines. Isolation and characterization of *AtFtsZ2-1* or *AtFtsZ2-2* Arabidopsis knockout mutants would greatly benefit studies aimed at determining the function of each FtsZ2 protein. Although knockout lines for each of the three Arabidopsis *FtsZ* genes are sought, *AtFtsZ2-2* is the only *FtsZ* gene for which we have knockout plants in our laboratory (Dr. Deena Kadirjan-Kalbach, unpublished results). The chloroplast phenotype of the *AtFtsZ2-2* knockout line is intermediate; it is characterized by about 15 enlarged chloroplasts per mesophyll cell. This phenotype would indicate the efficiency of plastid division is reduced but not inhibited by removal of AtFtsZ2-2 protein. This may reflect the fact that the total FtsZ2 protein level is

reduced by only about 20% in this mutant (McAndrew et al., 2003), personal communication)

Altered FtsZ1 or FtsZ2 Levels Disrupt Chloroplast Division

In addition to reducing *FtsZ* levels, we also asked whether increasing FtsZ1 or FtsZ2 protein levels would affect chloroplast division. For this purpose, I attempted to create transgenic plants overexpressing *AtFtsZ1-1*, *AtFtsZ2-1*, or *AtFtsZ2-2* transgenes. Analysis of the transgenic plants suggests there is a direct correlation between the *AtFtsZ1-1* overexpression level and the severity of the chloroplast division defect. Because the *AtFtsZ2-1* cDNA that was used in the overexpression construct was truncated, none of the transgenic plants can be described to be true overexpressors. However, a full-length *AtFtsZ2-1-cmyc* transgene was overexpressed by Vitha et al. (2001) who observed that slight increases in *AtFtsZ2-1* expression did not affect chloroplast division but that large increases did. These results indicate that altering the stoichiometric balance between the FtsZ1 or FtsZ2 proteins themselves, or between FtsZ proteins and other components of the chloroplast division apparatus, disrupts chloroplast division. However, there may be some flexibility in the stoichiometric balance since slight increases in FtsZ levels did not noticeably disrupt chloroplast division.

In bacteria, a slight increase in FtsZ protein level induces cell division abnormally at the cell poles because of FtsZ ring assembly at those sites (Ward Jr and Lutkenhaus, 1985). It was hypothesized that increased chloroplast division in plants would be manifested by increased numbers of smaller chloroplasts in mesophyll cells. None of the transgenic plants overexpressing *AtFtsZ1-1* or *AtFtsZ2-1* proteins had increased numbers

of chloroplasts. However, the experiments do not indicate that overexpression of the plant FtsZ proteins is unable to induce plastid division. One possible explanation for the fact that increased chloroplast division was not observed may be that a very narrow range of FtsZ overexpression is required to induce chloroplast division, and the FtsZ protein levels in the transgenic plants may not have been within that range. Related to this possibility is the use of the 35S promoter in all the overexpressiong constructs, which is typically a very strong promoter for gene expression. It is also possible that overexpression of two of the Arabidopsis FtsZ proteins together, or of all three, may be required before an increase in chloroplast division can be observed, since members of two families are required for chloroplast division. Therefore, increasing the level of only one of the plant FtsZ proteins may be insufficient to increase chloroplast division, no matter what level of overexpression is achieved. Furthermore, preserving the stoichiometric balance between the three FtsZ proteins, and possibly other components of the division apparatus, may also be necessary for the chloroplasts to divide.

To address whether FtsZ overexpression could induce chloroplast division, transgenic plants that overexpress combinations of two or even all three *FtsZ* genes could be generated. The stoichiometric ratios of the FtsZ proteins may be critical for increasing chloroplast division; therefore control of expression may be very important. Expression with the 35S promoter, which was used in the overexpression constructs, may be too strong for these experiments. Also, the strong expression of the *FtsZ* genes with the 35S promoter may have been a cause for the co-suppression that was observed in many of the transgenic plants. Therefore, it may be better to use the native *FtsZ* promoters or possibly an inducible promoter for studies of FtsZ function in transgenic plants.

FtsZ knockout plants would also benefit studies to determine whether the functions of the FtsZ2 proteins are redundant. One experiment might be to overexpress *AtFtsZ2-1* in the *AtFtsZ2-2* knockout line, and vice versa once an *AtFtsZ2-1* knockout line is found, and see if a normal chloroplast phenotype can be restored. Furthermore, experiments could be performed that investigate whether overexpression of FtsZ1 proteins could substitute for loss of FtsZ2, or vice verse. The results of these experiments would have implications for understanding the roles of FtsZ1 and FtsZ2 proteins in chloroplast division.

FtsZ1 and *FtsZ2* Genes are Coordinately Expressed

Expression of the Arabidopsis *FtsZ* genes in tissues where chloroplast division is occurring was expected due to the critical role FtsZ proteins have in this process. Histochemical staining of transgenic plants with the *FtsZ promoter-GUS* constructs, measurements of *FtsZ* transcript levels by real-time reverse transcription-polymerase chain reaction (RT-PCR), and measurements of protein by immunoblot analysis indicate that the three Arabidopsis *FtsZ* genes are coordinately expressed, especially in tissues where chloroplast division is occurring in a large proportion of the cell population. Transcript measurements of the *FtsZ* genes indicate *AtFtsZ2-2* is the least abundant, *AtFtsZ1-1* was intermediate, and *AtFtsZ2-1* was the most abundant. Furthermore, the ratio of *FtsZ1* to *FtsZ2* transcript was about 1 to 3, which correlates well with the 1 to 2 ratio of FtsZ1 to FtsZ2 protein that was determined by McAndrew et al. (in preparation). The *FtsZ* transcript ratios seem to be constant throughout leaf expansion, consistent with

the hypothesis that the stoichiometric balance between the FtsZ proteins may be important for chloroplast division.

One important experiment would be to identify and characterize the sequences that are required for *FtsZ* expression. This is especially important for *AtFtsZ1-1* since there are probably sequences downstream of the 5'-UTR that are required for expression, which were not present in our *promoter-GUS* constructs. Although the expression patterns observed with the *AtFtsZ2-1* and *AtFtsZ2-2* promoters were more defined, there may still be regulatory elements that are missing. An initial step to define the regulatory elements for the *FtsZ* genes would be to use computer programs to identify known regulatory elements that are involved in gene expression. The results of the computer analysis would allow, at least on a basic level, comparisons of potential regulatory elements among these *FtsZ* genes. Also, analysis of the sequences surrounding the *FtsZ* genes with these computer programs would help determine what regions may still be required in the *promoter-GUS* constructs to accurately represent endogenous *FtsZ* expression. Of course, mutation or deletion analysis would be required to determine the functionality of any identified elements.

In addition to computer analysis of the *FtsZ* promoters, the *promoter-GUS* plant lines and RNA quantification techniques can be used to determine the factors that affect *FtsZ* expression. By assaying the amount of GUS enzyme that is expressed with the *promoter-GUS* constructs, factors that change *FtsZ* expression in treated plants can be easily assayed. Similarly, changes in *FtsZ* transcript levels can be assayed using real-time RT-PCR or possibly northern blot analysis. Changes in *FtsZ* transcript amounts are

predicted to have an effect on chloroplast division, but the chloroplast phenotypes that result from any treatment will have to be characterized.

Some of the factors that should be tested for their effect on *FtsZ* expression include environmental conditions and growth hormone treatments. Environmental factors might include changes in the light intensity, photoperiod, temperature, nutritional factors, or water availability. There is already some evidence that light induces *FtsZ* expression and chloroplast division in peas (Gaikwad et al., 2000) and cucumber (Ullanat and Jayabaskaran, 2002). Although a variety of growth hormones should be tested, cytokinin has been shown to induce both cell expansion and chloroplast division in cultured spinach leaf disks (Possingham and Smith, 1972). Cytokinin treatment also increases transcript levels of one *FtsZ2* homologue in cucumber cotyledons, whereas auxin treatment did not increase transcript levels (Ullanat and Jayabaskaran, 2002). Although these studies with pea and cucumber *FtsZ* genes are important, they only investigated one of several *FtsZ* genes present in these organisms. Expression studies with the *FtsZ* genes from Arabidopsis would allow for analysis of the entire *FtsZ* gene complement of the plant. Throughout these studies different mutants could help determine the regulatory factors that are involved in *FtsZ* expression. For example, studies with mutants defective in light or cytokinin response could help characterize *FtsZ* regulation or help elucidate other regulatory factors involved in *FtsZ* expression.

During analysis of the *FtsZ2 promoter-GUS* transgenic plants, it was noticed that the older leaves were not stained. However, *FtsZ1* and *FtsZ2* transcripts and protein were detected in the older leaf tissues, indicating they are expressed. Furthermore, immunofluorescence and fluorescence microscopy experiments detected FtsZ1 and FtsZ2

protein rings in all the chloroplasts, including those of the older leaf cells (McAndrew et al., 2001; Vitha et al., 2001). The transcript and protein measurements suggest that FtsZ expression is reduced in the older leaves, and the GUS staining patterns are consistent with reduced FtsZ expression in these tissues. However, the microscopy data suggest there is still enough protein to maintain FtsZ rings in all the chloroplasts. The presence of rings in tissues where expression is reduced may indicate the FtsZ proteins are relatively stable and are not rapidly turned over, at least compared to the GUS protein. Stability could be inherent in the FtsZ proteins alone or a characteristic of the polymerized FtsZ structure. To test the stability of the FtsZ proteins, plants could be generated where an inducible promoter drives expression of an *FtsZ* transgene fused to a reporter gene or epitope tag. Treatment of transgenic plants with the inducers for only a short period would stimulate expression of the tagged FtsZ protein for a brief period, similar to a pulse labeling experiment. Following removal of the inducers, the stability of the tagged FtsZ proteins could be followed. Mutated FtsZ proteins unable to polymerize could be expressed similarly to determine whether FtsZ protein stability is affected by polymerization.

The regulation of *AtFtsZ2-2* expression may be different than the regulation of *AtFtsZ1-1* or *AtFtsZ2-1* expression. Three *AtFtsZ2-2* transcripts were isolated, all differentially spliced upstream of the start codon. Only a single RNA species was isolated for each of the *AtFtsZ1-1* and *AtFtsZ2-1* genes. The functionality and stability of the three *AtFtsZ2-2* species will need to be determined because their presence does not absolutely indicate they are functional. If there is more than one functional transcript, it is possible that they are expressed in different tissues or at different developmental

stages. Tissue- or developmental-specific expression could be tested by RT-PCR, *in situ* hybridization, or *in situ* RT-PCR. Although tissue- or developmental-specific expression could mean that *AtFtsZ2-2* has a regulatory role in chloroplast division, the total ratio of FtsZ1 to FtsZ2 protein may be the most important factor for chloroplast division and the splicing differences may just reflect the evolutionary history of the *FtsZ* genes.

FtsZ1 and FtsZ2 Sequence Comparisons Identify Potential Functional Differences

Phylogenetic analyses indicate that the *FtsZ1* and *FtsZ2* families diverged before the split between the chlorophycean and charophycean green algal lineages. Analysis of the intron positions in the *FtsZ* genes also supports the early divergence of the two families. The reason chloroplast division requires members of two FtsZ families are unclear, but one likely reason is that the functions of the FtsZ1 and FtsZ2 proteins differ. There are several pieces of evidence that support this hypothesis. First, antisense repression of either *FtsZ1* or *FtsZ2* genes inhibits chloroplast division indicating the proteins in the two families are not functionally redundant, at least at normal levels. However, it is possible that inhibition of chloroplast division in the antisense lines is due to reduction of the total FtsZ protein concentration below critical levels in the chloroplasts. Second, both families seem to be present in all green algae and higher plants. If the two families were functionally redundant, it would be expected that members of one family would be lost in at least one lineage. Third, differences in the FtsZ1 and FtsZ2 sequences also support specific roles for each family. For example, the C-terminal domain, the bacterial counterpart of which interacts with FtsA and ZipA in *E. coli* (Ma and Margolin, 1999), is present in the FtsZ2 but not the FtsZ1 proteins. This

sequence difference sugests FtsZ2 proteins may interact with a different set of proteins in the chloroplast division apparatus than FtsZ1. Mutation and deletion experiments would help to determine the functions of this C-terminal domain, as well as the numerous other sequence differences, in the plant FtsZ proteins and help to define the functional differences between the FtsZ1 and FtsZ2 proteins.

Phylogenetic analyses indicate that there are two or more FtsZ2 homologues in Arabidopsis, rice, tobacco, and moss. In contrast, the green alga *Chlamydomonas reinhardtii* only has one FtsZ2 homologue. Why do higher plants and moss have two FtsZ2 proteins while the green alga has only one? Is the chloroplast division apparatus different in single-celled green algae and multicellular plants? One interesting and important cellular difference between the green alga *C. reinhardtii* and Arabidopsis, rice, tobacco, and moss is that the former has only a single chloroplast in each cell, whereas the latter four organisms have numerous chloroplasts in each mesophyll cell. It is also interesting that only one of the rice FtsZ2 proteins has an identifiable C-terminal domain. Arabidopsis *FtsZ2* knockout plant experiments could be performed to investigate whether the green alga FtsZ2 protein, either rice FtsZ2 protein, or any of the FtsZ2 proteins from the various organisms could functionally complement the Arabidopsis FtsZ2 proteins. For instance, in one experiment the *C. reinhardtii* FtsZ2 protein could be expressed in *AtFtsZ2-1* and *AtFtsZ2-2* Arabidopsis knockout plants and in a double knockout plant. If AtFtsZ2-1 and AtFtsZ2-2 have different functions and expression of the *C. reinhardtii* protein restores chloroplast division in both FtsZ2 Arabidopsis knockout mutant, this would suggest the two FtsZ2 proteins in plants have divergent functions and the divergence occurred in only the plant lineage. Complementation studies where *FtsZ*

genes from different organisms like the archea bacteria, green algae, red algae, and primitive plants are transformed into Arabidopsis *FtsZ* knockout plants would provide valuable information into the functions of the FtsZ proteins as well as the structure of the FtsZ ring. This information may help in determining the reasons chloroplast division in plants evolved to have multiple FtsZ proteins from two families.

One weakness in the phylogenetic analyses was insufficient numbers of *FtsZ* sequences from diverse organisms. Therefore, additional genomic, RNA, and protein *FtsZ* sequences from a variety of photosynthetic organisms would benefit studies aimed at understanding functionally important regions of the FtsZ proteins, as well as the evolutionary events that led to the two FtsZ families in plants. Of particular interest are the *FtsZ* sequences from primitive plants like the hornworts and liverworts (Araki et al., 2003), which would provide information into the early evolutionary events of the plant *FtsZ* genes in land plants. Additional algal sequences are also important, especially from green algae since only the *C. reinhardtii* sequences have been reported. Adding more cyanobacterial *FtsZ* sequences to the analysis may help determine whether there are truly two clades for this group and whether those two clades could represent the sources from which the plant FtsZ1 and FtsZ2 clades evolved. Currently, all known cyanobacteria for which *FtsZ* sequence information is available have only a single *FtsZ* gene. However, as the genomes of more cyanobacteria are studied, one organism may be isolated with two different *FtsZ* genes; this could support the hypothesis that the origins of the FtsZ1 and FtsZ2 proteins extends back to the cyanobacterial endosymbiont.

APPENDICES

APPENDIX A

FtsZ Protein Alignment

The Nexus file with the FtsZ protein alignment that was used in the phylogenetic analyses that are discussed in Chapter 5 and used to produce the tree shown in Figure 1 of that chapter. Sequences are labeled with the organism initials followed by the accession or gene number.

```
#NEXUS
BEGIN DATA;
      DIMENSIONS  NTAX=40 NCHAR=322;
      FORMAT DATATYPE=PROTEIN  SYMBOLS = " 1 2 3 4"  MISSING=? GAP=-
INTERLEAVE ;
MATRIX

[                          10        20        30        40    ]
[                          .         .         .         .     ]

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NtCAC44257          AKIKVVGVGGGGSNAVNRMIESSMKGVEFWIVNTDIQAMRMS---   [42]
OsCLB17724_5        AKIKVVGVGGGGSNAVNRMIESSMNGVEFWIVNTDVQAIRMS---   [42]
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LlBAA96782          AKIKVIGVGGGGSNAVNRMIASSMDGVEFWIVNTDVQAMRMS---   [42]
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PpCAB54558          AKIKVIGVGGGGSNAVNRMLESEMQGVEFWIVNTDAQAMALS---   [42]
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GsBAA82091          CKIKVVGVGGAGGNAVQRMLESGLQDVEFLCANTDAQALGRFQEV   [45]
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Np61                ANIKVIGVGGGGGNAVNRMIESDVSGVEFWSINTDAQALTLA---   [42]
Tetll2382           ARIKVIGVGGGGGNAVNRMIASNVAGVEFWCVNTDAQAIAQS---   [42]
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```

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[                    50         60         70         80         90]
[                    .          .          .          .          .]

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NtCAC44257         --PVAAEQRLPIGQELTRGLGAGGNPDIGMNAANESKQAIEEAVY   [85]
OsCLB17724_5       --PVLPQNRLQIGQELTRGLGAGGNPDIGMNAAKESVESIQEALY   [85]
GlAAF23771         --PVYLENRLQIGQELTRGLGAGGNPDIGMNAAKESKEAIEEAVY   [85]
LlBAA96782         --PVYPENRLQIGQELTRGLGAGGNPDIGMNAAKESKVSIEESVS   [85]
AtAAC35987         --PVLPDNRLQIGKELTRGLGAGGNPEIGMNAARESKEVIEEALY   [85]
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PpCAB76386         --PVPAQNRLQIGQKLTRGLGAGGNPEIGCSAAEESKAMVEEALR   [85]
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CcBAA82871         ----AAHHTLGIGNKLTRGLGAGGNPEIGRKAAEESCDQIAEAVR   [83]
GtCAA07676         ----LAPNTCNIGAKLTRGLGAGGNPEIGRKAAEESRDLIAEAVS   [82]
GsBAA82090         ----KTSNSVTIGSEITRGLGAGGKPEVGRQAAEESQAAISSAVQ   [82]
MsAAF35433         ----SAKRRLNIGKVLSRGLGAGGNPAIGAKAAEESREEIMAVVK   [58]
GsBAA82091         YCQKTHHQVIQIGKQSCRGLGAGGNPEAGRVAAEESKEDIAKALQ   [90]
Te1949             ----RAPKRLQLGQKLTRGLGAGGNPAIGQKAAEESRDEIANALD   [83]
SsNP_440816        ----NAPDCIQIGQKLTRGLGAGGNPAIGQKAAEESRDEIARSLE   [83]
AsCAA83241         ----GAPSRLQIGQKLTRGLGAGGNPAIGQKAAEESRDEIATALE   [83]
Np61               ----GAPSRLQIGQKLTRGLGAGGNPAIGQKAAEESRDEIATALE   [83]
Tetll2382          ----QAHRCLQIGQKLTRGLGAGGNPAIGQKAAEESREDLAAALK   [83]
SyAAC26227         ----AAPKRMQLGQKLTRGLGAGGNPAIGMKAAEESREELIAALE   [83]
PsCAB56201         ----SADRRVQLGQNLTRGLGAGGNPSIGQKAAEESKDELQQTLE   [83]
Pm1658             ----SADRRVQLGQNLTRGLGAGGNPSIGQKAAEESKDELQQTLE   [83]
PmCAB95028         ----SAENRVQLGQTLTRGLGAGGNPSIGEKAAEESRAELQQALE   [83]
Sy549              ----QAQHRLQLGQTLTRGLGAGGNPTIGQKAAEESRTDLHDALQ   [83]
Pm1268             ----AASNRVQLGQTLTRGLGAGGNPSIGQKAAEESRAELQQALQ   [83]
NtCAB41987         ----AAENPLQIGELLTRGLGTGGNPLLGEQAAEESKEAIANSLK   [83]
NtCAB89287         ----AAENPLQIGELLTRGLGTGGNPLLGEQAAEESKEAIANSLK   [83]
TeAAF81220         ----VAHNPIQIGELLTRGLGTGGNPLLGEQAAEESKEAIGNALK   [83]
AtAAA82068         ----SAENPLQIGELLTRGLGTGGNPLLGEQAAEESKDAIANALK   [83]
PsCAA75603         ----AAENPIKIGELLTRGLGTGGNPLLGEQAAEESKEAIANALK   [83]
NtAAF23770         ----TVENPIQIGELLTRGLGTGGNPLLGEQAAEESKEHIANALK   [83]
NtCAB89286         ----TVENPIQIGELLTRGLGTGGNPLLGEQAAEESKEHIANALK   [83]
OsAAK64282         ----QAQYPLQIGEQLTRGLGTGGNPNLGEQAAEESKEAIANALK   [83]
CrBAB91150         ----QALNKVQIGSELTRGLGCGGNPELGRRAAMESEEALRRMVQ   [83]
CpAAC32266         ----RSSTKTQIGEKLTKGLGAGGNPEIGEKSVDETQDEIAQALH   [83]
EcP06138           ----AVGQTIQIGSGITKGLGAGANPEVGRNAADEDRDALRAALE   [83]
BsAAA22457         ----KAEVKMQIGAKLTRGLGAGANPEVGKKAAEESKEQIEEALK   [83]
```

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[                        100       110       120       130   ]
[                        .         .         .         .     ]

NtCAB89288          GADMVFVTAGMGGGTGTGAAPIIAGTAKSMGILTVGIVTTPFSFE   [130]
NtCAC44257          GADMVFVTAGMGGGTGTGAAPIIAGTAKSMGILTVGIVTTPFSFE   [130]
OsCLB17724_5        GADMVFVTAGMGGGTGTGGAPVIAGIAKSMGILTVGIVTTPFSFE   [130]
GlAAF23771          GADMVFVTAGMGGGTGTGGAPVIAGIAKSMGILTVGIVTTPFSFE   [130]
LlBAA96782          GADMVFVTAGMGGGTGTGGAPVIAGVAKSMGILTVGIVTTPFMFE   [130]
AtAAC35987          GSDMVFVTAGMGGGTGTGAAPVIAGIAKAMGILTVGIATTPFSFE   [130]
AtAAK63846          GSDMVFVTAGMGGGTGTGGAPIIAGVAKAMGILTVGIVTTPFSFE   [130]
OsCL005296_338      GADMIFVTAGMGGGTGTGGAPVIAGIAKSMGILTVGIVTTPFAFE   [130]
PpCAB76386          GADMVFVTAGMGGGTGSGAAPIIAGVAKQLGILTVGIVTTPFAFE   [130]
PpCAB54558          GADMVFVTAGMGGGTGSGAAPIIAGVAKQLGILTVGIVTTPFAFE   [130]
CrAAM22891          DTDMVFVTAGMGGGTGSGAAPVVAEVARELGILTVGIVTTPFTFE   [130]
CmBAA85116          GADLVFVTAGMGGGTGSGAAPVVAEAAREQGCLTVGVVTKPFAFE   [128]
CcBAA82871          GADLVFVTAGMGGGTGSGAAPVVAEAAREQGCLTVGVVTKPFAFE   [128]
GtCAA07676          AGDLVFVTAGMGGGTGSGAAPIVAEVAKEMGCLTVGVVTKPFAFE   [127]
GsBAA82090          GGDLVFVTAGMGGGTGSGAAPIVAKIAKEQGCLTVGVVTKPFSFE   [127]
MsAAF35433          NADLVFVTAGMGGGTGSGAAPVVAECAKEAGALTVGVVTKPFGFE   [103]
GsBAA82091          GGDLVFVTAGMGGGTGTGAAPIVADVARELGCLTVGVVTKPFAFE   [135]
Te1949              HPDLVFITAGMGGGTGTGAAPVIAEIAKEAGSLTVGVVTRPFTFE   [128]
SsNP_440816         GTDLVFITAGMGGGTGTGAAPIVAEVAKEMGCLTVGIVTRPFTFE   [128]
AsCAA83241          GADLVFITAGMGGGTGTGAAPIVAEVAKEMGALTVGVVTRPFVFE   [128]
Np61                GADLVFITAGMGGGTGTGAAPIVAEVAKEMGALTVGVVTRPFVFE   [128]
Tetll2382           DADLIFITCGMGGGTGTGAAPIVAEVAKEQGALTVAVVTRPFTFE   [128]
SyAAC26227          GADLVFITAGMGGGTGTGAAPIVAEVAKEVGALTVGIVTKPFTFE   [128]
PsCAB56201          GSDLVFIAAGMGGGTGTGAAPVVAEVAKQSGALTVGIVTKPFSFE   [128]
Pm1658              GSDLVFIAAGMGGGTGTGAAPVVAEVAKQSGALTVGIVTKPFSFE   [128]
PmCAB95028          GADLVFIAAGMGGGTGTGAAPVVAEVAKQSGALTVAIVTKPFSFE   [128]
Sy549               GSDLVFIAAGMGGGTGTGAAPVVAEVAREVGALTVGIVTKPFGFE   [128]
Pm1268              GVDLVFIAVGMGGGTGTGAAPVVAEVAKESGALTVGIVTKPFSFE   [128]
NtCAB41987          GSDMVFITAGMGGGTGSGAAPVVAQIAKEAGYLTVGVVTYPFSFE   [128]
NtCAB89287          GSDMVFITAGMGGGTGSGAAPVVAQIAKEAGYLTVGVVTYPFSFE   [128]
TeAAF81220          GSDLVFITAGMGGGTGSGAAPVVAQIAKEAGYLTVGVVTYPFSFE   [128]
AtAAA82068          GSDLVFITAGMGGGTGSGAAPVVAQISKDAGYLTVGVVTYPFSFE   [128]
PsCAA75603          GSDLVFITAGMGGGTGSGAAPVVAQISKEAGYLTVGVVTYPFSFE   [128]
NtAAF23770          GSDMVFITAGMGGGTGSGAAPVVAQIAKEAGYLTVGVVTYPFSFE   [128]
NtCAB89286          GSDMVFITAGMGGGTGSGAAPVVAQIAKEAGYLTVGVVTYPFSFE   [128]
OsAAK64282          DSDLVFITAGMGGGTGSGAAPVVAQISKEAGYLTVGVVTYPFSFE   [128]
CrBAB91150          GADLVFITAGMGGGTGTGAAPVVARLSKELGILTVGVVTYPFNFE   [128]
CpAAC32266          GSDMVFITAGMGGGTGTGAAPRIAAISKELGILTVGVVTKPFNFE   [128]
EcP06138            GADMVFIAAGMGGGTGTGAAPVVAEVAKDLGILTVAVVTKPFNFE   [128]
BsAAA22457          GADMVFVTAGMGGGTGTGAAPVIAQIAKDLGALTVGVVTRPFTFE   [128]
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[                    140       150       160       170       180]
[                    .         .         .         .         .]

NtCAB89288          GRRRAVQ--AQEGIAALRENVDTLIVIPNDKLLTAVSPSTPVTEA   [173]
NtCAC44257          GRRRAVQ--AQEGIAALRENVDTLIVIPNDKLLTAVSPSTPVTEA   [173]
OsCLB17724_5        GRRRAVQ--AQEGIAALRNSVDTLIVIPNDKLLSAVSPNTPVTEA   [173]
GlAAF23771          GRRRAVQ--AQEGIAALRDNVDTLIVIPNDKLLTAVSPSTPVTEA   [173]
LlBAA96782          GRRRTVQ--AQEGIAALRNNVDTLIVIPNDKLLTAVSPNTPVTEA   [173]
AtAAC35987          GRRRTVQ--AQEGLASLRDNVDTLIVIPNDKLLTAVSQSTPVTEA   [173]
AtAAK63846          GRRRALQ--AQEGIAALRDNVDTLIVIPNDKLLAAVSQSTPVTEA   [173]
OsCL005296_338      GRRRALQ--AQEGIASLRSNVDTLIVIPNDKLLTAVSPNTPVTEA   [173]
PpCAB76386          GRRRSVQ--AHEGIAALKNNVDTLITIPNNKLLTAVAQSTPVTEA   [173]
PpCAB54558          GRRRAVQ--AHEGIAALKNNVDTLITIPNNKLLTAVAQSTPVTEA   [173]
CrAAM22891          GRQRAQQ--ARSALANLRAAVDTLIVIPNDRLLSAMDSNVPIKDA   [173]
CmBAA85116          GRKRMNQ--ALEAIEALRESVDTLIVVSNDKLLQIVPENTPLQDA   [171]
CcBAA82871          GRRRMTQ--ALEAIEALRESVDTLIVVSNDKLLQIVPENTPLQDA   [171]
GtCAA07676          GKRRMQQ--ANDAILNLRNKVDTLIVVSNDKLLQIVPDNTPLQDA   [170]
GsBAA82090          GRRRMQQ--AEEAIEALRKEVDTLIVVSNDKLLEIVPENTALEKA   [170]
MsAAF35433          GRKRMQQ--ARNAILEMKDKVDTLIVVSNDKLLKIVPDNTPLTEA   [146]
GsBAA82091          GRRRLQQ--AVEGLANLREKVDTLIVISNDRLLETVPKDTPLTEA   [178]
Te1949              GRRRITQ--ADEGITALQTRVDTLIVIPNNRLLSVINDQTPVQEA   [171]
SsNP_440816         GRRRAKQ--AEEGINALQSRVDTLIVIPNNQLLSVIPAETPLQEA   [171]
AsCAA83241          GRRRTSQ--AEQGIEGLKSRVDTLIIIPNNKLLEVIPEQTPVQEA   [171]
Np61                GRRRTSQ--AEQGIEGLKSRVDTLIIIPNNKLLEVIPEQTPVQEA   [171]
Tetll2382           GRRRANQ--ADEGIEALQSRVDTLIVIPNDKILSVISEQTSVQDA   [171]
SyAAC26227          GRRRMKQ--AEEGTAALQSSVDTLITIPNDRLLHAISEQTPIQEA   [171]
PsCAB56201          GKRRMRQ--AEEGIARLAENVDTLIVIPNDRLKDVIAG-APLQEA   [170]
Pm1658              GKRRMRQ--AEEGIARLAENVDTLIVIPNDRLKDVIAG-APLQEA   [170]
PmCAB95028          GRRRMRQ--ADEGIAKLTESVDTLIVIPNDRLKDAIAG-APLQEA   [170]
Sy549               GRRRMRQ--ADEGIARLAEHVDTLIVIPNDRLREAIAG-APLQEA   [170]
Pm1268              GRRRMRQ--AAEGIGRLADHVDTLIVIPNDRIKDVISE-APLQEA   [170]
NtCAB41987          GRKRSVQ--ALEAIEKLQKNVDTLIVIPNDRLLDIADEQTPLQDA   [171]
NtCAB89287          GRKRSVQ--ALEAIEKLQKNVDTLIVIPNDRLLDIADEQTPLQDA   [171]
TeAAF81220          GRKRSVQ--ALEAIEKLQKNVDTLIVIPNDRLLDIADENTPLQDA   [171]
AtAAA82068          GRKRSLQ--ALEAIEKLQKNVDTLIVIPNDRLLDIADEQTPLQDA   [171]
PsCAA75603          GRKRSLQ--ALEAIEKLQKNVDTLIVIPNDRLLDIADEQMPLQDA   [171]
NtAAF23770          GRKRSLQ--ALEAIEKLQKNVDTLIVIPNDRLLDIADEQTPLQNA   [171]
NtCAB89286          GRKRSLQ--ALEAIEKLQKNVDTLIVIPNDRLLDIADEQTPLQNA   [171]
OsAAK64282          GRKRSLQASALEALEKLERSVDTLIVIPNDRLLDVVDENTPLQDA   [173]
CrBAB91150          GRRRAGQ--ALEGIEALREAVDSVIVIPNDRLLDVAGASTALQDA   [171]
CpAAC32266          GKKRMSN--AEKGIMELKKNVDTLVIIPNQRLLSIIDKKTTLTEA   [171]
EcP06138            GKKRMAF--AEQGITELSKHVDSLITIPNDKLLKVLGRGISLLDA   [171]
BsAAA22457          GRKRQLQ--AAGGISAMKEAVDTLIVIPNDRILEIVDKNTPMLEA   [171]
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[                          190        200        210        220    ]
[                          .          .          .          .      ]

NtCAB89288          FNLADDILRQGVRGISDIITIPGLVNVDFADVRAIMANAGSSLMG   [218]
NtCAC44257          FNLADDILRQGVRGISDIITIPGLVNVDFADVRAIMANAGSSLMG   [218]
OsCLB17724_5        FNLADDILRQGIRGISDIITVPGLVNVDFADVRAIMQNAGSSLMG   [218]
GlAAF23771          FNLADDILRQGVRGISDIITIPGLVNVDFADVRAIMANAGSSLMG   [218]
LlBAA96782          FNLADDILRQGVRGISDIITVPGLVNVDFADVRAIMANAGSSLMG   [218]
AtAAC35987          FNLADDILRQGVRGISDIITIPGLVNVDFADVRAIMANAGSSLMG   [218]
AtAAK63846          FNLADDILRQGVRGISDIITIPGLVNVDFADVRAIMANAGSSLMG   [218]
OsCL005296_338      FNLADDILRQGVRGISDIITVPGLVNVDFADVRSVMSDAGSSLMG   [218]
PpCAB76386          FNLADDILRQGVRGISDIITVPGLVNVDFADVRAIMANAGSSLMG   [218]
PpCAB54558          FNLADDILRQGVRGISDIITVPGLVNVDFADVRAIMANAGSSLMG   [218]
CrAAM22891          FKIADDVLRQGVKGISEIITVPGLVNVDFADVRAIMAGAGSSLMG   [218]
CmBAA85116          FRVADDILRQGVVGISDIIIRPGLINVDFADVRSVMAHAGSALMG   [216]
CcBAA82871          FRVADDILRQGVVGISDIIIRPGLINVDFADVRSVMAHAGSALMG   [216]
GtCAA07676          FSVADDILRQGVVGISEIIVRPGLINVDFADVRSVMADAGSALMG   [215]
GsBAA82090          FSVADDILRQGVVGISEIIVRPGLINVDFADVRSIMADAGSALMG   [215]
MsAAF35433          FLVADDILRQGVVGITEIIVKPGLVNVDFADVRTIMGNAGTALMG   [191]
GsBAA82091          FIFADEVLRQGVGGISDIITKPGLVNVDFADVRTVMAEKGFALLG   [223]
Te1949              FIIADDILRQGIQGISDIITVPGLVNVDFADVRAVMADAGSALMG   [216]
SsNP_440816         FRVADDILRQGVQGISDIIIIPGLVNVDFADVRAVMADAGSALMG   [216]
AsCAA83241          FRYADDVLRQGVQGISDIITIPGLVNVDFADVRAVMADAGSALMG   [216]
Np61                FRYADDVLRQGVQGISDIITIPGLVNVDFADVRAVMADAGSALMG   [216]
Tetll2382           FRVADDVLRQGVQGISDIINVPGLINVDFADIRSVMADAGSAMMG   [216]
SyAAC26227          FRVADDILRQGVQGISDIITIPGLVNVDFADVRAVMADAGSALMG   [216]
PsCAB56201          FRNADDVLRMGVKGISDIITCPGLVNVDFADVRSVMTEAGTALLG   [215]
Pm1658              FRNADDVLRMGVKGISDIITCPGLVNVDFADVRSVMTEAGTALLG   [215]
PmCAB95028          FKNADDVLRMGVKGITDIITLPGLVNVDFADVRSVMTEAGTSLLG   [215]
Sy549               FRSADDVLRMGVKGISDIITCPGLVNVDFADVRSVMTEAGTALLG   [215]
Pm1268              FRSADDILRMGVKGISDIITCPGLVNVDFADVRSVMTEAGTALLG   [215]
NtCAB41987          FLLADDVLRQGVQGISDIITIPGLVNVDFADVKAVMKDSGTAMLG   [216]
NtCAB89287          FLLADDVLRQGVQGISDIITIPGLVNVDFADVKAVMKDSGTAMLG   [216]
TeAAF81220          FLLADDVLRQGVQGISDIITIPGLVNVDFADVKAVMKDSGTAMLG   [216]
AtAAA82068          FLLADDVLRQGVQGISDIITIPGLVNVDFADVKAVMKDSGTAMLG   [216]
PsCAA75603          FRLADDVLRQGVQGISDIITIPGLVNVDFADVKAVMKDSGTAMLG   [216]
NtAAF23770          FLLADDVLCQGVQGISDIITIPGLVNVDFADVKAIMKDSGTAMLG   [216]
NtCAB89286          FLLADDVLCQGVQGISDIITIPGLVNVDFADVKAIMKDSGTAMLG   [216]
OsAAK64282          FLLADDVLRQGVQGISDIITIPGLVNVDFADVKAVMKNSGTAMLG   [218]
CrBAB91150          FALADDVLRQGVQGISDIITVPGLINVDFADVKAIMSNSGTAMLG   [216]
CpAAC32266          FKKADEILRQGVQGIADLISKPGVINLDFADVRTVMANKGIAHMG   [216]
EcP06138            FGAANDVLKGAVQGIAELITRPGLMNVDFADVRTVMSEMGYAMMG   [216]
BsAAA22457          FREADNVLRQGVQGISDLIATPGLINLDFADVKTIMSNKGSALMG   [216]
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[                    230        240        250        260        270]
[                    .          .          .          .          .]

NtCAB89288          IGTAT-------GKTRARDAALNAIQSPLLDIG-IERATGIVWNI    [255]
NtCAC44257          IGTAT-------GKTRARDAALNAIQSPLLDIG-IERATGIVWNI    [255]
OsCLB17724_5        IGTAT-------GKSRARDAALNAIQSPLLDIG-IERATGIVWNI    [255]
GlAAF23771          IGTAT-------GKTRARDAALNAIQSPLLDIG-IERATGIVWNI    [255]
LlBAA96782          IGTAT-------GKTRARDAALNAVQSPLLDIG-IERATGIVWNI    [255]
AtAAC35987          IGTAT-------GKSRARDAALNAIQSPLLDIG-IERATGIVWNI    [255]
AtAAK63846          IGTAT-------GKTRARDAALNAIQSPLLDIG-IERATGIVWNI    [255]
OsCL005296_338      IGTAT-------GKTRARDAALNAIQSPLLDIG-IERATGIVWNI    [255]
PpCAB76386          IGTAT-------GKSKAREAALSAIQSPLLDVG-IERATGIVWNI    [255]
PpCAB54558          IGTAT-------GKSRAREAALSAIQSPLLDVG-IERATGIVWNI    [255]
CrAAM22891          QGYGS-------GPRRASDAALRAISSPLLEVG-IERATGVVWNI    [255]
CmBAA85116          IGTGS-------GKSRAHDAAVAAISSPLLDFP-IERAKGIVFNV    [253]
CcBAA82871          IGTGS-------GKSRAHDAAVAAISSPLLDFP-IERAKGIVFNV    [253]
GtCAA07676          IGTGS-------GKTRAQDAAVAAISSPLLDFP-IEKARGIVFNI    [252]
GsBAA82090          IGSGS-------GKSRAKDAAVAAISSPLLDFP-IERAKGIVFNI    [252]
MsAAF35433          IGHGK-------GKNRAKDAALSAISSPLLDFP-ITRAKGIVFNI    [228]
GsBAA82091          IGTAS-------GDSRARNAATAAISSPLLDFP-ITSAKGAVFNI    [260]
Te1949              IGMGS-------GKSRAREAANAAISSPLLESS-IEGAKGVVFNI    [253]
SsNP_440816         IGVGS-------GKSRAKEAATAAISSPLLESS-IQGAKGVVFNV    [253]
AsCAA83241          IGVSS-------GKSRAREAAIAAISSPLLECS-IEGARGVVFNI    [253]
Np61                IGVSS-------GKSRAREAAIAAISSPLLECS-IEGARGVVFNI    [253]
Tetll2382           IGIAS-------GKSRATEAALSAISSPLLERS-IEGAKGVVFNI    [253]
SyAAC26227          IGSGS-------GKSRAREAAHAAISSPLLESS-IEGARGVVFNI    [253]
PsCAB56201          IGIGS-------GRSRALEAAQAAMNSPLLEAARIDGAKGCVINI    [253]
Pm1658              IGIGS-------GRSRALEAAQAAMNSPLLEAARIDGAKGCVINI    [253]
PmCAB95028          IGIGS-------GRSRAAEAAQAAINSPLLEAGRIDGAKGCVVNI    [253]
Sy549               IGIGS-------GRSRAVEAAQAAISSPLLETERIDGAKGCVINI    [253]
Pm1268              IGEGS-------GRSRAIEAAQAAISSPLLEAARIDGAKGCVINI    [253]
NtCAB41987          VGVSS-------SKNRAEEAAEQATLAPLIGSS-IQSATGVVYNI    [253]
NtCAB89287          VGVSS-------SKNRAEEAAEQATLAPLIGSS-IQSATGVVYNI    [253]
TeAAF81220          VGVSS-------SKNRAEEAAEQATLAPLIGSS-IQSATGVVYNI    [253]
AtAAA82068          VGVSS-------SKNRAEEAAEQATLAPLIGSS-IQSATGVVYNI    [253]
PsCAA75603          VGVSS-------GKNRAEEAAEQATLAPLIGSS-IQSATGVVYNI    [253]
NtAAF23770          VGVSS-------SRNRAEEAAEQATLAPLIGSS-IQSATGDVYNI    [253]
NtCAB89286          VGVSS-------SRNRAEEAAEQATLAPLIGLS-IQSATGVVYNI    [253]
OsAAK64282          VGVSS-------SKNRAQEAAEQATLAPLIGSS-IEAATGVVYNI    [255]
CrBAB91150          VGAASTATAAPGGPDRAEQAAVAATSAPLIQRS-IEKATGIVYNI    [260]
CpAAC32266          IGRAS-------GENKAEIAAKMAIQSPLLETT-IEGAKSVLINF    [253]
EcP06138            SGVAS-------GEDRAEEAAEMAISSPLLEDIDLSGARGVLVNI    [254]
BsAAA22457          IGIAT-------GENRAAEAAKKAISSPLLEAA-IDGAQGVLMNI    [253]
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[                    280        290        300        310    ]
[                    .          .          .          .      ]

NtCAB89288          TGGSDLTLFEVNAAAEVIYDLVDPSANLIFGAVIDPSIS-GQVSI   [299]
NtCAC44257          TGGSDLTLFEVNAAAEVIYDLVDPSANLIFGAVIDPSIS-GQVSI   [299]
OsCLB17724_5        TGGADMTLFEVNSAAEIIYDLVDPNANLIFGAVIDPSLN-GQVSI   [299]
GlAAF23771          TGGSDLTLFEVNAAAEVIYDLVDPSANLIFGAVVDPSLC-GQVSI   [299]
LlBAA96782          TGGNDLTLYEVNAAAEVIYDLVDPAANLIFGAVIDPSIS-GQVSI   [299]
AtAAC35987          TGGSDLTLFEVNAAAEVIYDLVDPTANLIFGAVVDPALS-GQVSI   [299]
AtAAK63846          TGGSDLTLFEVNAAAEVIYDLVDPTANLIFGAVVDPSYS-GQISI   [299]
OsCL005296_338      TGGNDLTLTEVNAAAEVIYDLVDPGANLIFGSVIDPSYT-GQVSI   [299]
PpCAB76386          TGGSDMTLFEVNAAAEVIYDLVDPNANLIFGAVVDEALH-DQISI   [299]
PpCAB54558          TGGSDMTLFEVNAAAEVIYDLVDPNANLIFGAVVDEALH-GQVSI   [299]
CrAAM22891          TGPPNMTLHEVNEAAEIIYDMVDPNANLIFGAVVDSTLPDDTVSI   [300]
CmBAA85116          TGGEDMTLHEINQAAEVIYEAVDPNANIIFGALIDQQME-SEISI   [297]
CcBAA82871          TGGEDMTLHEINQAAEVIYEAVDPNANIIFGALVDQQME-SEISI   [297]
GtCAA07676          TGGQDMTLHEINSAAEVIYEAVDSNANIIFGALVDDNME-NEISI   [296]
GsBAA82090          TGGHDMTLHEINAAAEVIYEAVDLNANIIFGALVDDSME-NELSI   [296]
MsAAF35433          VGGSDMSLQEINAAAEVIYENVDQDANIIFGAMVDDKMTSGEVSI   [273]
GsBAA82091          TGGTDMTLSEVNQAAQVIYDSVDSDANIIFGAVVDETFK-GKVSV   [304]
Te1949              TGGTDLTLHEVNAAAEIIYEVVDPNANIIFGAVIDDKLQ-GEIKI   [297]
SsNP_440816         TGGTDLTLHEVNVAAEIIYEVVDADANIIFGAVIDDRLQ-GEMRI   [297]
AsCAA83241          TGGSDLTLHEVNAAAETIYEVVDPNANIIFGAVIDDRLQ-GEVRI   [297]
Np61                TGGTDLTLHEVNAAAEAIYEVVDPNANIIFGAVIDDRLQ-GEVRI   [297]
Tet112382           TGGTDLSLHEVNAAADVIYNVADANANIIFGAVIDPQMQ-GEVQI   [297]
SyAAC26227          TGGRDMTLHEVNAAADAIYEVVDPEANIIFGAVIDDRLE-GELRI   [297]
PsCAB56201          TGGKDMTLEDMTSASEIIYDVVDPEANIIVGAVIDESME-GEIQV   [297]
Pm1658              TGGKDMTLEDMTSASEIIYDVVDPEANIIVGAVIDESME-GEIQV   [297]
PmCAB95028          TGGKDMTLEDMTSASEVIYDVVDPEANIIVGAVIDEALE-GEVQV   [297]
Sy549               SGGRDMTLEDMTTASEVIYDVVDPEANIIVGAVVDEALE-GEIHV   [297]
Pm1268              SGGRDMTLEDMTSASEVIYDVVDPEANIIVGAVVDEKLE-GEVHV   [297]
NtCAB41987          TGGKDITLQEVNRVSQVVTSLADPSANIIFGAVVDERYN-GEIHV   [297]
NtCAB89287          TGGKDITLQEVNRVSQVVTSLADPSANIIFGAVVDERYN-GEIHV   [297]
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NtAAF23770          TGGKDITLQEVNKVSQVVTSLADPSANIIFGAVVDERYN-GEIQV   [297]
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CrBAB91150          TGGRDLTLAEVNRVSEVVTALADPSCNIIFGAVVDEQYD-GELHV   [304]
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[                    .  ]

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AtAAC35987          TLIATGF   [306]
AtAAK63846          TLIATGF   [306]
OsCL005296_338      TLIATGF   [306]
PpCAB76386          TLIATGF   [306]
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GsBAA82090          TVIATGF   [303]
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GsBAA82091          TVVATGF   [311]
Te1949              TVIATGF   [304]
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Np61                TVIATGF   [304]
Tetll2382           TVIATGF   [304]
SyAAC26227          TVIATGF   [304]
PsCAB56201          TVIATGF   [304]
Pm1658              TVIATGF   [304]
PmCAB95028          TVIATGF   [304]
Sy549               TVIATGF   [304]
Pm1268              TVIATGF   [304]
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NtCAB89287          TIIATGF   [304]
TeAAF81220          TIVATGF   [304]
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NtCAB89286          TLIATGF   [304]
OsAAK64282          TIIATGF   [306]
CrBAB91150          TIIATGF   [311]
CpAAC32266          TVIATGL   [304]
EcP06138            TVVATGI   [305]
BsAAA22457          TVIATGF   [304]
;
END;
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APPENDIX B

FtsZ cDNA Alignment

The Nexus file with the FtsZ cDNA alignment that was used in the phylogenetic analyses that are discussed in Chapter 5 and used to produce the tree shown in Figure 2 of that chapter. Sequences are labeled with the organism initials followed by the accession or gene number.

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MATRIX

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OsCLB17724_5        GCCAAGATCAAGGTCGTCGGGGTCGGCGGAGGAGGCTCCAATGCC   [45]
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NtAJ311847          GCGAAGATCAAGGTGGTTGGTGTAGGAGGTGGCGGATCGAATGCA   [45]
GlAF205859          GCAAAGATCAAGGTAGTGGGCGTTGGAGGGGGTGGCTCGAATGCA   [45]
AtAF089738          GCGAGGATTAAGGTTATTGGTGTGGGAGGTGGTGGATCAAATGCT   [45]
AtAF384167          GCTAGGATTAAAGTTATCGGCGTTGGAGGTGGTGGTTCAAACGCT   [45]
PpAJ249139          GCGAAAATTAAAGTGATAGGCGTGGGGGGCGGGGGTTCCAACGCC   [45]
PpAJ249138          GCGAAAATTAAAGTAATAGGGGTCGGAGGTGGGGGTTCCAACGCC   [45]
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CcAB023962          TGTTTGATCAAGGTGATTGGTGTCGGCGGCGGCGGTGGCAACGCC   [45]
GtAJ007748          TGTGTTATTAAAGTTATTGGTGTTGGAGGTGGTGGTGGAAATGCA   [45]
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Pm1268              GCTCGCATTGAAGTAATTGGCGTCGGCGGAGGCGGCAGCAATGCC   [45]
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AsZ31371            GCCAATATCAAAGTAATTGGTGTTGGCGGTGGTGGTGGTAATGCT   [45]
Np61                GCCAACATCAAAGTGATTGGTGTCGGTGGCGGCGGTGGTAATGCC   [45]
Tet112382           GCACGCATCAAAGTGATTGGTGTCGGTGGTGGCGGTGGCAATGCA   [45]
Te1949              GCAAAAATTAAAGTAATTGGCGTAGGAGGAGGTGGCGGCAATGCT   [45]
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NtAJ271749          GCTAAGATTAAGGTTATCGGCGTCGGTGGCGGTGGTAACAATGCC   [45]
TeAF251346          GCAAAAATCAAAGTCGTTGGCGTCGGTGGTGGTGGCAACAATGCC   [45]
AtU39877            GCGAGAATTAAGGTGATTGGTGTCGGTGGTGGTGGTAACAATGCC   [45]
PsY15383            GCTAAGATTAAGGTCGTTGGAATTGGGGGTGGTGGTAACAATGCC   [45]
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NtAJ271748          GCCAAGATTAAGGTTGTCGGCGTCGGTGGCGGCGGCAACAATGCT   [45]
OsAF383876          GCGAGGATAAAGGTCGTGGGCGTCGGCGGCGGCGGGAACAACGCT   [45]
CrAB084236          GCCTGCATCAAGGTTATCGGGGTCGGCGGTGGTGGCGGCAATGCC   [45]
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OsCLB17724_5       GTCAACAGGATGATTGAGAGCTCCATGAACGGCGTCGAGTTCTGG    [90]
NtAJ271750         GTAAATCGCATGATTGAGAGTTCGATGAAGGGTGTAGAGTTTTGG    [90]
NtAJ311847         GTAAATCGCATGATTGAGAGTTCGATGAAGGGTGTAGAGTTTTGG    [90]
GlAF205859         GTTAATCGGATGATTGAAAGTGCTATGAAAGGCGTAGAGTTTTGG    [90]
AtAF089738         GTGAATCGTATGATAGAGAGTGAAATGTCAGGTGTGGAGTTCTGG    [90]
AtAF384167         GTGAATCGTATGATTGAGAGTGAGATGATTGGTGTGGAGTTTTGG    [90]
PpAJ249139         GTCAACCGAATGCTTGAGAGCGAAATGCAAGGTGTGGAATTCTGG    [90]
PpAJ249138         GTAAACCGAATGCTTGAGAGCGAGATGCAAGGGGTAGAATTCTGG    [90]
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CmAB032072         GTGAACCGTATGGCGGATACAGGTATTTCGGGTGTGGAGTTCTGG    [90]
CcAB023962         GTCAACCGTATGGCCGACACCGGCATCTCAGGCGTAGAGTTTTGG    [90]
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Np61               GTTAACCGCATGATCGAATCTGATGTCTCTGGTGTAGAGTTTTGG    [90]
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NtAJ311847          ------CCTGTAGCGGCTGAGCAACGACTGCCGATAGGTCAAGAA   [165]
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PsY15383            ------------GCGGCCGAGAATCCTATTAAAATTGGCGAGCTT   [159]
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NtAJ311847          CTTACAAGGGGACTTGGTGCAGGTGGTAATCCAGATATAGGGATG   [210]
GlAF205859          CTTACCAGAGGACTTGGAGCAGGTGGGAACCCTGATATTGGTATG   [210]
AtAF089738          TTGACTAGGGGTTTAGGTGCTGGAGGAAATCCAGAAATCGGTATG   [210]
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GtAJ007748          AAAGCAGCAGAAGAAAGTAGAGACTTAATTGCTGAAGCCGTCTCT   [246]
GsAB022594          CAGGCAGCTGAAGAGTCTCAGGCTGCAATAAGTTCTGCAGTTCAA   [246]
MsAF120117          AAAGCTGCCGAGGAAAGTCGGGAAGAAATCATGGCTGTTGTAAAG   [174]
GsAB022595          GTAGCAGCCGAAGAGTCCAAAGAGGATATAGCAAAAGCTCTTCAA   [270]
PsAJ011025          AAAGCTGCTGAGGAATCTAAAGATGAATTGCAACAAACCTTAGAG   [249]
Pm1658              AAAGCTGCTGAGGAATCTAAAGATGAATTGCAACAAACCTTAGAG   [249]
PmAJ237851          AAAGCTGCAGAAGAATCTAGAGCAGAGCTTCAACAAGCTTTAGAA   [249]
Sy549               AAGGCCGCCGAAGAGTCCCGAACAGACCTCCACGACGCCCTTCAG   [249]
Pm1268              AAGGCGGCGGAGGAATCTCGAGCAGAACTGCAACAAGCTCTGCAA   [249]
SyAF076530          AAAGCCGCTGAAGAATCGCGGGAAGAACTAATCGCCGCCTTGGAA   [249]
AsZ31371            AAAGCAGCTGAAGAATCACGCGATGAAATTGCTACAGCCTTAGAA   [249]
Np61                AAGGCAGCTGAGGAATCACGAGACGAAATTGCTACAGCTTTAGAG   [249]
Tetl12382           AAGGCGGCCGAAGAATCCCGCGAAGACCTGGCTGCGGCGCTCAAG   [249]
Te1949              AAAGCTGCTGAAGAATCTAGAGATGAAATAGCTAATGCTTTAGAC   [249]
SsNC_000911         AAAGCGGCGGAGGAATCCCGGGATGAAATTGCCCGTTCCCTTGAG   [249]
NtAJ133453          CAGGCAGCGGAGGAGTCGAAGGAAGCCATTGCAAATTCTCTAAAA   [249]
NtAJ271749          CAGGCAGCAGAGGAGTCGAAGGAAGCCATTGCAAATTCTCTAAAA   [249]
TeAF251346          CAGGCTGCGGAGGAGTCGAAGGAAGCGATTGGGAATGCGCTTAAA   [249]
AtU39877            CAAGCTGCAGAAGAATCAAAAGATGCAATTGCTAATGCTCTTAAA   [249]
PsY15383            CAAGCTGCGGAGGAATCGAAAGAGGCTATTGCTAATGCGCTTAAA   [249]
NtAF205858          CAGGCAGCAGAGGAGTCAAAGGAACACATTGCAAATGCTCTTAAA   [249]
NtAJ271748          CAGGCAGCAGAGGAGTCAAAGGAACACATTGCAAATGCTCTTAAA   [249]
OsAF383876          CAAGCTGCTGAGGAATCAAAAGAAGCCATAGCCAATGCCCTGAAG   [249]
CrAB084236          CGCGCTGCTATGGAGAGCGAGGAGGCGCTGCGCCGCATGGTGCAG   [249]
CpAF067823          AAATCCGTTGATGAAACCCAAGACGAAATTGCACAGGCTTTGCAT   [249]
BsM22630            AAAGCCGCTGAAGAAAGCAAAGAGCAGATTGAAGAAGCACTTAAA   [249]
EcX55034            AATGCGGCTGATGAGGATCGCGATGCATTGCGTGCGGCGCTGGAA   [249]
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[                    280        290        300        310    ]
[                    .          .          .          .      ]

LlAB042101          GGTGCTGACATGGTTTTCGTCACGGCTGGAATGGGTGGGGGTACA   [300]
OsCL005296_338      GGTGCTGATATGATTTTTGTGACTGCTGGAATGGGAGGAGGGACA   [300]
OsCLB17724_5        GGTGCTGATATGGTTTTTGTCACGGCTGGGATGGGTGGAGGCACT   [300]
NtAJ271750          GGCGCAGACATGGTTTTTGTTACTGCTGGAATGGGTGGAGGAACA   [300]
NtAJ311847          GGCGCAGACATGGTTTTTGTTACTGCTGGAATGGGTGGAGGAACA   [300]
GlAF205859          GGTGCAGATATGGTTTTTGTAACTGCTGGAATGGGTGGAGGAACA   [300]
AtAF089738          GGCTCAGATATGGTCTTTGTCACAGCTGGAATGGGCGGTGGAACT   [300]
AtAF384167          GGTTCAGATATGGTCTTTGTCACAGCTGGAATGGGTGGTGGAACT   [300]
PpAJ249139          GGAGCTGACATGGTTTTCGTTACAGCGGGCATGGGTGGTGGCACT   [300]
PpAJ249138          GGAGCTGACATGGTTTTCGTAACGGCGGGTATGGGTGGCGGCACT   [300]
CrAF449446          GATACTGACATGGTATTCGTGACGGCCGGAATGGGCGGCGGCACG   [300]
CmAB032072          GGCGCCGACCTGGTGTTTGTGACTGCCGGAATGGGCGGCGGCACT   [294]
CcAB023962          GGCGCTGACCTCGTCTTTGTGACAGCAGGCATGGGCGGCGGTACC   [294]
GtAJ007748          GCAGGTGATCTAGTTTTTGTGACAGCAGGAATGGGAGGAGGTACA   [291]
GsAB022594          GGTGGAGATCTCGTTTTTGTTACAGCCGGTATGGGAGGTGGAACA   [291]
MsAF120117          AACGCAGACCTGGTCTTTGTAACGGCCGGTATGGGTGGTGGCACG   [219]
GsAB022595          GGTGGAGATCTTGTGTTTGTTACTGCTGGTATGGGAGGTGGTACA   [315]
PsAJ011025          GGCTCTGACTTGGTTTTTATTGCTGCAGGTATGGGAGGAGGAACT   [294]
Pm1658              GGCTCTGACTTGGTTTTTATTGCTGCAGGTATGGGAGGAGGAACT   [294]
PmAJ237851          GGTGCCGATTTGGTATTTATTGCCGCTGGCATGGGTGGAGGAACA   [294]
Sy549               GGTTCCGATCTGGTGTTCATCGCTGCGGGTATGGGTGGCGGAACC   [294]
Pm1268              GGAGTCGATCTCGTCTTCATCGCCGTTGGCATGGGTGGCGGAACC   [294]
SyAF076530          GGGGCTGACCTCGTCTTTATCACGGCGGGGATGGGCGGTGGAACC   [294]
AsZ31371            GGTGCTGATTTAGTATTTATCACTGCTGGGATGGGAGGTGGTACT   [294]
Np61                GGTGCAGACCTAGTATTTATCACCGCTGGTATGGGTGGCGGTACT   [294]
Tetl12382           GACGCTGATTTGATTTTCATTACCTGTGGCATGGGGGGCGGCACT   [294]
Te1949              CATCCAGATCTAGTATTTATTACTGCTGGAATGGGAGGTGGTACA   [294]
SsNC_000911         GGTACGGATTTGGTCTTTATTACTGCGGGCATGGGGGGCGGCACT   [294]
NtAJ133453          GGTTCAGATATGGTGTTCATAACAGCAGGAATGGGTGGAGGTACA   [294]
NtAJ271749          GGTTCAGATATGGTGTTCATAACAGCAGGAATGGGTGGAGGTACA   [294]
TeAF251346          GGGTCGGATCTTGTGTTTATAACAGCAGGTATGGGTGGTGGGACG   [294]
AtU39877            GGATCAGACCTTGTTTTCATAACTGCTGGTATGGGTGGTGGAACA   [294]
PsY15383            GGATCGGATTTGGTGTTTATAACAGCTGGGATGGGTGGCGGTACA   [294]
NtAF205858          GGTTCGGATATGGTGTTTATAACAGCAGGCATGGGTGGCGGTACT   [294]
NtAJ271748          GGTTCGGATATGGTGTTTATAACAGCAGGCATGGGTGGCGGTACT   [294]
OsAF383876          GATTCTGACCTTGTCTTCATAACGGCTGGAATGGGAGGGGGTACT   [294]
CrAB084236          GGCGCTGATCTGGTGTTCATCACCGCGGGCATGGGCGGCGGTACC   [294]
CpAF067823          GGTTCTGACATGGTATTTATTACTGCCGGAATGGGTGGTGGCACC   [294]
BsM22630            GGTGCTGACATGGTATTCGTGACAGCTGGTATGGGCGGCGGAACA   [294]
EcX55034            GGTGCAGACATGGTCTTTATTGCTGCGGGTATGGGTGGTGGTACC   [294]
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[                    320        330        340        350        360]
[                    .          .          .          .          .]

LlAB042101           GGAACTGGTGGTGCTCCTGTAATTGCTGGAGTTGCCAAGTCAATG   [345]
OsCL005296_338       GGCACAGGCGGGGCCCCAGTTATAGCAGGGATTGCAAAGTCCATG   [345]
OsCLB17724_5         GGAACTGGAGGTGCCCCTGTAATCGCTGGAATTGCCAAGTCCATG   [345]
NtAJ271750           GGGACTGGTGCAGCTCCTATAATTGCAGGAACTGCTAAATCAATG   [345]
NtAJ311847           GGGACTGGTGCGGCTCCTATAATTGCAGGAACTGCCAAATCAATG   [345]
GlAF205859           GGAACTGGCGGGGCTCCAGTAATTGCGGGAATTGCTAAATCTATG   [345]
AtAF089738           GGCACTGGTGCAGCCCCTGTAATTGCAGGAATTGCCAAGGCGATG   [345]
AtAF384167           GGCACAGGTGGTGCTCCTATAATTGCAGGGGTTGCAAAAGCGATG   [345]
PpAJ249139           GGCAGCGGTGCTGCACCAATCATTGCTGGTGTAGCGAAGCAATTG   [345]
PpAJ249138           GGCAGCGGTGCAGCACCAATAATTGCGGGTGTGGCGAAGCAGTTG   [345]
CrAF449446           GGCAGTGGCGCCGCGCCCGTCGTGGCGGAGGTGGCGCGTGAATTG   [345]
CmAB032072           GGTTCCGGAGCCGCTCCAGTAGTTGCAGAGGCTGCCCGCGAGCAA   [339]
CcAB023962           GGCTCGGGTGCAGCACCGGTCGTCGCTGAGGCTGCCCGTGAACAG   [339]
GtAJ007748           GGATCAGGAGCTGCCCCGATAGTAGCTGAAGTTGCTAAAGAAATG   [336]
GsAB022594           GGCTCAGGTGCTGCTCCAATTGTTGCTAAAATAGCTAAAGAACAA   [336]
MsAF120117           GGATCTGGCGCTGCCCCTGTGGTGGCGGAATGTGCTAAGGAGGCT   [264]
GsAB022595           GGAACAGGAGCAGCTCCAATAGTTGCCGATGTTGCTAGAGAACTG   [360]
PsAJ011025           GGGACAGGAGCGGCTCCAGTAGTTGCTGAGGTTGCAAAGCAAAGT   [339]
Pm1658               GGGACAGGAGCGGCTCCAGTAGTTGCTGAGGTTGCAAAGCAAAGT   [339]
PmAJ237851           GGCACTGGAGCAGCACCAGTAGTTGCAGAAGTAGCCAAACAAAGC   [339]
Sy549                GGAACGGGTGCCGCCCCCGTGGTTGCGGAGGTCGCCCGTGAGGTC   [339]
Pm1268               GGCACTGGTGCCGCTCCAGTGGTTGCCGAAGTTGCCAAGGAAAGC   [339]
SyAF076530           GGCACTGGAGCTGCCCCGATCGTGGCAGAAGTCGCCAAAGAAGTG   [339]
AsZ31371             GGTACTGGTGCTGCACCAATTGTTGCGGAAGTGGCAAAGGAAATG   [339]
Np61                 GGGACTGGTGCAGCTCCAATCGTAGCAGAAGTAGCCAAAGAAATG   [339]
Tet112382            GGCACCGGCGCTGCCCCAATTGTGGCGGAAGTGGCCAAGGAACAG   [339]
Te1949               GGTACTGGTGCAGCTCCAGTTATAGCTGAAATTGCTAAAGAAGCA   [339]
SsNC_000911          GGCACTGGAGCAGCTCCCATTGTGGCCGAGGTGGCCAAAGAAATG   [339]
NtAJ133453           GGATCTGGTGCTGCTCCTGTTGTGGCTCAAATAGCAAAAGAAGCA   [339]
NtAJ271749           GGATCTGGTGCTGCTCCTGTTGTGGCTCAAATAGCAAAAGAAGCA   [339]
TeAF251346           GGTTCGGGTGCTGCTCCAGTTGTAGCGCAGATAGCGAAAGAAGCA   [339]
AtU39877             GGGTCTGGTGCTGCACCTGTGGTAGCTCAGATTTCGAAGGATGCT   [339]
PsY15383             GGGTCCGGTGCTGCCCCAGTTGTGGCTCAAATATCAAAAGAGGCA   [339]
NtAF205858           GGATCTGGTGCTGCTCCTGTTGTTGCTCAAATAGCCAAAGAAGCA   [339]
NtAJ271748           GGATCTGGTGCTGCTCCTGTTGTTGCTCAAATAGCCAAAGAAGCA   [339]
OsAF383876           GGATCTGGTGCTGCTCCAGTTGTTGCTCAGATATCAAAGGAAGCC   [339]
CrAB084236           GGCACCGGTGCCGCCCCCGTGGTGGCCCGCCTGTCCAAGGAGTTG   [339]
CpAF067823           GGTACAGGTGCGGCTCCCAGAATTGCCGCAATTTCCAAAGAACTA   [339]
BsM22630             GGAACAGGTGCCGCACCGGTTATCGCACAAATCGCGAAAGACTTA   [339]
EcX55034             GGTACAGGTGCGGCACCAGTCGTCGCTGAAGTGGCAAAAGATTTG   [339]
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[                    370        380        390        400    ]
[                    .          .          .          .      ]

LlAB042101          GGTATCTTGACTGTAGGCATTGTGACAACGCCATTTATGTTTGAA   [390]
OsCL005296_338      GGTATATTAACTGTTGGAATTGTGACAACCCCGTTTGCATTTGAG   [390]
OsCLB17724_5        GGTATACTGACAGTTGGCATCGTGACGACACCATTCTCATTTGAA   [390]
NtAJ271750          GGTATCTTAACTGTTGGTATTGTTACAACCCCCTTTTCTTTCGAG   [390]
NtAJ311847          GGTATCTTAACTGTTGGTATTGTTACAACCCCTTTTTCTTTCGAG   [390]
GlAF205859          GGTATCTTGACCGTTGGTATTGTCACAACACCTTTCTCCTTTGAA   [390]
AtAF089738          GGTATATTGACAGTTGGTATTGCCACTACGCCTTTCTCGTTTGAG   [390]
AtAF384167          GGTATATTAACGGTTGGTATTGTGACAACGCCTTTCTCATTTGAG   [390]
PpAJ249139          GGAATTCTTACCGTGGGAATAGTAACTACGCCTTTTGCCTTTGAA   [390]
PpAJ249138          GGAATTCTTACTGTAGGAATAGTTACTACTCCTTTCGCCTTTGAA   [390]
CrAF449446          GGCATCCTAACAGTTGGCATCGTCACCACCCCCTTCACCTTCGAG   [390]
CmAB032072          GGCTGCCTAACCGTGGGCGTTGTCACCAAGCCATTCGCGTTTGAA   [384]
CcAB023962          GGCTGCTTGACAGTCGGTGTCGTGACCAAGCCGTTTGCCTTCGAA   [384]
GtAJ007748          GGTTGTTTAACTGTTGGAGTTGTAACCAAACCTTTTGCTTTTGAA   [381]
GsAB022594          GGCTGTCTTACTGTTGGAGTTGTGACTAAACCTTTCAGCTTTGAG   [381]
MsAF120117          GGCGCGTTAACCGTAGGCGTAGTCACAAAGCCGTTCGGATTCGAA   [309]
GsAB022595          GGTTGTTTGACAGTTGGTGTTGTTACGAAACCTTTTGCTTTTGAA   [405]
PsAJ011025          GGTGCTTTAACTGTTGGGATAGTAACCAAGCCATTTTCATTTGAA   [384]
Pm1658              GGTGCTTTAACTGTTGGGATAGTAACCAAGCCATTTTCATTTGAA   [384]
PmAJ237851          GGAGCCCTTACTGTCGCAATAGTTACTAAACCATTTAGTTTTGAA   [384]
Sy549               GGAGCCCTCACCGTGGGAATCGTGACCAAACCCTTTGGTTTTGAA   [384]
Pm1268              GGTGCGCTCACTGTGGGCATCGTCACCAAACCCTTTAGCTTTGAG   [384]
SyAF076530          GGTGCGCTGACGGTTGGGATTGTCACCAAACCCTTCACCTTCGAA   [384]
AsZ31371            GGCGCTCTTACTGTTGGAGTGGTAACACGTCCTTTTGTTTTTGAA   [384]
Np61                GGCGCTCTCACTGTTGGGGTAGTCACACGTCCATTCGTCTTTGAA   [384]
Tetll2382           GGAGCCCTCACCGTTGCAGTGGTGACCCGCCCCTTTACCTTTGAG   [384]
Te1949              GGTTCTTTGACGGTGGGTGTTGTGACTCGCCCTTTTACTTTTGAG   [384]
SsNC_000911         GGCTGTTTGACGGTGGGCATTGTCACCCGTCCATTTACCTTTGAA   [384]
NtAJ133453          GGCTATTTGACTGTTGGTGTTGTCACATACCCATTCAGCTTTGAA   [384]
NtAJ271749          GGCTATTTGACTGTTGGTGTTGTCACATACCCATTCAGCTTTGAA   [384]
TeAF251346          GGGTATTTAACTGTTGGTGTTGTAACGTACCCATTCAGCTTTGAA   [384]
AtU39877            GGTTATTTGACTGTTGGTGTTGTTACCTATCCGTTTAGCTTTGAA   [384]
PsY15383            GGTTACTTGACTGTAGGTGTTGTTACATATCCTTTCAGTTTTGAA   [384]
NtAF205858          GGTTATTTGACTGTTGGTGTCGTCACGTATCCATTCAGCTTTGAA   [384]
NtAJ271748          GGTTATTTGACTGTTGGTGTCGTCACGTATCCATTCAGCTTTGAA   [384]
OsAF383876          GGTTATCTCACCGTTGGAGTTGTTACCTATCCATTCAGTTTTGAA   [384]
CrAB084236          GGCATCCTGACTGTGGGCGTCGTCACCTACCCCTTCAACTTCGAG   [384]
CpAF067823          GGCATCTTAACGGTAGGTGTTGTGACAAAGCCCTTTAACTTTGAG   [384]
BsM22630            GGCGCATTAACAGTCGGCGTTGTGACAAGACCGTTTACCTTCGAA   [384]
EcX55034            GGTATCCTGACCGTTGCTGTCGTCACTAAGCCTTTCAACTTTGAA   [384]
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[                    410        420        430        440        450]
[                    .          .          .          .          .]

LlAB042101         GGGCGAAGACGAACTGTTCAG------GCACAAGAAGGAATTGCA   [429]
OsCL005296_338     GGAAGGAGGCGTGCACTACAG------GCGCAAGAAGGAATTGCG   [429]
OsCLB17724_5       GGGAGGAGACGGGCAGTTCAG------GCTCAAGAAGGAATAGCA   [429]
NtAJ271750         GGGCGAAGACGAGCAGTTCAA------GCCCAAGAAGGTATTGCA   [429]
NtAJ311847         GGGCGAAGAAGAGCAGTTCAA------GCCCAAGAAGGGATTGCT   [429]
GlAF205859         GGTCGGAGAAGAGCAGTGCAA------GCACAAGAAGGCATCGCA   [429]
AtAF089738         GGTCGAAGAAGAACTGTTCAG------GCTCAAGAAGGGCTTGCA   [429]
AtAF384167         GGACGGAGAAGAGCACTCCAG------GCTCAGGAAGGGATTGCA   [429]
PpAJ249139         GGGCGGAGACGATCCGTTCAA------GCTCACGAAGGCATCGCG   [429]
PpAJ249138         GGGCGGAGACGAGCTGTCCAA------GCCCACGAGGGTATTGCA   [429]
CrAF449446         GGCCGCCAGCGCGCGCAGCAG------GCTCGCTCTGCTTTAGCC   [429]
CmAB032072         GGCCGGAAACGCATGAATCAG------GCGCTGGAGGCTATCGAG   [423]
CcAB023962         GGGCGGAGGCGTATGACGCAG------GCGCTCGAGGCTATCGAG   [423]
GtAJ007748         GGTAAACGAAGAATGCAACAA------GCAAATGACGCAATACTT   [420]
GsAB022594         GGAAGAAGGAGAATGCAACAG------GCAGAGGAAGCAATAGAA   [420]
MsAF120117         GGCCGAAAACGAATGCAACAA------GCGAGGAACGCTATTCTG   [348]
GsAB022595         GGACGTCGACGTCTGCAGCAA------GCAGTCGAAGGATTGGCA   [444]
PsAJ011025         GGTAAAAGGAGAATGCGTCAG------GCAGAAGAAGGGATTGCA   [423]
Pm1658             GGTAAAAGGAGAATGCGTCAG------GCAGAAGAAGGGATTGCA   [423]
PmAJ237851         GGTCGCCGCAGAATGCGTCAA------GCAGATGAAGGTATCGCC   [423]
Sy549              GGCCGCCGCCGCATGCGCCAG------GCCGATGAAGGCATCGCC   [423]
Pm1268             GGACGTCGTCGCATGCGACAG------GCCGCTGAAGGCATTGGC   [423]
SyAF076530         GGGCGTCGCCGAATGAAGCAG------GCGGAAGAAGGAACAGCC   [423]
AsZ31371           GGTCGTCGCCGGACTAGTCAG------GCAGAACAAGGAATTGAA   [423]
Np61               GGTCGCCGCCGTACCAGCCAA------GCCGAACAAGGGATCGAA   [423]
Tet112382          GGTCGTCGTCGAGCGAACCAA------GCCGATGAAGGGATTGAA   [423]
Te1949             GGACGACGCCGAATTACTCAG------GCTGATGAAGGAATTACT   [423]
SsNC_000911        GGCCGACGACGGGCTAAGCAA------GCTGAGGAAGGCATTAAT   [423]
NtAJ133453         GGACGTAAAAGATCCGTGCAG------GCTCTGGAAGCAATTGAA   [423]
NtAJ271749         GGACGCAAAAGATCCGTGCAG------GCTCTGGAAGCAATTGAA   [423]
TeAF251346         GGCCGTAAAAGATCAGTACAG------GCGTTAGAGGCTATTGAG   [423]
AtU39877           GGACGTAAAAGATCTTTGCAG------GCACTGGAAGCTATTGAA   [423]
PsY15383           GGACGGAAAAGATCCTTGCAG------GCACTTGAAGCGATTGAA   [423]
NtAF205858         GGACGTAAAAGATCTTTGCAG------GCTTTGGAAGCAATTGAA   [423]
NtAJ271748         GGACGTAAAAGATCTTTGCAG------GCTTTGGAAGCAATTGAA   [423]
OsAF383876         GGACGCAAGCGCTCTCTTCAGGCAAGTGCGTTGGAAGCATTAGAG   [429]
CrAB084236         GGCCGCCGCCGCGCCGGCCAG------GCTCTTGAGGGCATTGAG   [423]
CpAF067823         GGAAAGAAAAGAATGAGTAAT------GCCGAAAAAGGTATTATG   [423]
BsM22630           GGACGCAAAAGACAGCTTCAG------GCTGCAGGCGGAATCTCG   [423]
EcX55034           GGCAAGAAGCGTATGGCATTC------GCGGAGCAGGGGATCACT   [423]
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[                    460        470        480        490    ]
[                    .          .          .          .      ]

LlAB042101          GCTTTAAGAAATAACGTTGACACACTTATTGTCATCCCAAATGAC   [474]
OsCL005296_338      TCCTTAAGAAGCAATGTTGATACACTGATTGTAATTCCAAATGAC   [474]
OsCLB17724_5        GCCTTGAGAAATAGTGTGGACACCCTCATTGTCATCCCAAATGAC   [474]
NtAJ271750          GCTTTGAGAGAAAATGTCGATACTCTAATTGTCATTCCAAATGAC   [474]
NtAJ311847          GCTTTGAGAGAAAATGTCGATACTCTAATTGTCATTCCAAATGAC   [474]
GlAF205859          GCACTGAGAGATAATGTCGACACCCTAATTGTGATTCCAAATGAC   [474]
AtAF089738          TCTCTCAGAGACAATGTTGACACTCTCATCGTCATTCCAAATGAC   [474]
AtAF384167          GCCCTCAGAGATAATGTTGACACCCTCATTGTTATTCCAAACGAC   [474]
PpAJ249139          GCTCTCAAAAATAATGTTGACACTTTAATTACGATACCAAACAAC   [474]
PpAJ249138          GCTCTCAAAAATAACGTGGACACGTTAATTACGATTCCAAACAAC   [474]
CrAF449446          AACTTGCGTGCAGCGGTTGACACGCTCATTGTCATCCCGAACGAC   [474]
CmAB032072          GCCTTGCGCGAGAGCGTGGACACGCTGATTGTCGTGAGCAACGAC   [468]
CcAB023962          GCGCTGCGAGAGAGTGTGGACACGCTGATTGTGGTCAGCAATGAC   [468]
GtAJ007748          AACTTGAGAAACAAAGTTGATACACTTATTGTTGTATCTAATGAT   [465]
GsAB022594          GCTTTAAGAAAGGAAGTCGATACTTTGATTGTAGTTTCCAATGAT   [465]
MsAF120117          GAGATGAAGGACAAGGTGGACACGCTCATCGTCGTGTCCAACGAC   [393]
GsAB022595          AATTTGAGAGAAAAGGTCGATACTCTTATTGTTATTTCAAACGAT   [489]
PsAJ011025          AGATTAGCAGAAAACGTTGATACGCTTATTGTGATTCCAAATGAT   [468]
Pm1658              AGATTAGCAGAAAACGTTGATACGCTTATTGTGATTCCAAATGAT   [468]
PmAJ237851          AAGCTCACAGAAAGTGTTGACACTTTAATTGTCATCCCTAACGAT   [468]
Sy549               CGCTTGGCGGAACACGTGGATACCTTGATTGTGATTCCCAACGAT   [468]
Pm1268              CGCCTGGCCGATCATGTGGATACCTTGATTGTGATCCCCAACGAC   [468]
SyAF076530          GCACTGCAAAGCTCAGTCGACACTTTGATCACTATTCCTAATGAC   [468]
AsZ31371            GGGCTAAAAAGTAGAGTTGATACTTTAATTATTATTCCTAACAAT   [468]
Np61                GGCTTAAAAAGTAGGGTAGATACACTGATTATTATTCCTAACAAC   [468]
Tet112382           GCACTGCAAAGTCGCGTGGATACCCTAATCGTGATTCCCAATGAC   [468]
Te1949              GCTCTACAAACTAGAGTAGATACTTTAATTGTGATCCCCAATAAT   [468]
SsNC_000911         GCTCTCCAATCGCGGGTCGATACCCTAATTGTGATTCCTAATAAC   [468]
NtAJ133453          AAACTTCAGAAAAATGTAGATACCCTTATAGTAATTCCCAATGAC   [468]
NtAJ271749          AAACTTCAGAAAAATGTCGATACCCTTATAGTAATTCCCAATGAC   [468]
TeAF251346          AAGCTGCAAAAGAACGTTGACACACTTATAGTGATTCCAAATGAC   [468]
AtU39877            AAGCTCCAAAAGAATGTTGATACCCTTATCGTGATTCCAAATGAT   [468]
PsY15383            AAGCTTCAGAAAAATGTTGATACGCTTATTGTGATTCCAAATGAT   [468]
NtAF205858          AAACTTCAGAAAAATGTAGATACTCTTATAGTAATTCCCAATGAT   [468]
NtAJ271748          AAACTTCAGAAAAATGTAGATACTCTTATAGTAATTCCCAATGAT   [468]
OsAF383876          AAGCTGGAAAGGAGTGTAGACACACTTATTGTTATTCCAAATGAT   [474]
CrAB084236          GCGCTGCGTGAGGCCGTGGACTCCGTGATCGTCATCCCCAACGAC   [468]
CpAF067823          GAATTAAAGAAGAACGTTGATACTTTGGTTATTATTCCAAACCAA   [468]
BsM22630            GCAATGAAAGAAGCGGTGGATACACTGATCGTGATCCCGAACGAC   [468]
EcX55034            GAACTGTCCAAGCATGTGAACTCTCTGATCACTATCCCGAACGAC   [468]
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[                    500        510        520        530        540]
[                    .          .          .          .          .]

LlAB042101          AAGCTATTGACTGCCGTTTCCCCAAATACTCCTGTGACAGAGGCG   [519]
OsCL005296_338      AAATTGTTGACTGCTGTTTCTCCAAATACTCCTGTGACAGAAGCA   [519]
OsCLB17724_5        AAGCTGTTGTCTGCTGTTTCTCCAAATACTCCAGTAACCGAAGCA   [519]
NtAJ271750          AAATTATTGACAGCTGTTTCTCCATCGACCCCGGTAACTGAAGCT   [519]
NtAJ311847          AAATTGTTGACAGCTGTTTCTCCATCGACCCCAGTAACTGAAGCT   [519]
GlAF205859          AAATTACTCACTGCAGTTTCCCCATCTACTCCAGTCACAGAAGCA   [519]
AtAF089738          AAGTTGCTTACAGCTGTCTCTCAGTCTACTCCGGTAACAGAAGCA   [519]
AtAF384167          AAGTTACTAGCAGCAGTCTCTCAGTCTACTCCAGTTACAGAAGCA   [519]
PpAJ249139          AAGCTTTTGACTGCAGTTGCGCAGTCTACCCCCGTGACGGAAGCA   [519]
PpAJ249138          AAACTTTTGACTGCAGTTGCGCAGTCTACCCCAGTGACGGAAGCG   [519]
CrAF449446          CGGCTGCTGTCGGCCATGGACTCCAACGTGCCTATCAAGGACGCC   [519]
CmAB032072          AAGCTGCTTCAGATAGTCCCTGAGAACACACCATTGCAGGACGCA   [513]
CcAB023962          AAGCTGTTACAGATTGTACCAGAGAACACGCCATTGCAGGATGCA   [513]
GtAJ007748          AAATTATTACAGATAGTTCCAGATAATACGCCCCTTCAGGATGCA   [510]
GsAB022594          AAGTTACTCGAAATTGTTCCTGAAAATACAGCTTTGGAAAAGGCT   [510]
MsAF120117          AAGCTCTTGAAGATCGTGCCGGACAACACTCCCCTGACGGAAGCC   [438]
GsAB022595          CGACTCTTAGAAACAGTACCGAAAGATACTCCACTGACTGAGGCT   [534]
PsAJ011025          CGTTTAAAAGACGTAATTGCAGGA---GCTCCACTTCAAGAAGCC   [510]
Pm1658              CGTTTAAAAGACGTAATTGCAGGA---GCTCCACTTCAAGAAGCC   [510]
PmAJ237851          CGCCTTAAAGATGCAATTGCAGGA---GCGCCCCTTCAAGAAGCA   [510]
Sy549               CGTTTGCGGGAAGCGATCGCCGGG---GCTCCGCTTCAGGAGGCC   [510]
Pm1268              CGCATTAAAGACGTCATCTCCGAA---GCTCCGCTTCAAGAAGCC   [510]
SyAF076530          CGCCTACTCCACGCCATATCTGAGCAGACGCCGATTCAAGAAGCT   [513]
AsZ31371            AAATTATTGGAAGTGATTCCAGAACAAACACCAGTCCAAGAAGCG   [513]
Np61                AAACTACTGGAAGTGATCCCCGAACAAACACCTGTGCAAGAAGCT   [513]
Tetll2382           AAGATTCTCTCGGTCATCTCTGAGCAAACATCGGTTCAGGATGCG   [513]
Te1949              CGTTTGCTATCTGTAATTAATGACCAAACTCCAGTACAGGAGGCT   [513]
SsNC_000911         CAACTTTTGTCGGTTATTCCCGCCGAAACTCCTCTCCAGGAAGCT   [513]
NtAJ133453          CGTCTGCTAGATATTGCTGATGAGCAGACACCACTTCAAGATGCT   [513]
NtAJ271749          CGTCTGCTAGATATTGCTGATGAGCAGACACCACTTCAAGATGCT   [513]
TeAF251346          CGTTTGCTGGATATTGCTGATGAAAACACGCCTCTTCAGGATGCT   [513]
AtU39877            CGTCTGCTAGATATTGCTGATGAACAGACGCCACTTCAGGACGCG   [513]
PsY15383            CGTCTGCTTGACATAGCTGATGAGCAGATGCCCCTTCAAGATGCT   [513]
NtAF205858          CGTCTTCTGGATATTGCTGATGAGCAGACACCACTTCAAAATGCT   [513]
NtAJ271748          CGTCTTCTGGATATTGCTGATGAGCAGACACCACTTCAAAATGCT   [513]
OsAF383876          CGATTATTAGATGTTGTTGATGAAAACACGCCCTTGCAAGATGCG   [519]
CrAB084236          CGCCTGCTGGACGTGGCCGGCGCCAGCACCGCGCTGCAGGATGCC   [513]
CpAF067823          AGATTATTGAGCATTATTGATAAAAAGACGACACTCACCGAAGCT   [513]
BsM22630            CGTATCCTTGAAATTGTTGATAAAAACACACCGATGCTTGAAGCA   [513]
EcX55034            AAACTGCTGAAAGTTCTGGGCCGCGGTATCTCCCTGCTGGATGCG   [513]
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[                    550        560        570        580    ]
[                    .          .          .          .      ]

LlAB042101        TTTAACTTGGCTGATGATATACTTCGACAAGGTGTTCGTGGAATC   [564]
OsCL005296_338    TTTAATTTGGCAGATGATATACTTCGGCAAGGTGTCCGTGGAATC   [564]
OsCLB17724_5      TTCAACTTGGCTGATGATATTCTTCGACAAGGAATTCGTGGTATC   [564]
NtAJ271750        TTTAACCTGGCTGATGATATTCTTCGGCAAGGAGTTCGTGGAATT   [564]
NtAJ311847        TTTAACCTGGCTGATGATATTCTTCGGCAAGGAGTTCGTGGTATT   [564]
GlAF205859        TTTAACTTGGCTGATGATATTCTTCGACAAGGAGTTCGTGGAATC   [564]
AtAF089738        TTTAATCTAGCTGATGATATACTCCGTCAGGGGGTTCGTGGGATA   [564]
AtAF384167        TTTAATCTGGCAGATGATATACTTCGTCAAGGTGTCCGTGGAATA   [564]
PpAJ249139        TTCAATCTTGCCGATGACATCCTTCGGCAGGGAGTGCGGGGTATT   [564]
PpAJ249138        TTCAATCTTGCAGACGACATCCTTCGGCAGGGAGTGCGGGGTATT   [564]
CrAF449446        TTCAAAATTGCGGATGACGTACTGCGGCAGGGCGTAAAGGGCATC   [564]
CmAB032072        TTTCGAGTTGCAGATGACATCCTGCGGCAGGGTGTTGTGGGCATC   [558]
CcAB023962        TTTCGAGTAGCGGATGATATTCTGCGACAAGGTGTCGTTGGGATC   [558]
GtAJ007748        TTTTCTGTTGCTGATGATATTCTAAGACAAGGAGTTGTAGGAATA   [555]
GsAB022594        TTTTCTGTAGCGGATGATATTCTCAGACAAGGTGTGGTTGGAATC   [555]
MsAF120117        TTTCTGGTCGCAGACGACATCCTCAGACAGGGCGTGGTGGGCATC   [483]
GsAB022595        TTTATATTTGCGGACGAAGTTTTACGTCAAGGAGTTGGTGGAATT   [579]
PsAJ011025        TTTAGAAATGCTGATGATGTTTTAAGGATGGGAGTTAAAGGTATA   [555]
Pm1658            TTTAGAAATGCTGATGATGTTTTAAGGATGGGAGTTAAAGGTATA   [555]
PmAJ237851        TTTAAAAATGCAGATGATGTTTTACGAATGGGAGTGAAAGGCATA   [555]
Sy549             TTCCGCAGTGCCGATGACGTGCTTCGGATGGGTGTGAAAGGCATC   [555]
Pm1268            TTCCGAAGTGCAGATGACATCCTGCGTATGGGTGTTAAAGGTATC   [555]
SyAF076530        TTCCGGGTCGCCGACGATATTCTCCGGCAGGGTGTGCAAGGGATT   [558]
AsZ31371          TTTCGTTATGCAGATGACGTACTACGTCAAGGGGTACAAGGCATT   [558]
Np61              TTTCGCTATGCAGATGACGTGTTGCGTCAAGGGGTGCAAGGTATT   [558]
Tetll2382         TTTCGCGTTGCTGATGATGTGCTGCGCCAAGGGGTTCAGGGGATT   [558]
Te1949            TTCATAATTGCAGATGATATCTTACGTCAAGGTATACAGGGAATT   [558]
SsNC_000911       TTTCGGGTAGCCGATGATATTCTGCGCCAGGGGGTACAGGGTATT   [558]
NtAJ133453        TTTCTTCTTGCTGATGATGTATTACGCCAAGGTGTCCAAGGAATT   [558]
NtAJ271749        TTTCTTCTTGCTGATGATGTATTACGTCAAGGTGTCCAAGGAATT   [558]
TeAF251346        TTTCTTCTTGCTGATGATGTACTCCGCCAAGGAGTTCAAGGAATC   [558]
AtU39877          TTTCTTCTTGCAGATGATGTTTTACGCCAAGGAGTACAAGGAATC   [558]
PsY15383          TTTCGTCTTGCAGATGATGTTTTACGCCAAGGAGTTCAGGGAATA   [558]
NtAF205858        TTTCTTCTTGCTGATGATGTACTTTGTCAAGGCGTCCAAGGAATA   [558]
NtAJ271748        TTTCTTCTTGCTGATGATGTACTTTGTCAAGGCGTCCAAGGAATA   [558]
OsAF383876        TTTCTTCTTGCAGATGATGTTCTTCGTCAAGGTGTCCAAGGAATA   [564]
CrAB084236        TTCGCGTTGGCAGACGATGTGCTGCGCCAGGGTGTGCAGGGTATC   [558]
CpAF067823        TTCAAAAAGGCGGACGAAATTTTACGCCAAGGTGTTCAGGGTATT   [558]
BsM22630          TTCCGCGAAGCGGATAACGTACTTCGCCAAGGGGTTCAAGGTATT   [558]
EcX55034          TTTGGCGCAGCGAACGATGTACTGAAAGGCGCTGTGCAAGGTATC   [558]
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[                590       600       610       620       630]
[                .         .         .         .         .]

LlAB042101       TCTGATATAATCACGGTTCCTGGTCTAGTTAATGTTGATTTCGCT   [609]
OsCL005296_338   TCAGATATAATCACTGTGCCAGGTTTGGTCAATGTTGACTTTGCT   [609]
OsCLB17724_5     TCTGATATTATCACGGTTCCTGGGTTGGTTAATGTTGATTTTGCT   [609]
NtAJ271750       TCTGATATTATTACGATTCCTGGGCTAGTAAATGTGGATTTTGCT   [609]
NtAJ311847       TCTGATATAATTACGATTCCTGGGCTAGTAAATGTGGATTTTGCT   [609]
GlAF205859       TCTGATATAATTACGATCCCTGGGCTAGTAAATGTGGACTTTGCT   [609]
AtAF089738       TCTGATATCATTACGATTCCTGGTTTGGTGAATGTGGATTTTGCT   [609]
AtAF384167       TCTGATATTATTACGATTCCTGGATTGGTCAATGTGGATTTTGCT   [609]
PpAJ249139       TCAGATATTATCACTGTTCCTGGTCTCGTTAACGTGGACTTTGCG   [609]
PpAJ249138       TCAGATATTATCACGGTCCCTGGGCTGGTTAACGTAGATTTTGCC   [609]
CrAF449446       AGCGAAATTATCACAGTGCCCGGCCTAGTCAACGTGGACTTCGCG   [609]
CmAB032072       AGCGATATTATCATCCGCCCTGGTCTCATTAACGTAGACTTTGCC   [603]
CcAB023962       AGCGACATCATCATCCGCCCTGGACTGATTAATGTTGATTTTGCT   [603]
GtAJ007748       TCCGAGATTATTGTAAGACCAGGTTTAATTAATGTTGATTTTGCC   [600]
GsAB022594       TCAGAAATCATTGTTCGTCCAGGTCTGATTAATGTTGACTTTGCG   [600]
MsAF120117       ACCGAAATCATTGTGAAGCCAGGGCTCGTGAATGTTGATTTCGCT   [528]
GsAB022595       TCCGATATTATTACTAAACCGGGCTTAGTCAACGTAGATTTTGCA   [624]
PsAJ011025       AGTGACATAATTACATGCCCTGGATTAGTTAACGTTGATTTTGCT   [600]
Pm1658           AGTGACATAATTACATGCCCTGGATTAGTTAACGTTGATTTTGCT   [600]
PmAJ237851       ACCGACATAATCACTTTGCCTGGTCTTGTAAATGTGGACTTTGCG   [600]
Sy549            AGCGACATCATCACGTGCCCCGGTTTGGTGAACGTCGACTTCGCT   [600]
Pm1268           AGTGACATCATCACCTGCCCTGGCCTTGTCAATGTGGACTTCGCC   [600]
SyAF076530       TCTGACATCATCACGATCCCAGGTCTGGTCAACGTCGACTTTGCC   [603]
AsZ31371         TCCGACATCATTACTATTCCTGGTTTGGTAAATGTAGACTTTGCT   [603]
Np61             TCTGATATCATCACAATTCCCGGTTTGGTAAATGTTGACTTTGCT   [603]
Tetll2382        TCCGACATTATCAACGTGCCGGGGCTGATTAACGTGGATTTTGCC   [603]
Te1949           TCAGATATTATTACTGTACCTGGATTAGTAAATGTTGACTTTGCT   [603]
SsNC_000911      TCCGACATTATCATCATCCCCGGTTTGGTGAATGTGGACTTTGCG   [603]
NtAJ133453       TCCGATATAATTACTATACCTGGGCTTGTAAATGTGGATTTTGCC   [603]
NtAJ271749       TCCGATATAATTACTATACCTGGGCTTGTAAATGTGGATTTTGCC   [603]
TeAF251346       TCAGATATAATTACAATACCTGGGCTGGTAAATGTGGACTTTGCA   [603]
AtU39877         TCAGATATTATTACTATACCTGGACTAGTCAATGTGGATTTTGCA   [603]
PsY15383         TCGGACATTATAACAATACCTGGACTTGTAAATGTGGATTTTGCT   [603]
NtAF205858       TCTGATATAATCACTATACCTGGGCTGGTAAATGTGGATTTTGCA   [603]
NtAJ271748       TCTGATATAATCACTATACCTGGGCTGGTAAATGTGGATTTTGCA   [603]
OsAF383876       TCAGATATTATTACAATACCTGGACTTGTCAATGTTGATTTTGCT   [609]
CrAB084236       TCAGACATCATCACCGTGCCCGGCCTCATCAACGTTGACTTTGCG   [603]
CpAF067823       GCTGATTTGATTTCCAAGCCCGGCGTAATTAACTTGGACTTTGCC   [603]
BsM22630         TCTGACTTGATTGCTACACCTGGTCTTATCAACCTTGACTTTGCT   [603]
EcX55034         GCTGAACTGATTACTCGTCCGGGTTTGATGAACGTGGACTTTGCA   [603]
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[                    640        650        660        670    ]
[                    .          .          .          .      ]

LlAB042101          GATGTTAGAGCAATTATGGCAAATGCAGGCTCATCCCTAATGGGT   [654]
OsCL005296_338      GATGTCCGATCAGTTATGTCGGATGCAGGGTCATCTTTGATGGGC   [654]
OsCLB17724_5        GATGTTCGAGCCATCATGCAAAATGCAGGCTCATCCTTGATGGGT   [654]
NtAJ271750          GACGTGCGTGCTATTATGGCAAATGCTGGTTCTTCTTTAATGGGA   [654]
NtAJ311847          GACGTGCGTGCTATTATGGCAAATGCTGGTTCCTCTTTAATGGGA   [654]
GlAF205859          GATGTGCGGGCTATAATGGCCAATGCTGGGTCTTCCTTAATGGGC   [654]
AtAF089738          GATGTGAGAGCTATAATGGCAAATGCGGGGTCTTCATTGATGGGA   [654]
AtAF384167          GATGTGAGGGCGATAATGGCAAATGCAGGTTCTTCATTGATGGGA   [654]
PpAJ249139          GATGTGCGGGCGATCATGGCCAATGCAGGATCATCTTTGATGGGA   [654]
PpAJ249138          GACGTGCGGGCGATCATGGCTAATGCAGGATCATCTTTGATGGGC   [654]
CrAF449446          GACGTTCGTGCCATCATGGCGGGCGCCGGTAGCTCGCTCATGGGG   [654]
CmAB032072          GACGTCCGAAGTGTCATGGCACATGCGGGATCGGCCTTAATGGGC   [648]
CcAB023962          GACGTGCGGAGTGTTATGGCGCACGCAGGATCCGCGCTTATGGGC   [648]
GtAJ007748          GATGTAAGATCTGTCATGGCAGATGCGGGAAGCGCACTTATGGGT   [645]
GsAB022594          GATGTTCGTTCTATTATGGCAGATGCTGGTTCAGCTTTGATGGGC   [645]
MsAF120117          GACGTGCGGACAATCATGGGCAACGCAGGCACGGCCTTGATGGGC   [573]
GsAB022595          GATGTTCGTACGGTTATGGCAGAAAAAGGTTTTGCTTTGTTAGGC   [669]
PsAJ011025          GATGTTAGGTCTGTAATGACTGAAGCTGGCACTGCCCTGCTTGGT   [645]
Pm1658              GATGTTAGGTCTGTAATGACTGAAGCTGGCACTGCCCTGCTTGGT   [645]
PmAJ237851          GACGTTCGCTCTGTAATGACTGAAGCTGGAACATCATTACTTGGA   [645]
Sy549               GACGTGCGTTCCGTGATGACGGAAGCAGGAACAGCATTGCTAGGC   [645]
Pm1268              GACGTGCGTTCGGTGATGACCGAAGCCGGCACGGCCCTGCTAGGG   [645]
SyAF076530          GACGTTCGCGCCGTCATGGCCGATGCTGGATCAGCCCTGATGGGC   [648]
AsZ31371            GACGTGCGGGCTGTCATGGCCGATGCGGGATCAGCATTGATGGGT   [648]
Np61                GATGTCCGGGCTGTGATGGCAGATGCAGGATCGGCATTGATGGGA   [648]
Tetll2382           GATATTCGCTCGGTGATGGCTGATGCCGGTTCTGCCATGATGGGC   [648]
Te1949              GATGTTAGAGCAGTTATGGCCGATGCTGGTTCGGCATTGATGGGA   [648]
SsNC_000911         GACGTGCGGGCGGTGATGGCCGATGCTGGCTCCGCATTAATGGGC   [648]
NtAJ133453          GATGTAAAGGCAGTGATGAAAGATTCTGGAACTGCTATGCTTGGA   [648]
NtAJ271749          GATGTAAAGGCAGTGATGAAAGATTCTGGAACTGCTATGCTTGGA   [648]
TeAF251346          GACGTTAAAGCAGTCATGAAAGATTCTGGAACTGCAATGCTTGGT   [648]
AtU39877            GATGTGAAGGCAGTCATGAAAGATTCTGGAACTGCAATGCTCGGG   [648]
PsY15383            GATGTAAAAGCTGTGATGAAAGACTCTGGTACCGCAATGCTCGGA   [648]
NtAF205858          GATGTTAAGGCAATCATGAAAGATTCTGGAACTGCTATGCTCGGA   [648]
NtAJ271748          GATGTTAAGGCAATCATGAAAGATTCTGGAACTGCTATGCTCGGA   [648]
OsAF383876          GATGTGAAAGCTGTTATGAAAAACTCTGGAACTGCAATGCTTGGT   [654]
CrAB084236          GATGTCAAGGCCATCATGAGCAACAGCGGCACGGCCATGCTGGGT   [648]
CpAF067823          GATGTACGTACAGTAATGGCAAATAAAGGTATTGCCCATATGGGT   [648]
BsM22630            GATGTGAAAACAATCATGTCAAACAAAGGATCTGCTTTGATGGGT   [648]
EcX55034            GACGTACGCACCGTAATGTCTGAGATGGGCCACGCAATGATGGGT   [648]
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[                    680        690        700        710        720]
[                    .          .          .          .          .]

LlAB042101          ATAGGAACTGCAACA--------------------GGTAAGACA   [678]
OsCL005296_338      ATTGGAACTGCAACA--------------------GGTAAGACA   [678]
OsCLB17724_5        ATTGGAACGGCTACA--------------------GGGAAGTCA   [678]
NtAJ271750          ATAGGAACTGCTACT--------------------GGAAAGACC   [678]
NtAJ311847          ATAGGAACCGCTACG--------------------GGGAAGACC   [678]
GlAF205859          ATTGGGACAGCCACA--------------------GGGAAAACC   [678]
AtAF089738          ATAGGAACTGCGACA--------------------GGAAAGAGT   [678]
AtAF384167          ATAGGAACTGCAACA--------------------GGAAAGACC   [678]
PpAJ249139          ATTGGAACCGCTACA--------------------GGGAAGTCA   [678]
PpAJ249138          ATAGGGACCGCCACA--------------------GGTAAGTCA   [678]
CrAF449446          CAGGGCTATGGATCC--------------------GGTCCGCGG   [678]
CmAB032072          ATCGGCACCGGCAGT--------------------GGCAAGTCC   [672]
CcAB023962          ATCGGCACCGGCAGT--------------------GGCAAGTCC   [672]
GtAJ007748          ATAGGAACAGGATCT--------------------GGAAAAACT   [669]
GsAB022594          ATTGGAAGCGGTTCG--------------------GGAAAATCC   [669]
MsAF120117          ATCGGCCACGGCAAG--------------------GGAAAGAAC   [597]
GsAB022595          ATTGGAACTGCAAGT--------------------GGAGATTCG   [693]
PsAJ011025          ATAGGTATTGGTTCT--------------------GGTAGATCT   [669]
Pm1658              ATAGGTATTGGTTCT--------------------GGTAGATCT   [669]
PmAJ237851          ATAGGCATTGGATCT--------------------GGTCGTTCT   [669]
Sy549               ATCGGCATTGGATCC--------------------GGCCGCTCA   [669]
Pm1268              ATTGGTGAAGGTTCA--------------------GGACGTTCC   [669]
SyAF076530          ATCGGTAGCGGCTCT--------------------GGCAAGTCC   [672]
AsZ31371            ATTGGTGTGAGTTCA--------------------GGAAAATCT   [672]
Np61                ATTGGCGTTAGTTCT--------------------GGAAAATCT   [672]
Tet112382           ATTGGTATTGCCTCT--------------------GGAAAGTCA   [672]
Te1949              ATTGGTATGGGGTCT--------------------GGTAAGTCT   [672]
SsNC_000911         ATTGGGGTGGGTTCC--------------------GGCAAGTCC   [672]
NtAJ133453          GTTGGGGTTTCATCA--------------------AGCAAGAAC   [672]
NtAJ271749          GTTGGGGTTTCATCA--------------------AGCAAGAAC   [672]
TeAF251346          GTCGGTGTTTCCTCA--------------------AGTAAAAAC   [672]
AtU39877            GTAGGTGTTTCTTCC--------------------AGCAAAAAC   [672]
PsY15383            GTAGGTGTTTCATCC--------------------GGTAAAAAC   [672]
NtAF205858          GTTGGGGTTTCTTCC--------------------AGTAGGAAC   [672]
NtAJ271748          GTTGGGGTTTCTTCC--------------------AGTAGGAAC   [672]
OsAF383876          GTTGGTGTTTCTTCC--------------------AGCAAAAAT   [678]
CrAB084236          GTGGGCGCTGCCTCCACAGCCACCGCCGCCCCCGGCGGCCCCGAC   [693]
CpAF067823          ATCGGTCGTGCCAGT--------------------GGCGAAAAT   [672]
BsM22630            ATCGGTATTGCTACT--------------------GGGGAAAAT   [672]
EcX55034            TCTGGCGTGGCGAGC--------------------GGTGAAGAC   [672]
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[                    730        740        750        760    ]
[                    .          .          .          .      ]

LlAB042101          CGGGCAAGAGATGCTGCGTTAAATGCTGTTCAGTCACCATTACTG   [723]
OsCL005296_338      CGTGCCAGGGATGCTGCGCTTAATGCTATACAGTCTCCTCTTCTT   [723]
OsCLB17724_5        AGAGCAAGAGATGCTGCTCTTAATGCCATTCAGTCACCACTGCTA   [723]
NtAJ271750          AGAGCCAGAGATGCAGCATTGAACGCCATTCAATCTCCTTTACTG   [723]
NtAJ311847          AGAGCCAGAGATGCAGCGTTGAACGCTATTCAATCTCCTTTACTG   [723]
GlAF205859          AGGGCCAGAGATGCTGCCTTAAATGCGATCCAATCTCCCTTGCTA   [723]
AtAF089738          CGGGCAAGAGATGCTGCGCTAAATGCAATCCAATCCCCTTTGTTA   [723]
AtAF384167          CGAGCAAGAGATGCTGCATTAAACGCAATCCAATCCCCTTTATTA   [723]
PpAJ249139          AAAGCTAGAGAGGCAGCATTGAGTGCCATTCAGTCTCCATTGTTG   [723]
PpAJ249138          AGAGCTAGAGAAGCAGCATTGAGCGCAATCCAATCTCCTCTATTG   [723]
CrAF449446          CGTGCCTCTGACGCCGCGCTGCGCGCCATCAGCTCGCCGCTGCTG   [723]
CmAB032072          CGGGCCCATGACGCCGCCGTGGCAGCGATTTCCTCACCGCTGCTG   [717]
CcAB023962          CGAGCGCACGATGCTGCTGTCGCTGCCATCTCTTCTCCGCTCTTA   [717]
GtAJ007748          CGAGCACAAGATGCAGCAGTTGCAGCTATAAGTTCTCCTTTACTT   [714]
GsAB022594          CGTGCCAAGGATGCCGCAGTTGCTGCAATTTCCTCGCCATTACTT   [714]
MsAF120117          AGAGCCAAGGACGCGGCGCTGTCGGCCATCTCCTCCCCGCTGCTG   [642]
GsAB022595          AGAGCTCGAAATGCAGCAACTGCGGCTATTTCATCACCTCTTTTA   [738]
PsAJ011025          AGAGCATTAGAGGCTGCTCAAGCCGCAATGAATAGTCCTTTACTA   [714]
Pm1658              AGAGCATTAGAGGCTGCTCAAGCCGCAATGAATAGTCCTTTACTA   [714]
PmAJ237851          AGAGCCGCCGAAGCCGCTCAAGCAGCAATAAACAGTCCTTTATTA   [714]
Sy549               AGGGCGGTTGAGGCCGCGCAAGCTGCCATCAGCAGCCCGTTACTT   [714]
Pm1268              AGGGCGATAGAAGCAGCCCAGGCTGCCATCAGCAGTCCGCTACTG   [714]
SyAF076530          CGCGCTCGGGAAGCCGCTCATGCAGCCATTAGCTCACCGCTGCTG   [717]
AsZ31371            AGAGCCAGAGAAGCTGCGATCGCCGCTATATCTTCACCACTGCTA   [717]
Np61                AGAGCCAGAGAAGCTGCGATCGCAGCTATTTCTTCACCGTTACTA   [717]
Tetll2382           CGGGCCACTGAAGCTGCCCTCAGCGCCATTTCTTCACCTCTACTG   [717]
Te1949              AGGGCAAGGGAAGCAGCAAATGCGGCAATTTCTTCTCCTTTGCTT   [717]
SsNC_000911         CGGGCCAAGGAGGCGGCCACGGCGGCCATTTCCTCTCCTTTGTTG   [717]
NtAJ133453          CGTGCTGAAGAAGCAGCCGAACAAGCAACTCTTGCCCCTCTAATT   [717]
NtAJ271749          CGTGCTGAAGAAGCAGCCGAACAAGCAACTCTTGCCCCTCTAATT   [717]
TeAF251346          CGAGCTGAAGAAGCAGCTGAACAAGCAACTCTTGCTCCTTTGATT   [717]
AtU39877            CGGGCAGAAGAAGCAGCTGAACAAGCAACTTTGGCTCCATTGATC   [717]
PsY15383            CGAGCAGAAGAAGCAGCAGAACAGGCTACCTTGGCTCCTTTAATC   [717]
NtAF205858          CGTGCTGAGGAAGCAGCTGAACAAGCAACTCTGGCCCCTCTCATT   [717]
NtAJ271748          CGTGCTGAGGAAGCAGCTGAACAAGCAACTCTGGCCCCTCTCATT   [717]
OsAF383876          CGGGCCCAAGAAGCTGCAGAGCAGGCAACACTTGCTCCTTTAATC   [723]
CrAB084236          CGCGCGGAGCAGGCGGCCGTGGCCGCCACATCGGCACCGCTCATC   [738]
CpAF067823          AAAGCTGAAATTGCAGCGAAAATGGCAATTCAGAGCCCTCTTTTG   [717]
BsM22630            CGCGCGGCAGAGGCAGCAAAAAAAGCAATTTCCAGCCCGCTTCTT   [717]
EcX55034            CGTGCGGAAGAAGCTGCTGAAATGGCTATCTCTTCTCCGCTGCTG   [717]
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[                    770        780        790        800        810]
[                    .          .          .          .          .]

LlAB042101          GATATTGGT---ATTGAAAGAGCTACTGGAATAGTATGGAATATA   [765]
OsCL005296_338      GATATTGGC---ATTGAAAGAGCAACAGGAATTGTATGGAATATC   [765]
OsCLB17724_5        GACATTGGA---ATTGAAAGAGCTACAGGCATTGTGTGGAATATC   [765]
NtAJ271750          GACATTGGT---ATAGAGAGGGCTACTGGAATTGTGTGGAATATA   [765]
NtAJ311847          GATATTGGT---ATAGAGAGGGCTACTGGAATTGTGTGGAATATA   [765]
GlAF205859          GATATCGGT---ATCGAGAGAGCTACTGGTATTGTGTGGAATATT   [765]
AtAF089738          GATATTGGG---ATTGAGAGAGCCACTGGAATTGTTTGGAACATT   [765]
AtAF384167          GATATTGGC---ATTGAGAGAGCCACTGGAATAGTTTGGAACATA   [765]
PpAJ249139          GATGTGGGT---ATTGAGCGAGCCACAGGGATCGTTTGGAATATT   [765]
PpAJ249138          GATGTGGGT---ATTGAGCGAGCCACAGGGATAGTCTGGAATATC   [765]
CrAF449446          GAGGTGGGC---ATTGAGCGCGCCACTGGCGTGGTGTGGAACATC   [765]
CmAB032072          GATTTCCCT---ATCGAGCGTGCCAAAGGTATTGTTTTCAACGTC   [759]
CcAB023962          GATTTCCCT---ATAGAACGCGCCAAGGGCATCGTCTTCAATGTC   [759]
GtAJ007748          GATTTTCCA---ATCGAAAAAGCCAGAGGAATTGTATTTAATATC   [756]
GsAB022594          GACTTTCCT---ATTGAGCGTGCAAAAGGAATTGTTTTTAACATT   [756]
MsAF120117          GACTTCCCC---ATCACCCGCGCCAAAGGCATCGTTTTCAACATT   [684]
GsAB022595          GATTTTCCT---ATAACATCTGCGAAAGGTGCCGTTTTTAATATT   [780]
PsAJ011025          GAAGCGGCAAGAATTGATGGAGCTAAAGGTTGTGTGATAAATATT   [759]
Pm1658              GAAGCGGCAAGAATTGATGGAGCTAAAGGTTGTGTGATAAATATT   [759]
PmAJ237851          GAAGCTGGTCGTATAGATGGAGCAAAAGGCTGCGTAGTAAATATT   [759]
Sy549               GAAACCGAGCGAATCGATGGTGCCAAGGGCTGTGTGATCAACATC   [759]
Pm1268              GAAGCGGCCCGCATCGACGGAGCCAAAGGTTGCGTCATCAACATC   [759]
SyAF076530          GAGTCTTCG---ATCGAAGGGGCGCGCGGCGTTGTCTTCAACATC   [759]
AsZ31371            GAATGTTCT---ATCGAAGGTGCTAGAGGTGTTGTTTTTAACATC   [759]
Np61                GAATGTTCT---ATTGAAGGTGCTAGAGGGGTTGTATTTAATATT   [759]
Tet112382           GAGCGGTCT---ATCGAGGGTGCCAAGGGCGTTGTCTTTAACATT   [759]
Te1949              GAGTCTTCC---ATTGAAGGAGCAAAAGGGGTTGTATTTAATATT   [759]
SsNC_000911         GAATCTTCT---ATCCAGGGAGCTAAAGGAGTCGTATTTAATGTC   [759]
NtAJ133453          GGATCGTCC---ATTCAATCTGCAACTGGGGTAGTATACAACATT   [759]
NtAJ271749          GGATCGTCT---ATTCAATCTGCAACTGGGGTAGTATATAACATT   [759]
TeAF251346          GGATCATCA---ATTCAATCTGCTACAGGTGTTGTTTATAATATT   [759]
AtU39877            GGATCATCC---ATACAATCAGCTACTGGTGTCGTCTACAACATC   [759]
PsY15383            GGATCATCT---ATTCAATCAGCCACGGGAGTAGTGTATAATATC   [759]
NtAF205858          GGATCATCA---ATTCAATCTGCAACTGGTGATGTATATAACATT   [759]
NtAJ271748          GGATTATCA---ATTCAATCTGCAACTGGTGTTGTATATAACATT   [759]
OsAF383876          GGGTCGTCT---ATTGAGGCGGCTACTGGTGTTGTGTACAATATC   [765]
CrAB084236          CAGCGCAGC---ATCGAGAAGGCTACCGGCATTGTGTACAACATC   [780]
CpAF067823          GAAACTACC---ATTGAGGGAGCTAAGAGCGTATTGATTAATTTC   [759]
BsM22630            GAAGCGGCC---ATTGACGGTGCGCAAGGCGTCCTCATGAACATC   [759]
EcX55034            GAAGATATCGACCTGTCTGGCGCGCGCGGCGTGCTGGTTAACATC   [762]
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[                    820       830       840       850    ]
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L1AB042101          ACTGGGGGAAATGATTTAACTTTGTATGAGGTGAATGCTGCTGCA   [810]
OsCL005296_338      ACTGGAGGGAATGATCTAACCTTGACGGAGGTGAATGCAGCAGCT   [810]
OsCLB17724_5        ACTGGGGGAGCTGATATGACTTTGTTTGAGGTGAATTCTGCTGCT   [810]
NtAJ271750          ACTGGTGGTAGTGATCTAACATTATTTGAGGTAAATGCTGCAGCA   [810]
NtAJ311847          ACTGGTGGTAGTGATCTAACATTATTTGAGGTAAATGCCGCAGCA   [810]
GlAF205859          ACTGGCGGAAGTGATTTGACATTGTTTGAGGTCAATGCAGCTGCT   [810]
AtAF089738          ACTGGCGGAAGTGACTTGACATTGTTTGAGGTAAATGCTGCTGCG   [810]
AtAF384167          ACCGGTGGAAGTGACTTAACACTGTTCGAGGTAAATGCTGCTGCA   [810]
PpAJ249139          ACTGGGGGAAGCGACATGACCCTCTTTGAAGTCAATGCTGCAGCA   [810]
PpAJ249138          ACTGGGGGAAGCGACATGACTCTCTTTGAGGTAAATGCTGCAGCA   [810]
CrAF449446          ACCGGTCCGCCAAACATGACCCTGCACGAGGTGAACGAGGCGGCC   [810]
CmAB032072          ACCGGTGGAGAAGACATGACCCTGCACGAAATCAACCAGGCCGCT   [804]
CcAB023962          ACTGGCGGTGAAGACATGACGCTGCATGAGATTAACCAGGCTGCT   [804]
GtAJ007748          ACAGGTGGACAAGATATGACATTACATGAGATCAATTCTGCAGCA   [801]
GsAB022594          ACAGGTGGACATGATATGACGCTACACGAAATAAATGCCGCCGCA   [801]
MsAF120117          GTGGGCGGCTCCGACATGTCTCTGCAGGAAATCAACGCCGCCGCG   [729]
GsAB022595          ACAGGAGGAACGGATATGACCTTATCTGAAGTGAATCAGGCTGCA   [825]
PsAJ011025          ACAGGTGGGAAAGATATGACATTAGAAGATATGACCTCAGCTTCA   [804]
Pm1658              ACAGGTGGGAAAGATATGACATTAGAAGATATGACCTCAGCTTCA   [804]
PmAJ237851          ACAGGCGGGAAAGACATGACATTAGAAGACATGACCTCTGCTTCA   [804]
Sy549               AGCGGCGGCCGTGACATGACCCTCGAGGACATGACCACCGCCTCT   [804]
Pm1268              AGCGGCGGTCGCGACATGACCCTTGAGGACATGACCTCCGCATCG   [804]
SyAF076530          ACAGGCGGCCGCGATATGACCCTGCATGAGGTCAACGCAGCAGCG   [804]
AsZ31371            ACTGGTGGTAGTGACCTCACTCTACATGAAGTAAATGCTGCCGCC   [804]
Np61                ACAGGCGGTACCGATCTTACTTTACATGAAGTGAATGCAGCCGCA   [804]
Tet112382           ACAGGGGGCACCGATCTCAGTCTCCATGAAGTCAATGCTGCCGCA   [804]
Te1949              ACTGGTGGTACTGATTTAACTTTACATGAGGTTAATGCTGCTGCT   [804]
SsNC_000911         ACTGGTGGAACCGATCTGACCCTGCACGAAGTTAATGTTGCGGCT   [804]
NtAJ133453          ACAGGAGGAAAAGACATAACTTTGCAGGAAGTGAATAGGGTGTCT   [804]
NtAJ271749          ACAGGAGGAAAAGACATAACTTTGCAGGAAGTGAATAGGGTGTCT   [804]
TeAF251346          ACCGGAGGGAAGGACATAACTCTACAAGAAGTCAACAGGGTTTCT   [804]
AtU39877            ACTGGTGGAAAAGACATAACTTTGCAGGAAGTGAACCGAGTATCA   [804]
PsY15383            ACTGGAGGAAAGGACATCACCCTGCAGGAAGTCAACAGAGTATCT   [804]
NtAF205858          ACTGGAGGAAAAGATATTACTTTGCAGGAGGTGAATAAGGTGTCC   [804]
NtAJ271748          ACTGGAGGAAAAGATATTACTTTGCAGGAGGTGAATAAGGTGTCC   [804]
OsAF383876          ACTGGTGGAAAGGACATAACCTTGCAAGAAGTAAACAAAGTCTCT   [810]
CrAB084236          ACTGGTGGCCGTGACCTGACCCTGGCCGAGGTCAACCGCGTGTCC   [825]
CpAF067823          AGCGGTGATATGAACCTTGGTTTGATGGAAACAGAGGAAGCGGCT   [804]
BsM22630            ACTGGAGGAACAAACCTCAGCCTATATGAGGTTCAGGAAGCAGCA   [804]
EcX55034            ACGGCGGGCTTCGACCTGCGTCTGGATGAGTTCGAAACGGTAGGT   [807]
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OsCL005296_338      GAAGTGATATACGATCTTGTTGATCCTGGTGCAAATCTCATTTTT   [855]
OsCLB17724_5        GAGATCATCTATGACCTTGTTGATCCAAATGCTAATCTGATATTT   [855]
NtAJ271750          GAGGTTATATATGACCTTGTGGATCCTAGTGCGAACCTTATTTTC   [855]
NtAJ311847          GAGGTTATATATGACCTTGTGGATCCTAGTGCGAACCTTATTTTC   [855]
GlAF205859          GAAGTTATATATGATCTGGTAGATCCAAGTGCCAACTTAATTTTT   [855]
AtAF089738          GAAGTAATATATGATCTTGTCGATCCAACTGCCAATCTTATATTC   [855]
AtAF384167          GAAGTGATTTACGACCTCGTTGATCCAACAGCGAATCTTATATTT   [855]
PpAJ249139          GAGGTAATCTATGATTTGGTGGATCCTAACGCAAATCTTATTTTC   [855]
PpAJ249138          GAGGTGATTTATGATTTGGTCGATCCCAACGCAAATCTTATTTTT   [855]
CrAF449446          GAAATTATCTACGATATGGTGGACCCCAACGCCAACCTTATCTTT   [855]
CmAB032072          GAGGTAATCTACGAAGCAGTCGACCCCAACGCGAATATCATCTTC   [849]
CcAB023962          GAGGTGATCTACGAAGCGGTAGACCCGAATGCAAACATCATATTC   [849]
GtAJ007748          GAAGTAATTTATGAAGCAGTAGATTCTAATGCAAATATAATATTT   [846]
GsAB022594          GAAGTTATTTACGAAGCCGTGGATTTGAATGCCAATATAATCTTC   [846]
MsAF120117          GAGGTCATTTACGAGAACGTGGATCAGGACGCGAATATCATATTC   [774]
GsAB022595          CAAGTTATTTATGATAGTGTAGATTCTGATGCAAATATTATTTTT   [870]
PsAJ011025          GAAATTATTTATGATGTTGTAGATCCAGAAGCAAACATAATAGTA   [849]
Pm1658              GAAATTATTTATGATGTTGTAGATCCAGAAGCAAACATAATAGTA   [849]
PmAJ237851          GAGGTTATTTACGATGTTGTAGACCCAGAAGCAAATATTATTGTT   [849]
Sy549               GAAGTGATCTACGACGTGGTGGACCCAGAAGCCAACATCATTGTT   [849]
Pm1268              GAAGTGATCTACGACGTGGTCGATCCAGAAGCCAACATCATTGTT   [849]
SyAF076530          GATGCGATTTACGAAGTCGTCGATCCTGAAGCCAATATCATTTTC   [849]
AsZ31371            GAAACGATTTATGAAGTAGTTGATCCCAACGCCAACATTATTTTT   [849]
Np61                GAAGCAATCTATGAAGTAGTTGATCCCAACGCCAATATTATTTTT   [849]
Tetll2382           GACGTCATTTACAATGTGGCCGATGCCAACGCCAATATCATCTTT   [849]
Te1949              GAAATTATCTATGAAGTTGTAGATCCTAATGCTAATATTATTTTT   [849]
SsNC_000911         GAAATTATCTATGAAGTGGTGGATGCCGATGCCAACATCATCTTT   [849]
NtAJ133453          CAGGTTGTTACCAGTCTGGCTGATCCCTCCGCTAACATCATATTT   [849]
NtAJ271749          CAGGTTGTTACCAGTCTGGCTGATCCCTCCGCTAACATCATATTT   [849]
TeAF251346          CAGGTGGTAACAAGTTTGGCAGATCCATCAGCAAACATTATATTC   [849]
AtU39877            CAGGTCGTGACAAGTTTGGCAGACCCATCGGCCAACATCATATTT   [849]
PsY15383            CAGGTTGTGACTAGTTTGGCCGATCCTTCTGCCAATATTATATTT   [849]
NtAF205858          CAGGTTGTCACAAGCTTGGCTGATCCATCCGCCAACATCATATTC   [849]
NtAJ271748          CAGGTTGTCACAAGCTTGGCTGATCCATCCGCCAACATCATATTC   [849]
OsAF383876          CAGATTGTGACAAGCTTGGCCGATCCTTCTGCAAATATAATTTTC   [855]
CrAB084236          GAGGTGGTGACCGCCCTGGCCGACCCCTCATGCAACATTATCTTT   [870]
CpAF067823          GATTTAATCAGAGAGGCTATTGATCCTGATGCAGAAATCATCTTT   [849]
BsM22630            GACATTGTCGCTTCGGCGTCTGATCAAGACGTAAACATGATTTTC   [849]
EcX55034            AACACCATCCGTGCATTTGCTTCCGACAACGCGACTGTGGTTATC   [852]
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LlAB042101         GGAGCAGTGATTGATCCGTCGATTAGT---GGTCAAGTTAGCATA   [897]
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OsCLB17724_5       GGTGCTGTCATAGACCCATCACTCAAT---GGCCAAGTGAGCATA   [897]
NtAJ271750         GGGGCCGTGATAGACCCATCAATAAGT---GGACAGGTCAGCATA   [897]
NtAJ311847         GGGGCGGTGATAGACCCATCAATAAGT---GGACAGGTCAGCATA   [897]
GlAF205859         GGAGCTGTAGTAGATCCATCACTGTGT---GGTCAAGTCAGTATA   [897]
AtAF089738         GGTGCTGTTGTAGATCCAGCCCTCAGC---GGTCAAGTAAGCATA   [897]
AtAF384167         GGTGCTGTGGTAGATCCATCTTATAGT---GGTCAAATAAGTATT   [897]
PpAJ249139         GGAGCCGTAGTAGACGAAGCACTTCAT---GACCAAATTAGCATA   [897]
PpAJ249138         GGAGCCGTAGTAGACGAAGCACTTCAT---GGCCAAGTTAGTATA   [897]
CrAF449446         GGAGCCGTGGTGGACTCTACCCTGCCCGACGACACGGTGTCCATC   [900]
CmAB032072         GGTGCCCTTATTGATCAGCAAATGGAA---AGCGAGATATCCATA   [891]
CcAB023962         GGCGCCCTCGTTGACCAACAAATGGAG---AGCGAGATATCGATT   [891]
GtAJ007748         GGAGCGCTTGTTGATGATAATATGGAA---AATGAAATTTCAATT   [888]
GsAB022594         GGTGCTTTGGTCGATGATAGTATGGAA---AATGAATTATCCATT   [888]
MsAF120117         GGGGCGATGGTGGACGACAAGATGACCTCTGGAGAGGTGTCCATC   [819]
GsAB022595         GGTGCAGTTGTAGATGAGACATTCAAA---GGAAAAGTTTCGGTT   [912]
PsAJ011025         GGTGCTGTTATAGATGAATCAATGGAA---GGCGAAATACAGGTA   [891]
Pm1658             GGTGCTGTTATAGATGAATCAATGGAA---GGCGAAATACAGGTA   [891]
PmAJ237851         GGCGCAGTTATTGATGAAGCTCTTGAA---GGGGAAGTTCAAGTA   [891]
Sy549              GGTGCTGTGGTGGATGAAGCCCTAGAA---GGCGAAATCCACGTC   [891]
Pm1268             GGAGCTGTTGTAGACGAGAAACTCGAA---GGTGAAGTTCACGTC   [891]
SyAF076530         GGCGCCGTGATTGACGATCGATTGGAA---GGAGAGCTGCGGATC   [891]
AsZ31371           GGTGCCGTGATTGATGATAGGTTGCAA---GGAGAGGTGCGAATT   [891]
Np61               GGGGCTGTAATTGATGACAGACTCCAA---GGTGAGGTCAGAATT   [891]
Tet112382          GGTGCTGTCATTGATCCGCAAATGCAG---GGGGAAGTTCAAATC   [891]
Te1949             GGGGCTGTAATTGATGATAAACTTCAG---GGAGAAATTAAAATC   [891]
SsNC_000911        GGAGCGGTGATTGACGATCGCCTGCAG---GGAGAAATGAGAATT   [891]
NtAJ133453         GGTGCTGTTGTGGATGAGCGCTACAAT---GGCGAAATACACGTG   [891]
NtAJ271749         GGTGCTGTTGTGGATGAGCGCTACAAT---GGCGAAATACACGTG   [891]
TeAF251346         GGGGCAGTGGTAGATGAGAGATACAAC---GGGGAGATTCATGTG   [891]
AtU39877           GGAGCTGTTGTGGATGATCGCTACACC---GGAGAGATTCATGTA   [891]
PsY15383           GGAGCTGTAGTTGATGATCGTTACACC---GGAGAGATTCACGTG   [891]
NtAF205858         GGTGCTGTTGTGGATGAGCGCTATAAT---GGAGAGATCCAGGTG   [891]
NtAJ271748         GGTGCTGTTGTGGATGAGCGCTATAAT---GGAGAGATCCAGGTG   [891]
OsAF383876         GGGGCTGTTGTTGATGACCGGTACACT---GGTGAGATTCATGTG   [897]
CrAB084236         GGCGCCGTTGTGGACGAGCAGTACGAC---GGCGAGCTGCACGTG   [912]
CpAF067823         GGTACAACCATTAACGAAGATCTCAAT---AATGAGGTTGTTGTT   [891]
BsM22630           GGTTCTGTTATTAATGAAAATCTAAAA---GATGAGATTGTGGTG   [891]
EcX55034           GGTACTTCTCTTGACCCGGATATGAAT---GACGAGCTGCGCGTA   [894]
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[                    950        960     ]
[                    .          .       ]

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OsCLB17724_5        ACCTTGATTGCCACTGGCTTC   [918]
NtAJ271750          ACCCTAATCGCCACTGGTTTT   [918]
NtAJ311847          ACCCTAATCGCCACTGGTTTT   [918]
GlAF205859          ACCCTTATAGCCACGGGTTTT   [918]
AtAF089738          ACCCTGATAGCTACGGGTTTC   [918]
AtAF384167          ACCCTGATAGCAACTGGCTTC   [918]
PpAJ249139          ACCTTGATAGCAACAGGGTTT   [918]
PpAJ249138          ACTTTGATAGCAACAGGATTT   [918]
CrAF449446          ACCATCATTGCCACAGGCTTC   [921]
CmAB032072          ACGGTGGTTGCCACAGGCTTC   [912]
CcAB023962          ACAGTTGTTGCCACGGGCTTT   [912]
GtAJ007748          ACAGTTGTGGCAACAGGTTTT   [909]
GsAB022594          ACTGTCATTGCTACTGGTTTT   [909]
MsAF120117          ACAGTCCTGGCCACGGGCTTT   [840]
GsAB022595          ACCGTAGTTGCCACAGGCTTT   [933]
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Pm1658              ACTGTTATTGCAACAGGTTTC   [912]
PmAJ237851          ACTGTAATTGCAACTGGTTTT   [912]
Sy549               ACCGTAATCGCCACGGGATTT   [912]
Pm1268              ACCGTGATCGCCACAGGCTTT   [912]
SyAF076530          ACCGTGATCGCCACGGGCTTC   [912]
AsZ31371            ACCGTCATTGCTACTGGATTT   [912]
Np61                ACTGTAATTGCCACTGGGTTT   [912]
Tet112382           ACCGTTATCGCCACTGGCTTT   [912]
Te1949              ACTGTCATAGCTACTGGTTTT   [912]
SsNC_000911         ACCGTCATTGCCACGGGCTTC   [912]
NtAJ133453          ACCATAATTGCAACTGGTTTT   [912]
NtAJ271749          ACCATAATTGCAACTGGTTTT   [912]
TeAF251346          ACCATTGTTGCTACTGGCTTT   [912]
AtU39877            ACGATAATCGCCACAGGCTTC   [912]
PsY15383            ACTATCATCGCAACTGGCTTC   [912]
NtAF205858          ACTCTAATTGCAACTGGTTTC   [912]
NtAJ271748          ACTCTAATTGCAACTGGTTTC   [912]
OsAF383876          ACGATCATTGCCACAGGGTTT   [918]
CrAB084236          ACCATCATCGCTACCGGCTTC   [933]
CpAF067823          ACTGTAATTGCAACAGGGCTT   [912]
BsM22630            ACAGTGATTGCAACCGGCTTT   [912]
EcX55034            ACCGTTGTTGCGACAGGTATC   [915]
;
END;
```

APPENDIX C

Conserved Residues of the FtsZ Proteins

The Nexus file with the FtsZ protein alignment that was used to determine the conserved residues in each FtsZ family that is discussed in Chapter 5 and diagramed in Figure 5 of that chapter. Sequences are labeled with the organism initials followed by the accession or gene number.

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INTERLEAVE ;
MATRIX

[                          10        20        30        40    ]
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NtCAC44257          DSSRSNNFNEAKIKVVGVGGGGSNAVNRMIESSMKGVEFWIVNTD   [45]
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GlAAF23771          DSSSSNNYSEAKIKVVGVGGGGSNAVNRMIESAMKGVEFWIVNTD   [45]
LlBAA96782          SSVTSSDYNGAKIKVIGVGGGGSNAVNRMIASSMDGVEFWIVNTD   [45]
AtAAC35987          EPSAPSNYNEARIKVIGVGGGGSNAVNRMIESEMSGVEFWIVNTD   [45]
AtAAK63846          ELSTPNTYNEARIKVIGVGGGGSNAVNRMIESEMIGVEFWIVNTD   [45]
OsCL005296_338      DVSASHRYSEPRIKVIGVGGGGSNAVNRMIESDMKGVEFWIVNTD   [45]
PpCAB76386          NGDEYESSNEAKIKVIGVGGGGSNAVNRMLESEMQGVEFWIVNTD   [45]
PpCAB54558          SGDDTGSYNEAKIKVIGVGGGGSNAVNRMLESEMQGVEFWIVNTD   [45]
CrAAM22891          LVRLYASSDQAIIKVLGVGGGGSNAVNNMVNSDVQGVEFWIANTD   [45]
CmBAA85116          PLSSDSSAPPCLIKVIGVGGGGGNAVNRMADTGISGVEFWAINTD   [45]
CcBAA82871          PMSSDSSAPPCLIKVIGVGGGGGNAVNRMADTGISGVEFWAINTD   [45]
GtCAA07676          FFNQEISSSPCVIKVIGVGGGGGNAVNRMVG-GVEGVEFWSINTD   [45]
GsBAA82090          FVSSGGAVNPCIIKVVGVGGGGSNAVNRMCE-MVEGVEFWCINTD   [45]
MsAAF35433          ---------------------------------GVELWVVNTD   [45]
GsBAA82091          QSSTNLPQQQCKIKVVGVGGAGGNAVQRMLESGLQDVEFLCANTD   [45]
Te1949              RSDDIVPSNTAKIKVIGVGGGGGNAVNRMIASEVSGIEFWTVNTD   [45]
SsNP_440816         KRDQIVPSNIAKIKVIGVGGGGCNAVNRMIASGVTGIDFWAINTD   [45]
AsCAA83241          RIGEIVPGRVANIKVIGVGGGGGNAVNRMIESDVSGVEFWSINTD   [45]
Np61                RIGEIVPGRVANIKVIGVGGGGGNAVNRMIESDVSGVEFWSINTD   [45]
Tetll2382           SYDKLVETSAARIKVIGVGGGGGNAVNRMIASNVAGVEFWCVNTD   [45]
SyAAC26227          PEELIIPSSVARIKVIGVGGGGSNGVNRMISSDVSGVEFWALNTD   [45]
PsCAB56201          QSKDILPSQNAKIEVIGVGGGGSNAVNRMIDSDLEGVSFRVLNTD   [45]
Pm1658              QSKDILPSQNAKIEVIGVGGGGSNAVNRMIDSDLEGVSFRVLNTD   [45]
PmCAB95028          RSESIQPSQNARIEVIGVGGGGSNAVNRMILSDLQGVSYRVLNTD   [45]
Sy549               DATGIQPSQNAKIEVIGVGGGGSNAVNRMILSDLEGVAYRVLNTD   [45]
Pm1268              ETAGILPSQSARIEVIGVGGGGSNAVNRMILSDLDGVNYRVMNTD   [45]
NtCAB41987          ISSSFTPFDSAKIKVIGVGGGGNNAVNRMIGSGLQGVDFYAINTD   [45]
NtCAB89287          ISSSFTPFDSAKIKVIGVGGGGNNAVNRMIGSGLQGVDFYAINTD   [45]
TeAAF81220          VCCSFASLDSAKIKVVGVGGGGNNAVNRMIGSGLQGVDFYAINTD   [45]
AtAAA82068          LRCSFSPMESARIKVIGVGGGGNNAVNRMISSGLQSVDFYAINTD   [45]
PsCAA75603          VRCSLAYVDNAKIKVVGIGGGGNNAVNRMIGSGLQGVDFYAINTD   [45]
NtAAF23770          RFSICSSLSSAKIKVVGVGGGGNNAVNRMIGSGLQGVDFYAVNTD   [45]
NtCAB89286          RRSICSSLSSAKIKVVGVGGGGNNAVNRMIGSGLQGVDFYAVNTD   [45]
OsAAK64282          VRCSFAPVETARIKVVGVGGGGNNAVNRMIGSGLQGIEFYAINTD   [45]
CrBAB91150          STGYIPFGGDACIKVIGVGGGGGNALNRMINSGLQGVEFWAINTD   [45]
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[                    50         60         70         80         90]
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OsCLB17724_5        VQAIRMSPVLPQNRLQIGQELTRGLGAGGNPDIGMNAAKESVESI   [90]
GlAAF23771          VQAIKMSPVYLENRLQIGQELTRGLGAGGNPDIGMNAAKESKEAI   [90]
LlBAA96782          VQAMRMSPVYPENRLQIGQELTRGLGAGGNPDIGMNAAKESKVSI   [90]
AtAAC35987          IQAMRMSPVLPDNRLQIGKELTRGLGAGGNPEIGMNAARESKEVI   [90]
AtAAK63846          IQAMRISPVFPDNRLQIGKELTRGLGAGGNPEIGMNAATESKEAI   [90]
OsCL005296_338      FQAMRMSPIDPDNKLQIGQELTRGLGAGGNPEIGMNAAKESQELV   [90]
PpCAB76386          AQAMALSPVPAQNRLQIGQKLTRGLGAGGNPEIGCSAAEESKAMV   [90]
PpCAB54558          AQAMALSPVPAQNRLQIGQKLTRGLGAGGNPEIGCSAAEESKAMV   [90]
CrAAM22891          AQALATSPVNGKCKVQIGGKLTRGLGAGGNPEIGAKAAEESRDSI   [90]
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MsAAF35433          AQALSRS--SAKRRLNIGKVLSRGLGAGGNPAIGAKAAEESREEI   [90]
GsBAA82091          AQALGRFQKTHHQVIQIGKQSCRGLGAGGNPEAGRVAAEESKEDI   [90]
Te1949              AQALTLS--RAPKRLQLGQKLTRGLGAGGNPAIGQKAAEESRDEI   [90]
SsNP_440816         SQALTNT--NAPDCIQIGQKLTRGLGAGGNPAIGQKAAEESRDEI   [90]
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Np61                AQALTLA--GAPSRLQIGQKLTRGLGAGGNPAIGQKAAEESRDEI   [90]
Tetll2382           AQAIAQS--QAHRCLQIGQKLTRGLGAGGNPAIGQKAAEESREDL   [90]
SyAAC26227          AQALLHS--AAPKRMQLGQKLTRGLGAGGNPAIGMKAAEESREEL   [90]
PsCAB56201          AQALLQS--SADRRVQLGQNLTRGLGAGGNPSIGQKAAEESKDEL   [90]
Pm1658              AQALLQS--SADRRVQLGQNLTRGLGAGGNPSIGQKAAEESKDEL   [90]
PmCAB95028          AQALLQS--SAENRVQLGQTLTRGLGAGGNPSIGEKAAEESRAEL   [90]
Sy549               AQALIQS--QAQHRLQLGQTLTRGLGAGGNPTIGQKAAEESRTDL   [90]
Pm1268              AQALLQS--AASNRVQLGQTLTRGLGAGGNPSIGQKAAEESRAEL   [90]
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NtCAB89286          AQALLQS--TVENPIQIGELLTRGLGTGGNPLLGEQAAEESKEHI   [90]
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[                    100       110       120       130   ]
[                    .         .         .         .     ]

NtCAB89288           EEAVYGADMVFVTAGMGGGTGTGAAPIIAGTAKSMGILTVGIVTT   [135]
NtCAC44257           EEAVYGADMVFVTAGMGGGTGTGAAPIIAGTAKSMGILTVGIVTT   [135]
OsCLB17724_5         QEALYGADMVFVTAGMGGGTGTGGAPVIAGIAKSMGILTVGIVTT   [135]
GlAAF23771           EEAVYGADMVFVTAGMGGGTGTGGAPVIAGIAKSMGILTVGIVTT   [135]
LlBAA96782           EESVSGADMVFVTAGMGGGTGTGGAPVIAGVAKSMGILTVGIVTT   [135]
AtAAC35987           EEALYGSDMVFVTAGMGGGTGTGAAPVIAGIAKAMGILTVGIATT   [135]
AtAAK63846           QEALYGSDMVFVTAGMGGGTGTGGAPIIAGVAKAMGILTVGIVTT   [135]
OsCL005296_338       EQAVSGADMIFVTAGMGGGTGTGGAPVIAGIAKSMGILTVGIVTT   [135]
PpCAB76386           EEALRGADMVFVTAGMGGGTGSGAAPIIAGVAKQLGILTVGIVTT   [135]
PpCAB54558           EEALRGADMVFVTAGMGGGTGSGAAPIIAGVAKQLGILTVGIVTT   [135]
CrAAM22891           AAALQDTDMVFVTAGMGGGTGSGAAPVVAEVARELGILTVGIVTT   [135]
CmBAA85116           AEAVRGADLVFVTAGMGGGTGSGAAPVVAEAAREQGCLTVGVVTK   [135]
CcBAA82871           AEAVRGADLVFVTAGMGGGTGSGAAPVVAEAAREQGCLTVGVVTK   [135]
GtCAA07676           AEAVSAGDLVFVTAGMGGGTGSGAAPIVAEVAKEMGCLTVGVVTK   [135]
GsBAA82090           SSAVQGGDLVFVTAGMGGGTGSGAAPIVAKIAKEQGCLTVGVVTK   [135]
MsAAF35433           MAVVKNADLVFVTAGMGGGTGSGAAPVVAECAKEAGALTVGVVTK   [135]
GsBAA82091           AKALQGGDLVFVTAGMGGGTGTGAAPIVADVARELGCLTVGVVTK   [135]
Te1949               ANALDHPDLVFITAGMGGGTGTGAAPVIAEIAKEAGSLTVGVVTR   [135]
SsNP_440816          ARSLEGTDLVFITAGMGGGTGTGAAPIVAEVAKEMGCLTVGIVTR   [135]
AsCAA83241           ATALEGADLVFITAGMGGGTGTGAAPIVAEVAKEMGALTVGVVTR   [135]
Np61                 ATALEGADLVFITAGMGGGTGTGAAPIVAEVAKEMGALTVGVVTR   [135]
Tetll2382            AAALKDADLIFITCGMGGGTGTGAAPIVAEVAKEQGALTVAVVTR   [135]
SyAAC26227           IAALEGADLVFITAGMGGGTGTGAAPIVAEVAKEVGALTVGIVTK   [135]
PsCAB56201           QQTLEGSDLVFIAAGMGGGTGTGAAPVVAEVAKQSGALTVGIVTK   [135]
Pm1658               QQTLEGSDLVFIAAGMGGGTGTGAAPVVAEVAKQSGALTVGIVTK   [135]
PmCAB95028           QQALEGADLVFIAAGMGGGTGTGAAPVVAEVAKQSGALTVAIVTK   [135]
Sy549                HDALQGSDLVFIAAGMGGGTGTGAAPVVAEVAREVGALTVGIVTK   [135]
Pm1268               QQALQGVDLVFIAVGMGGGTGTGAAPVVAEVAKESGALTVGIVTK   [135]
NtCAB41987           ANSLKGSDMVFITAGMGGGTGSGAAPVVAQIAKEAGYLTVGVVTY   [135]
NtCAB89287           ANSLKGSDMVFITAGMGGGTGSGAAPVVAQIAKEAGYLTVGVVTY   [135]
TeAAF81220           GNALKGSDLVFITAGMGGGTGSGAAPVVAQIAKEAGYLTVGVVTY   [135]
AtAAA82068           ANALKGSDLVFITAGMGGGTGSGAAPVVAQISKDAGYLTVGVVTY   [135]
PsCAA75603           ANALKGSDLVFITAGMGGGTGSGAAPVVAQISKEAGYLTVGVVTY   [135]
NtAAF23770           ANALKGSDMVFITAGMGGGTGSGAAPVVAQIAKEAGYLTVGVVTY   [135]
NtCAB89286           ANALKGSDMVFITAGMGGGTGSGAAPVVAQIAKEAGYLTVGVVTY   [135]
OsAAK64282           ANALKDSDLVFITAGMGGGTGSGAAPVVAQISKEAGYLTVGVVTY   [135]
CrBAB91150           RRMVQGADLVFITAGMGGGTGTGAAPVVARLSKELGILTVGVVTY   [135]
EcP06138             RAALEGADMVFIAAGMGGGTGTGAAPVVAEVAKDLGILTVAVVTK   [135]
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[                    140        150        160        170        180]
[                    .          .          .          .          .]

NtCAB89288          PFSFEGRRRAVQAQEGIAALRENVDTLIVIPNDKLLTAVSPSTPV   [180]
NtCAC44257          PFSFEGRRRAVQAQEGIAALRENVDTLIVIPNDKLLTAVSPSTPV   [180]
OsCLB17724_5        PFSFEGRRRAVQAQEGIAALRNSVDTLIVIPNDKLLSAVSPNTPV   [180]
GlAAF23771          PFSFEGRRRAVQAQEGIAALRDNVDTLIVIPNDKLLTAVSPSTPV   [180]
LlBAA96782          PFMFEGRRRTVQAQEGIAALRNNVDTLIVIPNDKLLTAVSPNTPV   [180]
AtAAC35987          PFSFEGRRRTVQAQEGLASLRDNVDTLIVIPNDKLLTAVSQSTPV   [180]
AtAAK63846          PFSFEGRRRALQAQEGIAALRDNVDTLIVIPNDKLLAAVSQSTPV   [180]
OsCL005296_338      PFAFEGRRRALQAQEGIASLRSNVDTLIVIPNDKLLTAVSPNTPV   [180]
PpCAB76386          PFAFEGRRRSVQAHEGIAALKNNVDTLITIPNNKLLTAVAQSTPV   [180]
PpCAB54558          PFAFEGRRRAVQAHEGIAALKNNVDTLITIPNNKLLTAVAQSTPV   [180]
CrAAM22891          PFTFEGRQRAQQARSALANLRAAVDTLIVIPNDRLLSAMDSNVPI   [180]
CmBAA85116          PFAFEGRKRMNQALEAIEALRESVDTLIVVSNDKLLQIVPENTPL   [180]
CcBAA82871          PFAFEGRRRMTQALEAIEALRESVDTLIVVSNDKLLQIVPENTPL   [180]
GtCAA07676          PFAFEGKRRMQQANDAILNLRNKVDTLIVVSNDKLLQIVPDNTPL   [180]
GsBAA82090          PFSFEGRRRMQQAEEAIEALRKEVDTLIVVSNDKLLEIVPENTAL   [180]
MsAAF35433          PFGFEGRKRMQQARNAILEMKDKVDTLIVVSNDKLLKIVPDNTPL   [180]
GsBAA82091          PFAFEGRRRLQQAVEGLANLREKVDTLIVISNDRLLETVPKDTPL   [180]
Te1949              PFTFEGRRRITQADEGITALQTRVDTLIVIPNNRLLSVINDQTPV   [180]
SsNP_440816         PFTFEGRRRAKQAEEGINALQSRVDTLIVIPNNQLLSVIPAETPL   [180]
AsCAA83241          PFVFEGRRRTSQAEQGIEGLKSRVDTLIIIPNNKLLEVIPEQTPV   [180]
Np61                PFVFEGRRRTSQAEQGIEGLKSRVDTLIIIPNNKLLEVIPEQTPV   [180]
Tetll2382           PFTFEGRRRANQADEGIEALQSRVDTLIVIPNDKILSVISEQTSV   [180]
SyAAC26227          PFTFEGRRRMKQAEEGTAALQSSVDTLITIPNDRLLHAISEQTPI   [180]
PsCAB56201          PFSFEGKRRMRQAEEGIARLAENVDTLIVIPNDRLKDVIAG-APL   [180]
Pm1658              PFSFEGKRRMRQAEEGIARLAENVDTLIVIPNDRLKDVIAG-APL   [180]
PmCAB95028          PFSFEGRRRMRQADEGIAKLTESVDTLIVIPNDRLKDAIAG-APL   [180]
Sy549               PFGFEGRRRMRQADEGIARLAEHVDTLIVIPNDRLREAIAG-APL   [180]
Pm1268              PFSFEGRRRMRQAAEGIGRLADHVDTLIVIPNDRIKDVISE-APL   [180]
NtCAB41987          PFSFEGRKRSVQALEAIEKLQKNVDTLIVIPNDRLLDIADEQTPL   [180]
NtCAB89287          PFSFEGRKRSVQALEAIEKLQKNVDTLIVIPNDRLLDIADEQTPL   [180]
TeAAF81220          PFSFEGRKRSVQALEAIEKLQKNVDTLIVIPNDRLLDIADENTPL   [180]
AtAAA82068          PFSFEGRKRSLQALEAIEKLQKNVDTLIVIPNDRLLDIADEQTPL   [180]
PsCAA75603          PFSFEGRKRSLQALEAIEKLQKNVDTLIVIPNDRLLDIADEQMPL   [180]
NtAAF23770          PFSFEGRKRSLQALEAIEKLQKNVDTLIVIPNDRLLDIADEQTPL   [180]
NtCAB89286          PFSFEGRKRSLQALEAIEKLQKNVDTLIVIPNDRLLDIADEQTPL   [180]
OsAAK64282          PFSFEGRKRSLQALEALEKLERSVDTLIVIPNDRLLDVVDENTPL   [180]
CrBAB91150          PFNFEGRRRAGQALEGIEALREAVDSVIVIPNDRLLDVAGASTAL   [180]
EcP06138            PFNFEGKKRMAFAEQGITELSKHVDSLITIPNDKLLKVLGRGISL   [180]
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[                          190        200        210        220    ]
[                          .          .          .          .      ]

NtCAB89288          TEAFNLADDILRQGVRGISDIITIPGLVNVDFADVRAIMANAGSS   [225]
NtCAC44257          TEAFNLADDILRQGVRGISDIITIPGLVNVDFADVRAIMANAGSS   [225]
OsCLB17724_5        TEAFNLADDILRQGIRGISDIITVPGLVNVDFADVRAIMQNAGSS   [225]
GlAAF23771          TEAFNLADDILRQGVRGISDIITIPGLVNVDFADVRAIMANAGSS   [225]
LlBAA96782          TEAFNLADDILRQGVRGISDIITVPGLVNVDFADVRAIMANAGSS   [225]
AtAAC35987          TEAFNLADDILRQGVRGISDIITIPGLVNVDFADVRAIMANAGSS   [225]
AtAAK63846          TEAFNLADDILRQGVRGISDIITIPGLVNVDFADVRAIMANAGSS   [225]
OsCL005296_338      TEAFNLADDILRQGVRGISDIITVPGLVNVDFADVRSVMSDAGSS   [225]
PpCAB76386          TEAFNLADDILRQGVRGISDIITVPGLVNVDFADVRAIMANAGSS   [225]
PpCAB54558          TEAFNLADDILRQGVRGISDIITVPGLVNVDFADVRAIMANAGSS   [225]
CrAAM22891          KDAFKIADDVLRQGVKGISEIITVPGLVNVDFADVRAIMAGAGSS   [225]
CmBAA85116          QDAFRVADDILRQGVVGISDIIIRPGLINVDFADVRSVMAHAGSA   [225]
CcBAA82871          QDAFRVADDILRQGVVGISDIIIRPGLINVDFADVRSVMAHAGSA   [225]
GtCAA07676          QDAFSVADDILRQGVVGISEIIVRPGLINVDFADVRSVMADAGSA   [225]
GsBAA82090          EKAFSVADDILRQGVVGISEIIVRPGLINVDFADVRSIMADAGSA   [225]
MsAAF35433          TEAFLVADDILRQGVVGITEIIVKPGLVNVDFADVRTIMGNAGTA   [225]
GsBAA82091          TEAFIFADEVLRQGVGGISDIITKPGLVNVDFADVRTVMAEKGFA   [225]
Te1949              QEAFIIADDILRQGIQGISDIITVPGLVNVDFADVRAVMADAGSA   [225]
SsNP_440816         QEAFRVADDILRQGVQGISDIIIIPGLVNVDFADVRAVMADAGSA   [225]
AsCAA83241          QEAFRYADDVLRQGVQGISDIITIPGLVNVDFADVRAVMADAGSA   [225]
Np61                QEAFRYADDVLRQGVQGISDIITIPGLVNVDFADVRAVMADAGSA   [225]
Tetll2382           QDAFRVADDVLRQGVQGISDIINVPGLINVDFADIRSVMADAGSA   [225]
SyAAC26227          QEAFRVADDILRQGVQGISDIITIPGLVNVDFADVRAVMADAGSA   [225]
PsCAB56201          QEAFRNADDVLRMGVKGISDIITCPGLVNVDFADVRSVMTEAGTA   [225]
Pm1658              QEAFRNADDVLRMGVKGISDIITCPGLVNVDFADVRSVMTEAGTA   [225]
PmCAB95028          QEAFKNADDVLRMGVKGITDIITLPGLVNVDFADVRSVMTEAGTS   [225]
Sy549               QEAFRSADDVLRMGVKGISDIITCPGLVNVDFADVRSVMTEAGTA   [225]
Pm1268              QEAFRSADDILRMGVKGISDIITCPGLVNVDFADVRSVMTEAGTA   [225]
NtCAB41987          QDAFLLADDVLRQGVQGISDIITIPGLVNVDFADVKAVMKDSGTA   [225]
NtCAB89287          QDAFLLADDVLRQGVQGISDIITIPGLVNVDFADVKAVMKDSGTA   [225]
TeAAF81220          QDAFLLADDVLRQGVQGISDIITIPGLVNVDFADVKAVMKDSGTA   [225]
AtAAA82068          QDAFLLADDVLRQGVQGISDIITIPGLVNVDFADVKAVMKDSGTA   [225]
PsCAA75603          QDAFRLADDVLRQGVQGISDIITIPGLVNVDFADVKAVMKDSGTA   [225]
NtAAF23770          QNAFLLADDVLCQGVQGISDIITIPGLVNVDFADVKAIMKDSGTA   [225]
NtCAB89286          QNAFLLADDVLCQGVQGISDIITIPGLVNVDFADVKAIMKDSGTA   [225]
OsAAK64282          QDAFLLADDVLRQGVQGISDIITIPGLVNVDFADVKAVMKNSGTA   [225]
CrBAB91150          QDAFALADDVLRQGVQGISDIITVPGLINVDFADVKAIMSNSGTA   [225]
EcP06138            LDAFGAANDVLKGAVQGIAELITRPGLMNVDFADVRTVMSEMGYA   [225]
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[                    230        240        250        260        270]
[                    .          .          .          .          .]

NtCAB89288         LMGIGTATGKTRARDAALNAIQSPLLDIG-IERATGIVWNITGGS   [270]
NtCAC44257         LMGIGTATGKTRARDAALNAIQSPLLDIG-IERATGIVWNITGGS   [270]
OsCLB17724_5       LMGIGTATGKSRARDAALNAIQSPLLDIG-IERATGIVWNITGGA   [270]
GlAAF23771         LMGIGTATGKTRARDAALNAIQSPLLDIG-IERATGIVWNITGGS   [270]
LlBAA96782         LMGIGTATGKTRARDAALNAVQSPLLDIG-IERATGIVWNITGGN   [270]
AtAAC35987         LMGIGTATGKSRARDAALNAIQSPLLDIG-IERATGIVWNITGGS   [270]
AtAAK63846         LMGIGTATGKTRARDAALNAIQSPLLDIG-IERATGIVWNITGGS   [270]
OsCL005296_338     LMGIGTATGKTRARDAALNAIQSPLLDIG-IERATGIVWNITGGN   [270]
PpCAB76386         LMGIGTATGKSKAREAALSAIQSPLLDVG-IERATGIVWNITGGS   [270]
PpCAB54558         LMGIGTATGKSRAREAALSAIQSPLLDVG-IERATGIVWNITGGS   [270]
CrAAM22891         LMGQGYGSGPRRASDAALRAISSPLLEVG-IERATGVVWNITGPP   [270]
CmBAA85116         LMGIGTGSGKSRAHDAAVAAISSPLLDFP-IERAKGIVFNVTGGE   [270]
CcBAA82871         LMGIGTGSGKSRAHDAAVAAISSPLLDFP-IERAKGIVFNVTGGE   [270]
GtCAA07676         LMGIGTGSGKTRAQDAAVAAISSPLLDFP-IEKARGIVFNITGGQ   [270]
GsBAA82090         LMGIGSGSGKSRAKDAAVAAISSPLLDFP-IERAKGIVFNITGGH   [270]
MsAAF35433         LMGIGHGKGKNRAKDAALSAISSPLLDFP-ITRAKGIVFNIVGGS   [270]
GsBAA82091         LLGIGTASGDSRARNAATAAISSPLLDFP-ITSAKGAVFNITGGT   [270]
Te1949             LMGIGMGSGKSRAREAANAAISSPLLESS-IEGAKGVVFNITGGT   [270]
SsNP_440816        LMGIGVGSGKSRAKEAATAAISSPLLESS-IQGAKGVVFNVTGGT   [270]
AsCAA83241         LMGIGVSSGKSRAREAAIAAISSPLLECS-IEGARGVVFNITGGS   [270]
Np61               LMGIGVSSGKSRAREAAIAAISSPLLECS-IEGARGVVFNITGGT   [270]
Tet112382          MMGIGIASGKSRATEAALSAISSPLLERS-IEGAKGVVFNITGGT   [270]
SyAAC26227         LMGIGSGSGKSRAREAAHAAISSPLLESS-IEGARGVVFNITGGR   [270]
PsCAB56201         LLGIGIGSGRSRALEAAQAAMNSPLLEAARIDGAKGCVINITGGK   [270]
Pm1658             LLGIGIGSGRSRALEAAQAAMNSPLLEAARIDGAKGCVINITGGK   [270]
PmCAB95028         LLGIGIGSGRSRAAEAAQAAINSPLLEAGRIDGAKGCVVNITGGK   [270]
Sy549              LLGIGIGSGRSRAVEAAQAAISSPLLETERIDGAKGCVINISGGR   [270]
Pm1268             LLGIGEGSGRSRAIEAAQAAISSPLLEAARIDGAKGCVINISGGR   [270]
NtCAB41987         MLGVGVSSSKNRAEEAAEQATLAPLIGSS-IQSATGVVYNITGGK   [270]
NtCAB89287         MLGVGVSSSKNRAEEAAEQATLAPLIGSS-IQSATGVVYNITGGK   [270]
TeAAF81220         MLGVGVSSSKNRAEEAAEQATLAPLIGSS-IQSATGVVYNITGGK   [270]
AtAAA82068         MLGVGVSSSKNRAEEAAEQATLAPLIGSS-IQSATGVVYNITGGK   [270]
PsCAA75603         MLGVGVSSGKNRAEEAAEQATLAPLIGSS-IQSATGVVYNITGGK   [270]
NtAAF23770         MLGVGVSSSRNRAEEAAEQATLAPLIGSS-IQSATGDVYNITGGK   [270]
NtCAB89286         MLGVGVSSSRNRAEEAAEQATLAPLIGLS-IQSATGVVYNITGGK   [270]
OsAAK64282         MLGVGVSSSKNRAQEAAEQATLAPLIGSS-IEAATGVVYNITGGK   [270]
CrBAB91150         MLGVGAASTADRAEQAAVAATSAPLIQRS-IEKATGIVYNITGGR   [270]
EcP06138           MMGSGVASGEDRAEEAAEMAISSPLLEDIDLSGARGVLVNITAGF   [270]
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[                        280        290        300        310    ]
[                        .          .          .          .      ]

NtCAB89288          DLTLFEVNAAAEVIYDLVDPSANLIFGAVIDPSISGQVSITLIAT   [315]
NtCAC44257          DLTLFEVNAAAEVIYDLVDPSANLIFGAVIDPSISGQVSITLIAT   [315]
OsCLB17724_5        DMTLFEVNSAAEIIYDLVDPNANLIFGAVIDPSLNGQVSITLIAT   [315]
GlAAF23771          DLTLFEVNAAAEVIYDLVDPSANLIFGAVVDPSLCGQVSITLIAT   [315]
LlBAA96782          DLTLYEVNAAAEVIYDLVDPAANLIFGAVIDPSISGQVSITLIAT   [315]
AtAAC35987          DLTLFEVNAAAEVIYDLVDPTANLIFGAVVDPALSGQVSITLIAT   [315]
AtAAK63846          DLTLFEVNAAAEVIYDLVDPTANLIFGAVVDPSYSGQISITLIAT   [315]
OsCL005296_338      DLTLTEVNAAAEVIYDLVDPGANLIFGSVIDPSYTGQVSITLIAT   [315]
PpCAB76386          DMTLFEVNAAAEVIYDLVDPNANLIFGAVVDEALHDQISITLIAT   [315]
PpCAB54558          DMTLFEVNAAAEVIYDLVDPNANLIFGAVVDEALHGQVSITLIAT   [315]
CrAAM22891          NMTLHEVNEAAEIIYDMVDPNANLIFGAVVDSTLPDTVSITIIAT   [315]
CmBAA85116          DMTLHEINQAAEVIYEAVDPNANIIFGALIDQQMESEISITVVAT   [315]
CcBAA82871          DMTLHEINQAAEVIYEAVDPNANIIFGALVDQQMESEISITVVAT   [315]
GtCAA07676          DMTLHEINSAAEVIYEAVDSNANIIFGALVDDNMENEISITVVAT   [315]
GsBAA82090          DMTLHEINAAAEVIYEAVDLNANIIFGALVDDSMENELSITVIAT   [315]
MsAAF35433          DMSLQEINAAAEVIYENVDQDANIIFGAMVDDKMTSEVSITVLAT   [315]
GsBAA82091          DMTLSEVNQAAQVIYDSVDSDANIIFGAVVDETFKGKVSVTVVAT   [315]
Te1949              DLTLHEVNAAAEIIYEVVDPNANIIFGAVIDDKLQGEIKITVIAT   [315]
SsNP_440816         DLTLHEVNVAAEIIYEVVDADANIIFGAVIDDRLQGEMRITVIAT   [315]
AsCAA83241          DLTLHEVNAAAETIYEVVDPNANIIFGAVIDDRLQGEVRITVIAT   [315]
Np61                DLTLHEVNAAAEAIYEVVDPNANIIFGAVIDDRLQGEVRITVIAT   [315]
Tetll2382           DLSLHEVNAAADVIYNVADANANIIFGAVIDPQMQGEVQITVIAT   [315]
SyAAC26227          DMTLHEVNAAADAIYEVVDPEANIIFGAVIDDRLEGELRITVIAT   [315]
PsCAB56201          DMTLEDMTSASEIIYDVVDPEANIIVGAVIDESMEGEIQVTVIAT   [315]
Pm1658              DMTLEDMTSASEIIYDVVDPEANIIVGAVIDESMEGEIQVTVIAT   [315]
PmCAB95028          DMTLEDMTSASEVIYDVVDPEANIIVGAVIDEALEGEVQVTVIAT   [315]
Sy549               DMTLEDMTTASEVIYDVVDPEANIIVGAVVDEALEGEIHVTVIAT   [315]
Pm1268              DMTLEDMTSASEVIYDVVDPEANIIVGAVVDEKLEGEVHVTVIAT   [315]
NtCAB41987          DITLQEVNRVSQVVTSLADPSANIIFGAVVDERYNGEIHVTIIAT   [315]
NtCAB89287          DITLQEVNRVSQVVTSLADPSANIIFGAVVDERYNGEIHVTIIAT   [315]
TeAAF81220          DITLQEVNRVSQVVTSLADPSANIIFGAVVDERYNGEIHVTIVAT   [315]
AtAAA82068          DITLQEVNRVSQVVTSLADPSANIIFGAVVDDRYTGEIHVTIIAT   [315]
PsCAA75603          DITLQEVNRVSQVVTSLADPSANIIFGAVVDDRYTGEIHVTIIAT   [315]
NtAAF23770          DITLQEVNKVSQVVTSLADPSANIIFGAVVDERYNGEIQVTLIAT   [315]
NtCAB89286          DITLQEVNKVSQVVTSLADPSANIIFGAVVDERYNGEIQVTLIAT   [315]
OsAAK64282          DITLQEVNKVSQIVTSLADPSANIIFGAVVDDRYTGEIHVTIIAT   [315]
CrBAB91150          DLTLAEVNRVSEVVTALADPSCNIIFGAVVDEQYDGELHVTIIAT   [315]
EcP06138            DLRLDEFETVGNTIRAFASDNATVVIGTSLDPDMNDELRVTVVAT   [315]
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[                    320        330        340        350        360]
[                    .          .          .          .          .]

NtCAB89288          GFKRQE-------ESDGRPLQ-GNQLTQGDVS----------LGN   [360]
NtCAC44257          GFKRQE-------ESDGRPLQ-GNQLTQGDAS----------LGS   [360]
OsCLB17724_5        GFKRQD-------EPEGRTTK-----------------------    [360]
GlAAF23771          GFKRQE-------ESDKRSIQAGGQLAPGDAN----------QGI   [360]
LlBAA96782          GFKRQD-------ETEGQKSQ---GTQLGLGGN---------LGI   [360]
AtAAC35987          GFKRQE-------EGEGRTVQ----MVQADAAS---------VGA   [360]
AtAAK63846          GFKRQE-------EGEGRPLQ----ATQADAS----------MGA   [360]
OsCL005296_338      GFKRQE-------EAESRQEE-----------------------    [360]
PpCAB76386          GFSSQD-------DPDARSMQYASRVLEGQAG-----------R    [360]
PpCAB54558          GFSSQD-------EPDARSMQNVSRILDGQAG-----------R    [360]
CrAAM22891          GFGHVEPELGALADRGSRAAAAASPRVAAAANAPAAAAAVPPVTT   [360]
CmBAA85116          GFPQ---------PNESANSGGSSSTLNATANEFYQAG-PPNRQV   [360]
CcBAA82871          GFPQ---------PNESASNGGTSSTLNATASDFYQAG-PSARPA   [360]
GtCAA07676          GFTQ---------PNDSK--------------FFSTK-------    [360]
GsBAA82090          GFPQ---------PSDSPSSS-----------MTQTP-------    [360]
MsAAF35433          GFSTDY------FSNDGSGLENLPPNRLSPPKTVGSAK-KPKG--   [360]
GsBAA82091          GFS-----------------------------------------    [360]
Tel949              GFSGEVQT----QPIQEKVQP--------RRPVPNPTQN------   [360]
SsNP_440816         GFNGEKEK----PQAKTSSKPVLSGPPAGVETVPSTTTP------   [360]
AsCAA83241          GFTGEIQA----APQQNAANARVVSAPPKRTPTQTPLTN-----S   [360]
Np61                GFTGEVQA----AVQQSVASVRVAPNTSKRPTTQQPAVNPSSTPT   [360]
Tetll2382           GFSGEPMS----RTRATTKTTPLTN----RPLATTSPPP------   [360]
SyAAC26227          GFSTDRPN----LNTISTSTS---------QPTSQPSVS------   [360]
PsCAB56201          GFETNQPL----KQQRIKNR-----------LSNQPLYN------   [360]
Pm1658              GFETNQPL----KQQRIKNR-----------LSNQPLYN------   [360]
PmCAB95028          GFDGNQPY----TKQKAGAK-----------LSPQSLYR------   [360]
Sy549               GFDQGQQY----RSDRSSASG----------LPVQPQRS------   [360]
Pm1268              GFEGNQPY----RSERSINK-----------IASQSIYS------   [360]
NtCAB41987          GFTQSFQKTLLSDPRGAKLADKGPVIQESMASPVTLRS-------    [360]
NtCAB89287          GFTQSFQKTLLSDPRGAKLADKGPVIQESMASPVTLRS-------    [360]
TeAAF81220          GFAQSFQKSLLADPKGAKLVDRNQEPTQPLTSARSLTT-------    [360]
AtAAA82068          GFSQSFQKTLLTDPRAAKLLDKMGSSGQQENKGMSLPHQ------   [360]
PsCAA75603          GFSQSFQKKLLTDPRAAKLLDKVAEGKESKTVPPPLKSS------   [360]
NtAAF23770          GFAQSFQNSLLTDPRGAKLVDKSKGTTERTVSPDTLRS-------    [360]
NtCAB89286          GFAQSFQNSLLTDPRGAKLVDKSKGTTERTVSPDTLRS-------    [360]
OsAAK64282          GFPQSFQKSLLADPKGARIMEAKEKAANLTYKAVAAAT-------    [360]
CrBAB91150          GFAPTYENELLNGGNAQQQQARAARRASNQATAASLAPNPAVQPT    [360]
EcP06138            GIGMDKRPEITLVTNKQVQQPVMDRYQQHGMAPLTQEQKPVAKVV    [360]
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[                          370        380        390      ]
[                          .          .          .        ]

NtCAB89288          NRRPAS-FLEGGSVEIPEFLRKKGRSRYPRA------    [397]
NtCAC44257          NRRPAS-FLEGGSVEIPEFLRKKGRSRYPRA------    [397]
OsCLB17724_5        VQKLCVPMIGEIESGITVTIQRQRI------------    [397]
GlAAF23771          NRRPSS-FSESGSVEIPEFLRKKGRSRYPRA------    [397]
LlBAA96782          NRRPSSSMTMGGIVEIPHFLRKKAGSRNPRA------    [397]
AtAAC35987          TRRPSSSFRESGSVEIPEFLKKKGSSRYPRV------    [397]
AtAAK63846          TRRPSSSFTEGSSIEIPEFLKKKGRSRYPRL------    [397]
OsCL005296_338      ----------GPTLQIPEFLQRKGRSGFSRG------    [397]
PpCAB76386          SSMASSRGGNSSTINIPNFLRKRGQR-----------    [397]
PpCAB54558          SPTGLSQGSNGSAINIPSFLRKRGQTRH---------    [397]
CrAAM22891          AAPETPGGASSSGVEIPAFLRRRRVQGK---------    [397]
CmBAA85116          TPPPPQPGPSRTIGNIPDFLRRFQK------------    [397]
CcBAA82871          SQPPSQTGPSRSIGSIPDFLRRFQK------------    [397]
GtCAA07676          -----------------DLWKKFY-------------    [397]
GsBAA82090          --------------DIPDFLRRFQQENK---------    [397]
MsAAF35433          -------------GGFRGFIKRLFS------------    [397]
GsBAA82091          ------------------------------------    [397]
Te1949              PNSTPEPQRKLPGLDIPDFLQRRRNPSNK--------    [397]
SsNP_440816         EDPLGEIPMA-PELDIPDFLQKRRFPRR---------    [397]
AsCAA83241          PAPTPEPKEK-SGLDIPDFLQRRRPPKN---------    [397]
Np61                PTPTPEPKEK-PGLDIPDFLRNRRTPRN---------    [397]
Tetll2382           EAPAPEVEAK-PKLDIPEFLQRRRPTP----------    [397]
SyAAC26227          PNPASAPPASGGGLDIPAFLQRKIQNRP---------    [397]
PsCAB56201          -----ISDNKDTGTNIPEFLRLRQNKKDIE-------    [397]
Pm1658              -----ISDNKDTGTNIPEFLRLRQNKKDIE-------    [397]
PmCAB95028          -----QTPNKEPGASIPEFLRLRQLRRDQ--------    [397]
Sy549               -----AIE--ENGARIPEFLRQRQQQTNDPT------    [397]
Pm1268              -----QPEANESGARIPEFLRKRQPRNDNEI------    [397]
NtCAB41987          --------------STSPSTTSRTPTRRLFF------    [397]
NtCAB89287          --------------STSPSTTSRTPTRRLFF------    [397]
TeAAF81220          ---------------PSPAPSR---SRKLFF------    [397]
AtAAA82068          ------------KQSPSTISTKSSSPRRLFF------    [397]
PsCAA75603          ------------NFS-SKVESRPPPPRKLFF------    [397]
NtAAF23770          --------------SESPSTKPRPATRRLFF------    [397]
NtCAB89286          --------------SESPSTKPRPAARRLFF------    [397]
OsAAK64282          ----------------VQPAPAATWSRRLFS------    [397]
CrBAB91150          T-----------PPANPAAPWSRPNRAKLDFLGRSIL    [397]
EcP06138            NDNAPQTAKEPDYLDIPAFLRKQAD------------    [397]
;
END;
```

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