RESEARCH ARTICLE

Chloroplast Division in Higher Plants Requires Members of Two Functionally Divergent Gene Families with Homology to Bacterial *ftsZ*

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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 chloroplast-localized 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; Pyke et al., 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

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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 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, 1998; de Pereda et al., 1996; Erickson et al., 1996; Lowe and Amos, 1998), and it can undergo dynamic GTP-dependent assembly into long polymers in vitro (de Boer et al., 1992a; RayChaudhuri and Park, 1992; Mukherjee et al., 1993; Bramhill and Thompson, 1994; Mukherjee and Lutkenhaus, 1994, 1998; Bramhill, 1997; Yu and Margolin, 1997). 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 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.* Because 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., 1992a; 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
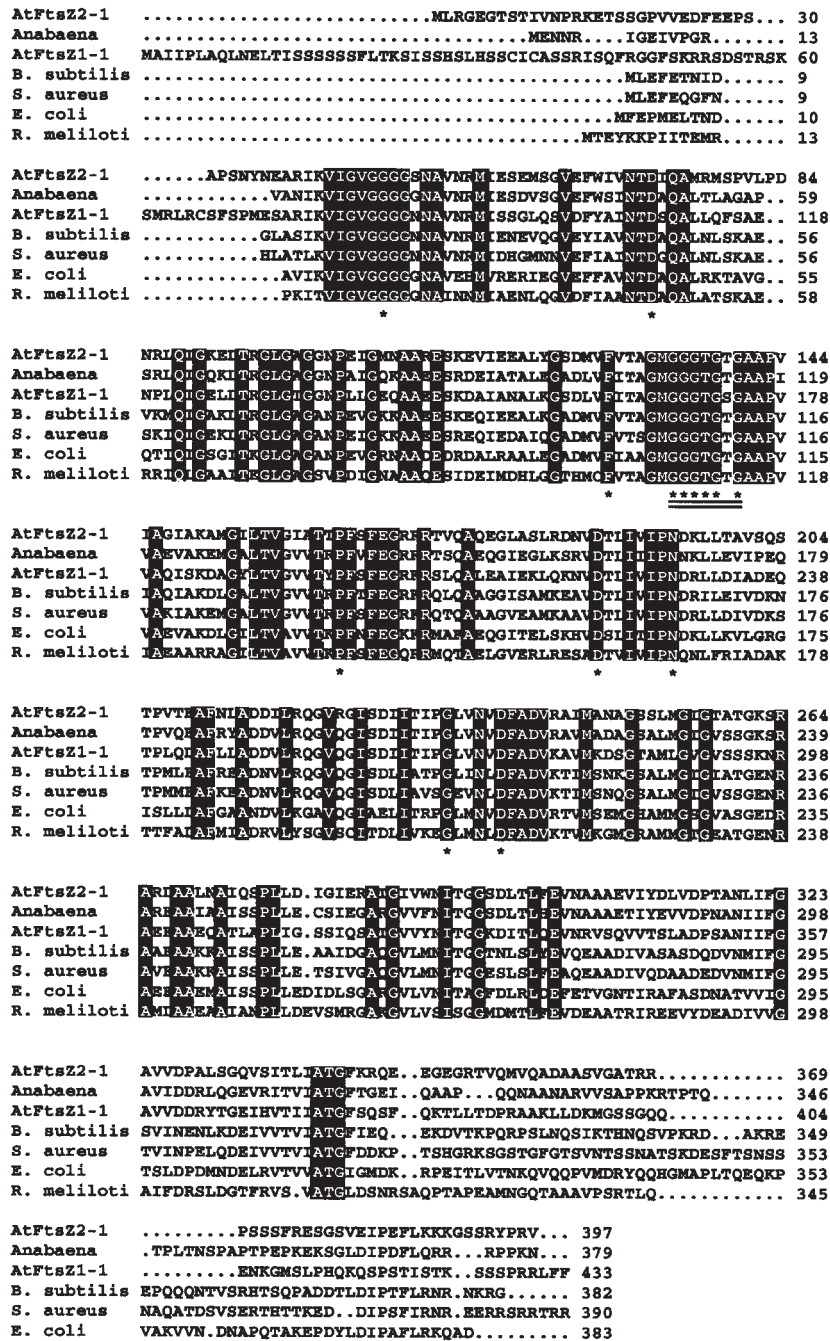
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AtFtsZ2-1   ........................MLRGEGTSTIVNPRKETSSGPVVEDFEEPS...  30
Anabaena    ........................MENNR....IGEIVPGR.......          13
AtFtsZ1-1   MAIIPLAQLNELTISSSSSSFLTKSISSHSLHSSCICASSRISQFRGGFSKRRSDSTRSK  60
B. subtilis ........................MLEFETNID......                    9
S. aureus   ........................MLEFEQGFN......                    9
E. coli     ........................MFEPMELTND......                  10
R. meliloti ........................MTEYKKPIITEMR......                13

AtFtsZ2-1   ......APSNYNEARIKVIGVGGGGSNAVNRMIESEMSGVEFWIVNTDIQAMRMSPVLPD  84
Anabaena    ............VANIKVIGVGGGGCNAVNRMIESDVSGVEFWSINTDIQALTLAGAP..  59
AtFtsZ1-1   SMRLRCSFSPMESARIKVIGVGGGGCNAVNRMISSGLQSVDFYAINTDIQALLQFSAE.. 118
B. subtilis ...........GLASIKVIGVGGGGNNAVNRMIENEVQGVEYIAVNTDIQALNLSKAE..  56
S. aureus   ...........HLATLKVIGVGGGGNNAVNRMIDHGMNNVEFIAINTDIQALNLSKAE..  56
E. coli     ..............AVIKVIGVGGGGGNAVEHMVRERIEGVEFFAVNTDIQALRKTAVG..  55
R. meliloti ..............PKITVIGVGGGGGNAINNMIAENLQGVDFIAANTDIQALATSKAE..  58
                                                          *                    *

AtFtsZ2-1   NRIQIGKEITRGLGAGGNPEIGMNAAREBKEVIEEALYGSDMVFVTAGMGGGTGTGAAPV 144
Anabaena    SRLQIGQKITRGLGAGGNPAIGQKAAEBSRDEIATALEGADLVFITAGMGGGTGTGAAPI 119
AtFtsZ1-1   NPIQIGELITRGLGTGGNPLLGEQAAEBSKDAIANALRGSDLVFITAGMGGGTGSGAAPV 178
B. subtilis VKMQIGAKITRGLGAGANPEVGKRAAEBSKEQIEEALRGADMVFVTAGMGGGTGTGAAPV 116
S. aureus   SKIQIGEKITRGLGAGANPEIGKRAAEBSREQIEDAIQGADMVFVTSGMGGGTGTGAAPV 116
E. coli     QTIQIGSGITRGLGAGANPEVGRNAADEDRDALRAALEGADMVFIAAGMGGGTGTGAAPV 115
R. meliloti RRIQIGAALTEGLGAGSVEDIGNAAAQEBIDEIMDHLGGTHMQFVTAGMGGGTGTGAAPV 118
                *                                         *      *****  *

AtFtsZ2-1   IAGIAKAMGILTVGIATTPFSFEGRRRTVQAQEGLASLRDNVDTIIVIPNDKLLTAVSQS 204
Anabaena    VAEVAKEMGALTVGVVTKPFVFEGRRRTSQAEQGIEGLKSRVDTIIDIPNNKLLEVIPEQ 179
AtFtsZ1-1   VAQISKDAGALTVGVVTKPFSFEGRRRSLQALEAIEKLQKNVDTIIVIPNDRLLDIADEQ 238
B. subtilis IAQIAKDLGALTVGVVTKPFTFEGRRRQLQAAGGISAMKEAVDTIIVIPNDRILEIVDKN 176
S. aureus   VAKIAKEMGALTVGVVTKPFSFEGRRRQTQAAAGVEAMKAAVDTIIVIPNDRLLDIVDKS 176
E. coli     VAEVAKDLGILTVAVVTKPFNFEGKRRMAKAEQGITELSKHVDSIITIPNDKLLKVLGRG 175
R. meliloti IAEAARRAGILTVAVVTRPFSFEGQKRMQTAELGVERLRESADTIVIPNQNLFRIADAK  178
                                     *                *      *

AtFtsZ2-1   TPVTEAFNIADDILRQGVRGISDIITIPGLVNVDFADVRAIMANAGBSLMGIGTATGKSR 264
Anabaena    TPVQEAFRYADDVLRQGVQGISDIITIPGLVNVDFADVRAVMADAGSALMGIGVSSGKSR 239
AtFtsZ1-1   TPLQDAFLIADDVLRQGVQGISDIITIPGLVNVDFADVKAVMKDSGTAMLGVGVSSSKNR 298
B. subtilis TPMLEAFREADNVLRQGVQGISDLIATRGLINIDFADVKTIMSNKGSALMGIGIATGENR 236
S. aureus   TPMMEAFKEADNVLRQGVQGISDLIAVSGEVNIDFADVKTIMSNQGSALMGIGVSSGENR 236
E. coli     ISLLIAFGAANDVLKGAVQGIAELITRFGLMNVDFADVRTVMSEMGYAMMGSGVASGEDR 235
R. meliloti TTFAIAFMIADRVLYSGVSCITDLIVKEGLMNJDFADVKTVMKGMGRAMMGIGEATGENR 238
                           *          *

AtFtsZ2-1   ARIAALNAIQSPLLD.IGIERATGIVWNITGGSDLTLFEVNAAAEVIYDLVDPTANLIFG 323
Anabaena    ARRAAIAAISSPLLE.CSIEGATGVVFNITCGSDLLHEVNAAAETIYEVVDPNANIIFG  298
AtFtsZ1-1   AEEAAEQATLAPLIG.SSIQSATCVVYNITCGKDITLQEVNRVSQVVTSLADPSANIIFG 357
B. subtilis AAEAAKKAISSPLLE.AAIDGATGVLMNITGGTNLSLYEVQEAADIVASASDQDVNMIFG 295
S. aureus   AVEAAKKAISSPLLE.TSIVGATGVLMNITCGESLSLFEAQEAADIVQDAADEDVNMIFG 295
E. coli     AEEAAEMAISSPLLEDIDLSGATGVLVNITAGFDLRLDEFETVGNTIRAFASDNATVVIG 295
R. meliloti AMIAAEAAIANPLLDEVSMRGATGVLVSISCGMDMTLFEVDEAATRIREEVYDEADIVVG 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
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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., 1992a); 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.

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.

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 AtFtsZ1-1 (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 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
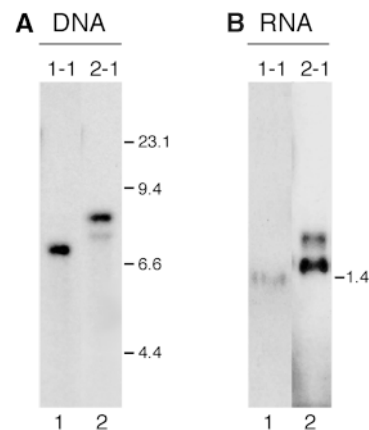

Figure 2. Hybridization Analysis of *AtFtsZ1-1* and *AtFtsZ2-1* in Arabidopsis.

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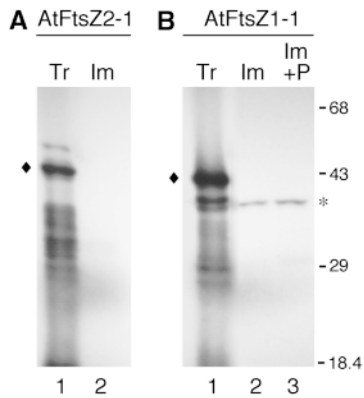
Figure 3. In Vitro Assay for Post-Translational Import of AtFtsZ2-1 to the Chloroplast.

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.

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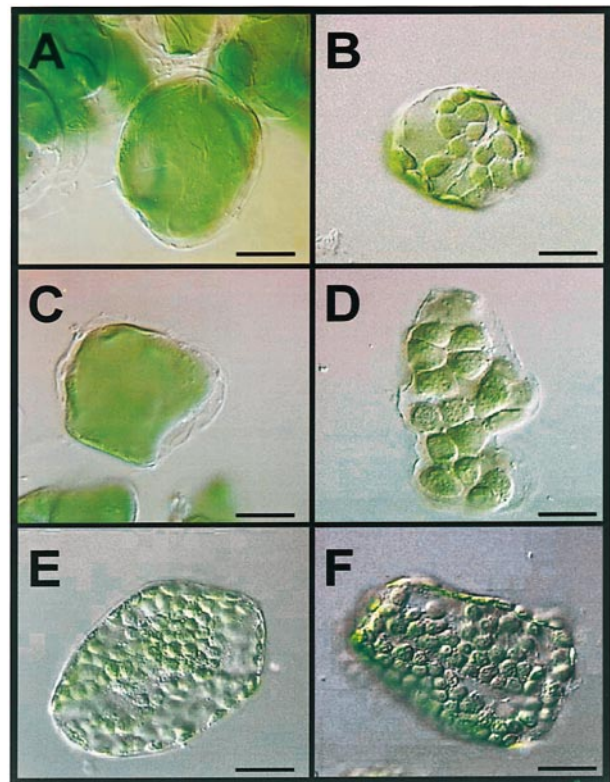
Figure 4. Phenotypes of Transgenic Plants Expressing Antisense Constructs of *AtFtsZ1-1* or *AtFtsZ2-1*.

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.

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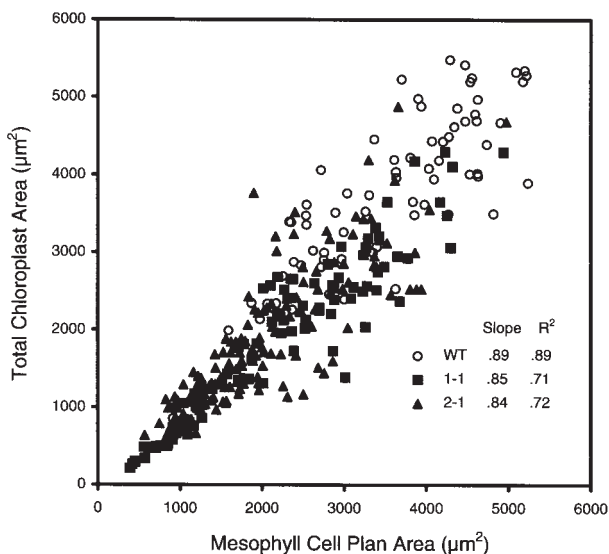
Figure 5. Relationship between Mesophyll Cell Plan Area and Total Chloroplast Plan Area in Transgenic and Wild-Type Plants.

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.

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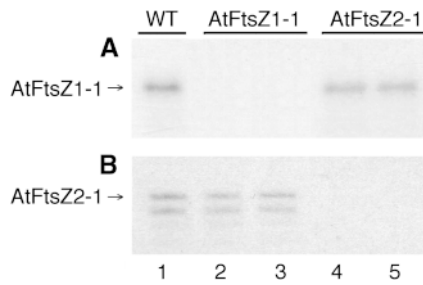
Figure 6. RNase Protection Assays of *AtFtsZ1-1* and *AtFtsZ2-1* Expression Levels in Independent Transgenic Antisense Lines.

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.

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 ≤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 cy-

tosol 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.

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.

A

```
AtFtsZ1-1  MA.II LAQL ELTIS S S SFLTK SIS SHSLH S CICA SRISQFRGG SK RSDSTRS  59
PsFtsZ     MA TLL STIS PNKLT Y S LFHNA SLS TS..P S LTTT VSIYPKTQR GR FG.....  53
AtFtsZ2-2  ................................ML NPRKETSSV PITEDLD.....           18
AtFtsZ2-1  ..................MLRGEGTSTIV NPRKETSSG PVVEDFE.....               27
PpFtsZ     ...................................MRN PVMAFEG.....               10


AtFtsZ1-1  KSMRL RCS FSPMESARIKVIGVGGGG NAVNRMISS GLG SV F IA NTDSQA IQFS AB.  118
PsFtsZ     ..S.V RCS LAYVDNAKIKVVGIGGGG NAVNRMIGS GLG GV F IA NTDAQA IHSA AE.  109
AtFtsZ2-2  ....ELSTPNT YNE ARIKVI GV GGGGS NAVNRMI ESEMIG EFWIV NTDIQA MRI SPVFP  74
AtFtsZ2-1  ....EPSAPSN YNE ARIKVI GV GGGGS NAVNRMI ESEMSG VEFWIV NTDIQA MRM SPVLP  83
PpFtsZ     ....SGDDTGS TNE AKIKVI GV GGGGS NAVNRMI ESEMIG VEFWIV NTDAQA MAI SPVPA  66
                   * *** *** IA GGGGS ***** ***  * *  ***  *   *** *** *


AtFtsZ1-1  .N LQIG LTRGLG GGNP TG AA ESKD I AL GSD VF TAGMGGGTG GAAP  177
PsFtsZ     .N IKIG LTRGLG GGNP TG AA ESKE I AL GSD VF TAGMGGGTG GAAP  168
AtFtsZ2-2  DNRLQIGKELTRGLGAGGNPETGMNAATESKEAIQEALYGSDMVFVTV             120
AtFtsZ2-1  DNRLQIGKELTRGLGAGGNPETGMNAARESKEVIEEALYGSDMVFVTAGMGGGTGTGAAP 143
PpFtsZ     QNRLQIGQKLTRGLGAGGNPETGCSAAEESKVIVEEALRGADMVFVTAGMGGGTGSGAAP 126
                 *  **  ****** ****  ** ** ***    ** ** *** * ******* ****


AtFtsZ1-1  A AQIS KD G LTVG VT PF FEGR R QA E AIEI L QKNVDTLI IPN RLL DIADE  237
PsFtsZ     A AQIS KE G LTVG VT PF FEGR R QA E AIEI L QKNVDTLI IPN RLL DIADE  228
AtFtsZ2-1  VI GIA KAMGILTVGIATTPSFEGRRRTVQAQEGLASLRDNVDTLIVIPNDKLLTAVSQ  203
PpFtsZ     IIGVAKQLGILTVGIVTTPFAFEGRRRAVQAHEGIAALKNNVDTLITIPNNKLLTAVAQ  186
                *  *  *   **** ***  *   ** ** ***      ****** ** * *


Rice EST               GISDIITIPGLVNVDFADV A M N G AMIG G G SK       40
AtFtsZ1-1  QTP QD AFLLADD LRQGV GISDIITIPGLVNVDFADV A M D G AMIG G G SK  297
PsFtsZ     MP QD AFRLADD LRQGV GISDIITIPGLVNVDFADV A M D G AMIG G G GK  288
AtFtsZ2-1  ST VTE AFNLADD ILRQGV RGISDIITIPGLVNVDFADV RAIMANA SSLMG IGTATG S  263
PpFtsZ     ST VTE AFNLADD ILRQGV RGISDIITVPGLVNVDFADV RAIMANA SSLMG IGTATG S  246
               * ** **** **** RGISDIIT*PGLVNVDFADV * *  *  ** *   * *


Rice EST   RAQEAAXRA TIA PL IGS IEAATG V NITGG D TL EVNK VSQ IVTSL DP AN IFG  99
AtFtsZ1-1  RAEEAAEQA TIA PL IGS IQSATG V NITGG D TL EVNR VSQ V TSL DP AN IFG  357
PsFtsZ     RAEEAAEQA TIA PL IGS IQSATG V NITGG D TL EVNR VSQ V TSL DP AN IFG  348
AtFtsZ2-1  RA RDAA LNA IQSPLLDG IERATG IVW NITGG SDLTI FEVN AAAE VIYDLV DPTAN LIFG  323
PpFtsZ     RA REAA LSA IQSPLLDVG IERATG IVW NITGG SDMTI FEVN AAAE VIYDLV DPNAN LIFG  306
             **  **   *   **     *  *** * *****  * **  ***       * ** ** ***


Rice EST   AVVD DRY G EIHVT IATGVS QS S A D K             132
AtFtsZ1-1  AVVD DRY G EIHVT IATGFS QSFQ T T D R AAKLLDR MGSSGQQ NKGMSL HQK.Q  416
PsFtsZ     AVVD DRY G EIHVT IATGFS QSFQ K LT D R AAKLLDR VAEG..K SKTVPP LKSSN  406
AtFtsZ2-1  AVVDPAL SG QVS IT LIATGFKR QPE GEGR TV QMV QADAASVGAT. RRPSSS FRESC SVE  381
PpFtsZ     AVVDEAL HG QVS IT LIATGFS QLE PDARS MQN VS RILDGQAGR. SPTGI SQGSNG SAIN  365
             ****      *    * ****


AtFtsZ1-1  SP TISTKSSS R RLFF  433
PsFtsZ     FS KVESRPPP R KLFF  423
AtFtsZ2-1  IP FI KKGSS RYPRV.  397
PpFtsZ     IP SFI RKRGQTR.....  377
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B

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

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 (over-lapping 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.

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 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.
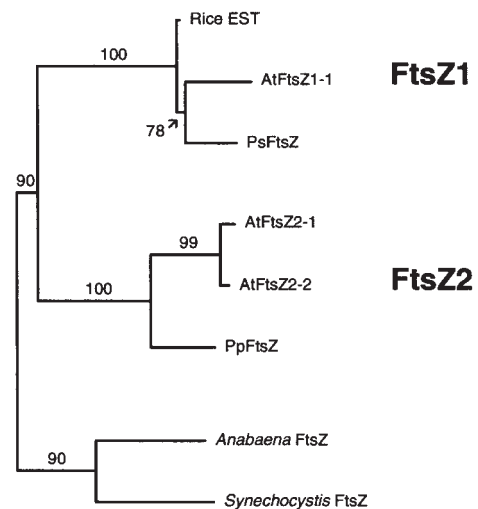

Figure 8. Phylogenetic Relationships among Plant FtsZ Proteins.

Weighted maximum parsimony analysis was performed with PAUP 3.1.1 (Swofford, 1993), 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.
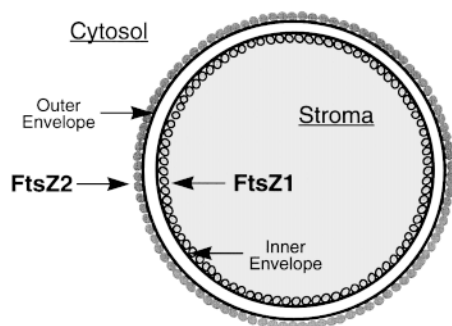
Figure 9. A Tentative Model for FtsZ Localization and Function in Division of Higher Plant Chloroplasts.

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.

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 eubacteria-like 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, 1992b), 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'-CCA-GGCTATGAGAATGTCT-3'; and reverse primer, 5'-CTGTGACAA-AGACCATATCTGAGC-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 μM each of ATP, GTP, and CTP, 1 mM DTT, 20 units of RNasin (Promega), 2 μL of 5 $\times$ 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 $\sim$5 $\times$ 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 $\sim$85 nucleotides on opposite strands. Pairwise comparisons, multiple sequence alignments, and parsimony analysis (Swofford, 1993) were performed as indicated in the legends for Figures 1, 7, and 8.

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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.