Filamentous temperature-sensitive Z (FtsZ) isoforms specifically interact in the chloroplasts and in the cytosol of *Physcomitrella patens*

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Summary

• Plant filamentous temperature-sensitive Z (FtsZ) proteins have been reported to be involved in biological processes related to plastids. However, the precise functions of distinct isoforms are still elusive. Here, the intracellular localization of the FtsZ1-1 isoform in a moss, *Physcomitrella patens*, was examined. Furthermore, the *in vivo* interaction behaviour of four distinct FtsZ isoforms was investigated.

• Localization studies of green fluorescent protein (GFP)-tagged FtsZ1-1 and fluorescence resonance energy transfer (FRET) analyses employing all dual combinations of four FtsZ isoforms were performed in transient protoplast transformation assays.

• FtsZ1-1 is localized to network structures inside the chloroplasts and exerts influence on plastid division. Interactions between FtsZ isoforms occur in distinct ordered structures in the chloroplasts as well as in the cytosol.

• The results expand the view of the involvement of *Physcomitrella* FtsZ proteins in chloroplast and cell division. It is concluded that duplication and diversification of *ftsZ* genes during plant evolution were the main prerequisites for the successful remodelling and integration of the prokaryotic FtsZ-dependent division mechanism into the cellular machineries of distinct complex processes in plants.

Key words: chloroplast division, filamentous temperature-sensitive Z (FtsZ) proteins, fluorescence resonance energy transfer (FRET), moss, *Physcomitrella patens*.

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Introduction

The chloroplasts of eukaryotes evolved from endosymbiotic cyanobacteria, and both divide by binary fission (Gray, 1999; McFadden, 2001). In almost all bacteria, filamentous temperature-sensitive Z (FtsZ), which is a tubulin-like protein that self-assembles into filaments (Mukherjee & Lutkenhaus, 1994), represents the major component of the complex division apparatus, the divisome. FtsZ assembles into a ring-like structure at the future division site and serves as a scaffold to which other proteins are recruited successively to achieve cell division (Aarsman *et al.*, 2005; Goehring & Beckwith, 2005). Plant FtsZ proteins are required for plastid division (Osteryoung *et al.*, 1998; Strepp *et al.*, 1998). During evolution, their genes were translocated to the nucleus where they experienced duplication (Vaughan *et al.*, 2004). Plant FtsZ proteins cluster in two clearly separated subfamilies (FtsZ1 and FtsZ2) (Stokes & Osteryoung, 2003; Rensing *et al.*, 2004). Despite its evolutionary descent from bacterial cytokinesis, which is well defined, the plastid division machinery remains only fragmentarily characterized (Margolin, 2005). Understandably, certain morphological innovations connected with the evolutionary adaptation of the endosymbionts to their new environment had to be complemented by novel strategies of division, while some other long-established strategies progressively became obsolete. In particular, the

necessity to generate force from the outside of a membranous envelope in the process of constriction required the development of distinct, though closely neighbouring, annular systems, the inner FtsZ ring and the outer plastid division ring(s), the latter being regarded as a donation from the endosymbiontic host (Miyagishima *et al.*, 2001; Kuroiwa *et al.*, 2002). Also, the abolishment of a rigid peptidoglycan cell wall in modern chloroplasts rendered the murein-synthesizing or -modifying functions of the prokaryotic divisome dispensable (Iino & Hashimoto, 2003; Sato *et al.*, 2005). Ultimately, except for FtsZ itself, none of the proteins localizing to the divisome of heterotrophic prokaryotes has been retained in plants harbouring chloroplasts (Miyagishima *et al.*, 2005). This raises the question of whether a complex structure representing an FtsZ-based divisome actually exists in plants. The issue of putative FtsZ interaction partners has been addressed in *Arabidopsis thaliana*, albeit by employing heterologous overexpression of *A. thaliana* genes in yeast and tobacco (*Nicotiana tabacum*) cells (Maple *et al.*, 2005). Interaction of AtFtsZ2-1 with AtFtsZ1-1 and AtARC6 (ARC, accumulation and replication of chloroplasts) was, however, observed in both systems. Intriguingly, ARC6 of *Arabidopsis* had previously been identified as an evolutionary descendant of Filamentation 2/Z-ring interacting protein N (Ftn2/ZipN) a component exclusive to the cyanobacterial divisome (Vitha *et al.*, 2003).

The duplication and diversification of *ftsZ* genes in the course of plant evolution and the obvious concomitant elimination of most prokaryote-derived divisomal FtsZ interaction partners suggest that functionality of FtsZ-based systems in plant cells relies largely on the interplay of the distinct plant FtsZ isoforms themselves.

In the moss *Physcomitrella patens*, two FtsZ isoforms have been described in each of the two subfamilies (Rensing *et al.*, 2004). Both members of the FtsZ2 subfamily (PpFtsZ2-1 and 2-2) reside exclusively within plastids, where in a fairly similar manner they polymerize into network-like structures on transient moderate overexpression as C-terminal green fluorescent protein (GFP) fusions. A proposed function of this 'plastoskeleton' is the maintenance of chloroplast integrity (Kiessling *et al.*, 2000; McFadden, 2000; Reski, 2002). Recent evidence suggests that prokaryotic FtsZ might have comparable functions in the cytoplasm of filamentous cyanobacteria (Klint *et al.*, 2007). At the amino acid level, the protein sequences of FtsZ2-1 and FtsZ2-2 are 94.7% identical. Despite this high degree of similarity, functional redundancy of FtsZ2-1 and FtsZ2-2 does not exist; the loss of FtsZ2-1 function cannot be compensated by FtsZ2-2 (Strepp *et al.*, 1998). PpFtsZ1-2 was found to be dually targeted to chloroplasts and the cytoplasm and assembled into rings in both cell compartments. Immunolocalization experiments revealed the formation of FtsZ1-2 ring structures at the division sites of cells and chloroplasts, indicating that this protein might be involved in chloroplast and cell division (Kiessling *et al.*, 2004).

Here it is shown that *in vivo* assembly of a fourth isoform, FtsZ1-1, is distinctly different from that of the other FtsZ proteins in *P. patens*, lending further support to the hypothesis of functional differences among FtsZ isoforms.

Addressing the assumption that the apparent functional diversification of FtsZ isoforms was an evolutionary prerequisite for the establishment of a eukaryotic interaction system derived from (and probably replacing) the prokaryotic divisome, it is demonstrated that the four FtsZ isoforms are indeed capable of specific *in vivo* interaction in *P. patens*. The results suggest that FtsZ functions related to cell division and maintenance of chloroplast integrity require cooperative action of distinct FtsZ isoforms in *P. patens*.

Materials and Methods

Plant material

Physcomitrella patens (Hedw.) B.S.G. was cultivated as described previously (Strepp *et al.*, 1998).

Isolation of *PpftsZ1-1* and sequence analysis

A partial cDNA clone was obtained upon a BLAST search of the *P. patens* expressed sequence tag (EST) database (Rensing *et al.*, 2002) using amino acid sequences of plant FtsZ isoforms. The missing 400 bp of the 5′ end were obtained by 5′ rapid amplification of cDNA ends (RACE)–polymerase chain reaction (RACE-PCR) with the FirstChoice™ RLM-RACE Kit (Ambion, Austin, TX, USA). To assess subcellular localization of FtsZ1-1, the sequence was analysed with TargetP (http://www.cbs.dtu.dk/services/TargetP/) (Nielsen *et al.*, 1997; Emanuelsson *et al.*, 2000). A plastid targeting probability of 0.719 and a putative cleavage site at aa 33 were predicted.

RNA isolation and reverse transcriptase–polymerase chain reaction (RT-PCR)

RNA isolation was performed as described previously (Bierfreund *et al.*, 2003). First-strand synthesis was performed with 2 μg of total RNA from wild-type protonema or gametophores, respectively. SuperscriptII Reverse Transcriptase (Invitrogen GmbH, Karlsruhe, Germany) was used according to the manufacturer's protocol. PCR reactions were performed according to standard procedures (Sambrook & Russell, 2001). See Supplementary Material Table S1 for sequences of oligonucleotide primers.

Cloning of *PpftsZ* fusions and transient transfection of *P. patens* protoplasts

For construction of full-length FtsZ1-1-GFP (aa 1–444), the coding region of *ftsZ1-1* was PCR-amplified with primers F1 and R1. The PCR product was cleaved and cloned into the

GFP-reporter plasmid mAV4 (Kircher *et al.*, 1999). The (aa 1–84) truncated plasmid was built using primers F1 and R2. The (aa 85–444) vector was constructed using primers F2 and R1. For construction of cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) fusion reporter plasmids, mGFP4 from mAV4 was replaced by ECFP or EYFP (Clontech, Heidelberg, Germany), respectively. FtsZ1-1-CFP and FtsZ1-1-YFP were constructed using primers F3 and R3. FtsZ1-2-YFP was constructed with oligos F4 and R4 and FtsZ2-1-CFP and FtsZ2-1-YFP with primers F5 and R5. FtsZ2-2-CFP and FtsZ2-2-YFP were constructed using primers F6 and R6. The PCR products were cloned into the CFP- and YFP-reporter vector, respectively. Construction of FtsZ1-2-GFP, FtsZ2-1-GFP and FtsZ2-2-GFP has been described previously (Kiessling *et al.*, 2000, 2004). The YFP-CFP fusion was constructed by amplifying the coding region of *yfp* with oligos Y1 and Y2. Isolation and transfection of protoplasts from moss protonema were carried out as described previously (Rother *et al.*, 1994; Kiessling *et al.*, 2004). See Supplementary Material Table S2 for sequences of oligonucleotide primers.

Cloning of *EcftsZ*

The bacterial *ftsZ* gene was amplified from *Escherichia coli* XL-1 blue (Stratagene, Heidelberg, Germany) with primers E1 and E2. The PCR product was cloned into *cfp*-mAV4. For the construction of the transit-*EcftsZ-cfp*, the DNA sequence corresponding to amino acids 1–93 of FtsZ2-1 was amplified with oligonucleotides T1 and T2. This region covers the predicted N-terminal chloroplast transit peptide of 31 amino acids. The PCR product was cloned into *EcftsZ-cfp*-mAV4. See Supplementary Material Table S2 for sequences of oligonucleotide primers.

Subcellular localization of FtsZ-GFP fusion proteins and fluorescence resonance energy transfer (FRET) analysis

Subcellular localization of FtsZ-GFP fusion proteins was analysed by confocal laser scanning microscopy (TCS 4D; Leica, Solms, Germany) using 488-nm excitation and two-channel measurement of emission from 510 to 580 nm (green/GFP) as well as > 590 nm (red/chlorophyll). Cells were examined with a × 100 1.4 PL-APO oil immersion objective (Leica). Images represent overlays of optical sections obtained at 1-μm intervals from top to bottom of the corresponding object. FRET analysis was performed using a LSM510 META confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) essentially as described (Karpova *et al.*, 2003), with objective lens C-Apochromat × 63/1.2 W corr. (Carl Zeiss MicroImaging GmbH). Software for image acquisition and subsequent improvement of brightness and contrast was LSM510 software (Carl Zeiss MicroImaging GmbH) and Java image processing program ImageJ 1.34 g (National Institutes of Health, Bethesda, MD, USA). With this system, spectral imaging and subsequent linear unmixing can be performed, which allow a clear discrimination between the two fluorescent proteins (CFP and YFP) used in this study. For linear unmixing, the different spectra of CFP and YFP had to be saved into the system using protoplasts that contained only one of the proteins. To obtain the single spectra, fluorescent proteins were excited with 458-nm (CFP) and 514-nm (YFP) argon laser lines. Emission spectra were taken by the META channel in the range of 473–558 nm. Using these saved spectra for comparison, emission spectra consisting of two different fluorophores could be unmixed with the linear unmixing mode of the software (LSM510 Meta; Zeiss). Subsequently, protoplasts were embedded in agarose, then bleached in the acceptor YFP channel by scanning a region of interest (ROI) approx. 15 times using the 514-nm argon laser line at 100% intensity from an output of 25% (10 mW laser power). Within each protoplast, a ROI was chosen for bleaching (red areas in the respective figures), whereas a similar region within the same protoplast served as a nonbleached control (white areas in the respective figures). Bleach time ranged from 40 to 55 s, depending on the size of the bleached area. Before and after the bleach, images were collected to assess changes in donor and acceptor fluorescence. The images were collected using the λ mode and an excitation wavelength of 458 nm, which primarily excites CFP. As a result of FRET, YFP fluorescence was also detected. To calculate the FRET energy transfer efficiency as a percentage (E_F), the formula $E_F = (I_3 - I_2) \times 100/I_3$ was used, where I_2 and I_3 are the CFP intensities at time points 2 and 3, respectively (I_2 = CFP intensity before bleaching; I_3 = CFP intensity after bleaching) (Karpova *et al.*, 2003). Thus, this formula yields the increase in CFP fluorescence following a YFP bleach normalized by CFP fluorescence after the bleach. As controls, similar calculations were carried out using values obtained for nonbleached areas.

Results and Discussion

All four *ftsZ* genes are expressed simultaneously

It is intriguing that the bacterial divisome only requires one isoform of the FtsZ proteins in order to perform binary fission, while in plants several FtsZ proteins are involved in plastid division. Possible explanations for this difference might be functional redundancy, functional diversification or tissue specificity of the isoforms. In order to determine whether the multiplicity of *ftsZ* genes in *P. patens* is connected to the tissue specificity of the different isoforms, RT-PCR was performed for two different developmental stages of the growth cycle of the moss: the filamentous protonema and the leafy gametophores. The results clearly indicated that each of the four *ftsZ* genes was transcribed in both tissues (Fig. 1). It can therefore be assumed that the presence of all four isoforms

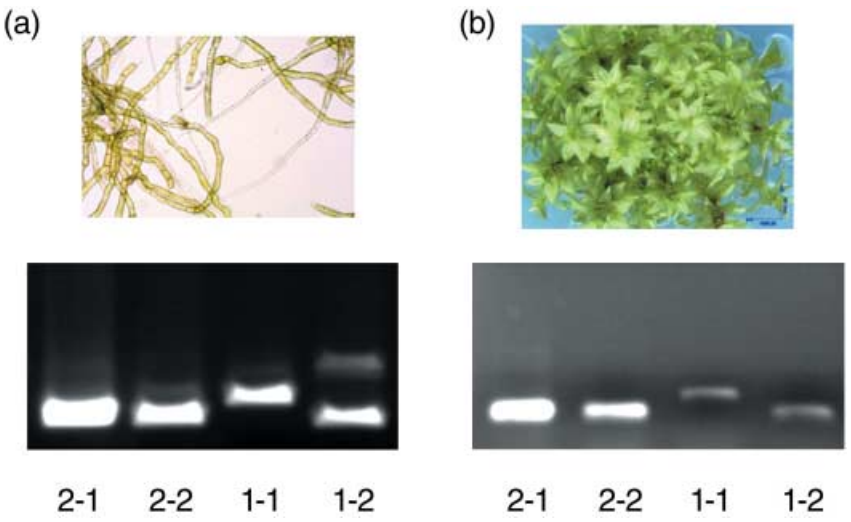

Fig. 1 All four *Physcomitrella patens* filamentous temperature-sensitive Z (*PpftsZ*) homologues are expressed in gametophytic tissues of *P. patens*. Reverse transcriptase–polymerase chain reaction (RT-PCR) using 200 ng of total RNA from (a) protonema and (b) gametophores, respectively, demonstrated that all *PpftsZ* homologues are expressed in these tissues. Gene-specific PCR primers were designed to span intron regions in the genomic DNA. In lane four (a), the upper band represents the corresponding genomic fragment of *ftsZ1-2*. Here, 100 ng of genomic DNA was added to the PCR reaction to demonstrate that oligos spanning an intron were used to clearly distinguish between genomic and cDNA. See Supplementary Material Table S1 for sequences of oligonucleotide primers.

of the FtsZ proteins is required in both tissues. Furthermore, these results show that protonema protoplasts used for transient transformation experiments were prepared from tissue that actually expresses all four *ftsZ* genes, which excludes the risk of ectopic gene expression.

The FtsZ1-1 protein is located in chloroplasts

Apart from the three FtsZ proteins described previously (Kiessling *et al.*, 2000, 2004; Reski, 2002), *P. patens* possesses a fourth FtsZ which belongs to the FtsZ1 subfamily and was designated FtsZ1-1. The two members of the FtsZ1 subfamily show an identity of only 56.5%, which equals their relatedness to the FtsZ2 subfamily members (56.3%). Furthermore, their genes are rather distinct in terms of genomic organization (Rensing *et al.*, 2004). In order to address the so far unknown function of this FtsZ, we set about determining the *in vivo* localization of FtsZ1-1 proteins in *P. patens* and the way in which they are assembled. To this end, protoplasts were transiently transfected with a construct of *ftsZ1-1-gfp* under the control of the cauliflower mosaic virus (CaMV) 35S promoter. In contrast to seed plants, the CaMV 35S is not a strong promoter in *P. patens* (Horstmann *et al.*, 2004). The resulting moderate concentrations of the GFP fusion protein render the detection of fluorescence signals difficult. At the same time, the risk of overexpression artefacts is reduced compared with experiments performed with seed plants. Three days after transfection, the fluorescent label could be observed inside the chloroplasts where it assembled into filamentous networks of strands traversing the organelles (Fig. 2a,b). These structures were distinctively different from those known to be built by the other FtsZ proteins in *P. patens*. While FtsZ1-2 forms annular structures within the chloroplasts and in the cytosol (Fig. 2c,d), the plastoskeleton networks formed by the members of the FtsZ2 family are clearly node structured (Fig. 2e–h). This observation suggests clear functional diversifications within the protein family. The observed plastidic localization of FtsZ1-1 is in accordance with the prediction of an N-terminal chloroplast transit peptide of 33 amino acids. This finding was furthermore confirmed experimentally by ligating the DNA sequence representing the N-terminal 84 amino acids to the 5′-terminus of the *gfp*-cDNA and placing the construct under the control of the 35S promoter. Three days after transient transfection of protoplasts, the fluorescence was restricted to the chloroplasts (Fig. 3a,b). At the same time, the loss of the respective N-terminal amino acid sequence from the FtsZ1-1-GFP fusion resulted in an exclusively cytosolic localization, where the label could be found in network structures (Fig. 3c,d). It is therefore concluded that the ability of FtsZ1-1 to assemble into networks is independent of factors that are exclusive to chloroplasts. In *P. patens*, this feature probably distinguishes FtsZ1 proteins from FtsZ2 proteins which aggregated upon forced mistargeting to the cytosol (Kiessling *et al.*, 2004).

Occasionally, FtsZ1-1-GFP was not only detectable inside the chloroplasts but could also be observed in thin filaments connecting the organelles (arrows in Fig. 3e,f). In *A. thaliana*, the respective protein AtFtsZ1-1 could be found in strands interconnecting the chloroplasts upon overexpression of a *gfp* fusion (Vitha *et al.*, 2001). It is conceivable that these structures represent stromules (stroma-filled tubules). The exact function of stromules, which have been observed with all plastid types examined to date, is still unresolved (Kwok & Hanson, 2004b; Natesan *et al.*, 2005). Proteins up to the size of 550 kDa have been reported to enter the stromules (Kwok & Hanson, 2004a). Nonetheless, no stromules were ever detected when the GFP protein alone was targeted to the chloroplasts (Fig. 3a,b) but their occurrence depended on the presence of FtsZ1-1 in the much larger fusion protein. However, at present it cannot be concluded whether overexpression of

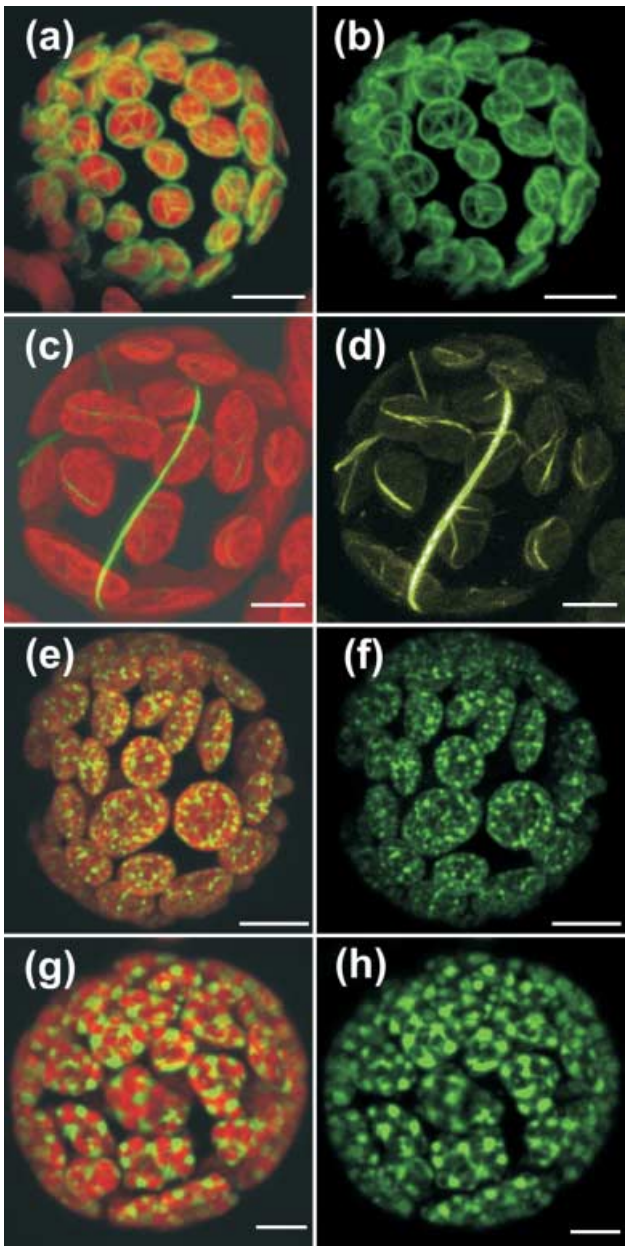

Fig. 2 Subcellular localization of the four filamentous temperature-sensitive Z (FtsZ) isoforms in *Physcomitrella patens*. Wild-type protoplasts from *P. patens* were transiently transfected with full-length *PpftsZ-gfp* (GFP, green fluorescent protein). Three days after transfection, samples were analysed by confocal microscopy, demonstrating that FtsZ1-1 forms a network inside chloroplasts (a, b) distinctively different from those formed by other FtsZ proteins. FtsZ1-2 forms ring-like structures within the chloroplasts and in the cytosol (c, d) whereas members of the FtsZ2 family, FtsZ2-1 (e, f) and FtsZ2-2 (g, h), form the plastoskeleton. Merging of chlorophyll and GFP channels (a, c, e, g) and the GFP channel only (b, d, f, h) are shown. Bars, 10 μm.

ftsZ1-1 indirectly induces the formation of the stromules or whether FtsZ1-1 is a structural component of the stromules themselves.

FtsZ1-1 is involved in chloroplast division

In bacteria, FtsZ acts by combinatorial assembly of a variety of associated proteins to form the cell division apparatus. The functionality of the cell division apparatus is known to be dependent on the molecular ratios of its components: while low levels of *ftsZ* overexpression induce the formation of minicells in *E. coli* (Ward & Lutkenhaus, 1985), cell division is inhibited upon further increases in *ftsZ* expression (Dai & Lutkenhaus, 1992). Like their bacterial ancestors, members of the FtsZ2 family and FtsZ1-2 have been shown to act in a dose-dependent manner on plastid division, strongly indicating their involvement in the process (Kiessling *et al.*, 2000, 2004). Upon overexpression of *ftsZ1-1* in transiently transformed protoplasts, a dependence of chloroplast division on the amount of FtsZ1-1-GFP protein present in the organelles was clear. Whenever fluorescence responses were weak, the number of chloroplasts per cell increased markedly. While a chloronema cell usually contains on average 48 chloroplasts with diameters of around 5 μm, transfected protoplasts contained up to 80 chloroplasts with diameters of only 2.0–2.5 μm (Fig. 3g,h). This observation suggests an acceleration of chloroplast division at low concentrations of FtsZ1-1. Intriguingly, the fusion protein never assembled into ordered structures within these mini-chloroplasts but showed a rather irregular distribution. In contrast to Fig. 2(a,b), Fig. 3(g,h) show chloroplasts that have just completed division. It is clear that, under these circumstances, the network structure built by FtsZ1-1 is far less ordered compared with the undisturbed chloroplasts shown in Fig. 2(a,b). This finding suggests that the FtsZ1-1 plastoskeleton undergoes reorganization during plastokinesis, an event that might either be a trigger or a consequence of chloroplast division. Promotion of chloroplast division is probably attributable to the participation of FtsZ1-1 in the formation of the plastidic divisional apparatus. Very bright fluorescence signals were usually accompanied by an impairment of chloroplast division. This effect and its attenuation upon the decrease of overexpression were observed in regenerating protoplast. The emerging thread-shaped protonema grows by division of the tip cell. Because of the transience of transfection, each cell division further dilutes the number of incorporated plasmids and hence the concentration of the fusion protein in the cell. This approach provides us with a dilution series of the fusion protein observable in a single plant cell lineage. This is obvious from a reduction of fluorescence intensity along the filament. In the outgrowing protonema, chloroplast division is resumed when the amount of FtsZ1-1-GFP decreases below an inhibitory threshold (Fig. 3i,j). Increased numbers of (mini-)chloroplasts were never observed in these experiments,

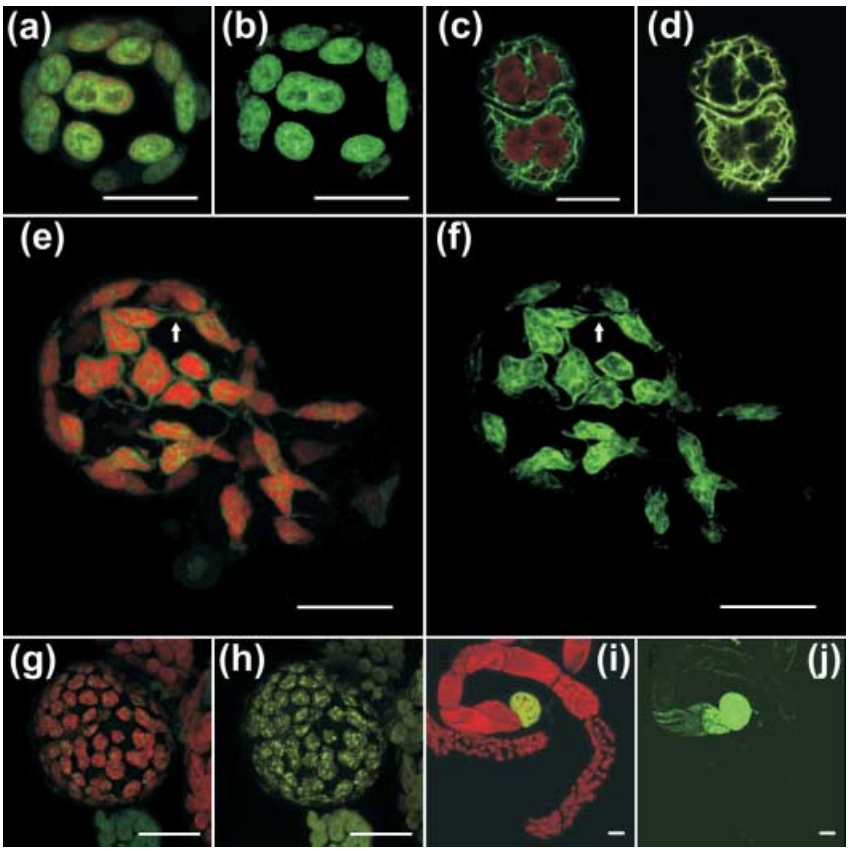

Fig. 3 Filamentous temperature-sensitive Z1-1 (FtsZ1-1) is targeted to the chloroplasts of *Physcomitrella patens* and affects plastid division. The N-terminal 84 amino acids of FtsZ1-1 are responsible for targeting of (FtsZ1-1(aa 1–84)-GFP) (GFP, green fluorescent protein) to the chloroplasts (a, b). Deletion of the N-terminal 84 amino acids results in (FtsZ1-1(aa 85–444)-GFP) being retained in the cytosol (c, d). Analysis of protoplasts transiently transfected with the full-length construct demonstrated a localization within chloroplasts. In addition, PpFtsZ1-1-GFP seemed to connect chloroplasts via filaments (e, f; indicated by arrows). Low concentrations of the FtsZ1-1-GFP fusion proteins in transiently transfected protoplasts resulted in 'mini-chloroplasts'. Because of the low amount of GFP in these protoplasts, excitation laser energy had to be increased by a factor of 20 in order to record fluorescence (g, h). High concentrations of FtsZ1-1-GFP (indicated by strong GFP fluorescence) resulted in a block of plastid division, leading to a single plastid or a few huge plastids per cell. Chloroplast division was resumed upon dilution of FtsZ1-1-GFP during protoplast regeneration (i, j). Merging of the chlorophyll and GFP channels (a, c, e, g, h) and the GFP channel only (b, d, f, i, j) are shown. Bars, 10 μm.

which suggests that promotion of chloroplast division occurs within small ranges of FtsZ1-1 concentrations and increases in chloroplast numbers and are levelled out as the cells divide. Experimental data presented here and published previously (Kiessling *et al.*, 2000, 2004) show that, despite their obvious functional differentiation, all four FtsZ proteins analysed are involved in the process of chloroplast division.

Identification of *P. patens* FtsZ protein interactions in living plant cells

A diversification of plant FtsZ proteins could be expected to reflect their functional specialization within a divisome-like complex. To test whether the initial prerequisite for this assumption is actually fulfilled, *in vivo* protein–protein interactions among the four *P. patens* FtsZ proteins were analysed using the FRET technique. FRET occurs when a donor fluorophore is excited by incident light and energy is transferred by radiationless processes to an acceptor fluorophore, the excitation spectrum of which overlaps with the emission spectrum of the donor fluorophore. The two fluorophores must be closely juxtaposed for the energy transfer to take place and allow the acceptor to fluoresce (usually < 100 angstroms) (Karpova *et al.*, 2003). To analyse all possible dual interactions between members of both FtsZ families, protoplasts were cotransfected with the respective *cfp* (donor) and *yfp* (acceptor) fusion constructs. Three days after transfection, localization of both fluorophores could be determined by confocal laser scanning microscopy (Fig. 4). Here, it is demonstrated that gene expression under the control of the moderate CaMV 35S promoter is sufficient to perform FRET experiments in *P. patens*. The results show that different members of the *P. patens* FtsZ family are actually capable of interacting specifically *in vivo*.

When members of the same FtsZ families were assayed, it was found that FstZ1-1-YFP received energy transferred from FtsZ1-2-CFP within the cytosolic ring and in the chloroplasts, where networks resembling those known to be formed by FtsZ1-1 were observed. No annular structures could be found in the chloroplast. Here, the network structure expected to be formed by FtsZ1-1 evidently dominates and prevents ring formation by FtsZ1-2. FRET between FtsZ2-1-YFP and FtsZ2-2-CFP occurred within the plastoskeleton, the assembly of which is the characteristic feature of the *P. patens* FtsZ2 subfamily proteins. FRET experiments between the two different FtsZ families were also performed. The results indicated that the interaction behaviours of FtsZ1-1 with both members of the FtsZ2 family were fairly similar. The occurrence of the isoforms was restricted to the chloroplasts, where FRET was observed in structures that in both cases closely resembled the plastoskeleton networks of the

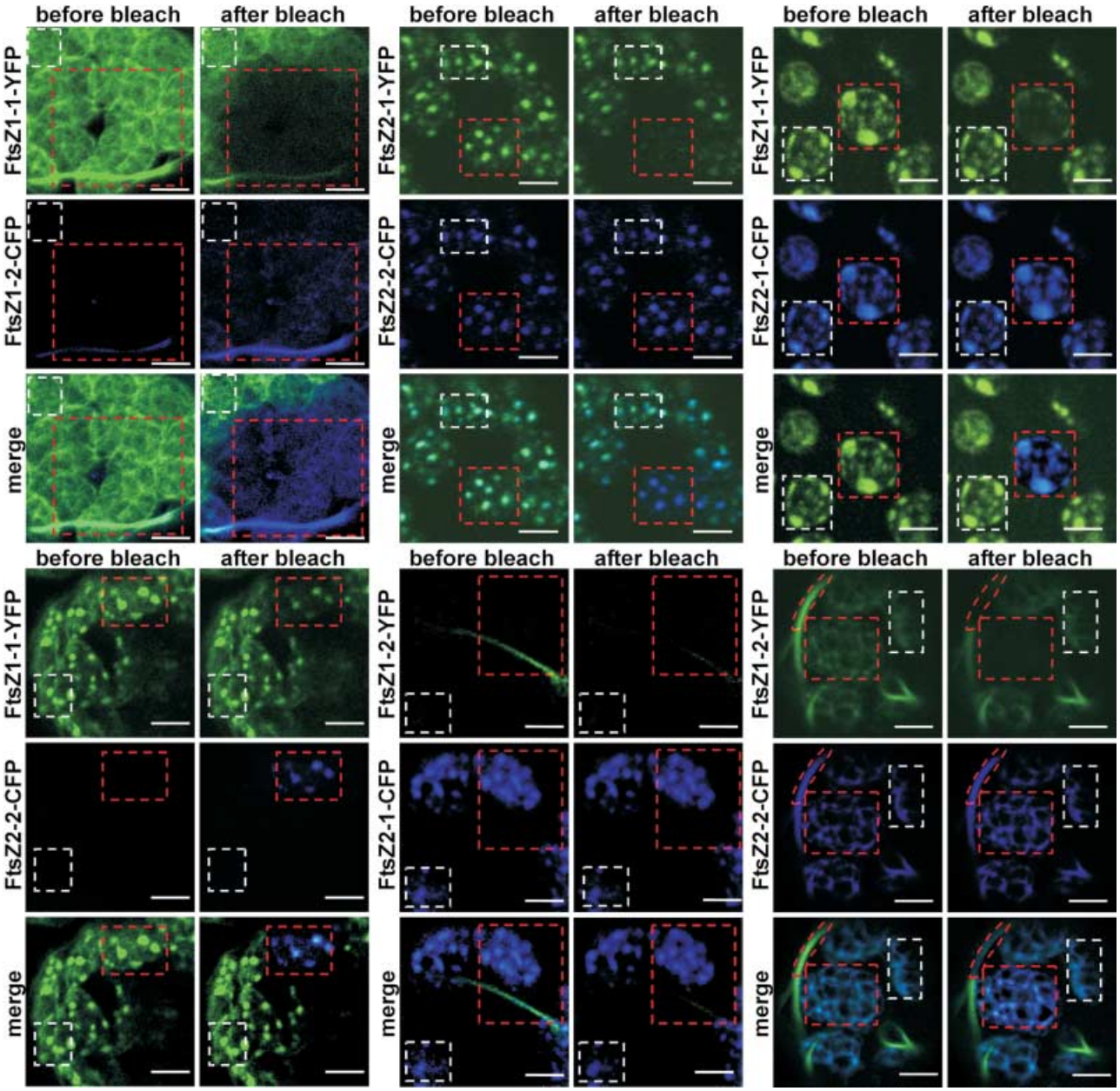

Fig. 4 *In vivo* interaction of filamentous temperature-sensitive Z (FtsZ) isoforms is detected via fluorescence resonance energy transfer (FRET). *Physcomitrella patens* protoplasts were transiently cotransfected with *ftsZ-cfp* and *-yfp* fusion constructs (CFP, cyan fluorescent protein; YFP, yellow fluorescent protein). At close proximity (< 100 Å) of the two fluorophores, a portion of the emission energy of CFP is quenched through radiationless transfer to the YFP fluorophore. The occurrence of fluorescence resonance energy transfer from the donor CFP to the acceptor YFP is detected via acceptor photobleaching at a wavelength of 514 nm. In all parts of the figure, the red rectangle marks the region of YFP photobleaching and the white rectangle marks the unbleached control area. Inactivation of YFP, confirmed by a decrease in light emission at 530 nm, is accompanied by an increase in CFP fluorescence at 490 nm that is indicative of FRET. Single channel CFP and YFP fluorescence and merging of the two wavelengths before and after acceptor photobleaching are shown for each of the six dual combinations. See Supplementary Material for FRET efficiencies (Fig. S1 and Table S3). Bars, 4 μm.

respective FtsZ2 isoform. FtsZ1-2, in contrast, displayed a rather distinctive behaviour with respect to the different members of the FtsZ2 family. Striking similarities in both cases were the absence of annular structures in the plastids and the formation of a cytosolic ring. Again, the filaments of FtsZ2 plastoskeletons pervaded the chloroplasts. In the case of the FtsZ1-2-YFP/FtsZ2-2-CFP combination, FRET indicated the interaction of the two isoforms in the cytosolic ring as well as in the plastoskeleton. Intriguingly, there was no evidence for any *in vivo* interaction of FtsZ1-2 with FtsZ2-1. This

incapability of FtsZ1-2 to interact with the FtsZ2-1 isoform further supports the view that in *P. patens* the members of the FtsZ2 family are functionally nonredundant in spite of their clear similarity. As both isoforms interact with FtsZ1-1 in a comparable manner, it must be concluded that the specific interactions with FtsZ1-1 or FtsZ1-2 involve distinct regions of the FtsZ2 family members. FRET efficiencies calculated after acceptor photobleaching (Karpova *et al.*, 2003) are provided in Supplementary Material Fig. S1 and Table S3 for all dual combinations

In the FRET assay, FtsZ1-2 was capable of interacting with FtsZ1-1 and FtsZ2-2. Intriguingly, in both cases energy transfer also occurred within the cytosolic annular structure, even though the predictable targeting of FtsZ1-1 as well as FtsZ2-2 to the chloroplast could be confirmed experimentally. This finding suggests that a cytosolic localization of FtsZ1-1 and FtsZ2-2 depends on the presence of FtsZ1-2, the attractive force of which probably competes with the plastidic import machinery for the recruitment of the respective FtsZ isoforms. When concentrations of FtsZ1-2 and other single FtsZ isoforms were increased simultaneously, ring formation in the chloroplasts was never observed. It is tempting to speculate whether an elaborate plastoskeleton could be a feature of static (nondividing) chloroplasts and whether the dominance of this structure could in turn prevent the formation of a then superfluous division ring. It has to be mentioned that cotransfection of protoplasts with gene constructs encoding fusions of different FtsZ isoforms did not result in the formation of novel structures that had not been observed in previous experiments with only one isoform. At the same time, the experiments revealed that, with the exception of FtsZ2-1, the occurrence of the isoforms was not restricted to the structures observed in single transfection experiments (see Fig. 2). The ability of single proteins to interact with diverse partners sets the prerequisite for the establishment of protein interaction networks. The issue of which protein partner interacts with a protein determines the biological function(s) of the protein within a protein network (Tucker *et al.*, 2001; Cho *et al.*, 2004). We have shown that FtsZ proteins are capable of self-interaction, as expected (Kiessling *et al.*, 2000, 2004). In addition, evidence has now been provided that FtsZ proteins interact with other FtsZ proteins in *P. patens*. In the experimental system, the type of interaction in which an FtsZ protein engages is obviously a function of the availability of its interaction partners; that is, an excess of one isoform only will favour self-interaction (respectively the formation of structures indicative of FtsZ homomerization). For example, we must conclude from the results of the present study that, in the case of a transient transfection of protoplasts with *ftsZ-1-2-gfp* (Fig. 2c), it is most likely that the isoforms FtsZ1-1 and FtsZ2-2 are also recruited to the cytosolic ring. This interaction goes unnoticed, however, as only FtsZ1-2 carries the GFP marker. At the same time, incorporation of FtsZ1-2-GFP into the plastoskeleton structures built by FtsZ1-1 or FtsZ2-2,

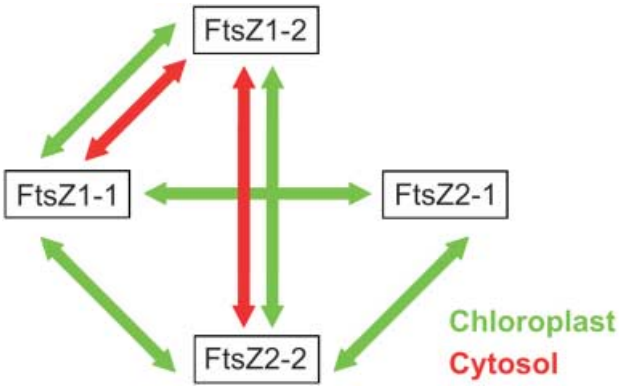

Fig. 5 Model of filamentous temperature-sensitive Z (FtsZ) interactions based on fluorescence resonance energy transfer (FRET) analysis. Arrows represent the interactions that occur between FtsZ proteins within the chloroplasts (green) and in the cytosol (red).

which occurs in the FRET experiments (Fig. 4), is not observed upon single transfection with *ftsZ1-2-gfp* (Fig. 2c). It is probable that these structures are not built at all, as isoforms capable of interaction with FtsZ1-2 are recruited to the dominant annular FtsZ1-2-GFP structures. Alternatively, the amount of FtsZ1-2-GFP molecules incorporated into the plastoskeleton structures might be too low to allow its detection in the presence of the bright GFP fluorescence signals obtained from the FtsZ1-2 annular structures. Independent of interaction events, the ring was the only structure that was formed in the cytosol, whereas the plastoskeleton network was built in the chloroplast whenever a member of the FtsZ2 family was present. Figure 5 gives an overview of FtsZ interactions in *P. patens*. Furthermore, FRET efficiencies were calculated after acceptor photobleaching (Karpova *et al.*, 2003) (Supplementary Material Fig. S1 and Table S3).

Identification of interactions between PpFtsZ and bacterial FtsZ proteins

Even in prokaryotes, FtsZ function is dependent on its ability to self-interact, which allows the formation of the Z-ring (Wang *et al.*, 1997; Yan *et al.*, 2001). While this feature might have predestined novel eukaryotic FtsZ isoforms to co-engage in functional complexes, we are aware of the fact that a general tendency of FtsZ proteins to stick together could yield false positive results in a FRET assay. To address this potential risk, FRET experiments were repeated with the introduction of a bacterial FtsZ protein. For this purpose, the full-length *ftsZ* gene from *E. coli* strain XL1 Blue was fused to the 3′ end of the *PpftsZ2-1* transit peptide sequence and cloned into a CFP-reporter plasmid driven by the CaMV 35S promoter (*EcftsZ-cfp*). In transiently transfected protoplasts, EcFtsZ-CFP was found to localize in a diffuse manner inside plastids and failed to assemble into filaments or cytoskeletal structures on its own (Fig. 6a,b). At the same time, overexpression of

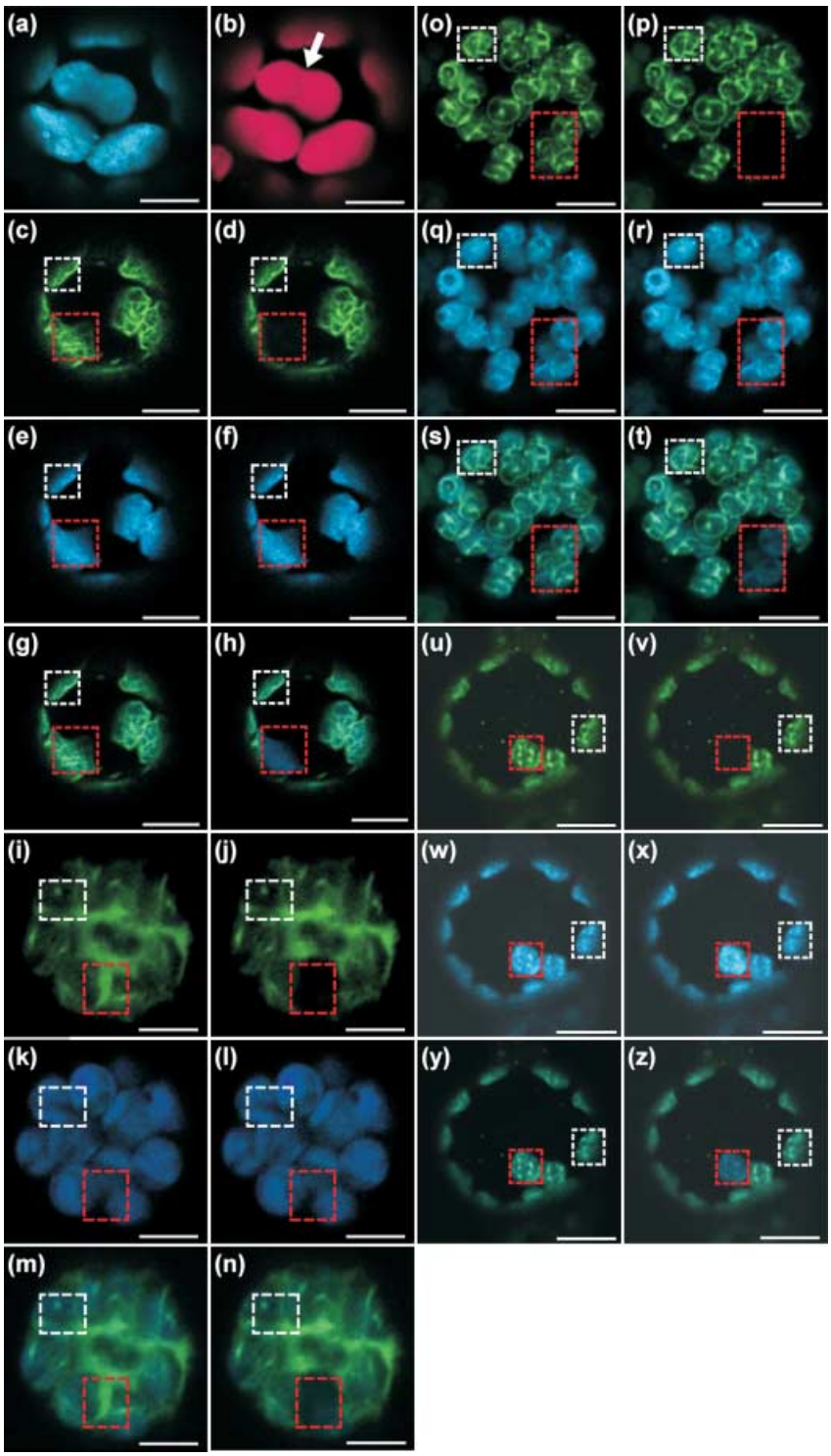

Fig. 6 Fluorescence resonance energy transfer (FRET) analysis of the interactions between *Physcomitrella patens* filamentous temperature-sensitive Z (PpFtsZ) and FtsZ from *Escherichia coli*. EcFtsZ-CFP (CFP, cyan fluorescent protein) localized inside chloroplasts and its overexpression did not inhibit chloroplast division (a, CFP channel; b, chlorophyll channel). The arrow in (b) indicates a site of chloroplast constriction. FRET assays between EcFtsZ and PpFtsZ1-1 (c–h), PpFtsZ1-2 (i–l) or PpFtsZ2-1 (o–t) did not show energy transfer. The only positive FRET assay was found between EcFtsZ and PpFtsZ2-2 (u–z). In (c–z), the red rectangle marks the regions of yellow fluorescent protein (YFP) photobleaching and the white rectangles mark the unbleached control area (see legend of Fig. 4 for details). The images show the single YFP fluorescence channel (c, d, i, j, o, p, u, v), the single CFP fluorescence channel (e, f, k, l, q, r, w, x) and merging of the two wavelengths (g, h, m, n, s, t, y, z). Bars, 10 μm.

EcftsZ-cfp did not seem to inhibit chloroplast division (arrow, Fig. 6b). These findings indicate that FtsZ from *E. coli* is nonfunctional in *P. patens*.

When EcFtsZ-CFP was assayed as an energy donor in combination with PpFtsZ-YFP fusion proteins, no FRET could be detected between the bacterial protein and the isoforms FtsZ1-1, FtsZ1-2 and FtsZ2-1 (Fig. 6c–t). Although these findings speak against a direct interaction of the respective proteins, the mere presence of the heterologous protein seemed to exert an influence upon the assembly of the different

FtsZ isoforms in *P. patens*. The usually very distinct formation of cytosolic and plastidic annular structures by FtsZ1-2 was clearly perturbed in protoplasts cotransfected with *EcftsZ-cfp* (Fig. 6i–n). The presence of the bacterial protein in the chloroplasts also affected the FtsZ2-1 plastoskeleton insofar as the number of network nodes was reduced considerably (Fig. 6o–t). This might be the result of competition for interaction partners that are sequestered from the plastoskeleton by the bacterial protein. Alternatively, the very process of avoiding direct interaction might result in a distortion of the plastoskeleton. The only evidence for direct interaction with the heterologous protein was found for FtsZ2-2. In this case, however, FtsZ from *E. coli* seemed to be incorporated into the plastoskeleton without displaying any effect on its pristine structure (Fig. 6u–z; Supplementary Material Fig. S1).

There is evidence for the conservation of basic ancestral functions in plant FtsZ proteins. Previous studies demonstrated that *ftsZ1* and *ftsZ2* cDNAs from the green alga *Nannochloris bacillaris* (Koide *et al.*, 2004) or *ftsZ1* from pea (*Pisum sativum*; Gaikwad *et al.*, 2000) could complement the thermosensitive division defect of *E. coli ftsZ* mutants. Intriguingly, targeting of the mini-cell C (MinC) protein from *E. coli* to chloroplasts in *N. tabaccum* resulted in abnormally large plastids in the transgenic plants (Tavva *et al.*, 2006). In *E. coli*, MinC and MinD are inhibitors of FtsZ polymerization (De Boer *et al.*, 1992; Pichoff & Lutkenhaus, 2001). Homologues of the genes *minD*, *minE* and suppressor of *lon* A (*sulA*), the products of which do not belong to the core divisome but regulate the formation and positioning of the FtsZ ring in prokaryotes, are found in seed plants. However, homologues of prokaryotic *minC* genes have never been detected (Osteryoung & Nunnari, 2003; Miyagishima *et al.*, 2005). Presumably, the chloroplast division machinery retained the capability of interaction even with erstwhile partners that were lost during evolution. Results obtained here, however, speak against arbitrary interactions of *P. patens* FtsZ proteins with their evolutionary ancestor, which, conversely, strongly suggests that the FtsZ/FtsZ interactions of the four *P. patens* isoforms are indeed specific.

Concluding remarks

The introduction of photosynthetic endosymbionts not only provided the host with the possibility of performing photosynthesis. In the course of evolution, mechanisms of symbiontic and host origin merged as the genomes and their functions were extensively reshuffled. Endosymbiontic genes were translocated in large numbers to the nucleus and subsequently protein import into the chloroplast evolved. However, > 50% of the proteins introduced by the endosymbiont are predicted not to be targeted to the chloroplast. Furthermore, a considerable number of proteins targeted to the chloroplast clearly do not originate from the genome of the endosymbiont (Martin *et al.*, 2002; Timmis *et al.*, 2004; Lopez-Juez & Pyke, 2005). In the light of these evolutionary considerations, it does not seem implausible that FtsZ was recruited to function in the cytoplasm of the eukaryotic cell in *P. patens*.

The chloroplast division factor ARC3 isolated from *A. thaliana* has even been reported to be a chimera of prokaryotic FtsZ and parts of a eukaryotic phosphatidylinositol-4-phosphate 5-kinase (Shimada *et al.*, 2004). It was shown very recently that ARC3 interacts specifically with *A. thaliana* FtsZ1, MinE1 and MinD1 and probably fulfils a MinC-like function. (Maple *et al.*, 2007). From their findings, Maple *et al.* (2007) concluded that divergence of plant FtsZ proteins mainly occurred to allow interaction of different accessory proteins with the Z-ring. In this respect, however, chloroplast division in *P. patens* obviously differs from that in *A. thaliana* as both *minC* and *arc3* homologues are missing from the *P. patens* genome.

While most of the prokaryotic divisomal FtsZ interaction partners were lost during plant evolution, evidence is provided suggesting that this loss has been compensated by the evolution of diverse FtsZ isoforms capable of specific interaction. In *P. patens*, FtsZ interactions occur in distinct structures in two compartments, the chloroplast and the cytosol. While evidence for the involvement of single FtsZ isoforms in cell division and maintenance of chloroplast shape has been presented before, the findings of the present study suggest that these cellular functions require co-ordinated interactions of FtsZ isoforms in *P. patens*.

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Supplementary Material

The following supplementary material is available for this article online:

Fig. S1 Efficiencies of fluorescence resonance energy transfer (FRET) between the filamentous temperature-sensitive Z (FtsZ) fusion proteins at distinct subcellular locations.

Table S1 Oligonucleotide primers used for filamentous temperature-sensitive Z (*ftsZ*) reverse transcriptase–polymerase chain reactions (RT-PCRs)

Table S2 Oligonucleotide primers used for cloning of filamentous temperature-sensitive Z (*ftsZ*) fusion genes

Table S3 *In vivo* interaction of filamentous temperature-sensitive Z (FtsZ) isoforms in *Physcomitrella patens* revealed via fluorescence resonance energy transfer (FRET)

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