

Chapter 6

Plastid Division

Jodi Maple-Grødem and Cécile Raynaud

Abstract Plastids evolved from free-living cyanobacteria that were engulfed by a host cell. One critical element for the stable establishment of endosymbiosis was the acquisition of the ability of the endosymbiont to multiply inside the host cell. In all plants, plastids divide by binary fission via the assembly and subsequent constriction of a multiproteic scaffold called the plastid division ring. This highly complex structure associates proteins that have been retained from the bacterial ancestor, such as FtsZ that forms a ring inside the plastid, with proteins brought by the host cell, that form a ring outside the plastid. Over the past 20 years, dramatic progress has been made in our understanding of the mechanisms underlying plastid division, thanks to the combination of various approaches including direct and reverse genetics, electron microscopy and biochemistry. In this review we describe in detail the different steps of plastid division, from the choice of the division site to the sequential assembly of all constituents of the plastid division ring. We also discuss the current knowledge of the regulation of this process, which is still in its infancy but raises fascinating questions for future research.

Keywords Plastid division ring • FtsZ • Min

Abbreviations

CGA1	Cytokinin-responsive GATA1
CJD1	Chloroplast J-like domain 1
CLMP1	Clumped chloroplast 1
CLS-8	Crinkled leaves 8
CpDNA	Chloroplast DNA
Cpn60	Chaperonin 60
CRL	Crumpled leaf
DGDG	Digalactosyldiacylglycerol
DRP5B	Dynamamin-related protein 5B

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FP	Fluorescent protein
FRP	FtsZ regulatory proteins
FtsZ	Filamenting temperature-sensitive mutant Z
GED	GTPase effector domain
HP1	High pigment 1
IMS	Inter membrane space
MCD1	Multiple chloroplast division site 1
MD	Middle domain
MGDG	Monogalactosyldiacylglycerol
Min	Mini-cell
MORN	Membrane Occupation and Recognition Nexus
MSC	Mechano-sensitive channel
nDNA	Nuclear DNA
PARC6	Paralog of ARC6
PD	Plastid dividing
PDR1	Plastid dividing ring 1
PH	Pleckstrin homology
RNR	Ribonucleotide reductase
TEM	Transmission electron microscopy

6.1 Introduction

Although the hypothesis that plastid and mitochondria derived from a free living prokaryote was first formulated at the end of the nineteenth century, the question of the biogenesis of plastids in plant cells was still debated in the 1960s: some authors postulated that plastids could arise from small submicroscopic vesicles called proplastid precursors, even though a wealth of microscopic observations provided evidence for plastid division by binary fission [118]. It is now clear that plant cells cannot generate plastids *de novo*, and that binary fission is the main process allowing the multiplication of these organelles, although budding has been reported in ripening tomato fruits [30]. When observed under the microscope, the chloroplast division process can be described as follows: initially spherical chloroplasts become more ovoid, and constriction becomes visible at chloroplast midpoint. As constriction proceeds chloroplasts become dumbbell shaped and finally deeply constricted with a narrow neck between the two future organelles. At the final stage of division the two chloroplasts can twist around this neck and finally become completely separated. Observation of dividing chloroplasts was reported in the nineteenth century, but only in the last 20 years have we come to understand how plastid division actually takes place.

The study of plastid division has highly benefited from three types of approach: transmission electron microscopy (TEM), forward genetics and reverse genetics. TEM images of dividing *Cyanidium caldarium* cells revealed the existence of an electron dense structure at the division site of chloroplasts [90]. This observation was subsequently generalized to all plants, demonstrating that a complex structure

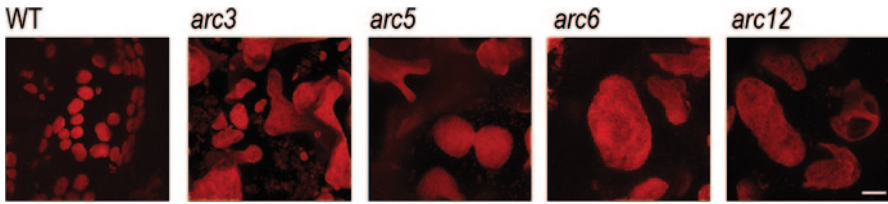


Fig. 6.1 Leaf mesophyll cells from fully expanded leaves of wild-type *Arabidopsis* (WT) and *arc* mutants *arc3*, *arc5*, *arc6* and *arc12*. Chlorophyll autofluorescence was detected, and extended focus images were generated. Scale bar = 10 μ m

is responsible for plastid division. Genetic studies were then required to identify the constituents of this structure. In the early 1990s, K. Pyke and R. Leech isolated several mutants specifically affected for plastid division which were called *arc* for Accumulation and Replication of Chloroplasts [122, 123]. A total of 12 *arc* mutants have been described, most of which show a reduction of chloroplast number compensated by an increase in chloroplast size (Fig. 6.1). Because positional cloning is a long-term approach, especially when dealing with mutants whose phenotype must be studied under the microscope, more than 10 years passed before the first *ARC* gene (*ARC5*) was identified [40]. In the meantime, complete sequencing of the *Arabidopsis* genome allowed K. Osteryoung and E. Vierling to discover homologues of the *FtsZ* gene [113]; the bacterial *FtsZ* protein is distantly related to tubulin and can polymerize in a GTP-dependent manner. Its ability to form contractile rings allows cell division by binary fission. Since then, several studies have demonstrated that plastid division does require homologues of *FtsZ* and involves several other proteins of prokaryotic origin, but also proteins that were encoded by the genome of the host cell. The resulting machinery is extremely complex, and how this multi-proteic scaffold functions at the molecular level is only beginning to be elucidated.

In this chapter, we will focus mainly on chloroplast division in higher plants, because it is the best described model in many respects, but it is generally assumed that the division mechanisms are common to all plastid types. Over the past 15 years, our knowledge of the mechanisms allowing plastid division has made considerable progress. By contrast, very little is known at present regarding the regulation of plastid division. However, a few recent studies provide insights into this regulation and can now allow new interpretation of older reports regarding the factors that influence plastid division. In the last paragraph of this chapter we will therefore try to summarize these reports, illustrating how the recent discoveries about plastid division can allow a new understanding of early microscopic analyses.

6.2 Placement of the Plastid Division Site

Our knowledge of bacterial cell division has greatly contributed to the understanding of all aspects of chloroplast division, including division ring placement. To date, the best characterized model is *Escherichia coli* in which two distinct

mechanisms allow the division machinery to assemble at midcell: the nucleoid occlusion system prevents FtsZ ring formation in the close vicinity of chromosomes (i.e. all sites except midcell and cell poles) and the Min system avoids FtsZ polymerization at cell poles [24]. In the absence of Min proteins, division frequently takes place at cell poles, giving rise to minicells. MinC is the inhibitor of FtsZ polymerization, while MinD and MinE regulate its location in the cell and thereby its activity. To affect FtsZ polymerization, MinC needs to be attached to the membrane, and this association requires MinD, an ATPase which forms dimers and engages its C-terminus into the membrane upon ATP binding. After ATP hydrolysis and release of ADP, MinD dissociates from the membrane. MinCD complexes oscillate from pole to pole, owing to the topological factor MinE. MinE forms a ring that appears to cap the Min polar zone and prevent it from extending past midcell: MinE stimulates the ATPase activity of MinD, thus promoting its dissociation from the membrane. The MinE ring then moves towards the pole, until most proteins are released from the membrane and assemble again at the opposite pole [11]. In other rod-shaped bacteria such as *Bacillus subtilis*, the Min system is conserved, but MinCD proteins and their topology factors DivIVA and MinJ do not appear to oscillate from pole to pole. They rather seem to accumulate at the poles at early steps of division and to be dynamically localized to the mature division apparatus, thereby labeling the new cell poles after division is complete [11]. The Min system is also conserved in cyanobacteria, which are the closest relatives of chloroplasts, but their dynamic behavior during the division process has not been elucidated [85].

6.2.1 *Many Proteins are Involved in the Choice of the Division Site*

Homologues of MinD and MinE have been identified in Arabidopsis and appear to have antagonistic functions towards FtsZ polymerization. Mesophyll cells of AtMinE over-expressers and *atminD/arc11* mutants contain chloroplasts of heterogeneous size, showing aberrant Z-ring positioning and multiple Z-rings in one chloroplast [20, 34, 55, 77]. By contrast, AtMinD overexpressers and *atmine/arc12* show inhibition of plastid division [45]. These opposite effects of AtMinD and AtMinE on chloroplast division are due to their impact on FtsZ polymerization: FtsZ proteins form multiple rings in *atminD* mutants and AtMinE overexpressers whereas they form short filaments in *atminE* mutants and AtMinD overexpressers, indicating that AtMinD inhibits FtsZ polymerization whereas AtMinE promotes it [37, 45, 147]. Proper balance between AtMinD and AtMinE activities is therefore required for Z-ring formation at midplastid [35]. Molecular data further supports the notion that the Min system operates in chloroplasts in a similar way as in bacterial cells. As observed for their bacterial homologues, AtMinD and AtMinE can form homo or heterodimers [79]. Furthermore, the ATPase activity of AtMinD seems to be important for its proper localization in chloroplasts, and AtMinE, like its bacterial counterpart, stimulates this activity [4]. Finally, neither AtMinD nor AtMinE seem

to interact with FtsZ proteins, suggesting that as observed in bacteria, a third component is required to regulate Z-ring positioning.

Although MinC is conserved in cyanobacteria [85] and the chloroplast division machinery is sensitive to overexpression of the *E. coli* MinC protein [143], plant genomes lack *MinC* homologues. ARC3 has been suggested to replace MinC functionally. The phenotype of *arc3* mutants and over-expressers is similar to *arc11* and AtMinD over-expressers respectively [80, 134], providing evidence for an inhibitory function of ARC3 on Z-ring assembly away from the mid-plastid point. ARC3 interacts with AtMinE and AtMinD via its central domain and with AtFtsZ1 via its N-terminal FtsZ-like domain, although this domain lacks the tubulin signature and amino acids crucial for GTP hydrolysis [80]. Taken together with the division site misplacement in the *arc3* mutant, the presence of ARC3 in an FtsZ/Min complex suggests that ARC3 plays a central role in division site placement.

Two more Arabidopsis proteins participate in division site placement, bringing further complexity to the system compared with bacterial cell division: MCD1 [100] and PARC6/CDP1 [46, 155]. PARC6 is a paralogue of the ARC6 protein which destabilizes FtsZ ring (see below). PARC6 is conserved from algae to land plants whereas MCD1 is specific to land plants. Both proteins appear to be negative regulators of Z-ring formation, but how they achieve this function is not completely clear. In *mcd1* mutants, FtsZ proteins form long filaments, and overexpression of AtMinD has little effect on FtsZ polymerization. In this background the AtMinD protein accumulates at wild-type level, but shows diffuse localization in chloroplasts; taken together, these results suggest that MCD1 may enhance the inhibitory effect of AtMinD on FtsZ polymerization by modulating its intra-plastidial localization [100]. PARC6/CDP1 negatively regulates FtsZ polymerization, possibly by interacting with ARC3 [46, 155]. Interestingly, this interaction appears to be mediated by the C-terminal MORN domain of ARC3 [46]. Mesophyll cells of *parc6* mutants contain chloroplasts of heterogeneous sizes suggesting that PARC6 participates in the choice of the division site, but also constricted chloroplasts blocked at a later stage of division, pointing at additional roles of PARC6 ([46], see below).

Finally, mechano-sensitive channel (MSC) proteins appear to be involved in division site positioning [49]. A systematic functional analysis of MSC of the S family (Msc-S Like) led to the observation that MSL2 and MSL3 play redundant roles in chloroplast division: *msl2* and *msl3* mutants are identical to the wild-type whereas *msl2msl3* double mutants are variegated and show abnormal chloroplast size and shape [49]. Further genetic analyses revealed that MSL2 and 3 proteins function as inhibitors of Z-ring formation in a Min/ARC3 dependent manner, although their molecular mechanism of action is not clear [149]. Strikingly, in *B. subtilis*, the MinE functional equivalent DivIVA can recognize curved membrane regions and has therefore been suggested to have the intrinsic capacity to bind and accumulate at negatively curved membranes [11]. MSL proteins, by regulating ion fluxes to or from the chloroplast may affect membrane curvature and in this way influence the polarity of the organelle.

6.2.2 *How Do Proteins Involved in the Choice of the Division Site Work Together?*

Although many players of the Z-ring placement have been identified in the past 10 years, our understanding of the underlying mechanism remains very incomplete and the molecular functions of all the identified players are not fully elucidated yet. Based on interaction data (summarized in Fig. 6.2), and on the effect of these proteins on FtsZ polymerization, AtMinD, AtMinE and ARC3 may be the functional equivalent of the bacterial MinCDE complex. However, it is not clear whether AtMinD engages the chloroplast envelope upon ATP binding and dimerization, like its bacterial counterpart. By contrast, MCD1 is a membrane spanning protein which seems to be required for proper AtMinD localization and function [100]. It is therefore tempting to speculate that MCD1 may be required to attach the MinDE/ARC3 complex to the chloroplast inner envelope. PARC6 may also play a similar role since it is a transmembrane protein and can interact with ARC3 [46]. All these proteins may hence form a complex attached to the chloroplast inner membrane as pictured on Fig. 6.2a, but it is not clear whether a single complex harboring all proteins or several sub-complexes differing by their composition exist in chloroplasts.

Even more elusive is how and where in the chloroplast all these proteins function. This gap in our knowledge can be attributed to the lack of live imaging of proteins regulating Z-ring assembly, and to conflicting results regarding their localization in dividing chloroplasts. Depending on the technique used for localization experiments, two types of distributions have been reported for division site placement machinery. According to fluorescent protein (FP) fusions and BiFC assays, AtMinD and AtMinE co-localize into large dots at chloroplast poles [77, 79]. The same localization was reported for MSL2 and MSL3 [49]. PARC6 [46] was also found in pole localized dots, but also at the division site, where it seems to function at later steps of the division process. The distribution of ARC3 seems more complex since it was found to co-localize with FtsZ1 in ring-like structures and to form discrete dots containing MinD [80], or multiple dots in which it interacts with PARC6/CDP1 [155]. By contrast, immuno-fluorescence experiments suggest that MCD1 [100] and AtMinD [37] are localized in ring-like structures at the chloroplast midpoint as well as punctuate structures dispersed throughout the envelope, whereas ARC3 is localized at the division ring during early and middle steps of chloroplast division [134]. Several hypotheses can explain these discrepancies. In some of these studies, localization of FP-fusion proteins was performed in transient expression assays using tobacco leaves in which chloroplast division may have stopped, and the localization of these proteins might therefore be altered. Furthermore, Fujiwara et al reported that the AtMinD-YFP fusion cannot complement the *arc11/minD* mutant [36], suggesting that the reported localization of the fusion protein may not reflect the proper distribution of AtMinD. Reciprocally, some of the observed immuno-fluorescence signals are very faint, and the inferred distribution of the target protein may not be accurate.

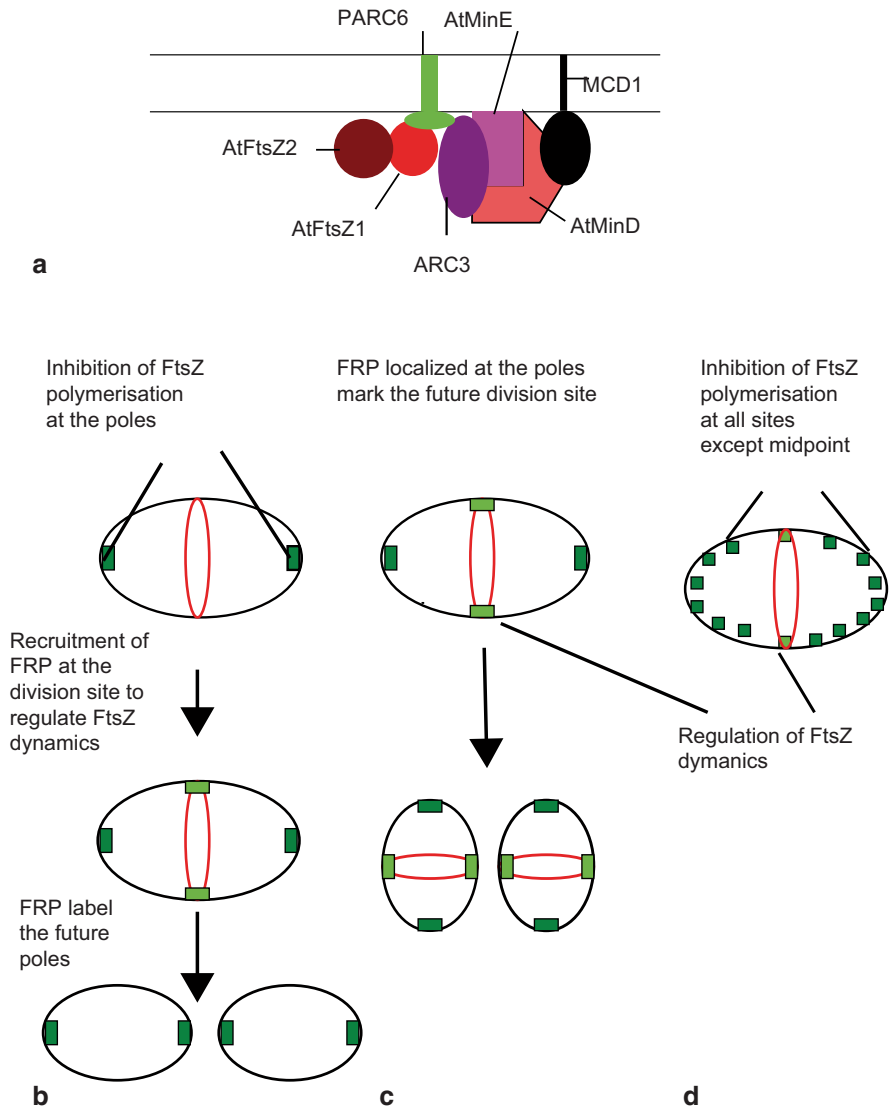


Fig. 6.2 Regulation of Z-ring positioning during chloroplast division. **a** The Z-ring regulatory complex. This picture summarizes the reported interactions between proteins and their membrane association. It is not clear however whether such a complex exists in chloroplasts, or several sub-complexes differing by their composition co-exist. **b-d** Three alternative models for Z-ring positioning based on localization data. *Dark green* and *light green* boxes represent complexes of FtsZ regulatory proteins. *Dark green* boxes are for a combination of proteins that completely inhibit FtsZ polymerization, whereas *light green* boxes represent a complex capable to modulate FtsZ dynamics at the division site. **b** FtsZ regulatory proteins (FRP) operate predominantly at chloroplast poles to inhibit ectopic Z-ring formation. After Z-ring assembly (*red* ring), a different set of FRP may be recruited at the division site and regulate FtsZ dynamics at the division site. FRP could also be required to label the newly formed poles, thereby maintaining chloroplast polarity. **c** Alternatively, the polar localization of FRP may mark the site of the future division site. **d** FRP inhibit Z-ring formation at all sites except midpoint. At the division site, another regulatory complex differing from the previous one by its composition regulates the dynamics of the Z-ring

Based on these localization experiments, several models can be proposed for the choice of chloroplast division site (Fig. 6.2b–d). All these models assume that the Z-ring regulatory complex is present at least transiently at the division site, implying that a minimum of two complexes differing by their proteic composition must exist in chloroplasts, one of them being an inhibitor of Z-ring formation (Fig. 6.2b–d; dark green boxes), and the other a regulator of FtsZ dynamics (Fig. 6.2b–d; light green boxes). One possibility would be that inhibitors of FtsZ polymerization act predominantly at the poles, as suggested by FP-fusion experiments (Fig. 6.2b, c). Recognition of the poles by this inhibitory complex may involve membrane curvature and require MSL proteins. In addition, accumulation of proteins involved in the choice of the division site at the poles may reflect their role in the regulation of chloroplast polarity [44]. Indeed, in *B. subtilis*, Min proteins are associated to the cell poles, and are recruited at the division site to mark the newly formed poles after division (Fig. 6.2b). Another possibility would be that Min-containing dots mark the site of the future division (Fig. 6.2c). Indeed, the cyanobacteria *Synechocystis* *sp* divides in alternating perpendicular planes [85]. Finally, as suggested by immuno-fluorescence analysis, inhibitors of FtsZ polymerisation may be distributed both in discrete dots dispersed throughout the inner membrane and at the division site (Fig. 6.2d). If this were the case, the discrete dots would represent inhibitory complexes preventing ectopic Z-ring formation, whereas the inhibitory action of AtMinD, ARC3, MCD1 and PARC6 would somehow be alleviated by AtMinE or another yet unidentified factor at the division site (Fig. 6.2d). Furthermore, the presence of ARC3 and PARC6 at the division site may be required to modulate the dynamics of AtFtsZ polymerization.

Models illustrated by Fig. 6.2c, d have the advantage that they may better account for the choice of the division site. Indeed, unlike bacteria in which the nucleoid occlusion system prevents the formation of the division apparatus everywhere except at midcell and at the poles, chloroplasts contain numerous small nucleoids that are distributed throughout the stroma [67]. Therefore, preventing Z-ring assembly at the poles may not be sufficient to ensure symmetric division of plastids. To clarify the model for Z-ring positioning, time-course analysis of the localization of all the above mentioned proteins during chloroplast division is absolutely indispensable.

6.3 Assembly and Dynamics of the FtsZ Ring

FtsZ is a stable protein and in bacteria the intracellular concentration changes little during cell division, therefore, rapid responses required for regulation of the Z-ring occur through control over FtsZ assembly. Recent evidence indicates that regulation of Z-ring assembly/maintenance in plastids may be achieved through the opposing effects of ARC6 and PARC6 and also through the action of the plastid chaperone system. Further complexity is added to the potential regulatory mechanisms in plants because at variance with prokaryotes, plant genomes encode several FtsZ isoforms.

6.3.1 Z-ring Assembly Requires Two Isoforms of FtsZ

In prokaryotes, FtsZ is usually encoded by a single gene even though exceptions have been reported [82]. By contrast, three homologues of FtsZ distributed in two families are present in *Arabidopsis* (AtFtsZ1, AtFtsZ2-1 and AtFtsZ2-2). Divergence between the two FtsZ families occurred early during evolution, probably before the divergence between Chlorophycean and Charophycean [138], and the number of isoforms in each family seems to vary from one plant to another. For example, only one FtsZ1 isoform can be found in *Arabidopsis thaliana* versus four in *Nicotiana tabacum*. AtFtsZ1 and AtFtsZ2 are assumed to play distinct role in chloroplast division because mutants cannot be complemented by overexpression of a member of the other family [132] and AtFtsZ1 and AtFtsZ2 differ by their ability to interact with other plastid division proteins: ARC3 interacts specifically with AtFtsZ1 [80] whereas ARC6 binds only to AtFtsZ2 ([147] see below). In *Arabidopsis*, *ftsZ2-2* null mutants show only mild plastid division defects, and this phenotype can be complemented by expression of AtFtsZ2-1. Reciprocally, the more severe phenotype of *ftsZ2-1* mutants can be rescued by AtFtsZ2-2 expression, suggesting that the two proteins are functionally redundant but differ by their expression level [132]. However, the two FtsZ2 isoforms may differ in their role in thylakoid development [59], see below).

AtFtsZ1 and AtFtsZ2-1 proteins co-localize and form rings at chloroplast mid-point (Fig. 6.3) [79, 86]. FtsZ proteins are structural homologues of tubulins, and can form both longitudinal and lateral interactions. Longitudinal interactions are stimulated by GTP binding and allow the formation of proto-filaments that consist of a head-to-tail linear polymer of FtsZ. However, unlike tubulin, FtsZ proto-filaments do not associate into microtubules but instead form bundles or sheets. Whether FtsZ1 and FtsZ2 proteins form distinct proto-filaments or hetero-multimeric ones is not clear. In chloroplasts, AtFtsZ1 and AtFtsZ2-1 can form curved filaments in the absence of the other protein, even if these structures are often disordered and more numerous than in the wild-type [146]. Purification of FtsZ complexes from *Arabidopsis* suggests that Z-rings contain both AtFtsZ1 and AtFtsZ2 at a 1:2 ratio [87], and the stoichiometry between the two proteins is probably important to their function because overexpression of either isoform severely inhibits chloroplast division [114]. Interestingly, AtFtsZ1 and AtFtsZ2-1 can form hetero-polymers *in vitro* and FtsZ1 promotes co-assembly, suggesting it can favor lateral interactions between proto-filaments [109]. Finally, phosphorylation of AtFtsZ2 proteins has recently been reported to somewhat affect their ability to interact with AtFtsZ1 and ARC6 and to form rings *in vivo*, providing evidence for an additional layer of complexity in the mechanisms governing Z-ring assembly [41].

6.3.2 Opposing Roles of ARC6 and PARC6 on Z-Ring Formation

One major difference between AtFtsZ1 and AtFtsZ2 is that only AtFtsZ2 possesses a C-terminal extension harboring a CORE domain allowing its interaction with ARC6 (Fig. 6.3) [79]. *arc6* mutants are the most severely affected of all *arc* mutants,

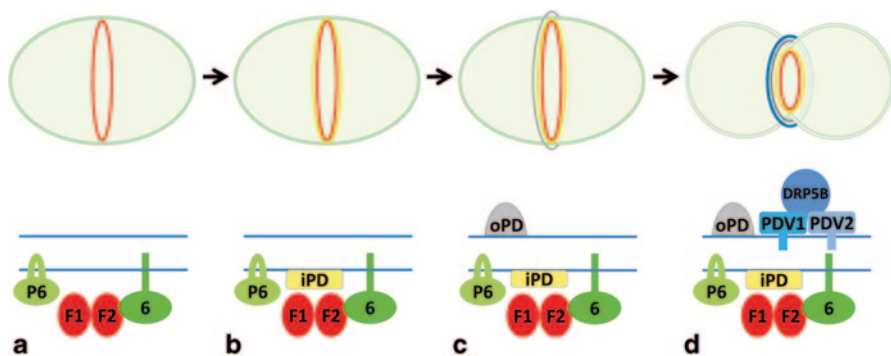


Fig. 6.3 The stromal and cytoplasmic division machineries of chloroplasts are assembled in a step-wise manner. **a** In Arabidopsis plastid division is initiated by the assembly of AtFtsZ1-1 (F1), AtFtsZ2-1 and AtFtsZ2-2 (F2) at the centre of chloroplasts to form the Z-ring. Z-ring assembly/maintenance may be achieved through the opposing effects of ARC6 (6) and PARC6 (P6). **b** After completion of the Z-ring the inner PD ring (iPD) assembles on the inside of the Z-ring. This is followed by the middle PD ring in the inter membrane space, if present (not shown). **c** Subsequently the outer PD ring (oPD) forms on the cytosolic face of the chloroplast. In *C. merolae* the oPD is composed of PRD1 and polyglucan filaments but the composition in higher plants is unknown. **d** Finally dynamin is recruited to the division site. In Arabidopsis this is achieved by the combined action of PDV1 and PDV2. DRP5B is faintly detected on un-constricted but only forms ring-like structures after constriction has begun. The timings of the assembly of the rings are inferred from microscopy studies in both Arabidopsis and *C. merolae*

containing only 2 chloroplasts per mesophyll cell on average [129]. ARC6 is an integral membrane protein homologous to the Cyanobacterial protein Ftn2 [64, 147], which appears to affect FtsZ polymerization positively: FtsZ2 forms short filaments in *arc6* mutants whereas it forms long polymers in ARC6 over-expressing lines [147]. In this respect, ARC6 seems to function similarly to AtMinE. Nevertheless, chloroplast division is almost completely abolished by ARC6 overexpression or loss of function, and asymmetric division was never observed: ARC6 is therefore a component of the chloroplast division machinery and not involved in the choice of the division site. ARC6 has been reported to form a discontinuous ring at the chloroplast division site [79], and is likely to function as a Z-ring anchor, stabilizing FtsZ polymers and associating them with the inner envelope of the chloroplast. This is similar to the role played by Zip and FtsA in *E. coli* which interact with FtsZ through the CORE domain and play a role in maintaining/stabilizing the Z-ring [117].

Unlike ARC6, PARC6 lacks a functional J-domain and subsequent associated co-chaperone activity [46]. This observation has led to the suggestion that PARC6 arose in vascular plants as a new functional class of plastid division proteins and evidence suggests that PARC6 carries out at least two, potentially overlapping functions, both playing a role in Z-ring placement (see above) and influencing Z-ring assembly and/or maintenance. PARC6 appears to act antagonistically to ARC6 in its role in Z-ring stability; in *arc6* chloroplasts FtsZ filaments are short, indicating that ARC6 acts to stabilize the Z-ring, but in contrast to this in *parc6* chloroplasts FtsZ filaments are relatively long and appear as multiple rings or spirals, indicating that

PARC6 inhibits Z-ring assembly [46, 147]. These data point to ARC6 and PARC6 having opposing effects on the formation/stability of the Z-ring, despite the high levels of homology between them.

ARC6 is proposed to mediate its effects on the Z-ring by anchoring the ring to the membrane through a direct interaction of the N-terminal conserved domain of ARC6 with AtFtsZ2-1 and AtFtsZ2-2 [79, 132]. Given the high levels of similarity between the N-terminal conserved domains of ARC6 and PARC6, the possibility that PARC6 directly interacted with AtFtsZ1 or AtFtsZ2-1 was investigated by yeast two-hybrid assays, but no interactions were detected [46]. Unlike ARC6, PARC6 is predicted to contain two trans-membrane domains, which would also orientate the C-terminal domain of PARC6 in the stroma and it will be important to test if the C-terminal domain can interact with any of the plastid FtsZ proteins to determine if PARC6 can directly influence the Z-ring.

The N-terminal stromal domain of PARC6 has been found to interact with ARC3. This interaction is dependent on the C-terminal MORN domain of ARC3, which is the same domain of ARC3 which inhibits the interaction of ARC3 with AtFtsZ1 in yeast two-hybrid studies [46, 80]. If PARC6 exerts the destabilizing effects on the Z-ring through ARC3, the availability of the MORN domains could play a vital regulatory role. The interaction of PARC6 with ARC3 could sequester the MORN domain, thus enabling ARC3 to interact with the Z-ring and render the Z-ring unstable. In the absence of the PARC6-ARC3 interaction, the MORN domain could inhibit the interaction of ARC3 with AtFtsZ1, thus rendering the Z-ring more stable, as seen in the *parc6* mutant [46]. In an additional level of complexity the availability of ARC3 to interact with PARC6 could also be influenced by the other interacting partners of ARC3, including MinD, MinE, and MCD1 [80, 100], and much work will be required to unravel the role of PARC6 on the placement and assembly of the Z-ring.

6.3.3 Chaperone-Regulation of Z-Ring Assembly/Maintenance

Recent evidence has shed light on the possible role of molecular chaperones in the assembly and/or maintenance of the Z-ring. Chaperones assist in the folding or unfolding of proteins and the assembly or disassembly of protein complexes. The first chaperones shown to play a direct role in the process of plastid division were members of the chaperonin 60 (cpn60, hsp60) family in *Arabidopsis* [142]. The Cpn60 family of chaperonins is highly conserved and found in bacteria, mitochondria and plastids [51]. The best characterized members include the GroEL complex in *E. coli*, which together with the cochaperonin GroES forms a large (~1 MDa) chaperone complex termed GroE. In *E. coli* GroE function is required for the formation of the cell division machinery and cells with impaired GroE activity exhibit filamentous cell morphology, characteristic of cell division inhibition [33, 104]. The filamentous phenotypes are also observed in GroE-depleted *Streptococcus mutans* and *Caulobacter crescentus*, suggesting that GroE plays a universal role in cell division in bacteria [72, 141].

In *Arabidopsis* at least two members of the Cpn60 family, ptCpn60 α 1 and ptCpn60 β 1, are required for chloroplast division [142]. A null mutation in *ptCpn60 α 1* or the double *ptcpn60 β 1-1 ptcpn60 β 2* mutant results in albino and dwarf seedlings harboring small, colorless plastids, indicating Cpn60 function is vital for plant development [5, 142]. However a moderate reduction in the levels of activity of ptCpn60 β B (caused by a premature stop codon in the ptCpn60 β 1) or of ptCpn60 α 1 (caused by a missense mutation, Ala-342-Val), results in the modest impairment of plastid division and mesophyll cells that harbor fewer but larger chloroplasts than those in wild type cells [142]. In *E. coli*, treatment of cells with penicillin has been shown to trigger the localization of GroE to possible division sites in an FtsZ dependent manner [104], although insults with other stresses did not replicate these results [16]. ptCpn60 β is localized as speckles throughout the chloroplasts in *Arabidopsis* [142] and there is no evidence to date of localization to the division machinery. However In both the ptCpn60 α 1 and ptCpn60 β 1 mutants FtsZ forms long, disorganized filaments, similar to those observed in *Arabidopsis* plants in which ARC6 is over-expressed, suggesting that reduced ptCpn60 levels result in excessively stable FtsZ filaments.

How changes in the levels of Cpn60 function might alter FtsZ filament stability in plastids is not yet clear. In bacteria proteome-wide analysis of GroE substrates have revealed several cell division proteins as putative targets of GroE [15, 52, 62]. To date GroE has been shown to assist in the folding of the FtsE [33]. FtsE interacts with FtsZ and works together with FtsX to support septal ring assembly and most likely ring constriction [6, 21]. FtsE is absent in plant and algal genomes and in *Arabidopsis* FtsZ is properly imported and processed in the ptCpn60 β 1-1 mutant, suggesting that the plastid Cpn60 system targets a different plastid division substrate(s) [142]. However, it is attractive to speculate that akin to bacterial system, the impaired chloroplast division phenotype and excessively stable FtsZ filament phenotype observed in the *cpn60* mutants are as a result of changes in the stability of a regulator of Z-ring formation and identification of possible targets of Cpn60 chaperonin activity will be important.

Given the striking similarity in the structure of the long, disorganized FtsZ filaments observed the *cpn60* mutants and in *Arabidopsis* plants overexpressing ARC6, it is interesting to observe that ARC6 might also play a role in the chloroplast chaperone systems. ARC6 harbors a J domain characteristic of co-chaperones that interact with the Hsp70 family of chaperone proteins [17, 147, 148]. J domains can associate with unfolded polypeptide chains and deliver them to Hsp70 chaperones for processing and also regulate the activity of the Hsp70 chaperones [148]. Most J domains harbor a central His-Pro-Asp motif, but in ARC6 and its orthologues only the central Pro is conserved uniformly. The His-Pro-Asp motif is commonly crucial for interaction with the Hsp70 chaperone partners, although exceptions exist [50]. Z-ring formation in *E. coli* is known to be influenced by Hsp70 [145] and it is possible that in addition to the proposed role in anchoring the Z-ring, ARC6 may have additional functions.

Whilst it remains to be shown whether ARC6 can interact with a chaperone through its atypical J-domain, recently it was shown that ARC6 can interact with

a second chloroplast protein harboring a J domain, Chloroplast J-like Domain 1 (CJD1) [3]. Like ARC6, CJD1 is an inner envelope protein and harbors an N-terminal J-like domain that resides in the stroma. The J-domain of CJD1 interacts with the ARC6 J-like domain and the adjacent conserved region. Interestingly, during mitochondrial protein import in yeast the dynamic interaction of two J-domain containing proteins (the J co-chaperone Tim14 and the J-like protein Tim16) has been shown to regulate the co-chaperone activity of Tim14 on mtHsp70, which plays an important regulatory role [98] and it is possible that the ARC6-CJD1 interaction has a similar regulatory role.

CJD1 plants were identified as containing altered levels of the leaf galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) [3]. These two galactolipids commonly constitute up to 80% of the total chloroplast lipids and play an essential role in chloroplast membrane biogenesis and are indispensable for correct protein import into chloroplasts [31]. Mutant analysis failed to reveal evidence for a direct link between the function of CJD1 and ARC6, however in the *tgd-1-1, fad6-1* and *tgd-4-3, fad6-1* double mutants, a strong reduction in the levels of MGDG and DGDG is coupled with a reduction in the number of chloroplasts per cell [29]. The authors suggest that an inadequate lipid supply for membrane proliferation during chloroplast division is responsible. This hypothesis is supported by the recent finding that impairment of very-long-chain fatty acids biosynthesis via genetic or pharmacologic approaches affects plastid division [103]. Whilst the picture is complicated by the lack of strong linkage between lipid mutants and chloroplast division mutants phenotypes, it will be interesting to determine the functional significance of interaction between CJD1 and ARC6.

6.4 Assembly of the Plastid Division Machinery

In bacteria the Z-ring is known to act as a scaffold protein, playing an essential role in the sequential and coordinated recruitment of up to nine additional cell division proteins to the division site (FtsX, FtsE, FtsK, FtsQ, FtsL, FtsB, FtsI, FtsW and FtsN) to form the divisome or septal ring (reviewed in [24]). Studies in *Arabidopsis* and algae have also shown that assembly of the chloroplast division machinery appears to occur in a linear order. After the formation of the Z-ring the timed addition of division machinery components results in assembly of up to four additional ring-like structures at the division site (Fig. 6.3).

6.4.1 The Plastid Dividing Rings

Many algae have just one chloroplast per cell and have been used extensively for the study of the coordinated assembly of the chloroplast division machinery because, unlike in *Arabidopsis* in which each cell can contain as many as 100 chloroplasts

that divide asynchronously, algal cell and organelle division can be highly synchronized by light/dark cycles. The completion of Z-ring formation is followed by the appearance of ring like structures known as plastid dividing (PD) rings. PD rings were first identified as fuzzy plaques in electron microscopy images in young wheat leaves [71]. This fuzzy plaque was subsequently shown to be an electron-dense ring-like structure in the alga *C. caldarium* [90]. Studies in both *Avena sativa* and *Cyanidioschyzon merolae* further resolved this ring to be composed of two or three electron-dense rings: an inner ring on the stromal face of the inner envelope, an outer ring on the cytosolic face of the outer envelope, and occasionally a middle ring in the intermembrane space. PD rings have subsequently been detected in many plant and algae species and are thought to be a universal feature of plastid division machineries [47, 68, 90–92, 94].

The first PD ring to assemble after the completion of the Z-ring is the inner PD ring, which forms between the inner envelope membrane and the Z-ring [93, 94]. This is followed by the middle PD ring in the inter membrane space (IMS), if present [91]. The components of the inner and middle PD ring are as yet unidentified. Finally the outer PD ring, which is composed of a bundle of 5–7 nm filaments, forms on the outer chloroplast envelope (Fig. 6.3) [95].

The identity of the components of the PD rings remained elusive for many years. It was found that isolated PD machineries from *C. merolae* could not be fully decomposed with proteases, indicating a non-proteic component, and electron dense deposits appeared on the PD rings after staining with periodic acid–horseradish peroxidase, indicating that it is saccharic in nature [153]. Proteomic analysis of the isolated PD machinery identified more than 20 putative components, one of which was a glycogenin-like protein and was designated Plastid Dividing Ring 1 (PDR1) [153]. PDR1 forms a ring that is recruited to the division site after the formation of the Z-ring and down-regulation of *C. merolae PRD1* by antisense suppression prevents the formation of the PDR1 ring, but does not affect the assembly of the Z-ring. PDR1 harbors a glycosyltransferase domain in the C-terminal region, putatively associated with the transfer of a sugar moiety from an activated donor sugar onto an acceptor molecule. Immunoblot analysis of synchronized cell cultures revealed that during chloroplast division the band corresponding to PDR1 shifted downwards before decreasing in intensity. The authors propose that PDR1 synthesizes the sugars that constitute the outer PD ring and is glycosylated during chloroplast division before decomposing [153].

The precise role of PRD1 and the polyglucan filaments is unclear, but down-regulation of *PRD1* leads to a decrease in the frequency of chloroplast division in *C. merolae* compared with control cells [153], indicating that the correct formation of the PD ring is essential for chloroplast division. PDR1 is widely conserved from red alga to land plants, and in *Arabidopsis* there is a family of 6 PDR1 proteins (PGSIP1–6). PGSIP1 and PGSIP3 are located in the Golgi [105], but the remaining proteins are yet to be investigated. It will be interesting to determine if any members of the family are involved in chloroplast division and if the outer PD ring is also a bundle of PDR1-mediated polyglucan filaments in higher plants.

6.4.2 *PDV1, PDV2 and Dynamin Recruitment to the Division Site*

Although the outer PD ring is not well characterized in higher plants, microscopy studies have revealed that the formation of the Z-ring is followed by the recruitment of two novel proteins, Plastid Division 1 and Plastid Division 2 (PDV1 and PDV2) to the division site on the outer envelope [97]. PDV1 and PDV2 are integral outer envelope proteins that show no significant sequence similarity to known proteins. PDV1 and PDV2 have similar domain structure and are composed of a large N-terminal domain residing in the cytosol, a single transmembrane domain and a small C-terminal domain residing in the in the IMS [44, 97]. The cytosolic domains harbor two short conserved regions and a coiled-coil domain, whilst the IMS domains both terminate in a conserved C-terminal glycine. The PDV1 family of proteins is found in only higher plants, whilst the PDV2 family is found in higher plants and in moss [97]. The primary defining feature between the two PDV families is the presence of a 28 amino acid extension in the C-terminal domain of the PDV2 proteins, that is absent in all PDV1 proteins [44].

Both PDV1 and PDV2 localize to patches on the outer membrane of the chloroplasts and are recruited to ring-like structures at the division site after Z-ring formation. The analysis of the localization of PDV1 and PDV2 in the *pdv2* and *pdv1* mutants, respectively, reveals that both proteins are recruited to the division site independently of each other [46]. PDV2 is recruited to the division site by a direct interaction of the IMS domain of PDV2 with the IMS domain of ARC6 [44]. Disruption of this interaction, by deletion of the conserved C-terminal glycine of PDV2 (PDV2_{G307D}) destroys PDV function *in vivo*, as demonstrated by the inability of PDV2_{G307D} to complement the *pdv2* mutant [44]. The IMS of PDV1 does not interact with ARC6 and it is possible that the unique 28 amino acid extension present in the PDV2 family of proteins mediates this interaction [44]. The recruitment of PDV1 to the division site is dependent on PARC6, although no direct interaction has been detected between the IMS domains of PDV1 and PARC6 [46]. Consequently the mechanism by which PDV1 is recruited to the division site is not clear.

A mutation of the C-terminal conserved glycine residue of PDV1 (PDV1_{G272D}) does not disrupt the insertion of PDV1 into the outer chloroplast membrane but, PDV1_{G272D} is localized dispersed throughout the membrane and is unable to be recruited to the division site [97]. It will be important to determine the protein interacting partner that mediates the ability of PDV1 to identify and/or localize to the division site through the C-terminal Gly. Interestingly, analysis of a truncated form of ARC6 lacking the IMS domain (ARC6_{ΔIMS}) found that ARC6_{ΔIMS} is able to insert into the inner chloroplast envelope and to correctly localize to the division site [44]. Furthermore, whilst the ARC6 ring is detected in both the *pdv1* and *pdv2* mutant background, neither PDV1 nor PDV2 are recruited to the division site in *arc6* mutant [44]. These data indicate that ARC6 acts upstream of PDV1 and PDV2 and is necessary for the membrane recruitment of both proteins. The link between the PDV proteins and ARC6 (and potentially PARC6) is the first clue as to how

the cytosolic and stromal division machineries can be coordinated across the inner and outer chloroplast envelope. In addition to conveying topological information regarding the location of the division site, it may also act to convey signals concerning the status of the stroma to the cytosolic division machineries.

Disruption or deletion of either *PDV1* or *PDV2* results in mesophyll cells containing chloroplasts that are fewer in number and larger than those in the wild type, and which frequently show prevalent constrictions [97]. This phenotype suggests that plants lacking functional PDV proteins are able to initiate constriction of the division sites but are unable to complete division. A strikingly similar phenotype is observed in Arabidopsis plants expressing a truncated form of DRP5B (Dynamamin-related protein 5B; ARC5) [40], the last chloroplast division protein known to be recruited to the division site, and recent work has shown these three proteins work together as part of the cytosolic chloroplast division machinery.

DRP5B was first identified through cloning of the *arc5* mutant and around the same time a dynamin-like protein in *C. merolae* (CmDnm2) was also identified as a late stage chloroplast division protein [40, 96]. The dynamin and dynamin-like protein superfamily of self-assembling GTPases are multidomain proteins and are well documented to participate in fission and fusion events of intracellular membrane structures (such as endocytosis, intracellular vesicle trafficking, cytokinesis, mitochondrial fusion and fission and peroxisome division) reviewed in [22]. How dynamin proteins function to bring about such a diverse range of events has been the focus of much study.

There are 16 dynamin-like proteins in Arabidopsis and only two in *C. merolae*. CmDnm2 and AtDRP5B, along with AtDRP5A which is involved in cytokinesis, represent a novel plant-specific group of dynamin-related proteins. This group harbor the three domains central to dynamin proteins: N-terminal GTPase domain, a middle domain (MD) and a C-terminal GTPase effector domain (GED), thought to interact directly with the GTPase domain and to mediate self-assembly [22]. Both AtDRP5B and CmDnm2 also have a pleckstrin homology (PH) domain, which have been shown to mediate lipid binding of other dynamin-like proteins. DRP5B is expressed as two alternatively spliced forms in Arabidopsis, with the shorter protein (714 amino acids) harboring a shorter PH domain than the longer variant (777 amino acids), although the functional significance of this is not yet apparent [40].

Both CmDnm2 and DRP5B relocate from the cytosol, where they localize as patches, to the division site where they form a ring like structure on the surface of the chloroplast [40, 96]. In Arabidopsis this dynamin ring is faintly detected in un-constricted chloroplasts but in *C. merolae* immunoblot analysis has shown that the dynamin ring only associates with chloroplasts during the division phase [96]. DRP5B is recruited to the division site by the combined action of PDV1 and PDV2. DRP5B is observed to localize to the division site in both the single *pdv1* and *pdv2* mutants, but not in the double *pdv1, pdv2* mutant, indicating that PDV1 and PDV2 function independently to recruit DRP5B to the division site [97]. The precise mechanism of recruitment is unclear and no interaction has been detected between DRP5B and PDV1 or PDV2 [97, 107]. However, both PDV proteins harbor cytoplasmic coiled-coil domains and there are several examples of proteins that

recruit dynamin proteins to the mitochondrial division site via a direct interaction with their coiled-coil domain, for example Mdv1p recruits Dnm1p during mitochondrial division [144].

In *C. merolae* the PDV families of proteins have not been identified, indicating that a novel mechanism may exist to recruit CmDnm2 to the chloroplasts division site in algae. Immunofluorescence microscopy demonstrated that PDR1 appears earlier than CmDnm2 in the cytoplasm and forms a ring from the beginning to the end of chloroplast division. CmDnm2 forms a ring between the outer most rings of the PD1/polyglucan filaments, leading to the hypothesis that PD ring plays a role in organizing the dynamin ring [153].

Recently it was found that DRP5B is also required for peroxisome division in Arabidopsis [154]. Akin to the phenotype observed in the chloroplasts of the *arc5* mutant, these plants exhibit enlarged peroxisomes and peroxisomes that have undergone membrane constriction but failed to complete fission and therefore are unable to separate from each other [154]. Two other dynamin-like proteins, two dynamin-related proteins (DRP3A and DRP3B) are also implicated in peroxisome division [32] and it will be interesting to see both how these they are coordinated with DRP5B and also how peroxisome division is coordinated with chloroplast division.

To date the dynamin-like proteins are the final component known to be recruited to the chloroplast division machinery, however it is doubtful that we have identified all of the components of the stromal and cytosolic division machineries. In the future the collection of *arc* mutants may provide novel insights into chloroplast division, as the mutations in at least four of the original *arc* mutants are yet to be identified. Furthermore, disruptions of several Arabidopsis genes have been demonstrated to lead to defects in chloroplast division, for example GC1/AtSula, CRL (crumpled leaf) and ARTEMIS, although a direct link to the division machinery has yet to be demonstrated [8, 39, 78, 126, 140]. Likewise, the negative regulator of chloroplast division ARC1 has recently been identified as FtsHi, but does not affect accumulation of chloroplast division proteins or Z-ring formation [58]. Further characterization of these proteins will be important to identify the full complement of chloroplast division machinery proteins.

6.5 Constriction and Separation of Chloroplasts

After formation of the division machinery on the inner and outer envelope, microscopic observations reveal that constriction of the plastid is initiated and the septum progressively tightens to eventually separate the two new daughter plastids. Whilst events mediating the placement and the temporal assembly of the machinery are beginning to be elucidated, less clear is how the chloroplast division machinery is initiated to begin constriction, how the force for constriction is generated and which molecules/mechanisms mediate the final scission event.

The analysis of plants in which the mechanism of chloroplast division is disrupted, either through the absence of a functional form of a division protein or through

overexpression of a division protein, has revealed distinct classes of chloroplast division phenotypes. Most striking are the presence of either (i) enlarged chloroplasts which are globular/round in shape or (ii) enlarged chloroplasts which are frequently constricted and have a dumbbell-shaped appearance. The first category are commonly caused by diminution of functional stromal ring division components, such as ARC6 or FtsZ [86, 125, 139], whilst the second class are only caused by diminution of the cytosolic division components [40, 97] (Fig. 6.1). These phenotypic differences suggest that the early and late stages of plastid constriction are governed by different forces, generated by either the stromal or the cytosolic division machineries.

6.5.1 Force Generation by the Stromal Plastid Division Machinery

It is thought that FtsZ is necessary and sufficient to generate the force for constriction of the septum during bacterial cell division [9, 76]. In support of this in some bacteria the sole cell division protein is FtsZ, suggesting that FtsZ alone is sufficient to bring about cell division, furthermore FtsZ protofilaments are observed to undergo a conformational change from straight to curved that would be capable of generating a force [75, 81]. This hypothesis was strengthened when membrane tethered FtsZ successfully assembled into Z-rings on the inside of liposomes [110, 111]. The Z-rings formed were dynamic and capable of constricting the liposomes in a GTP dependent manner, proving that FtsZ alone is capable of self-assembly and force generation. How FtsZ might generate the force for constriction in bacteria is still controversial but years of genetic, biochemical and structural studies have revealed many properties of the protein.

FtsZ has the ability to bind GTP and FtsZ proteins from at least seven bacteria, as well as both AtFtsZ1 and AtFtsZ2-1 from Arabidopsis, have been shown to be functional GTPase enzymes, although both AtFtsZ1 and AtFtsZ2-1 hydrolyze GTP at a slower rate than their bacterial counterparts [25, 74, 109, 137]. Monomeric FtsZ hydrolyses GTP very slowly but can self-assemble into filaments. When assembled into a protofilaments the bottom interface of one FtsZ makes contact with the GTP pocket of the subunit below [131].

FtsZ filaments have three preferred conformations; straight, intermediate curved and highly curved [28]. Whilst the structure of the Z-ring in cell or chloroplast division is not yet clear, it is thought to be formed from small, overlapping subunits of curved FtsZ filaments that are tethered to the membrane via interaction with FtsA, ZipA or ARC6 [79, 117]. GTP hydrolysis is not necessary for FtsZ self-assembly and FtsZ polymers from bacteria have been shown to contain a substantial amount of GTP, suggesting that hydrolysis takes place with some lag after polymer formation [28, 108]. Despite many years of research it is not known what happens following GTP hydrolysis in a protofilament. It is known that GDP substantially destabilizes the protofilaments and one suggestion is that after hydrolysis the protofilament

fragments, however it has been possible to detect FtsZ protofilaments that contain a 1:1 ratio of GTP:GDP and many believe that the polymers fragment only at the ends [18, 130].

Several mathematical and physical models have been proposed to explain how the physical properties of FtsZ can generate the force necessary to constrict a membrane. It is proposed that the membrane tethered Z-ring generates force by the protofilaments exerting a bending force on the membrane as they are induced into a curved conformation. In support of this elegant experiments were performed in which Z-rings were reconstituted in liposomes by either targeting the C-terminal end of FtsZ (FtsZ-c) to the membrane (to mimic the *in vivo* orientation of FtsZ tethered to the membrane) or by targeting the N-terminal end of FtsZ (FtsZ-n) to the membrane (to reverse the orientation). As described above, the Z-rings reconstituted from FtsZ-c were capable in inducing concave constrictions of the liposomes. In stark contrast, the Z-rings formed from FtsZ-n caused convex bulges to form on the liposomes, indicating that the bending force was generated in the opposing direction [111, 112]. It will be extremely interesting to determine if each of the three Arabidopsis FtsZ proteins is capable of generating such bending forces alone and whether they act cooperatively.

If FtsZ is not (solely) responsible for generating the force necessary to initiate constriction of the chloroplast division site, what other proteins could play a role? To date the best candidates are the components of the PD rings. During constriction the inner PD ring has been observed to remain a constant thickness and loses components as constriction proceeds, disassembling late in constriction, indicating that any role played in force generation is not related to final constriction/scission [93]. Identification of the components of these rings and analysis of the phenotype of mutants lacking functional components will be vital in determining possible roles.

6.5.2 Force Generation by the Cytosolic Plastid Division Machinery

The principal candidates to generate force for constriction on the outer envelope are the chloroplast division dynamin-like proteins. Dynamin-like proteins are well established to be essential for constriction during the division of organelles in many species. For example mitochondrial division is known to employ dynamin-like proteins in mammals (Drp1/Dlp1), yeast (Dnm1), higher plants (ADL1 and ADL2) and *C. merolae* (CmDnm1) (reviewed in [69]), and the involvement of dynamin-related proteins in peroxisome division has been demonstrated in Arabidopsis (DRP5B, DRP3A, DRP3B), humans (DLP1, Fis1) and yeast (Vps1, Dnm1) (reviewed in [61]). Best characterized is the role of Dnm1p/Drp1 in mitochondrial division and current models leave no doubt that dynamin is the key player mediating membrane scission.

The study of how the dynamin chloroplast division proteins function during division is in its relative infancy but many parallels can be drawn between the

mechanisms of chloroplast division and mitochondrial division. Like the chloroplast dynamin division proteins, Dnm1p/Drp1 interacts with other proteins to form the mitochondrial division machinery (reviewed in [116]). Furthermore, Dnm1p/Drp1 are believed to be involved in late stages of division: *C. elegans* with mutant *drp-1* have constrictions in the outer mitochondrial membranes but fail to complete division, a phenotype reminiscent of the chloroplasts in the *arc5* mutant, which are frequently constricted and dumbbell shaped [70]. Also, the majority of Dnm1p/Drp1 is present in the cytosol and is recruited to the mitochondrial outer membrane during the late stage of division [136]. Similarly, CmDnm2 and DRP5B also relocate from cytosolic patches to associate with the chloroplast division site [40, 101].

Drp1 exists as small oligomers (dimers/tetramers) that can assemble into larger multimeric structures at the mitochondrial outer membrane [38, 135]. DRP5B can interact with itself *in vivo* [154], suggesting DRP5B can form higher order structures on chloroplasts. Drp1 can polymerize *in vivo* into spirals with a diameter of approximately 100 nm, which is considerably smaller than the diameter of mitochondria (400 nm) [54] and it is thought that as higher order structures of dynamin are only observed at later stages of mitochondrial division, they may only form once the membrane has undergone some constriction. In *C. merolae* small dynamin patches are discontinuously localized at the division site at the onset of constriction and only form a continuous ring at later stages, suggesting that the same mechanism may be in place in chloroplast division [96].

Once assembled, dynamin can undergo GTPase activity, which is stimulated by interactions between the GTPase domain, the MD and the GED [14, 54]. The GTPase activity is stimulated once a ‘critical mass’ is reached and the resulting activity resembles a chain reaction. When dynamin hydrolyses GTP there is a conformation change in the protein which results in considerable bending of the filament, sufficient to bring about constriction of the membrane [88]. Although the identified dynamin-like proteins from Arabidopsis and *C. merolae* are yet to be proven to be GTPases, given the high levels of conservation in the domain architecture it is likely that they are functional enzymes. Additionally the membrane-free PD machineries isolated from *C. merolae* formed super-twisted rings, circular rings and spirals which is attributed to the motive force generated for contraction by CmDnm2 [152].

What is the role of the outer PD ring? PDR1 is only glycosylated during division and after constriction has been initiated the outer PD ring is observed to widen and thicken, and remains attached until division has been completed [94, 153]. It is possible that the PDR1 and sugar ring is required to organize the dynamin ring throughout the division process, but it could also play a role in force generation.

The mechanism behind the final scission of chloroplasts remains unknown. It is possible that dynamin is necessary and sufficient to bring about this stage, but other factors may also be required. After constriction is completed, plastids are separated and can move inside the cell via the actin cytoskeleton [66, 133]. Very recently, the CLUMPED CHLOROPLAST 1 protein (CLMP1) has been identified as an important factor for chloroplast separation [151]. In *clmp1* mutants, chloroplasts remain interconnected and form clumps instead of being distributed throughout the cells, resulting in the formation of some aplastidic cells. This phenotype is transient and

almost completely disappears as leaves age. Interestingly, both early and late steps of chloroplast division appear to occur normally since FtsZ and DRP5B localization is normal in this mutant. Ultrastructure analysis revealed that some chloroplasts within the clumps are held together by thin membranous-connections, similar in structure to the isthmus, suggesting that only the final scission of chloroplasts is impaired in this mutant [151]. CLMP1 does not localize at the division site, and further analysis will be required to fully understand possible roles in scission and how its activity affects chloroplast separation.

6.6 Regulation of Plastid Division

It is now clear that all plastids originate from the division of a pre-existing organelle, and fusion between plastids appears to occur very rarely if at all [7]. Because plastid number is maintained constant depending on the species and the cell type, plastid division must be tightly regulated to achieve the correct number of organelles in a given cell. Exogenous cues such as light, temperature or nutrition have also been reported to affect chloroplast division (reviewed in [118]), but the underlying mechanisms remain largely unknown.

6.6.1 Regulation During Cell Proliferation

Regulation of chloroplast division during the cell cycle has been extensively studied in unicellular algae. In such model organisms cells contain one or a few chloroplasts, and the requirement for a strict regulation of chloroplast division during the cell cycle is obvious: if chloroplast division did not occur prior to cell division, aplastidic daughter cells would be generated. This can be observed in the case of *Hatena sp.*, a protist that hosts an endosymbiotic green algal partner but inherits it unevenly because the algae does not divide inside the host cell [106]. The ability of the host cell to promote and control the division of the endosymbiont has hence clearly been a major step during the evolution of chloroplasts. In the red algae *C. merolae*, accumulation of chloroplast division proteins CmFtsZ1 and CmFtsZ2 precedes accumulation of tubulin during mitosis [102]. In the brown algae *Seminavis robusta*, chloroplast division has been shown to occur during the S/G2 phase of the cell cycle, and this regulation may involve transcriptional regulation of *FtsZ* genes expression [43]. In *Chlamydomonas reinhardtii*, expression of *FtsZ* and *Min* genes is also cell cycle regulated [2, 53].

In higher plants, the relationship between cell and plastid division is far more complex because (i) cells contain numerous plastids, and a strict coordination between cell cycle and plastid division is therefore no longer required to avoid the formation of aplastidic cells, and (ii) plastid division can occur in two contrasting cellular contexts: in proliferating cells or in differentiating cells. Indeed, mature

Arabidopsis mesophyll cells contain on average 120 chloroplasts in the *L-er* background, whereas meristematic cells contain about 10 proplastids [83]. Hence, proplastid division is required to keep pace with cell division, and in developing leaves, differentiated chloroplasts can divide both in dividing and in expanding cells.

The existence of mechanisms regulating plastid division during the cell cycle in higher plants is debated. Early observations of dividing chloroplasts in various plant species do not provide evidence for synchrony of chloroplast division in a given cell. Furthermore, chloroplast division can occur independently of the cell cycle in wheat leaves where the maximum rate of chloroplast division is observed in a region of the leaf where cell division has ceased [10]. However, plastid division might be regulated depending on nuclear DNA (nDNA) content (or synthesis) because there is a correlation between chloroplast number and cell ploidy: chloroplast number is increased in a polyploid plant compared to a diploid plant of the same species, and chloroplast number in a given cell type has also been proposed to vary according to endoreduplication (a particular type of cell cycle during which the cell undergoes subsequent phases of DNA replication without mitosis) [12]. In agreement with this hypothesis, in tomato fruit, in which endoreduplication is particularly high, chromoplast number can be up to 1000 per cell [121].

To date, data is lacking to firmly establish that chloroplast division is cell cycle regulated in higher plants, or to provide a molecular mechanism underlying this regulation, but some studies have provided insights into this process. In algae, cell cycle-dependent regulation of FtsZ seems to be the rule, but is not so clear in higher plants: it was observed in BY-2 cells [27], but not in micro-arrays on synchronized *Arabidopsis* cells [89]. Furthermore, expression of genes encoding proteins involved at early steps of plastid division such as FtsZ or ARC6 does not seem to vary much in developing leaves [107]. In *Arabidopsis*, silencing of the *AtCDT1a* gene, encoding a protein involved in nuclear DNA replication licensing, resulted in an inhibition of cell cycle progression and chloroplast division. *AtCDT1a* was also found to interact with ARC6, suggesting that this factor may not only be a key factor for nuclear DNA replication but also play a direct role in plastid division, and providing evidence for a common regulatory pathway to both processes at the onset of S-phase [127]. In tomato, Caspi et al (2008) reported that the *high pigment 1* (*hp1*) mutation, affecting the DDB1 protein, results in increased chloroplast number per cell [13]. Interestingly, DDB1 is a sub-unit of an ubiquitin-E3 ligase complex and CDT1 is one of its targets; this result may hence support the view of a CDT1-mediated coordination of cell cycle and chloroplast division at the onset of S-phase.

6.6.2 Plastid Division and Plastid DNA Replication

To date, it is not clear whether plastid DNA replication and plastid division are strictly coupled processes. Plastids contain multiple copies of their genome, and replication of plastid DNA is therefore not a pre-requisite to plastid division. In some instances, chloroplast division can occur independently of cpDNA replication since in the latest stages of leaf development DNA copy number per chloroplast

decreases [48]. However, in dividing chloroplasts, nucleoid replication usually occurs before chloroplast division [121]. The phenotype of the *crinkled leaves 8* mutant (*cls-8*) may provide further evidence for a link between cpDNA replication and chloroplast division. These plants are deficient for the large sub-unit of ribonucleotide reductase (RNR), but display severe inhibition of chloroplast division. Despite a clear reduction in dTTP and dATP pools, little effect was observed on nDNA replication whereas chloroplast DNA (CpDNA) copy number was drastically reduced [42]. Finally, the YlmG protein was recently identified in cyanobacteria and the red alga *C. merolae* for its putative role in nucleoid partitioning [57]. Its overexpression inhibits chloroplast division whereas loss of function only affects nucleoid localization, suggesting that partitioning of chloroplast DNA may somehow be related to the chloroplast division process.

As illustrated by the above paragraphs, several lines of evidence suggest that plastid division may be influenced by nuclear and plastidial DNA replication, although further work is needed to further support this hypothesis and to decipher the molecular mechanisms involved.

6.6.3 *Plastid Division Is Regulated by Cell Differentiation, and Cell Expansion*

Plastid number strikingly differs depending on the cell type: for example in *Arabidopsis*, meristematic cells contain about 10 proplastids [83] whereas stomata guard cells contain only 4 [129], petal cells 15–20 [124], and mesophyll cells more than 100 [83]. How plastid division is regulated according to cell type remains largely unknown, but cytokinins are probably responsible for the stimulation of chloroplast division in young developing leaves. Early reports supporting this hypothesis came from the isolation of the *PC22* mutant in the moss *Physcomitrella patens*. In this mutant, chloroplast division is severely impaired, but can be partially restored by exogenously applied cytokinins [1, 60]. A positive effect of cytokinins on chloroplast division was also reported in bean leaves [99] and *Brassica rapa* leaf disks [150], but not in spinach leaf disk [119]. The molecular basis for this regulation is beginning to be elucidated and is mediated at least partly by PDV proteins. Indeed, unlike FtsZ or ARC6, accumulation of PDV1 and PDV2 is higher in young leaves, where chloroplast division is most active, than in older leaves, and their overexpression is sufficient to stimulate chloroplast division, like exogenous cytokinin application. Furthermore, the cytokinin response factor CRF2 was shown to stimulate *PDV* genes expression and chloroplast division [107]. More recently, two cytokinin responsive transcription factors, GATA Nitrate-inducible Carbon metabolism involved (GNC) and Cytokinin-Responsive GATA1 (CGA1) have also been involved in the cytokinin-dependent stimulation of chloroplast division downstream of B-type ARRs [19]. Whether these factors regulate chloroplast division via PDV proteins or via an independent mechanism remains unclear, but PDV proteins nevertheless appear to be key regulators of chloroplast division in developing leaves. The mechanisms regulating plastid division in other organs such as roots, flowers,

tuber or seeds have not been investigated, but it is tempting to speculate that other phytohormones such as gibberellins in the case of flowers [23] may play a role in regulating plastid division in non-green tissues.

Additional regulatory factors probably affect chloroplast division in leaves. Indeed in mesophyll cells, chloroplast number clearly depends on cell area: in *Arabidopsis*, a very nice correlation is observed between cell size and chloroplast content in the mesophyll cells [123]. This correlation is retained between different species: for example cocoa mesophyll cells contain on average 3 chloroplasts but are very small [121]. Isolation of *arc* mutants led to the conclusion that total chloroplast area, rather than chloroplast number is under tight control in mesophyll cells. Diminution of chloroplast number due to impaired chloroplast division is compensated by an increase in chloroplast size, so that the total chloroplast plan area remains almost constant [123]. Reciprocally, in the few *arc* mutants characterized by an increased chloroplast number, chloroplast size is reduced [120]. Finally, comparison of mesophyll cell size, chloroplast number and chloroplast size in different species demonstrated that total chloroplast area in cells is strictly related to cell size over a tenfold range of cell sizes [121]. Because cell size is generally related to cell ploidy [65], some authors postulate that chloroplast division is regulated directly by cell size and independently of DNA replication. In agreement with this hypothesis, chloroplast number still correlates with cell size in transgenic plants over-expressing an inhibitor of cell cycle regulation, resulting in increased cell size and reduced cell ploidy [56].

As shown by the above summary, several studies have tackled the issue of the regulation of plastid division during development, but little is known yet regarding the exogenous control of this cellular process. Due to their sessile nature, external conditions affect many aspects of plant growth and development, and in this respect are also likely to affect chloroplast division, but evidence for such regulatory pathways is scarce.

6.6.4 Chloroplast Division Is Regulated by Exogenous Cues

The most obvious external signal that could affect chloroplast division is probably light. Evidence for light regulation of chloroplast division has been provided long ago by Hashimoto and Possingham [48], in spinach leaf disks they found chloroplast generation time to be more than 2 times longer in the dark. Interestingly, the late phases of chloroplast division are most affected: chloroplasts remain constricted (at the dumbbell stage) for 22 h instead of 3.5 h [48]. Recently *DRP5B* expression has been shown to be regulated by FHY3, a transcription factor involved in phytochrome A signaling [115]. This result fits nicely with early microscopic observations since chloroplasts in *arc5* mutants are arrested at the dumbbell stage of division [83]. FHY3 is specifically involved in far-red signaling and appears to stimulate *DRP5B* expression in far-red light. In agreement with this observation, mutants deficient for FHY3 or its homolog are affected in chloroplast division, and

accumulation of DRP5B at the division site is no longer detected in *phy3*. Chloroplast division is even more severely impaired in *phy3 far1* double mutants, demonstrating that FHY3 and FAR1 act partially redundantly to promote chloroplast division in response to far-red light. Nevertheless, other light dependent signaling pathways regulating chloroplast division may exist. Indeed, the chloroplast division phenotype of the PC22 mutant in *P. patens* can be reverted by blue light treatment [128], suggesting that other photoreceptors such as cryptochromes may play a role in regulating chloroplast division.

Capture of light energy by chloroplasts and active photosynthesis can modify the redox status of the plastoquinone pool in the chloroplast, and the redox state of PQ is likely to regulate nuclear gene expression [63]. In addition, chloroplasts generate high amounts of reactive oxygen species, especially when submitted to excess light, which can result in severe photo-oxidative stress. Because the chloroplast redox balance is most important for the survival and function of plant cells, redox regulation of chloroplast division may well occur. In agreement with such a hypothesis, *ntrc* mutants deficient for the chloroplast NADPH-thioredoxin reductase appear to accumulate fewer chloroplasts than the wild-type during their first month of growth [73], but further work is needed to confirm this observation and determine how this regulation may function.

Finally, many other factors have been reported to affect chloroplast division including temperature [119] or mineral nutrition [118], but it is not clear whether these effects are direct or indirect, and the underlying molecular mechanisms have yet to be investigated.

Now that the mechanism for chloroplast division is better understood, an increasing number of studies have investigated its regulation, and are providing molecular basis accounting for early cytological observations. Regulation of plastid division by external cues is only beginning to be unraveled but the role of internal cues is more thoroughly described. Two contrasting situations can be distinguished: in unicellular algae containing one or a few chloroplasts, cell cycle regulation of chloroplast division appears to be the rule, and seems to involve transcriptional regulation of genes involved at early steps of the process such as *FtsZ* or *Min*. By contrast in higher plants, cell cycle regulation of plastid division is still debated, although several lines of evidence suggest that plastid division may be modulated by nuclear DNA replication, the only consensus is that total chloroplast area is modulated according to cell area, but the molecular mechanisms for this regulation have yet to be described. One striking feature of plastid division in higher plants is that expression of *FtsZ* proteins or ARC6 does not seem to vary a lot and proteins involved at later stages of division such as ARC5 or PDV seem to be targets for regulatory pathways. This may point to additional functions for *FtsZ* proteins unrelated to plastid division. Indeed, in *Arabidopsis*, *FtsZ* proteins are associated with thylakoid membranes [26, 59], and *fsZ2-2* mutants show defects in chloroplast shape and thylakoid development [59]. Likewise, in *P. patens*, the five *FtsZ* isoforms appear to play distinctive roles in cell patterning, chloroplast shaping, plant development and gravity sensing [84] suggesting that in multi-cellular plants, the functions of *FtsZ* proteins may have diversified.

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