The Molecular Machinery of Chloroplast Division[1][OPEN]

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Chloroplasts descended from a free-living cyanobacterium acquired through endosymbiosis roughly one billion years ago. Three major groups comprising the Archaeplastida, photosynthetic eukaryotes bearing primary plastids, arose subsequently: the glaucophytes, red algae (red lineage), and Viridiplantae, also called Chloroplastida, encompassing green algae and land plants (green lineage; Keeling, 2010; Zimorski et al., 2014). Beyond carrying out photosynthesis, plastids perform many other vital functions, such as fatty acid and amino acid synthesis, and are therefore essential organelles (Pyke, 2009). Plastid division increases chloroplast populations during leaf development, crucial for photosynthetic capacity (Leech and Baker, 1983), and ensures that plastids are faithfully inherited during cytokinesis.

Similar to their free-living ancestors, plastids are propagated through division of preexisting organelles. This process is powered by a macromolecular machine with ring-shaped contractile complexes on both the inner and outer envelope membranes (for review, see Miyagishima et al., 2011; Falconet, 2012; Yoshida et al., 2012; Osteryoung and Pyke, 2014). The division machinery is a mosaic of components of both endosymbiotic and host origin that must cooperate to divide the organelle (Fig. 1B). The stromal components are largely endosymbiont derived, whereas the cytosolic components are strictly eukaryotic. In land plants and many algae, all the plastid division proteins are encoded in the nucleus, but some algae retain a few that are plastid encoded (Onuma et al., 2017). Here, we highlight developments in the division of chloroplasts, particularly in land plants, with an emphasis on findings published since the previous *Plant Physiology* Update on this topic (Miyagishima, 2011). We refer readers to other reviews for information on the division of other plastid types, including secondary plastids, and for a more evolutionary perspective (for review, see Miyagishima, 2011; Miyagishima et al., 2011, 2014; Pyke, 2016).

ADVANCES

- The two chloroplast FtsZ types in the green lineage (FtsZ2 and FtsZ1) and red lineage (FtsZA and FtsZB) copolymerize and have conserved functions; one provides the structural framework for the Z ring and the other enhances Z-ring dynamics.
- A peptidoglycan wall surrounds the chloroplasts in moss and likely many other organisms, and the presence of a complete chloroplast peptidoglycan synthesis pathway is correlated with the occurrence of a third type of FtsZ, FtsZ3.
- ARC3 replaced MinC as the direct inhibitor of FtsZ assembly in the angiosperm chloroplast Min system, acting with MinD, MinE and MCD1 to restrict Z-ring placement to the division site.
- PDV2 dimerization in the OEM induces dimerization of ARC6 in the IEM, thereby transmitting information from the external to the internal division machinery.
- In unicellular algae, the cell cycle restricts expression of nucleus-encoded chloroplast division proteins and Z-ring assembly to the S phase; disrupted assembly of the chloroplast division machinery arrests cell-cycle progression.

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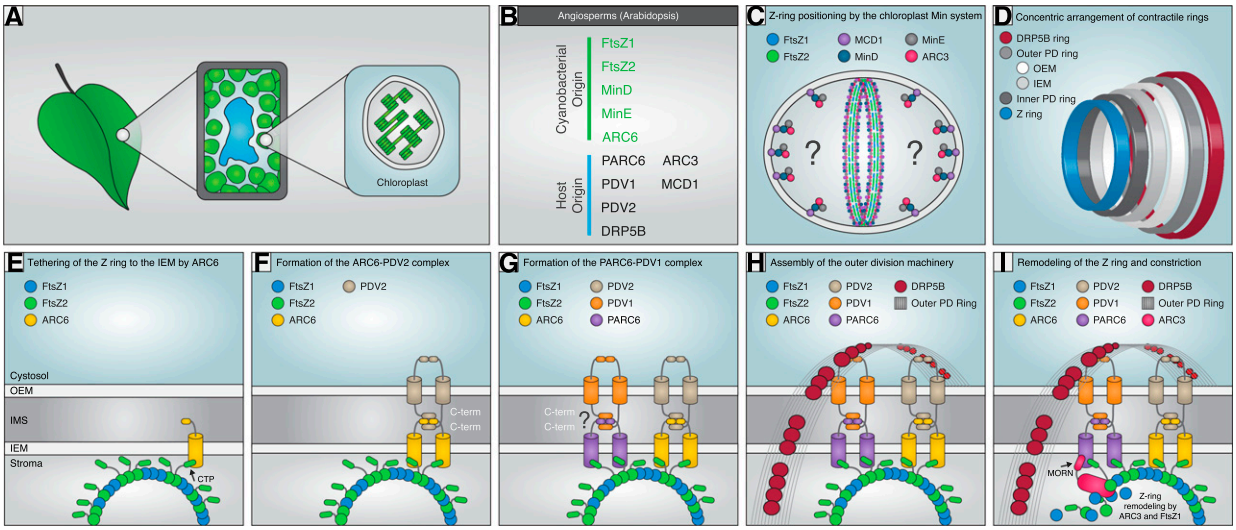


Figure 1. Working model of the positioning, assembly, and dynamics of the chloroplast division machinery in angiosperms based primarily on studies in Arabidopsis but informed by studies in *C. merolae* and other organisms. A, Diagram showing chloroplasts within a leaf mesophyll cell. B, Cyanobacterial (endosymbiotic) or host (eukaryotic) origin of chloroplast division components in angiosperms. C, FtsZ1 and FtsZ2 self-assemble as dynamic heteropolymers, possibly with mixed stoichiometry (Olson et al., 2010; Chen et al., 2017); protofilaments may possibly associate laterally to form the Z ring at the division site (Lutkenhaus and Du, 2017). Positioning of the Z ring is confined to the midzone by the chloroplast Min system, comprising ARC3, MCD1, MinD, and MinE, which inhibits Z-ring assembly at nondivision sites. ARC3 acts downstream of MinD and MinE as the direct inhibitor of Z-ring assembly (Zhang et al., 2013). MCD1, a transmembrane IEM protein, recruits MinD to the membrane (Nakanishi et al., 2009), where MinE is also colocalized (Miyagishima et al., 2011). ARC3 interacts with both MinD and MinE (Maple et al., 2007). Thus, we hypothesize that ARC3 forms a complex with MinD and MinE that is tethered to the membrane by MCD1. The exact localization pattern of ARC3 and the inhibitory mechanisms of ARC3 on Z-ring assembly are unclear (indicated by the question marks). The chloroplast Min-system components also localize partly to the division site (Shimada et al., 2004; Nakanishi et al., 2009; Miyagishima et al., 2011), where they may promote Z-ring remodeling during division (Johnson et al., 2015). D, Overview of the four contractile ring structures formed across the two envelope membranes. The composition of the inner PD ring is unknown and is not shown in further panels. The outer PD ring is synthesized by PDR1 (not shown) in the red alga *C. merolae* (Yoshida et al., 2010). The order of assembly based on studies in *C. merolae* is Z ring, inner PD ring, outer PD ring, DRP5B ring (Miyagishima et al., 2001, 2003). E to I, Stepwise assembly and dynamics of the division complex at the middle of the chloroplast. E, Tethering of the Z ring to the IEM is achieved mainly through interaction of the conserved FtsZ2 C-terminal peptide (CTP) with ARC6 (Maple et al., 2005), which probably stabilizes the Z ring and facilitates its assembly (Vitha et al., 2003; Johnson et al., 2013). F, ARC6 recruits PDV2 to the division site through direct interaction between their C-terminal IMS regions (Glynn et al., 2008). Dimerization of the cytosolic regions of two PDV2 molecules induces dimerization of two ARC6 molecules (Wang et al., 2017). G, PARC6 acts downstream of ARC6 to localize PDV1 to the division site through direct interaction between their C-terminal IMS regions (Glynn et al., 2009; Zhang et al., 2016). Based on ARC6-PDV2 studies (Wang et al., 2017), PDV1 dimerization might also promote PARC6 dimerization (indicated by question mark). H, In *C. merolae*, PDR1 (not shown) is recruited from the cytosol to construct the outer PD ring, composed of polyglucan fibrils (Yoshida et al., 2010). In Arabidopsis, PDV1 and PDV2 function together to recruit DRP5B from the cytosol (Miyagishima et al., 2006) to form the DRP5B ring (Gao et al., 2003; Miyagishima et al., 2003; Holtsmark et al., 2013). Coordination of the stromal Z ring and the cytosolic DRP5B ring is established through the ARC6-PDV2 and PARC6-PDV1 complexes. Whether the outer PD ring interacts with the PDV proteins is unclear. I, Remodeling of the Z ring and constriction. We speculate that PARC6 recruits ARC3 to the division site via interaction with the ARC3 MORN domain, enabling ARC3 to interact with FtsZ in the Z ring. The latter interaction may be facilitated by interaction of PARC6 with the FtsZ2 CTP (Zhang et al., 2016). As an FtsZ assembly inhibitor, ARC3 activation at the division site may promote Z-ring remodeling. MinD, MinE, and MCD1 also localize partly to the division site (Nakanishi et al., 2009; Miyagishima et al., 2011; not shown in I). Dynamic remodeling of the Z ring probably also depends on FtsZ1 (TerBush and Osteryoung, 2012; Yoshida et al., 2016; Terbush et al., 2018). OEM, Outer envelope membrane; IMS, intermembrane space; IEM, inner envelope membrane; MORN, membrane occupation and recognition nexus domain of ARC3; PD ring, plastid-dividing ring.

FUNCTION, ASSEMBLY, AND DYNAMICS OF FTSZ

A central and nearly ubiquitous component of the chloroplast division machinery is FtsZ, a tubulin-like cytoskeletal GTPase that descended from the cyanobacterial ancestor of chloroplasts, where it functioned in cell division (Erickson et al., 2010; Miyagishima et al., 2011; TerBush et al., 2013; Osteryoung and Pyke, 2014; Haeusser and Margolin, 2016). In both bacteria and chloroplasts, FtsZ assembles into a contractile "Z ring" inside the cell or organelle that defines the division site

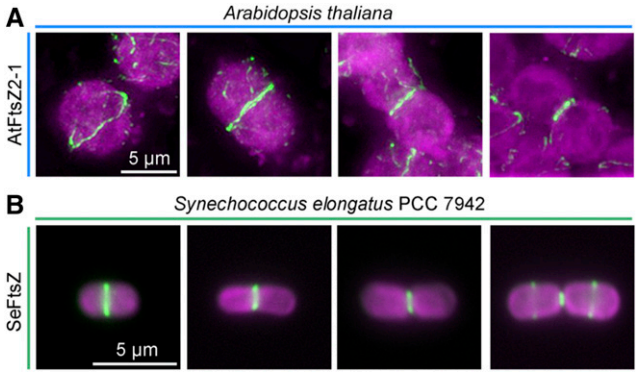


Figure 2. Immunofluorescence localization of the contractile Z ring in chloroplasts and cyanobacterial cells during division. A, Arabidopsis FtsZ2-1 (AtFtsZ2-1) detected with an anti-AtFtsZ2-1 antibody (McAndrew et al., 2001) in mesophyll cells of a fully expanded leaf obtained from a 3-week-old plant (Col-0). B, FtsZ in the cyanobacterium *Synechococcus elongatus* PCC 7942 (SeFtsZ) detected with an anti-*Anabaena* FtsZ antibody (Agrisera). Green, FtsZ; magenta, chlorophyll fluorescence. Bars, 5 μm.

(Fig. 2; Box 1; Friedman and Nunnari, 2014; Addinall et al., 1996; Beech et al., 2000; Gilson et al., 2003; Bilsson-Filho et al., 2017; Osawa et al., 2009; Rothfield et al., 2005; Yang et al., 2017; Wagstaff et al., 2017; Strepp et al., 1997; Nishida et al., 2003; Lutkenhaus et al., 1980; Wang et al., 2017). Purified FtsZ undergoes GTP-dependent self-assembly into single-stranded polymers called protofilaments. Because the GTPase active site is formed within the subunit interface by the interaction of two monomers, polymerization catalyzes GTP hydrolysis. Hydrolysis destabilizes the interface, leading to protofilament fragmentation and subunit dissociation (Mukherjee and Lutkenhaus, 1998; Scheffers et al., 2002; Redick et al., 2005; Huecas et al., 2007; Chen and Erickson, 2009; Erickson et al., 2010). Released subunits can exchange nucleotides and recycle back into protofilaments. This dynamic GTPase-dependent turnover was essential for constriction of Z rings reconstituted on tubular liposomes (Osawa et al., 2008; Osawa and Erickson, 2011) and is likely required for Z-ring constriction in vivo.

Because most bacteria have only a single *FtsZ* gene, bacterial Z rings are composed of homopolymers. In contrast, the majority of photosynthetic eukaryotes appear to encode two plastid-targeted, stroma-localized paralogs of FtsZ (Osteryoung and Vierling 1995; McAndrew et al., 2001; Leger et al., 2015; Box 1). These are called FtsZ1 and FtsZ2 in the green lineage, where they appear to be universally conserved, and FtsZA and FtsZB in red algae (Miyagishima et al., 2004; TerBush et al., 2013). FtsZ1 and FtsZ2 colocalize to Z rings in vivo (McAndrew et al., 2001; Vitha et al., 2001), are both required for normal chloroplast division in Arabidopsis (*Arabidopsis thaliana*; Osteryoung et al., 1998; Yoder et al., 2007; Schmitz et al., 2009), and coassemble in heteropolymers (Olson et al., 2010; TerBush and Osteryoung, 2012; Yoshida et al., 2016; Fig. 1). FtsZ2 and FtsZA are structurally more similar to bacterial FtsZs, in that they both retain a conserved C-terminal peptide (CTP), which in bacteria interacts with membrane proteins to anchor Z rings to the plasma membrane (Ma and Margolin, 1999; Vaughan et al., 2004; Margolin, 2005; Haeusser and Margolin, 2016). Similarly, the Arabidopsis FtsZ2 CTP interacts with two proteins in the chloroplast inner envelope membrane (IEM; Fig. 1, E–G; described below). In contrast, FtsZ1 and FtsZB lack the CTP, and no interaction between FtsZ1 and any membrane protein has been detected, suggesting its presence in the Z ring is a consequence of coassembly with FtsZ2 (Maple et al., 2005; Glynn et al., 2008; Zhang et al., 2016).

Recently, FtsZA and FtsZB from the red alga *Galdieria sulphuraria* were also shown to copolymerize in vitro (Chen et al., 2017), indicating that FtsZ heteropolymerization is a conserved feature and may be the physiologically relevant state. Studies of FtsZ dynamics in heterologous yeast systems are beginning to provide insight into the functional significance of heteropolymerization. These systems lack FtsZ and any native assembly regulators, allowing the intrinsic assembly and dynamic properties of FtsZ proteins to be investigated (Srinivasan et al., 2008; TerBush et al., 2016; Yoshida et al., 2016). When fluorescent fusions of Arabidopsis FtsZ2 (AtFtsZ2) and AtFtsZ1 were expressed separately in the fission yeast *Schizosaccharomyces pombe*, each assembled homopolymeric filamentous structures (homofilaments) in the cytosol (TerBush and Osteryoung, 2012), as do purified FtsZ2 and FtsZ1 in vitro (El-Kafafi et al., 2005; Lohse et al., 2006; Olson et al., 2010; Smith et al., 2010). Fluorescence recovery after photobleaching (FRAP) experiments showed that AtFtsZ2 homofilaments exhibited a much lower degree of subunit turnover than AtFtsZ1 homofilaments. When coexpressed, AtFtsZ2 and AtFtsZ1 colocalized in heterofilaments, which were considerably more dynamic than AtFtsZ2 homofilaments. Similar results were obtained for *G. sulphuraria* FtsZA (GsFtsZA) and GsFtsZB expressed in *S. pombe*; GsFtsZA homofilaments were less dynamic than GsFtsZB homofilaments, and GsFtsZA/GsFtsZB heterofilaments were more dynamic than GsFtsZA homofilaments (Terbush et al., 2017). In a related study, Yoshida et al. (2016) reconstituted Z rings in the yeast *Pichia pastoris* by fusing a membrane-tethering sequence (MTS; Osawa et al., 2008) to the C terminus of AtFtsZ2, enabling it to bind directly to the plasma membrane in the yeast cells. MTS-tagged AtFtsZ2 assembled into a well-defined ring, but MTS-tagged AtFtsZ1 did not. When expressed together, MTS-tagged AtFtsZ2 and AtFtsZ1 (without the MTS) coassembled in the membrane-tethered ring, and FRAP showed these rings were more dynamic than AtFtZ2 rings. Further, both types of rings could be induced to constrict, and coassembled rings constricted more rapidly. These findings, along with studies of Arabidopsis *ftsZ* mutants (Yoder et al., 2007; McAndrew et al., 2008; Schmitz et al., 2009), suggest that the more bacterial-like FtsZ2 and FtsZA proteins establish

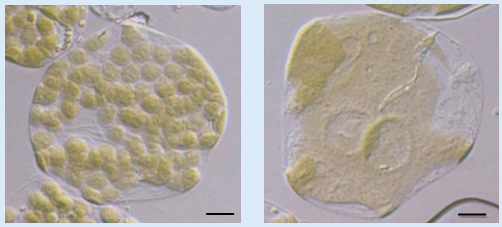
BOX 1. FtsZ Function in the Division of Bacteria and Endosymbiotic Organelles

FtsZ in Bacteria

FtsZ (Filamentous temperature-sensitive Z), first identified in *E. coli,* is essential for cell division in most prokaryotes. It was so-named because temperature-sensitive mutants formed long filaments at the restrictive temperature (Lutkenhaus et al., 1980; Addinall et al., 1996). The Z ring acts partly as a scaffold for recruitment of downstream division proteins and it pulls the plasma membrane inward during constriction (Osawa et al., 2009; Erickson et al., 2010; Haeusser and Margolin, 2016). Recently, it was shown that protofilaments in bacterial Z rings undergo GTPase-dependent treadmilling, which pilots the peptidoglycan cell-wall synthesis machinery (Box 2) around the division site (Bisson-Filho et al., 2017; Yang et al., 2017). The Z ring may be composed of short overlapping protofilaments *in vivo*, but its architecture remains unclear (Lutkenhaus and Du 2017; Wagstaff et al., 2017).

FtsZ in Eukaryotic Organelles

The first-discovered organellar FtsZ was identified in Arabidopsis, where it is encoded in the nucleus and targeted to the chloroplast stroma. Its similarity to cyanobacterial FtsZs implied an endosymbiotic origin (Osteryoung and Vierling, 1995). FtsZ mutants in *Physcomitrella patens* and Arabidopsis displayed grossly enlarged chloroplasts (Box 1 Fig.), analogous to the bacterial filamentation phenotype, proving a role for FtsZ in chloroplast division (Osteryoung et al., 1998; Strepp et al., 1998). Mitochondria also arose by endosymbiosis and in many organisms retain nuclear-encoded FtsZs that likely function in their division (Beech et al., 2000; Gilson et al., 2003; Nishida et al., 2003; Leger et al., 2015). However, mitochondrial FtsZ has been lost in fungi, animals, green algae and land plants (Leger et al., 2015), which seem to use primarily dynamin-related proteins to constrict their mitochondria from the outside (Friedman and Nunnari, 2014; Purkanti and Thattai, 2015; Arimura, 2018). Although most bacteria bear only a single FtsZ, two distinct types of plastidic and/or mitochondrial FtsZ are found in many eukaryotes (Miyagishima et al., 2004; Leger et al., 2015). The two FtsZ types in plastids copolymerize, which promotes protofilament and probably Z-ring dynamics (Olson et al., 2010; TerBush and Osteryoung, 2012; Yoshida et al., 2016; Chen et al., 2017; Terbush et al., 2017). The assembly and dynamic properties of mitochondrial FtsZs have yet to be investigated.



Box 1 Figure . Images of Arabidopsis leaf mesophyll cells from a wild-type Col-0 plant (left) and a mutant null for *FtsZ* (right; Schmitz et al., 2009). The enlarged morphology in the mutant is the chloroplast equivalent of the filamentation phenotype of bacterial *ftsZ* mutants. Bars, 10 μm.

the structural framework and impart stability to chloroplast Z rings, while FtsZ1 and FtsZB enhance Z-ring dynamics through copolymerization.

The biochemical mechanism by which FtsZ1 and FtsZB increase protofilament turnover is not yet clear, but one possibility is that they introduce lower-affinity FtsZ-FtsZ interfaces into heteropolymers. This is suggested partly by experiments showing that coassembled GsFtsZA/GsFtsZB protofilaments were much more dynamic than GsFtsZA protofilaments in vitro (Chen et al., 2017). Additionally, mutation of a conserved residue required for GTP hydrolysis drastically reduced turnover of AtFtsZ2 and GsFtsZA homofilaments in *S. pombe*, as expected based on the GTPase-dependent turnover of bacterial FtsZ described above. Surprisingly, however, the equivalent mutations in AtFtsZ1 and GsFtsZB reduced but did not abolish turnover of these homofilaments, suggesting their dynamic behavior is not solely dependent on GTPase activity (TerBush and Osteryoung, 2012; Terbush et al., 2017). Collectively, these studies provide evidence that the duplication, functional divergence, and coassembly of plastid FtsZs imparted a new mechanism for facilitating Z-ring dynamics in red- and green-lineage chloroplasts.

Interestingly, two FtsZ types are also widespread in organisms bearing secondary plastids or that retain mitochondrial FtsZ (Miyagishima et al., 2004; Leger et al., 2015; Box 1). Additionally, a third FtsZ family,

FtsZ3, is found in a subset of photosynthetic eukaryotes, where its occurrence may be correlated with the retention of a chloroplast peptidoglycan wall (Box 2; Cassier-Chauvat and Chauvat, 2014; Grosche and Rensing, 2017; Hirano et al., 2016; Kasten et al., 1997; Katayama et al., 2003; Homi et al., 2009; Takano and Takechi, 2010; Martin et al., 2009b, 2009b; Machida et al., 2006; Lin et al., 2017).

SPATIAL REGULATION OF DIVISION: THE CHLOROPLAST MIN SYSTEM

The Z ring is the first structure to assemble at the division site (Miyagishima et al., 2001; Fig. 1), and its placement likely establishes the placement of downstream components. In bacteria, Z-ring positioning is controlled by a negative regulatory system called the Min system that prevents self-assembly of Z rings everywhere but at the division site (Box 3; Miyagishima et al., 2005; Monahan et al., 2014; de Boer et al., 1989; reviewed in Lutkenhaus, 2007; Rowlett and Margolin, 2013). In *E. coli* and cyanobacteria (MacCready et al., 2017), the Min system concentrates MinC, the direct inhibitor of FtsZ polymerization, near the cell poles through a remarkable oscillatory mechanism driven by MinD and MinE, which function as regulators of MinC localization (Box 3). Homologs of cyanobacterial MinD and MinE acquired through endosymbiosis have been retained in the green lineage and localize to the stroma, where they play roles in the spatial regulation of chloroplast division and Z-ring placement analogous to those in bacteria (Colletti et al., 2000; Itoh et al., 2001; Vitha et al., 2003; Fujiwara et al., 2004; Aldridge and Møller, 2005; Glynn et al., 2007; Fujiwara et al., 2008). However, in many species, MinC has been lost and instead replaced by the stromal protein ARC3 (Accumulation and Replication of Chloroplasts3; Shimada et al., 2004; Maple et al., 2007). A MinC-like role for ARC3 was initially suggested by the phenotypes of Arabidopsis *arc3* mutants (Pyke and Leech, 1992), which displayed multiple chloroplast constrictions, multiple Z rings, and mispositioning of chloroplast division sites, leading to heterogeneity in chloroplast size and number (Glynn et al., 2007; Maple et al., 2007; Fig. 3), reminiscent of bacterial *minicell* phenotypes (de Boer et al., 1990; Yu and Margolin, 1999; Box 3). ARC3 interacts directly with AtFtsZ1 and AtFtsZ2 and inhibits their assembly in *S. pombe* (Maple et al., 2007; TerBush and Osteryoung, 2012; Zhang et al., 2013). Its overexpression in Arabidopsis produces dose-dependent chloroplast enlargement and fragmented FtsZ filaments (Maple et al., 2007; Zhang et al., 2013), resembling MinC overexpression in *E. coli* and cyanobacteria (de Boer et al., 1990; MacCready et al., 2017; Box 3). Finally, the large-chloroplast phenotypes in Arabidopsis MinD overexpressors and *minE* mutants (Colletti et al., 2000; Glynn et al., 2007; Fig. 3) were completely suppressed in the absence of ARC3, consistent with a role for these proteins as ARC3 regulators (Zhang et al., 2013). These findings established ARC3 as a functional replacement for MinC and the proximal inhibitor of Z-ring assembly in Arabidopsis chloroplasts. However, ARC3 is not found in all green-lineage organisms; some, including *Physcomitrella patens* bear sequences with partial similarity to MinC (Yang et al., 2008; Osteryoung and Pyke, 2014), whose functions have not been tested.

ARC3 and MinC share little sequence similarity and may inhibit FtsZ assembly by different mechanisms. MinC acts by promoting protofilament breakage at GDP-bound subunit interfaces, competing with Z-ring anchoring proteins for FtsZ binding and inhibiting protofilament bundling (Shen and Lutkenhaus, 2009, 2010; Lutkenhaus and Du, 2017). Unlike MinC, ARC3 bears an FtsZ-like region, though it lacks conserved residues required for GTP binding and hydrolysis (Shimada et al., 2004). This region may interact with FtsZ (Maple et al., 2007), perhaps sequestering FtsZ subunits and thereby antagonizing polymerization. Like MinC, ARC3 may also have multiple assembly inhibitory activities.

Although it is unknown whether MinD, MinE, and ARC3 oscillate in chloroplasts and the full localization of ARC3 is not entirely clear, the MinC-like role of ARC3 in preventing Z-ring formation at nondivision sites suggests that ARC3 and MinD must localize at least partly to membrane regions away from the midplastid. Such localization is suggested by immunostaining showing that MinD and MinE localize partly to punctate structures dispersed over the envelope membrane (Nakanishi et al., 2009; Miyagishima et al., 2011; Fig. 1C). However, the chloroplast Min system in Arabidopsis differs from bacterial systems in several other respects. In bacteria, MinD binds directly to the membrane toward polar zones, where it recruits MinC (Lutkenhaus, 2007). In Arabidopsis, MinD association with the membrane requires the green-lineage-specific transmembrane protein MCD1 (Nakanishi et al., 2009; Fig. 1C). ARC3 might be recruited to the membrane by an MCD1-MinD complex, but this is not yet known. Additionally, MinC only interacts with MinD, whereas ARC3 interacts with both MinD and MinE (Maple et al., 2007), which colocalize in vivo (Miyagishima et al., 2011). Defining the complex interactions among ARC3, MinD, MinE, MCD1, and FtsZ will be important for understanding how the chloroplast Min system spatially regulates Z-ring formation.

ARC3 also colocalizes with FtsZ to the midplastid (Shimada et al., 2004), likely through interaction with the IEM transmembrane protein PARALOG OF ARC6 (PARC6; also called CDP1; Fig. 1, C and I). Chloroplasts in Arabidopsis *parc6* mutants displayed multiple and misplaced constrictions, multiple Z rings (though less well defined than in *arc3* and *minD* mutants; Fig. 3) and excessively long FtsZ filaments. Further, PARC6 overexpression produced enlarged chloroplasts with small FtsZ fragments, similar to ARC3 overexpression. These phenotypes implicated PARC6 as an additional negative regulator of FtsZ assembly and Z-ring positioning factor (Glynn et al., 2009; Zhang et al., 2009). The stromal region of PARC6 interacts with both FtsZ2 and ARC3 (Zhang et al., 2016; Fig. 1I), and in vivo FRAP

BOX 2. Co-occurrence of a Chloroplast Peptidoglycan Wall and FtsZ3

Like other bacteria, cyanobacteria are surrounded by a cell wall composed of peptidoglycan (PG) (Cassier-Chauvat and Chauvat, 2014). PG synthesis at the midcell causes cell-wall ingrowth and is essential for cytokinesis. The plastids of glaucophytes, the most basal group in Archaeplastida, retain the PG wall from the endosymbiont, but until recently PG could not be detected in other photosynthetic eukaryotes (reviewed in Takano and Takechi, 2010). However, plastid division in *P. patens* (moss), but not tomato, an angiosperm, was inhibited by antibiotics that specifically inhibit PG synthesis in bacteria, resulting in the formation of macrochloroplasts (Kasten et al., 1997; Katayama et al., 2003). Further, homologs of the full set of *Mur* and other genes required for synthesis of bacterial PG (also called murein) were identified in moss (Machida et al., 2006; Homi et al., 2009), and a PG wall has now been detected surrounding moss chloroplasts (Hirano et al., 2016). All the pathway genes are also found in the streptophyte green alga *Klebsormidium flaccidum*, which is closely related to land plants, in the basal vascular plant *Selaginella moellendorffii*, and potentially in two gymnosperms, suggesting retention of a PG wall in these groups as well (Machida et al., 2006; Homi et al., 2009; Lin et al., 2017). The full gene set is missing in red algae, chlorophyte green algae such as *Chlamydomonas*, and angiosperms, suggesting multiple independent losses of plastid PG synthesis.

Intriguingly, a third type of FtsZ, FtsZ3 (see nomenclature in Martin et al., 2009b), is found in the same subset of organisms that bear the full set of PG-synthesis genes, suggesting a functional link (Grosche and Rensing, 2017). Supporting this, glaucophyte FtsZs and green-lineage FtsZ3s form a distinct clade. FtsZ3 function has only been studied in moss, where it localizes to the Z ring and is required for normal chloroplast division (Martin et al., 2009a; Martin et al., 2009b). Because much of the bacterial PG synthesis machinery is recruited to the midcell by FtsZ, FtsZ3 could conceivably play a specific role in recruiting the plastid PG-synthesis machinery to the division site. Another possibility is that FtsZ3 guides the machinery around the division site (see Box 1). However, moss FtsZ3-GFP also localizes to a cytosolic ring and has been implicated in cell division (Martin et al., 2009a; Martin et al., 2009b). This is an area ripe for investigation.

data suggest ARC3 may accelerate Z-ring remodeling (Johnson et al., 2015). PARC6-ARC3 interaction is mediated by a C-terminal region of ARC3 called the Membrane Occupation and Recognition Nexus (MORN) domain (Glynn et al., 2009; Zhang et al., 2016; Fig. 1I). However, the MORN domain also inhibits ARC3 interaction with FtsZ (Maple et al., 2007; Zhang et al., 2013). These and related findings suggested a model in which PARC6 may bring ARC3 and FtsZ into close proximity at the division site, where PARC6-ARC3 interaction would sequester the MORN domain, enabling ARC3 to interact with FtsZ (Zhang et al., 2016). The negative effect of ARC3 on FtsZ assembly would facilitate Z-ring remodeling during constriction. This effect would presumably be less pronounced than at non-division sites, where Z-ring formation is fully inhibited (Fig. 1C). MinD, MinE, and MCD1 may also contribute to ARC3-mediated Z-ring remodeling because they also localize partly to the midplastid (Nakanishi et al., 2009; Miyagishima et al., 2011; Fig. 1C).

PARC6 has also been detected near plastid poles, which may indicate its retention at newly formed poles immediately following division (Glynn et al., 2009). If ARC3 is also retained or concentrated at these positions following division, this could potentially prevent premature Z-ring assembly and misplacement prior to reestablishment of a new division site (Osteryoung and Pyke, 2014; Zhang et al., 2016). These ideas are speculative but suggest avenues for future research.

OUTER DIVISION COMPONENTS

The outer chloroplast division machinery comprises two key contractile rings, the DYNAMIN-RELATED PROTEIN5B (DRP5B) ring, and the outer plastid-dividing (PD) ring (Fig. 1, H and I). DRP5B, also called ACCUMULATION AND REPLICATION OF CHLOROPLASTS5 (ARC5; Gao et al., 2003), is a plant-specific subfamily of the dynamin GTPases (Gao et al., 2003; Miyagishima et al., 2003), which mediate constriction of many organelle types (Purkanti and Thattai, 2015). Arabidopsis *arc5* mutants have fewer chloroplasts than wild type that display an enlarged dumbbell shape, suggesting DRP5B is required for sustaining and/or completing

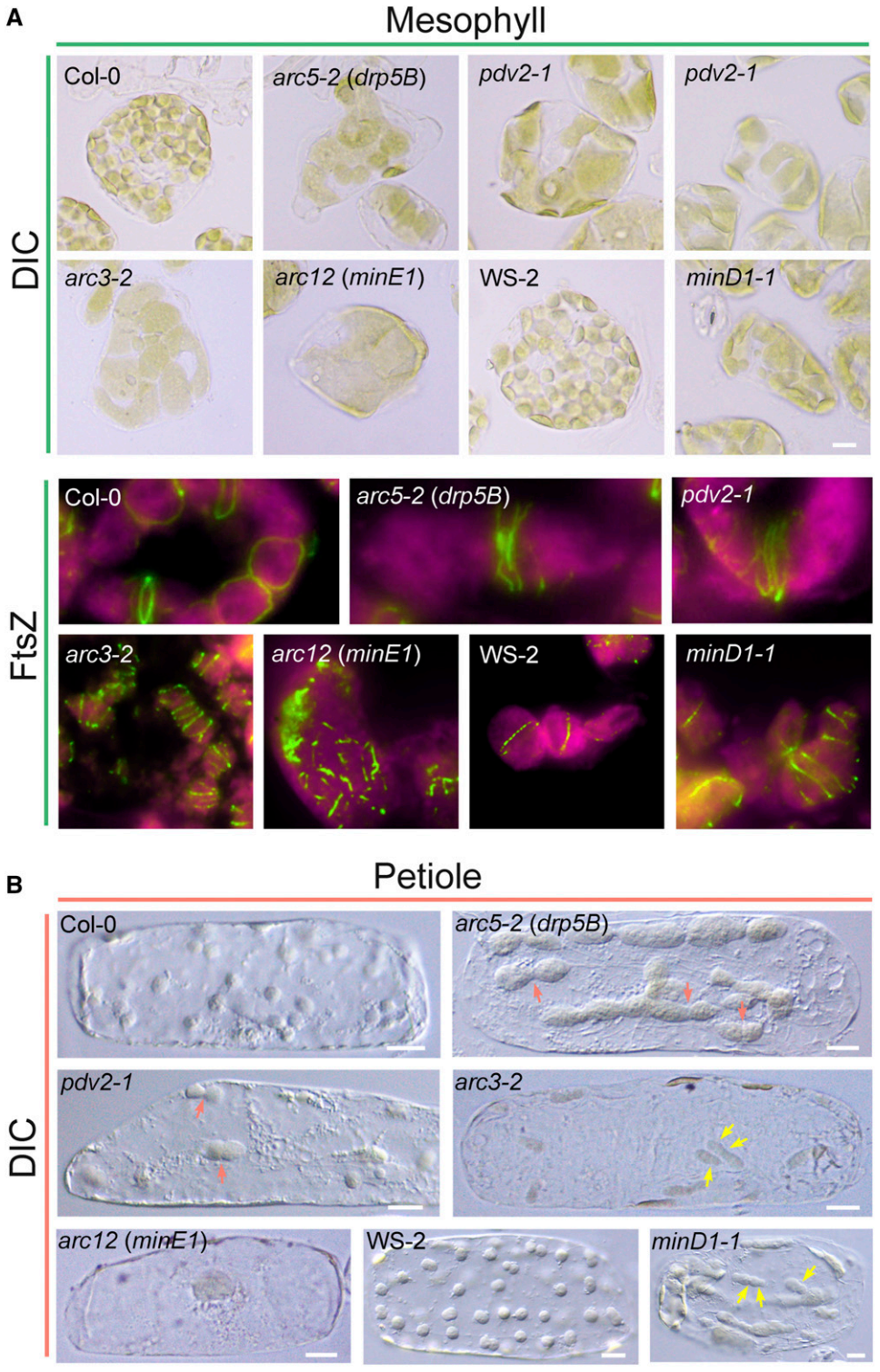


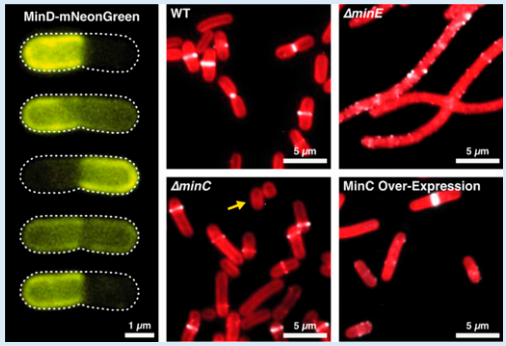
Figure 3. Chloroplast morphology and Z-ring localization patterns in Arabidopsis wild-type plants and various chloroplast division mutants. Although phenotypes vary, most mutants display reduced numbers of enlarged chloroplasts (Osteryoung and Pyke, 2014). A, Chloroplast morphology observed by differential interference contrast (DIC) microscopy (top) and Z-ring localization detected by immunofluorescence staining (bottom) in mesophyll cells. The *minD1-1* mutant (Zhang et al., 2013) is in the WS-2 background; all others are in Col-0. Images of FtsZ localization were adapted from Miyagishima et al. (2006) (Col-0, *pdv2-1* and *arc5-2)* and Zhang et al. (2013) (*arc12* and *minD1-1*) with permission; copyright © 2006 and 2013 by the American Society of Plant Biologists. Green, FtsZ; magenta/red, chlorophyll fluorescence. B, Chloroplast morphology in cells isolated from leaf petioles. Petiole cells contain fewer chloroplasts than mesophyll cells, and the morphology phenotypes are more evident. Red arrows indicate dumbbell-shaped chloroplasts in *pdv2-1* and *arc5-2* mutants. Yellow arrows denote asymmetric division planes in *arc3-2* and *minD1-1* mutants. Bars, 10 μm.

constriction (Pyke and Leech, 1994). DRP5B is recruited from cytosolic patches to the external surface of the chloroplast by the OEM proteins PDV1 and PDV2 (Gao et al., 2003; Miyagishima et al., 2006; Fig. 1H), which may regulate its GTPase activity (Holtsmark et al., 2013). DRP5B also participates in the division of peroxisomes and mitochondria, indicating possible cross-talk in the replication of these organelles (Zhang and Hu, 2010; Aung and Hu, 2012; Kao et al., 2018; Arimura, 2018).

The outer PD ring (Fig. 1, D, H, and I) is an electron-dense contractile structure observed in algae and land plants and characterized most extensively in the unicellular red alga *Cyanidioschyzon merolae* (Kuroiwa

BOX 3. Bacterial Min System Function

The core function of the Min system is to confine FtsZ polymerization and Z-ring assembly solely to the midzone. Min proteins are so named because their mutation leads to mispositioned division planes and asymmetrical division in bacteria, causing a characteristic "mini-cell" phenotype (de Boer et al., 1989). In rod-shaped *E. coli*, Z-ring formation is inhibited at cell poles by the dynamic pole-to-pole oscillation of MinC, MinD, and MinE (Rothfield et al., 2005). MinD is a membrane-binding ATPase that first associates with one end of the cell and recruits MinC, a direct inhibitor of FtsZ assembly. MinE then interacts with membrane-bound MinCD, stimulating MinD ATPase activity and accelerating membrane dissociation of MinCD. A higher-order behavior emerges from these interactions in which MinE "chases" MinCD from one pole to the other. This dynamic localization produces a time-averaged gradient of MinCD concentration that is highest at the poles, antagonizing FtsZ polymerization, and lowest at midcell, enabling centralized Z-ring assembly (Lutkenhaus, 2007). Although Min-system composition varies between species (Monahan et al, 2014), cyanobacteria possess *minC*, *minD*, and *minE* genes functionally similar to those in *E. coli* (Mazouni et al., 2004; Miyagishima et al., 2005; Nakanishi et al., 2009; Cassier-Chauvat and Chauvat, 2014; MacCready et al., 2017). Loss of Min function in the cyanobacteria *Synechocystis sp. PCC6803* (Mazouni et al., 2004) and *Synechococcus elongatus* (MacCready et al., 2017) leads to aberrant Z-ring formation and/or positioning. For example, *minE* deletion in rod-shaped *S. elongatus* results in loss of MinCD oscillation, disorganized FtsZ polymerization, and cell elongation, consistent with positionally uncontrolled FtsZ inhibition throughout the cell (Box 3 Fig.). Conversely, *minC* deletion removes the direct antagonist of FtsZ polymerization, allowing formation of misplaced Z rings and misplaced divisions that yield a broad distribution of cell lengths. Overproduction of MinC inhibits FtsZ polymerization globally, producing more elongated cells (MacCready et al., 2017) (Box 3 Fig.). These phenotypes are reminiscent of those observed in chloroplasts of analogous Arabidopsis mutants (Zhang et al, 2013). It should be noted that the extensive thylakoid membrane networks within cyanobacteria and chloroplasts might be expected to disrupt Min-system function and/or oscillation. Yet Min proteins oscillate from pole to pole in cyanobacteria (MacCready et al., 2017) (Box 3 Fig.), suggesting this might also occur in chloroplasts.



Box 3 Figure. The bacterial Min system in *S. elongatus*. Left, time-lapse imaging showing oscillation of MinD-mNeonGreen over 2 minutes. Right panels, FtsZ immunostaining (white) overlaid on chlorophyll autofluorescence (red) in cells of the indicated genotypes. Yellow arrow indicates a putative minicell produced by misplacement of Z rings and the cell division plane. Scale bars as indicated. Adapted from MacCready et al. (2017) with permission. Copyright 2016 John Wiley & Sons, Ltd.

et al., 2008; Miyagishima et al., 2011; Yoshida et al., 2012), where it is composed primarily of fine polyglucan fibrils. These are probably synthesized by Plastid-Dividing Ring1 (PDR1), a putative glycosyltransferase that relocalizes from the cytosol to a ring at the chloroplast-division site and disperses following division (Yoshida et al., 2010). DRP5B is recruited to the chloroplast after appearance of the outer PD ring and was speculated to provide the motive force for sliding of PD-ring filaments during constriction (Yoshida et al., 2006; Yoshida et al., 2010). To date, neither the functions of PDR1 homologs nor outer PD-ring composition have been investigated in other organisms.

COORDINATION OF DIVISION COMPLEXES ACROSS THE ENVELOPE MEMBRANES

Chloroplast division requires the coordinated formation and constriction of the stromal and cytosolic contractile machineries across the two envelope membranes. The stromal machinery appears to include an additional contractile structure termed the inner PD ring (Fig. 1D) of unknown composition (Kuroiwa et al., 2002, 2008; Hashimoto, 2005). At present, no data exist on how coordination of the PD rings is achieved, but in Arabidopsis, coordination between the Z ring and DRP5B ring is governed by two sets of paralogous proteins: ARC6

and PARC6 in the IEM, and PDV1 and PDV2 in the OEM (Fig. 1, E–H).

ARC6 descended from the cyanobacterial cell division protein Ftn2 (also called ZipN) and is conserved throughout the green lineage (Koksharova and Wolk, 2002; Vitha et al., 2003; Mazouni et al., 2004; Marbouty et al., 2009). *arc6* mutants (Pyke et al., 1994) possess one or two giant chloroplasts with fragmented FtsZ filaments, while ARC6 overexpressors, though still bearing enlarged chloroplasts, exhibit exceptionally long FtsZ filaments. These phenotypes indicated that ARC6 promotes FtsZ assembly and probably stabilizes the Z ring (Pyke et al., 1994; Vitha et al., 2003). ARC6 spans the IEM with its large N terminus exposed to the stroma and its smaller C terminus protruding into the intermembrane space (IMS; Fig. 1E). The stromal region of ARC6 binds specifically to FtsZ2 via the CTP and probably acts as the primary membrane tether for the Z ring (Johnson et al., 2013; Fig. 1E). The IMS region interacts with the C-terminal IMS region of the land-plant-specific OEM protein PDV2 (Fig. 1F), and this interaction is required for PDV2 localization to the division site (Glynn et al., 2008). A similar relationship exists between PARC6 and PDV1 (Fig. 1G), which arose by duplication and divergence of ARC6 and PDV2, respectively, and may both be confined to vascular plants (Miyagishima et al., 2006; Glynn et al., 2009). In turn, PDV2 and PDV1 recruit DRP5B. Thus, ARC6 and PARC6 convey positional information from the stromal Z ring to the outside of the chloroplast through PDV2 and PDV1 to localize the DRP5B ring.

The dumbbell-shaped appearance and presence of multiple, laterally associated Z rings near the division site in the enlarged chloroplasts of Arabidopsis *pdv1* and *pdv2* mutants (Fig. 3) indicates that division is initiated but not completed in these mutants (Miyagishima et al., 2006) and suggests that information is also relayed from the external to internal division complexes during normal chloroplast division. A recent study combining x-ray crystallography, interaction assays, and genetic analysis in Arabidopsis provides evidence for this hypothesis (Wang et al., 2017). The authors demonstrated that part of the IMS region of PDV2 ($PDV2_{IMS}$) inserts into a pocket formed by a highly conserved region of the $ARC6_{IMS}$ (Kumar et al., 2016) and that a second PDV2 molecule induces $ARC6_{IMS}$ dimerization, resulting in the formation of a heterotetramer (Fig. 1F). $ARC6_{IMS}$ dimerization depended on interaction between the cytosolic regions of the two PDV2 molecules. A dimerization-deficient mutant of PDV2 produced multiple, uncondensed ARC6 rings in vivo instead of the single ring observed in wild type (Wang et al., 2017). Thus, PDV2 dimerization on the cytosolic surface transmits information inside the chloroplast, resulting in the formation of a single ARC6 ring. Though not specifically tested in this study, PDV2-induced ARC6 dimerization may also contribute, along with the chloroplast Min system, to the formation or maintenance of a single, condensed Z ring in the stroma, though by an unknown mechanism (Wang et al., 2017).

There are many additional questions regarding coordination of inner and outer complexes. One concerns the role of DRP5B in the division process. DRP5B recruitment to the chloroplast probably involves direct interaction with PDV proteins (Miyagishima et al., 2006; Holtsmark et al., 2013). Therefore, DRP5B might facilitate the dimerization of PDV2, leading to condensed ARC6 and Z rings. This possibility is suggested by the phenotype of *arc5*, a *DRP5B* mutant (Robertson et al., 1996; Gao et al., 2003), that also exhibits multiple, uncondensed Z rings near the middle of its dumbbell-shaped chloroplasts (Miyagishima et al., 2006; Fig. 3). Another question is whether PDV1-PARC6 interaction (Fig. 1G) might also relay information from outside to inside the chloroplast. Assuming so, the effect might be to destabilize rather than stabilize Z rings, since PARC6 negatively regulates Z-ring formation, probably at least partly through its interaction with ARC3 (Glynn et al., 2009; Zhang et al., 2009, 2016; Fig. 1I).

OTHER CHLOROPLAST DIVISION PROTEINS

A few other proteins with less well-defined functions also contribute to chloroplast division (Basak and Møller, 2013; Osteryoung and Pyke, 2014). Final separation of chloroplasts appears to involve two proteins: CLUMPED CHLOROPLASTS1, a cystolic protein localized partly near the plasma membrane and partly on the chloroplast (Yang et al., 2011), and CRUMPLED LEAF, located in the OEM (Asano et al., 2004; Chen et al., 2009; Sugita et al., 2012). Both proteins were speculated to mediate chloroplast attachment to the cytoskeleton. GIANT CHLOROPLAST1 is a stromal protein associated with the IEM that bears some similarity to the bacterial cell-division inhibitor SulA and may play an indirect role in division-site placement (Maple et al., 2004; Raynaud et al., 2004).

REGULATION OF CHLOROPLAST DIVISION

Knowledge on how chloroplast division is regulated is still rudimentary, particularly in land plants with multiple chloroplasts per cell whose division is not tightly coordinated with cell division (Miyagishima, 2011; Pedroza-Garcia et al., 2016). Here, we highlight recent work on selected aspects of division regulation.

To achieve a permanent endosymbiotic relationship and ensure faithful organelle inheritance, the eukaryotic host needed to establish synchrony between endosymbiont division and host cell cycles (Pedroza-Garcia et al., 2016). Massive gene transfer to the nucleus and loss from the chloroplast genome following endosymbiosis solved part of this problem by bringing most genes under host control (Keeling, 2010). Miyagishima et al. (2012) used synchronized cultures of several unicellular organisms representing the major lineages of primary plastid-bearing algae to investigate the expression patterns of key chloroplast division genes and proteins during the cell cycle. They found that all the

nuclear genes were expressed during S phase, when Z-ring formation and chloroplast division were initiated, except *FtsZ* from the glaucophyte *Cyanophora paradoxa*, which was constitutively expressed (Miyagishima et al., 2012). Nevertheless, Z-ring formation in *C. paradoxa* was still confined to the S phase. In contrast, *minD* and other chloroplast division genes in the plastid genomes of the green algae *Chlorella vulgaris* and *Mesostgma viride* were expressed constitutively, but chloroplast division still began during S phase. The results suggested that expression of endosymbiont-derived plastid-division genes that reside in the nucleus is under tighter host control than expression of genes retained in the plastid genome and that nuclear-gene expression governs the timing of chloroplast division (Miyagishima et al., 2012). The molecular mechanisms controlling the phase-specific timing of gene expression and chloroplast division remain unclear.

Accumulating evidence indicates that retrograde signals from the chloroplast also exert control over the host cell cycle (Garton et al., 2007; Kobayashi et al., 2009, 2011; Pedroza-Garcia et al., 2016). A recent study in *C. merolae* (Sumiya et al., 2016) revealed that cell-cycle progression was arrested in prophase when chloroplast division, which is sensitive to FtsZ level (Vitha et al., 2001), was disrupted by FtsZ overexpression prior to assembly of the mature division machinery. Two indicators of the G2-to-M transition, increased cyclin B expression and relocalization of cyclin-dependent kinase B, were blocked in the arrested cells. However, once the assembled chloroplast division complex began to constrict, disruption of chloroplast division no longer impeded cell-cycle progression. These results revealed that insufficient assembly of the chloroplast division machinery imposes a checkpoint on the cell cycle. Further analysis suggested the checkpoint is sensed between Z-ring formation and DRP5B recruitment (Sumiya et al., 2016). How this retrograde signal is conveyed to the nucleus is unknown.

Cytokinin has been implicated in the control of chloroplast division in *P. patens* (Abel et al., 1989; Reutter et al., 1998), and recent work indicates this is partly through regulation of *PDV* gene expression. Overexpression of the PDV proteins results in increased chloroplast number and decreased chloroplast size in Arabidopsis and *P. patens*, indicating that PDV1 and PDV2 levels influence the frequency of chloroplast division (Okazaki et al., 2009; Chang et al., 2017). Overexpression of Cytokinin-Responsive Transcription Factor2 or exogenous cytokinin treatment produced a similar phenotype in Arabidopsis, and these plants had elevated levels of PDV1 and PDV2 but not other division proteins. Similarly, treatment of moss with cytokinin specifically increased *PDV2* transcript levels and chloroplast division (Okazaki et al., 2009). Recently, Vercruyssen et al. (2015) reported that overexpression of another transcription factor, GROWTH REGULATING FACTOR5 (GRF5), stimulates chloroplast and cell division. Because cytokinin has similar effects, they proposed that GRF5 and cytokinin cooperate in the control of chloroplast division. However, *PDV2* expression was not increased in GRF5 overexpressors, suggesting GRF5 promotes chloroplast division by a different mechanism (Vercruyssen et al., 2015).

Another study showed that chloroplast division is impaired in mutants deficient in gibberllins (GA) (Jiang et al., 2012). *FtsZ2*, *ARC6*, *DRP5B*, and *PDV* transcript levels were dramatically decreased in the mutant. Exogenous GA treatment restored wild-type chloroplast division and transcript levels. Based on additional mutant studies, the authors proposed that GA might indirectly stimulate chloroplast division through promoting the degradation of DELLA protein family members, which negatively regulate chloroplast division (Jiang et al., 2012).

A mutant screen in Arabidopsis identified the transcription factor FHY3/CPD45, a key regulator of far-red light signaling, and its homolog FRS4/CPD25, as coactivators of *ARC5* (*DRP5B*) expression and chloroplast division (Gao et al., 2013). *fhy3/cpd45* and *frs4/cpd25* mutants had enlarged dumbbell-shaped chloroplasts, similar to *arc5* (Fig. 3). Expression of a downstream target of *FHY3/CPD45* or of *ARC5* in *fhy3/cpd45* rescued only the far-red light signaling or chloroplast division defect, respectively, suggesting that *FHY3/CPD45* activates *ARC5* expression and far-red light signaling through independent pathways (Chang et al., 2015).

Recently, Okazaki et al. (2015) reported that the phosphoinositide phosphatidylinositol 4-phosphate (PI4P) negatively regulates chloroplast division in Arabidopsis. Inhibition of phosphatidylinositol 4-kinase to reduce PI4P levels in chloroplast membranes accelerated chloroplast division, producing a larger population of smaller chloroplasts, similar to overexpression of PDV proteins (Okazaki et al., 2009). However, this effect was due primarily to increased recruitment of DRP5B from the cytosol to the chloroplast surface and not to increased PDV protein levels. PDV1 and PDV2 both bound to PI4P, but the effect of PI4P depletion on chloroplast division was largely abolished in *pdv1* but not *pdv2* mutants. Based on these and other results, the authors proposed that PDV1 interaction with PI4P in the chloroplast envelope alters PDV1 affinity for DRP5B, which may alter the rate of chloroplast division. These findings implicate phosphoinositide signaling in the regulation of chloroplast division (Okazaki et al., 2015).

CONCLUDING REMARKS

While many of the players in chloroplast division have been identified, particularly in Arabidopsis, we still lack detailed mechanistic understanding of many of their biochemical activities and functional interactions as components of a dynamic molecular machine. Moreover, phylogenomic and functional studies indicate significant evolutionary diversity in the composition of the division machinery (Miyagishima et al., 2011). For example, although *ARC6* descended from

OUTSTANDING QUESTIONS

- How do FtsZ1 and FtsZB enhance chloroplast Z-ring dynamics?
- What mechanisms govern ARC3-mediated inhibition of Z-ring assembly and Z-ring placement?
- What are the roles of ARC3, MinD, MinE, and MCD1 at the division site?
- What is the role of MinC-like proteins in plants and algae lacking ARC3?
- What is the connection between FtsZ3 and plastid peptidoglycan synthesis?
- Is outer PD-ring composition conserved, and how does the outer PD ring constrict?
- What is the composition and function of the inner PD ring?
- How are the FtsZ and DRP5B rings coordinated across membranes in organisms lacking homologs of proteins involved in this coordination?
- How is chloroplast division triggered?
- How is chloroplast division integrated with cell division and expansion?
- How do other plastid types divide?
- How are plastid size and number in different cell types controlled?
- How do thylakoids divide?

a closely related cyanobacterial cell division gene (Koksharova and Wolk, 2002; Vitha et al., 2003), no ARC6 homolog or other membrane-tethering protein for the Z ring has been reported in red algae. We still know absolutely nothing about how thylakoids divide; ultrastructural evidence suggests it may occur independently from division of the envelope membranes (Whatley, 1988). We have little information about the division of plastid types other than chloroplasts or about other potential modes of plastid replication, such as a reported budding mechanism (Forth and Pyke, 2006; Pyke, 2016). The mechanisms regulating the control of chloroplast compartment size remain mostly unidentified (Pyke, 1999; Larkin et al., 2016). The extent to which division is coordinated with lipid biosynthesis (Wu and Xue, 2010; Fan and Xu, 2011; Nobusawa and Umeda, 2012) and other metabolic processes is unknown. Finally, how plastid division is regulated and integrated with cell division and expansion, chloroplast biogenesis, and plant growth and development remain poorly understood (Jarvis and López-Juez, 2013; Pedroza-Garcia et al., 2016; see "Outstanding Questions"). Clearly, it is still early days in the study of plastid division—many rich avenues remain to be explored.

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