The molecular biology of plastid division in higher plants

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Abstract

Plastids are essential plant organelles vital for life on earth, responsible not only for photosynthesis but for many fundamental intermediary metabolic reactions. Plastids are not formed de novo but arise by binary fission from pre-existing plastids, and plastid division therefore represents an important process for the maintenance of appropriate plastid populations in plant cells. Plastid division comprises an elaborate pathway of co-ordinated events which include division machinery assembly at the division site, the constriction of envelope membranes, membrane fusion and, ultimately, the separation of the two new organelles. Because of their prokaryotic origin bacterial cell division has been successfully used as a paradigm for plastid division. This has resulted in the identification of the key plastid division components FtsZ, MinD, and MinE, as well as novel proteins with similarities to prokaryotic cell division proteins. Through a combination of approaches involving molecular genetics, cell biology, and biochemistry, it is now becoming clear that these proteins act in concert during plastid division, exhibiting both similarities and differences compared with their bacterial counterparts. Recent efforts in the cloning of the disrupted loci in several of the accumulation and replication of chloroplasts mutants has further revealed that the division of plastids is controlled by a combination of prokaryote-derived and host eukaryote-derived proteins residing not only in the plastid stroma but also in the cytoplasm. Based on the available data to date, a working model is presented showing the protein components involved in plastid division, their subcellular localization, and their protein interaction properties.

Key words: Arabidopsis, arc mutants, cell biology, Min proteins, Plastid division.

Introduction

Plant cells contain plastids which represent one of the principal features that differentiate plant cells from other eukaryotic cells and it is now widely accepted that plastids have arisen from an endosymbiotic event between a proto-eukaryote and a photosynthetic prokaryote (Gray, 1999; McFadden, 2001). Plastids develop from small, colourless, undifferentiated proplastids in the cytoplasm through binary fission. Because of this, plastid division is of paramount importance, not only for the maintenance of plastid populations in dividing plant cells but also in the accumulation of large numbers of chloroplasts in photosynthetic cells, presumably to maximize photosynthesis. Although the basic ultrastructural features of plastid division may seem relatively simple, it is becoming increasingly clear that the cellular processes that govern plastid division represent elaborate pathways of highly co-ordinated events involving proteins of both prokaryotic and eukaryotic origin.

Although dividing chloroplasts were observed back in the late 1960s (Possingham and Saurer, 1969), it is only during the last 5–6 years that the cellular events underlying the division process have started to become unravelled. Not only have a number of new protein components of the division machinery been identified, but insight into biochemical
activities and inter-protein relationships has shed light on the intricate complexity of plastid division in higher plants. This review focuses on the molecular biology of plastid division with the objective of integrating previous knowledge with recent findings in the field.

The overall basic morphology of plastid division

Although most research has centred on the ultrastructural changes that take place during chloroplast division, some information exists, although limited, regarding proplastid division. Proplastids are small, undifferentiated, colourless plastids found in dividing meristematic cells from which all plastids in differentiated cells originate. Meristematic cells contain between 10 and 20 proplastids (Juniper and Clowes, 1965; Lyndon and Robertson, 1976), and these differentiate into a variety of plastid types as cell differentiation proceeds (Cran and Possingham, 1972). In order to maintain appropriate proplastid segregation during cell division, proplastids must divide prior to cytokinesis. Indeed, dumbbell-shaped proplastids containing central constrictions have been observed, indicative of proplastid division (Chaly and Possingham, 1981; Whatley, 1983). In addition, and by contrast with the wild type, the Arabidopsis chloroplast division mutant arc6 (see later sections) has only two enlarged proplastids present in apical meristematic cells, demonstrating that proplastid division is under cellular regulation. In meristematic cells, proplastid division keeps pace with cell division but, as cells differentiate, the number of plastids per cell increases, suggesting that the release of plastid division constraints is an early event during plant cell differentiation (Lyndon and Robertson, 1976). This further suggests that the regulation of proplastid division is different from that of differentiated plastids, although up to now limited evidence exists to support this.

The first documented evidence for chloroplast division came in 1969 when dumbbell-shaped chloroplasts observed in spinach were followed by a subsequent increase in chloroplast number per cell (Possingham and Saurer, 1969). Further observations of dumbbell-shaped chloroplasts in other plant species such as tobacco and sesame established that chloroplasts replicate by constriction division (Bousson et al., 1972; Platt-Aloia and Thompson, 1977) and that the division process can be separated into four distinct but basic stages (Leech et al., 1981; Possingham and Lawrence, 1983): (i) slight plastid elongation; (ii) plastid constriction and dumbbell formation; (iii) further constriction, isthmus formation, and thylakoid membrane separation; and (iv) isthmus breakage, plastid separation, and envelope resealing (Fig. 1).

Initial insight into ultrastructural changes during plastid division came from electron microscopy studies that identified fuzzy plaques of electron-dense material covering or displacing the constricting isthmus of dividing chloroplasts (Leech et al., 1981). Later studies using the alga Cyanidium caldarium, a unicellular organism containing a single chloroplast, revealed this fuzzy plaque to be an electron-dense ring-like structure on the cytosolic face of the membrane encircling the constricting isthmus. This structure was termed the plastid-dividing ring or PD ring (Mita et al., 1986). Subsequently, in Avena sativa, the PD-ring structure was resolved and found actually to consist of two rings with an inner PD ring on the stromal face of the inner envelope and an outer PD ring on the cytosolic face of the outer envelope (Hashimoto, 1986). These two PD rings have now been detected in numerous plant and algal species and are thought to represent a universal feature of dividing chloroplasts in all plant cells. Interestingly, but perhaps unexpectedly, a third middle PD ring was identified in the intermembrane space of single chloroplasts found in the unicellular red alga Cyanidioschyzon merolae (Miyagishima et al., 2001a). Although this third PD ring has only been observed in C. merolae it is highly possible that it is ubiquitous throughout plant species.

Structural analysis utilizing synchronized cultures of C. merolae have provided a model of how the three PD rings may function together, demonstrating that the timing of assembly and the behaviour of each ring is different (Miyagishima et al., 2001c). The inner PD ring forms first, followed by the middle and outer PD rings; however, all of the rings form before any visual constriction at the division site. During constriction, the inner and middle PD rings remain a constant thickness but decrease in overall volume. This volume decrease is in proportion with constriction, suggesting a steady loss of components from these two rings as constriction proceeds. By contrast, the outer PD ring widens and thickens during constriction indicating no loss of components and eventually becomes a wide, thick, and rigid structure. Late in constriction the middle and inner PD rings disassemble before the daughter plastids are severed, while the outer PD ring remains attached until after completion of division and then disassembles (Miyagishima et al., 2001c). The distinct behaviour of the rings implies that PD ring protein composition is different. The thickening of the outer PD ring during constriction suggests that this may provide the driving force necessary for central plastid constriction. Furthermore, the late disassembly of the outer PD ring suggests that it is involved in the completion of division, whereas the inner and middle PD rings are not (Miyagishima et al., 2001c). The outer PD ring has been shown in C. merolae to consist of a bundle of unidentified 5 nm filaments that coil around the constriction site (Miyagishima et al., 2001b). Further analysis identified a 56 kDa protein that is thought to be the main component of the 5 nm bundles (Miyagishima et al., 2001b); however, further evidence is needed to confirm this. The isolation and cloning of arc5 has revealed that ARC5 encodes a dynamin-like protein that localizes into a ring-like structure at the division site (Gao et al., 2003). Because some dynamin
strands have an approximate diameter of 6 nm (Klockow et al., 2002) the idea has been raised that ARC5 might represent the observed 5 nm filaments of the outer PD ring (Gao et al., 2003); however at 87 kDa it is larger than expected. Because plastids have arisen from a cyanobacterial endosymbiont (Gray, 1999) it was speculated relatively early that plastid division may share common features with bacterial cell division. Bacterial cell division is initiated at mid-cell by the polymerization of the FtsZ protein (Bi and Lutkenhaus, 1991; Lutkenhaus and Addinall, 1997) to form a contractile Z ring. FtsZ is a structural homologue of tubulin (Loewe and Amos, 1998) and its assembly at mid-cell is mediated by a complex interplay of cell division proteins encoded by the minB operon (de Boer et al., 1989; Bi and Lutkenhaus, 1993; see later sections). The first evidence for the bacterial ancestry of plastid division came with the identification of a nuclear FtsZ gene in Arabidopsis encoding a protein with over 40% amino acid identity to many bacterial FtsZ proteins (Osteryoung and Vierling, 1995). This protein was shown to have a functional chloroplast targeting transit peptide, and subsequent studies demonstrated that, by contrast with most bacteria encoding a single FtsZ protein, Arabidopsis and other plant species harbour two families of plastid-targeted FtsZs. FtsZ proteins from both of these families were found to co-localize into a Z ring at the division site in Arabidopsis, pea, and tobacco (Fujiwara and Yoshida, 2001; McAndrew et al., 2001; Vitha et al., 2001; J Maple et al., unpublished results). Because of the stromal Z-ring localization it was first thought that FtsZ might have been the main component of the inner PD ring. Further analyses have shown that the Z ring is distinct from components of the inner PD ring and actually forms a ring on the stromal side of the inner PD ring (Miyagishima et al., 2001c). Analysis of Z-ring formation in C. merolae revealed that it forms 3–4 h before the formation of the PD rings (Kuroiwa et al., 2002), suggesting that the Z ring determines the site of division after which there is recruitment and assembly of the PD rings (Kuroiwa et al., 2002). It has been postulated that the Z ring-based system evolved from a cyanobacterial endosymbiont, whereas the PD ring-system has probably been recruited from the eukaryotic host (Miyagishima et al., 2001c).

Accumulation and replication of chloroplast mutants

Although the ultrastructural events that occur during chloroplast division have been studied since the late 1960s, it was not until the 1990s that work began to investigate the nature of the molecular control of plastid division. It was realized that in order to gain a non-biased molecular handle on plastid division, a collection of mutants defective in plastid division was required. A genetic screen was developed based on the visual identification of Arabidopsis ethyl methanesulphonate (EMS)-mutagenized, and later T-DNA-mutagenized, seedlings with altered numbers of chloroplasts in mesophyll cells (Pyke and Leech, 1991; Rutherford, 1996). The resulting collection of arc (accumulation and replication of chloroplasts) mutants define at least 12 loci with greatly reduced (~95%) or greatly increased (+50%) chloroplast numbers per mesophyll cell. They define loci which are important in both the process of plastid division and in the control of plastid population size within a cell during development. The diverse range of phenotypes exhibited by the arc mutants suggest that they encode nuclear genes with several unique roles in the plastid division process and hence represent a rich source of new plastid division genes (Table 1).

arc mutant physiology

The most extreme mutant in the collection is arc6 which has between one and four greatly enlarged chloroplasts per mesophyll cell instead of >100 in wild-type cells (Pyke et al., 1994; Robertson et al., 1995; Vitha et al., 2003). The
low number of chloroplasts per cell is maintained throughout development, and there is no evidence of division events occurring. arc6 seedlings are unique as they are the only mutant in the collection in which proplastids, in both shoot and root meristems, are reduced in number, and all differentiated plastid types within the plant appear to be affected, including mesophyll and epidermal cell chloroplasts, root plastids, guard cell plastids, and petal chloroplasts (Pyke et al., 1994; Robertson et al., 1995; Pyke and Page, 1998). It is believed that these plastids can function normally since the growth of arc6 plants is not severely affected compared with the wild type in controlled growth conditions. Ultrastructure analysis has revealed that, although arc6 chloroplasts are very elongated, the arrangement and distribution of the thylakoid membranes are not drastically different compared with the wild type (Pyke et al., 1994).

The lack of plastid-less cells in the arc6 mutant indicates that segregation of plastids in new cells still occurs and, although the mechanism by which this occurs is still unclear, it may simply involve physical breakage of the plastids during cytokinesis. The controlled segregation of plastids is apparently less stringent in stomatal development since c. 30% of stomata lack plastids in one or both guard cells (Robertson et al., 1995). This phenotype appears to be due to perturbations in proplastid populations leading to plastids segregating abnormally during stomatal development. arc12 is not allelic to arc6 but shows a similar phenotype (Pyke, 1999; Yamamoto et al., 2002).

arc3 and arc5 mutants also show significant reductions in the number of chloroplasts per cell, both containing between 13 and 15 chloroplasts per mesophyll cell (Pyke and Leech, 1992, 1994; Robertson et al., 1996; Morrison et al., 1999). Because in both mutants the number of chloroplasts is the same as the number of proplastids, it is thought that they specifically affect chloroplast division. However, recent studies have shown that the arc5 mesophyll cells can contain between 3 and 15 chloroplasts depending on the growth conditions (Gao et al., 2003), and it will be interesting to examine whether proplastid numbers are affected in the same way.

In both mutants the number of chloroplasts per cell does not increase significantly as the mesophyll cells expand during leaf growth, indicating that arc3 and arc5 chloroplasts do not divide during mesophyll development. Indeed, in arc3, no division profiles have been found, and ARC3 is believed to be involved in the initiation of chloroplast division (Pyke and Leech, 1992, 1994; Morrison et al., 1999). It is speculated that ARC3 plays a role in regulating the expansion of chloroplasts before division, such that in the arc3 mutant expansion goes unchecked and the chloroplasts become too large to divide. arc3 chloroplasts show some irregularity in shape and are elongated and thin, but the stromal thylakoids and grana thylakoids are retained (Shimada et al., 2004).

In arc5 mesophyll cells chloroplasts enter division and arrest when they have become centrally constricted, never completing the division process (Robertson et al., 1996). Analysis of arc5 mesophyll cells shows that a majority of chloroplasts enter division early in cellular development and that the proportion of chloroplasts showing a central constriction rises close to 100% in mature cells. Continued expansion of these chloroplasts results in a mature population of large dumbbell chloroplasts. Interestingly, in wild-type plants a large proportion of plastids at the base of

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**Table 1. A summary of 11 Arabidopsis arc mutants indicating their phenotype, chloroplast size and chloroplast number**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Ecotype</th>
<th>Chloroplast size (μm²)</th>
<th>Chloroplast number/cell</th>
<th>Chloroplasts/l mm² mesophyll cell plan area</th>
<th>Notes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Ler</td>
<td>50</td>
<td>120</td>
<td>25</td>
<td>Spherical</td>
<td>Pyke and Leech, 1992</td>
</tr>
<tr>
<td>WT</td>
<td>Ws</td>
<td>50</td>
<td>80–90</td>
<td>20–23</td>
<td>Spherical</td>
<td>Pyke et al., 1994; Rutherford, 1996</td>
</tr>
<tr>
<td>WT</td>
<td>Col</td>
<td>50</td>
<td>100</td>
<td>23</td>
<td>Spherical</td>
<td>Osteryoung et al., 1998</td>
</tr>
<tr>
<td>arc1</td>
<td>Ler</td>
<td>25</td>
<td>108</td>
<td>32</td>
<td>Increased number of smaller chloroplasts</td>
<td>Pyke and Leech, 1992; Morrison et al., 1999</td>
</tr>
<tr>
<td>arc2</td>
<td>Ler</td>
<td>110</td>
<td>40</td>
<td>9</td>
<td>Fewer chloroplasts/cell than WT</td>
<td>Pyke and Leech, 1992</td>
</tr>
<tr>
<td>arc3</td>
<td>Ler</td>
<td>200–300</td>
<td>18</td>
<td>4–5</td>
<td>Heterogeneous chloroplast size</td>
<td>Pyke and Leech, 1992; Pyke and Leech, 1994; Morrison et al., 1999; Pyke and Leech, 1994; Robertson et al., 1996; Morrison et al., 1999; Gao et al., 2003</td>
</tr>
<tr>
<td>arc5</td>
<td>Ler</td>
<td>300–900</td>
<td>3–15</td>
<td>1–4</td>
<td>Dumbbell-shaped chloroplasts</td>
<td>Pyke and Leech, 1992; Pyke and Leech, 1994; Morrison et al., 1999; Pyke and Leech, 1994; Robertson et al., 1996; Morrison et al., 1999; Gao et al., 2003</td>
</tr>
<tr>
<td>arc6</td>
<td>Ws</td>
<td>1000</td>
<td>2</td>
<td>0.5</td>
<td>One or two large chloroplasts</td>
<td>Pyke et al., 1994; Robertson et al., 1995; Vitha et al., 2003</td>
</tr>
<tr>
<td>arc7</td>
<td>Ws</td>
<td>40</td>
<td>80</td>
<td>26</td>
<td>Pale first leaves</td>
<td>Rutherford, 1996; Pyke, 1999</td>
</tr>
<tr>
<td>arc8</td>
<td>Ws</td>
<td>110</td>
<td>45</td>
<td>10</td>
<td>Moderately enlarged chloroplasts</td>
<td>Rutherford, 1996</td>
</tr>
<tr>
<td>arc9</td>
<td>Ws</td>
<td>140</td>
<td>34</td>
<td>12</td>
<td>Moderately enlarged chloroplasts</td>
<td>Rutherford, 1996</td>
</tr>
<tr>
<td>arc10</td>
<td>Ws</td>
<td>170</td>
<td>38</td>
<td>6</td>
<td>Highly variable in size</td>
<td>Rutherford, 1996; Pyke, 1999</td>
</tr>
<tr>
<td>arc11</td>
<td>Ler</td>
<td>110</td>
<td>30</td>
<td>7</td>
<td>Heterogeneous chloroplast size</td>
<td>Morrison et al., 1999; Colletti et al., 2000; Fujiwara et al., 2004</td>
</tr>
<tr>
<td>arc12</td>
<td>Col</td>
<td>ND</td>
<td>1–2</td>
<td>ND</td>
<td>Similar to arc6</td>
<td>Pyke, 1999; Yamamoto et al., 2002</td>
</tr>
</tbody>
</table>

*WT=wild type.*
petals are dumbbell shaped and do not appear to complete division (Pyke and Page, 1998). It is possible that if ARC5 is required to complete the division process it may be expressed in a cell-specific manner.

arc11 chloroplasts, as observed for arc10 chloroplasts (Rutherford et al., 1996; Marrison et al., 1999; Pyke, 1999), show a highly heterogeneous population of chloroplast size and shape within a single cell. Approximately half of the chloroplasts in each mesophyll cell are within wild-type size whilst half are larger than the wild type. This phenotype could result from either the presence of a subpopulation of chloroplasts which cannot divide or from the occurrence of asymmetric division events. In the case of the arc11 mutant, the latter is clearly the case with the observation of aberrant division events giving rise to ‘budding’ chloroplasts (Marrison et al., 1999), multiple-arrayed chloroplasts, and spherical mini-chloroplasts (Fujiwara et al., 2004).

arc1 and arc7 both have a larger number of smaller chloroplasts per cell than the wild type, and both are pale as young seedlings and are slow to green. arc1 and arc7 are both recessive mutations and are believed to affect chloroplast development or chlorophyll biosynthesis rather than division directly (Pyke and Leech, 1992; Rutherford, 1996; Marrison et al., 1999; Pyke, 1999). The increase in number of chloroplasts per cell is considered to be a compensatory mechanism for the decreased chloroplast growth. Double-mutant studies have shown that ARC1 down-regulates proplastid division and is in a separate pathway from ARC5. ARC5 has been shown to localize to a ring-like structure at the site of constriction on the cytosolic surface of chloroplasts (Gao et al., 2003). ARC5 represents the first chloroplast division component to be located on the cytosolic surface, raising the possibility that it is a component of the outer PD ring. ARC5 has no bacterial homologues, in keeping with the proposed eukaryotic origin of the PD rings; however, ARC5 is considerably larger than the proposed 56 kDa size of the outer PD ring component. GFP–ARC5 can be detected as a speckled ring indicating that the ring may be discontinuous or non-uniform in conformation. Interestingly, GFP–ARC5 can be faintly detected in unconstrained chloroplasts indicating that ARC5 may play a role in chloroplast division earlier than was previously suggested from analysis of the arc5 mutant. The localization of ARC5 resembles that of the dynamin-like protein CmDnm2 from C. merolae which has been implicated in chloroplast division (Miyagishima et al., 2003); however, during early division stages, CmDnm2 localizes to cytosolic patches and is recruited to the cytosolic side of the division site only after outer PD ring formation.

The Arabidopsis genome contains 11 dynamin-like (and related phragmoplastin-like) homologues. Phylogenetic analysis has demonstrated that ARC5 is distantly related to dynamin-like proteins shown to play a role in mitochondrial division in higher plants (ADL2b: Arimura and Tsutsumi, 2002), yeast (Dnm1p: Bleazard et al., 1999), mammals (Drp1: Smirnova et al., 2001), and red algae (CmDnm1: Nishida et al., 2003). Several of these proteins (ADL2b, CmDnm1, and Dnm1p) have been shown to localize to mitochondrial constriction sites, and ARC5 appears to represents a new class of dynamin-like proteins unique to chloroplast division in Arabidopsis. Until recently, it was thought that chloroplasts divided by a prokaryotic mechanism using machinery conserved from their cyanobacterial ancestors. Evidence for the involvement of dynamins in plastid division is exciting and could represent an important step in allowing the eukaryotic host to gain nuclear control over the process.

ARC6

Prominent in the collection of arc mutants is arc6, which shows the most striking phenotype with an average of two greatly enlarged chloroplasts per mesophyll cell (Pyke et al., 1994; Robertson et al., 1995; Vitha et al., 2003). The mutation in arc6 was mapped to chromosome V (Marrison et al., 1999) close to a gene showing homology to the division of a small number of proplasts in the meristem, but that increased levels of ARC5 (and the homologue) are required for the proliferation of large populations of chloroplasts in expanding mesophyll cells. Further studies into the role of ARC5 and its homologue will shed light in this matter.
cyanobacterial cell division gene *Ftn2*, which was identified by transposon mutagenesis (Koksharova and Wolk, 2002). Sequencing of this gene in the *arc6-1* mutant revealed that nucleotide 1141 of its open reading frame is mutated, resulting in a premature stop codon (Vitha et al., 2003). Alignment of the *ARC6* sequence has revealed homologues in fern, moss, and green alga, but not in non-cyanobacterial prokaryotes, indicating that *ARC6* is a descendant of the cyanobacterial *Ftn2* gene. *ARC6* encodes an 810 amino acid protein that contains an N-terminal J-domain motif characteristic of DNAJ chaperones. Proteins containing J domains are found in most organisms and their J domains are responsible for their interaction with Hsp70 chaperones to stimulate Hsp70 ATPase activity necessary for stable binding of Hsp70 to its protein substrates (Bukau and Horwich, 1998). It has been suggested that the J domain is involved in recruiting an Hsp70 interaction with a specific set of substrates defined by other domains in the protein. In *Escherichia coli*, HscA (an Hsp70 family protein) is involved in Z-ring formation, through a chaperon-like interaction with FtsZ (Uehara et al., 2001), and it is attractive to speculate that *ARC6* may play a similar role in *Arabidopsis*, acting as a plastid division-specific Hsp70 co-chaperone.

A putative transmembrane region was identified in *ARC6* and *in vitro* chloroplast import and protease protection assays suggest that *ARC6* spans the inner chloroplast envelope membrane with the N-terminus, including the J domain, extending into the chloroplast stroma (Vitha et al., 2003). Using GFP fusion protein experiments, *ARC6* has been shown to localize to a ring structure at the chloroplast division site prior to and during constriction similar to the localization of FtsZ1 and FtsZ2 (Fig. 2A). It has been suggested that *ARC6* is involved in Z-ring assembly or maintenance. Consistent with this idea, the *arc6* defect is accompanied by the fragmentation of FtsZ polymers (Fig. 2B), and *arc6* chloroplasts contain lower levels of FtsZ. Additionally, in plants over-expressing *ARC6*, FtsZ filaments are more numerous and occasionally form spiral or ring patterns around the enlarged chloroplast, suggesting *ARC6*-governed excessive FtsZ polymerization and/or stabilization (Fig. 2D). In *E. coli*, two cell division proteins, FtsA and ZipA, are believed to be involved in regulating or controlling the FtsZ polymerization reaction. In *E. coli* mutants lacking both of these components, cell division is blocked and FtsZ forms arcs and dots instead of rings (Pichoff and Lutkenhaus, 2002; see later sections). Although no homologues of these bacterial proteins have been identified in the *Arabidopsis* genome, *ARC6* may prove to play a function analogous to FtsA and ZipA, anchoring and/or stabilizing the FtsZ ring at the plastid division site.

**ARC11**

The asymmetrical division and mini-chloroplast phenotype of *arc11* chloroplasts resembles that of the bacterial *min* phenotype (Bi and Lutkenhaus, 1993). This chloroplast division defect is also very similar to that observed in *Arabidopsis AtMinD1* antisense plants (Colletti et al., 2000), and mapping data placed the *arc11* mutation on chromosome V (Marrison et al., 1999) in close proximity to *AtMinD1*. Genomic sequence analysis revealed that *arc11* contains a single missense mutation at nucleotide 887 of its open reading frame, changing an alanine to glycine at position 296 in a predicted α-helix towards the extreme C-terminus of the protein (Fujiwara et al., 2004). Although this substitution does not alter the endogenous *AtMinD1* transcript level in *arc11*, complementation analysis using the wild-type *AtMinD1* cDNA under the control of the endogenous promoter demonstrates that it is the cause of the *arc11* chloroplast phenotype.

Interestingly, alignment analysis of MinD amino acid sequences from bacteria to higher plants reveals that alanine 296 represents a plant-specific conserved residue. Functional analyses and crystal structure studies of bacterial MinD proteins places Ala296 distantly from important motifs and amino acid residues required for nucleotide binding and interaction with MinC (see later sections), and no functional role for this domain has previously been proposed in any species. Recently, studies by Fujiwara et al. (2004) have shown that the single point mutation in α-helix 11 in *arc11* disrupts normal intraplastidic localization patterns where, in sharp contrast with wild-type AtMinD1 which localizes to one or two discrete spots at polar regions in chloroplasts (Maple et al., 2002), AtMinD1(A269G)–YFP fusion protein localizes to large and distorted fluorescent aggregates and/or multiple fluorescent spots. The extreme C-terminal part of MinD, which is conserved from eu-bacteria to chloroplasts, is surface exposed and, in bacteria, is responsible for membrane localization of MinD through an amphipathic...
helix (Szeto et al., 2002). It appears that correct localization of AtMinD1 is also governed by the extreme C-terminal domain. In *E. coli* the membrane localization of MinD is mediated by an ATP-driven dimerization/polymerization reaction (de Boer et al., 1991). AtMinD1 has been shown to form homodimers using both the yeast two-hybrid system and fluorescence resonance energy transfer (FRET) assays in living plant cells (Fig. 3) and this dimerization capacity is abolished by the single point mutation in AtMinD1 (A269G) (Fujiwara et al., 2004). This suggests that the C-terminal domain is also involved in dimerization, but further studies are required to elucidate if the point mutation in α-helix 11 results in mislocalization, through loss of interaction with the membrane region itself or as a secondary consequence of the mutant proteins inability to dimerize. The identification of a point mutant of AtMinD1 has made available novel tools to investigate this protein’s mode of action in the chloroplast division machinery, expanding and reinforcing previous studies.

**ARC3**

It is thought that ARC3 plays an important role in the initiation of chloroplast division, since the number of chloroplasts in an arc3 mutant cell is the same as the final proplastid number, indicating that no chloroplast division occurs (Pyke and Leech, 1992, 1994; Marrison et al., 1999). The ARC3 locus was mapped to a 97 kb region of a BAC clone (F9E10) and database searches revealed that a gene within this BAC, At1g75010, had a region of homology to FtsZ-like proteins (Shimada et al., 2004). 3′-RACE and 5′-RACE were used to obtain a full-length cDNA for both ARC3 and arc3-1. Subsequent sequence analysis revealed that, in arc3-1, a single base-pair mutation at nucleotide position 2001 results in the conversion of a tryptophan at position 667 of ARC3 to a stop codon (Shimada et al., 2004). To confirm that this mutation was responsible for the arc3 chloroplast phenotype, both forward and reverse genetic approaches were taken. Firstly, a T-DNA insertion line was identified with an insertion in intron 13 of ARC3, and analysis showed that this line had a phenotype very similar to that of arc3. Furthermore, successful complementation of arc3-1 with the wild-type ARC3 gene confirmed that the arc3 gene was At1g75010.

The ARC3 protein contains 742 amino acids and sequence alignments reveal that it is a chimera of a prokaryotic gene, FtsZ, and a eukaryotic gene, phosphatidylinositol-4-phosphate 5-kinase (PIP5K). The N-terminal FtsZ-like domain of ARC3 (1–419 amino acids) lacks complete GTP-binding and hydrolysis motifs highly conserved among bacteria and archaea, suggesting that the FtsZ domain of ARC3 may have a different function to AtFtsZ1-1 and AtFtsZ2-1. The C-terminal portion of ARC3 (603–730 amino acids) has high identity with PIP5K proteins, suggesting that this region might be important for ARC3 function, although there is no catalytic domain and no kinase activity has been detected in biochemical assays (Shimada et al., 2004). However, this domain does contain MORN (membrane occupation and recognition nexus) repeat motifs only found in a relatively small number of proteins from various organisms. In animal cells, MORN repeat motifs in junctophilin proteins, which are components of the junctional complexes present between the plasma membrane and the endoplasmic reticulum, are necessary for binding to the plasma membrane (Takehima et al., 2000). Whether the MORN domain of ARC3 plays a role in the subcellular localization determination is not known. A second possibility is that the MORN repeat motifs play a role in stabilizing the protein as the truncated arc3 protein.
lacks these motifs and cannot be detected in western blotting experiments (Shimada et al., 2004). ARC3 might have arisen by fusion of a prokaryotic FtsZ and part of the eukaryotic PIP5K. The FtsZ domain may be important in the correct localization of the protein at the chloroplast division site and the PIP5K-homologous region may play a role in nuclear control of the chloroplast division process. This remains to be seen.

Immunofluorescence microscopy has demonstrated that ARC3 is located at the site of chloroplast division in a ring-like structure at early and middle stages of the process, but not during later stages of division (Shimada et al., 2004). The ARC3 protein is predicted to have a transit peptide; however, chloroplast import and protease protection assays have shown that the ARC3 protein is located on the cytosolic surface of the outer chloroplast envelope, like ARC5. It will be interesting to analyse the timing of ARC3 ring assembly to investigate whether this protein acts in concert with ARC5.

**Bacterial cell division versus plastid division**

Because of the endosymbiont origins of chloroplasts and the involvement of prokaryote-derived proteins in chloroplast division, bacterial cell division is often used as a paradigm for chloroplast division. Bacteria divide symmetrically during normal growth and have a central constriction to bring about binary fission of the cell. FtsZ mediates bacterial cell division by assembly into a contractile ring (Z ring) at the mid-cell division site, which is required for the subsequent localization of all other division components (Bi and Lutkenhaus, 1991; Lukenhaus and Addinall, 1997). FtsZ polymerization can occur at any point along the *E. coli* cell membrane; however, mid-cell placement of the Z ring is essential for the equal distribution of cellular components during division: Asymmetric division leads to the formation of mini-cells which generally lack chromosomes and fail to divide (de Boer et al., 1989). The correct division site placement in *E. coli* requires the components of the minB operon and mutations within this operon lead to the formation of mini-cells (de Boer et al., 1990). The minB operon encodes three proteins; MinD, MinE, and MinC, that act in concert to limit the placement of the Z ring to mid-cell. MinC acts as an antagonist to FtsZ polymerization, preventing formation of the Z ring; however, MinC lacks site specificity so it will inhibit FtsZ polymerization anywhere in the cell (de Boer et al., 1992a; Pichoff and Lutkenhaus, 2001). Topological specificity is conferred on MinC by the co-ordinated action of MinD and MinE (Hu and Lutkenhaus, 1999). Although homologues of both MinD and MinE proteins have been identified in the nuclear genome of *Arabidopsis*, to date no MinC homologue has been discovered.

Division of cyanobacteria has been much less studied than division in other heterotrophic bacteria, although homologues of most *E. coli* cell division proteins are present in the cyanobacterial genome. The morphological similarities between dividing cyanobacteria and dividing chloroplasts are striking and further knowledge of cyanobacterial division will undoubtedly benefit plastid division research.

**FtsZ**

A homologue of the *E. coli* cell division protein FtsZ was identified in *Arabidopsis* through homology searches (Osteryoung and Vierling, 1995). *Arabidopsis* FtsZ shares over 40% amino acid identity to many bacterial FtsZ proteins but is more closely related to those from cyanobacteria compared with other prokaryotes, sharing 50% protein similarity with the cyanobacterial FtsZ (Osteryoung and Vierling, 1995). After the identification of the first FtsZ homologue in *Arabidopsis* two further homologues were discovered. By contrast with bacteria that encode a single ftsZ gene it became clear that there were two distinct families of FtsZ proteins in *Arabidopsis* and other plant species (Osteryoung et al., 1998; Osteroyung and McAndrew, 2001; Stokes and Osteroyung, 2003). These two families of FtsZ proteins have been termed FtsZ1 and FtsZ2, and it was originally thought that they arose by a duplication event from a single ftsZ gene present in the cyanobacterial ancestor of chloroplasts (Osteryoung and McAndrew, 2001). However, recent phylogenetic analysis seems to contradict this, indicating that the divergence of the two families occurred between the divergence of red and green algae, signifying that the duplication of the ftsZ gene may have happened in the cyanobacterial progenitor of chloroplasts (Stokes and Osteroyung, 2003). FtsZ proteins from the two families are distinguished by conserved differences in amino acid sequences. Plant FtsZ proteins share most of the structural features common to the bacterial proteins, and all FtsZ proteins can be divided into two structural domains, a highly conserved N-terminal domain which is sufficient for polymerization (Wang et al., 1997), and a more variable C-terminal domain. One of the most important features of the N-terminal domain is the Rossmann fold, a motif frequently found in nucleotide-binding proteins (Löwe and Amos, 1998). The Rossmann fold, essential for GTP-hydrolysis, harbours the GTP-binding tubulin signature motif GGGTG(T/S)G (de Boer et al., 1992b; RayChaudhuri and Park, 1992) and contains additional residues that contact the guanine nucleotide (Wang et al., 1997; Löwe and Amos, 1999; Osteroyung and McAndrew, 2001). In FtsZ1, but not FtsZ2, one of these residues is substituted, possibly representing an important divergence between these two protein families. Despite this, the secondary and tertiary structures of the N-terminal domain throughout the FtsZ1/2 families and the bacterial FtsZ proteins are almost identical (Osteroyung and McAndrew, 2001).

The C-terminal domain of FtsZ is more variable among different organisms. Important features present in the C-terminal domain include highly conserved ‘synergy’ residues
believed to regulate GTP-hydrolysis and loop structures that are possibly involved in calcium binding (Löwe and Amos, 1998). Interestingly, calcium binding has been shown not to be essential for FtsZ assembly, at least in E. coli (Mukherjee and Lutkenhaus, 1999). Also present in the C-terminal domain is the core domain that is required in E. coli FtsZ for direct interactions with ZipA and FtsA (Wang et al., 1997; Din et al., 1998; Liu et al., 1999; Hale et al., 2000; Mosyak et al., 2000; Yan et al., 2000). ZipA is thought to function by anchoring FtsZ to the membrane and supporting the Z-ring structure, while FtsA is thought to stabilize FtsZ proto-filaments preventing depolymerization of the Z ring. The core domain is conserved in the plant FtsZ2 proteins but not in FtsZ1 proteins, and it is possible that FtsA- and ZipA-like proteins interact specifically with FtsZ2 and not FtsZ1. However, no homologues of FtsA or ZipA have as yet been identified in Arabidopsis or in other plant species.

FtsZ was first demonstrated to be an essential chloroplast division component in the moss Physcomitrella patens in which a knockout of the FtsZ homologue caused the inhibition of chloroplast division, resulting in cells containing only one large chloroplast (Strepp et al., 1998). The same phenotype is observed in Arabidopsis in which reduced expression of FtsZ from either family causes inhibition of chloroplast division (Osteryoung et al., 1998). This demonstrates that both FtsZ1 and FtsZ2 are essential for chloroplast division and over-expression studies further revealed that the correct stoichiometric amount is paramount for correct division to occur. Levels of FtsZ1 elevated as little as 3-fold are enough to cause the inhibition of chloroplast division (Stokes et al., 2000). Similarly, in P. patens over-expression of FtsZ2 causes plastid division inhibition in a dose-dependent manner (Kriessling et al., 2000). This dose dependency is also witnessed in E. coli; a slight increase in the level of FtsZ actually increases cell division, whereas a high level of FtsZ inhibits cell division producing long filamentous cells (Ward and Lutkenhaus, 1985). In Arabidopsis, FtsZ proteins from both families were found to form a ring structure at the plastid midpoint suggesting that both families have a role as part of the Z ring (Fujiiwara and Yoshida, 2001; McAndrew et al., 2001; Vitha et al., 2001).

AtMinD1

E. coli MinD is a peripheral membrane protein (de Boer et al., 1989). MinD belongs to a large family of ATPases that contain a deviant Walker A motif involved in the binding and hydrolysis of ATP (de Boer et al., 1991). ATP-bound MinD interacts with MinC and activates MinC-mediated division inhibition (de Boer et al., 1991; Zhou and Lutkenhaus, 2004). MinD also recruits MinC to the membrane where together they form a stable inhibition complex at the polar zone of the cell (Huang and Lutkenhaus, 1996; Huang et al., 2003). MinE imparts topological specificity to this inhibition complex through its interactions with MinD. MinE binds to the MinD/C complex stimulating the ATPase activity of MinD. This causes dissociation of MinD from the membrane and oscillation to the opposite cell pole, again forming a stable complex with MinC and the membrane until again being released by MinE. In this way MinE acts as a topological specificity factor, constantly redistributing MinD and MinC so that all three components repeatedly oscillate from one cell pole to the other (Fu et al., 2001; Huang et al., 2003). This oscillation means that the time-averaged concentration of MinC and MinD is lowest at mid-cell, allowing Z-ring formation to occur here. At the membrane, MinD is able to polymerize, thus creating a polar zone of MinD.

The possibility that a Min-based system may operate in the division site-selection in plastid division was initially indicated by the identification of homologues of minD and minE in the plastid genome of the unicellular chlorophyte Chlorella vulgaris (Wakasugi et al., 1997). This was followed by the identification of a MinD homologue in the nuclear genome of Arabidopsis (Colletti et al., 2000), rice, and marigold (Moehs et al., 2001). The Arabidopsis MinD was identified through homology searches using the protein sequence of the C. vulgaris MinD as the query input. The Arabidopsis homologue, referred to as AtMinD1, shares 65% amino acid similarity to MinD from C. vulgaris and greater than 40% amino acid identity with other bacterial MinD proteins (Colletti et al., 2000). The AtMinD1 gene encodes a protein of 326 amino acids which includes an N-terminal chloroplast-targeting transit peptide.

The importance of AtMinD1 in plastid division in Arabidopsis was established by the phenotypes observed when AtMinD1 expression is altered. Reduced levels of AtMinD1 gives rise to chloroplast size heterogeneity within individual cells, and chloroplasts are consistently fewer and larger in size compared with wild-type cells (Colletti et al., 2000). The heterogeneity in chloroplast size is reminiscent of the asymmetric division and subsequent mini-cell formation in E. coli when MinD is inactivated (de Boer et al., 1989), suggesting functional conservation between the Arabidopsis AtMinD1 and the E. coli MinD. Increased levels of AtMinD1 leads to a dramatic reduction in the number of chloroplasts per cell, indicating that over-expression of AtMinD1 inhibits chloroplast division (Colletti et al., 2000). The few chloroplasts present are greatly enlarged resembling the filamentation phenotype observed in E. coli when MinD is expressed at high levels (de Boer et al., 1989). AtMinD1 mode of action also seems to be conserved amongst different plant species in that AtMinD1 over-expression in transgenic tobacco plants results in inhibition of chloroplast division (Dinkins et al., 2001).

AtMinD1–GFP fusion protein experiments reveal a distinct interplastidic localization of AtMinD1, often forming one or two discrete spots at polar zones of chloroplast (Fig. 4B) (Maple et al., 2002; Fujiiwara et al., 2004). AtMinD1 is
always observed in close proximity to the envelope region, suggesting it is a membrane-associated protein (Maple et al., 2002). The localization pattern is similar to that observed in E. coli, and it is expected that AtMinD1 exhibits dynamic behaviour analogous to E. coli MinD, although this has not yet been observed. AtMinD1 contains a deviant Walker A motif responsible for ATP binding and hydrolysis and, although AtMinD1 has been shown to be an ATPase (C Aldridge, SG Møller, unpublished results), the physiological role of AtMinD1-mediated ATP hydrolysis remains to be investigated.

**AtMinE1**

AtMinE1 was identified in Arabidopsis on chromosome 1, based on its similarity to prokaryotic and chloroplast-encoded MinE proteins (Itoh et al., 2001; Maple et al., 2002; Reddy et al., 2002). AtMinE1 encodes a protein of 229 amino acids with no known functional domains and is predicted to be soluble, similar to its bacterial counterpart. In bacteria, MinE is composed of two domains: an N-terminal anti-MinCD domain (AMD) and a C-terminal topological specificity factor domain (TSD) (Pichoff et al., 1995; Zhao et al., 1995). The TSD domain is required for the ability of MinE to prevent mini-cell formation, to induce formation of MinCD polar zones, and to form MinE rings, but it does not counteract the action of the MinC division inhibitor in MinCD+ cells (Zhao et al., 1995). By contrast, the AMD domain is similar to full-length MinE in its ability to counteract the action of the MinC division inhibitor in the presence of MinD, but it is unable to prevent mini-cell formation (Zhao et al., 1995). Sequence alignments of AtMinE1 with the E. coli MinE sequence suggest that the Arabidopsis protein harbours an N-terminal AMD domain; however, the C-terminal TSD domain region is less conserved. TSD domains from various species show limited similarity, suggesting evolutionary divergence of TSD function, possibly to integrate the MinE protein into the division machineries of different species.

AtMinE1 has been shown to have retained some of its ancestral functions because over-expression of AtMinE1 in E. coli leads to a classical minicelling phenotype as observed when overexpressing the endogenous E. coli MinE protein in bacteria (Maple et al., 2002). In E. coli this is due to the suppression of MinC/D activity at potential division sites, leading to a loss of topological specificity (de Boer et al., 1989). Remarkably, although AtMinE1 is only c. 20% similar to E. coli MinE, expression of AtMinE1 can clearly counteract the division inhibition induced by MinCD in an E. coli strain null for MinE (J Maple, SG Møller, unpublished results). However, expression of AtMinE1 is unable to prevent minicell formation seen in the parent strain, indicating that the Arabidopsis TSD domain is not fully functional in bacteria.

The role of AtMinE1 in chloroplast division was demonstrated in Arabidopsis and tobacco plants containing elevated levels of AtMinE1 (Itoh et al., 2001; Maple et al., 2002; Reddy et al., 2002). In these plants, mesophyll cells contained a reduced number of enlarged chloroplasts which showed striking size heterogeneity within single cells. It was predicted that if AtMinE1 acted as a topological specificity factor in Arabidopsis, then over-expression of the protein should lead to asymmetric plastid division events. Detailed analysis of the chloroplast division phenotype in hypocotyls of seedlings over-expressing AtMinE1 showed the presence of chloroplasts with misplaced constriction sites towards one pole of the plastid, giving rise to a ‘mini-cell’ phenotype similar to that of E. coli over-expressing AtMinE1 or bacterial MinE (de Boer et al., 1989; Maple et al., 2002). In addition, elongated plastids displaying up to five constriction sites were observed, indicating that AtMinE1 over-expression leads to a complete loss of topological specificity and to multiple aberrant division sites (Fig. 4A).

Intraplastidic localization analysis reveals that, in a fashion similar to AtMinD1 (Fig. 4B), AtMinE1 has a distinct intraplastidic localization pattern, localizing as a single spot or as two spots in close proximity towards one end of the chloroplast (Fig. 4C). During E. coli division the MinE ring moves from mid-cell position towards the pole where MinC/D is present, and by so doing triggers the relocalization of MinC/D to the opposite pole (Hu et al., 2002). The similarity in localization patterns of AtMinE1 and

**Fig. 4.** (A) Typical phenotype of chloroplast in hypocotyl cells of transgenic Arabidopsis seedlings over-expressing AtMinE1. AtMinE1 over-expression results in elongated chloroplasts displaying multiple constriction sites (black arrowheads) compared with a central constriction site in wild-type dividing chloroplasts (inset). Intraplastidic localization patterns of (B) an AtMinD1–YFP fusion protein and (C) an AtMinE1–YFP fusion protein in Arabidopsis mesophyll chloroplasts. Scale bar = 5 μm.
AtMinD1 suggests that these two proteins might act in concert in a way analogous to the Min complex in bacteria. In line with this hypothesis, studies carried out, both in the yeast two-hybrid assays and in planta, have demonstrated that AtMinE1 can interact with AtMinD1 (J Maple, C Aldridge, SG Møller, unpublished results).

**Novel plastid division proteins**

Recently a number of non-arc-related plastid division proteins have been identified which do not seem to form part of the classical Min protein-mediated division pathway in Arabidopsis. The identification and characterization of these proteins has clearly strengthened the idea that plastid division in higher plants represents a complex interplay between prokaryotic and eukaryotic protein components.

**ARTEMIS**

ARTEMIS (Arabidopsis thaliana envelope membrane integrase) was identified in a search for proteins involved in chloroplast biogenesis (Fulgosi et al., 2002). ARTEMIS is a 1013 amino acid protein, encoded for by a gene on chromosome I, and is an integral membrane protein localized to the inner envelope membrane of chloroplasts. It has a unique molecular structure combining a C-terminal domain similar to the Alb3 and Oxa1 proteins with conserved YidC translocase elements and an N-terminal region similar to receptor protein kinases. These are separated by a region harbouring a predicted ATP/GTP-binding motif (P-loop), which has been demonstrated to bind GTP, indicating that GTP may regulate ARTEMIS function.

The role of ARTEMIS in chloroplast division was discovered from studies using transposon insertion Arabidopsis plants with greatly reduced levels of the ARTEMIS protein (Fulgosi et al., 2002). These plants have similar growth characteristics to wild-type plants, but ultrastructural analysis revealed extended, duplicated, or triplicated, undividing chloroplasts. Interestingly, despite the failure of the envelope membranes to complete constriction, the thylakoid membranes are visibly constricted at the centre of the chloroplasts and are apparently partitioned between the two halves of the organelle.

The Alb3/Oxa1/YidC family comprises proteins important for the integration of membrane proteins (reviewed in Kuhn et al., 2003). YidC is a bacterial translocase which plays an essential role in the insertion of bacterial membrane proteins (Scotti et al., 2000). Both Oxa1p and Alb3 are thought to have originated from YidC and, in chloroplasts, Alb3 is involved in the insertion of light-harvesting antenna proteins into the thylakoid membrane (Moore et al., 2000), whilst in mitochondria Oxa1p protein is involved in the insertion of a subset of inner membrane proteins from the mitochondrial matrix (Hell et al., 2001). Expression of a chimeric version of the Alb3/Oxa1/YidC-like domain that is targeted to the inner membrane of mitochondria in yeast was shown to be able to partially complement the function of yeast Oxa1 in the insertion and assembly of mitochondrial membrane proteins, demonstrating that ARTEMIS is a functional member of the Alb3/Oxa1/YidC family (Funes et al., 2004). The prospect of a novel protein-sorting pathway in chloroplasts which integrates polypeptides from the stroma into the inner envelope by an evolutionary conserved process is exciting. PtsQ is a transmembrane bacterial division protein, and it has been shown to interact with the YidC translocase (van der Laan et al., 2001) inviting the possibility that ARTEMIS may function late in the division process to insert plastid division components into the envelope membrane by the YidC/Alb3-like translocase motif.

Using the YidC/Alb3-like translocase domain, a homologue of ARTEMIS has been identified in Synechocystis PCC6803, and a deletion mutant cell line for this gene (slr1471) has altered cell morphology, with the formation of tetrameric or hexameric clusters of cells indicative of late cell division arrest (Fulgosi et al., 2002). Cells also seem to initiate their fission events unevenly, leading to cells of irregular shape. The evolutionary conservation of ARTEMIS has been demonstrated by the rescue of wild-type division characteristics in the slr1471 cell line with the YidC/Alb3-like domain of ARTEMIS.

The receptor and GTPase domains are not present in the cyanobacterial homologue and their function in plastid division is not clear. It is possible that, like ARC3, ARTEMIS is a modular protein and that these domains were added to the YidC/Oxa1/Alb3-like domain during evolution of the chloroplast division machinery, representing an important step in the process whereby the cell nucleus acquired control over organelle division. Further analysis of the role of the ARTEMIS receptor domain will be crucial in understanding the evolution of the chloroplast division machinery.

**GIANT CHLOROPLAST 1**

GIANT CHLOROPLAST 1 (GC1) (also called AtSulA) was originally identified based on its similarity to putative cell division inhibitor SulA proteins in Anabaena sp. PCC 7120 (all2390) and Synechocystis sp. PCC 6803 (slr1223), although no function had been reported for the cyanobacterial proteins (Maple et al., 2004; Raynaud et al., 2004). GC1 is located on chromosome II and encodes a protein of 347 amino acids which has an N-terminal plastid-targeting transit peptide absent in the cyanobacterial protein. Phylogenetic analysis of GC1 homologues indicates a clear cyanobacterial origin of GC1. This was verified recently through the analysis of a Synechocystis slr1223 deletion mutant, showing that slr1223 is essential for cell survival as homoploids could not be identified (Raynaud et al., 2004). Microscopic analysis of heteroploid clones revealed that up to 40% initiated but failed to complete cell division, resulting in cloverleaf-like structures, demonstrating that slr1223 is
required for correct cell division in *Synechocystis*. At the secondary structure level, GC1 has high (80–90%) structural similarity to nucleotide-sugar epimerases (Maple *et al*., 2004). Epimerases control and change the stereochemistry of carbohydrate-hydroxyl substitutions, often modifying protein activity or surface recognition (Baker *et al*., 1998); however, to date no data exist to support GC1 as having epimerase activity.

GC1 is located in plastids and is most probably anchored to the stromal surface of the chloroplast inner envelope by a C-terminal amphipathic helix (Maple *et al*., 2004). GC1–YFP is located uniformly on the entire chloroplast envelope in transgenic wild-type *Arabidopsis* plants as well as in a number of *arc* mutants, indicating that the entire *Arabidopsis* chloroplast envelope is competent for GC1 recruitment and, furthermore, that the GC1 mode of action is probably associated with the envelope. A role for GC1 in chloroplast division was initially demonstrated through analysis of transgenic *Arabidopsis* plants with greatly reduced (c. 95%) levels of *GC1* transcript (Maple *et al*., 2004). It was found that GC1-deficiency by co-suppression results in mesophyll cells harbouring one or two giant chloroplasts. Interestingly, antisense transgenic lines with as much as a 70% reduction in *GC1* transcript levels showed wild-type chloroplast division profiles, indicating that transcript levels must be severely reduced to affect chloroplast division, possibly supporting the hypothesis that GC1 encodes an enzyme. By striking contrast, GC1 over-expression has no effect on chloroplast division in mesophyll cells. In addition, analysis of an *Arabidopsis* GC1 insertion mutant expressing a truncated N-terminal version of GC1 revealed wild-type chloroplast division characteristics, suggesting that the N-terminal region is responsible for GC1 functionality (M Fujiwara; SG Møller, unpublished results).

More recently, a report has shown that over-expression of a GC1–YFP fusion protein in *Arabidopsis* transgenic plants can also lead to a chloroplast division defect, with lines harbouring a range of chloroplast numbers per cell [60% with 80 (wild-type-like); 11% with 40–60, 24% with 10–40, and 4% with 1 chloroplast per cell] (Raynaud *et al*., 2004). Interestingly, in this study, over-expression did not alter plastid division in the same way in all plants from the same line, or indeed in all cells or in all plastids. Also, a root plastid division phenotype was observed, indicating that the GC1 mode of action might not be limited to photosynthetic tissues where it is primarily expressed. The observed differences in phenotypes amongst different lines and within lines are intriguing and may reflect differences in protein abundance and stability in different cell types. Clearly further functional analysis of GC1 during plastid division is required to resolve these discrepancies.

Ultrastructural analysis reveals that in GC1-deficient giant chloroplasts thylakoid biogenesis is normal, but that grana are more densely packed and there is a reduction in starch grains (Maple *et al*., 2004). GC1-deficient leaves have also been shown to exhibit lower rates of CO₂ assimilation compared with the wild type, possibly due to a greater average path length for CO₂ diffusion from the cytoplasm to Rubisco carboxylation sites in the GC1-deficient large chloroplasts.

In *E. coli*, SulA is synthesized in large amounts when the SOS response is induced (Huisman *et al*., 1984). This is a mechanism to inhibit cell division in the event of DNA damage, allowing cells time to repair their DNA before cell division recommences. SulA is sufficient to halt cell division by directly binding to FtsZ, and induction of the SOS response causes the disappearance of Z rings (Bi and Lutkenhaus, 1990, 1993). Also, over-expression of FtsZ has been shown to result in SulA resistance. GC1 shows limited homology to the *E. coli* SulA protein, raising the possibility that an analogous SOS response pathway could exist in plants. Over-expression of GC1 or GC1-YFP can rescue the plastid division defect caused by over-expression of AtFtsZ1-1 or AtFtsZ2-1 (Raynaud *et al*., 2004), possibly by balancing the increased levels of the FtsZ proteins by titration, indicating that these three proteins may play a role in the same pathway. However, no interaction between GC1 and AtFtsZ1-1 or AtFtsZ2-1 is detected in the yeast two-hybrid system, although GC1 can clearly form homodimers in this system (Maple *et al*., 2004). In addition, *E. coli* sulA mutants show no cell division phenotype (Huisman *et al*., 1984) and this is in contrast with the results obtained in the *Synechocystis slr1223* gene deletion experiments. Consequently, GC1 may not act as a SulA-like protein in *Arabidopsis* and the co-suppression phenotypes indicate that GC1 does not act as a plastid division inhibitor, but rather as a positive factor at an early stage of the division process. Further experiments will help to clarify the exact relationships between GC1 and AtFtsZ at the molecular level.

**How do the different plastid division proteins act together?**

Now that many proteins involved in plastid division have been identified the next step is to understand how the different protein components act together during division. As a first step towards this it will be important to dissect how the different proteins interact with each other at the protein level. Double-immunofluorescence labelling experiments have revealed tight co-localization of FtsZ1 and FtsZ2 proteins, even when their levels and assembly patterns are perturbed (McAndrew *et al*., 2001). Interactions between FtsZ proteins are necessary for the polymerization of FtsZ to form the Z ring, and interaction studies reveal that proteins from both FtsZ families are not only able to interact with themselves to form homodimers but are also able to interact with each other forming heterodimers (J Maple, C Aldridge, SG Møller, unpublished results). The
co-localization of the two FtsZ proteins has led to three different models being proposed as to how the FtsZ proteins may function together in forming the Z ring. In the first model, FtsZ1 and FtsZ2 form separate homopolymeric protofilaments that associate laterally to form the plastid Z ring. In the second model, FtsZ1 and FtsZ2 co-assemble as heteropolymeric filaments, a situation which would be analogous to the association of α- and β-tubulin in microtubules (McAndrew et al., 2001). A third model that would satisfy these interactions proposes that FtsZ proteins could form both heterodimers and homodimers in any given Z ring, leading to a more disorganized composition of the Z ring.

AtMinD1 is able to interact with itself to form homodimers (Fujiwara et al., 2004). From evidence provided by the arc11 mutation, in which AtMinD1 is unable to dimerize, it is likely that dimerization is important in the correct localization of AtMinD1 and ultimately necessary for correct division site placement (Fujiwara et al., 2004). E. coli MinD has been shown to undergo self-assembly on phospholipid vesicles (Hu et al., 2002), and in vivo studies have revealed that MinD is organized into an extended coiled structure that winds around the E. coli cell (Shih et al., 2003). The possibility that AtMinD1 has to undergo self-assembly in order to function validates how essential the interaction of AtMinD1 with itself is for the correct division placement in plastids.

Using a combination of the yeast two-hybrid system and co-localization studies it has been established that AtMinE1 can interact with AtMinD1 (J Maple, C Aldridge, SG Møller, unpublished results). In E. coli this interaction is important for the oscillation of MinD and the topological specificity of the Min system to bring about correct division site placement. In E. coli, MinE stimulates the ATPase activity of MinD, causing MinD to disassociate from the membrane and redistribute to the opposite pole of the cell (Hu et al., 2002). It will indeed be interesting to examine whether this is the case in plastids. Like AtMinD1, AtMinE1 can form homodimers (J Maple et al., unpublished results). In E. coli, MinE has been shown to form a ring structure (E ring) at the leading edge of the MinD polar zone (Fu et al., 2001; Hale et al., 2001) and, like MinD, is part of an extended coiled structure. A ring structure has never been observed for AtMinE1 in plastids but this may simply be a reflection of the difference in shape between the rod shape of the E. coli cell and the ovoid shape of the plastid.

GC1 is capable of forming homodimers, but is unable to interact with the Arabidopsis plastid division proteins FtsZ1, FtsZ2, AtMinD1, AtMinE1, or ARC6. This suggests that GC1 is probably not directly involved in the FtsZ-mediated plastid division pathway in Arabidopsis. However, GC1 and ARC6 are both envelope-associated plastid division proteins, and the arc6 phenotype is almost indistinguishable from the GC1-deficient phenotype. Consequently it is possible these proteins may act in concert during plastid division even though they do not directly interact.

Although increased insight is being gained into the mode of actions of the proteins involved in plastid division it is still not possible to determine whether there are several interconnected division pathways or whether there are simply different control points in the same pathway. It is known that plastid division involves both prokaryotic-derived and eukaryotic-derived proteins, but how they might act together remains unclear. The evidence does point towards the formation of the Z ring being the initial step in chloroplast division, with the site placement of the Z ring being determined by components of a plant Min system, but how the other plastid division components such as ARC6 and GC1 fit into this network is yet to be resolved. With the discovery of new plastid division components and further characterization of the arc mutants and the components of the PD rings, complete understanding of the networks that make up plastid division should become achievable.

Conclusions and future perspectives

Since the realization, approximately 30 years ago, that plastids divide inside plant cells, our understanding of the plastid division process in higher plants has increased considerably, particularly during the last 5–6 years. Through a combination of two main approaches, using bacterial cell division as a paradigm and the cloning of several arc mutants, it is now possible to start constructing meaningful working models of the process (Fig. 5). Several important key points emerge when combining findings to date. First, it is clear that plants have retained crucial prokaryote-derived plastid division proteins such as FtsZ, MinD, and MinE (Osteryoung and Vierling, 1995; Colletti et al., 2000; Maple et al., 2002) and that these show distinct intracellular localization patterns (McAndrew et al., 2001; Maple et al., 2002; Fujiwara et al., 2004). Secondly, protein interaction studies have shown that these proteins do not act in isolation but rather as protein complexes, presumably governing appropriate plastid division (J Maple et al., unpublished results). Thirdly, through the characterization of several ARC proteins it is evident that plastid division is controlled by a complex interplay between prokaryote- and eukaryote-derived protein components (Gao et al., 2003; Shimada et al., 2004). Fourthly, subcellular localization studies have demonstrated that plastid division is controlled not only by proteins residing in the plastid stroma but also by cytoplasmic proteins (Gao et al., 2003; Shimada et al., 2004).

Although there is now a solid foundation from which to base future studies it is important critically to evaluate future approaches and directions in the field. The use of known bacterial cell division proteins as input queries in homology searches is rapidly reaching saturation. However, it may be possible to perform genetic screens which aim to identify novel bacterial and cyanobacterial cell
division mutants, followed by the identification of the corresponding plant proteins. Clearly, the cloning of the remaining arc mutants represents a valuable source of yet undiscovered proteins involved in plastid division; however, it is likely that the initial arc screen was not performed to saturation, posing the question whether a new plastid division screen in Arabidopsis should be performed. Another approach towards identifying new proteins involved in plastid division would be through yeast two-hybrid screening programmes.

The identification of new plastid division proteins will clearly widen our knowledge of how plastid division is controlled in higher plants. However, it is becoming increasingly evident that plastid division proteins do not act independently but rather in concert during division. It will therefore be important, alongside the discovery of new protein components, to assemble the different plastid division programmes.

There are still numerous questions that need to be answered before the complexity of plastid division in higher plants can be fully appreciated. However, combining the recent exciting discoveries in the field, together with the existing tools and new innovative approaches, has provided a fantastic position from which to address these questions.

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