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Review

Challenges to our current view on chloroplasts

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Abstract

Chloroplasts are the co-evolution product of three different genetic compartments. This review compiles reports about bacteria and various photosynthetically active eukaryotes that challenge our current view on the structure of chloroplasts. It highlights their structurally dynamic nature and their differences in various groups of the *Archaeplastida*. Based on these reports, it argues in favor of an evolutionary view on bacterial as well as on plastid cell biology.

Keywords: *Acetabularia*; *Arabidopsis*; *Chlamydomonas*; chloroplast division; endosymbiosis; FtsZ; *Mesostigma*; *Physcomitrella*; plastoskeleton; stromule.

Introduction

The ability of photosynthetic organisms to store solar energy in carbon bonds is the basis of current life on earth. Consequently, research focused on the process of photosynthesis and the biochemistry of chloroplasts in general. Subsequently, researchers analyzed the reciprocal control of plastid and nuclear gene expression to gain a better understanding of the regulatory networks underlying the biochemistry of chloroplasts. It has, however, been unclear for a long time, how these important cell organelles maintain their integrity, change their shape, and divide.

Answers to these questions come from an evolutionary perspective (Kuroiwa et al., 2008) in combination with novel cell biology tools applied to bacteria (Graumann, 2007) and to chloroplasts (Reski, 2002; Hanson and Sattarzadeh, 2008), as the latter are a product of evolution, and evolved from the former. In this brief review, I can only concentrate on some aspects of chloroplast cell biology. Therefore, I will try to highlight and to connect those findings which, to my understanding, challenge our current view on chloroplasts. And I apologize to those colleagues whose important findings are not cited here.

Evolution: cyanobacteria, cyanelles, and chloroplasts

Photosynthetic eukaryotes evolved from a eukaryotic cell that already had the typical compartmentalization: its organelles were surrounded by single (e.g., endoplasmic reticulum, Golgi apparatus, and functionally different vacuoles) and double membranes (nucleus and mitochondria), respectively (Kuroiwa et al., 2008). This progenitor cell engulfed a photosynthetic prokaryote, i.e., a cyanobacterium, and did not digest but integrated it. Subsequently, a massive rearrangement of genes, metabolite fluxes, and structures occurred to evolve a 'domesticated' chloroplast from a once free-living bacterium. The most important event was the transfer of cyanobacterial genes to the host nucleus. Here, the expression of the formerly bacterial genes came under the control of eukaryotic promoter elements, and a plethora of the encoded proteins were, although now being synthesized in the cytoplasm, re-imported into the chloroplast after acquisition of short N-terminal transit peptides that function as targeting signals. Other genes were transferred to mitochondria or were lost (Martin et al., 1998).

All photosynthetic eukaryotes with plastids surrounded by one double membrane are descendants of a single endocytotic event. This group, the *Archaeplastida*, comprise the land plants, the green algae (together known as *Viridiplantae*), the red algae, and a small group of protists called Glaucophytes (Adl et al., 2005). In all other photosynthetic eukaryotes the plastids are surrounded by three or four membranes and originate from a secondary endosymbiosis. Most probably the original founder cell – the last common ancestor – of *Archaeplastida* lived at least 1.5 billion years ago (Zimmer et al., 2007) and also had one single nuclear and one single mitochondrial DNA. Therefore, the genetic and morphological diversity of algae and plants observed today is the consequence of separate evolution after that single event (Lang et al., 2008).

This primary event of phagocytizing but not digesting a free-living cyanobacterium was followed by an integration of three different genetic compartments with different evolutionary histories (the mitochondria are descendants of once free-living α-proteobacteria), leading to a radically altered flux of information between organelles (Martin et al., 1998). The barriers against the integration of the third genetic compartment were enormous. Not only a double membrane had to be crossed for gene transfer, but also a rigid bacterial cell wall consisting of peptidoglycan had to be overcome. So far, it is unclear how these passages were achieved. Possible routes for this gene transfer are stromules which are described later.

Notably, the peptidoglycan layer that gave cyanobacteria shelter and helped them to maintain their shape as a type of exoskeleton can in a reduced form be found in one group of the *Archaeplastida*, in the Glaucophytes. Therefore, their photosynthetic organelles are called cyanelles, not chloroplasts. These cyanelles are evolutionary intermediates between the free-living cyanobacteria and

the 'domesticated' chloroplasts (Wasmann et al., 1987). In contrast to cyanelles, no wall-like structures have been detected in plastids from other sources. Nevertheless, a couple of sequence data and experiments provide evidence that the genes encoding enzymes for synthesis of the peptidoglycan layer were not abolished during evolution into the *Viridiplantae* but were retained to some extent and have acquired different functionalities. These experiments are reviewed here as well.

Plastid differentiation: a variety of forms and functions

It appears to be a simple line from a cyanobacterium to the lens-shaped chloroplasts of flowering plants. However, plastids are the DNA-containing organelles with the widest range of different forms and functions. Interconversion between different plastid types implies marked changes in their architecture and physiology. Furthermore, these changes occur alongside differentiation processes of cells and organs during plant development (reviewed, e.g., by Reski, 1994).

Two major lines of modifications appear to be important: (1) during evolution of flowering plants with their huge complexity of specialized tissues and organs, a structural and biochemical diversity of plastids coevolved. Especially angiosperms harbor a variety of specialized plastid forms: proplastids, leucoplasts, chromoplasts, amyloplasts, gerontoplasts, and chloroplasts. Each form has its specific ultrastructure and biochemistry. However, this variability is only found in highly developed plant families. All other plants that, for example, do not have colored flowers do not have chromoplasts either. Plants with roots, probably never exposed to light, possess leucoplasts in these organs. Consequently, plants without roots, such as mosses, do not possess leucoplasts. Even more dramatic: while the seeds inherit plastids in their minimal form, the proplastids, the spores of mosses contain green chloroplasts (Reski, 1998). (2) During radiation of the *Archaeplastidae* some groups developed significant deviations from the 'normal' shape of chloroplasts. Huge cup-shaped plastids can be found especially in non-vascular plants. Most prominent examples are the alga *Chlamydomonas* with its giant chloroplast which is highly compartmentalized (Uniacke and Zerges, 2007), another is the moss *Anthoceros* which also has the largest plastid DNA genome reported for any land plant so far, that, moreover, is the subject of intensive RNA-editing (Kugita et al., 2003). Most importantly, however, the flagellate *Mesostigma viride* is regarded as among the earliest diverging lineage of the monophyletic group leading to land plants (Qiu, 2008), shares more genes with them than with *Chlamydomonas*, and has one single discoid chloroplast per cell (Simon et al., 2006). Thus, it seems likely that the most basal form of plastids is such a discoid macrochloroplast, while the well-known lens-shaped plastids of most land plants are a derived feature.

Plastids connected: stromules

The current textbook view on the structure of chloroplasts is rather simplistic: mostly the organelles are drawn as lens-shaped entities surrounded by a double membrane of lipid bilayers. Obviously, it is an enigma how such entities could retain or even change their shape. Moreover, the situation became more complex with the (at least) third re-discovery of 'stromules' – stroma-filled tubules – by Köhler et al. (1997).

When the flowering plants tobacco and petunia were stably transformed with DNA, encoding a plastid-targeted green fluorescent protein (GFP), fluorescence was found in the stroma of the plastids. Surprisingly, in addition, long, thin GFP-labeled tubules emanating from chloroplasts became visible. These structures did not contain chlorophyll and were extending outwards dynamically from the plastid and then retracting. Likewise, these authors observed tubules emanating from non-transgenic spinach chloroplasts (Köhler et al., 1997).

Upon finding these structures, Köhler et al. (1997) were, according to Hanson and Sattarzadeh (2008), concerned that they might see artifacts of transgene expression. However, they noticed that similar structures had been reported sporadically in the prevalent literature for many years (reviewed in Gray et al., 2001; Kwok and Hanson, 2004a). One such publication was that of Menzel (1994), describing an interconnected plastidome in the giant single-celled green alga *Acetabularia*. Before that, several authors described protrusions and tubular structures emanating from the chloroplasts of different plant species (e.g., Wildman et al., 1962). However, these findings were largely forgotten. For this reason, what was probably the first discovery of these structures is rarely appreciated. In his book on dynamics of chloroplast movement and change of chloroplast shape, Senn (1908) already pointed out that both processes are accompanied by filamentous structures emanating from the chloroplast envelope, at least in the moss *Funaria hygrometrica*. As these filaments did not contain the green color, he misinterpreted them as filaments of the cytoplasm but already describes their temporary nature in young tissues and their persistence in older tissues, especially in older leaves where they connect all chloroplasts of a cell with each other. Senn (1908) noticed that his observations confirmed those of Klebs (1888) with the same moss. Transferred to recent knowledge these reports are the first description of 'stromules'.

Obviously stromules increase the surface area of the plastid envelope, and thus the interface between plastids and the cytoplasm. More strikingly, stromules are often found in connection with other cell organelles. Therefore, they may facilitate the exchange of lipids between plastids and mitochondria, e.g., the transport of the glycerolipid digalactosyldiacylglycerol to mitochondria (Block et al., 2007) and the transfer of proteins passing through the secretory pathway to plastids (Villarejo et al., 2005). Most striking, however, was a report from Kwok and Hanson (2004b) who found penetration of stromules into grooves of the plant nucleus. Thus, these tubules may also facilitate plastid-to-nucleus signaling (Nott et al.,

2006). It is tempting to speculate, however, that they also facilitate the otherwise unexplainable massive gene transfer from the plastid to the nucleus during evolution as well as the extraordinary high rate of gene transfer from the chloroplast genome to the nucleus after plastid transformation, as observed by Stegemann et al. (2003) and others.

So far, it is unclear how stromules are built and what mechanisms provide their dynamics. Theoretically, these tubular structures could be formed either by proteins pushing out from within the plastid ('pushing-out hypothesis') or by proteins outside the plastids attached to the envelope membrane and pulling it away from the main plastid body. This 'pull-away hypothesis' would require that the inner and outer membranes be grasped together (Hanson and Sattarzadeh, 2008), which makes it rather unlikely.

The bacterial cytoskeleton: end of a dogma

It has long been believed that one fundamental difference between bacteria and eukaryotes is the presence of a cytoskeleton only in the latter. It seemed plausible that small cells like bacteria do not need structural elements, and they appeared as 'plasma-filled tiny droplets', although microbiologists were well aware of the different cell shapes bacteria may encounter. Finally, molecular biology on one hand and modern fluorescence microscopic methods on the other have revolutionized our view on bacterial cell biology. Many proteins are now known to be targeted, as in eukaryotic cells, to specific locations in the bacterial cell or to undergo rapid directed changes in localization. It has been shown that all cytoskeletal proteins known from eukaryotic cells are also present and functional in prokaryotes. Bacterial tubulin (FtsZ), actin (MreB) and intermediate filament (IF) proteins are key players in cell division, chromosome segregation, maintenance of cell-shape and cell-polarity, and in the assembly of intracellular organelle-like structures. In addition, some bacteria encode another bacterial tubulin homolog (TubA/TubZ) for plasmid segregation. Although similar in their overall tasks, eukaryotic and prokaryotic cytoskeletal orthologs have their individual functions, revealing a striking evolutionary plasticity of cytoskeletal proteins. Taken together, it is evident by now that the cytoskeleton is an old invention dating well back before the existence of eukaryotes (reviewed by Ausmees et al., 2003; Errington, 2003; Lutkenhaus, 2003; Lowe et al., 2004; Graumann, 2007; Lowe and Amos, 2009).

FtsZ: ancient tubulin and tubules within chloroplasts

Interestingly, FtsZ is not simply the bacterial ortholog of the eukaryotic tubulin, but also a protein found in recent plants in addition to their tubulins. While most bacteria encode one single FtsZ protein, most plants encode at least three different FtsZ proteins in two small protein families, FtsZ1 and FtsZ2. Phylogenetic analyses reveal that all plant FtsZ proteins can be traced back to their cyanobacterial ancestor and that the split into different families occurred during land plant evolution (Figure 1). Surprisingly, lower plants encode a third FtsZ family, making the moss *Physcomitrella patens* currently the organism with the most different FtsZ proteins, namely five (Martin et al., 2009).

FtsZ was the first bacterial cytoskeletal protein to be identified. It polymerizes to filaments, minirings and tubules, and is, according to fluorescence microscopy with FtsZ::GFP-fusion proteins, the backbone of the bacterial cell-division machinery localized midcell in the Z-ring (Lowe and Amos, 1998; Margolin, 1998; Nogales et al., 1998; Lu et al., 2000). Likewise, the plant FtsZ was the first protein that was shown to be instrumental for the division of chloroplasts (Osteryoung et al., 1998; Strepp et al., 1998). It is clear from a variety of publications that plant FtsZ builds a backbone of the complex ring-like plastid division machinery (Kwok and Hanson, 2004a; Glynn et al., 2007; Maple and Moller, 2007; Yang et al., 2008).

Transfecting the moss *Physcomitrella* with FtsZ2::GFP constructs we were surprised to find not only a chloroplast division ring but also filamentous networks in chloroplasts, leading to the suggestion that FtsZ proteins may serve as one part of a filamentous plastoskeleton which helps chloroplasts in maintaining their integrity and their shape, including division (Kiessling et al., 2000; Reski, 2002). Although tubules had not been visualized in bacteria then, McFadden (2000) pointed out that the cytoskeleton-like structures observed in *Physcomitrella* chloroplasts with FtsZ::GFP fusions correlate well with the tubules observed in other chloroplasts, which are described as forming anastomosing or reticulated networks ramifying throughout the entire chloroplast (Hoffman, 1967; Pickett-Heaps, 1968; Rivera and Arnott, 1982; Lawrence and Possingham, 1984). Intriguingly, chloroplast tubules were described long before the discovery of FtsZ. At this time, eukaryotic cytosolic microtubules were only beginning to be characterized, and Hoffman (1967) and Pickett-Heaps (1968) related the chloroplast tubules to cytoplasmic microtubules, although at the same time they recognized key differences in the substructure of the two types of tubules (McFadden, 2000).

According to FRET analysis, the different FtsZ isomers specifically interact with each other in a defined hierarchy to build the plastoskeleton in *Physcomitrella* (Gremillon et al., 2007). And to make the story even more complex, the moss FtsZ with no ortholog in seed plants (FtsZ3) is dually targeted to the cytosol and to chloroplasts and is functional in both cellular compartments (Kiessling et al., 2004). This complexity of up to five different FtsZ isoforms in a single plant cell poses the question on what their functions beside the division process and forming a plastoskeleton might be.

Common to *Arabidopsis* and to *Physcomitrella*, however, is that FtsZ1::GFP fusion proteins, but not those of the FtsZ2-family, are not only found in rings or networks but also in stromules (Vitha et al., 2001; Gremillon et al., 2007; Martin et al., 2009). Therefore, it seems plausible

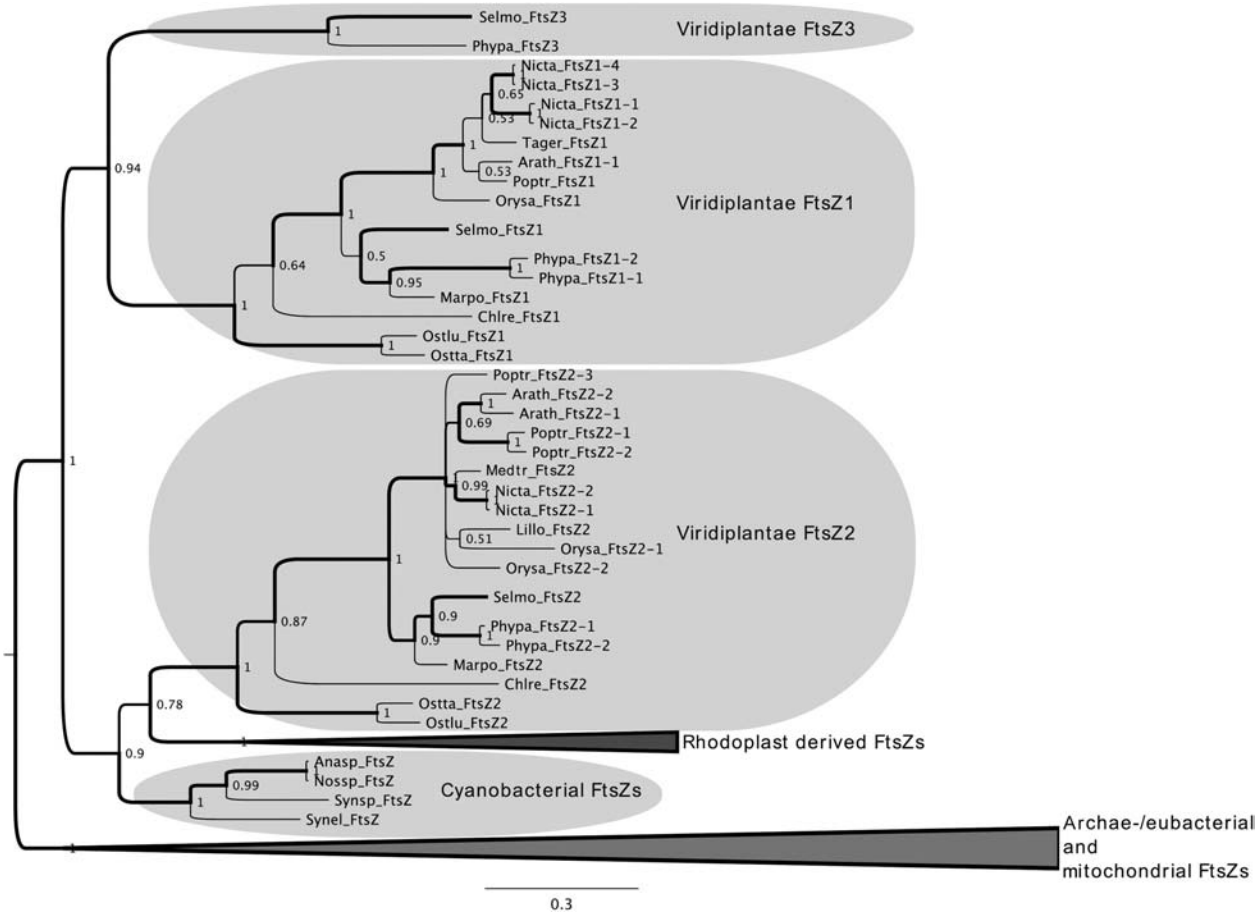

Figure 1 Phylogenetic tree of FtsZ proteins modified according to Martin et al. (2009).
The FtsZ sequences for each organism are indicated by a five letter code as follows: Anasp: *Anabaena* sp. (PCC 7120); Arath: *Arabidopsis thaliana*; Chlre: *Chlamydomonas reinhardtii*; Lillo: *Lilium longiflorum*; Marpo: *Marchantia polymorpha*; Medtr: *Medicago truncatula*; Nicta: *Nicotiana tabacum*; Nossp: *Nostoc* sp. PCC 7120; Orysa: *Oryza sativa*; Ostlu: *Ostreococcus lucimarinus*; Ostta: *Ostreococcus tauri*; Phypa: *Physcomitrella patens*; Poptr: *Populus trichocarpa*; Selmo: *Selaginella moellendorffii*; Synel: *Synechococcus elongatus* PCC 6301; Synsp: *Synechocystis* sp. PCC 6803; Tager: *Tagetes erecta*.

that these dynamic FtsZ1-filaments are the structural basis for stromule-building and -dynamics according to the 'pushing-out hypothesis' (see above), which would be another functionality of the plastoskeleton and an explanation for FtsZ diversity within a given plant.

Pharmacology of plastid division

The cell division of bacteria, including cyanobacteria, can be blocked by β-lactam antibiotics, possibly masking a penicillin-binding protein (PBp3, FtsI) that interacts with the FtsZ protein during the division process (Lutkenhaus, 1990). The same was described for cyanelles (Berenguer et al., 1987; Kies, 1988).

Surprisingly, β-lactam antibiotics also inhibit chloroplast division in *Physcomitrella*, although moss plastids have no peptidoglycan wall. As expected, the drugs had no effect on chloroplasts from tomato (*Lycopersicon esculentum*), indicating physiological similarities between the division of cyanobacteria, cyanelles, and moss chloroplasts on one hand, and differences to the plastid division process of flowering plants on the other (Kasten and Reski, 1997).

Already at low concentrations ampicillin, cefotaxim, and penicillin, respectively, led to enlarged chloroplasts in dividing cells of *Physcomitrella*, but had no effect on plastid shape or number in resting cells. These findings revealed a direct effect of the drugs on the organelle division process itself, as they had no secondary effect on plastids, e.g., organelles did not fuse after drug treatment. Furthermore, the comparison with tomato reveals an evolution of the chloroplast division machinery (Kasten and Reski, 1997). Interestingly, the drugs acted specifically as the major plastid protein composition remained unaffected, whereas in a mutant (Abel et al., 1989), plastid division was accompanied by increasing amounts of energy converting enzymes (Kasten et al., 1997).

Subsequently, Katayama et al. (2003) analyzed the effects of drugs that inhibit bacterial peptidoglycan synthesis (ampicillin, D-cycloserine, fosfomycin, vancomycin, and bacitracin) on chloroplast division in *Physcomitrella*. Independently confirming Kasten et al. (1997), Katayama et al. (2003) found that active antibiotics block chloroplast division and did not lead to fusion of the organelles. Division to normal size started upon removal of the drugs (Katayama et al., 2003). In the same year,

Iino and Hashimoto (2003) reported that cyanelles of *Cyanophora paradoxa* divide by ingrowth of the septum at the cleavage site. Unlike plastid division in higher plants, the inner and outer envelopes of cyanelles, the latter containing polysaccharides, do not constrict simultaneously. Further, they visualized a single electron-dense ring on the stromal face of the inner envelope membrane at the isthmus, but no ring-like structures on the outer envelope. Such a single, stromal ring is unique and also distinct from FtsZ rings, which are not detectable by electron microscopy. These features significantly add to the conclusion that cyanelle division represents an intermediate between cyanobacterial and plastid division (Iino and Hashimoto, 2003).

Subsequently, Sato et al. (2007) described a connection of the drugs with FtsZ-formation during cyanelle division. Utilizing anti-FtsZ antibodies and immunofluorescence microscopy, they demonstrated that an FtsZ arc and a split FtsZ ring emerged during the early and late stages of cyanelle division, respectively. FtsZ did not surround the division plane at an early stage of division, but formed an FtsZ arc at the constriction site. The constriction spread around the cyanelle, which gradually became dumbbell-shaped. After invagination of the envelope the ring split parallel to the division plane. Addition of ampicillin led to spherical cyanelles with an FtsZ arc or ring on the division plane. From that, Sato et al. (2007) concluded that the inhibition of peptidoglycan synthesis by ampicillin caused the inhibition of septum formation and a marked delay in constriction development.

Similarly, Suppanz et al. (2007) treated *Physcomitrella* with ampicillin and transfected protoplasts from these cultures with FtsZ::GFP fusion constructs. Plastid division rings were not found in such cells, probably because the FtsZ ring could not attach to the chloroplast inner membrane. Conversely, the FtsZ-based plastoskeleton was not obviously affected although the amount of FtsZ-filled stromules was significantly higher in drug-treated macrochloroplasts, supporting their putative function in regulating the amount of interface between plastids and the cytosol. Further, Suppanz et al. (2007) compared these results with the *Physcomitrella pdi* mutant that exhibits macrochloroplasts (Abel et al., 1989), which can start to divide upon treatment with blue light as well as after addition of the phytohormone cytokinin (Reski et al., 1991; Kasten et al., 1997). Transfected protoplasts from this mutant exhibited massive FtsZ1-filled stromules as well as plastoskeletal FtsZ2 networks (Figure 2). In contrast to drug treatment, these macrochloroplasts showed FtsZ rings as well, supporting the conclusion of Reutter et al. (1998) that in this mutant the defect in chloroplast division is due to a mutation in signaling rather than in the division process itself.

In contrast to cyanelles, no wall-like structures have been detected in plastids from other sources. It was surprising therefore that Machida et al. (2006) found five genes homologous to bacterial genes essential for peptidoglycan synthesis [MurE, MurG, two genes for D-Ala-D-Ala ligase (Ddl), and the gene for translocase I (MraY)] in the *Arabidopsis* genome and even nine such homologs

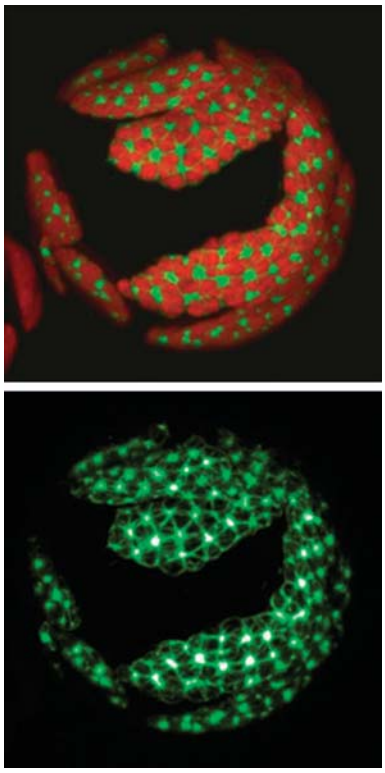

Figure 2 Confocal laser scanning micrograph of a protoplast from the *Physcomitrella pdi* mutant transfected with an FtsZ2-1::GFP fusion according to Suppanz et al. (2007). Top: overlay of chlorophyll fluorescence (red) and GFP signal (green). Bottom: GFP signal alone.

[MurA, B, C, D, E, and F, Ddl, genes for the penicillin-binding protein Pbp, and DD-carboxypeptidase (Dac)] in the *Physcomitrella* genome. Of these, at least the MurE- and the Pbp-protein are targeted to moss chloroplasts, as revealed by GFP-fusion proteins. As the *Physcomitrella* genome is highly efficient in gene targeting (Hohe et al., 2004), the loci of MurE and of Pbp were disrupted independently, resulting in macrochloroplasts in both loss-of-function mutants (Machida et al., 2006). As homologs of the bacterial penicillin-binding protein (Pbp) were found in *Physcomitrella* but not in *Arabidopsis*, Machida et al. (2006) thus elegantly explained why penicillin and other β-lactam antibiotics block division of bacteria, cyanelles, and moss chloroplasts, but do not affect plastid division in flowering plants.

In contrast to Pbp, MurE is encoded by bacteria, *Physcomitrella* and *Arabidopsis*. In bacteria, this protein catalyzes the ATP-dependent formation of uridine diphosphate-N-acetylmuramic acid-tripeptide in peptidoglycan biosynthesis. In *Arabidopsis*, the MurE gene is expressed in leaves and flowers, but not in roots or stems, and the protein is imported into chloroplasts (Garcia et al., 2008). AtMurE mutants exhibit a white phenotype and are inhibited in thylakoid membrane development, suggesting that MurE in flowering plants is involved in chloroplast biogenesis (Garcia et al., 2008). To analyze the functional relationships between the MurE genes of cyanobacteria, *Physcomitrella* and *Arabidopsis*, these authors tried to complement the macrochloroplast

phenoptype of the *Physcomitrella* PpMurE knockout mutant. While the bacterial *Anabaena* MurE gene, fused to the plastid targeting signal of PpMurE, restored plastid division in the moss mutant, transformation with AtMurE did not (Garcia et al., 2008). These results suggest that the MurE gene is important for peptidoglycan synthesis in bacteria, for chloroplast division in moss, and has assumed a different functionality (in chloroplast biogenesis) during evolution to seed plants, confirming the striking differences in the evolution of chloroplast division in land plants.

Future prospects

It is becoming increasingly clear that bacteria possess a cytoskeleton and chloroplasts a plastoskeleton, the latter derived from the former. Both help to maintain integrity, facilitate changes in shape and are the functional backbone for division. Furthermore, members of the plastoskeleton (FtsZ proteins) may be the driving force for stromules in plants. These dynamic structures may have functions in intercellular signaling and in gene transfer. Whether stromules are homologous to the bacterial pili, which have similar biological roles to those proposed for stromules, remains to be elucidated.

Likewise, several more topics in chloroplast biology are underexplored. For example, phosphate-starvation influences cell division, the number of FtsZ rings per chloroplasts and chloroplast replication in the alga *Nannochloris bacillaris* (Sumiya et al., 2008), whereas such data is missing for land plants. Further, it is evident that the bacterial cytoskeleton interacts with chromosome replication in *Bacillus subtilis* (Graumann, 2007), but less stringently with chromosome segregation in the cyanobacterium *Synechocystis* (Schneider et al., 2007), whereas comparable data on plastid DNA replication and segregation is lacking.

Taken together, plastids are structurally highly dynamic organelles, well integrated into growth and development of the cell. They are co-evolution products and consequently specific genes have acquired different functionalities during land plant radiation. Therefore, an evolutionary perspective is important in unraveling the physiology and cell biology of chloroplasts.

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