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INVITED REVIEW

Stromules and the dynamic nature of plastid morphology

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Summary

Investigation of plastids via green fluorescent protein (GFP) has led to the rediscovery of tubular extensions of the plastid membrane, termed stromules, for stroma-filled tubules. These unique structures are challenging our understanding of plastid structure and function. Stromules are highly dynamic, branching and elongating across the plant cell. Recent experiments indicate that cytoplasmic microtubules and microfilaments control the shape and motility of stromules. Whether stromule formation involves plastid-specific structural systems, such as the plastid division machinery, remains open to debate. Fluorescence photobleaching experiments have revealed that GFP can traffic between plastids joined by stromules. As a result, interest has grown in whether other macromolecules can also travel through these connections. Although the function of stromules is unknown, several aspects of their biology suggest they play a role in molecular exchange between plastids and other organelles.

1. Introduction

Stroma-filled tubules, or stromules, are tubular extensions of the plastid envelope membrane. In the past, these structures have been observed in a variety of species using conventional light and electron microscopy (EM). However, recent use of green fluorescent protein (GFP) targeted to plastids has allowed for the viewing of stromules *in vivo* in all parts of plants and has greatly facilitated the study of their structure and function. Using GFP, stromules have been studied in a number of higher plants including *Nicotiana tabacum*, *Petunia parodii*, *Arabidopsis thaliana*, *Triticum aestivum*, *Oryza sativa*, *Commelina communis*, *Allium cepa* and *Lycopersicon esculentum* (Köhler *et al.*, 1997; Tirlapur *et al.*, 1999; Langeveld *et al.*, 2000; Arimura *et al.*, 2001; Gray *et al.*, 2001; Pyke & Howells, 2002). Historically, research on stromules has focused on chloroplasts in leaves although, in fact, stromules occur less frequently in mesophyll cells than in other parts of higher plants (Fig. 1A). In angiosperms, stromules are most often encountered in flower petals, roots, hair cells, etiolated hypocotyls and liquid-cultured suspension cells (Fig. 1; Köhler & Hanson, 2000). In addition to their tissue specificity, stromules appear to be developmentally regulated. When leaves of *Nicotiana tabacum* are cultured on callus-inducing medium, the proportion of plastids carrying stromules increases as cells dedifferentiate and chloroplasts become leucoplasts (Köhler & Hanson, 2000). The nature of this regulation, as with many other properties of stromule biology, is still unknown.

This review will attempt to bring the reader up to date on the current status of research on stromules. We will then address several aspects of plastid and plant cell biology that are relevant to the understanding of how stromules are maintained in the cell and what their possible functions may be.

2. History

Reports of stromules appear only sporadically in the research literature: few systematic efforts have been made to understand their function. The lack of attention to these structures is understandable in light of the fact that stromules occur at a low frequency in chloroplasts, but chloroplasts are the most easily observed form of plastids. The large size, well-characterized ultrastructure and pigmentation of chloroplasts make them amenable to study using most forms of microscopy. Conversely, non-green plastids are more difficult to observe and identify, although their structure has been studied with EM. Stromules are particularly challenging to observe by conventional light and electron microscopy because their delicate structure and their ability to move in and out of the plane of sectioning make preservation and identification difficult. These complications notwithstanding, a number of studies on stromules have been conducted and the results have led to a variety of hypotheses on their function.

Senn (1908) produced an impressive monograph on chloroplasts in a variety of plant and algal species. In this early work,

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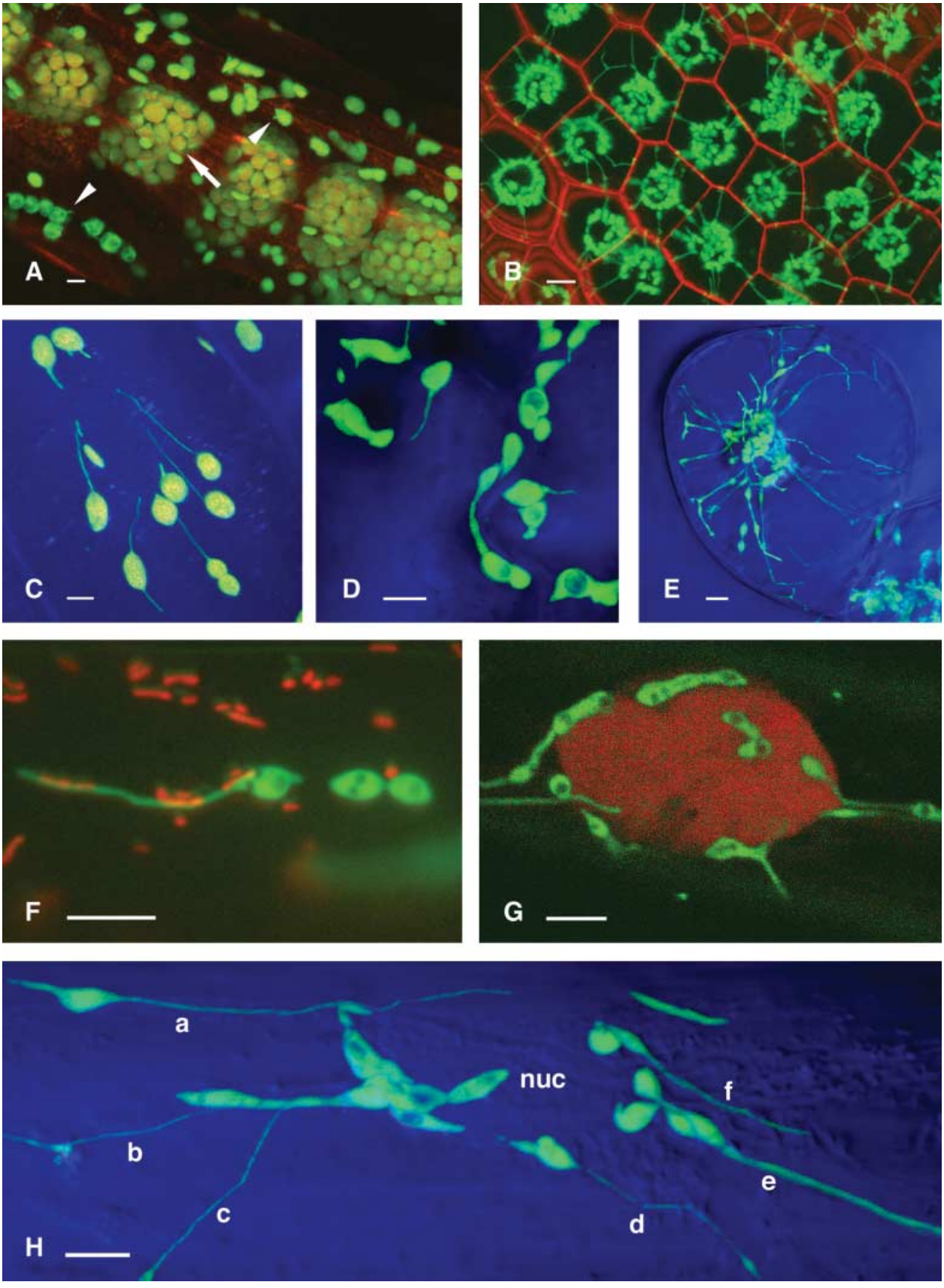
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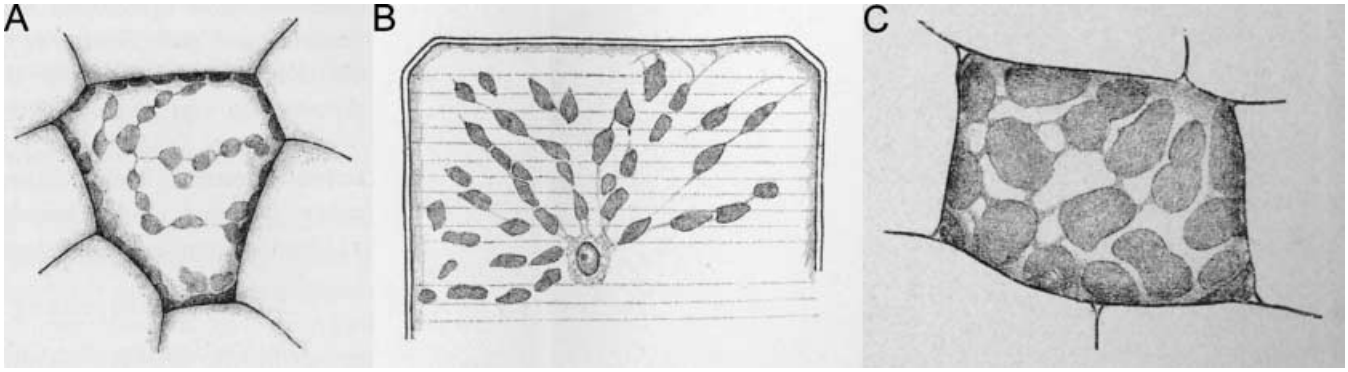

Fig. 2. Images suggestive of stromules obtained by Senn (1908). (A) Prothallus cell of the fern *Anemia phyllitis*, (B) diatom *Striatella unipunctata*, (C) moss *Funaria hygrometrica*.

Senn described his microscopic observations of structures that appear to correspond to the 'protuberances' described in later literature, as well as to the stromules visualized by GFP (Fig. 2). At the time of Senn's work, the structure of the chloroplast was still being debated. Based on his observations of chloroplast protuberances, Senn proposed the existence of the 'peristromium', a mobile structure surrounding the plastid. He described chloroplasts that had a colourless envelope from which pseudopods could emanate and retract. A discussion of stromule observations in the early literature can also be found in Gray *et al*. (2001). More recently, other groups have also interpreted chloroplast stromules as mechanisms of motility. Menzel (1994) found numerous long connections between chloroplasts in the unicellular green alga *Acetabularia*. Menzel noted that these connections appeared capable of bearing tension in motile strands of chloroplasts and suggested that stromules had a role in movement. Indeed, the motility of stromules does suggest they have a role in plastid movement (see below).

The dynamic nature of stromules has also led to theories associating stromules with mitochondria, which are also highly motile in the plant cytoplasm. Wildman *et al*. (1962) first proposed this theory on the basis of their work on chloroplasts of *Spinacia oleracea*. Their phase contrast cinephotomicrographs showed long, rapidly moving protuberances that often broke off from the chloroplast body, resulting in structures indistinguishable from mitochondria. More recently, Gunning (2003) has also recorded rapid movement of stromules in chloroplasts and chromoplasts. The phase contrast micrographs of Spencer & Unt (1965) showed isolated chloroplasts from *Spinacia* with what appear to be envelope protrusions. However, the morphology of the isolated chloroplasts suggests the organelles may not have been well preserved during isolation. Wildman (1967) went on to propose that chloroplasts were actually the source of mitochondria and that these two compartments were in a state of equilibrium. This theory was supported by electron micrographs of *Zea mays*, *Hordeum vulgare* and *Spinacia oleracea*, in which chloroplast protuberances contained membranes resembling mitochondrial cristae (Vesk *et al*., 1965). However, Weier & Thomson (1962) studied *Nicotiana rustica* and *Phaseolus vulgaris* with EM and described protrusions similar to those observed by Vesk and colleagues, but without the internal cristae-like membranes. Current understanding of the biogenesis of plastids and mitochondria rules out the possibility that mitochondria form from the stroma of chloroplasts. However, the hypothesis of Wildman and his collaborators is understandable when one has actually viewed chloroplast stromules and mitochondria in the phase contrast microscope and seen the remarkable similarity in appearance of the two structures.

Observation of irregularly shaped, or amoeboid, plastids under the electron microscope prompted the theory that stromules were one phase of plastid development. This theory was most comprehensively outlined by J. M. Whatley and

Fig. 1. Stromules are found in many different tissues of higher plants and exhibit highly variable morphology. (A–H) Fluorescence micrographs of *Nicotiana tabacum* expressing plastid-targeted fluorescent proteins. In all images, plastid-localized fluorescent proteins are pseudocoloured green whereas transmitted light images are pseudocolored blue. (A) Lateral view of cotyledon chloroplasts. Cotyledon is orientated with adaxial side up. Mesophyll chloroplasts (arrow) show few stromules whereas stomatal and epidermal chloroplasts show some short stromules (arrowheads) Chlorophyll autofluorescence is pseudocoloured red. (B) Developing cotyledon petiole showing plastids clustered around nuclei with stromules branching out to cell periphery. Propidium idodide staining of cell walls is pseudocoloured red. (C) Hypocotyl epidermis of light-grown seedling near the cotyledons. Overlap of GFP fluorescence and chlorophyll autofluorescence is pseudocoloured yellow. (D) Leucoplasts in pink region of corolla. (E) Liquid-cultured suspension cell. (F,G) Dark-grown hypocotyl epidermis expressing plastid-targeted CFP (pseudocoloured green) and GFP targeted to mitochondria (F) and nuclei (G) (both pseudocoloured red). (H) Dark-grown hypocotyl of seedling expressing plastid-targeted GFP showing many plastids clustered about the nucleus (nuc). 'a' to 'f' were measured to give the following stromule widths: a, 590 nm; b, 400 nm; c, 560 nm; d, 590 nm; e, 1080 nm; f, 560 nm. Scale bars = 5 μm.

colleagues. Whatley's group observed amoeboid plastids by EM in *Phaseolus* and a number of other species (Whatley, 1974, 1977, 1983a). Other groups observed amoeboid plastids in the scutellum of *Triticum* and *Secale* (O'Brien, 1951), leaves of *Lilium* and *Convallaria* (Steffen, 1964, as cited in Whatley, 1974), root tips of *Phaseolus* (Newcomb, 1967), leaves of *Spinacia* (Chaly *et al.*, 1980) and callus cultures of *Vitis* (Jasik & Hudak, 1987). Chaly & Possingham (1981) pointed out the distinction between amoeboid plastids and dividing plastids in root apices, thus providing evidence that the irregularly shaped plastids were a discreet phase of development and not merely an intermediate leading to plastid replication. In grain endosperm, amoeboid plastids were thought to be a stage of amyloplast differentiation (Buttrose, 1960; Parker, 1985). Several recent experiments in endosperm tissues have supported this theory (Langeveld *et al.*, 2000; Bechtel & Wilson, 2003). Thomson and Whatley recognized that amoeboid plastids were considered environmental responses by some groups (see below) but insisted they were a normal part of development, specifically in the transition from amyloplast to leucoplast in roots (Thomson & Whatley, 1980; Whatley, 1983b). They also theorized that formation of stromules could be due to a number of factors including changes in the viscosity of the stroma, changes in fluidity of the plastid envelope membrane, and changes in the ratio of the surface area of the envelope to plastid volume (Thomson & Whatley, 1980).

Another theory of stromules was based on the induction of stromule formation by various biotic and abiotic stresses. Plastid projections have been correlated with viral infection in both *Beta vulgaris* and *Lycopersicon* (Esau, 1944; Shalla, 1964). Drought and salt stress have been implicated in stromule formation in *Triticum* and *Hordeum* (Freeman & Duysen, 1975; Huang & van Steveninck, 1990; Yan, 1995). In *Spinacia*, manganese deficiency was believed to increase the number of amoeboid plastids (Possingham *et al.*, 1964). X-ray treatment of fern gametophytes was found to cause an array of morphological changes in chloroplasts, including the formation of stromule connections (Knudson, 1940). However, researchers also often noted that stromules were visible, but to a lesser degree, in unstressed conditions. In many cases, the statistical significance of the apparent induction was not dealt with rigorously. Furthermore, in all of these stress induction studies, researchers were looking specifically for ultrastructural changes when they described stromules. Researchers studying cells under unstressed conditions may also have observed stromules but disregarded them as artefacts of preservation. Therefore, early data linking stromules to stress must be interpreted carefully.

Presence of stromules has also been tied to plastid function in certain cells. With the growing recognition of the myriad biochemical pathways localized to plastids, several theories connecting stromule structure to enhanced metabolic activity were formulated. For instance, Yan (1995), who observed an increase in the occurrence of amoeboid plastids under salt stress, postulated that the change in plastid structure might be for the sake of increased metabolic activity to adapt to high salt conditions. Newcomb (1967) noticed that amoeboid plastids often encircled areas of cytoplasm that stained less densely than neighbouring regions of cytoplasm. He proposed that the amoeboid stage of plastids was a 'feeding stage' in which the protrusions allowed for rapid uptake of cytoplasmic contents. Laetsch & Prince (1969) observed a high frequency of protrusions from chloroplasts in the bundle sheath of *Saccharum* and proposed the structure was related to C4 photoassimilation. Lütz & Moser (1977) suggested a relationship between the formation of chloroplast 'proliferations' in alpine plants and their ability to grow at high altitude. Whatley & Whatley (1987) claimed that plastid protrusions might be related to chromoplast activity in secretory cells. This hypothesis was also offered by Charon *et al.* (1987), who saw stromules in pine resin duct cells. They proposed that the increase in surface area relative to volume might promote the mobilization of terpenes from their site of synthesis in plastids.

Stromules have also been associated with symbiotic activity. In root cells of *Alnus glutinosa* infected with *Frankia* actinomycetes, amyloplasts lost their starch and became amoeboid (Gardner *et al.*, 1989). Gardner and colleagues recognized that the change in structure might be related to a change in plastid activity as these cells engaged in symbiotic metabolism. Recently, stromules were found to form during mycorrhizal arbuscule development in *Nicotiana* (Fester *et al.*, 2001).

3. Structure

Research on the structure of plastids has advanced with every new development in microscopy and cell biology. There is now a large body of work on the structure of the envelope and thylakoid membranes and the ultrastructure of other features of the stroma such as plastoglobuli and prolamellar bodies (Sarafis, 1998). A variety of techniques have also been used to uncover the many different forms that plastids take in plants. Indeed, the name plastid comes from the organelle's plasticity of shape during development (Kirk & Tilney-Bassett, 1978). However, there is very little information available regarding how the overall structure of plastids is maintained and altered during development.

Plastid-targeted GFP allows for *in vivo* observation of stromules in three dimensions. In addition, the ability to target GFP to specific compartments of plastids has provided data on the exact composition of stromules. GFP targeted to the stroma accumulates in stromules, an observation that suggests that stromules are also bounded by the plastid envelope membrane (Köhler *et al.*, 1997). Expression of GFP fused to integral membrane proteins of the plastid envelope has confirmed this hypothesis. Gray *et al.* (2001) reported that in cells expressing GFP fused to the plastid outer envelope protein OEP14, fluorescence was observed surrounding both plastid bodies and stromules. Similar results were obtained with a fusion

between yellow fluorescent protein and TOC34, a component of the chloroplast outer envelope protein import machinery (R. H. Köhler and M. R. Hanson, unpublished observations). Furthermore, plants expressing a fusion between GFP and a plastid inner envelope phosphate translocator also exhibit fluorescence around stromules (Gray *et al.*, 2001).

The macrostructure of stromules is highly variable. Stromule length and width vary between tissues within a single species, and even within a single cell. In the recent literature, stromule width has been reported to range from less than 100 nm in *Lycopersicon* chromoplasts to 850 nm in *Nicotiana* chloroplasts (Köhler *et al.*, 1997; Gray *et al.*, 1999; Pyke & Howells, 2002). Köhler & Hanson (2000) have proposed that stromules be defined as extensions of the plastid envelope that are less than 800 nm wide to distinguish stromules from irregularly shaped plastids. Reports of stromule lengths include short, bud-like protrusions of *Oryza* chloroplasts and extensions up to 50 μm long in *Nicotiana* epidermis (Bourett *et al.*, 1999; Arimura *et al.*, 2001). An example of the variability of stromule lengths and widths is seen in the dark-grown hypocotyl epidermis of *Nicotiana tabacum* (Fig. 1H). In this tissue, thick stromules that could be considered irregularly shaped plastid bodies and long thin stromules that are clearly distinct from the main plastid body occur in the same cell.

In addition to these long thin projections of the plastid body, other forms of plastid envelope modifications have been documented. In *Lycopersicon*, Pyke & Howells (2002) observed stromules that resembled a string of beads. In some instances, it was impossible to detect GFP between the beads, although the authors recognized that very thin stromules might still be present. Clusters of bead-like structures are also observable in *Arabidopsis* and *Nicotiana* hypocotyls near the root–hypocotyl junction (E. Y. Kwok and M. R. Hanson, unpublished observations). Pyke & Howells (2002) also reported long thin stromules not attached to any plastid bodies. Stromules unconnected to plastid bodies were also described in *Nicotiana* leaf epidermis (Arimura *et al.*, 2001).

Thus stromules, much like plastids themselves, exist in many different forms. A wide variety of stromule-type structures have been documented in many species. However, nothing is known of how these structures are maintained. Current research in two areas, plastid division and chloroplast motility, provides clues to possible mechanisms for how plastid structure is regulated and may shed light on how stromules are formed.

4. Chloroplast division

4.1. Plastid division rings

Chloroplasts divide by fission: a chloroplast forms a constriction at its centre that narrows until the two halves pinch apart, yielding two daughters of similar size and shape. Kuroiwa *et al.* (1998) have made a thorough study of chloroplast division in the red alga *Cyanidioschyzon merolae*. When observed by EM, *C. merolae* constriction sites are marked by a set of electron-opaque rings, termed the plastid-dividing (PD) rings. Three PD rings are observable in *C. merolae*: the outer ring, associated with the outer envelope membrane's cytoplasmic face; the middle ring, in the intermembrane space of the plastid envelope; and the inner ring, associated with the inner envelope membrane's stromal face. In higher plants, only the inner and outer PD rings are visible by EM (Kuroiwa *et al.*, 1998). Little is known regarding the protein composition of the PD rings, except that the outer ring is composed of 5-nm filaments (Miyagishima *et al.*, 2001). However, two proteins associated with the PD rings, FtsZ and ARC5, have been identified and are required for chloroplast division. In addition, these two proteins are related to cytoskeletal proteins.

4.2. FtsZ-like proteins

FtsZ was originally identified in bacteria, where loss of FtsZ function results in bacteria that cannot divide, but continue to expand, forming filaments (Lutkenhaus *et al.*, 1980). FtsZ forms a ring, the Z ring, around the midpoint of the bacterium before fission, and constricts with the septum until the daughters separate (Bi & Lutkenhaus, 1991). FtsZ polymerizes *in vitro* to form filaments and sheets, reminiscent of microtubules (Erickson *et al.*, 1996). X-ray crystallography of FtsZ reveals that its structure is very similar to tubulin, although amino acid similarity between the two proteins is limited (Faguy & Doolittle, 1998). Some evidence suggests that FtsZ has mechanochemical activity that would give it the power to reshape the bacterial cell at the constriction site (Lu *et al.*, 2000).

Study of FtsZ in regard to chloroplasts was initiated by Osteryoung & Vierling (1995) based on the hypothesis that chloroplasts, which divide by fission and are descended from cyanobacteria, might also use FtsZ for division. FtsZ proteins are indeed found in many plants and play critical roles in chloroplast division (reviewed in Osteryoung & McAndrew, 2001). In *Arabidopsis*, three FtsZ genes are found in the nucleus and all three gene products are targeted to chloroplasts, where they form ring structures at the chloroplast midpoint (Osteryoung & Vierling, 1995; McAndrew *et al.*, 2001). Overexpression or silencing of these genes produces drastic changes in chloroplast number and size in mesophyll cells (Osteryoung *et al.*, 1998; Stokes *et al.*, 2000). Similar results were observed in *Nicotiana* and the moss *Physcomitrella patens* (Strepp *et al.*, 1998; Kiessling *et al.*, 2000; Jeong *et al.*, 2002). In all of these transgenic plants, cells contained only one to a few chloroplasts each, whereas the wild-type cells contained over 50 chloroplasts. In addition, the chloroplasts of the mutants were larger, preserving the ratio between chloroplast area and cell area found in wild-type plants. Thus, even though chloroplast division was perturbed, the chloroplasts still expanded to fill up the cell volume, as would wild-type chloroplasts. The fact that both reductions

and increases of FtsZ levels resulted in the same phenotype shows that careful control of FtsZ protein accumulation in the stroma is critical for correct division. When FtsZ proteins were fused to GFP and then overexpressed in *Arabidopsis*, fluorescent filaments formed around the edges of the chloroplast interior (Vitha *et al.*, 2001). Similar experiments in *P. patens* resulted in the appearance of a network of FtsZ–GFP in chloroplasts (Kiessling *et al.*, 2000). It is tempting to speculate that FtsZ may form an internal skeleton in chloroplasts, but it should be noted that these filamentous structures are only found after FtsZ is overexpressed and chloroplast morphology is perturbed. Nonetheless, in *Arabidopsis*, when FtsZ–GFP is overexpressed, epidermal cell chloroplasts show no change in number or morphology. In these wild-type-like epidermal chloroplasts, Vitha *et al.* (2001) observed stromules containing FtsZ–GFP filaments. Does FtsZ accumulate in stromules in wild-type plants? The localization and activity of FtsZ proteins in non-green tissues have not been determined and thus the role of these proteins in stromule formation is unknown.

In bacteria, proper placement of FtsZ at the bacterial midpoint is dependent on the Min proteins: mutants in these genes divide asymmetrically and produce minicells (Addinall & Holland, 2002). Homologues of Min proteins have been detected in higher plants. In *Arabidopsis*, overexpressing either MinD or MinE gives drastic chloroplast division changes (Colletti *et al.*, 2000; Kanamaru *et al.*, 2000; Dinkins *et al.*, 2001; Itoh *et al.*, 2001; Maple *et al.*, 2002; Reddy *et al.*, 2002). In both cases, there were fewer chloroplasts per cell, and the chloroplasts were a mixture of very large and very small chloroplasts, reminiscent of the bacterial minicell phenotype. In petal and leaf epidermal cells of these lines, chloroplasts were observed dividing asymmetrically (Colletti *et al.*, 2000). Thus Min proteins regulate placement of the division site in both bacteria and in chloroplasts. Is it possible that Min proteins also specify the location of stromule initiation on the plastid body?

The fact that bacterial FtsZ and Min proteins are involved in chloroplast division suggests that bacterial morphology proteins might also have roles in stromule structure. Bacteria were previously assumed to be without cytoskeletons, depending instead on their peptidoglycan walls for structure. However, new lines of research concerning bacterial morphology and secretion are providing information about how bacterial shape is controlled. The bacterial MreB protein polymerizes into filaments and has a three-dimensional structure similar to actin (reviewed in van den Ent *et al.*, 2001). Mutation of MreB causes rod-shaped bacteria to become spherical (reviewed in Egelman, 2003). ParM, another bacterial actin-like protein with filament-forming activity, is involved in plasmid segregation during cell division (van den Ent *et al.*, 2002). Bacterial pili, long thin protein tubes projecting from the bacterial cell membrane, are morphologically similar to stromules. In addition, the pili of type three secretion systems have been found to act as conduits for protein secretion. Pili and stromules therefore also share the ability to traffic proteins (He & Jin, 2003).

4.3. Dynamin-like proteins

ARC5 is a second protein whose role in plastid division and apparent cytoskeletal properties makes it a candidate for controlling plastid and stromule morphology. The *ARC5* gene is affected in the *arc5* mutant of *Arabidopsis*, which exhibits a reduction in the number of chloroplasts per mesophyll cell (Gao *et al.*, 2003). The mesophyll chloroplasts of *arc5* mutants are larger than wild-type equivalents and exhibit a constricted appearance, indicating an inability to complete division (Pyke & Leech, 1994). However, in meristems of *arc5* mutants, proplastid division is not disturbed (Robertson *et al.*, 1996). Therefore, ARC5 is only one of a number of factors regulating plastid division. This conclusion is supported by the phenotype observed in double mutants of *arc5* and *arc1*. The *arc1* mutant has an increased number of mesophyll chloroplasts relative to wild-type, with mutant chloroplasts having a smaller diameter than wild-type (Pyke & Leech, 1992). Double mutants of *arc5* and *arc1* have an intermediate phenotype, indicating that chloroplast division is not completely blocked in the *arc5* background (Pyke & Leech, 1994). The ARC5 protein is a dynamin-like protein that localizes to ring structures in dividing and non-dividing chloroplasts and resides on the cytoplasmic surface of the chloroplast envelope (Gao *et al.*, 2003). A dynamin-like protein similar to ARC5 has also been found in *C. merolae*. This protein forms rings associated with, but unique from, the outer PD ring (Miyagishima *et al.*, 2003).

The dynamins are a family of proteins found across eukaryotes. They participate in a number of different activities but share the capacity to manipulate membranes mechanically (Danino & Hinshaw, 2001). A number of dynamin-like proteins are found in plants. In *Arabidopsis*, ADL1A participates in cell plate formation and ADL6 has been implicated in Golgi vesicle trafficking (Lauber *et al.*, 1997; Jin *et al.*, 2001; Kang *et al.*, 2001). ADL2A has been shown to accumulate in plastids and is associated with plastid envelope membranes, although it is distinct from ARC5 and its function is as yet unknown (Kim *et al.*, 2001). ADL2B localizes to mitochondria, and mutations in this protein cause abnormal mitochondrial morphology (Arimura & Tsutsumi, 2002). The *Arabidopsis* genome also contains several other dynamin-like genes about which nothing is known. Whether any of these proteins have roles in stromule or plastid structure is unknown.

arc5 is just one of several chloroplast division mutants isolated by Pyke & Leech (1992). Analysis of other ARC mutants may also provide insight into the structural control of plastids. In particular, mesophyll chloroplasts of the *arc6* mutant are very similar to the large chloroplasts found in FtsZ mutants (Pyke *et al.*, 1994). Imaging of plastids in the vegetative meristems of *arc6* mutants shows that the mutation also reduces division of proplastids (Robertson *et al.*, 1995). Therefore, ARC6 may be required for division of all plastids. Although seriously compromised in plastid division, *arc6* mutants still contain plastids capable of forming stromules (Gray *et al.*, 2001). ARC6

has recently been cloned and is a DnaJ-like chaperone that resides in the chloroplast inner envelope membrane (Vitha *et al.*, 2003). ARC6 appears to localize to the midpoint of chloroplasts and is theorized to promote FtsZ filament formation (Vitha *et al.*, 2003). Thus ARC6 may also play a role in controlling stromule formation and morphology.

4.4. Artemis

ARTEMIS is another protein implicated in chloroplast division (Fulgosi *et al.*, 2002). The protein shares domains with proteins that direct peptide insertion into the mitochondrial inner membrane and chloroplast thylakoid membranes. ARTEMIS localizes to chloroplast inner envelope membranes and is an integral membrane protein. Insertion mutants of ARTEMIS have no gross phenotypic differences relative to wild-type, but their chloroplasts do not complete division, resulting in triangle-shaped chloroplasts. Mutant chloroplasts sometimes form long filaments with thylakoid and envelope membranes that appear wild-type. It has been proposed that ARTEMIS functions in assembly of the plastid division apparatus along the inner envelope membrane (Fulgosi *et al.*, 2002). Although no structural activities have been found for ARTEMIS, the ability to regulate inner envelope membrane architecture might allow ARTEMIS to dictate the shape of plastids and stromules.

Thus, FtsZ and ARC5 highlight the endosymbiotic past of chloroplasts. That is, division is controlled by both a bacterial division protein, FtsZ, which has been relocated to the nucleus, as well as a eukaryotic cytoskeletal protein, the dynamin-like ARC5. Continuing investigation of the ARC proteins, chloroplast FtsZ, ARTEMIS and perhaps bacterial morphology proteins will illuminate many aspects of plastid structure and function in the future, and perhaps shed light on the question of stromule structure. Another avenue of research that may inform on stromules is the study of chloroplast motility.

5. Chloroplast motility

Stromules are highly motile in most of the cells in which they are observed. They extend and contract from the plastid body, and undulate within the cytoplasm (see movies at http://www.mbg.cornell.edu/hanson/JMicro.html). In phase contrast cinephotomicrographs made by Wildman *et al.* (1962), as well as by Gunning (2003), stromules can be seen breaking off from one chloroplast and fusing with neighbouring chloroplasts. There is no truly stereotypical form of stromule movement: they move when connected to stationary plastids, they appear to anchor actively moving plastids, they translate across large regions of the cell along with their plastids and, sometimes, the stromules and plastid body are completely stationary (Kwok & Hanson, 2003). The question then arises: how is this motility achieved, and how is it regulated? One possibility is that motility is based on a plastid-specific structural system, perhaps associated with the division apparatus. Motility could be controlled by some internal cytoskeleton or a dynamin-like protein working on the outer envelope from the cytoplasmic side. However, the cytoplasm of plant cells is a highly kinetic environment, and so possibilities abound there as well. The activity of both actin- and tubulin-based cytoskeletal systems is well documented in plants. Plant cytoskeletal activity is dramatically illustrated by the cytoplasmic streaming visible in many plant cells.

Cytoplasmic streaming aids in mixing the cytoplasm of large vacuolate plant cells, ensuring that macromolecules and metabolites are evenly distributed. Streaming is best understood in the Characean algae, *Nitella* and *Chara*, in which streaming rates of up to 100 $\mu m\ s^{-1}$ are observed (reviewed in Shimmen & Yokota, 1994). In *Nitella*, the motile force for streaming is located at the interface between the immobile cortical ectoplasm and the motile endoplasm (Kamiya & Kuroda, 1956). Bundled actin microfilaments lie at this junction and support the movement of both myosin-coated beads and purified organelles in the same direction as that observed for endogenous streaming (Palevitz & Hepler, 1975; Shimmen & Tazawa, 1982). Based on these observations and many others, it is believed that streaming in Characean algae is accomplished by myosin movement along actin cables (reviewed in Shimmen & Yokota, 1994). Theoretical calculations suggest that localization of myosins to the membranes of vesicles and organelles, most likely the endoplasmic reticulum, could promote the rates of cytoplasmic streaming observed in these cells (Nothnagel & Webb, 1982). In higher plants, most cytoplasmic streaming, as well as organelle movements, is associated with actin and myosin, although some microtubule-based systems have been reported (reviewed in Williamson, 1993). In *Chara* and *Nitella*, chloroplasts are immobile and fixed in the ectoplasm, but in many plants such as *Vaucheria* and *Elodea*, chloroplasts move along with cytoplasmic streaming (reviewed in Haupt & Scheuerlein, 1990). In these systems, it is likely that chloroplasts are actively driven by an acto-myosin interaction, as the large size of chloroplasts would make it difficult for them to be borne passively by the streaming cytoplasm. Stromules could therefore use the same acto-myosin mechanism to move. In addition, because stromules are small, they could also be moved passively by collisions with streaming organelles and the action of the bulk cytoplasm. However, observation of stromules *in vivo* reveals that they often move against, or oblique to, the current of cytoplasmic streaming, indicating that stromules have some structural or motile system independent of streaming.

Chloroplasts also change their orientation and location within cells in response to a variety of environmental factors. Directed chloroplast movement is best studied in response to light, where chloroplasts show two different types of behaviour: an accumulation response, in which chloroplasts orientate themselves to maximize light absorption for efficient photosynthesis; and an avoidance response, in which chloroplasts orientate themselves to minimize light absorption to prevent light damage (reviewed in Wada *et al.*, 2003). Photorelocation

has been studied in a number of algal systems where both actin-dependent mechanisms, as in *Mougeotia* and *Vaucheria*, and tubulin-dependent systems, as in *Bryopsis* and *Ulva*, have been observed (Wagner *et al.*, 1972; Britz, 1979; Blatt *et al.*, 1980; Mizukami & Wada, 1981) In higher plants, such as the angiosperm *Lemna* and the fern *Adiantum*, photorelocation is believed to be exclusively actin-based (Kadota & Wada, 1992b; Tlalka & Gabrys, 1993). By contrast, in the moss *Physcomitrella*, chloroplast relocation is dependent on a combination of microfilaments and microtubules (Sato *et al.*, 2001). In a number of species, microfilaments have been observed in ring structures around chloroplasts, adding to the evidence that actin regulates chloroplast mobility (Kadota & Wada, 1992a; Kandasamy & Meagher, 1999; Sato *et al.*, 2001). Chloroplasts dominate the discussion of plastid motility because of their ease of observation, but some work has also been performed on non-green plastids. In pollen tubes, which show a very high rate of streaming, organelles, including amyloplasts, are moved by an acto-myosin system (Cai *et al.*, 2000). In roots, amyloplasts have been found associated with actin filaments (Collings *et al.*, 2001).

Do stromules move by actin filaments or microtubules? Is movement of stromules linked to their morphology? Cytoskeletal inhibitors have been used to test these questions in dark-grown hypocotyl epidermis of *Nicotiana* (Kwok & Hanson, 2003). Actin inhibitors cytochalasin D and latrunculin B stopped cytoplasmic streaming in these cells and also inhibited nearly all stromule movement. N-ethylmalemide (NEM), and 2,3-butanedione monoxime (BDM), which inhibit the ATPase activity of myosins, also blocked stromule and plastid movement (E. Y. Kwok and M. R. Hanson, unpublished results; Gray *et al.*, 2001). Thus non-green plastids and stromules rely on an acto-myosin system for motility. This motility is not merely a result of cytoplasmic streaming because plastids and stromules often move in the opposite direction of streaming. Treatment of stromules with the microtubule inhibitors amiprophosmethyl and oryzalin resulted in an increase in plastid and stromule motility, indicating that microtubules normally inhibit movement. Microtubule inhibition of organelle movement has also been observed for Golgi stacks in *Arabidopsis* as well as for chloroplast orientation in *Mougeotia* (Serlin & Ferrell, 1989; Nebenführ *et al.*, 1999) In the hypocotyls of a number of plants, microtubules form a dense meshwork in the cell cortex (Sakoda *et al.*, 1992; Ueda *et al.*, 1999; Hejnowicz *et al.*, 2000). This dense array of microtubules could inhibit movement of stromules and plastids as well as other organelles. We therefore propose a model for movement in which stromules and non-green plastids move along actin microfilaments via myosin motors, presumably attached to the plastid outer envelope. This movement is inhibited by the network of cortical microtubules. Whether this inhibition is purely due to steric interactions or is the product of a direct interaction between microtubules and plastids has yet to be determined.

An interesting result of the aforementioned motility experiments was the observation that stromule morphology also changed after treatment with cytoskeleton inhibitors (Kwok & Hanson, 2003). Inhibition of microfilaments resulted in a significant reduction in average plastid length. In these treated cells, many plastids took on an elliptical or bi-lobed appearance and the stromules that remained were always of the thick variety. Inhibition of microtubules gave a similar reduction in plastid length, but morphology was affected differently. As with actin inhibitors, many plastids took on an elliptical or bi-lobed shape. However, thin stromules were still visible. Thus, in hypocotyl epidermis, microfilaments are necessary for the maintenance of thin stromules. In addition, when actin inhibitors were rinsed out and cells were allowed to recover cytoplasmic streaming and plastid movement, the thin stromules did not reappear within 5 h. This evidence shows that thin stromules are not simply created by cytoplasmic streaming. Apparently, some interaction between the actin cytoskeleton and plastids is lost during inhibitor treatment and does not recover immediately after cytoplasmic streaming is re-established. Furthermore, thin stromules are not necessary for the recovery of plastid movement in the cell.

Thus it appears that motility and morphology of stromules can be accounted for in large part by the cytoskeletal systems of actin and tubulin. Indeed, joint treatment of both microtubule and microfilament inhibitors resulted in the near complete loss of both plastid motility and stromules (Kwok & Hanson, 2003). These conclusions are in keeping with what is known about chloroplast motility and the structure of the cytoskeleton in relation to plastids. However, many questions concerning stromule morphology and dynamics remain. For instance, if stromule shape and movement are maintained by cytoplasmic proteins, how is force transmitted to both the inner and the outer envelope membranes? Gray *et al.* (2001) have postulated that an internal plastid apparatus is necessary to join the envelopes. Future studies will need to determine exactly where motile and structural forces are applied to plastids and stromules.

6. Macromolecular trafficking

Although very little is known concerning stromule function, one activity of stromules has been characterized: protein trafficking. Early observation of stromules via plastid-targeted GFP revealed that GFP could move between plastids that were connected by stromules (Köhler *et al.*, 1997). GFP trafficking was observed by fluorescence recovery after photobleaching (FRAP; Klonis *et al.*, 2002). In a typical FRAP experiment (Fig. 3), one plastid in a pair of connected plastids is bleached of fluorescence by laser light. Fluorescence of both plastids is then monitored over time. If GFP molecules were unable to move through stromules, the fluorescence of the plastids would be expected to remain stable after bleaching. However, in most cases, GFP travels from the unbleached plastid to the bleached plastid via the stromule, resulting in fluorescence recovery in the bleached plastid and loss of fluorescence in the unbleached plastid.

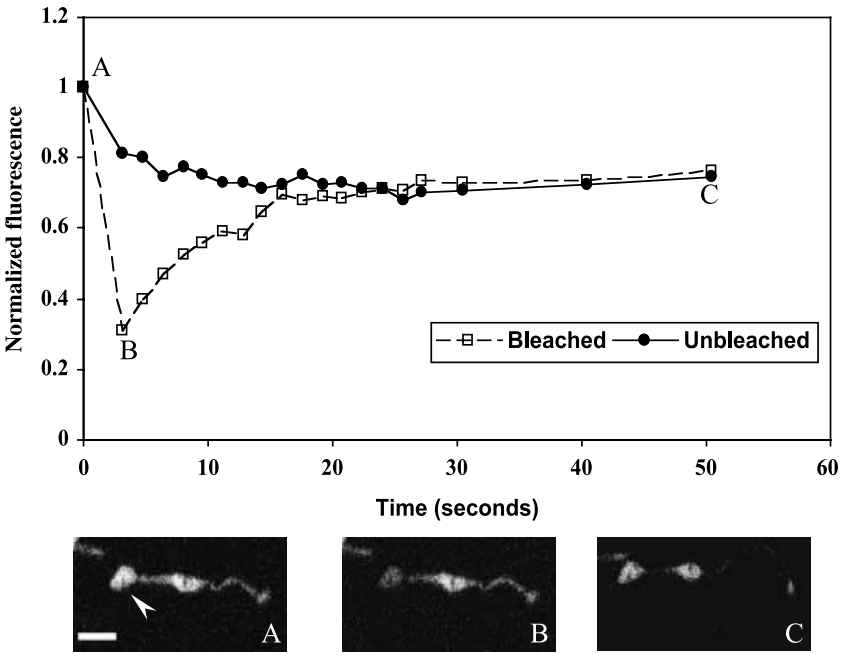

Fig. 3. Fluorescence recovery after photobleaching (FRAP) in a pair of plastids joined by a stromule. Graph represents change in fluorescence over time during a bleaching experiment conducted on the plastid pair depicted in A–C. Images A–C were taken at the points indicated by the corresponding letters on the graph. Fluorescence values at each time point were normalized to the starting fluorescence. (A) Pre-bleach image. Arrow indicates bleached plastid. (B) Post-bleach image. (C) Recovered image. Scale bar = 5 μm.

The ability of proteins to move through stromules implies that other molecules may traffic as well. The mobility of GFP, a foreign protein with molecular weight of 30 kDa, indicates that small metabolites should pass freely through stromules from plastid to plastid. Can plastid genomes, or RNA transcribed from those genomes, also move through stromules? Evidence in favour of protein or RNA exchange between mesophyll chloroplasts was obtained by Knoblauch *et al.* (1999) after microinjecting GFP expression vectors into chloroplasts. In these experiments, a single chloroplast in a *Vicia faba* mesophyll cell was injected with plasmids carrying the gene for GFP. Three days later, GFP fluorescence was detectable in at least 12 chloroplasts in the injected cells. Because the expression vectors carried plastid-specific promoters and because control experiments in which plasmids were injected into the cytosol did not result in GFP expression, it is unlikely that the observed GFP expression was a result of incorporation of the vector into the nucleus. Furthermore, the authors calculated that each injection resulted in the release of about three plasmids into the chloroplast, thereby ruling out the possibility that multiple chloroplasts were transformed. The fact that mature mesophyll cells were used in the experiments meant that fluorescence could not be transferred by division of the injected chloroplast. The authors therefore concluded that GFP travelled from chloroplast to chloroplast via stromules (Knoblauch *et al.*, 1999). It is also reasonable to consider that exchange of RNA molecules between chloroplasts could have caused the spread of GFP expression.

Motility of plastid genomes is a more complex issue. In general, plastid genomes map as circular chromosomes 100–200 kb in length (Gillham, 1994). Each plastid contains multiple copies of the genome that associate with proteins to form compact nucleoids in the stroma (reviewed in Kuroiwa, 1991). Recently, a histone-like protein was found associated with chloroplast nucleoids in *C. merolae*, suggesting a mechanism for genome packaging (Kobayashi *et al.*, 2002). Fluorescent staining of nucleoids with 4′,6-diamidino-2-phenylindole (DAPI) shows them to have a diameter of about 200 nm in higher plants (Kuroiwa *et al.*, 1981; Kuroiwa, 1991). This large size would prohibit the movement of nucleoids through smaller diameter stromules. However, our understanding of nucleoid biology is insufficient to rule out the possibility that individual DNA molecules might leave nucleoids and thus be capable of trafficking through stromules. Nonetheless, a strong body of genetic data further argues against the movement of plastid genomes through stromules.

The phenomenon of sorting out is perhaps the most obvious evidence that genomes are not exchanged freely between plastids of vascular plants. Sorting out is the process by which cells that contain a mixture of different plastid genomes eventually give rise to cells that have a single chloroplast genotype; that is, the different genomes segregate until only cells of single genotypes, representing each of the original genotypes, remain (reviewed in Birky, 2001). Sorting out is observable in species in which plastid inheritance is biparental and one of the parental lines is marked by a visible plastid mutation (reviewed in Kirk & Tilney-Bassett, 1978). The young cells of the offspring have a mixture of the parental chloroplasts. Mixed cells containing both mutant and wild-type plastids have been observed by microscopy (Kirk & Tilney-Bassett, 1978). Eventually, as the cells and chloroplasts divide, the two segregate until only the pure parental types are visible. This sorting out behaviour is believed to be random. Another example occurs when spontaneous mutations arise in the chloroplast genome that give

rise to visible phenotypes. Here, a single genome in one chloroplast is affected, but this genome, if it can still replicate, is passed on to progeny by cell division and chloroplast division. Over time, the mutation sorts out, leaving cells carrying only mutant chloroplasts or cells containing only wild-type chloroplasts. Thus sorting out implies that chloroplasts behave as individuals, and sharing of genomes does not occur. Lack of genome exchange between chloroplasts has also been shown in protoplast fusion experiments. In these experiments, protoplasts of cells containing different plastid genomes were fused together (Clark *et al.*, 1986; Hanson *et al.*, 1987). Subsequent analysis of the chloroplast genomes in the progeny of the fused cells showed no recombination. Recombination between chloroplast genomes of vascular plants is only observed at extremely low frequency when recombination is selected for with antibiotics (Medgyesy *et al.*, 1985).

Sorting out and the existence of mixed cells also suggests that significant amounts of RNA and protein are not exchanged either, because RNA or protein should be able to complement mutant plastids. How can the observation of GFP trafficking between plastids be reconciled with the phenomenon of sorting out? One possibility is that mutant chloroplasts for which sorting out is observed are so compromised that they are incapable of exchanging proteins or ribosomes with wild-type plastids. Another possibility is that exchange of proteins or RNA does occur, but the rate of exchange between mutant and wild-type plastids is not great enough to overcome certain genetic lesions. This would be particularly likely in tissues in which stromules are rare, such as mesophyll cells. A lack of exchange between mesophyll chloroplasts was demonstrated by Shiina *et al.* (2000), who fused mesophyll protoplasts containing chloroplasts transformed with GFP with wild-type protoplasts. They observed no transfer of GFP from the transformed chloroplasts to the wild-type chloroplasts. Finally, there do appear to be some instances in which the process of sorting out is masked by 'dominance' of one chloroplast genotype over another (reviewed in Kirk & Tilney-Bassett, 1978). This dominance could be achieved by the complementation of a mutant genotype by exchange of proteins or nucleic acids.

In contrast to the apparent lack of movement of plastid genomes through stromules, there is some evidence that small DNAs and RNAs may traffic between plastids. Hibberd *et al.* (1998) transiently transformed the chloroplast genomes of a number of angiosperms with GFP by particle bombardment. A few days thereafter, cells were observed in which, as expected, a single chloroplast was transformed and accumulated GFP. However, in about one-fifth of the positively transformed cells, every chloroplast in the cell contained GFP. Because plastid-specific regulatory sequences were used, it was unlikely that GFP was being distributed to plastids via expression in the nucleus and subsequent transit to chloroplasts. Likewise, it was improbable that every chloroplast in the cell had been transformed by an independent bombardment event. Hibberd and colleagues therefore proposed that GFP protein or genetic material had moved from chloroplast to chloroplast. Because each particle used in bombardment is coated with many plasmids, it is possible that the plasmids carrying the GFP construct spread between chloroplasts through stromules. In this manner, stromules may promote the production of homoplasmic chloroplast transformants following particle bombardment or plasmid uptake into protoplasts. Initially, chloroplast transformation results in only one or a few chloroplasts incorporating a transgene (Svab & Maliga, 1993). Homoplasmic plants are recovered after sorting out of the transformed and untransformed chloroplasts. Transfer of plasmids from bombarded chloroplasts to their untransformed neighbours could accelerate this sorting out process.

7. Proposed functions

Although the role of stromules in the plant cell is still largely unknown, several possible functions have been proposed. One function is suggested by the increased surface area to volume ratio that stromules provide: stromules may enhance the plastids' ability to exchange materials with the cytosol and other organelles (Köhler & Hanson, 2000). Plastids are the sites of many metabolic processes crucial to the cell. Their activity depends on efficient exchange of reactants and products across the envelope membrane with the cytoplasm and with other organelles. For example, photorespiration involves a circuit of exchange between chloroplasts, mitochondria and peroxisomes. Likewise, lipid synthesis requires a tight exchange between plastids and the endoplasmic reticulum. Close interactions between chloroplasts and other cytoplasmic organelles have been documented before by EM (Crotty & Ledbetter, 1973; Lütz & Moser, 1977; Whatley *et al.*, 1991). Recently, the use of GFP spectral variants targeted to different organelles has allowed the observation of organelle–organelle interactions *in vivo* (Fig. 1F,G). In cells in which mitochondria are marked with GFP and plastids are marked with cyan fluorescent protein, clusters of mitochondria are found around stromules (Fig. 1F). Gunning (2003) has also observed close interactions between mitochondria and plastid stromules. This theory of exchange between plastids and other organelles mirrors several of the historic hypotheses regarding stromule activity discussed above. Plastids carrying stromules are often found surrounding the nucleus (Fig. 1G). This association may allow exchange of molecules between plastid and nucleus. As in the past, stromules have been postulated to participate in secretion of plastid products. Pyke & Howells (2002) saw bead-like stromules in trichomes of *Lycopersicon* and suggested these beads might be a vehicle for mobilizing metabolites generated in the plastid.

The long length of stromules and their projection into distant parts of the plant cell has also suggested that stromules may allow plastids to sense the environment of the cell. Pyke and Howells (2002) point out that stromule frequency is inversely proportional to plastid frequency. They propose that stromules may allow plastids to reach out into the cell to sense the total

number of plastids in the cell and thus control plastid division (Pyke & Howells, 2002). In some cells, such as cultured suspension cells and developing leaf cells, stromules extend from plastid bodies clustered about the nucleus to the cell membrane (Fig. 1B,E). This configuration might allow sensation of light or electrical signals from the cell periphery (Tirlapur *et al*., 1999). These stromules could also be a pathway for plastids to deliver biosynthetic products to the plasma membrane or cell wall. Shiina *et al*. (2000) proposed that stromules might provide a conduit for mixing the contents of different plastids within a cell.

The involvement of stromules in plastid division has not been ruled out. Stromules in some cells may participate in steps leading to division. Some stromules may represent a remnant of past division. Although chloroplast division is well characterized and stromules do not appear to play a role, leucoplast and proplastid division is less well documented. In the case of amyloplasts, Bechtel & Wilson (2003) proposed that stromules might be necessary for division because the presence of large starch grains in endosperm amyloplasts prevents division by the conventional constriction mechanism of chloroplasts.

8. Conclusion

Stromules are a fascinating aspect of plastid biology that have been rediscovered thanks to the application of targeted fluorescent proteins. Understanding of their structure and function is still rudimentary at this point, but information is growing. A promising area of research is aimed at stromule structure and how it relates to the plastid division machinery and the cytoskeleton. Research on stromules will probably provide a better understanding of the control of structure, motility and division of plastids.

Note added in proof

Experiments relevant to the discussion of stromule motility in this review have recently appeared in Kwok, E. Y. & Hanson, M. R. (2004) *In vivo* analysis of interactions between GFP-labeled microfilaments and plastid stromules. *BMC Plant Biology*, **4**, 2.

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