Stromules: Recent Insights into a Long Neglected Feature of Plastid Morphology and Function [W]

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Extensions and protrusions of the plastid envelope had been described a number of times in the pre-1990s literature, including a report in 1888 and a 1908 monograph on plastids (for review, see Gray et al., 2001; Kwok and Hanson, 2004a), but they were never generally accepted features of chloroplasts and nongreen plastids until the era of fluorescent protein technology. The first GFP-labeled plastids in transgenic plants were visualized in 1996 (Köhler et al., 1997), leading to the rediscovery of the sometimes motile tubular structures extending from chloroplasts that were previously best documented by Sam Wildman and colleagues by phase contrast microscopy in the 1960s (Wildman et al., 1962). Plastids were occasionally observed that were connected by the long, thin extensions (Fig. 1), raising the possibility that molecules could be exchanged through them. Inspired by a report from the Lippincott-Schwartz group that utilized a photobleaching method to observe protein flow in the Golgi (Cole et al., 1996), we performed photodestruction of the green fluorescent signal present in a plastid connected to another plastid. Photobleaching eliminated the fluorescence of the targeted plastid, but green fluorescence quickly reappeared, due to the flow of GFP from the connected, unbleached plastid to the plastid body where GFP fluorescence had been abolished (fluorescence recovery after photobleaching [FRAP]; Köhler et al., 1997). GFP fluorescence could be eliminated from two different plastid bodies by directing the laser at a tubule connecting them (fluorescence loss in photobleaching; Hanson and Köhler, 2001). An example of a FRAP experiment is shown in Figure 1 and can be found as a time-lapse movie in Supplemental Movie S1.

After a science writer called with a request for an interview concerning the “microtubules” that had been found on chloroplasts, it became evident that a name was needed for the plastid tubules that would prevent confusion with other subcellular structures. In 1999, we chose the name “stromules” for these stroma-filled tubules, and it appeared first in 2000 in several of our papers (Köhler and Hanson, 2000; Köhler et al., 2000). Ten years later, this name now seems generally accepted and has appeared in a number of papers and reviews (Gray et al., 2001; Hanson and Köhler, 2001; Kwok and Hanson, 2004a; Natesan et al., 2005; Hanson and Sattarzadeh, 2008). A 2006 Web essay on stromules that accompanies a plant physiology textbook (Taiz and Zeiger, 2010) is available at http://5e.plantphys.net/article.php?ch=7&id=122.

CONTENT AND STRUCTURE

While the original GFP observed within tubules carried only the recA transit sequence, fusions of GFP to genuine chloroplast proteins have since shown that many different soluble proteins and protein complexes enter stromules. FRAP experiments demonstrated that a chloroplast enzyme and Rubisco traffic between plastids (Kwok and Hanson, 2004b), making it likely that many other molecules, including solutes and RNA, maybe be transferred as well. By our somewhat arbitrary definition, stromules are less than 1 μm in diameter; however, they are often much less than 1 μm. Thin fluorescent stromules less than 100 nm in diameter were observed on tomato (Solanum lycopersicum) chromoplasts (Pyke and Howells, 2002), and in Arabidopsis (Arabidopsis thaliana), they have even been measured at only 40 to 50 nm wide by electron microscopy (Holzinger et al., 2008). Nevertheless, most stromules should readily be able to accommodate multiprotein complexes as large as ribosomes. There is no evidence that thylakoid membranes enter stromules, as chlorophyll autofluorescence is not visible; in fact, the lack of chlorophyll is one reason that the presence of tubules escaped attention for so long. Chloroplast genomes are not likely to routinely be transported through stromules. Protoplast fusion experiments in the 1980s performed by a number of laboratories showed that plastid genomes normally segregate during the regeneration of somatic hybrid plants (Clark et al., 1986). Were stromules able to convey the nucleoid-localized plastid DNA from one plastid to organelle, recombined genomes in somatic hybrid plants would be expected not to be as rare as they actually are following protoplast fusion (Medgyesy et al., 1985; Clark et al., 1986). However, the much smaller plasmid DNAs that enter plastids...
through microinjection, protoplast uptake, or bombardment might traffic through stromules between plastids and thus participate in the generation of transplastomic plants. Propagation of the GFP signal within chloroplasts of a cell following microinjection of a single plastid with the encoding gene could have resulted from the transfer of small DNA molecules through stromules (Knoblauch et al., 1999).

The mechanism for the movement of proteins within stromules remains uncertain, but certainly diffusion plays a role. Measurements by fluorescence correlation spectroscopy indicated that GFP diffuses 50 times slower in the stroma than in the cytosol (Köhler et al., 2000), consistent with prior studies that found the stroma to be quite viscous. Observations with fluorescence correlation spectroscopy also indicated an active component to the movement of "packets" of GFP (Köhler et al., 2000). Small bodies of GFP can often be seen within a stromule, giving the appearance of a thin snake that has swallowed a small rodent (Fig. 2F). Such swellings along the length of a long stromule are also visible in the differential interference contrast images obtained by Gunning (2005). Chromoplasts of tomato fruit were observed to carry stromules with many such fluorescent bodies, producing a beaded appearance (Pyke and Howells, 2002). A movie of such a GFP mass proceeding in one direction through a stromule toward a large plastid body accompanied a previous review (Hanson and Sattarzadeh, 2011).

DETECTION AND DISTRIBUTION

Plastids and stromules can be labeled with fluorescent proteins expressed from either the nuclear or plastid genome in stably transformed plants or by transient expression through particle bombardment, agroinfiltration, or DNA uptake into protoplasts (Hanson and Sattarzadeh, 2011). Due to greater ease of generation of stable transgenic lines expressing fluorescent protein, dicot model systems such as Nicotiana and Arabidopsis have most often been used for studies of stromules, but they have also been observed by light or electron microscopy in monocots such as onion (Allium cepa), maize (Zea mays), iris (Iris sp.), rice (Oryza sativa), and wheat (Triticum aestivum; Bourett et al., 1999; Langeveld et al., 2000; Gray et al., 2001; Gunning, 2005; Sattarzadeh et al., 2010; Hanson and Sattarzadeh, 2011). The presence of stromules is not limited to vascular plants; they have been described in Euglena, Acetabularia, and in a variety of lower plants in the prefluorescent protein literature (for review, see Gray et al., 2001; Kwok and Hanson, 2004a). Stromules are well documented to be found more frequently on nongreen plastids than on chloroplasts (Köhler and Hanson, 2000; Pyke and Howells, 2002; Waters et al., 2004; Natesan et al., 2005; Figs. 2 and 3). GFP labeling of plastids has made evident the irregular shapes of the entire plastid sometimes found in certain tissue types such as petal (Fig. 2D). In addition to stromules, smaller protrusions lacking chlorophyll are often evident; these may be incipient or collapsed stromules (Fig. 2E). Rapidly growing suspension culture cells have particularly abundant, long stromules extending from plastid bodies clustered around the nucleus to the plasma membrane and other plastids at the cell periphery (Fig. 4B). Despite the appearance of a network in many suspension cultured cells, fluorescence loss in photobleaching experiments showed that only a few plastids and stromules were connected to one another and thus do not form an interconnected organelle (Köhler and Hanson, 2000), in contrast to the endoplasmic reticulum.

FORMATION AND MOBILITY

How stromules form has not been elucidated; theoretically they could result from pressure from within...
or by grasping of the envelope membrane from outside. Electron micrographs obtained by Holzinger et al. (2007a, 2008) reveal the presence of both the outer and inner envelope membrane surrounding stromules. These micrographs are consistent with fluorescent microscopic observations of stromules in plants, with envelopes labeled by fluorescent protein fusions to either inner or outer envelope membrane proteins (Gray et al., 2001; Hanson and Sattarzadeh, 2008).

Whether members of the Filamenting temperature-sensitive mutant Z (FtsZ) family of proteins are involved in stromule formation in vascular plants is unclear. Two families of FtsZ proteins, FtsZ-1 and FtsZ-2, are components of the plastid division apparatus and can form bundled protofilaments in vitro (Olson et al., 2010). The plastid-targeted stroma-located FtsZ proteins are obvious candidates for cytoskeleton-like elements possibly involved in stromule formation (Reski, 2009). In the moss Physcomitrella patens and in Arabidopsis, FtsZ-GFP fusions expressed at high levels have resulted in visualization of filamentous networks (Viitha et al., 2001; Martin et al., 2009b); however, whether endogenous FtsZ also forms a “plastoskeleton” is still uncertain. In moss, there is strong evidence for a role of FtsZ proteins in maintaining chloroplast shape; knockout mutants exhibit abnormal chloroplast morphology (Martin et al., 2009a). Further work will be needed to determine whether ftsZ and accumulation and replication of chloroplasts (arc) genes involved in plastid division may also play a role in stromule formation in vascular plants. Stromules in some cells of Arabidopsis arc3 and arc6 mutants are not only present but are more abundant and larger than in the wild type (Holzinger et al., 2008).

In vascular plants, both internal pressure and external attachment to the actin cytoskeleton may be important to maintain linear stromules. When the actin cytoskeleton is disrupted by cytochalasin D, most linear stromules disappear, but fluorescently labeled plastids assumed a bilobed shape that could result from a detached stromule falling back onto the main plastid body (Kwok and Hanson, 2003). Stromules sometimes appear to be anchored at a particular spot within the cell (Gunning, 2005; Hanson and Sattarzadeh, 2008). Detachment from a tethering point was captured by Gunning (2009) and illustrates the looping back and self-attachment that could result in the bilobed appearance of stromules that were observed following dissolution of the actin cytoskeleton (Kwok and Hanson, 2003). Double labeling of the actin cytoskeleton and stromules has revealed contact between microfilaments and stromules that may constitute anchor points for stromules (Kwok and Hanson, 2004c). The effect on chloroplast...
morphology of disruption of the actin cytoskeleton by cytochalasin D has also been monitored in the artic/alpine plant *Oxyria digyna*; according to electron microscopy, chloroplast protrusions decreased in number but were not entirely eliminated (Holzinger et al., 2007b).

While plastids move more slowly within plant cells than mitochondria and Golgi, plastids in both non-green tissues and green tissues of the leaf can be observed to travel within the cell. Stromules move rapidly in other cell types, often more rapidly than the main plastid bodies; spectacular movies of active stromules moving within the cytoplasmic stream were captured by video microscopy (Wildman et al., 1962; Gunning, 2005, 2009). A time-lapse movie of slowly moving stromules in a cluster of suspension culture cells is provided in Supplemental Movie S3.

The actin cytoskeleton is not only important for tethering of stromules but also for their movement. Several lines of evidence indicate that myosin motors, which operate on actin microfilaments, may be involved in stromule motility. Treatment of cells with the myosin ATPase inhibitor 2,3-butanedion 2-monoxime resulted in a loss of cytoplasmic streaming and stromule movement and a decrease in stromule length and abundance (Natesan et al., 2009). When myosin motor domains in a virus-induced silencing vector were introduced by agroinfiltration into *Nicotiana benthamiana*, expression of myosin XI was reduced and chloroplast positioning became abnormal (Sattarzadeh et al., 2009). Similar transient RNA silencing experiments, except with a construct carrying an inverted repeat of a myosin XI cargo domain, resulted in reduced abundance of stromules in leaf epidermal cells (Natesan et al., 2009). Transient expression of a GFP fused to a myosin XI tail from *N. benthamiana* introduced into tobacco *Nicotiana tabacum* leaf epidermis by biolistic transformation resulted in a loss of stromules and labeling of the chloroplasts and cell periphery (Natesan et al., 2009). In contrast, transient expression of a different yellow fluorescent protein (YFP) fusion, to an Arabidopsis or *Nicotiana* myosin XI-F tail region, by agroinfiltration resulted in the decoration of both chloroplasts and stromules (Sattarzadeh et al., 2009).

**FUNCTIONS**

**Increase in Envelope Surface Area for Exchange of Molecules**

Stromules are notably more abundant in cells with low plastid density (cultured cells, elongated nongreen cells in the plant) than those with many chloroplasts, such as mesophyll cells (Figs. 2 and 3). Stromules increase as cell length increases within the root (Fig. 4A; Köhler and Hanson, 2000). Measurements of stromule length in tobacco hypocotyls has confirmed that cells with lower density of plastids exhibit longer stromules (Waters et al., 2004). These observations suggest that one role they may play is to increase the surface area of the plastid compartment for import and export of molecules from other organelles or to sample a larger region of the cytoplasm in large cells. Stromules increase in number during dedifferentiation of leaf cells into callus cells (Köhler and Hanson, 2000) and also in cells that are forming...
Facilitating Transfer between Compartments

Most plastids are not connected by stromules at any one time, although over the course of a day, it is possible that many plastids within a cell establish transient contacts with one another through stromules. Nevertheless, transfer of materials among plastids is not likely to be their major function. Whether stromules ever fuse with other organelles is not known. Plastids and stromules are often observed in close proximity to other organelles and the endoplasmic reticulum (Kwok and Hanson, 2003, 2004d) and may facilitate the biochemical pathways that require the transfer of substrates and products between organelles, such as photospereiration and lipid synthesis. The close association of plastid bodies and stromules with the endoplasmic reticulum that has frequently been noted may assist in the import of proteins that flow from the secretory pathway into plastids (Radhamony and Theg, 2006). Stromules may function to reduce diffusion distance between organelles that exchange materials or provide a “highway” through which molecules must pass from one location to another, rather than wandering randomly “off road.” Furthermore, stromules may anchor plastids to a particular location within the cell in order to foster interactions between plastids and other cellular components. Proliferation of stromules in arbuscules may be important for the transfer of materials to the symbiont (Fester et al., 2007).

Signal Transduction

While it is not known whether stromules are involved in signal transduction, observations of their close associations with nuclei, including passage through nuclear grooves and channels (Kwok and Hanson, 2004d), raise the question of whether they might be conduits for signaling. The long stromules that pass from clusters of plastid bodies around the nucleus and extend toward the cell membrane, present not only in cultured cells (Fig. 4B) but also sometimes in the intact plant (Kwok and Hanson, 2004d), could be channels through which signals from the environment pass to plastids and then to the nucleus. There are some stromal proteins that unexpectedly appear to be involved in sugar and pathogen sensing (Huang et al., 2006; Krenz et al., 2010; Wangdi et al., 2010); whether their presence in stromules helps to mediate the signaling pathway can only be speculation at present.

Recycling of Chloroplast Content

Stromules may be involved in a mechanism to recycle chloroplast proteins during times of starvation or reduced photosynthesis. Wildman et al. (1962) sometimes observed that chloroplast tubules fragmented and vesicular structures floated away in the cytoplasmic stream. Gunning (2005) also documented this phenomenon and described it as “tip-shedding.” We and others (Pyke and Howells, 2002) have also observed circular bodies labeled with fluorescent chloroplast-targeted proteins that may not be attached to a main plastid body. The fate of the vesicles shed from stromules is unknown; possibly, they might come into contact with another plastid or stromule by chance and fuse with them, delivering their contents into a new plastid body. Alternatively, the vesicles might be shuttled to the vacuole for degradation.

As the most abundant stromal protein, Rubisco is a likely target for recycling of nutrients should some be needed for survival of the plant under suboptimal environmental conditions. Small vesicles containing Rubisco were found by immunoelectron microscopy to be located outside the chloroplasts in senescing wheat leaves (Chiba et al., 2003). In mature tobacco leaves, where stromal protein breakdown had begun, stroma-targeted GFP was observed to appear in 1-µm-diameter punctate loci within vacuoles of leaves treated with concanavalin A, an inhibitor of the vacuolar ATPase that had been shown to cause an accumulation of GFP-ATG8 autophagic bodies in vacuoles, likely due to inhibition of their breakdown. In order to determine whether these vacuolar bodies, which contain stromal protein but lack chlorophyll, might be autophagosomes, both a stroma-targeted DsRed and the GFP-ATG8 fusion were expressed in the same plant. Colocalization of the DsRed and GFP signal confirmed the identity of the vacuole bodies as autophagosomes containing stromal protein (Ishida et al., 2008). Furthermore, no such bodies were observed in a mutant with a disrupted ATG5 gene, which is essential for autophagy. Stromules also increased in abundance in the atg5 mutant (Ishida et al., 2008). A possible scenario, therefore, is that in wild-type plants under nutrient stress, tips or segments of stromules break off and enter the autophagic pathway, resulting in retention of the primary chloroplast body while allowing recycling of some of the plastid’s contents. An obvious advantage to recycling only a portion of the chloroplast and retaining the thylakoid membranes is that if conditions improve, photosynthesis could resume.

Recent analysis of starchless mutants indicates that the carbohydrate rather than the nitrogen status of the plant is likely what is sensed by the plant cell to determine whether stromal proteins should be recycled (Izumi et al., 2010).

CONCLUSIONS

Stromules are now established as genuine features of plastids in a variety of cell types in plants. Much remains to be learned about their function and formation. Quite possibly, these plastid appendages play more than one role in the cell. Most mutants that have
been analyzed with respect to stromule formation have been found to have more and/or longer stromules in certain cell types rather than fewer. As yet, no vascular plant mutant has been identified that completely lacks or exhibits greatly reduced stromule formation in all cells. If viable, such mutants would be valuable for determining which cellular processes are impaired in the absence of stromules and which molecules are required for their formation.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Movie S1. Photobleaching and recovery of green fluorescence within a plastid connected to another by a stromule.

Supplemental Movie S2. Tethering and streaming of stromules in hypocotyl of a dark-grown Nicotiana tabacum plant.

Supplemental Movie S3. Time-lapse movie of plastid and stromule movement in a tobacco suspension culture cell.

Supplemental Movie Legends S1.

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