Stromules: recent insights into a long neglected feature of plastid morphology and function

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Extensions and protrusions of the plastid envelope had been described a number of times in the pre-1990’s literature, including a report in 1888 and a 1908 monograph on plastids [reviewed in (Gray et al., 2001) and (Kwok and Hanson, 2004a), but never were generally accepted features of chloroplasts and non-green plastids until the era of fluorescent protein technology. The first green fluorescent protein (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 his colleagues by phase contrast microscopy in the 1960’s (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 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 (FLIM) (Hanson and Köhler, 2001). An example of a FRAP experiment is shown in Fig. 1 and can be found as a time-lapse movie available online (Supplementary Movie 1).

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


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 1µm; thin fluorescent stromules less than 100 nm in diameter were observed on tomato chromoplasts (Pyke and Howells, 2002) and in Arabidopsis have even been measured at only 40-50 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 1980’s performed by a number of labs showed that plastid genomes normally segregate during regeneration of somatic hybrid plants (Clark et al., 1986). Were stromules able to convey the nucleoid-localized plastid DNA from one chloroplast 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 transfer of small DNA molecules through stromules (Knoblauch et al., 1999).

The mechanism for movement of proteins within stromules remains uncertain, but certainly diffusion plays a role. Measurements by fluorescence correlation spectroscopy (FCS) indicated that GFP diffuses 50x 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 FCS 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 DIC images obtained by Gunning (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, 2008).
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, maize, iris, rice and wheat (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 pre-fluorescent protein literature [reviewed in (Gray et al., 2001; Kwok and Hanson, 2004a)]. Stromules are well-documented to be found more frequently on non-green plastids than on chloroplasts (Köhler and Hanson, 2000; Pyke and Howells, 2002; Waters et al., 2004; Natesan et al., 2005) (Fig. 2, 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. 3B). Despite the appearance of a network in many suspension cultured cells, FLIM 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 and colleagues (Holzinger et al., 2007a; Holzinger et al., 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 to outer envelope membrane proteins (Gray et al., 2001; Hanson and Sattarzadeh, 2008).

Whether members of the 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 (Vitha 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 *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 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 bi-lobed 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 (Gunning, 2009) and illustrates the looping-back and self-attachment that could result in the bi-lobed 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; Gunning, 2009). A time-lapse movie of slowly moving stromules in a cluster of suspension culture cells is provided as Supplementary Movie 3.

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 loss of cytoplasmic streaming and stromule movement and 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 leaf epidermis by biolistic transformation resulted in loss of stromules and labeling of the chloroplasts and cell periphery (Natesan et al., 2009). In contrast, transient expression of a different YFP fusion--to an Arabidopsis or Nicotiana myosin XI-F tail region--by agroinfiltration resulted in 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 non-green cells in the plant) than those with many chloroplasts such as mesophyll cells (Fig. 2, 3). Stromules increase as cell length increases within the root (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 de-differentiation of leaf cells into callus cells (Köhler and Hanson, 2000), and also in cells that are forming arbuscules due to infection with mycorrhizae (Fester et al., 2007).

**Facilitating transfer between compartments**

Most plastids are not connected by stromules at any one time, though 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 transfer of substrates and products between organelles, such as photorespiration and lipid synthesis. The close association of plastid bodies and stromules with the ER that has frequently been noted may assist in 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 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. 3B), but also seen 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 and his colleagues (Wildman et al., 1962) sometimes observed that chloroplast tubules fragmented and vesicular structures floated away in the cytoplasmic stream. Gunning (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, stromal-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 that contain stromal protein, but that lack chlorophyll, might be
autophagosomes, both a stromal- 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 or not stromal proteins should be recycled (Izumi et al.,
2010).

CONCLUSION

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.

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Figure Legends

Figure 1. Quick recovery of green fluorescence after localized photodestruction of GFP in a tobacco cultured cell plastid that is connected to another plastid via a stromule. A, Prebleach image. Arrow indicates region that will undergo localized photobleaching. B, Bleach image. The circled area was photobleached for 109 ms with a 488 Argon laser. C, After photobleaching the plastid within the circle is not fluorescent. D, GFP has recovered in the bleached plastid within the circle after 28 sec. Photobleaching and imaging was performed with an inverted Olympus FluoView1000 (Olympus America, Center Valley, PA). See also Supplementary Movie 1.

Figure 2. Plastid and stromule morphology in various tissues of transgenic plants encoding plastid-targeted GFP. A-C, tobacco trichome. D, Petal epidermis cells. E and H, tobacco epidermal cell in stem, F-G, tobacco hypocotyls. GFP fluorescence is shown as green and Red as chlorophyll auto-fluorescence. E, Yellow color is merged color of green and red. C, DIC image of tobacco trichome. F, A single image from the supplementary movie S2. Bar 10 μm. Laser scanning confocal microscopy (LSCM) was performed with a Leica TCS-SP2 (Leica Microsystems, Wetzlar, Germany) scanning head mounted on a Leica DMRE-7 upright microscope.
**Figure 3.** Distribution of stromules in different tissues of transgenic plants carrying a nuclear transgene encoding plastid–targeted GFP. A, Roots of *Arabidopsis thaliana*. B, tobacco suspension culture cells. A, Stromule numbers increase as cell length increases within the root. B, Plastid bodies surround the nucleus and stromules radiate outward. Cell walls are stained with propidium iodide (red). Images are maximum projection of 20 LSCM optical sections taken along the z-axis. Bar: 15 µm. LSCM was performed with (A) a Leica TCS-SP2 (Leica Microsystems, Wetzlar, Germany) (B) an inverted Zeiss LSM 710 Laser Scanning Microscope (Zeiss USA, Thornwood, NY).

**Figure 4.** Labeling of plastid envelope and stromules by a YFP::myosin XI fusion. Transient expression of YFP::myosin XI-F subdomain from *N. benthamiana* agroinfiltrated into *N. benthamiana* leaves. A, YFP signal (yellow). C, Chlorophyll autofluorescence (red). B, Merged images of YFP and chlorophyll autofluorescence. Images are maximum projections of 10 confocal images taken along the z-axis. Scale bar = 10 µm. LSCM was performed with a Leica TCS-SP2.

**Movie S1.** Photobleaching and recovery of green fluorescence within a plastid connected to another by a stromule. Photobleaching and imaging was performed with an inverted Olympus FluoView1000 (Olympus America, Center Valley, PA). See also Fig. 1.

**Movie S2.** Tethering and streaming of stromules in hypocotyl of a dark-grown *Nicotiana tobacum*. Note directional movement of a packet of plastid-targeted green fluorescent protein (GFP) through a stromule (left). Time-lapse series of 20 frames, 3 sec intervals. Time indicated in seconds. Images obtained by LSCM (Leica TCS-SP2).

**Movie S3.** Time-lapse movie of plastid and stromule movement in a tobacco suspension culture cell. Suspension cell cultures were derived from a tobacco plant carrying a nuclear
transgene encoding plastid GFP. Frames were taken as 30 minute intervals for a total of 8 h. Imaging performed using Andor Revolution® XD spinning disk laser microscopy system (Andor, South Windsor, CT) mounted on a Nikon A1 confocal scanning microscope (Nikon, Melville, NY).
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