

Cover image



STROMULES DO NOT FORM PLASTID NETWORKS

Stromules are tentacle-like protrusions of plastids that have been hypothesized to connect individual plastids and shuttle molecules between them. Schattat et al. (pages 1465-1477) re-examine this hypothesis using a photoconvertible fluorescent protein, which enabled differential coloring of the plastids in a cell, and directly monitoring the putative transfer of fluorescent proteins between plastids. Using this technique, the authors show that stromules extended by independent plastids do not fuse or allow exchange of fluorescent proteins between plastids. The cover image shows the differential coloring of plastids and their stromules in leaf epidermal cells. Chlorophyll autofluorescence is rendered in blue.

Differential Coloring Reveals That Plastids Do Not Form Networks for Exchanging Macromolecules

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Stroma-filled tubules named stromules are sporadic extensions of plastids. Earlier, photobleaching was used to demonstrate fluorescent protein diffusion between already interconnected plastids and formed the basis for suggesting that all plastids are able to form networks for exchanging macromolecules. However, a critical appraisal of literature shows that this conjecture is not supported by unequivocal experimental evidence. Here, using photoconvertible mEosFP, we created color differences between similar organelles that enabled us to distinguish clearly between organelle fusion and nonfusion events. Individual plastids, despite conveying a strong impression of interactivity and fusion, maintained well-defined boundaries and did not exchange fluorescent proteins. Moreover, the high pleomorphy of etioplasts from dark-grown seedlings, leucoplasts from roots, and assorted plastids in the *accumulation and replication of chloroplasts5 (arc5), arc6,* and *phosphoglucomutase1* mutants of *Arabidopsis thaliana* suggested that a single plastid unit might be easily mistaken for interconnected plastids. Our observations provide succinct evidence to refute the long-standing dogma of interplastid connectivity. The ability to create and maintain a large number of unique biochemical factories in the form of singular plastids might be a key feature underlying the versatility of green plants as it provides increased internal diversity for them to combat a wide range of environmental fluctuations and stresses.

INTRODUCTION

Both mitochondria and chloroplasts are considered organelles of endosymbiont derivation and contain internal membranes arranged within a bilayered envelope (Margulis and Stolz, 1984; Perkins et al., 1998). Plastids, especially chloroplasts, are responsible for the trapping of solar energy into carbon rich compounds, whereas mitochondria are involved in releasing energy from metabolites to drive active processes within the cell. The two organelles also transduce energy similarly through a chemiosmotic mechanism involving an electron transport chain (Mitchell, 1961; Allen et al., 2005). Sporadically, mitochondria and plastids extend tubules (Wildman et al., 1962; Menzel, 1994; Logan et al., 2004), named matrixules and stromules, respectively (Köhler et al., 1997; Scott et al., 2007). Visualization of these extensions in living cells strongly suggests that both organelles connect with similar organelles and exchange macromolecules. Indeed, fusion and fission are readily observed in mitochondria, and several genes involved in the process have already been characterized (Hoppins and Nunnari, 2009; van der Bliek, 2009). In the case of plastids, whereas the earliest suggestions of their possible interconnectivity appeared in the late 1880s

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(Haberlandt, 1888), it was only much later that photobleaching of green fluorescent protein (GFP) was used to demonstrate that fluorescent proteins could diffuse between interconnected plastids (Köhler et al., 1997; Kwok and Hanson, 2004a, 2004b). However, details of the precise mechanism by which two or more independent plastids can interconnect for exchanging proteins are lacking.

Nevertheless, lack of detail and critical evidence has not stopped the phenomenon of protein exchange between plastids via stromules from being presented as an established fact in reviews (Gray et al., 2001; Hanson and Sattarzadeh, 2011) and standard textbooks. According to Buchanan et al. (2000), "stromules not only have the ability to grow and contract; they can also fuse with other plastids. This creates stromal bridges between plastids through which genetic material can be exchanged." Similarly, Hanson and Köhler (2006), describe stromules as "long thin protuberances [that] sometimes form and extend from the main plastid body into the cytosol, occasionally touching and fusing with projections extending from other chloroplasts."

While trying to formulate our experimental approach through a perusal of relevant primary literature, we realized that the use of "touching and fusion" for describing stromule behavior is completely unfounded. The techniques used for observing stromules in living plant cells have either employed phase contrast and video-enhanced differential interference contrast microscopy (Wildman et al., 1962; Gunning, 2005) or depended upon time-lapse imaging of membrane tubules highlighted with a fluorescent protein (Köhler et al., 1997, 2000; Tirlapur et al., 1999; Köhler and Hanson, 2000; Shiina et al., 2000; Gray et al., 2001; Arimura et al., 2001; Pyke and Howells, 2002; Kwok and Hanson, 2004a;

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Waters et al., 2004; Shaw and Gray, 2011). In describing the behavior of dynamic stromules, these studies freely used the words fusion, connection, and interaction but have provided no parameters, other than proximity, as the basis for using these words. We realized that before seeking the mechanism of plastid fusion, we would have to establish that they actually fuse with each other.

To date, observations of fluorescently highlighted stromules and their behavior have relied largely on single-colored fluorescent proteins (Köhler et al., 1997; Tirlapur et al., 1999; Arimura et al., 2001; Kwok and Hanson, 2004a; Shaw and Gray, 2011). The use of single-colored proteins does not allow resolving between actual and apparent contact. Recent additions to the live imaging toolbox include novel fluorescent proteins (FPs) that change their initial fluorescence into another color upon photoconversion (Wiedenmann et al., 2004). The green-to-red photoconvertible EosFP (mEosFP), which has been used successfully in plants (Mathur et al., 2010; Wozny et al., 2012), allowed us to differentially color similar targeted organelles. Our experiments using this novel color-based approach for understanding organelle fusion have been performed in wild-type Arabidopsis thaliana and tobacco (Nicotiana tabacum) plants and on the plastids of Arabidopsis mutants, such as the accumulation and replication of chloroplasts5 (arc5) and arc6 (Pyke et al., 1994; Pyke and Leech, 1994) as well as phosphoglucomutase1 (pgm1; Casper et al., 1985), which are impaired in different aspects of plastid division and morphology. Our observations contradict earlier findings and suggest a major reappraisal of views on interplastid connectivity.

RESULTS

A Photoconvertible FP Allows Differential Coloring of Targeted Organelles

The targeting of mEosFP-based probes to mitochondria and peroxisomes has been reported earlier (Mathur et al., 2010). A novel probe was created for highlighting plastids by fusing the Nterminal transit peptide sequence of plastid ferredoxin NADP(H) oxidoreductase (Marques et al., 2003, 2004) to mEosFP and driving its expression under a cauliflower mosaic virus 35S promoter. Agroinfiltration of Nicotiana benthamiana leaves with tpFNR:mEosFP showed that each of the photoconvertible probes efficiently labeled their target organelle in a bright fluorescent green color. Photoconversion changed the green fluorescence of each organelle irreversibly to red (Figures 1A to 1C; see Supplemental Movies 1 to 3 online). By varying the duration of irradiation with violet blue light within a target area, different ratios of green and red fluorescent mEosFP molecules can be created (Mathur et al., 2010). As shown in Figure 1D (see Supplemental Figure 1 online), this property was especially useful for highlighting individual plastids clustered together in green (nonphotoconverted), yellow, orange, or red. Whereas it was not possible to differentiate between green colored plastids clustered in small groups (Figure 1E), differential coloring clearly highlighted individual plastids (Figure 1F). Retention of the specific fluorescent color over several hours suggested that each plastid constituted an independent unit.

Following successful demonstration of differential coloring, several *Arabidopsis* lines stably expressing Pro35S-tpFNR: mEosFP were created. All plastid types were highlighted in these plants, and nearly 30% (n = 200 cells) of chloroplasts in leaf epidermal cells in transgenic *Arabidopsis* tpFNR:mEosFP lines were found to extend stromules. Photoconversion of a small region of a stromule extended by a plastid caused a rapid color change within the entire plastid. Individual plastids maintained their color over several hours and stromules produced by each plastid (n = 500 observed) invariably possessed the same color as the parent body.

Earlier reports demonstrating the flow of GFP between interconnected plastids have not required setting up standards for arriving at conclusions of organelle fusion. Therefore, we used probes for mitochondria and peroxisomes for first establishing parameters that could be used to discriminate between true fusion and nonfusion of organelles.

Differentially Colored Mitochondria, but Not Peroxisomes, Undergo Fusion and FP Exchange

Whereas mitochondria are known to undergo frequent fusion and exchange proteins (Arimura et al., 2004), there are no reports showing the fusion of peroxisomes. We hypothesized that upon fusion, differentially colored red and green organelles would show an altered morphology accompanied by a rapid change in color to an intermediate hue. Alternatively, it may be concluded that fusion has not occurred when independent colors persist over time, despite apparent interactivity between organelles. Since a change in morphology of \sim 1- μ m diameter peroxisomes is not easily detectable, we used drp3A mutants of Arabidopsis that display abnormally elongated peroxisomes that often intermingle and cluster (Mano et al., 2004). The 15 \pm 7 μm length of peroxisomes in the drp3a mutant is also similar to the length of stromules extended by plastids. Accordingly, drp3A mutant (SALK_066958) plants stably expressing mEosFP carrying a carboxy terminal peroxisome targeting signal sequence type 1 (mEosFP-PTS1) were generated and showed elongated green fluorescent photoconvertible peroxisomes.

Observations of cells expressing mito-EosFP (Mathur et al., 2010) showed subpopulations of small (0.5 to 1.2 µm diameter) and elongated (3 to 7 µm long) mitochondria. Numerous mitochondrial fusions were recorded in five separate experiments (Figure 1G; see Supplemental Movie 2 online). Many fusions took place within a few seconds of each other and as many as six fusion events could be observed within 8 min of viewing. In each case, as predicted, the shape and color of mitochondria changed. Notably, in the same experiments, mitochondria that did not fuse maintained their individual colors (Figure 1G, panel 7, arrowhead). By contrast, when a subpopulation of tubular peroxisomes in the drp3A mutant were photoconverted and allowed to intermingle, they exhibited frequent aggregations and appeared to contact each other at numerous points (Figure 1H). However, after more than 7 h of imaging, the apparently interacting tubular peroxisomes failed to fuse or exchange FPs (Figure 1H; see Supplemental Movie 3 online).

The observations convincingly demonstrated that mEosFP could be used to observe fusion events and that these events



Figure 1. Photoconvertible Probes Allow Differential Coloring of Similar Organelles.

(A) Representative image of fluorescently highlighted mitochondria in a cell transiently expressing mito-mEosFP. A single mitochondrion (arrowhead) photoconverted to red color.

(B) Representative image of green and red (photoconverted; e.g., arrowheads) peroxisomes fluorescently highlighted using mEosFP-PTS1.

(C) Chloroplasts expressing the stroma-targeted tpFNR:mEosFP probe appear green before and red (arrow) after photoconversion (see Supplemental Movie 1 online).

(D) Differential coloring of chloroplasts packed around the nucleus achieved by varying the duration of photoconversion. Circles depict green, yellow, and orange-red based on a 0 to 255 scale (see Supplemental Figure 1 online).

(E) An interconnected plastid network (circle) suggested by a group of six green fluorescent chloroplasts with stromules before differential coloring.

(F) Irreversible photoconversion to a red color distinguishes a single chloroplast (asterisk) and differentiates its stromule from others in the group. (G) Representative, sequential snapshots from a 4-min time-lapse series (see Supplemental Movie 2 online) depict alignment (panels 1, 2, 4, and 6) and fusion (arrows, panels 3 and 7) of nonphotoconverted green and irreversibly photoconverted red mitochondria. Fusion results in altered mitochondrial morphology with an intermediate color, whereas nonfused mitochondria (arrowheads, panel 8) maintain their initial color.

(H) Representative snapshots from a series of time-lapse images (see Supplemental Movie 3 online) of differentially colored elongated peroxisomes in a *drp3A* mutant observed over 40 min. Despite appearing to intermingle and interact (e.g., arrowhead, panels 1 to 7), the tubules do not exchange FPs and separate (panel 8) while retaining their independent coloration.

would lead to a rapid exchange of FPs, resulting in a detectable color change.

Stromules from Differentially Colored Chloroplasts Appear to Interact but Do Not Exchange FPs

Based on our observations of fusing mitochondria and nonfusing peroxisomes, it was decided that an interaction between stromules involving a membrane fusion event was to be concluded only when an exchange of FPs leading to an intermediate color was observed between two physically separate, different-colored plastids (Figure 1I). Alternatively, nonconnectivity and the lack of fusion were to be concluded when plastids did not exchange FP during 2 h of coalignment and apparent contact. The 2-h limit was reached by evaluating the time that a plastid pair could be observed repeatedly without photobleaching the green fluorescence of mEosFP.

Observations of green fluorescent stromules in transiently expressing N. benthamiana leaves and transgenic Arabidopsis seedlings taken before photoconversion suggested several different positions in which stromules might contact each other (Figure 1I). FP exchange might be expected in each of these situations.

We actively searched for and subjected these positions to investigation. Representative observations depict a thin stromule that appears to connect two plastids before photoconversion (Figures 2A [panel 1], 2B [panel 1], and 2C; see Supplemental Movie 4 online). However, photoconversion of either plastid in a pair connected by a thin stromule generally revealed that the red fluorescence extended for only a short distance within the apparently single tubule (Figure 2A, panels 2 to 6). In other cases (Figures 2B [panel 2] and 2C), the stromule extension was traced to just one of the two plastids. These spatial relationships were maintained in some cases over 25 \pm 10 min without any exchange of FPs between the two plastids. In yet other cases, the stromules retracted and the plastids moved away independently within the general cytoplasmic stream (e.g., Figure 2A, panels 2 to 8; see Supplemental Movie 4 online). Alternatively, stromules could align to form several possible lateral interaction points (Figure 2D; see Supplemental Movies 5 to 7 online). Color differentiation also showed stromules being extended and retracted by plastids that appeared to create and break contact intermittently but did not exchange FPs (Figure 2E; see Supplemental Movie 5 online). Dynamic stromules entwined within clusters displayed multiple interaction points at places where they crossed each other (Figure 2F, panels 1 to 5; see Supplemental Movie 6 online). Although the time during which the stromules appeared to interact ranged from a few seconds to as long as 50 min and displayed clear regions of overlap (Figure 2F, panels 2 to 4, arrowheads), the stromules did not fuse, change shape, or exchange FPs. In addition to the dynamic tubular stromules, we also investigated plastids clustered around the nucleus or at other locations that could be expected to exchange FPs due to their close proximity. These plastids also extended stromules sporadically but maintained their color identity for up to 5 h of observation (Figures 1D and 2G).

Increased Frequency of Plastid Stromules in a Cell Does Not Lead to FP Exchange

One of the reasons cited for the inability to draw conclusions on exchange of FPs between chloroplasts is the low incidence of stromule formation from chloroplasts in light-grown plants (Kwok and Hanson, 2004a). In our hands, agroinfiltration of young tobacco tpFNR:GFP leaves results on average in nearly a twofold increase in the frequency of stromules from chloroplasts in epidermal cells compared with mock-treated leaves (n = 500plastids \times three independent experiments; Figures 2H to 2J). Moreover, some of the infiltrations resulted in cells in which up to 70% plastids, many of them clustered around the nucleus (Figure 2J) developed stromules. Similar stromule frequencies were obtained upon agroinfiltration of wild-type tobacco leaves with tpFNR:mEosFP and thus provided us with sufficient plastids extending stromules for carrying out a large number of experiments. In more than 10 different agroinfiltration experiments and observations of dynamic stromules in more than 300 chloroplasts, covering more than 13 h of time-lapse movies, we were unable to find even a single event of interplastid fusion and FP exchange.

A second approach aimed at increasing the chances of observing stromule interactions while not depending upon transient protein expression involved the arc6 mutant of Arabidopsis (Pyke et al., 1994). In accordance with literature (Holzinger et al., 2008), arc6 stably expressing tpFNR:mEosFP displayed two to three giant chloroplasts and three to four smaller plastids in epidermal cells (Figure 2K), while multiple stromules extended from the large plastids to form large overlapping loops. Photoconversion allowed each plastid and its stromules to be colored differently (Figure 2K). Observations performed over 5 to 12 h did not reveal any stromule membrane fusions or change in fluorescent color of stromules in transgenic arc6 seedlings. The continued cytoplasmic streaming and the dynamic extension and retraction of stromules throughout the observation period showed that general cellular processes were not inhibited in these mutant cells.

The two sets of experiments presented above allowed us to conclude that despite the strong impression of interplastid connectivity, there is no exchange of FPs between chloroplasts. Since the present evidence for FP diffusion between plastids via

Figure 1. (continued).

⁽I) Schematic based on (G) and (H) creates a set of guidelines for observations that might lead to conclusions of fusion or nonfusion between independent plastids. 1, The different situations suggesting plastid interactions before color differentiation; 2, differential coloring followed by maintenance of colors within discrete domains and indicates lack of plastid fusion; 3, differential coloring followed by exchange of FPs that results in an intermediate color and represents true interplastid connectivity and fusion.

Bars = 2.5 μ m in (A), (B), and (G) and 5 μ m in (C) to (F) and (H).



Figure 2. Each Plastid Unit Maintains Its Discrete Identity without Exchanging FPs.

(A) Sequential snapshots from Supplemental Movie 4 online showing a single stromule apparently linking two chloroplasts (panel 1). Photoconversion (panel 2) splits the tubule into two independent stromules of varying lengths (arrowhead). Panels 2 to 7 depict stromule retraction along the cytoplasmic streaming–induced movement of the plastid pair suggested by arrow in panel 6.

(B) Two plastids appear to be linked by a stromule (panel 1), but photoconversion of the lower plastid shows the long stromule is extended by the upper plastid only (panel 2).

(C) Dividing plastid (top) linked to another by a stromule observed 30 min after photoconversion shows no FP exchange.

(D) Lateral alignment of differentially colored stromules (panels 1 and 2) without protein exchange (see Supplemental Movie 5 online).

(E) Sequential, representative snapshots of three plastids (letters a to c) taken from Supplemental Movie 7 online extending and retracting stromules over 25 min without exchanging proteins. Panels 2 and 4 suggest contact between plastids, while panels 7 and 8 depict plastids moving away from each other without any apparent exchange of proteins.

(F) Snapshots from a time-lapse series (see Supplemental Movie 6 online) of a photoconverted red stromule in close contact with a nonphotoconverted green stromule (asterisk; panel 1). Panels 2 to 5 present an enlarged view of the box in panel 1. Despite apparent local contact (arrowheads in panels 2 to 4), each stromule maintains its independent color.

(G) Chloroplasts aggregated around a nucleus (n) extend stromules independently. A single yellow stromule (arrowhead) appears to be in contact with two plastids (blue autofluorescence) but does not exchange proteins over 30 min.

(H) A twofold increase in stromule extension observed upon agroinfiltration of tobacco leaves. AIM, *Agrobacterium* infiltration medium only; GV, *Agrobacterium* infiltrated; NI, noninfiltrated. Sample size = 75 cells per treatment.

(I) and (J) Representative snapshots of mock-treated and agroinfiltrated leaves show the difference in clustering of plastids around the nucleus (n; white circle) and increase in stromule frequency from the chloroplasts.

(K) Giant plastids in the *Arabidopsis arc6* mutant expressing tpFNR:mEosFP with elaborate stromules that appear to interact. Photoconverted plastids (asterisks) and their stromules highlighted in red do not exchange FPs with nonphotoconverted plastids (green) over 12 h of observation. Bars = 2.5μ m in (A) to (E), 10 μ m in (F1) and (K), 2.5μ m in (F2) to (F5), and 5μ m in (G), (I), and (J).

stroma-filled tubules is based largely on observations from tubular plastids, our investigations were extended to similar plastids.

Dark-Grown Seedlings Display Pleomorphic Plastids That Maintain Independent Color Identities

Following procedures described in earlier publications (Kwok and Hanson, 2004a, 2004b; Newell et al., 2012), seeds of tobacco (tpFNR:GFP) and *Arabidopsis* (tpFNR:GFP and tpFNR: mEosFP) germinated in the dark for 5 to 7 d were observed immediately after unwrapping. Figure 3A (see Supplemental Table 1 online) shows the variation in plastid morphology under dark and light conditions. Seedlings maintained in light showed normal oblate chloroplasts, whereas etioplasts of dark-grown seedlings were significantly elongated and displayed a higher frequency of stromule formation (see Supplemental Table 1 online). Whereas none of the chloroplasts (n = 500 plastids) in light-grown seedlings of tobacco and *Arabidopsis* displayed elongation, 26% ± 2% of the plastids in dark-grown *Arabidopsis* seedlings and 21% ± 7% from *N. benthamiana* were elongated.



Figure 3. Darkness Promotes Plastid Elongation and Pleomorphy.

(A) Representative snapshots of light- and dark-grown tobacco and *Arabidopsis* seedlings showing changes in plastid morphology.
(B) and (C) Elaborate stromules seen in *Arabidopsis* expressing tpFNR:mEosFP plastids maintained in the dark convey the impression of stromule

Elaborate stromules were observed when transgenic *Arabidopsis* seedlings grown in light were transferred to dark for 5 d. A varying number of plastids clustered, and, with their stromules, this conveyed an impression of networks (Figure 3B). Photoconversion initiated at any point in a plastid body or its stromule caused rapid color change, which despite apparent interactions with contiguous stromules over several hours did not diffuse into the entire cluster (Figure 3C). Similar observations on leucoplasts in tobacco Bright Yellow 2 (BY2) cells (see Supplemental Figure 2 online) established that tubular plastids maintain a clear boundary beyond which FPs do not diffuse.

A strong assumption common to many publications on stromules is that two bulbous regions linked by a stroma-filled tubule represent two interconnected plastids. Since plastid fusion was not observed by us, we investigated whether a single plastid might be misconstrued as interconnected plastids.

A Single Pleomorphic Plastid Can Be Misinterpreted as Two Interconnected Plastids

Plastids in hypocotyl and root tissue of dark-grown *Arabidopsis* expressing tpFNR:mEosFP and *N. benthamiana* expressing tpFNR:GFP were examined by time-lapse imaging. We sought out organelles with bulbous ends that looked similar to published images of so-called interconnected plastids (Köhler et al., 1997; Kwok and Hanson, 2004a; see Supplemental Figure 3 online). Photoconversion at either dilated end changed the color of the entire plastid, indicating that it represented a single compartment (Figure 3D; see Supplemental Movie 8 online). In some cases, the color diffusion could be followed easily (e.g., Figure 3E; see Supplemental Movie 9 online). Interestingly, time-lapse imaging showed that over a duration of several minutes, almost every elongated plastid changes its morphology, with new dilations conveying the impression of two or more interconnected plastids (e.g., Figure 3D; see Supplemental Movie 8 online).

Additional support for a possible role for plastid pleomorphy in creating misinterpretations came from the *pgm1* mutant of *Arabidopsis* that possesses chloroplasts with an abnormal elongated morphology (Casper et al., 1985). Time-lapse observations of single green fluorescent plastids in *pgm1* transformed with tpFNR:mGFP (Figure 3F; see Supplemental Movie 10 online) showed that the formation of new dilations and their redistribution within tubular plastids can be quite misleading.

The pleomorphy shown by elongated plastids was traced to differences in the internal membrane organization between light-

and dark-grown plastids. Whereas chloroplasts exhibited the typical stacked thylakoids (Figure 4A), dark-grown etioplasts were characterized by loosely clustered, amorphous prolameller bodies that appear to lack the structural rigidity of grana (Figures 4B and 4C). In a few instances (e.g., Figure 4D), two large bodies connected by a thin stroma-filled tubule were sectioned, and while one end (Figure 4D, labeled a) had features of a chloroplast with well-defined, stacked thylakoids, the other bulbous end (Figure 4D, labeled b) contained bulked-up stroma only. Such bulbous bodies with bulked up stroma could easily suggest interconnected plastids.

Based on a careful appraisal of experimental conditions and comparison of images presented for the plastids on which photobleaching experiments have been reported by Köhler et al. (1997) and Kwok and Hanson (2004a), we conclude that they represent single, pleomorphic leucoplasts and etioplasts. Plastids undergoing division can also appear as interconnected plastids and were explored next.

Concomitant Plastid Division and Elongation Suggest Interconnected Plastids

Throughout our experiments with hypocotyl cells from lightgrown plants, we observed plastids in different stages of division (Figures 5A to 5D). Dividing plastids were distinguished from nondividing plastids by the presence of a medial constriction (Figure 5B). By late stages of division, a stroma-filled isthmus became visible and linked two well-separated regions of chlorophyll autofluorescence (Figures 5C and 5D). Irrespective of the distance between the chlorophyll containing regions or the narrowness of the isthmus, photocoversion invariably colored the entire plastid. However, if judged on the basis of chlorophyll fluorescence, these plastids can also be interpreted as two plastids linked by a stroma-filled region. Discrimination between dividing and nondividing plastids became even more difficult in tubular etioplasts lacking chlorophyll.

To eliminate the possibility of interplastid fusion, we investigated chloroplasts in *arc5* and *arc6* mutants that are unable to complete the division process. Division is initiated in *arc5* plastids, but the two daughter plastids fail to separate. Consequently, *arc5* plants exhibit a high frequency of constricted, dumbbell-shaped plastids (Pyke and Leech, 1994; Robertson et al., 1996). In the light-grown hypocotyl epidermis cells of the *arc5* mutant transformed with tpFNR:mEosFP, we found that 43% \pm 7% of the plastids were dumbbell shaped compared with

Figure 3. (continued).

networks, which is dispelled following photoconversion of a few plastids (asterisks).

⁽D) Representative snapshots from a time-lapse series of a pleomorphic photoconverted tubular etioplast (see Supplemental Movie 8 online). Whereas separate plastid bodies (e.g., arrowheads pointing to dilated regions) were suggested, photoconversion highlighted the entire tubule uniformly, indicating that it represented a single plastid unit.

⁽E) Two plastids appear to be interconnected, and fluorescent color is exchanged between them within ~ 2 min. The change in fluorescent color (based on pixel brightness values on 0 to 255 RGB color planes; ImageJ), suggesting the diffusion and intermixing of fluorescent forms of mEosFP within the narrow strip shown in panels 1 to 6, is depicted in the line tracings for fluorescent pixels in the green and red channels.

⁽F) Representative, sequential snapshots from a time-lapse series (see Supplemental Movie 10 online) of a pleomorphic plastid in the *pgm1* mutant changing from an elongated tubule (panels 1 to 4) into two globular bodies (panels 6 to 8) and again into a stromule-extending tubule (panels 11 to 13). Bars = 5 μ m in (A) to (F).



Figure 4. Transmission Electron Microscopy of Chloroplasts and Etioplasts Reveals Differences in Internal Membrane Organization.

(A) to (C) Well-organized thylakoid stacks (g) in chloroplasts compared with prolamellar bodies (pb) in etioplasts ([B] and [C]). pg, plastoglobuli; s, starch.

(D) A single plastid with a bulbous stroma-filled region (labeled b) connected to the thylakoid-containing region (labeled a) through a thin stroma-filled tubule.

Bars = 0.5 μ m.

14% \pm 10% in wild-type *Arabidopsis* and 28% \pm 7% in tobacco. The division-impaired *arc5* plastids underwent rapid color change upon the initiation of photoconversion in any region of the plastid, confirming them as a single plastid compartment. Subsequent observations of plastids in dark-grown *arc5* hypocotyl cells also revealed more than 50% plastids with bulbous ends, suggesting that dividing plastids, although constituting a single unit, can convey an impression of interconnected plastids.

Chloroplasts in *arc6* mutants provided additional support, as they do not enter the constriction stage and are consequently nearly 20-fold larger than wild-type plastids (Pyke et al., 1994; Marrison et al., 1999). We speculated that plastids in dark-grown *arc6* plants will merely elongate and exhibit dilated pleomorphic tubules but not show any phenotype to suggest fused plastids or dividing plastids. Our observations of *arc6* plants matched these expectations, since only elongated tubular plastids were observed (n = 200 plastids). Observations of the *arc6* mutant showed that forms suggestive of interconnected plastids cannot be obtained without the presence of dumbbell-shaped plastids. We conclude that at least some of the earlier impressions of interconnected plastids might have been drawn from dividing and elongating plastids in dark-grown seedlings.

DISCUSSION

Plastid Fusion: Assumption versus Evidence

As early as the 1880s, it was cautiously suggested that some chloroplasts in *Selaginella* spp appear to be interconnected but might well represent late stages of division (Haberlandt, 1888). However, the idea of interplastid connectivity was revived in 1997 through the rediscovery of stroma-filled extensions from

plastids that were highlighted by a stroma-targeted GFP. Photobleaching suggested that proteins were exchanged through connections between higher plant plastids (Köhler et al., 1997). In subsequent years, despite the high interest generated by stromules, the precise mechanisms underlying interplastid connectivity and protein exchange were not elucidated. These questions formed the initial focus of our investigations.

Surprisingly and to our disappointment, we found that the concepts on stromules being promoted through reviews and textbooks are grossly misstated as they are based on strong assumptions rather than experimental evidence (Gray et al., 2001; Hanson and Köhler, 2006).

Interactions, contacts, and fusions often have been mentioned in publications on plastids and stromules. Unfortunately, each of these words was found to present a perception that was not supported by evidence. By contrast, mitochondria observed by us and shown in numerous studies on other eukaryotes (Hoppins and Nunnari, 2009, and references therein) clearly interact and their contact results in fusion that is readily visible as a change in morphology and an intermixing of proteins.

An assumption was also made when stromules were presented as forming networks through which proteins might spread. Despite semblances due to overlapping plastids and stromules, the presence of such networks has never been actually demonstrated. Notably, light microscopy does not provide sufficient spatial resolution to discriminate between real contact and the perception of touch between organelles occupying the same subcellular region. Thus, stromules from independent plastids fluorescently highlighted in the same color appear to form networks when their fluorescence overlaps. A direct contradiction of this observation came from our use of the differential coloring technique, which clearly differentiated between plastids and



Figure 5. Dividing Plastids Are Connected by a Stroma-Filled Isthmus.

(A) to (D) Sequential stages in plastid division show the presence of a medial constriction in (B) and a thin stroma-filled isthmus (arrows in [C] and [D]). Chlorophyll autofluorescence (ch) indicates regions with separated thylakoids, but color uniformity following photoconversion points to stromal continuity within each dividing plastid. en, envelope; pb, plastid body.

(E) to (H) Chloroplasts from light-grown and dark-grown seedlings of tobacco expressing tpFNR:GFP (E) and of Arabidopsis (F), arc5 (G), and arc6 (H) expressing tpFNR:mEosFP. Dark-grown plastids in (E) to (G) represent a single unit connected by a stroma-filled isthmus, while arc6 plastids do not initiate division and simply elongate.

Bars = 2.5 μ m in (A) to (D) and 5 μ m in (E) to (H).

[See online article for color version of this figure.]

showed that each plastid maintains its unique coloration even when it seems to interact with other plastids. Clearly, a continuous network was not formed through stromules.

Another discrepancy became apparent through a critical appraisal of the literature. Gray et al. (2001) defined stromules as stroma-filled tubules that appear as extensions and connections between plastids. The definition emphasizes the stromal contents of plastidic tubules but also equates the phenomenon of tubule extension with the process of tubule connection. The innate assumption is that stroma-filled tubules extended de novo by plastids are similar to the stroma-filled regions that appear between plastids. Notably, such regions are created when a plastid elongates or divides and are called the isthmus (Waters and Pyke, 2005). Under the extant definition of stromules, the isthmus connecting two halves of a dividing plastid should also be called a stromule. Nevertheless, for a majority of plant biologists, the term stromule conjures the picture of a tubule extending from an independent plastid. In fluorescently highlighted plastids, such an extension might appear to connect with another but it has never been shown to exchange proteins. Most scientists do not realize that since the term also covers stromafilled tubules connecting two regions of a plastid, it is through such connections that proteins as large as 550 kD have been shown to flow (Köhler et al., 1997; Kwok and Hanson, 2004a). In other words, observations on the diffusion of proteins from one portion of a membrane-bound compartment to another region of the same compartment resulted in establishing the concept of interplastid connectivity.

The consequences of the oversimplified definition are also apparent in the way other experiments were conducted. Given the simple definition of stromules, it is possible that the presence of a stroma-filled tubule extending between two bulbous regions resulted in the assumption that it represented two plastids. Interestingly, while presenting stromules as tubular extensions from individual plastids, including chloroplasts, the first report of photobleaching of interconnected plastids actually used tubular leucoplasts from tobacco roots (Figure 4 in Köhler et al., 1997). As shown by Köhler et al. (1997), the leucoplasts are quite elongated and have bulbous ends. In subsequent reports (e.g., Kwok and Hanson, 2004a, 2004c; Newell et al., 2012), darkgrown seedlings were used for observing plastids with stromules. For unknown reasons, the researchers appear to have overlooked the fact that they were using etioplasts. These plastids are described in numerous publications as immature chloroplasts produced under conditions of dark growth. Like leucoplasts, the etioplasts are long, flexible plastids that lack the structural rigidity of chloroplasts. Etioplasts are characterized by the presence of amorphous prolamellar bodies (Gunning, 1965; Kahn, 1968; Mackender, 1978; Murakami et al., 1985; Gunning, 2001; Solymosi and Schoefs, 2010). Our electron microscopybased observations performed on plants that were grown under conditions defined in previous reports confirm these differences in internal membrane organization between chloroplasts and etioplasts. It appears plausible that single pleomorphic etioplasts might have been misconstrued as two interconnected plastids. Interestingly, etioplasts also divide as they undergo elongation in the dark. As shown by us through the use of division-impaired mutants, different stages of plastid division can also easily convey the impression of interconnected plastids.

A single report of GFP exchange between interconnected chloroplasts in guard cells refrains from providing any evidence to show that the two plastids in this singular instance of photobleaching were ever independent (Tirlapur et al., 1999). Therefore, the critical question that remained unanswered until now was whether independent plastids, with or without stromules, had ever been observed fusing like mitochondria for exchanging proteins. Our observations made through the innovative use of differential coloring of similar organelles strongly suggest that independent plastids neither fuse nor exchange proteins. Nevertheless, the involvement of stromules in forming conduits through which genetic material can be exchanged (Taiz and Zeiger, 2010) is considered a rare event. General stress has been shown to increase stromule induction (Gray et al., 2011, 2012). Therefore, we tried to combat the possible rarity of the fusion event by increasing stromule induction frequency through agroinfiltration. Alternatively, we used arc5 and arc6 mutants that have elaborate stromules (Holzinger et al., 2008). In both situations, we did not observe exchange of FPs. Interestingly, a succinct conclusion by Newell et al. (2012) states that "transfer of genetic information by this route is likely to be a rare event, if it occurs at all." It is always possible that we too might have missed the rare fusion event between independent plastids. While the significance of such an elusive event is questionable, it is important to consider whether the absence of fusion could be advantageous for plastids and the eukaryotic cell in general.

Nonfusing Plastids versus Fusing Mitochondria: Significance

Organelle fusion generally leads to homogeneity within the organelle population. Mitochondrial fusion occurs routinely and has been suggested as a means of minimizing oxidative damage to the mitochondrial genome and preventing the accumulation of mutations (Meeusen and Nunnari, 2005; Hoppins and Nunnari, 2009). A similar explanation might apply to plastids if evidence is found for their fusion. In this context, we asked why the two organelles should display opposite behaviors.

A fundamental difference between the two organelles is the way that energy flows through them. Plastids, notably chloroplasts, trap solar energy by transducing it through an electron transport chain and progressively channeling it into the production of an energy-rich carbon compound. The reverse happens in mitochondria, where energy is released during progression through an electron transport chain (Buchanan et al., 2000; Allen et al., 2005; Taiz and Zeiger, 2010). Interestingly, the internal thylakoid lumen in chloroplasts is proton rich and relatively acidic, whereas in mitochondria, it is the intermembrane space, external to the mitochondrial matrix, that becomes progressively acidic. Consequently, mitochondria are subject to a respiratory control (Mattenberger et al., 2003; Gnaiger, 2009) wherein their continued production of energy is dependent upon the maintenance of a chemiosmotic gradient across the intermembrane space and the matrix. Possibly, mitochondrial fusion is a way to regenerate electrochemical gradients and maintain energy flow. By contrast, energy is internalized and fixed in large carbon compounds within the chloroplast. While carbohydrate production affects intraplastid osmotics, it does not apparently affect the inward directed chemical gradients involved in energy transduction.

Nonetheless, for the plastid, the dispersal of end products requires increased surface contact with other cytoplasmic components. Stromules are possibly able to act as conduits for metabolite dispersal through their strong alignment with endoplasmic reticulum tubules (Schattat et al., 2011). In addition to functioning as cellular biochemical factories, each plastid needs to maintain a degree of uniqueness that would be lost if they fused and exchanged material. Contrary to the mitochondrial need to fuse, plastid fusion would not serve an apparent useful purpose. Our work implies that maintaining plastid uniqueness might be the key to increasing biochemical diversity within a cell and thus account for the higher survival ability and versatility of plants.

Conclusion

As far as the lack of FP exchange between plastids suggests, this work strongly refutes the dogma of interplastid connectivity through stromules. However, our observations do not negate the possibility that smaller proteins, ions, and metabolites might still be exchanged between plastids by other, as yet undiscovered, means. In addition, in the interest of preventing confusion in the future, we recommend that the term "stromule" be qualified further to depict a "stroma-filled tubule extended by an independent plastid," while "isthmus" should be maintained as the correct term for stroma-filled tubular regions within an elongated and/or dividing plastid.

METHODS

Molecular Methods

The mito:mEosFP and mEosFP-PTS1 constructs were described earlier (Mathur et al., 2010). For labeling the plastid stroma with mEosFP, the enhanced GFP (EGFP) coding sequence of the previously reported tpFNR:EGFP fusion (Marques et al., 2003, 2004) was replaced by mEosFP and placed in the binary T-DNA vector pCAMBIA1300 (http:// www.cambia.org.au). Standard molecular biology protocols were followed for the cloning (Sambrook and Russell, 2001).

Plant Material and Expression in Plant Cells

Transient expression of tpFNR:mEosFP, mEosFP-PTS1, mito:mEosFP (Mathur et al., 2010), and pCP60:dsRed2 (untagged dsRed2 in binary vector pCP60 kindly provided by Conny Papst) was performed through infiltration of *Agrobacterium tumefaciens* (strain GV3101 at an optical density of 0.8 at 600 nm), harboring the respective plasmid, into leaves of soil-grown *Nicotiana benthamiana* plants according to Sparkes et al. (2006). Observations of the infiltrated leaves were taken 2 to 3 d after infiltration.

Tobacco (*Nicotiana tabacum*) BY2 cells (Nagata et al., 2003) expressing tpFNR:mEosFP were obtained through biolistic bombardment using DNA-coated gold particles according to Schenkel et al. (2008). Transient expressing cells were observed 16 to 48 h after bombardment.

For creation of transgenic lines of *arc5* (CS486), *arc6-1* (CS286), *pgm-1* (CS210), and *drp3A* (SALK_066958), seeds were obtained from the ABRC (The Ohio State University, Columbus, OH). Plants were stably transformed with tpFNR:mEosFP using the floral dip transformation method (Clough and Bent, 1998). tpFNR:EGFP was introduced in the *arc5*, *arc6-1*, and *pgm-1* mutants by crossing with FNR:EGFP plants (Marques et al., 2004).

The creation of transgenic *N. benthamiana* plants expressing tpFNR: EGFP was described previously (Schattat et al., 2011).

Plant Growth Conditions

Plants on soil (*Arabidopsis thaliana and N. benthamiana*) were grown in a controlled environment growth chamber maintained at 21 \pm 2°C with an 8-h/16-h light/dark regime using cool-white light of \sim 100 to 140 μ mol m $^{-2}$ s $^{-2}$. To induce flowering, plants were transferred to a 16-h/8-h light/dark regime after the formation of a well-developed rosette.

Plants in sterile culture (*Arabidopsis* and *N. benthamiana*) were grown in Petri dishes in an incubator maintained at $21 \pm 2^{\circ}$ C with a 16-h/8-h light/dark regime using cool-white light of ~80 to 100 µmol m⁻² s⁻². To break dormancy, *Arabidopsis* seeds where incubated at 4°C for 48 h and *N. benthamiana* seeds for 16 h. Growth medium for *Arabidopsis* wild-type and mutant seedlings consisted of 1% agar-gelled Murashige and Skoog (1962) basal medium containing Gamborg vitamins (M404; PhytoTechnology Labs) supplemented with 3% Suc, pH 5.8. *N. benthamiana* seedlings were grown on similar medium but lacking the Gamborg vitamin supplement.

To obtain etiolated *Arabidopsis* seedlings, Petri dishes were wrapped in several layers of aluminum foil right after the cold treatment; *N. benthamiana* seeds were exposed for 30 min to light in the growth chamber before transfer to complete darkness.

Microscopy

Preparation for Microscopy

To observe plant tissue, seedlings or leaf discs were mounted in tap water on a glass depression slide and placed under a cover slip. To limit water evaporation and cover slip drift during long-term observations, an open ring of silicon grease was applied on the slide before adding the water and applying the cover slip. Gold particle bombarded tobacco BY2 cells were collected from filter paper, mounted on a glass slide in culture medium, and screened for FP expression.

Photoconversion and Imaging

Photoconversion time varied according to the brightness of the respective organelles in transient or transgenic material. In general, exposure times between 7 and 10 s resulted in bright red organelles. However, to minimize photodamage, photoconversion was limited to a maximum of 30 s. A second exposure was performed after at least a 30-s pause if a more intense red color was desired. The light source for photoconversion was an HBO 100 W/2 Mercury Short Arc lamp and the Leica fluorescence filter set D (excitation filter, 355 to 425 nm; dichromatic mirror, 455 nm; suppression filter, long-pass 470 nm). The epifluorescence setup consisted of a Leica DM-6000CS microscope. Photoconversion was performed manually by controlling the diaphragm as described earlier (Mathur et al., 2010).

Subsequent simultaneous imaging of unphotoconverted and photoconverted probes was performed using a Leica TCS-SP5 confocal laser scanning unit equipped with a 488-nm argon and a 543-nm helium-neon laser. To avoid photobleaching of nonphotoconverted green mEosFP, the 488-nm laser intensity was kept at a minimum. For visualizing mEosFP probes, the respective probe was excited using a 488- and a 458-nm laser, and emissions were collected between 511 and 540 nm for unphotoconverted green mEosFP and between 568 and 600 nm for photoconverted red mEosFP. Plants expressing tpFNR:EGFP were imaged using the 488-nm argon laser, emission for GFP was collected between 410 and 435 nm, and the red autofluorescence of chlorophyll was collected in the range of 619 to 730 nm. All images were captured at a color depth of 24-bit RGB.

Postacquisition and Image Processing

All images and movies (see Supplemental Movies 1 to 10 online) were cropped and processed for brightness/contrast as complete images or stacks using either Adobe Photoshop CS3 (http://www.adobe.com) or the ImageJ distribution package Fiji (http://pacific.mpi-cbg.de/wiki/index.php/ Fiji). Adobe Photoshop was used for annotation of movies. Figures were assembled using Adobe Illustrator CS3 (http://www.adobe.com).

For analysis of hypocotyl plastid morphology of dark-grown and lightgrown seedlings, plastids of hypocotyl cells of *N. benthamiana* and *Arabidopsis* wild-type seedlings as well as of *arc5* and *arc6* mutants were recorded by taking z-stacks that captured multiple plastids. At least three such z-stacks were taken and analyzed for three seedlings of each plant line for each treatment (grown in the dark or in a 16-h/8-h light cycle). By browsing through these z-stacks with the LSMImageBrowser (Zeiss), plastids were grouped in different morphological classes and marked according to the class with a unique color. Subsequent processing of data was performed according to Schattat and Klösgen (2011).

Supplemental Data

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

Supplemental Figure 1. Differential Coloration of Chloroplasts Surrounding a Nucleus Depicted through Numeric Fluorescence Intensity Values and Line Tracings for the Circled Regions.

Supplemental Figure 2. Tobacco Callus and Cell Suspension Cultures Constitute Another System Used for Observing Clusters of Stromules That Apparently Interact with Each Other.

Supplemental Figure 3. A Comparison of Plastid Shapes.

Supplemental Table 1. Frequency of Elongated Plastids and Stromules in Etiolated Seedlings and Seedlings Grown under a Normal Light-Dark Cycle.

Supplemental Movie 1. Photoconversion Allows Differential Coloring.

Supplemental Movie 2. Labeling by Mito:mEosFP Shows Green (Nonphotoconverted) and Red (Photoconverted) Mitochondria Coaligning and Fusing with Each Other in Rapid Succession.

Supplemental Movie 3. Tubular Peroxisomes in the drp3A Mutant of *Arabidopsis* Labeled by mEosFP-PTS1 Undergo Photoconversion Readily into Red and Green Subpopulations.

Supplemental Movie 4. A Thin Stroma-Filled Tubule Seemed to Connect Two Plastids before Photoconversion, but Two Distinct Stromules with Strict Color Boundaries Become Visible Following Photoconversion.

Supplemental Movie 5. Two Differentially Colored Stromules Interact Laterally for >4 min without Exchanging Fluorescent Proteins.

Supplemental Movie 6. Stromules Crossing Each Other and Apparently Forming Junctions.

Supplemental Movie 7. Three Chloroplasts Transiently Occupying a Small Region of the Cell Colored Differently Exhibit Possible Contact but Do Not Exchange Fluorescent Proteins before Moving Apart.

Supplemental Movie 8. Tubular Plastids are Pleomorphic and, as Shown in This Movie of a Single Etioplast from a Hypocotyl Cell of *Arabidopsis*, Can Give the Impression of Multiple Plastids Fused Together.

Supplemental Movie 9. Fluorescent Protein Diffusion Leading to Rapid Color Change of a Tubular Etioplast That Gave the Impression of Two Plastids Connected to Each Other by a Stroma-Filled Region.

Supplemental Movie 10. The pgm1 Mutant of *Arabidopsis* Exhibits Pleomorphic Plastids.

Supplemental Movie Legends 1. Legends for Supplemental Movies 1 through 10.

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AUTHOR CONTRIBUTIONS

S.G. and M.H.S. created FNR:mEosFP. K.B. provided data on mitochondria. N.M. created and maintained transgenic plants. M.R.W. performed experiments on tobacco BY2 cells. J.S.G. and N.D. performed electron microscopy. J.M. and M.H.S. planned the work, performed the experiments, and cowrote the article.

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IN BRIEF

Plastids Do Not Form Interconnected Networks

Tentacle-like protrusions have long been recognized as a feature of plastids. In 1888. Haberlandt reported that chloroplasts could link together via thin filaments (Haberlandt, 1888). These dynamic stroma-filled tubules, named stromules (Köhler and Hanson, 2000), sporadically extend from the plastids of most cell types and plant species and sometimes appear to connect with each other (see figure, left). Photobleaching experiments demonstrated that green fluorescent protein travels between plastids connected by these structures (Köhler et al., 1997) and gave rise to the notion that stromules shuttle molecules within an interplastid communication system.

Now, Schattat et al. (pages 1465–1477) have used a photoconvertible fluorescent protein (mEosFP) isolated from stony coral to reexamine the widely held view that plastids form an interconnected network. The emission color of mEosFP changes from green to red upon violet-blue illumination. By differentially coloring the plastids in a cell, transfer of fluorescent proteins between plastids can be tested directly. The researchers produced Arabidopsis thaliana lines that stably expressed plastid-targeted mEosFP. Photoconversion of a target region for various durations resulted in plastids containing different ratios of green and red fluorescent proteins, thus appearing green, yellow, orange, or red. The exchange of fluorescent proteins between plastids would result in plastids of intermediate color. Although the differentially colored plastids sporadically extended stromules that came into contact with each other for up to 50 min, the plastids maintained their color identity over several hours of observation (see figure, right). Therefore, fluorescent proteins are not exchanged between plastids. Similar results were found in Nicotiana tabacum leaves transiently expressing plastid-targeted mEosFP.

Furthermore, the authors expressed plastid-targeted mEosFP in *arc6*, an *Arabidopsis* mutant harboring giant chloroplasts.

They reasoned that fusion of stromules would result in a change in morphology and that such a change would be amplified in the enlarged stromules of this mutant. Once again, there was no indication of stromule fusion or exchange of fluorescent proteins.

Finally, the authors noted that interconnected plastids can be difficult to distinguish from a single plastid with bulbous ends and suggested that the earlier photobleaching experiments (Köhler et al., 1997) may have tracked movement of proteins within a single, convoluted plastid and not between neighboring plastids.

Although this study does not rule out the possibility that stromules transport small molecules and ions between plastids, it dispels the view that macromolecules are exchanged between plastid networks. The function of these mysterious protuberances remains to be determined.

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Stromules come into close contact with each other but do not transfer proteins between neighboring plastids. Left: An apparent network of chloroplasts expressing mEosFP (before photoconversion). Right: Photoconversion of plastid-targeted mEosFP revealed that stromules extending from neighboring plastids are physically separate and remained so throughout the observation period. Bars = 5 μ m (left) and 2.5 μ m (right).

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Differential Coloring Reveals That Plastids Do Not Form Networks for Exchanging Macromolecules

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Lab profile

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The Mathur lab uses a variety of targeted fluorescent proteins for live-imaging of plant cells with the aim of uncovering rapid subcellular responses such as the formation of organelle extensions like stromules and peroxules. Stromules are believed to connect plastids and act as conduits for inter-plastid exchange of proteins. Since the basic mechanism underlying protein exchange between plastids is unknown the lab focused on the role of stromules in this process. However, contrary to expectations the findings using a photo-convertible probe clearly indicated that plastid stromules do not fuse at all or aid protein exchange between plastids. The findings strongly reinforce the lab's commitment towards understanding rapidly induced organelle responses to environmental stimuli and their implications for achieving and maintaining homeostasis in plant cells.