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Chloroplasts extend stromules independently and in response to internal redox signals

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Contributed by Patricia C. Zambryski, June 12, 2015 (sent for review April 15, 2015); reviewed by Winslow R. Briggs and Howard M. Goodman

A fundamental mystery of plant cell biology is the occurrence of "stromules," stroma-filled tubular extensions from plastids (such as chloroplasts) that are universally observed in plants but whose functions are, in effect, completely unknown. One prevalent hypothesis is that stromules exchange signals or metabolites between plastids and other subcellular compartments, and that stromules are induced during stress. Until now, no signaling mechanisms originating within the plastid have been identified that regulate stromule activity, a critical missing link in this hypothesis. Using confocal and superresolution 3D microscopy, we have shown that stromules form in response to light-sensitive redox signals within the chloroplast. Stromule frequency increased during the day or after treatment with chemicals that produce reactive oxygen species specifically in the chloroplast. Silencing expression of the chloroplast NADPH-dependent thioredoxin reductase, a central hub in chloroplast redox signaling pathways, increased chloroplast stromule frequency, whereas silencing expression of nuclear genes related to plastid genome expression and tetrapyrrole biosynthesis had no impact on stromules. Leucoplasts, which are not photosynthetic, also made more stromules in the daytime. Leucoplasts did not respond to the same redox signaling pathway but instead increased stromule formation when exposed to sucrose, a major product of photosynthesis, although sucrose has no impact on chloroplast stromule frequency. Thus, different types of plastids make stromules in response to distinct signals. Finally, isolated chloroplasts could make stromules independently after extraction from the cytoplasm, suggesting that chloroplast-associated factors are sufficient to generate stromules. These discoveries demonstrate that chloroplasts are remarkably autonomous organelles that alter their stromule frequency in reaction to internal signal transduction pathways.

chloroplasts | stromules | redox signaling | light signaling | leucoplasts

Chloroplasts, the descendants of ancient bacterial endosymbionts, exert impressive influence over processes that are not directly related to their metabolic roles. In recent years, forward genetic screens have led to the discoveries that chloroplasts are critical regulators of leaf shape, cell–cell signaling through plasmodesmata, pathogen defense, and even alternative splicing in the nucleus (1–8); however, in almost all of these pathways, the signaling route between the chloroplast and the nucleus is unknown. This is a pressing question for plant biology and cell biology in general: how do organelles communicate with the nucleus to coordinate genetic programs and cellular function? One possible route for this communication is through "stromules," stroma-filled tubular extensions of unknown function from plastids (9–11).

Stromules were first observed in spinach cells (12), and have since been observed in every cell type and land plant species investigated to date (13). Several studies have identified conditions that can induce or decrease stromule formation (14–18), concluding that stromule frequency can change in response to abiotic stress, phytohormone signaling, and massive disruption of cellular function (e.g., strong inhibition of cytosolic translation or of actin microfilament dynamics). Almost nothing is known about the genetics of stromules; some mutants with strong morphological defects in plastids, such as mutants with improper plastid division or lacking plastid mechanosensitive channels, cannot form stromules at normal frequencies, but these plastids are so severely misshapen that their stromule frequencies cannot be directly compared with wild-type plastids (19, 20). To date, few experiments have tested whether signals inside plastids can affect stromule frequency, and all of those experiments (e.g., treatment with antibiotics that interfere with plastid genome expression; ref. 17) have suggested that stromule frequency is not regulated by internal plastid biology. Here we test whether light-sensitive redox signaling pathways initiated within chloroplasts regulate stromule activity.

Results and Discussion

Chloroplasts Make More Stromules During the Day. We began our study by conducting a time course to determine the effects of light on chloroplast stromule formation. For all *in planta* experiments (except where noted otherwise), we observed stromules in the proximal abaxial epidermis of cotyledons of young *N. benthamiana* or *A. thaliana* plants, or in the proximal abaxial epidermis of young leaves at 2 wk after silencing gene expression with virus-induced gene silencing (VIGS). We collected a single *z*-stack of confocal images of only one leaf of each plant, and considered plants to be independent samples. (The number of plants observed for each treatment for an experiment is designated "*n*" throughout.) Thus, each experiment considered the stromule frequency determined from hundreds, thousands, or even tens of thousands of plastids.

Over the course of 2 d, we measured stromule frequency in cotyledons from young *N. benthamiana* seedlings every 4 h, and found significantly more stromules during the daytime than at

Significance

Chloroplasts are critical, dynamic organelles in plant cells responsible for photosynthesis and myriad other aspects of metabolism. In recent years, plant cell biologists have increasingly focused on the formation of thin, long extensions from plastids called "stromules." Although stromules have been observed in all land plant species and cell types investigated, we do not know why these projections form or what they do. Here we demonstrate that stromules form in response to light-related redox signals inside the chloroplast. We then show that chloroplasts extracted from plant cells can make stromules independently. These discoveries suggest that stromules may be involved in transmitting signals from within the chloroplast to other subcellular compartments.

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The authors declare no conflict of interest.

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nighttime; 20.8 ± 1.8% of chloroplasts had stromules in the day, compared with only 12.8 ± 0.9% at night ($n \geq 22$, $P < 0.0005$) (Fig. 1 and Fig. S1). (Throughout the paper, stromule frequency is reported as percentage ± SE.) There was no significant difference in stromule frequency between the first and second days or between the first and second nights, indicating that the observed changes were reactions to the changing light environment rather than a progressive developmental change over 48 h.

Previous studies investigating the relationship between chloroplast stromule frequency and light reported that light decreases stromule frequency in seedling hypocotyls during de-etiolation after skotomorphogenesis, and that constant darkness or exposure to only blue light increases stromule frequency after photomorphogenesis (17). The apparent discrepancy between those conclusions and our results showing that light promotes chloroplast stromule formation is explained by the different plastid types used in the de-etiolation experiment (etioplasts transitioning to become chloroplasts) and the dramatic developmental and physiological transitions used in both experiments (constant darkness to constant light, or vice versa), which are not reflective of typical chloroplast stromule behavior in normal, healthy plants. We conclude that light promotes chloroplast stromule formation during the day.

Reactive Oxygen Species Inside Chloroplasts Promote Stromule Formation. Plants sense light with the pigments of the photosynthetic electron transport chain (pETC) in the chloroplast or with photoreceptors elsewhere in the cell (21). We tested whether stromule frequency responds specifically to light sensed by the chloroplast itself by chemically inhibiting pETC activity. We used two pETC inhibitors, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone (DBMIB). DCMU prevents reduction of plastoquinone at photosystem II and generates singlet oxygen, whereas DBMIB prevents plastoquinols from reducing the cytochrome b_6f complex and generates superoxide (8).

We measured the effects of DCMU and DBMIB on the chloroplast stromal redox status, as monitored by a stromal redox-sensitive transgenic GFP biosensor, pt-roGFP2 (4), to find very low active concentrations of each compound with our treatment technique, and found that 10 μM DCMU or 12 μM DBMIB was sufficient to strongly oxidize redox buffers in the chloroplast stroma. The normalized proportion of oxidized pt-roGFP2 rose from 20.0 ± 3.5% in control conditions to 68.9 ± 3.0% after 10 μM DCMU treatment and to 41.5 ± 7.1% after 12 μM DBMIB treatment ($n \geq 28$, $P < 0.01$) (Fig. S2).

We assessed stromule frequency at 2 h after treating *N. benthamiana* cotyledons with either of the photosynthesis inhibitors (Fig. 2 *A–C* and Fig. S2 *A–C*). In the epidermal chloroplasts of mock-treated cotyledons, the average chloroplast stromule frequency was 9.2 ± 1.4%. After treatment with DCMU or DBMIB, stromule frequency increased by more than 50%, to 15.5 ± 2.6% with DCMU and 16.8 ± 2.9% with DBMIB ($n \geq 20$, $P < 0.05$). This finding suggests that stromule formation responds to light-sensitive redox signals inside the chloroplast, to our knowledge the first demonstration that internal chloroplast pathways may regulate stromules.

Unlike *N. benthamiana*, the epidermis of *A. thaliana* has two distinct types of plastids: chloroplasts in the guard cells and leucoplasts in the pavement cells (Fig. S3). Leucoplasts are not photosynthetic, but like chloroplasts, they have many other roles in metabolism and storage. As in *N. benthamiana* epidermal chloroplasts, DCMU and DBMIB promote stromule formation in guard cell chloroplasts of *A. thaliana* cotyledons, raising stromule frequency from 15.7 ± 3.8% to 28.1 ± 4.0% for DCMU ($n \geq 16$, $P < 0.05$) and to 48.1 ± 6.3% for DBMIB ($n \geq 10$, $P < 0.0005$) (Fig. 2*D*). This demonstrates that the induction of chloroplast stromules by DCMU and DBMIB is conserved in evolutionarily divergent plants, since *N. benthamiana* is an asterid

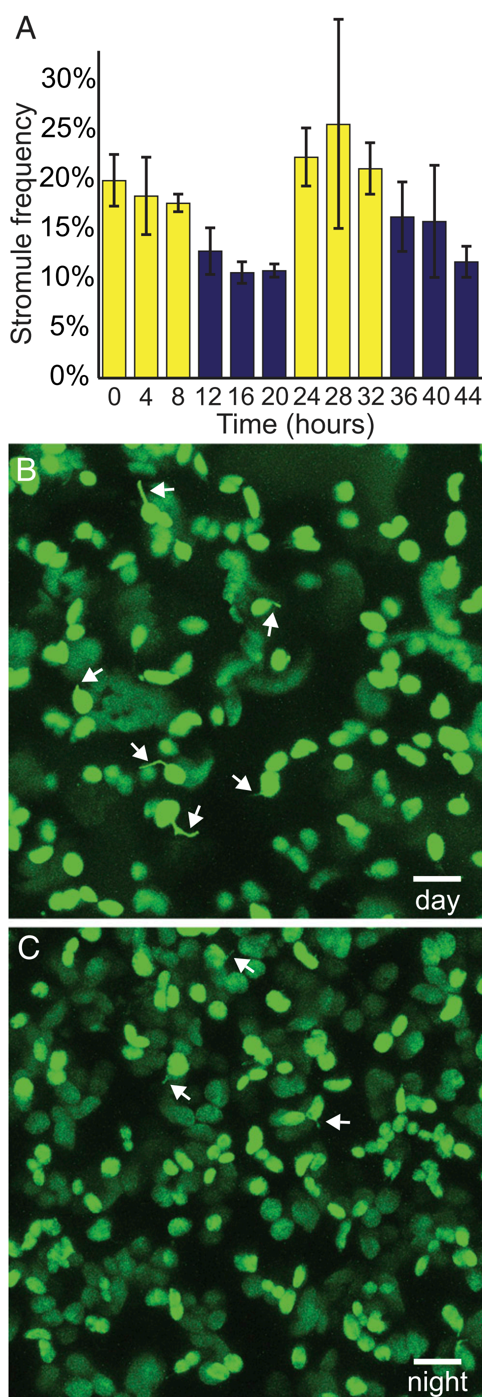

Fig. 1. Chloroplast stromule frequency varies with diurnal cycles. (*A*) Stromule frequency rises in the day (yellow bars) and decreases at night (blue bars) in chloroplasts of *N. benthamiana* seedlings ($n \geq 22$, $P < 0.0005$). (*B* and *C*) Representative images of *N. benthamiana* epidermal chloroplasts labeled with stromal GFP (green) in the day (*B*) and at night (*C*). Some stromules are indicated by white arrows. As a visual aid, here and in other figures; not all stromules are indicated, and the indicated stromules were selected at random. Error bars indicate SE. (Scale bars: 10 μm.)

and *A. thaliana* is a rosid, with the last common ancestor of the two species living more than 100 million years ago (22). In contrast, leucoplasts in the epidermis of *A. thaliana* were unaffected by DCMU and DBMIB treatment [14.6 ± 3.4% in control conditions vs. 14.6 ± 2.8% with DCMU ($n \geq 16$, $P = 0.98$) and 13.5 ± 4.3%

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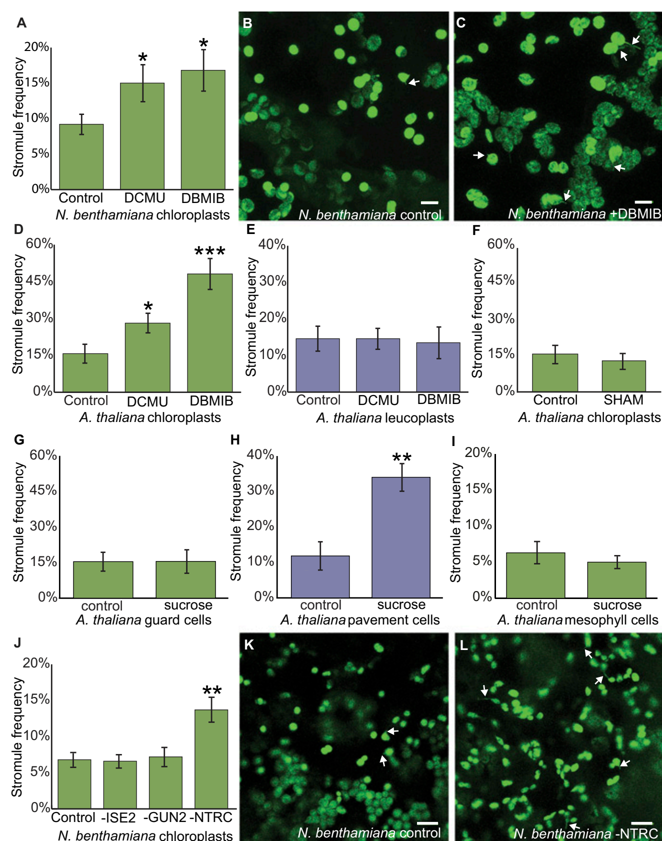

Fig. 2. ROS in the chloroplast induce stromules. (*A*) DCMU and DBMIB treatments both increase stromule frequency in chloroplasts of *N. benthamiana* seedlings ($n \geq 20$, $P < 0.05$). (*B* and *C*) Representative images of *N. benthamiana* chloroplasts treated with control (*B*) or with DBMIB (*C*). (*D*) *A. thaliana* epidermal chloroplast stromule frequency increases after DCMU or DBMIB treatment (DCMU, $n \geq 16$, $P < 0.05$; DBMIB, $n \geq 10$, $P < 0.0005$). (*E*) Stromule frequency in *A. thaliana* epidermal leucoplasts is unaffected by DCMU or DBMIB treatment (DCMU, $n \geq 16$, $P = 0.98$; DBMIB, $n \geq 10$, $P = 0.84$). (*F*) *A. thaliana* epidermal chloroplast stromule frequency is unaffected by SHAM ($n \geq 13$, $P = 0.57$). (*G*) *A. thaliana* epidermal chloroplast frequency is similar in chloroplasts with or without sucrose treatment ($n \geq 8$, $P = 0.96$). (*H*) *A. thaliana* epidermal leucoplast stromule frequency increases after sucrose treatment ($n \geq 8$, $P < 0.01$). (*I*) Stromule frequency is not affected by sucrose treatment in *A. thaliana* mesophyll chloroplasts ($n \geq 8$, $P = 0.52$). (*J*) Silencing *NbNTRC* increases stromule frequency in *N. benthamiana* leaves ($n = 8$, $P < 0.01$), but silencing *NbISE2* or *NbGUN2* does not affect stromule frequency ($n = 8$, $P > 0.82$). (*K* and *L*) Representative images of *N. benthamiana* chloroplasts in control (*K*) or after silencing *NbNTRC* (*L*). Chloroplasts and stromules in are labeled with GFP. Some stromules are indicated by white arrows. Error bars indicate SE. *$P < 0.05$; **$P < 0.01$; ***$P < 0.0005$. (Scale bars: 10 μm.)

with DBMIB ($n \geq 10$, $P = 0.84$)], showing that the effects of DCMU and DBMIB are specific responses to their roles interfering with the pETC (Fig. 2*E*).

To further test whether stromules form in response to the chloroplast redox status specifically, as opposed to any oxidative stress in the cell, we also treated *A. thaliana* cotyledons with salicylhydroxamic acid (SHAM). SHAM inhibits the mitochondrial alternative oxidase, which leads to rapid and strong oxidation of mitochondrial redox buffers (4). SHAM did not impact chloroplast stromule formation in *A. thaliana* (15.7 ± 3.8% in control vs. 12.8 ± 3.3% with SHAM; $n \geq 13$, $P = 0.57$), supporting the hypothesis that chloroplast stromule frequency is specifically regulated by the redox status of the chloroplast (Fig. 2*F* and Fig. S4).

In summary, low concentrations of DCMU or DBMIB are sufficient to induce significant increases in stromule frequency within only 2 h. This is apparently not a secondary effect of broad disruption of cellular metabolism or redox homeostasis, because leucoplasts, which are not photosynthetic, are unaffected by the treatments, and disrupting mitochondrial function and generating reactive oxygen species (ROS) in mitochondria by SHAM treatment does not affect chloroplast stromule frequency. Thus, light-sensitive redox cues inside chloroplasts specifically affect stromule frequency.

NADPH-Dependent Thioredoxin Reductase c Regulates Chloroplast Stromule Frequency. Signaling from chloroplasts to other organelles within the plant cell is critical for plant survival and development (23, 24). Chloroplast-to-nucleus signaling is transduced through several pathways, some of which are light-sensitive. We used VIGS in *N. benthamiana* as a reverse genetic approach (25) to determine whether disrupting the light-sensitive chloroplast-to-nucleus signal transduction pathways impacts stromule formation. VIGS strongly reduces gene expression in young leaves within 1–2 wk of infection by generating small RNAs that specifically target a gene for posttranscriptional silencing (25).

Chloroplasts contain their own genomes encoding approximately 80 proteins (mostly related to photosynthesis or transcription and translation), and light exerts control over plastid genome expression (PGE) at transcriptional and posttranscriptional levels (26). Thus, we first focused on NbISE2, an essential plastid RNA helicase required for healthy chloroplast biogenesis and PGE (3). Without *ISE2*, hundreds of nuclear genes involved in photosynthesis are strongly down-regulated (3). Silencing *NbISE2* gene expression had no impact on stromule frequency, however (6.8 ± 1.0% in controls vs. 6.6 ± 0.9% after silencing *NbISE2*; $n = 8$, $P = 0.85$) (Fig. 2*J* and Fig. S5), in agreement with previous reports that antibiotics directly interfering with PGE, such as lincomycin, have no effect on stromule frequency (17).

PGE coordinates the expression of photosynthesis-associated nuclear genes through a signal transduction pathway mediated by tetrapyrrole metabolism (23, 24, 27). Genetic disruptions to tetrapyrrole metabolism, specifically defects in the branch point between heme and chlorophyll biosynthesis, interfere with chloroplast biogenesis and photosynthesis (27). We next tested whether loss of NbGUN2, a chloroplast heme oxygenase that participates in chloroplast-to-nucleus communication, impacts stromule formation. As with *NbISE2*, silencing *NbGUN2* gene expression had no impact on chloroplast stromule frequency (7.2 ± 1.3% after silencing *NbGUN2*; $n = 8$, $P = 0.82$) despite causing clear physiological stress and chlorosis (Fig. 2*J* and Figs. S5, S6, and S7).

We then silenced the expression of the chloroplast NADPH-dependent thioredoxin reductase (*NbNTRC*) (Figs. S8 and S9), which regulates the redox status and activity of myriad chloroplast proteins and is a critical hub in chloroplast redox signal transduction (28). Silencing *NbNTRC* more than doubled the stromule frequency (13.7 ± 1.7%; $n = 8$, $P < 0.01$), providing genetic evidence that redox signaling within the chloroplast regulates stromule formation (Fig. 2 *J*–*L* and Figs. S5, S8, and S9). Moreover, to our knowledge, *NbNTRC* is now the first gene identified that regulates stromule frequency without other apparent effects on chloroplast shape.

Sucrose Promotes Stromule Formation in Epidermal Leucoplasts, but Not in Chloroplasts. Schattat et al. (29) reported that stromule frequency increases during the day in the epidermal leucoplasts of *A. thaliana*. Because leucoplasts do not contain pigments and do not respond to DCMU or DBMIB, we sought another hypothesis to explain why leucoplast stromule frequency is light-responsive. Physiologically, one of the major impacts of light on epidermal pavement cells is an increase in sucrose imported from underlying cells that contain photosynthesizing chloroplasts. Previous reports have indicated that stromule frequency is sensitive to sugar levels, but with inconsistent conclusions (16). We found that epidermal

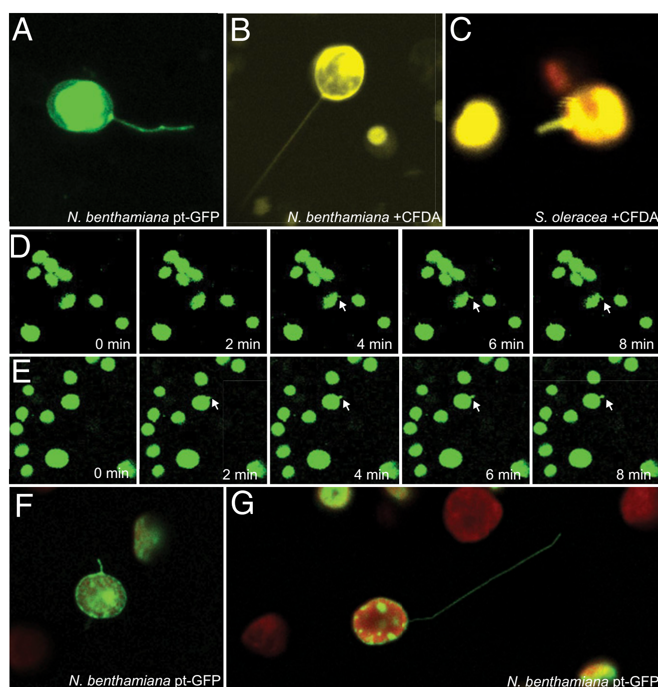

Fig. 3. Isolated chloroplasts form stromules. *N. benthamiana* chloroplasts labeled with stromal GFP (*A*, green) or CFDA staining (*B*, yellow) show stromules after isolation from their cellular environment. (*C*) Chloroplasts isolated from *S. oleracea* and stained with CFDA (yellow; chlorophyll autofluorescence, red) also have stromules. (*D* and *E*) Isolated *N. benthamiana* chloroplasts labeled with stromal GFP form new stromules over time. Newly forming stromules are indicated by white arrows. (*F* and *G*) *N. benthamiana* chloroplasts isolated with a Percoll purification step and labeled with stromal GFP (green; chlorophyll autofluorescence, red) also have stromules.

leucoplast stromule frequency rises remarkably following sucrose treatments in *A. thaliana* (33.9 ± 3.8% with sucrose vs. 11.8 ± 3.9% without sucrose; $n \geq 8$, $P < 0.01$) (Fig. 2*H* and Fig. S10). In contrast, chloroplast stromule frequency did not respond to sucrose treatments in either the epidermal guard cells or mesophyll of *A. thaliana* [in the epidermis, 15.4 ± 3.9% with sucrose vs. 15.7 ± 3.8% without ($n \geq 8$, $P = 0.96$); in the mesophyll, 5.0 ± 0.9% with sucrose vs. 6.3 ± 1.7% without ($n \geq 8$, $P = 0.52$)] (Fig. 2 *G* and *I* and Fig. S10). These results imply that different plastid types use separate signaling pathways to induce stromule formation.

Isolated Chloroplasts Can Form Stromules. With the finding that signals originating within the chloroplast can trigger stromule formation, we then explored whether stromule formation is dependent on cytosolic structures (e.g., the cytoskeleton), as has been suggested previously (10, 15), or if chloroplasts can make stromules on their own. Previous studies have argued that stromule formation is guided and supported by the cytoskeleton and endoplasmic reticulum, but whether stromule formation requires these external factors is unknown (10, 30). To address this question, we extracted chloroplasts from leaves of *N. benthamiana*, *A. thaliana*, and *Spinacia oleracea* using well-established methods for isolating functional, undamaged chloroplasts (31). We visualized chloroplast stroma either with GFP by extracting chloroplasts from leaves expressing plastid-targeted GFP or with a supravital stain, carboxyfluorescein diacetate (CFDA), which fluoresces only after hydrolysis by carboxylesterases in the chloroplast stroma (32). We readily found isolated chloroplasts with intact stromules in all three species and regardless of staining technique (Fig. 3 and Fig. S11). The stromules were dynamic and could grow very long, sometimes extending more than 150 μm from chloroplasts only 4–6 μm in diameter (Fig. S11). As in plant cells, many stromules were bent and curved along their length, whereas some were very long and straight (Fig. 3 and Fig. S11). Using time-lapse microscopy, we repeatedly observed isolated chloroplasts from apparently new stromules, validating that chloroplasts can generate stromules independently. Stromules are absent in the first frames of chloroplasts shown in Fig. 3 *D* and *E*, but they appear and lengthen over the course of 8 min (Movies S1 and S2).

Superresolution Microscopy Illuminates Stromule Ultrastructure. Because stromules can form in isolation after extraction of chloroplasts from their cellular context, we decided to further investigate stromules using superresolution microscopy to gain new insight into their ultrastructure, which will inform future efforts at identifying the chloroplast-associated structural components responsible for stromule formation. The diameter of stromules is postulated to be <200 nm, but this is below the diffraction limit of conventional light microscopy (10, 33), even under optimal conditions. Visualizing stromules by transmission electron microscopy is challenging, because stromule membranes are not easily distinguished from other membranes in thin sections required for conventional electron microscopy. We used 3D structured illumination microscopy (3D-SIM) to obtain the highest-resolution images of wild-type stromules to date (10, 33), and present some representative examples in Fig. 4 and Movie S3. The improved resolution of 3D-SIM is illustrated by the well-defined thylakoid grana in 3D-SIM images (Fig. 4 *B*–*E*) compared with thylakoids visualized by more conventional confocal scanning laser microscopy (Fig. 4*A*).

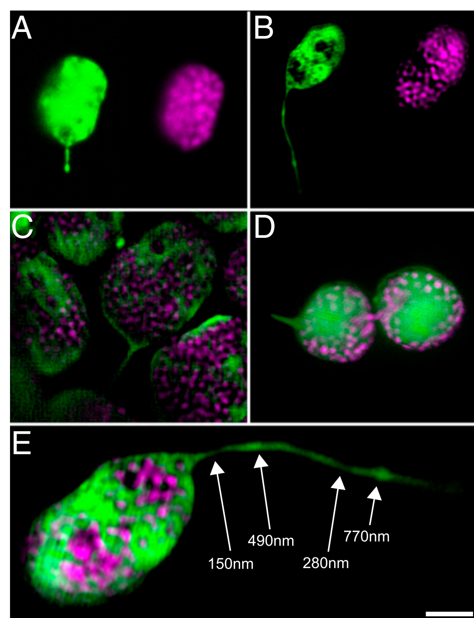

Fig. 4. Examples of fluorescent stromules in *N. benthamiana* chloroplasts visualized by 3D structured illumination microscopy (3D-SIM). (*A* and *B*) Comparison of confocal laser scanning microscopy (*A*; also shown in Fig. S11) and 3D-SIM (*B*; also shown in *E* and in Movie S3) to visualize chloroplast structure (*Left*: stromal GFP, green; *Right*: thylakoid chlorophyll, magenta). In particular, note the improved resolution of stromule width and the clarity of the thylakoid grana in the 3D-SIM *z*-slice (*B*). (*C*) 3D-SIM *z*-slice image of mesophyll chloroplasts with stromules. (*D*) An epidermal chloroplast connected by a thin bridge that contains both stroma and thylakoids also has a stromule (*Left*), as shown by SIM. (*E*) 3D-SIM reveals variability in stromule width. Stromal GFP, green; chlorophyll autofluorescence, magenta. (Scale bar: 2 μm.) One *z*-slice from a 3D-SIM reconstruction is shown, with measured stromule diameters labeled at indicated positions (white arrows).

At their smallest, we observed stromules <150 nm in diameter (Fig. 4*E*); given that this is approximately the resolution of 3D-SIM, stromules could be even narrower. 3D-SIM also revealed striking variability in stromule diameter along the length of an individual stromule. Stromules often were narrowest near the chloroplast body, and then typically varied between approximately 200 and 600 nm wide at different positions along their lengths, as shown in Fig. 4*E*. The variability in stromule width was apparent whether we observed chloroplasts *in planta* or after isolation, suggesting that structural factors inside the chloroplast could be responsible for the heterogeneous diameter of stromules.

Conclusions

Chloroplasts are extraordinarily independent organelles with their own genomes, as many as 3,000 different proteins, and an array of biochemical activities ranging from photosynthesis and carbon fixation to the synthesis of amino acids, fatty acids, hormones, and pigments. Here we have shown that chloroplasts are even more independent, generating stromules in response to changes in the internal chloroplast redox status in a pathway regulated by the chloroplast NADPH-dependent thioredoxin reductase, NTRC. Leucoplasts, nonphotosynthetic plastids, do not make stromules in response to the same redox cues as chloroplasts, but instead are responsive to sucrose concentration, demonstrating that different types of plastids form stromules in response to different signals. We propose a model consistent with these findings that light promotes stromule formation in leaves by increasing ROS in chloroplasts (34) and by increasing sucrose levels in cells with leucoplasts (Fig. 5).

Previous reports have investigated stromules using a variety of plastid types in a broad range of species and tissues, generally assuming that stromules act similarly in all cells (9–19). In light of the clear differences in signals that influence leucoplast and chloroplast stromule formation (Figs. 2 and 5), future work will need to carefully consider the biological context of stromule activity. With the discovery that stromules extend from chloroplasts independently of external structures, analogies to cytonemes could help reveal the roles of stromules, because cytonemes are comparable thin, tubular projections that extend from animal cells to facilitate intercellular communication during development (35, 36). Although the function of stromules remains unknown, it may be speculated that they similarly facilitate signal transduction between organelles, given that stromules have been observed associating with the nucleus, plasma membrane, endoplasmic reticulum, and other plastids (10, 11, 30).

Numerous studies in just the past few years have demonstrated the vital importance of chloroplast-to-nucleus signaling in plant growth and responses to stress (1–8, 23, 24, 27), with critical agricultural implications, but the structural pathways underlying this signal transduction remain largely uncharacterized. Stromules may contribute to these pathways, because they dynamically respond to physiological signals inside the chloroplast. Continued study of stromules may illuminate how chloroplasts physically interact with their environment to coordinate cellular function.

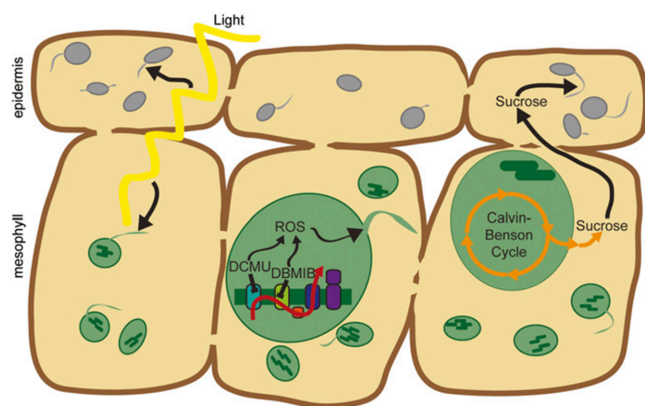

Fig. 5. Stromules are initiated by signals within the chloroplast. (*Left*) Stromule frequency increases in the light (daytime) in both chloroplasts and leucoplasts. (*Center*) ROS generated from the pETC trigger stromule formation in chloroplasts. (*Right*) Sucrose promotes stromule formation in leucoplasts, but not chloroplasts. Sucrose is synthesized in the cytosol from products of the Calvin–Benson cycle in chloroplasts and then moves into neighboring heterotrophic pavement cells via plasmodesmata. For simplicity of presentation, only photosynthetic mesophyll cells are shown (and not photosynthetic guard cells), because there is no evidence suggesting that stromules in these cell types behave differently.

Methods

Plant and Chemical Materials. For most *N. benthamiana* studies, we used a stable transgenic stromal fluorescent marker line, *$35S_{PRO}$:FNRtp:EGFP*, designated pt-GFP herein (30). We also used the *N. benthamiana* wild-type accession Nb-1 for isolation of wild-type chloroplasts. For *A. thaliana* studies, we used a stable transgenic stromal fluorescent marker line (4), *$35S_{PRO}$:RbcStp:roGFP2*, designated pt-roGFP2 herein. pt-roGFP2 was also used for measuring stromal redox status.

We obtained DCMU (also known as Diuron; product no. D2425), DBMIB (product no. 271993), and SHAM (product no. S607) from Sigma-Aldrich. Concentrated stock solutions were prepared in DMSO.

Microscopy. All standard stromule visualization experiments were performed using a Zeiss LSM 710 confocal microscope equipped with an acousto-optical tunable filter to tightly control laser power. GFP was excited with a 488-nm laser with <0.25 mW original power, and emissions from 500 to 530 nm were detected. The 3D-SIM was performed using a Zeiss Elyra PS.1 microscope equipped with standard GFP and Cy5 filter sets.

Diurnal Time Course Experiment. *N. benthamiana* stably expressing pt-GFP was grown for 5 d under 100 μmol of photons $m^{-2}s^{-1}$ (measured with a LI-COR 250A light meter with a LI-190R quantum sensor that detects photosynthetically active radiation, 400–700 nm) with 12-h day length. Epidermal chloroplasts of cotyledons of intact plants were observed every 4 h over the course of 48 h. A green light-emitting diode was used during night time points to prevent exposure to photosynthetically active radiation.

pETC Inhibitor Treatments. *A. thaliana* pt-roGFP2 plants were stratified for 3 d at 4 °C and then grown for 14 d under 100 μmol of photons $m^{-2}s^{-1}$ of light with 16 h day length. The redox status of pt-roGFP2 in *A. thaliana* cotyledons was measured after treatment with 10 μM DCMU, 12 μM DBMIB, or 0.1% DMSO (control treatment), following the protocol described by Stonebloom et al. (4) but using a Zeiss LSM 710 confocal microscope with 405-nm and 488-nm lasers, collecting emissions ranging from 500 to 530 nm.

N. benthamiana stably expressing pt-GFP was grown for 14 d under 100 μmol of photons $m^{-2}s^{-1}$ of light with a 16-h day length. Cotyledons were painted with DCMU (10 μM from 10 mM stock solution in DMSO), DBMIB (12 μM from 12 mM stock solution in DMSO), or control 2 h before observation of epidermal chloroplasts.

To measure stromule frequency in *A. thaliana* (grown as above to measure redox status), pt-roGFP2 cotyledons were removed and placed on 0.5× Murashige and Skoog plates (0.8% agar, pH 5.6) with DCMU (10 μM from 10 mM stock solution in DMSO), DBMIB (12 μM from 12 mM stock solution in DMSO), SHAM (200 μM in DMSO), sucrose (30 mM, or 1% wt/vol), or control for 2 h before imaging of cotyledon epidermal leucoplasts (of pavement cells) and chloroplasts (of guard cells).

VIGS. pt-GFP plants were grown for 3 wk under 100 μmol of photons $m^{-2}s^{-1}$ of light with a 16-h day length before agroinfiltration with the appropriate VIGS vectors. Details of VIGS vector construction are provided in *SI Methods*. Two weeks later, young leaves with silenced gene expression were cut, and the epidermal chloroplasts of the basal region of the leaf were visualized immediately.

Chloroplast Extraction. Intact chloroplasts were extracted from mature leaves of pt-GFP (*N. benthamiana*), Nb-1 (wild-type *N. benthamiana*), pt-roGFP2 (*A. thaliana*), and spinach by grinding leaves in extraction buffer (50 mM Hepes NaOH, 330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, pH

6.9), filtering the homogenate through several layers of cheesecloth, and then pelleting by centrifugation and resuspending in isolation buffer (50 mM Hepes NaOH, 330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 10 mM KCl, 1 mM NaCl, pH 7.6) as described previously (31). More complex protocols, such as inclusion of a Percoll gradient for purification (35% vol/vol), had no discernable effect on chloroplast stromule formation. Isolated spinach or wild-type *N. benthamiana* chloroplasts were incubated with an equal volume of 25 mg/L 5 (6)-carboxyfluorescein diacetate (Sigma-Aldrich, product no. 21879) for 5 min (32), centrifuged at $700 \times g$ for another 60 s, and resuspended in isolation buffer. Chloroplasts were visualized by confocal or structured illumination microscopy immediately after isolation.

Data Analysis. Stromule frequencies were counted using ImageJ software (imagej.nih.gov/ij/) to scan through *z*-stacks of confocal images using a focal depth of 1 Airy unit, which allowed us to visualize stromules extended in any axis from the plastid. Stromules were counted regardless of length but only if <1 μm in diameter, as described by Hanson and Sattarzadeh (10). Most previous studies reported stromule frequencies per cell, considering multiple cells from a single leaf as independent samples. Schattat and Klösgen (16), for example, found little variation in stromule frequencies within an individual leaf, but dramatic variation in stromule frequencies between different leaves. This led them to treat separate fields of view within a single leaf as distinct samples, reducing the apparent variation and effectively increasing statistical power. We also found that stromule frequency varied very little among cells within a leaf, but varied notably among leaves of the same age and condition from different plants. Therefore, we considered one leaf per plant as an individual sample (*n*), and observed many plants for each experiment. Throughout an experiment, all of the analyzed leaves experienced the same growth conditions and were observed at the same age and size.

We conducted power analysis ($\alpha = 0.05$; $\beta = 0.20$) on pilot studies under our growth conditions to determine the sufficient sample size to confidently assert whether or not a treatment caused changes in stromule frequency, and found an approximate minimal $n \geq 16$ for *N. benthamiana* time course and chemical treatment experiments, and $n \geq 8$ for all other experiments. Per treatment, we counted more than 5,000 plastids, at least 100 cells, and ∼20 plants for each chemical treatment or 8 plants for each silencing experiment. Mean stromule frequencies were compared with the Student *t* test, with significance indicated at $P < 0.05$.

We also analyzed all data using angular transformations to account for differences in variation in datasets with very high or low stromule frequencies, but the transformation had no impact on the statistical significance of our results, so we present the data as raw frequencies for the purpose of clear presentation. SEs are presented throughout to describe stromule frequencies. R (www.r-project.org) was used for all statistical analyses.

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