Reactive oxygen species signal chloroplasts to extend themselves

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When Sam Wildman's group first reported his phase-contrast microscopic observations of chloroplast tubular extensions in 1962 (1), he was greeted with skepticism by some plant biologists, who dismissed his findings of membrane protrusions as artifacts of stress caused by long observation of leaf cells under the microscope. Following their rediscovery through GFP technology (2), it is now known that stress actually can induce the tubular formations (3), subsequently named stromules (4). But stromules are no artifact; instead, they are a feature exhibited by plastids in many different types of plant cells (4–7). Probable functions for stromules have been hypothesized for many years (4, 5, 8–10), but there has been no proof of function and little has been known about how they form. In PNAS, Brunkard et al. (11) describe their discovery that stromules do not require external structures for their formation. Furthermore, the authors demonstrate that increase in reactive oxygen species (ROS) by inhibition of photosynthesis induces stromule formation in chloroplasts.

Brunkard et al. (11) used two model plants, *Nicotiana benthamiana* and *Arabidopsis thaliana*, to examine stromule formation and frequency when tissues were placed under various environmental regimes and stresses. When the photosynthetic electron transport chain was inhibited by exogenously supplied chemicals, resulting in generation of ROS, stromule frequency in *N. benthamiana* epidermal chloroplasts and in *A. thaliana* guard cell chloroplasts significantly increased. However, stromule frequency was not altered in naturally nonphotosynthetic leucoplasts present in *A. thaliana* epidermal cells, demonstrating that ROS generated by photosynthesis is responsible for the effect. Brunkard et al. also took advantage of a transient RNA silencing method in *N. benthamiana* to reduce the expression of the nuclear-encoded chloroplast NADPH-dependent thioredoxin reductase. Following this disruption of redox signal transduction within the chloroplast, stromule frequency more than doubled. Notably, RNA silencing of a gene involved in plastid gene expression and another involved in tetrapyrrole biosynthesis did not affect stromule frequency, indicating that not all disturbances in chloroplast function lead to an increase in stromules. Treating *Arabidopsis* cotyledons with an inhibitor that causes ROS generation in mitochondria also did not affect stromule frequency. The experiments by Brunkard et al. in PNAS establish that ROS generated within chloroplasts stimulate stromule formation (Fig. 1). These results extend the findings of Caplan et al. (12), who observed that stromule formation is induced in *N. benthamiana* following ROS generation in chloroplasts during the defense response. Thus, both pathogen effectors and photosynthetic function can lead to production of ROS and the ensuing increase in stromules.

By microscopic examination every 4 h for 44 h, Brunkard et al. (11) observed that stromule frequency was higher in chloroplasts in the cotyledon epidermis of *N. benthamiana* during illumination during the day vs. a nighttime dark period. Suspecting that increases in

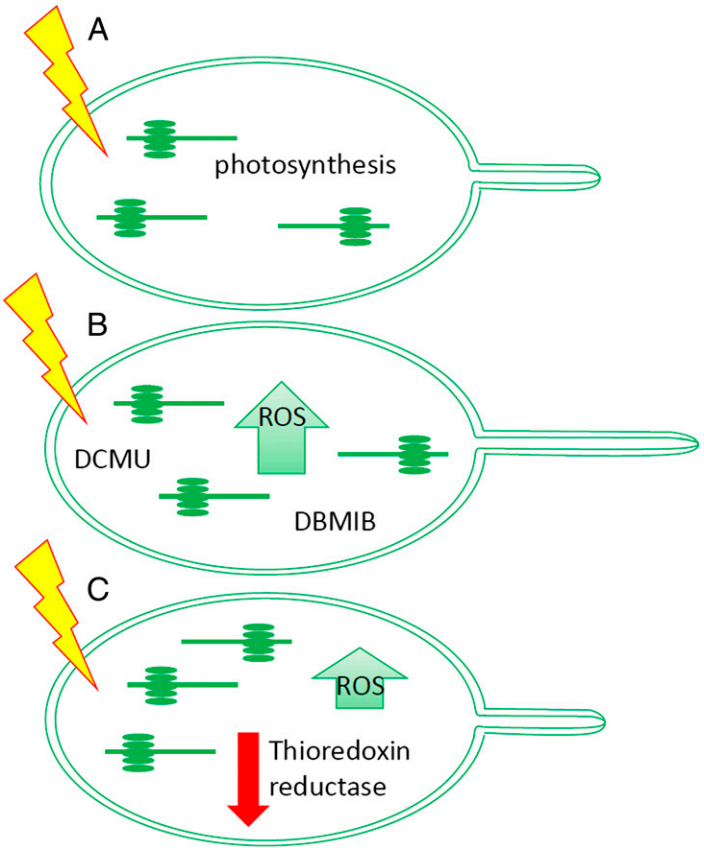

Fig. 1. ROS signaling and stromule formation. (*A*) Light induces stromule formation, presumably because of ROS generated during photosynthesis. (*B*) Inhibition of the photosynthetic electron transport chain by DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] or DBMIB (2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone) increases ROS production and promotes stromule formation. (*C*) When ROS increases because of silencing of thioredoxin reductase, stromules are induced.

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leucoplasts might be a result of the provision of sucrose from mesophyll cells, the authors examined the effect of sucrose treatment on stromule formation in *A. thaliana*. As previously observed by Schattat and Klösgen (13), Brunkard et al. (11) documented a large increase in stromules on leucoplasts in sucrose-treated epidermal cells. However, whereas the previous study detected an increase in stromule frequency in *Arabidopsis* mesophyll cells upon sucrose treatment (13), Brunkard et al. (11) found no effect of sucrose on either guard cell or mesophyll stromule frequency.

An important observation made by Brunkard et al. (11) is the formation of stromules on cell-free chloroplasts, even after purification on a density gradient. Although many investigators have routinely examined the quality of their chloroplast preparations by phase or differential interference contrast microscopy, the presence of stromules would typically have gone unnoticed without specific labeling. To determine whether isolated chloroplasts could form stromules, Brunkard et al. used transgenic plants expressing chloroplast-targeted GFP or stained wild-type chloroplasts with carboxyfluorescein diacetate. Being able to observe stromule formation on isolated chloroplasts opens a way to examine what factors affect stromule formation in vitro. Furthermore, this discovery indicates that cytoskeletal elements outside the chloroplast are not required for formation of stromules. A remaining riddle is the identity of the structure within the chloroplast that is responsible for extending the narrow tubular structures, sometimes to considerable length. A general pressure from within the plastid would not be adequate to form thin stromules; a constriction of the envelope membrane is also necessary for the tubules to remain narrow as they leave the main plastid body. How these constrictions form and how they are maintained are open questions. Without some mechanism for constriction of the envelope, the broad, round, or beak-like protrusions sometimes observed under some conditions (14) would be predicted to result from a force from within the chloroplast.

Brunkard et al.'s (11) finding that isolated chloroplasts form stromules does not exclude the possibility that cytoskeletal elements and other subcellular structures may often be involved in their formation or profoundly affect their location and morphology. There is abundant evidence for the involvement of the actin cytoskeleton and myosin XI in stromule positioning and movement (6, 12, 15–17). Stromules often are attached to unknown structures and can be seen to recoil or flip when the attachment is broken (6, 15, 16). Therefore, although the authors have shown that chloroplasts are able to form stromules in the absence of the intact cell, it remains possible that within the living cell, close interactions or attachment to external structures affects the location on the plastid body they form and play a complementary role in their formation and maintenance.

The work by Brunkard et al. (11) provides further clues to the possible functions of stromules. The observation that ROS generated internally induces stromules raises the possibility that one purpose is to increase contact with nuclei and thereby signal their oxidative stress. Stromules have previously been documented to border nuclei or other organelles (4, 12, 18), leading to the hypothesis that they may facilitate exchange of signaling molecules or metabolites. During the innate immune response, Caplan et al. (12) observed stromules in close proximity to nuclei and that H_2O_2 increased within nuclei at the point of contact with the stromules. Proteins are known to flow through stromules, so small molecules undoubtedly are distributed through them as well (2, 19). Whereas two plastid bodies have occasionally been observed to be joined together through stromules and to exchange molecules (2, 19), GFP photobleaching experiments performed soon after their rediscovery (4) demonstrated that there is no network of interconnected plastids in those cells that were examined. Thus, stromules are more likely to function in reducing diffusion distance of molecules between plastids and other subcellular locations rather than communication among plastids. The current work (11), along with that of Caplan et al. (12), implicates ROS as molecules that may emanate from stromules to other components of the cell. Brunkard et al. (11) provide the first published images of stromules acquired by superresolution microscopy; such methods should facilitate further probing of the interactions of stromules and other parts of the cell.

There is no reason to believe that stromules have a single function, nor that their function is identical in different cell types, on different types of plastid, or under different environmental conditions. As well as involvement in ROS signaling from chloroplasts and sucrose signaling on nonphotosynthetic plastids, both implicated in the present study (11), prior work has suggested that stromules might break off—releasing small vesicles that enter the vacuole—to allow autophagic recycling of a portion of the protein content of a chloroplast during times of nutrient limitation (20). Brunkard et al.'s (11) finding of increased stromule formation following inhibition of the photosynthetic electron transport chain suggests another possible new function for the "tip-shedding" of stromules that has sometimes been observed (6): perhaps toxic ROS are released from the chloroplast to disperse them or to detoxify them elsewhere in the cell.

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