Stromules, functional extensions of plastids within the plant cell

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ABSTRACT

Stromules are thin tubular extensions of the plastid compartment surrounded by the envelope membrane. A myriad of functions have been proposed for them, and they likely have multiple roles. Recent work has illuminated aspects of their formation, especially the important of microtubules in their movement and microfilaments in anchoring. A variety of biotic and abiotic stresses result in induction of stromule formation, and in recent years, stromule formation has been strongly implicated as part of the innate immune response. Both stromules and chloroplasts relocate to surround the nucleus when pathogens are sensed, possibly to supply signaling molecules such as reactive oxygen species. In addition to the nucleus, stromules have been observed in close proximity to other compartments such as mitochondria, endoplasmic reticulum, and the plasma membrane, potentially facilitating exchange of substrates and products to carry out important biosynthetic pathways. Much remains to be learned about the identity of proteins and other molecules released from chloroplasts and stromules and how they function in plant development and defense.

Abbreviations:

endoplasmic reticulum (ER), tobacco mosaic virus (TMV), tobacco rattle virus (TRV)

INTRODUCTION

Cells coordinate the interactions of separate compartments in order to function optimally. Signals are sent and received from different parts of the cells, and substrates and products also flow in a controlled manner. One mechanism that appears to be involved in communication of plastids with other parts of the plant cell is production of tubular extensions of the stroma, the soluble portion of the organelle. These appendages, termed stromules, have been implicated in a variety of cellular processes and are hypothesized to have a variety of functions (Fig. 1). There is no reason to suspect that any molecule that moves freely within the stroma cannot enter a stromule. Chlorophyll-containing thylakoid membranes, however, have never been observed within stromules, and thus it is likely that molecules—such as chloroplast DNA—bound to these membranes are likely to be retained within the main chloroplast body.

This review will focus primarily on work that has appeared in the last three years since our group and others last completed a review [1,2]. Earlier literature and perspectives on the mechanism of stromule formation have recently been discussed [3], as well as the possible role of stromules and chloroplasts in retrograde signaling [4]. Several still earlier reviews provide an overview of the literature that preceded the GFP era, before it became possible to readily visualize stromules in a wide variety of cell types and plant species [5,6]. Remarkably, these structures have been observed in occasional reports for over 100 years yet were largely neglected until fluorescent protein technology became available. The movies produced by Wildman and his collaborators [7] and by Gunning [8,9] are some of the most stunning ever produced without the aid of fluorescent protein labeling.

Chloroplast and stromule positioning and movement

An extremely thorough study of the involvement of the cytoskeleton in stromule movement and positioning appeared in 2019 [10]. Kumar et al. (2019) used a variety of fluorescent protein labels for the stroma, microtubules, and actin filaments in order to follow stromule and cytoskeletal dynamics under normal conditions and after activation of the N receptor by the Tobacco mosaic virus (TMV)-derived protein. Previously, some of the same investigators had shown that activation of N by p50-induced stromules caused induction of stromules, which then assumed a perinuclear location along with numerous chloroplasts [11]. In the follow-up study, painstaking microscopic imaging was used to follow the formation and movement of both stromules and chloroplasts as they migrated within the cell, before and after treatment with a variety of cytoskeletal inhibitors or stabilizers, at varying concentrations and times following treatment. Stromules were demonstrated to extend along microtubules, and to have anchor points on microfilaments, which appear to result in the eventual clustering of chloroplasts and stromules at the nucleus [10]. The findings agree with another report that also used labeling of both stromules and microtubules and concluded the stromules can move along them [12]. Kumar et al. (2019) also observed microtubule stromule-guided chloroplast movement and indicated that over 50% of all chloroplast movement was directed by stromules. These authors thus propose that one function of stromules could be to escort chloroplasts to the nucleus (Fig. 1D), either by providing a highway (stromules) or by towing nuclei in the appropriate direction. This hypothesis is also consistent with the novel observation by Erickson et al. (2018) [12] that

expression of the XopL effector encoded by *Xanthomonas campestris* in *N. benthamiana* could eliminate both stromules and movement of chloroplasts to the nucleus. The XopL effector appears to be unique, given that other *X. campestris* effectors induce, rather than inhibit stromules [11,12]. Given the evidence the perinuclear clustering is important for innate immune response, a pathogen that can remove stromules and thereby abolish proper chloroplast positioning could potentially have an advantage during infection.

A report by Ding et al. (2019) on geminivirus-induced stromule formation has added to our knowledge of stromule-associated chloroplast positioning [13]. Earlier, Krenz et al (2012) had seen induction of stromules by the geminivirus *Abutilon* mosaic virus [14] and Wang et al (2017) had observed that transient expression of the Tomato yellow leaf curl virus' Rep (replication-associated) protein causes perinuclear clustering of chloroplasts [15]. In 2019, the Rep protein was transiently expressed in *Nicotiana benthamiana* along with a stromal GFP, and both stromules and chloroplasts were observed surrounding nuclei of mesophyll cells [13]. The fact that it is Rep that is inducing the clustering, rather than a side effect of Agroinfiltration, was demonstrated when other geminivirus proteins were transiently expressed, but no perinuclear localization of chloroplasts was observed.

Ding et al. (2019) then infected *N. benthamiana* leaves with two additional geminiviruses or their Rep proteins, and again observed chloroplasts encircling the nucleus [13]. Inspired by the geminivirus results, the investigators then examined the effect of infection with TMV, tobacco rattle virus (TRV), and two *Pseudomonas syringae* strains. TMV (as expected), TRV, and the pathogenic bacteria all induced perinuclear chloroplast positioning. Furthermore, the same response could be induced with a bacterial flagellin peptide or the Arabidopsis RPS2 protein, which confers resistance to *P. syringae*. The authors provide video images that make it clear that not only chloroplasts, but also stromules, are encircling nuclei during these responses [13]. Previously, Caplan et al. (2015) had observed that effectors from *P. syringae* pv tomato induced stromule formation. However, when plants were infiltrated with a non-pathogenic bacterium, *P. syringae* lacking the ability to send effectors into plant cells [11], induction of stromules and chloroplast relocation did not occur, indicating that the mere presence of the bacteria was not sufficient to cause formation of stromules.

In addition to repositioning during innate immune responses, chloroplasts are known to cluster around nuclei in dividing cells, which may have evolved as a mechanism to ensure that daughter cells each receive appropriate portions of the organelles [16]. A recent paper demonstrated that the ACT7 gene, which encodes one isoform of actin, is required for the perinuclear positioning of chloroplasts [17]. Mutation of two other actin genes did not affect chloroplast positioning in cultured Arabidopsis mesophyll protoplasts. The actin disrupter latrunculin B eliminated perinuclear positions, but microtubule depolymerization with oryzalin did not [17]. These results are consistent with the actin-driven positioning of chloroplasts and stromules described above in response to pathogens and pathogen-encoded proteins, suggesting that ACT7 might also be required for the perinuclear response elicited by pathogens.

The function of the perinuclear clustering phenomenon is not known, but generally is hypothesized as a means to target signaling molecules to nuclei by reducing diffusion distance.

Stromules can not only surround the surface of nuclei, but also can be found within furrows and channels that run through nuclei [18], making proximity-facilitated signaling a significant possibility. However, because chloroplasts are part of the defense system of plant cells, is it possible that the chloroplasts and stromules are also creating a physical barrier around the nucleus in an attempt to keep out invaders or their protein minions?

Molecules that may pass between stromules and other compartments that are in close proximity

Stromules, chloroplasts, and other types of plastids have often been observed in close juxtaposition to other organelles (Fig 1B, E), and stromules have often been visualized connecting two or more plastids (Fig 2). Whether any or all of these associations actually allow transfer of molecules remains an area of active investigation. In the case of connection of distant plastids by stromules, flow of proteins has been directly demonstrated. Using photobleaching or photoconversion methods, a variety of fluorescent protein-labeled chloroplast targeted proteins have been shown to move from one plastid body to another, as well as GFP itself or the photoconvertible mEosGFP [1,19,20]. Nevertheless, plastids are only infrequently visibly connected by stromules, suggesting such transmission is not their major function, even though such connections have been visualized in a wide variety of cell types [5,21,22]. Undoubtedly there are incidental interactions of stromules within other membrane-bound compartments in the cell that do not result in membrane fusion events. A recent review describes the methods that can be used to determine whether organelle interactions lead to functional membrane contact sites [23].

Interactions between the endoplasmic reticulum (ER) and stromules or chloroplasts have often been observed. Fatty acids are synthesized within plastids, and lipids must traffic in and out of the organelle (Fig 1B); there is no reason to suspect that this trafficking would not occur between a stromule and the ER as well. In order to test whether molecules could move between ER and plastids, enzymes for tocopherol synthesis were placed in the ER and were able to complement Arabidopsis mutants lacking them in the plastid, thus indicating that non-polar compounds could move from ER to plastid [24]. The authors hypothesized that hemifusion between ER and the plastid envelope could allow such exchange. Another possible function of stromule/ER interactions comes from a recent study of a plastid-localized biosynthetic pathway. The enzymes of the 2*C*-methyl-D-erythritol 4-phosphate pathway that produces gerionol were demonstrated to all be located in plastids and stromules. Gerionol is then exported from the plastid compartment to the ER, where an enzyme is located that catalyzes the next step in the synthesis of valuable monoterpene indole alkaloids found in the Madagascar periwinkle [25].

A number of molecules have been shown or hypothesized to pass from chloroplasts/stromules to nuclei during abiotic and biotic stress in a form of retrograde signaling. In the case of nuclear-encoded, chloroplast-targeted proteins, it can be difficult to prove that a protein was actually transmitted from the chloroplast to the nucleus, rather than entering the nucleus from the cytosol following synthesis on cytoplasmic ribosomes. An arogenate dehydratase, AGT5, was observed to be present in chloroplast and stromules in *N. benthamiana*. Bross et al. (2017)

were able to obtain circumstantial evidence that the enzyme might be moving from stromules to nuclei by inhibiting stromule formation through expression of myosin XI tail domains. As well as reduced stromule frequency, the investigators observed less fluorescently labeled ADT5 in the nucleus [26].

Strong supportive evidence for the release of a protein from chloroplasts was provided by a creative strategy carried out by Caplan et al. (2015) to find whether the nuclear-localized host protein NRIP1, involved in TMV defense, actually was derived from the chloroplast [11]. NRIP1 was expressed with a nuclear export signal on its N-terminus, so that unless the signal was cleaved off during transit peptide removal in the chloroplast, none of the protein could be retained in the nucleus. Because NRIP1 (labeled with a fluorescent protein) was indeed found in the nucleus after expression of a TMV protein effector, the protein must have been released from chloroplasts and stromules, both of which were observed in close proximity to nuclei [11]. This test system could be used for any plastid protein that is normally encoded by the nucleus and targeted to plastids. Because of its importance in the response to pathogens, it has been proposed that the ferredoxin Fd2 may be transferred from chloroplast to nuclei [27], but more evidence is needed to determine whether such transfer occurs and has functional significance. A recent review has pointed out that a large number of proteins are thought to be located in more than one compartment, often relocating due to some stimulus such as changes in redox state [28]. There is much more known about the mechanism of import of molecules into plastids than export of proteins and other molecules, and the identities of many signals emanating from plastids await experimental assessment.

Another strategy to determine where a protein is exported from within chloroplasts is to engineer its gene into the chloroplast genome; the mRNA will be translated on chloroplast ribosomes and thus the protein will never pass through the cytoplasm unless released from the chloroplast. When a tagged Whirly1 protein was produced in transgenic plastids, it could be found in both chloroplasts and nuclei [29]. This strategy for examining plastid-to-nuclear protein transfer is feasible in any plant for which plastid transformation is possible [30].

In addition to export of proteins (as detailed above) and the substrates that chloroplasts supply for a number of pathways completed in other subcellular locations, small signaling molecules also emanate from plastids (Fig 1C) [31]. A variety of insults, such as high light stress, blockage of photosynthesis, or pathogen attack, result in production of reactive oxygen species (ROS), especially H_2O_2 [28,32-34]. H_2O_2 is also known to induce stromules, suggesting their possible involvement in ROS-mediated signal transduction [11,35]. It is well known that H_2O_2 accumulates in chloroplasts when they are exposed to high light [4]. In order to determine whether H_2O_2 found in nuclei after light stress was derived from chloroplasts rather than from other organelles, Exposito-Rodriguez et al. (2017) expressed an H_2O_2 protein biosensor in the nucleus, stroma, and cytoplasm, and found that the rate of nuclear accumulation of H_2O_2 was correlated with its appearance in the stroma [36]. These investigators were able to reduce H_2O_2 production in the nucleus by expressing a scavenger enzyme in the chloroplast, but not when it was localized to the cytosol, thus further implicating chloroplasts as the source of the H_2O_2. This evidence seems quite compelling, though some of the same authors have also pointed out

that chloroplast H_2O_2 could instead be inducing some other molecule that then goes to the nucleus, where it then triggers *de novo* H_2O_2 synthesis [4].

Stromules may also be sending molecules to vacuoles for recycling (Fig. 1E). Several groups have observed Rubisco-containing bodies (RCBs) in vacuoles, often after some sort of stress. One hypothesis is that pieces of stromules break off—possibly by tip-shedding—and enter the vacuole in order to recycle proteins or remove toxic molecules from the chloroplast [37-39] (Fig. 1E). Recently, the formation of RCBs in rice experiencing osmotic stress was investigated by electron microscopy, including 3D reconstruction. The authors were able to observe formation of chloroplast protrusions and an RCB being released into the cytoplasm [40], illustrating the value of electron tomography for investigation of stromule and RCB formation.

Conclusions and future prospects

Most studies that have observed altered size or frequency of stromules have compared cells from different tissues types or under various types of stresses or hormonal treatment. There likely are additional phenomena that increase or decrease stromule abundance. These could be used to induce stromules in order to study their formation and dynamics. A particularly interesting method to induce stromules discovered by Breuers et al. (2012) [41] is to overexpress a chloroplast outer envelope protein (Fig. 2), as it was demonstrated that such expression-induced structures contained both the outer and inner envelope membranes. Induction of abundant fluorescent protein-labeled stromules might allow researchers to observe, by photobleaching or photoconversion methods, initially separate stromules connecting to allow exchange of proteins. Even though plastids already connected at a distance have been shown to exchange proteins, because of their low frequency, it has not been technically feasible to observe stromules undergoing fusion with each other or other plastids and subsequently check whether molecules are moving between them.

Another promising technology and cell-type for studying stromules was discovered by Dvorak et al. (2020) [42], who were studying the localization of the superoxide dismutase FDS1 by labeling it with GFP. Using light-sheet and Airyscan confocal microscopy, the investigators found FDS1-GFP in nuclei, cytosol and the chloroplast stroma in Arabidopsis. Notably, they were able to observe abundant stromules in some lateral root cells that appeared to be reaching out and contacting each other, then retracting [42]. Thus, Airyscan confocal microscopy, along with photobleaching or photoconversion of lateral root-expressed stroma-targeted fluorescent protein, might also allow researchers to determine whether the stromule contacts represent actual fusion events that could permit exchange of molecules.

Additional high-resolution microscopy methods may complement prior technology for fluorescent protein probing of stromule formation and morphology [43,44]. Brunkard et al. (2015) [33] were the first to observe stromules by 3D structured illumination microscopy. They could detect *N. benthamiana* stromules that were less that <150 in diameter and could discern regions of stromules that were wider compared to other parts of the same stromules. Previously, by video confocal microscopy, we were able to observe what appeared to be a bolus of GFP moving down an *N. tabacum* hypocotyl stromule, giving the appearance of a thin snake that had swallowed a mouse [45].

The number of mutants known to affect stromule morphology and number are few in number, but likely to increase in the future. Arabidopsis mutants altered in plastid number due to mutation in the ARC3, ARC5, and ARC7 plastid cell division factors have previously been reported to exhibit longer or more abundant stromules [46]. Recently, mutation of the PARC6 plastid division factor was shown to increase stromule frequency [47]. Possibly, additional genes that affect chloroplast division [48] may be found to affect stromule formation and morphology, provided that researchers examine mutant lines with the aid of fluorescent protein labeling of plastids. However, since a viable plant with a complete loss of stromules may not exist, methods to inhibit as well as to induce stromules could be helpful to probe their functions in lieu of mutants. As discussed above, stromule formation can be inhibited by transgenic expression of myosin XI tails [26] or chloroplast movement protein CHUP1 [11].

Stromules are clearly part of the plant's response to biotic stress and require investigation by anyone seeking to understand host response to a particular pathogen. Labeling and confocal imaging of plastids through nuclear-encoded chloroplast-targeted fluorescent proteins is straightforward, though adequate expression of the transgenic protein is essential to observe thin and often transient stromules [43]. Achieving extremely high-level expression of a stromal fluorescent protein-tagged chloroplast protein can easily be achieved by encoding the transgene in the chloroplast genome rather than in the nucleus [30,49]. Encoding of fluorescent proteins within the chloroplast to observe relocalization under abiotic and biotic stress is likely to allow new discoveries of multifunctional proteins that are only part-time residents in plastids, and sometimes move elsewhere to take up new jobs.

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Author Contributions

M.R.H. drafted the manuscript, P.L.C. edited the manuscript and performed transient expression and microscopic analysis of stromules and chloroplasts.

Figures

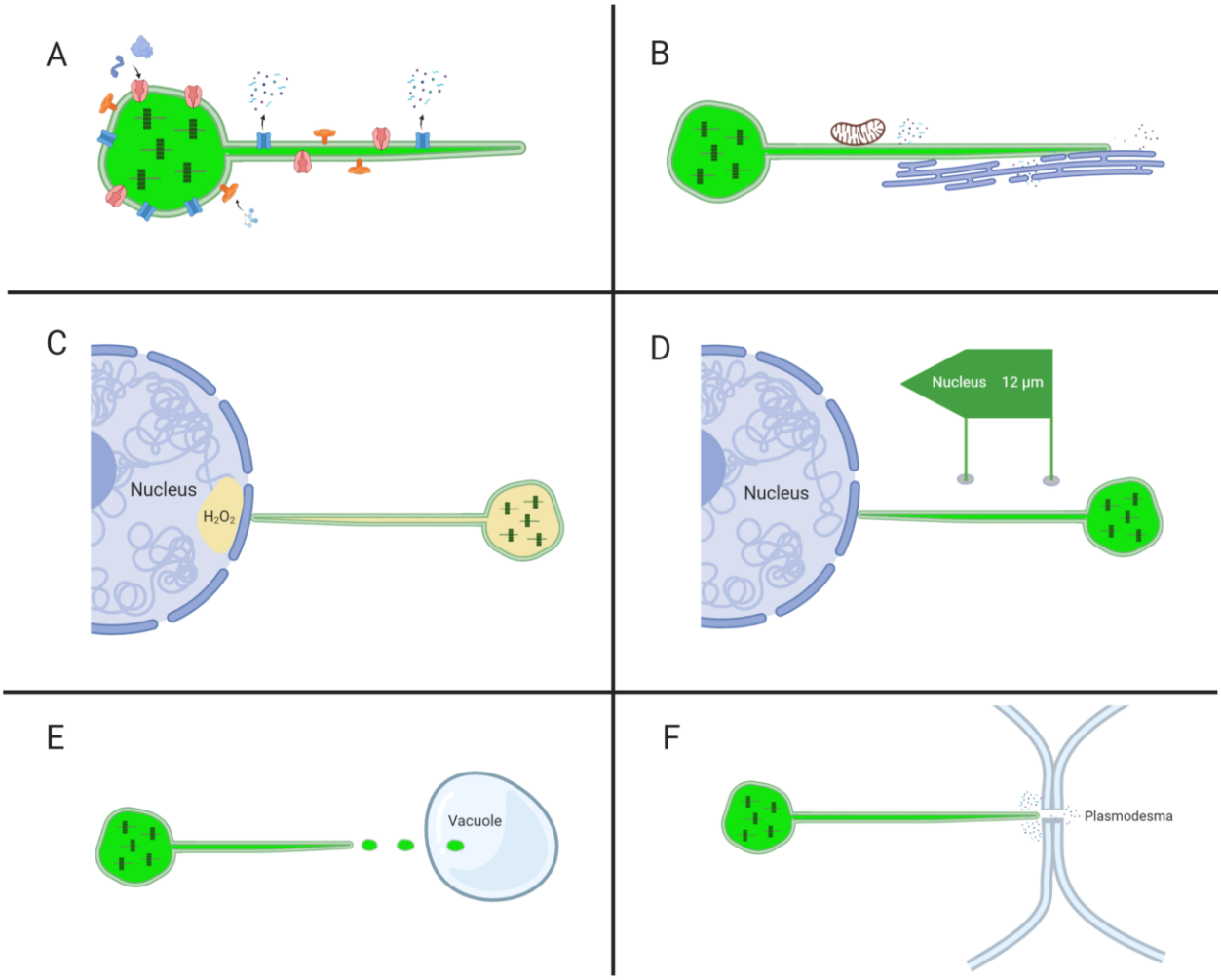

Figure 1. Possible roles of stromules A) Stromules increase the surface area of the envelope membrane and disperse the compartment further within the cytoplasm, possibly facilitating import and export. (B) Chloroplasts exchange molecules with a variety of other compartments; stromules may place the stromal compartment in close proximity to reduce diffusion distance. C) Reactive oxygen species are induced within chloroplasts when plant cells are under pathogen attack, and stromules may transmit them to the nucleus, resulting in altered gene expression. D) Stromules may function to gather chloroplasts at the nucleus by providing directional information or pulling them there. E) Tips of stromules may break off and enter the vacuole for recycling of proteins or removal of toxic molecules F) Stromules may send signals to other cells through close proximity to plasmodesmata. Created with Biorender.com.

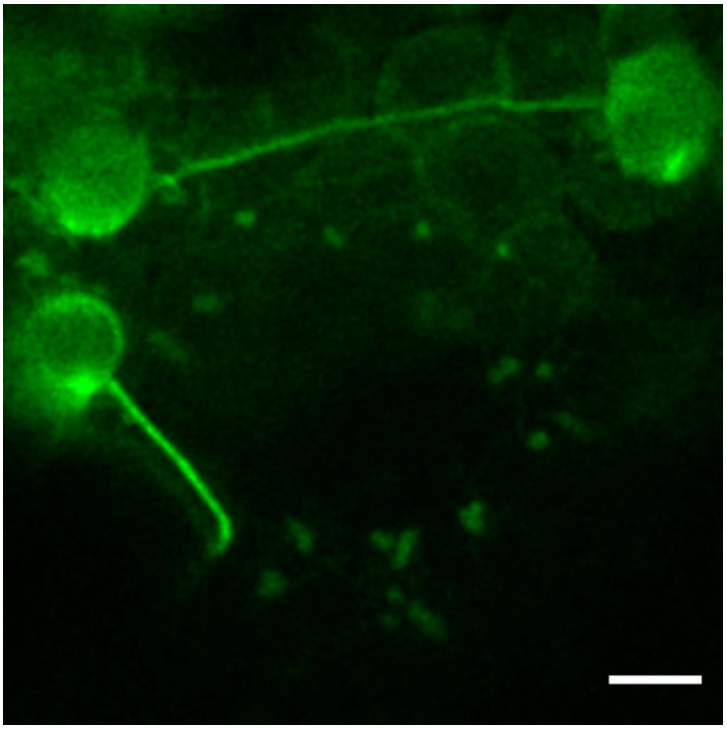

Figure 2. Chloroplasts apparently connected by a stromule. In addition to stromules, small green bodies can be seen that may be excess membrane that has broken off due to overexpression of LACS9. *N. benthamiana* leaf epidermal cells infiltrated with 0.4 OD units each of *Agrobacterium* carrying AtLACS9-GFP [41], and 0.8 OD units of a vector expressing P19, the tomato bushy stunt virus silencing inhibitor [50]. The image was taken 96 hours post-infiltration with a Zeiss 710 confocal microscope. Scale bar: 5 μm.

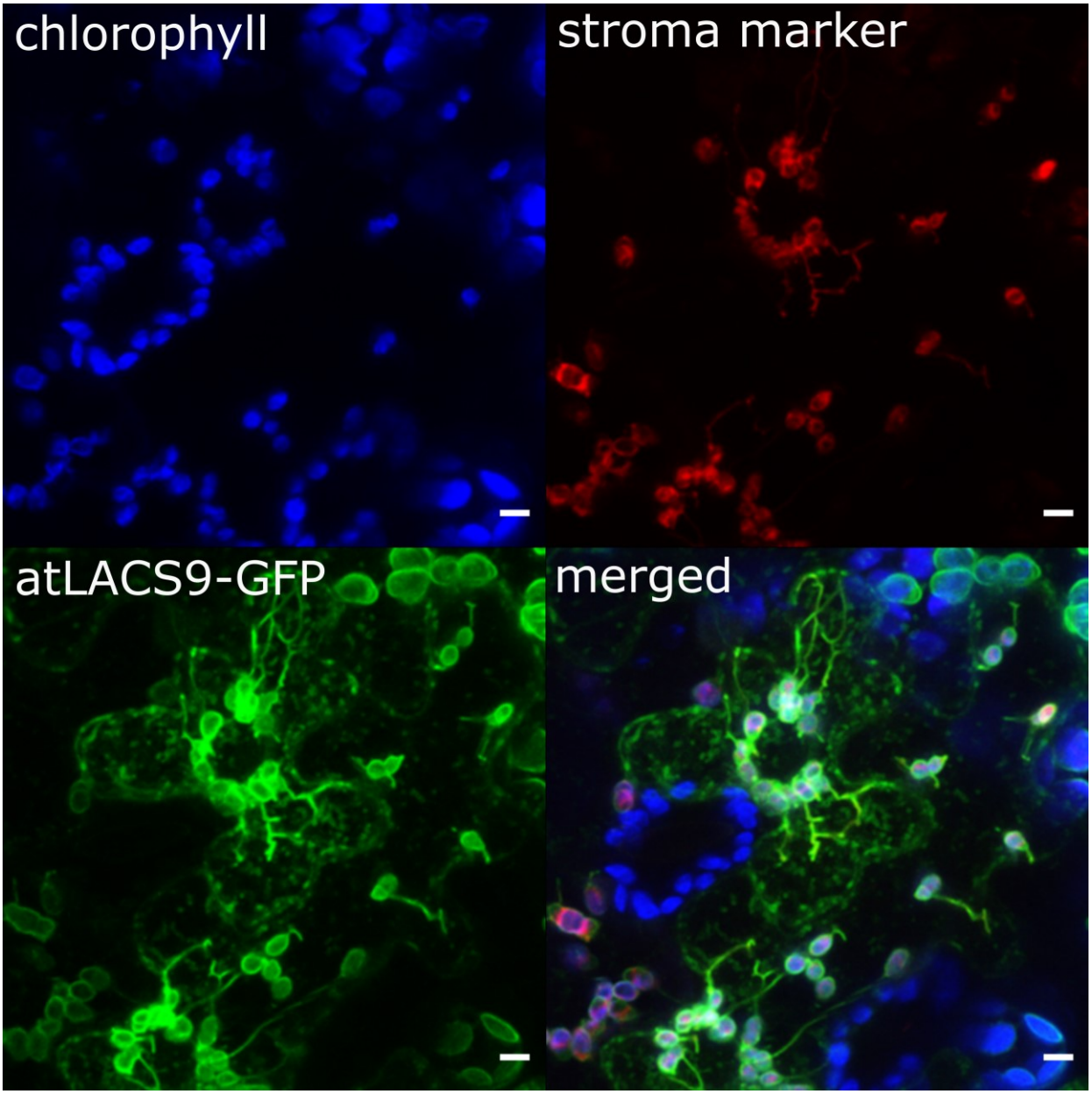

Figure 3. Induced projections from plastids transiently expressing the outer membrane protein AtLACS9-GFP contain stromal protein. Agroinfilitration of N. benthamiana leaf epidermal cells with 0.4 OD units each of Agrobacterium carrying AtLACS9-GFP [37], the PT-RK vector (expresses stromal-mCherry marker [43]), and 0.8 OD units of a vector expressing P19, the tomato bushy stunt virus silencing inhibitor. Images were taken 96 hours post-infiltration with a Zeiss 710 confocal microscope and are maximum projections from 12 images taken along the Z-axis. Images colored as follows: Chlorophyll (blue), mCherry (Red), GFP (green). Scale bar: 5 μm.

References

1. *Hanson MR, Hines KM: **Stromules: probing formation and function**. *Plant Physiol* 2018, **176**:128-137.

This publication represents the most recent comprehensive review of the hypotheses for the function of stromules and the data that support them.

2. Brunkard JO, Burch-Smith TM: **Ties that bind: the integration of plastid signalling pathways in plant cell metabolism**. *Essays Biochem* 2018, **62**:95-107.

3. Erickson JL, Schattat MH: **Shaping plastid stromules-principles of in vitro membrane tubulation applied in planta**. *Curr Opin Plant Biol* 2018, **46**:48-54.

4. Mullineaux PM, Exposito-Rodriguez M, Laissue PP, Smirnoff N, Park E: **Spatial chloroplast-to-nucleus signalling involving plastid-nuclear complexes and stromules**. *Philos Trans R Soc Lond B Biol Sci* 2020, **375**:20190405.

5. Kwok EY, Hanson MR: **Stromules and the dynamic nature of plastid morphology**. *J Microsc* 2004a, **214**:124-137.

6. Natesan SK, Sullivan JA, Gray JC: **Stromules: a characteristic cell-specific feature of plastid morphology**. *J Exp Bot* 2005, **56**:787-797.

7. Wildman SG, Hongladarom T, Honda SI: **Chloroplasts and mitochondria in living plant cells: cinephotomicrographic studies**. *Science* 1962, **138**:434-436.

8. Gunning BE: **Plastid stromules: video microscopy of their outgrowth, retraction, tensioning, anchoring, branching, bridging, and tip-shedding**. *Protoplasma* 2005, **225**:33-42.

9. Gunning BES: **Plant Cell Biology on DVD**. Edited by. Heidelberg, Germany: Springer-Verlag; 2009.

10. **Kumar AS, Park E, Nedo A, Alqarni A, Ren L, Hoban K, Modla S, McDonald JH, Kambhamettu C, Dinesh-Kumar SP, et al.: **Stromule extension along microtubules coordinated with actin-mediated anchoring guides perinuclear chloroplast movement during innate immunity**. *Elife* 2018, **7**.

This wide-ranging and carefully performed study demonstrates movement of stromules along microtubules and anchoring of stromules onto actin microfilaments and contains evidence that stromules may direct chloroplasts to the nucleus.

11. **Caplan JL, Kumar AS, Park E, Padmanabhan MS, Hoban K, Modla S, Czymmek K, Dinesh-Kumar SP: **Chloroplast stromules function during innate immunity**. *Dev Cell* 2015, **34**:45-57.

A well-documented study demonstrating formation of stromules during pathogen response and following treatment with defense signals such as salicylic acid and hydrogen peroxide.

12. *Erickson JL, Adlung N, Lampe C, Bonas U, Schattat MH: **The Xanthomonas effector XopL uncovers the role of microtubules in stromule extension and dynamics in *Nicotiana benthamiana***. *Plant J* 2018, **93**:856-870.

The study finds microtubules to be involved in stromule formation and movement, and also contains the novel observation that a pathogen effector can eliminate stromule formation during infection.

13. Ding X, Jimenez-Gongora T, Krenz B, Lozano-Duran R: **Chloroplast clustering around the nucleus is a general response to pathogen perception in *Nicotiana benthamiana***. *Mol Plant Pathol* 2019, **20**:1298-1306.

14. Krenz B, Jeske H, Kleinow T: **The induction of stromule formation by a plant DNA-virus in epidermal leaf tissues suggests a novel intra- and intercellular macromolecular trafficking route**. *Front Plant Sci* 2012, **3**:291.

15. Wang L, Ding X, Xiao J, Jimenez-Gomicronngora T, Liu R, Lozano-Duran R: **Inference of a geminivirus-host protein-protein interaction network through affinity purification and mass spectrometry analysis**. *Viruses* 2017, **9**.

16. Sheahan MB, Rose RJ, McCurdy DW: **Organelle inheritance in plant cell division: the actin cytoskeleton is required for unbiased inheritance of chloroplasts, mitochondria and endoplasmic reticulum in dividing protoplasts**. *Plant J* 2004, **37**:379-390.
17. Sheahan MB, Collings DA, Rose RJ, McCurdy ADW: **ACTIN7 Is required for perinuclear clustering of chloroplasts during Arabidopsis protoplast culture**. *Plants (Basel)* 2020, **9**.

18. Kwok EY, Hanson MR: **Plastids and stromules interact with the nucleus and cell membrane in vascular plants**. *Plant Cell Rep* 2004d, **23**:188-195.

19. *Hanson MR, Sattarzadeh A: **Trafficking of proteins through plastid stromules**. *Plant Cell* 2013, **25**:2774-2782.

This paper represents a response to a report challenging our prior work that demonstrated flow of proteins between chloroplasts. Fluorescence microscopic imaging data is provided to show that proteins can indeed move between chloroplast bodies separated at a distance.

20. Kohler RH, Cao J, Zipfel WR, Webb WW, Hanson MR: **Exchange of protein molecules through connections between higher plant plastids**. *Science* 1997, **276**:2039-2042.

21. Pyke KA, Howells CA: **Plastid and stromule morphogenesis in tomato**. *Ann Bot* 2002, **90**:559-566.

22. Pyke KA: **Plastid Biology**. Edited by. Cambridge, UK: Cambridge University Press; 2009.

23. Baillie AL, Falz AL, Muller-Schussele SJ, Sparkes I: **It started with a kiss: monitoring organelle interactions and identifying membrane contact site components in plants**. *Front Plant Sci* 2020, **11**:517.

24. Mehrshahi P, Stefano G, Andaloro JM, Brandizzi F, Froehlich JE, DellaPenna D: **Transorganellar complementation redefines the biochemical continuity of endoplasmic reticulum and chloroplasts**. *Proc Natl Acad Sci U S A* 2013, **110**:12126-12131.

25. Guirimand G, Guihur A, Perello C, Phillips M, Mahroug S, Oudin A, Duge de Bernonville T, Besseau S, Lanoue A, Giglioli-Guivarc'h N, et al.: **Cellular and subcellular compartmentation of the 2C-methyl-D-erythritol 4-phosphate pathway in the Madagascar periwinkle**. *Plants (Basel)* 2020, **9**.

26. Bross CD, Howes TR, Abolhassani Rad S, Kljakic O, Kohalmi SE: **Subcellular localization of Arabidopsis arogenate dehydratases suggests novel and non-enzymatic roles**. *J Exp Bot* 2017, **68**:1425-1440.

27. Wang M, Rui L, Yan H, Shi H, Zhao W, Lin JE, Zhang K, Blakeslee JJ, Mackey D, Tang D, et al.: **The major leaf ferredoxin Fd2 regulates plant innate immunity in Arabidopsis**. *Mol Plant Pathol* 2018, **19**:1377-1390.

28. **Foyer CH, Baker A, Wright M, Sparkes IA, Mhamdi A, Schippers JHM, Van Breusegem F: **On the move: redox-dependent protein relocation in plants**. *J Exp Bot* 2020, **71**:620-631.

A thought-provoking review of moonlighting, multi-compartmented proteins and redox-regulated post-translational modifications that may mediate protein relocation.

29. Isemer R, Mulisch M, Schafer A, Kirchner S, Koop HU, Krupinska K: **Recombinant Whirly1 translocates from transplastomic chloroplasts to the nucleus**. *FEBS Lett* 2012, **586**:85-88.

30. **Chloroplast Biotechnology: Tools for Plastd Genome Engineering**. edn 2. Edited by Maliga P. New York: Springer Science+Business Media, LLC; 2021:in press. Methods in Molecular Biology, vol 40.]

31. Fernandez JC, Burch-Smith TM: **Chloroplasts as mediators of plant biotic interactions over short and long distances**. *Curr Opin Plant Biol* 2019, **50**:148-155.

32. Noctor G, Foyer CH: **Intracellular redox compartmentation and ROS-related communication in regulation and signaling**. *Plant Physiol* 2016, **171**:1581-1592.

33. Brunkard JO, Runkel AM, Zambryski PC: **Chloroplasts extend stromules independently and in response to internal redox signals**. *Proc Natl Acad Sci U S A* 2015, **112**:10044-10049.

34. Park E, Nedo A, Caplan JL, Dinesh-Kumar SP: **Plant-microbe interactions: organelles and the cytoskeleton in action**. *New Phytol* 2018, **217**:1012-1028.

35. Gray JC, Hansen MR, Shaw DJ, Graham K, Dale R, Smallman P, Natesan SK, Newell CA: **Plastid stromules are induced by stress treatments acting through abscisic acid**. *Plant J* 2012, **69**:387-398.

36. *Exposito-Rodriguez M, Laissue PP, Yvon-Durocher G, Smirnoff N, Mullineaux PM: **Photosynthesis-dependent H_2O_2 transfer from chloroplasts to nuclei provides a high-light signalling mechanism**. *Nat Commun* 2017, **8**:49.

An H2O2 sensor is exploited to observe its presence in nuclei after induction within chloroplasts during a defense response.

37. Ishida H, Yoshimoto K, Izumi M, Reisen D, Yano Y, Makino A, Ohsumi Y, Hanson MR, Mae T: **Mobilization of rubisco and stroma-localized fluorescent proteins of chloroplasts to the vacuole by an ATG gene-dependent autophagic process**. *Plant Physiol* 2008, **148**:142-155.

38. Izumi M, Hidema J, Wada S, Kondo E, Kurusu T, Kuchitsu K, Makino A, Ishida H: **Establishment of monitoring methods for autophagy in rice reveals autophagic recycling of chloroplasts and root plastids during energy limitation**. *Plant Physiol* 2015, **167**:1307-1320.

39. Wada S, Ishida H, Izumi M, Yoshimoto K, Ohsumi Y, Mae T, Makino A: **Autophagy plays a role in chloroplast degradation during senescence in individually darkened leaves**. *Plant Physiol* 2009, **149**:885-893.

40. Yamane K, Oi T, Taniguchi M: **Three-dimensional analysis of chloroplast protrusion formed under osmotic stress by polyethylene glycol in rice leaves**. *Plant Production Science* 2020, **23**:160-171.

41. Breuers FK, Brautigam A, Geimer S, Welzel UY, Stefano G, Renna L, Brandizzi F, Weber AP: **Dynamic remodeling of the plastid envelope membranes - a tool for chloroplast envelope in vivo localizations**. *Front Plant Sci* 2012, **3**:7.

42. **Dvorak P, Krasylenko Y, Ovecka M, Basheer J, Zapletalova V, Samaj J, Takac T: **In-vivo light-sheet microscopy resolves localisation patterns of FSD1, a superoxide dismutase with function in root development and osmoprotection**. *Plant Cell Environ* 2020.

While the emphasis of this study is on the function of FSD1, the authors provide striking images of plastids and stromules with the use of Airyscan confocal microscope. One of the supplementary videos shows stromules extending to contact each other and then retracting.

43. Hanson MR, Conklin PL, Sattarzadeh A: **Flurescoent labeling and confocal microscopic imaging of chloroplasts and non-green plastids (protocol)**. In *Chloroplast Biotechnology: Tools for Plastid Genome Engineering*, edn 2. Edited by Maliga P. New York: Springer Science+Business Media, LLC; 2021:in press. vol 40

44. Brunkard JO, Runkel AM, Zambryski P: **Visualizing stromule frequency with fluorescence microscopy**. *J Vis Exp* 2016.

45. Hanson MR, Sattarzadeh A: **Dynamic morphology of plastids and stromules in angiosperm plants**. *Plant Cell Environ* 2008, **31**:646-657.

46. Holzinger A, Kwok EY, Hanson MR: **Effects of *arc3, arc5* and *arc6* mutations on plastid morphology and stromule formation in green and nongreen tissues of *Arabidopsis thaliana***. *Photochem Photobiol* 2008, **84**:1324-1335.

47. Itoh RD, Ishikawa H, Nakajima KP, Moriyama S, Fujiwara MT: **Isolation and analysis of a stromule-overproducing Arabidopsis mutant suggest the role of PARC6 in plastid morphology maintenance in the leaf epidermis**. *Physiol Plant* 2018, **162**:479-494.

48. Osteryoung KW, Pyke KA: **Division and dynamic morphology of plastids**. *Annu Rev Plant Biol* 2014, **65**:443-472.

49. Reed ML, Wilson SK, Sutton CA, Hanson MR: **High-level expression of a synthetic red-shifted GFP coding region incorporated into transgenic chloroplasts**. *Plant J* 2001, **27**:257-265.

50. Dhillon T, Chiera JM, Lindbo JA, Finer JJ: **Quantitative evaluation of six different viral suppressors of silencing using image analysis of transient GFP expression**. *Plant Cell Rep* 2009, **28**:639-647.

* Of special interest

** of outstanding interest