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Talk to your neighbors in an emergency: Stromule-mediated chloroplast-nucleus communication in plant immunity

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Abstract

Hypersensitive response-programmed cell death (HR-PCD) is a response mounted by plants to defend themselves against pathogens. Communication between the chloroplast and the nucleus is critical for the progression of HR-PCD. Tubular protrusions of chloroplasts, known as stromules, are tightly associated with the HR-PCD progression. There is emerging evidence that signaling molecules originating from chloroplasts are transferred to the nucleus through stromules. The translocation of signaling molecules from the chloroplast to the nucleus might trigger defense responses, including transcriptional reprogramming. In this review, we discuss the possible functions of stromules in the rapid transfer of signaling molecules in the chloroplast-nucleus communication.

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Stromule, Organelle communication, Changes in organelle morphology, Plant immunity, Translocation, Retrograde signaling.

Introduction

Plants have evolved many responses for maintaining homeostasis and protecting themselves from biotic and abiotic threats at the organelle level. Inter-organellar communication is a general strategy for translating various stimuli into cellular acclimation responses by adjusting metabolic and/or catabolic activities to ensure optimal growth under specific conditions [1]. Such communication involves structural and morphological modifications of individual organelles [2–6]. For example, stroma-filled tubular structures called stromules extend from chloroplasts and associate with the nucleus, the endoplasmic reticulum (ER), and the plasma membrane (PM) in *Arabidopsis thaliana* [2,3]. These structures extend and retract dynamically in response to abiotic and biotic stimuli such as high light, heat, reactive oxygen species (ROS), phytohormones, sugars, and pathogens [4]. While the length of the stromule is more diverse, stromule width was measured within the range of 0.15–1.5 μm in *Nicotiana benthamiana* chloroplasts, in agreement with the detection capacity of confocal microscopy or super-resolution structural illumination microscopy [7]. Stromules are thought to influence inter-organelle communication by exchanging signaling molecules produced in the chloroplasts with other organelles [1]. The genetic regulators governing the morphological dynamics of these tubular structures are largely unknown.

Stromules are observed in various cell types, including guard cells, trichomes, parenchyma cells, mesophyll cells, and epidermal cells [7,8]. The nonphotosynthetic leucoplasts of cells in fresh calli, roots, and hypocotyls contain abundant stromules [8]. By contrast, the chloroplasts of fully differentiated mesophyll and epidermal cells in mature leaves contain relatively few stromules in the absence of a stress stimulus [9,10], suggesting that stromule biogenesis might be regulated by both environmental stimuli and intrinsic developmental cues [8].

The biogenesis of these highly plastic structures is intertwined with chloroplast division. Indeed, *A. thaliana* mutants harboring defects in *accumulation and replication of chloroplast* (*ARC*) genes such as *arc3*, *arc5*, and *arc6* show increased stromule frequency on plastids at different developmental stages [11]. In addition, an *Arabidopsis* mutant in the chloroplast division site regulator, *paralog of ARC6* (*PARC6*), shows grape-like clustering of chloroplasts with long and excessively developed stromules in pavement cells [12], indicating that stromule biogenesis is influenced by chloroplast development.

Stromules are sometimes associated with other subcellular compartments other than the nucleus [13–18].

Specifically, the associations of stromules with the PM and the plasmodesmata might be important for cell-to-cell communication, especially in the context of immune responses [18]. However, a detailed understanding of how stromules communicate with the organelles is lacking. In this review, we highlight the current understanding of inter-organellar communication, focusing on the latest findings in the communication between the chloroplast and the nucleus via stromules during plant immune responses (Figure 1). We also briefly introduce stress-induced morphological changes in other organelles, such as peroxisomes and mitochondria, providing insight into the potential roles of organelle dynamics and communications in stress responses.

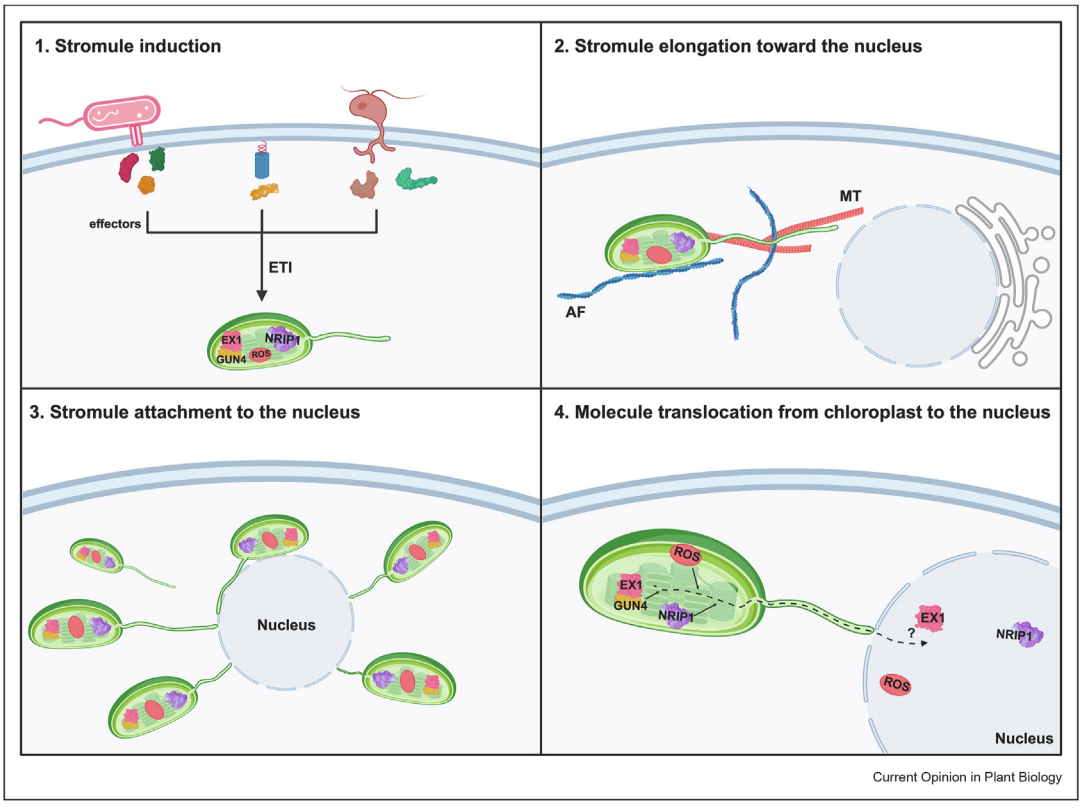
The four stages of the chloroplast-nucleus communication in plant immunity

While observations of stromules have been reported for more than 100 years [10], stromule formation under biotic stress has become prevalent within a decade despite limited evidence and postulation regarding stromule formation. However, it is clear that stromules contribute to the complex signaling network of the immune response [13,19–22]. Long and highly dynamic stromules have been observed during immune responses, and they were proposed to translocate signaling molecules from chloroplasts to the nucleus [13]. We present a conceptual framework of stromule-mediated chloroplast-nucleus communication in immunity, divided into four major stages to organize our discussion of previous findings. This organization helps highlight open questions for future research in the field (Box 1, Figure 1).

Stage 1: Plant immune responses induce morphological changes in chloroplasts

Stromules were observed in the early stage of the effector triggered immunity (ETI). Effectors from pathogens can be recognized by the cytosolic nucleotide-binding leucine-rich repeat (NLR)-type immune receptors in resistant plant cells. Several effector (Avr) and receptor (R) pairs have been characterized to trigger robust plant immunity accompanied by a stage of hypersensitive response-programmed cell death (HR-PCD) when these Avr-R pairs are co-expressed transiently in one cell [23]. Co-expression of AvrBs2 from *Xanthomonas oryzae* and Bs2 (R) in *N. benthamiana* significantly induces stromules

Figure 1



Stages of stromule-mediated chloroplast-nucleus communication. Stage 1: Effector-triggered immunity causes morphological changes of chloroplasts. Stage 2: Collaborative regulation of stromule dynamics by the cytoskeletons. Stage 3: Attachment of stromule tips to the nucleus. It is uncertain whether this attachment is direct. Stage 4: Translocation of materials from the chloroplast to the nucleus during immune responses. See the text for abbreviations.

Box 1. Summary of the current understanding of stromules and chloroplast dynamics for discussion about the conceptual framework of the four stages of stromule-mediated chloroplast-nucleus communication in plant immunity.

Relevant stage	Key findings	Reference
Stage 1	A tight connection between stromule induction and plant immunity	[13,20]
	The infection of *Phytophthora infestans* induces stromules	[15]
	First identification of the bacterial effector XopL that negatively regulates stromules	[22]
Stage 2	Collaborative contribution of actin filament (AF) and microtubule (MT) in stromule dynamics	[21,22]
	MT is a major track of stromules, distinct from AF-based chloroplast movement	[21]
	KIS1, a MT motor kinesin, is required for stromule formation in immunity	[29]
Stage 3	Potential stromule function as a driving force for perinuclear clustering of chloroplasts	[21]
	Uncouple stromule induction and perinuclear clustering in immunity	[22]
Stage 4	The first demonstration (directly or indirectly) of proteins translocated from the chloroplast to the nucleus in plant immunity	[13]

[13]. In addition, expression of *Xanthomonas* effector *Xanrhomonas* outer protein Q 1 (XopQ1) increases stromule formation through the endogenous expression of the cognate immune receptor Recognition of XOPQ1 (ROQ1), a Toll/Interleukin-1 receptor domain (TIR)-type NLR immune receptor in *N. benthamiana* [20]. *A. thaliana* Col-0 ecotype also encodes several R proteins to recognize various Avr proteins from *Pseudomonas syringae* pv. *tomato* (*Pst)* DC3000. Stromules were induced when *Pst* DC3000 containing AvrRpt2, AvrRpm1, or AvrRps4 infected the leaves of the *A. thaliana* Col-0 ecotype [13]. Finally, transient expression of the viral elicitor p50, a 50 kDa helicase fragment from tobacco mosaic virus (TMV), in transgenic *N. benthamiana* leaves expressing the cognate R protein N and its co-factor N receptor interacting protein 1 (NRIP1) to trigger the ETI resulted in a vigorous stromule induction [13]. All these cases clearly show that stromules are induced in the ETI responses. Although the exact timing for stromule induction has not been determined, the infection of *Pst* expressing AvrRpt2 induces stromules within 6 h before observable dead cells, suggesting that stromule induction occurs in the early stage of the ETI [11]. During *Phytophthora infestans* infection, stromule formation is induced in chloroplasts near the haustoria [15]. In addition, stromules respond to the exogenous application of immune signaling regulators such as hydrogen peroxide (H_2O_2) and salicylic acid (SA) [13], suggesting a tight connection between stromule induction and early immune response. Based on the observations, stromule biogenesis appears to be active where immune responses are active.

However, stromule formation can be uncoupled with HR-PCD triggered by ETI [20]. Stromule formation is attenuated in the *N. benthamiana roq1* and *eds1* mutants [20]. Since Enhanced disease susceptibility 1 (EDS1) is essential for resistance and cell death mediated by ROQ1, the signaling cascade regulated by ROQ1-EDS1 contributes to stromule induction during ETI signaling. However, in the mutant lacking *N-required gene 1* (*NRG1*), a downstream regulator of this immune signaling pathway, stromule induction was significantly increased, while HR-PCD triggered by active ROQ1 upon recognition of XopQ was completely abolished [20]. This phenomenon indicates that stromule formation appears to be regulated by additional pathways beyond the ETI-triggered HR-PCD signaling cascade activated by ROQ1 (R) activation upon XopQ (Avr) recognition. Recently, structural and genetic studies of EDS1 function in plant immunity over decades have enabled us to propose a separate mechanism of defense gene regulation from the cell death pathways in the plant immune network [24], implicating that the stromule regulation mechanism in ETI might be part of the pathway to regulate defense gene expressions during immune responses.

Moreover, the induction of stromule formation by pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) is also observed [13,15]. PTI activated by direct treatment with the flg22 peptide, a well-known PAMP, induces stromule formation within 30 min as well as after 8 h, matching the physiological response of ROS burst during PTI [13]. Together with stromule induction by exogenous application of H_2O_2 [13], the induction of stromules by both PTI and ETI responses suggests that stromule induction is tightly associated with physiological responses during plant immunity. However, due to highly dynamic and interconnected changes in physiological status during plant immunity [1], it is difficult to characterize the direct

signal of the stromule induction during plant immune responses. Comprehensive time-course observations of stromule induction, the dynamics of ROS flux, and other intertwined physiological changes such as calcium flux in different subcellular compartments help to provide detailed molecular insights into the signaling cascade of stromule induction.

Stage 2: Regulation of stromule extension toward the nucleus

Cytoskeleton rearrangement is important to regulate the length of stromules and their direction of growth. While chloroplast movements are regulated by actin filaments (AFs) [25], microtubules (MTs) and AFs collaboratively regulate stromule dynamics [21,22,26,27]. During the active immune response mediated by the TMV resistant immune receptor, N, MTs provide a track for stromule elongation and retraction, while AFs serve as anchor points, not only restricting stromule growth but also changing their direction [21]. This study also demonstrated that stromule dynamics could be regulated by MTs, as observed in live-cell imaging aimed at co-visualizing stromules and MTs [21]. Stromules elongate along MTs to anchor points on AFs, creating kinks that facilitate changes in their direction by switching to grow on another MT track [21]. Genetic and pharmaceutical disruptions of MTs and AFs have been used to analyze the effect of cytoskeleton dynamics on stromule biogenesis [21,22,26,27]. In higher concentrations of AF disrupting drugs, severe stromule retractions were observed, while the effect of MT disrupting drugs on stromule formation was marginal, suggesting the importance of the acto-myosin cytoskeleton to stromule formation [22,27]. However, later comprehensive live-cell time-lapse imaging analyses clearly support that both AF and MT cytoskeletons coordinate in regulating stromules through distinct mechanisms [21]. In the treatment of higher concentrations of both AF inhibitors and MT inhibitors, cross disruption of both cytoskeletons was demonstrated [28]. Notably, at low concentrations of drug treatments, MT inhibitor oryzalin increased the velocity of stromule extension significantly, while AF inhibitor cytochalasin D significantly decreased the velocities of stromule extension and retractions, thereby regulating the length of the stromule [21].

Manipulation of cytoskeletons affects both stromule number and length, suggesting that the cytoskeleton has the potential to be involved in the stromule induction (Stage 1). However, the observation of stromule formation in the *Arabidopsis kis1* mutant suggests that cytoskeletons play a role in the regulation of stromule dynamics [29]. KIS1 is a member of the kinesin 14 family, which contains an actin-binding calponin homology domain [30]. Kinesin motor proteins function to move various cargos, including organelles, along the MT track. Overexpression of *KIS1* vigorously induced stromules without activation of immunity, while the *KIS1* mutation compromised stromule formation as well as HR-PCD in ETI [29]. Although stromule elongation during immune responses was clearly compromised in the *kis1* mutant, a basal level of stromules was always observed, supporting the independent regulation of stromule initiation (Stage 1) from the regulation of stromule dynamics (Stage 2). However, we cannot exclude the possibility of the involvement of other kinesins. In addition, considering the role of AF in stromule dynamics, the contribution of the acto-myosin cytoskeleton to stromule induction is feasible. Interestingly, isolated chloroplasts can induce short stromule formations with cytosolic extracts but have no effect upon the addition of ATPs, which are required for the motor activity of myosins and kinesins [7,31], suggesting that the cytoskeleton might play a major role in the regulation of stromule dynamics (Stage 2).

The recent characterization of the XopL effector function in *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) virulence is of interest. XopL has E3 ligase activity, enhancing *Xcv* virulence by manipulating host autophagic activity [32]. Interestingly, overexpression of XopL in *N. benthamiana* epidermal cells significantly suppressed stromule formation [22]. Moreover, a XopL mutant defective in E3 ligase activity lost its ability to suppress stromule formation [22], indicating an important negative regulatory role for the E3 ligase activity of XopL on the stromule induction. Since the E3 ligase activity of XopL is important to suppress host autophagic activity [32], the significant suppression of stromules by XopL overexpression raises questions about the contribution of autophagy to stromule formation. In fact, multiple pathways for chloroplast degradation by autophagy have been characterized [33]. Furthermore, the regulation of MTs by autophagy through XopL is also feasible, as evidenced by the change in XopL localization from the cytosolic puncta, representing potential autophagosomes, to MTs when its E3 ligase activity is defective [22]. This suggests that the changes in MT dynamics caused by XopL consequently affect stromule formation. Considering its highly dynamic nature and the pleotropic observations of stromules, thorough observation of cytoskeleton dynamics should be accompanied by monitoring stromule dynamics under potential stimuli over time to conclude the regulatory mechanism of stromule initiation and elongation.

Stromules are often observed to orient toward the nucleus during ETI [13,20–22,34]. It has been suggested that an unknown signal guides stromules toward the nucleus. However, it is difficult to identify such a signal, as many cellular and physiological changes occur, including changes in ROS, calcium flux and gradients, phytohormone signaling cascades, cytoskeletal rearrangement, and organelle dynamics during ETI. Advanced live-cell imaging and quantitative imaging analyses capable of dissecting cellular and physiological

changes concurrent with stromule formation throughout the ETI response will provide clues about the regulatory mechanism and potential directionality of stromules in the cell.

Stage 3: Attachment of stromule tips to the nucleus

During the N-mediated ETI responses, stromules even encage the nucleus [13]. The induction of stromule formation and the association of the tip with the nucleus provide a driving force for chloroplast movement toward the nucleus [13,21,34]. The force responsible for chloroplast movements appears to be generated by the extension and retraction of stromules along MT tracks [21]. The tips of stromules appear to form tight attachments to the nucleus, pulling chloroplasts toward the nucleus [21,34]. This movement might contribute to the perinuclear clustering of chloroplasts (PNC) during the later stage of ETI [21]. The PNC has been observed under stressful conditions [34,35]. However, under high-light stress conditions, although the PNC is still present, vigorous stromule formation is not observed. Importantly, XopL suppressed stromule formation and induced the PNC [22]. This suggests that the clustering of chloroplasts may be regulated by an additional mechanism not involved in stromule formation. In addition, genetic studies of the signaling involved in XopQ-Roq1-mediated ETI responses showed that stromule induction is uncoupled from the PNC [20]. However, in the N-mediated ETI responses, stromule dynamics significantly contribute to the PNC [21,34], suggesting that stromule regulation and the PNC are controlled by independent mechanisms but are intertwined in the ETI responses. Recent findings of KIS1 function in stromule formation and the PNC clearly support the notion of the separated but intertwined regulations of stromule formation with the PNC [29]. Overexpression of KIS1 induced stromule formation as well as the PNC. However, a serial deletion of domains in KIS1 shows kinesin motor activity is critical for stromule formation, while both actin binding and motor activity on the MT are critical for the PNC. Given previous studies characterizing the function of the kinesin 14 family in the nuclear movement [36,37], KIS1 might be a key regulator of the PNC, which is required for the nuclear movement. Meier et al. clearly confirmed the involvement of KIS1 kinesin in stromule regulation as well as in TIR-containing receptor (TNL)-mediated ETI [29]. However, the detailed relationship among stromule, PNC, and ETI regulated by other types of receptors is still unclear. A comprehensive observation and quantification of stromules and stromule-mediated PNC using live cell imaging should be conducted in ETI conditions triggered by various effectors to elucidate the relationship among stromule, PNC, and plant immune responses.

The PNC is suggested to play a role in decreasing the distance between chloroplasts and the nucleus, facilitating the rapid transfer of signaling molecules in chloroplast-nucleus communication [34]. Although the fusion of the chloroplast outer membrane to the ER membrane was necessary for membrane lipid biosynthesis [38], electron microscopy revealed that no membrane–membrane exchange took place between the chloroplast (or stromule) and the nuclear envelope during plant immune responses [13]. Remarkably, the wrapping of stromules around the nucleus causes abnormal curvature of the nuclear envelope when the chloroplasts are less than 200 nm away, and many nuclear membrane perforations, implying nuclear pores, are observed at the interface between stromules and the nucleus [Figure 5D in Ref. [13]]. This peculiar formation raises the question of whether a channel is formed between the tip of the stromule and the nuclear pore complex. If a channel exists at the interface, it would be interesting to investigate which genetic factors participate in the formation of channels. However, our current understanding of protein export from the chloroplast is limited. Interestingly, overexpression of HP22, one of the preprotein and amino acid transporter (PRAT) protein families that contains several translocases, enhances Green Fluorescent protein (GFP) leakage from the chloroplast to the cytoplasm during *Arabidopsis* leaf senescence [39]. Unlike other HP20 family proteins, HP22 is not involved in the import of transit peptide-less precursor proteins into chloroplasts. Instead, it has been suggested that this protein might have the potential to transport chloroplastic proteins from stromules into the nucleus via nuclear pores. It is worth examining the possibility that HP22 plays a role in transferring signal molecules between stromules and the nucleus.

Stage 4: Transfer of molecules from the chloroplast to the nucleus

Several chloroplast-localized proteins tagged with fluorescent proteins are detected in the nucleus during stress conditions, including the ETI responses [13,34,40,41]. NRIP1 accumulates at a high level in the nucleus during N-mediated immune responses [13]. NRIP1 translocation into the nucleus is frequently coincident with stromule attachment to the nucleus as well as the PNC in the later timepoint [21], suggesting that stromules contribute to this translocation of NRIP1 proteins from the chloroplast to the nucleus. Despite the observations, why the macromolecules are transferred from the chloroplast to the nucleus remains unclear. One hypothesis is that they serve *bona fide* functions in the chloroplast during normal growth conditions when there is no threat from the environment, such as abiotic stress and pathogen attacks. In emergency situations, organelle communication becomes necessary to maintain homeostasis and to acclimate in response to stress conditions.

Chloroplasts are a major source of pro-death signals such as ROS, nitric oxide, and SA [42]. These compounds

accumulate at considerable levels, triggering HR-PCD along with stromule formation. Stromules are thought to function as a path for rapid communication between the chloroplast and the nucleus. They might enable the putative regulators to be redistributed, and the regulators play important roles in different cellular compartments under diverse stress conditions.

The accumulation of H_2O_2 in stromules has been visualized using a synthetic H_2O_2 sensor, RecA(cTP)-HyPer H_2O_2 [13]. Furthermore, an increase of H_2O_2 levels was detected by the sensor at the contact regions between chloroplasts and the nucleus over time [13], suggesting that stromules may facilitate the transfer of signaling molecules between the chloroplast and the nucleus during the immune response. Additionally, a significant increase of H_2O_2 levels was observed in the chloroplast body [13], suggesting the potential transfer of *de novo* synthesized signaling molecules from the chloroplasts to the nucleus. This implies the possibility of molecular movement from the perinuclear clustered chloroplast bodies to the nucleus. Furthermore, it is plausible that the transfer of ROS from other subcellular compartments besides chloroplasts may also contribute to increased ROS levels in the nucleus. Thus, live-cell time-lapse imaging using sensors to monitor ROS dynamics across all subcellular compartments is necessary to dissect their individual contributions to immune responses. In addition, super-resolution imaging may be employed to provide evidence for stromule function as a pathway for transferring proteins from the chloroplasts to the nucleus.

The role of stromules in the translocation of chloroplast-residing proteins into the nucleus remains obscure. Arogenate dehydratases (ADTs) catalyze the decarboxylation/dehydration of arogenate to produce the aromatic amino acid phenylalanine [43], which serves as a building block for protein biosynthesis and a precursor for many secondary metabolites [44]. ADT5 was observed in chloroplasts, stromules, and the nucleus [40]. However, the function of ADT5 in the nucleus is currently unknown, and its translocation from the chloroplast to the nucleus has not been observed during either plant-microbe interactions or stress conditions. Therefore, it is important to carefully examine whether ADT5 translocates from the chloroplast to the nucleus using transgenic lines expressing *ADT5* under the control of its native promoter to avoid potential artifacts caused by overexpression. More precise experimental evidence is required to determine if ADT5 indeed translocates to the nucleus and to elucidate its biological significance, if any.

While WHIRLY (WHY) is considered a candidate signaling modulator in the crosstalk between organelles [45], its translocation from chloroplasts to the nucleus through stromules has not been demonstrated. The WHY family is conserved throughout the plant kingdom, and its members are predicted to bind to single-stranded DNA to modulate growth and defense responses [46,47]. *Arabidopsis* WHY1 exhibits dual localization in chloroplasts and the nucleus, whereas *At*WHY2 and *At*WHY3 localize to mitochondria and chloroplasts, respectively [48–50]. Translocation of WHY1 from the chloroplast to the nucleus has been demonstrated in transplastomic tobacco plants expressing the *WHY1* open reading frame integrated into the plastid genome [51]. This plastid-produced recombinant WHY1 stimulates the expression of pathogen response genes upon its translocation to the nucleus. Moreover, *WHY1* is implicated as a downstream component of the SA signaling pathway. The mutation of *Arabidopsis WHY1* not only decreases the expression of the SA-responsive gene *pathogenesis-related 1* (*PR1*) but also influences disease susceptibility to *Phytophthora parasitica* [46], indicating that WHY1 contributes to defense responses through the transcriptional reprograming of defense-related genes. Despite investigations into the molecular function and compartmentalization of WHY1, the mechanism by which it is translocated from the chloroplast to the nucleus remains elusive.

Another example of molecular crosstalk involves EXECUTER1 (EX1), a nucleus-encoded chloroplast-localized protein in plants. EX1 plays a crucial role in regulating the singlet-oxygen-triggered retrograde signaling pathway, which helps plants acclimate to changing light conditions. EX1 interacts with Genomes Uncoupled 4 (GUN4) exclusively localized in chloroplasts [41]. In the chloroplast, the interaction between EX1 and GUN4 inhibits the GUN4 function involved in chlorophyll biosynthesis. However, exposure to high light or treatment with Rose Bengal (RB, singlet oxygen generator) results in the accumulation of GFP-tagged EX1 in the nuclei, while GUN4 remains in the chloroplasts. This translocation is attributed to the weakening of the interaction between GUN4 and EX1 under high-light exposure and RB treatment, allowing EX1 to move from the chloroplasts to the nucleus [41]. Subsequently, EX1 binds to WRKY18 and 40 to promote the expression of genes responsive to singlet oxygen during high-light stress [41]. This case illustrates the dual function of chloroplast-localizing proteins in the nucleus during stress responses. However, the reasons for the initial targeting of these proteins to the chloroplasts and their subsequent translocation to the nucleus remain unknown. Notably, EX1 and EX2 also contribute to defense responses in plants, as evidenced by the failure of the *Arabidopsis ex1ex2* double knockout mutant to induce programmed cell death (PCD) under high-light/low temperature stress and its reduced defense against the bacterial pathogen *Pst* DC3000 [52]. Comprehensive observation of stromules under high-light stress and singlet oxygen accumulation is needed to determine whether EX1 is translocated to the nucleus via stromules, despite the observed accumulation of EX1 in the nuclei in response to high-light exposure and singlet oxygen.

Current knowledge is limited to explain the phenomena of stromule-mediated inter-organelle communications, highlighting the complexity of organelle communication. While WHY proteins are involved in regulating gene transcription in both the chloroplast and the nucleus [45], the translocation of WHY proteins from the chloroplast to the nucleus in response to environmental stress has not yet been observed. Conversely, NRIP1 is known to translocate from the chloroplast to the nucleus, playing a role in plant immunity. However, the specific biochemical function of NRIP1 in the chloroplast remains unknown. These observations emphasize the importance of elucidating the molecular mechanisms underlying chloroplast-nucleus communication during host-microbe interactions. Further research endeavors are imperative to deepen our understanding of cellular responses during plant immunity and to potentially identify targets for enhancing plant defense responses.

Morphological changes in other organelles

The dynamic morphological changes in peroxisomes and mitochondria have been observed under various stress conditions (Figure 2). Peroxisomes have a spherical shape with a diameter of ~1 μm under normal conditions but change their morphology and abundance to sustain cellular functions under stress conditions [53,54]. Peroxisome proliferation is a key response mechanism to control ROS and reactive nitrogen species (RNS) levels. This proliferation is often accompanied by morphological changes such as elongation, constriction, and fission [55,56]. During their transition from a spherical to a tubular form, peroxisomes develop thin

Figure 2

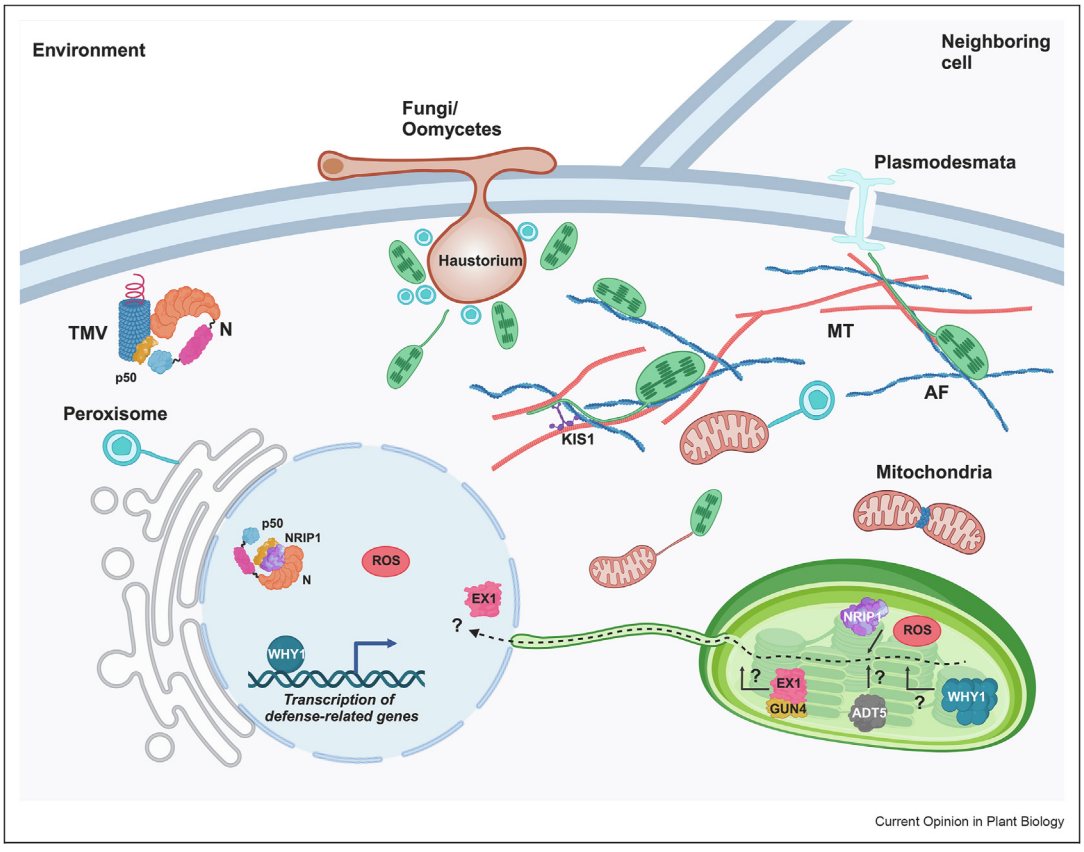


Diagram of inter-organelle communications in association with morphological changes in plant cells.
Organelles dynamically change their morphologies in response to pathogen infection. Chloroplasts, mitochondria, and peroxisomes send out thin, tubular structures known as stromules, matrixules, and peroxules, respectively. Matrixules from highly plastic mitochondria undergoing fusion/fission of mitochondria are often observed in response to stresses. The association of peroxules with the endoplasmic reticulum and mitochondria is often observed under stress conditions. Upon *P. infestans* infection, chloroplasts and peroxisomes gather near the haustorium. The extension and retraction of stromules occur on microtubules, while actin filaments function as anchors. Stromules are often associated with various subcellular compartments, including plasmodesmata. The kinesin KIS1 contributes to stromule regulation and perinuclear clustering of chloroplasts.
NRIP1 translocates from the chloroplast and recognizes p50 from TMV. The assembly of p50-NRIP1 with N in the nucleus triggers the N-mediated defense response. During host-microbe interactions, both NRIP1 and ROS translocate from the chloroplast to the nucleus through stromules. Additionally, chloroplast-localized proteins such as WHY1 and EX1 are excellent candidates for signaling molecules capable of transcriptional reprogramming following their translocation from the chloroplast to the nucleus. However, there is currently no experimental evidence supporting their translocation through stromules. TMV, tobacco mosaic virus; ROS, reactive oxygen species.

and dynamic protrusions called peroxules [56–58]. The formation of peroxules is influenced by the levels of hydroxyl ROS. Low levels of hydroxyl ROS induce peroxule formation, whereas higher levels lead to peroxisome tubulation [58]. Peroxisomes communicate with other organelles via peroxules, which often connect to the mitochondrial outer membrane under high-light conditions [54]. Despite these observations, it remains unclear whether peroxisomes undergo dynamic changes in their morphology and establish tight connections with other organelles similar to those observed with chloroplasts during plant immunity.

Morphological changes associated with mitochondrial functions have been comprehensively demonstrated during vertebrate immunity [59]. Recent studies have also revealed that plant mitochondria respond to stress signals, highlighting their coordination with other organelles [60] by extending tubular structures called matrixules [61,62]. Mitochondria exhibit a high degree of plasticity through fission and fusion cycles, allowing them to repair damaged mitochondria and generate new ones [63]. The mutation of *dynamin-related protein 3A* (*DRP3A*) increases matrixule formation in *Arabidopsis* [64], demonstrating that dynamin-like proteins are essential for matrixule formation [65–67]. Thus, it is possible that mitochondrial biogenesis is connected to the regulation of mitochondrial morphology. While levels of mitochondrial ROS and RNS influence mitochondrial morphology and function [63], the precise mechanisms linking matrixule formation to mitochondrial biogenesis and morphological changes remain unclear. Further research is needed to elucidate how matrixules contribute to mitochondrial dynamics and their role in response to pathogen invasion.

Arabidopsis FISSION1A (FIS1A) was shown to localize to three distinct compartments (mitochondria, chloroplasts, and peroxisomes) as well as their corresponding tubular protrusions such as matrixules, stromules, and peroxules, respectively, in overexpressed cells [68]. Intriguingly, this study also showed that matrixules interacted with chloroplasts and other mitochondria. Furthermore, FIS1A was anchored to the outer membranes of chloroplasts and mitochondria and to the membranes of peroxisomes [Figure1A in Ref. [69]]. However, other studies have not observed the localization of FIS1A at the chloroplast membrane [69,70], suggesting that the overexpression of *FIS1A* might have caused mislocalization of the protein. Other experiments, such as immunohistochemistry using a specific anti-FIS1A antibody, might be needed to clarify the true location of FIS1A. Regardless of its true location within the cell, FIS1A might be an interesting tool for exploring the mechanism of organelle–organelle interactions in immunity.

Conclusion and future perspectives

We are just beginning to understand how individual organelles interact and communicate to be engaged in mutual assistance by exchanging signaling molecules and/or metabolites. Evidence of highly dynamic morphological changes and interactions among organelles under stress conditions has been accumulated in recent years. However, the detailed molecular mechanisms for these organelle–organelle interactions and their functions in stress responses remain largely unknown.

Plant immune responses cause dynamic morphological changes in a variety of organelles. These changes can be transmitted rapidly from cell to cell upon host microbe interactions. The dynamic morphological changes are accompanied by the spatio-temporal translocation of putative signaling molecules. Although we have gained a considerable understanding of molecular interactions between pathogens and host plants in recent decades [23], how organelle–organelle communication contributes to the plant immune response through the exchange of putative signaling molecules has been neglected. Stromule-mediated transfer of signaling molecules from chloroplasts to the nucleus might offer a good starting point for an exploration of the cellular responses facilitating plant immunity.

Stromules may be a potential conduit for the transfer of ROS, SA, and other signaling macromolecules originating from the chloroplast to the nucleus, where they activate defense signaling or maintain cellular homeostasis for proper responses to external stimuli [13]. In addition, stromules might provide a driving force to rapidly move chloroplasts toward the nucleus during the immune response [21], which would be important for a particularly rapid response such as HR-PCD. However, several key questions must be answered to understand the role of the stromule-mediated chloroplast-nucleus communication in plant cells. Careful experimental design to examine the dynamics of organelles using a combination of microscopy, genetic, and biochemical approaches will allow us to understand spatial and temporal responses in cells and to clarify the role of stromules in plant immunity [Box 2].

In addition, how putative signaling molecules are transferred through stromules from the chloroplast into the nucleus still remains elusive. This simple question can be further dissected into several subquestions: Is this translocation an outcome of an active and selective process? Or does it occur through a simple, passive, and bulky transfer? Is a channel formed at the contact site between stromules and the nucleus? Is there a change in membrane potential to facilitate the movement of signaling molecules away from the chloroplast at the

Box 2. Remaining outstanding questions and potential experiments for understanding each stage of the stromule-mediated chloroplast-nucleus communication during plant immunity.

	Main question	Remaining questions	Potential experiments
Stage 1	**What is the direct signal triggering stromule initiation?**	Can we specify (a) the genetic pathway(s) regulating stromule biogenesis? In subcellular compartments, do ROS mainly regulate stromule formation?	Comprehensive characterization of mutants with abnormal stromule phenotypes and further identification of stromule-specific regulatory pathway Comprehensive live-cell imaging of subcellular ROS dynamics and stromule dynamics using synthetic sensors
Stage 2	**How do stromules find their way to the nucleus?**	What controls the cytoskeleton to guide stromules toward the nucleus?	Live-cell imaging of calcium gradients and corresponding cytoskeleton dynamics near the nucleus during stress responses
Stage 3	**How do stromules make a connection with the nucleus?**	Do protein–protein interactions occur at stromule-nucleus contact sites? Are there other mechanisms regulating the perinuclear clustering of chloroplasts besides stromule dynamics?	Genetic and proteomic analyses to identify interacting candidates originating from two subcellular compartments, such as the chloroplast and the nuclear outer membrane, by proximity labeling Single-cell imaging after bacterial infection using XopQ and other available mutants to uncouple PTI-ETI crosstalk
Stage 4	**Which molecules are transferred from the chloroplast to the nucleus?**	Do moonlighting proteins function in the nucleus during plant immune responses?	Proteomic analysis to identify and characterize proteins that translocate from the chloroplast to the nucleus during plant immune responses

ROS, reactive oxygen species; PTI, PAMP-triggered immunity; ETI, effector triggered immunity.

contact region? The identification and characterization of genes that regulate stromule biogenesis and signaling pathways will provide answers to these questions.

Lastly, emerging evidence supports the involvement of multiple signaling pathways in the control of various aspects of stromule biogenesis, such as induction, elongation, and growth direction, as well as stromule attachment to nuclei or other organelles. Notably, quantitative imaging analysis during plant immune responses proposes that stromules provide a driving force for the clustering of chloroplasts around the nucleus [21]. However, other studies reveal an absence of a robust correlation between stromule formation and the PNC under distinct stress conditions [22,29]. Moreover, a study involving a series of deletion mutants of the stromule-specific kinesin, KIS1, supports the notion that stromules play a minor role in the context of the PNC [22,29], suggesting that their primary function during immune responses might be to transfer pro-defense molecules for the induction of rapid immune responses. Further comprehensive observations of stromule dynamics under various experimental conditions are needed. Additionally, multiomics approaches should be employed to identify stromule regulatory genes and signaling pathways. These efforts will enhance our understanding of this dynamic structure and its function in plant immunity. A current knowledge-based model, recently developed through a comprehensive observation of stromules, might provide a valuable tool for screening stromule-defective mutants [70]. Furthermore, conducting proteomic analysis to identify putative candidate proteins that undergo translocation from chloroplasts to the nucleus during plant immune responses will offer crucial insights into decoding the complex molecular mechanisms underlying chloroplast function in plant immunity.

Author Contributions

Seungmee Jung: Conceptualization, Reference collection, Original draft preparation, Manuscript reviewing and editing, Visualization. **Jongchan Woo:** Conceptualization, Reference collection, Visualization, Original

draft preparation, Manuscript reviewing and editing. **Eunsook Park:** Conceptualization, Reference collection, Manuscript reviewing and editing, Manuscript revision, Visualization, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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- •• of outstanding interest

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