EXAMINING THE ROLE OF MICROTUBULES AND KINESINS DURING
STROMULE DYNAMICS

by

Ali Owaydhah Alqarni

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fulfillment of the requirements for the Master of Science in Plant and Soil Sciences

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ABSTRACT

The innate immunity of plants requires the transport of defense factors that are synthesized from the chloroplasts to the nucleus. Insight into the role of dynamic tubular extensions from chloroplasts called stromules, in this process, is quintessential to our understanding of plant innate immunity. These defense factors enhance the hypersensitive response-programmed cell death (HR-PCD) that can help plants to survive. The mechanism of the stromule movement is still not well understood. This movement can potentially involve either of the two plant cytoskeletal elements: actin microfilaments (AF) or microtubules (MT). A few research groups have previously claimed that stromules use myosin motors and move along actin microfilaments. However, our lab findings suggest that stromules dynamically extend and move along microtubules and are stabilized by actin microfilaments. Using cytoskeleton distributions for both AFs and MTs, and by quantifying the number of stromules and their length, we demonstrated that stromules extend and move along microtubules (MTs). Furthermore, to understand how stromules extend and move along microtubules to the nucleus, a MT kinesin motor (KSE2) was investigated to see how that will affect stromule driven movement. The results support that MTs are necessary for stromule formation and KSE2 can play a role in stromules extention and movement.
Chapter 1

LITERATURE REVIEW: FORMAT AND FUNCTION OF CHLOROPLASTS

1.1 Plant Innate Immunity

A plant pathogen is an organism that can cause a disease; for example, Tobacco Mosaic Virus (TMV) produces mosaic viruses in Nicotiana plants, bacteria such as Pseudomonas syringae, and fungi. These pathogens affect the health of a plant, which in turn affects humans and animals that eat those plants. Humans and animals have their own defense factors against pathogens, but how do plants defend themselves from pathogens? The innate immune system of plants constitutes the first line of host defense against infection, and it consists of two branches in response to invading pathogens (Figure 1.1) (Jones and Dangl 2006). The first line of defense is pathogen-associated molecular patterns (PAMPs), PAMPs are molecules associated with groups of pathogens and are recognized by pattern recognition receptors (PRRs) (Janeway 1989). PAMPs are nonspecific as to the pathogens they attack and use transmembrane PRRs that respond slowly to evolving pathogens, such as flagellin (Zipfel and Felix 2005). The second innate immune system of plants, effector-triggered immunity (ETI), are more specific and recognize a specific immune receptor on different pathogens (Jones and Dangl 2006). ETI uses binding (NB) and leucine-rich repeat (LRR)
domains as the polymorphic NB-LRR protein products encoded by specific plant-encoded immune receptors called resistance (R) genes (Jones and Dangl 2006). Plant NB-LRR proteins have a Toll-inter-leukin-1 receptor (TIR) homology domain that contributes to cell death signaling role during resistance responses (Swiderski, Birker, and Jones 2009). N, an immune receptor is localized to the cytoplasm and the nucleus that contains TIR-NB-LRR. Pathogen effectors from different taxonomic kingdoms are recognized by N immune receptor and activate defense responses (Burch-Smith et al. 2007). For example, *Tobacco mosaic virus* (TMV) is a positive-sense single-stranded RNA virus that infects a wide range of plants, especially tobacco (Burch-Smith et al. 2007). *Tobacco mosaic virus* expresses p50 domain in plant cells that recognize N immune receptor and defense factors such as NRIP1 to activate Hypersensitive response (HR) (Figure 1.2) (Caplan et al. 2008). Hypersensitive response is a type of programmed cell death (HR-PCD), a mechanism used by plants to prevent the spread of pathogens to other parts of the plant (Morel and Dangl 1997). A plant will sacrifice the area that is affected by the pathogen in order to save the rest of the plant (Morel and Dangl 1997). HR-PCD occurs by oxidative burst, which frees up highly reactive oxygen inside the cell and kills it (Morel and Dangl 1997). All of the cells in and around the affected area are destroyed, and those cells immediately around the infected area change their appearance. This prevents them from becoming infected in the future (Morel and Dangl 1997).
Pathogen-associated molecular patterns, or PAMPs, are molecules associated with groups of pathogens and are recognized by pattern recognition receptors (PRRs). This one is non-specific immunity. The second type, Effector-triggered immunity, is more specific in that it recognizes different pathogens by a specific immune receptor.

**Figure 1.1 Plant Innate Immunity**

Pathogen-associated molecular patterns, or PAMPs, are molecules associated with groups of pathogens and are recognized by pattern recognition receptors (PRRs). This one is non-specific immunity. The second type, Effector-triggered immunity, is more specific in that it recognizes different pathogens by a specific immune receptor.
When *Tobacco mosaic virus* (TMV) infects the plant, TMV expresses p50 domain in the plant cells that recognize by N Immune receptor with an interaction of defense factor such as NRIP1 that is generated by chloroplasts that will lead to active HR-PCD (Caplan et al. 2008).

**Figure 1.2 Progression of an Innate Immune Response to Pathogens**
1.2 Chloroplasts Play an Important Role During Plant Innate Immunity

Chloroplasts are a primary production site of important defense factors such as protein N receptor interacting protein 1 (NRIP1) and pro defense molecule hydrogen peroxide (H$_2$O$_2$), which play a significant role during plant innate immunity (Figure 1.3). NRIP1 and H$_2$O$_2$ are required for hypersensitive response programmed cell-death (HR-PCD) to occur (Caplan et al. 2015). Our lab is considering how chloroplasts function during this process. We found that when any pathogen affects plants, there is a tubular structure that extends from a chloroplast called a stromule (Figure 1.3).

Stromules was discovered for the first time in 1962 by Sam Wildman’s group using an electronic microscope (SEM) (Wildman, Hongladarom, and Honda 1962). In 1997, researchers marked a stromule by GFP, which allowed stromules to be seen alive by using confocal microscopy (Morel and Dangl 1997). In turn, researchers were more able to see how stromules function and how they are induced during HR-PCD (Morel and Dangl 1997; Caplan et al. 2015). Stromules have been observed in several genera in the plant kingdom, and are most common in non-green plastids (Gray et al. 2001; Hanson and Sattarzadeh 2008; Köhler and Hanson 2000; Natesan, Sullivan, and Gray 2005). Stromules are developmentally regulated and induced in response to biotic and abiotic stress, symbiotic association, and changes in plastid number and size (Brunkard, Runkel, and Zambryski 2015; Caplan et al. 2008, 2015; Schattat et al. 2011; Waters, Fray, and Pyke 2004). The role of stromule extension is to increase the surface area of chloroplasts to transport defense signals to the nucleus (Caplan et al. 2015; E. Y. Kwok and Hanson 2004; Ernest Y Kwok and Hanson 2004). When
stromules are induced during an immune response, they make connections with nuclei to transport defense protein NRIP1 (N receptor-interacting protein 1) and the pro-defense molecule, hydrogen peroxide (H$_2$O$_2$), from chloroplasts into nuclei (Figure 1.4) (Caplan et al., 2015). However, the mechanism(s) by which stromules extend from a chloroplast and move to nuclei is poorly understood.
A chloroplast is a double membraned structure containing outer and the inner membranes. The inner membrane surrounds the stroma and also includes the thylakoid membrane and thylakoid stacks (granum). Also, there stromules extend from the chloroplast and are filled with stroma.

**Figure 1.3 Chloroplast and Stromules**
Figure 1.4 Stromules Extend From Chloroplasts to Induce HR-PCD

The figure shows how stromules extend from chloroplasts in order to transport the defense factors to the nucleus resulting in enhancement of hypersensitive response-programmed cell-death (Caplan et al. 2015).
1.3 Potential Cytoskeletal Elements Used for Stromules Formation

Several research groups investigated cytoskeletal elements to understand how stromules form and move. One possible microfilament responsible for the movement of stromules is actin filaments (AFs). AFs are the thinnest filament structures in the cytoplasm of eukaryotic cells and provide strength, architecture, and location within cells (P. W. Gunning et al. 2015) (Figure 1.5). Microtubules (MTs) are another filament that could be responsible for stromule formation and movement. MTs are dynamic polymers assembled of the cytoskeleton made of α/β/γ tubulin dimers, which play a crucial role in intracellular space and cell division (Conduit 2016) (Figure 1.5). Additionally, γ-tubulin is the most important protein within the MTs structure because it is abundant and there are several subunits of γ-tubulin complex such as GCP2, GCP3, GCP4, GCP5, GCP6 (Conduit 2016) (Figure 1.6). Some research supports each cytoskeletal element has a unique motor protein; microfilaments (AFs) have myosin and microtubules have kinesin (Figure 1.5). On the contrary, some researchers support that stromules move along AFs using myosin motors, while others claim that stromules dynamically extend along actin microfilaments (B. E. S. Gunning 2005; Ernest Y. Kwok and Hanson 2003; Ernest Y Kwok and Hanson 2004).

To support their findings, Gunning and his colleagues used Cytochalasin D (CytD) and Latrunculin B (LatB) as inhibitors on AF in non-green tissue to observe the effect on stromule formation, movement, and length (B. E. S. Gunning 2005; Ernest Y. Kwok and Hanson 2003; Ernest Y Kwok and Hanson 2004). Using the AF inhibitors
treatment, their findings show a reduction of stromule frequency in tobacco epidermal cells (Ernest Y. Kwok and Hanson 2003). In another related study, it was found that stromules extend along AFs and the tips of stromules contact AFs in Arabidopsis epidermal cells (Ernest Y Kwok and Hanson 2004).

Other research has shown that myosins are required for stromule formation (Natesan, Sullivan, and Gray 2009; Amirali Sattarzadeh, Schmelzer, and Hanson 2013). In their experiment, myosin was treated with ATPase inhibitor 2,3 butanedione 2-monoxime (BDM) to observe the effects on stromule movement and length (Natesan, Sullivan, and Gray 2009; Amir Sattarzadeh et al. 2009). As a result, they found that Myosin XI family motor proteins played a role and are involved in stromule movement in Nicotiana tabacum.

However, Dr. Dinesh-Kumar and Dr. Caplan discovered that MTs, not AFs, are required for stromule extension and movement (Kumar et. al., eLife, under revision). Kumar et. al. examined the function of stromules in chloroplasts of green leaf tissue during innate immunity (Kumar et. al., eLife, under revision). The results suggest that MTs are required for stromules to extend and move along them (Kumar et. al., eLife, under revision).
The two cytoskeleton elements that plants have are microfilaments (AFs) that made up of actin and microtubules that are made of tubulin (MTs) (Lodish H, Berk A, Zipursky SL 2008). In addition, each cytoskeleton element has a motor protein. Microfilament (AFs) has myosin and microtubule (MTs) has kinesin (Carter 2013).

Figure 1.5 Cytoskeleton Elements of Plants and Their Motor Proteins

The two cytoskeleton elements that plants have are microfilaments (AFs) that made up of actin and microtubules that are made of tubulin (MTs) (Lodish H, Berk A, Zipursky SL 2008). In addition, each cytoskeleton element has a motor protein. Microfilament (AFs) has myosin and microtubule (MTs) has kinesin (Carter 2013).
Figure 1.6 Initiation and Extension of Microtubules

MTs are made of $\alpha/\beta/\gamma$ tubulin dimers, and $\gamma$-tubulin complex (blue) made of several subunits such as $GCP2$, $GCP3$, $GCP4$, $GCP5$, and $GCP6$. All complexes participate in the formation of the MTs structure (Teixidó-Travesa, Roig, and Lüders 2012).
1.4 Rationale and Goals

Our understanding is that MT depolymerization led to stromule retraction and MT stabilization increased stromule frequency immunity (Kumar et. al., eLife, under revision). By silencing the gene for γ-tubulin complex protein 4 (GCP4), it was found that the MTs are bundling and disrupting stromules dynamic. Additionally, the length of stromules increased but the movement of stromules extension and retraction decrease. These data suggest that stromules extend and move along microtubules and microtubules motors, such as kinesins, may contribute to extension and movement of stromules along plant microtubule arrays. Hence, the hypothesis of my research is that stromules extend along microtubules using kinesins. My research had two specific aims:

1. Examine the role of microtubules during stromule dynamics by
   A. Knockdown of GCP4 protein of microtubules on stromule dynamics.
   B. Determine changes in stromule dynamics along microtubules during plant defense.

2. Examine the function of kinesins during stromule dynamics.
   A. Examine the effect of overexpression kinesin 14 on stromule movement.
   B. Quantify the effect of overexpressing KSE2 on stromules.
Chapter 2

MATERIALS AND METHODS

2.1 Plasmids

Plasmids used in this research include; Lifeact-TagRFP (SPDK2209), TagRFP-MAP-CKL6 (SPDK2386), NRIP1(cTP)-TagBFP (SPDK3168), TRV2-EV (SPDKYY13), TRV-\textit{NbGCP4} (SPDK3111), Citrine alone (TBS67), p50-Citrine (TBS66), Citrine-p50 (SPDK1939), tRFP-KAC1 (SPDK2984), tRFP-KAC2 (SPDK2985), Citrine-KAC1 (SPDK2986), Citrine-KAC2 (SPDK2987), tRFP-At1g09170 (SPDK3027), Citrine-At1g09170 (SPDK3028), tRFP-At1g63640 or KES1 (SPDK3029), Citrine-At1g63640 or KES1 (SPDK3030), tRFP-At3g10310 (SPDK3031), Citrine-At3g10310 (SPDK3032), tRFP-At3g44730 (SPDK3033), Citrine-At3g44730 (SPDK3034), tRFP-At5g27000 (SPDK3035), Citrine-At5g27000 (SPDK3036), tRFP-At5g41310 or KES2 (SPDK3037), Citrine-At5g41310 or KES2 (SPDK3038), and tRFP-sfGFP11 (SPDK2422) (Table 2.1)
Table 2.1 The Plasmids

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SSRC (Spectinomycin, Streptomycin, Rifampicin, Carbenicillin)

K SRC (Kanamycin, Spectinomycin, Rifampicin, Carbenicillin)

SPDK (Savithramma P. Dinesh-Kumar)

TBS (Tessa Burch-Smith)
2.2 Plant Material and Growth Condition

Seeds for transgenic *N. benthamiana* plants expressing the NRIP1- Cerulean (NN 59.8.3) zz. The plants were grown under lights at 20°C in growth carts for 4-6 weeks (Figure 2.1).

![Figure 2.1 Leaf-Stage *Nicotiana Benthamiana* Plant](image)
2.3 Agrobacterium Preparation

Cultures of Agrobacterium containing the recombinant plasmids were maintained in plates containing the appropriate antibiotics such as; Spectinomycin 100 µg/ml, Streptomycin 100 µg/ml, Rifampicin 25 µg/ml, Carbenicillin 50 µg/ml, and Kanamycin 50 µg/ml.

2.3.1 Stock Solutions of Antibiotics

- 50-100 mg/ml Spectinomycin Stock Solution:
  1. Weigh 0.5-1 g of Spectinomycin.
  2. Add 10 ml of sterile H2O. Dissolve completely.
  3. Prewet a 0.22 µm syringe filter by drawing through 5-10 ml of sterile H2O and discard water.
  4. Sterilize spectinomycin stock through the prepared 0.22 µm syringe filter.
  5. The stock may be kept at -20°C for one year.

- 50 mg/ml Streptomycin Stock Solution:
  1. 0.5 g of Streptomycin.
  2. 10 ml of sterile H2O.
3. 0.22 µm syringe filter by drawing through 5-10 ml of sterile H2O and discard water.

4. Sterilize streptomycin stock through the prepared 0.22 µm syringe filter.

- 50 mg/ml Rifampicin Stock Solution:
  1. 0.5 g of Rifampicin.
  2. 10 ml of 100% methanol.

- 50-100 mg/ml Carbenicillin Stock Solution:
  1. 0.5-1 g of Carbenicillin.
  2. 10 ml of sterile H2O.
  3. 0.22 µm syringe filter by drawing through 5-10 ml of sterile H2O and discard water.
  4. Sterilize Carbenicillin Stock through the prepared 0.22 µm syringe filter.

- 50 mg/ml Kanamycin Stock Solution:
  1. 0.5 g of Kanamycin
  2. 10 ml of sterile H2O.
3. 0.22 µm syringe filter by drawing through 5-10 ml of sterile H2O and discard water.

5. Sterilize Kanamycin Stock through the prepared 0.22 µm syringe filter.

### 2.3.2 Protocol to Make Plates of Antibiotics

- This recipe is for 500 mL of LB agar. This makes about 20 plates (1 bag).

1. Adding solids (12.5 g LB Broth 7.5 g agar) and mix them well with 500 mL of deionized water in a large bottle or flask. 2. Autoclave 20 minutes on LIQUID cycle.

2. Allow the flask to cool on a bench.

3. Adding antibiotics. Mix well by swirling.

### 2.4 Leaf Preparation and Inhibitor Treatment

The leaves used for this research were 20 to 30 days old *N. benthamiana* or transgenic *N. benthamiana* NRIP1-Cerulean plants. For agro infiltration, fully expanded leaves were used, however the leaf excisions were cut to approximately 4mm² and used for imaging. The concentrations used in the microtubule depolymerization did not have lethal effects on the leaves because the leaves were used for further experiments.
Infiltration medium, containing:

- 10 ml of 1 M MgCl₂ (10 mM)
- 10 ml of 1 M MES (10 mM)
- 0.75 ml of 200 mM acetosyringone (150 µM).

2.5 Laser Scanning Confocal Microscopy

*N. benthamiana* leaf sections (4 mm²) were used for imaging at indicated time points. The *N. benthamiana* leaf section was infiltrated with water and imaged on a Zeiss LSM 710 confocal microscope or LSM 880 confocal microscope fitted with 40X C-Apochromat water immersion objective (NA=1.2) used for live cell imaging (Carl Zeiss). The 405 nm, 458 nm, 488 nm, 514 nm, or 561 nm laser line was used for TagBFP, Cerulean, Citrine, or TagRFP, respectively. TagBFP and Cerulean were pseudo colored cyan, Lifeact-TagRFP and TagRFP-Kinesins were pseudo colored magenta, and GFP-TUA6, Citrine-Kinesins, and TagRFP-MAP-CKL6 were pseudo colored yellow throughout the manuscript for consistency of data presentation.

2.6 Image Processing

A program called Huygens Professional (Scientific Volume Imaging) was used to deconvolve most of my images using a Classical Maximum Likelihood Estimation (CLME) restoration method. For fixing the drift, the object stabilizer algorithm was used to correct photo bleaching across time lapsed images and to equalize brightness.
and contrast. Noise was removed from images not suited for deconvolution using a 3 x 3 median filter. Volocity (Perkin Elmer) was used to generate images, kymographs and videos.

2.7 Quantification of Stromules

Stromules were manually counted by using ImageJ (Schindelin et al. 2012) from the maximum intensity projections of the confocal images. The average of stromule ratios was determined by counting the total number of stromules and then dividing by the total number of chloroplasts. Images were captured by confocal microscopy as Z series at the indicated time points. Experiments were repeated four or eight times and analyzed by GraphPad Prism 7.

2.8 Manual Stromule Tracking

The velocity of stromule dynamics was calculated by using the FIJI version of ImageJ (Schindelin et al. 2012) via frame-by-frame analysis (Sixty frames of time-lapse z-stack images every 10 sec). The movement of stromules between frames of a temporal stack was quantified using 2D and 3D images. Images were generated in Zen software (Carl Zeiss) as maximum intensity projections of time-lapse z-stacks. The different types of stromule movement (constant smooth, sudden erratic, and side) and extension and retraction velocity were manually counted. Also, the maximum and minimum stromule lengths were manually measured using the ImageJ.
2.9 Statistical Analysis

All statistical analysis graph generations were performed using Microsoft Excel and GraphPad Prism 7.

2.10 Abbreviations List

MT, microtubules; ER, endoplasmic reticulum; AF, actin filament; CytD, Cytochalasin D; BDM, 2,3 butanedione 2-monoxime; APM, amiprophosmethyl; CLSM, Confocal laser-scanning microscopy; TMV, Tobacco Mosaic Virus.
Chapter 3

RESULTS

3.1 Introduction

Previous research has shown that AFs can regulate chloroplast movement and stromules to extend (Ernest Y. Kwok and Hanson 2003; Ernest Y Kwok and Hanson 2004). Our lab tested if stromules extend and move along AFs by using Lifeact-TagRFP, an AF marker, which could show whether stromules extend and move along AFs (Riedl et al. 2008; Era et al. 2009). Transgenic N. benthamiana plants expressing NRIP1-Cerulean were used to mark stromules (Caplan et al. 2008, 2015). It was found that stromules associate with AFs, but did not move and extend along AFs (Kumar et. al., eLife, under revision).

To see if stromules interact and extend along microtubules, the role of stromules with MTs was examined using a marker that expressed TagRFP to the C-terminal microtubule associated protein domain of CKL6 (Ben-Nissan et al. 2008) (TagRFP-MAP-CKL6) in transgenic N. benthamiana plants, expresses NRIP1-Cerulean and marks stromules (Caplan et al. 2008, 2015). This showed stromules extend from a chloroplast as beak-like structures and move along MTs (Kumar et. al., eLife, under revision).
To investigate further how stromules move along MTs, they tested transgenic *N. benthamiana* plants that include MT marker lines expressing green fluorescent protein fused with the tubulin alpha 6 (GFP-TUA6) (Gillespie 2002). TagRFP [NRIP1(cTP)-TagRFP] was added to mark stromules with MT line (Kumar et. al., eLife, under revision). As a result, time-lapse imaging of GFP-TUA6 and [NRIP1(cTP)-TagRFP] showed that stromules dynamically extended along MTs (Kumar et. al., eLife, under revision).

Secondly, EB1-Citrine was used to mark MTs to examine the direction of movement, where EB1 fused to Citrine at the positive end of MTs (Chan et al. 2003). This showed that the tip of stromules move and extend along MTs (Kumar et. al., eLife, under revision).

An experiment was designed to show MTs are required for stromules to form and extend by using MTs disruptions such as APM or Oryzalin (Kumar et. al., eLife, under revision). TagRFP-MAP-CKL6 was used as an MT marker in NRIP1-Cerulean transgenic *N. benthamiana* plants (Kumar et. al., eLife, under revision). Depolymerization of MTs occurred and the number of stromules decreased at fifteen minutes with APM and Oryzalin treatments (Kumar et. al., eLife, under revision). In other words, the number of stromules with the mock control (DMSO treatment) increased compared to the APM or Oryzalin disruption treatments of MTs after fifteen minutes (Kumar et. al., eLife, under revision).

So far, the results showed that stromules extend and move along MTs. Because of this, the effect of MT stabilization was tested using Taxol (Schiff and Horwitz 1980).
Paclitaxel-BODIPY was used to infiltrate leaves of NRIP1-Cerulean *N. benthamiana* plants (Kumar et. al., eLife, under revision). Extensive MT stabilization and increased stromules with Paclitaxel treatment compared to the mock control was observed thirty minutes after treatment (Kumar et. al., eLife, under revision). Furthermore, stromule numbers increased significantly per chloroplast with Paclitaxel treatment (Kumar et. al., eLife, under revision). For this reason, it is suggested that MT stabilization can play a significant role in increased stromules induction (Kumar et. al., eLife, under revision). What is known so far is that MTs are required for stromules to form and extend and increasing the stability of MTs leads to increase the number of stromules per chloroplasts. These results suggest that stromules extend and move along microtubules and microtubules motors, such as kinesins. Additionally, kinesins may play a role in stromules to extend and move along plant microtubule arrays.
3.2 Aim 1: Examine The Role of Microtubules During Stromule Dynamics:

3.2.1 Sub Aim A: Examine the Effect of Knockdown of GCP4 Protein of MT on Stromule Dynamics:

3.2.1.1 The γ-tubulin Complex Protein GCP4 Is Required for Organizing Functional Microtubule

To visualize the effect of MTs on stromule dynamics, a virus induced gene silencing (VIGS) approach was used on *N. benthamiana* (Dinesh-Kumar et al. 2003). GCP4 is a subunit of the γ-tubulin complex and artificial microRNA (amiR)-mediated knockdown of *Arabidopsis* GCP4 resulted in bundled MTs in leaf epidermal cells (Kong et al. 2010). With amiR-GCP4 leafs, MTs showed an increased bundled parallel configuration compared to control leaves (Figure 3.1) (Kong et al. 2010).

3.2.1.2 *NbGCP4*-Silenced Plants Increased Bundling of Long MTs

To determine the effect of MTs dynamics on stromule formation, *NbGCP4* was silenced in NRIP1-Cerulean transgenic *N. benthamiana* plants that mark stromules and in GFP-TUA6 transgenic *N. benthamiana* leaves that mark MTs. After four days-post silencing using VIGS-NbGCP4, it was decided to test the plants, giving VIGS time to occur and minimize any potential physiological changes that could harm further experiments. It was found that MT organization was significantly changed and affected in the leaves of *o*-silenced plants compared to the control (Figure 3.2).
Figure 3.1 Microtubules Organization in Epidermal Cells of the Wild-Type Control and amiR-GCP4 Leaves Expressing GFP-TUB6 in Arabidopsis Plants

(A) In a control leaf, MTs with non-uniform directions with each cell show great cortical MTs (Kong et al. 2010). (B) In an amiR-GCP4 leaf, MTs showed an increased bundled parallel configuration (Kong et al. 2010). Scale bars equal 10 µm (Kong et al. 2010).
Figure 3.2 *NbGCP4*-Silenced Plants Increased Bundling of Long MTs

*NbGCP4* silencing resulted in increased bundling of MTs (VIGS-*NbGCP4*, right) compared to the VIGS vector control (VIGS-EV, left) in GFP-TUA6 transgenic *N. benthamiana* leaves that mark MTs (yellow). The chosen images are maximum intensity projections of confocal z-stacks. Scale bar = 40 µm.
3.2.1.3 *NbGCP4* Silencing Induces Microtubule Stabilization

Counting stromules is one of the most significant factors to assess when attempting to visualize the effect of MTs on stromules dynamics. Because of this, we counted stromules four days-post silencing of *NbGCP4*. The number of stromules more than doubled in *NbGCP4*-silenced plants compared to the VIGS vector control, which is statistically significant (Figure 3.3, top panels and Figure 3.4, compare mock bars). Also, the length of stromules in *NbGCP4*-silenced plants was significantly longer compared to the VIGS empty vector control (Figure 3.3, top the panels and Figure 3.5 A, compare mock bars). In addition, we saw a greater percentage of stromules that were longer than three micrometers (Figure 3.5). Knowing that stromules can extend and form during an active immune response by pathogens (Caplan et al. 2015), we tested the activation of immune response in *NbGCP4*-silenced plants. An effector from *Tobacco Mosaic Virus* (TMV), P50, was used to infiltrate three days *NbGCP4*-silenced plants. The plants examined one day after infiltration. The number of stromules were significantly increased with the p50 and VIGS vector control plants compared to mock-treatment without p50 (Figure 3.3 and Figure 3.4, compare green bars). In addition, I found that the length of stromules and the percentage of stromules longer than three μm were increased when we activated the immune response of the plants by the p50 VIGS vector control (Figure 3.5 A, green bars and Figure 3.5 B). However, with *NbGCP4*-silenced plants there was no significant change in the number of stromules with p50-treated compared to the mock-treated *NbGCP4*-silenced plants without p50-treated (Figure 3.3, right panels and
Figure 3.4, compare red bars). Also, with the length of stromules and without p50-treated, NbGCP4-silenced plants showed longer stromules compared to VIGS vector control plants (Figure 3.5 A). Similarly, p50-treated VIGS vector control plants showed significantly longer stromules compared to mock-treated plants without p50-treated (Figure 3.5 A, compare green bars). However, with NbGCP4-silenced plants with p50-treated or without, there was no significant changes in stromule length (Figure 3.5 A, compare red bars). These results show that with the activation of the immune response, the number of stromules and the length in NbGCP4-silenced plants did not increase as we saw with VIGS vector control plants.
Figure 3.3 *NbGCP4* Silencing Plants Increase the Number of Stromules and the Length

Infiltration of the TMV-p50 effector in plants induced stromules after one day as Dr. Caplan described in his paper (Caplan et al., 2015). He compared the image in the bottom left to a mock control in the top left image. As a result, stromules were induced in the bottom left image with p50 to a mock control in the top left image. However, with *NbGCP4* silencing, I found that stromules were induced in both mock (top right) and p50 infiltrated (bottom right). For the length of stromules, *NbGCP4*-silenced plants showed longer stromules (top right) compared to the VIGS-EV control (top left). Scale bar = 40 µm.
Figure 3.4 *NbGCP4* Silencing Plants Increase the Number of Stromules

The number of stromules per chloroplasts was significantly induced in mock-treated *NbGCP4*-silenced plants compared to the mock-treated VIGS-EV control. Also, with the p50 effector, the number of stromules was significantly increased in VIGS-EV control plants compared to the mock (green bars). However, there was no significant change in the number of stromules in *NbGCP4*-silenced mock-treated plants compared to the p50-treated plants (magenta bars). Four images per leaf were generated for quantification from the total of twelve leaves for each condition. The data represented as the mean + standard error of the average, ***P <0.001 (Student's t-test with Welch's correction).
Figure 3.5 *NbGCP4* Silencing Plants Increase the Length of Stromules

(A) The length of stromules was significantly increased with mock-treated VIGS-*NbGCP4* plants compared to the VIGS-EV control (left bars). Also, with p50 effector-induced immune response, the length of stromules increased with VIGS vector control plants compared to the mock-treated VIGS-EV control plants (green bars). However, there was no significant change with p50-treated and mock-treated VIGS-*NbGCP4* plants without p50 (red bars). The box and whisker plot was drawn with rank transformation. Also, the box covers from first to third quartiles while a bar in the middle of the box indicates median. Whiskers show from 5 to 95% of the ranking. ***P<0.001, ♦ comparison with VIGS-EV control, ♦♦♦ p<0.001 by Mann-Whitney test. Dots in the graph indicate outliers. (B) ImageJ was used to measured the length of stromules manually. The length of stromules was longer in VIGS-*NbGCP4* plants compared to empty vector control plants. The graph shows a higher percentage of stromules in *NbGCP4*-silenced plants that were three µm or more extended compared to empty vector control plants. Total of 302, 162, 137, and 109 stromules were measured for VIGS-EV, VIGS-*NbGCP4*, with and without TMV p50. Fisher’s exact test was performed for comparison. *P<0.05 and ***P<0.0001.
3.2.1.4 Stromule Velocity Shows Inverse Relationship Between EV-Control and NbGCP4-Silenced Plants

Next, the velocity of stromules was tested by looking to stromule extension and retraction. The extension and retraction of stromule rates decreased in the NbGCP4-silenced plants compared to the EV control (Figure 3.6). This shows stromules were less dynamic and more stable in the NbGCP4-silenced plants compared to the EV control (Figure 3.6).
Figure 3.6 Stromule Velocity Shows Inverse Relationship Between EV-Control and NbGCP4 Lines

The graph shows the velocity of stromule extension and retraction in VIGS-EV control and VIGS-NbGCP4 plants with or without TMV-p50-induced immune response. The speed of stromule extension and retraction increased in VIGS-EV control compared to the NbGCP4-silenced plants. Data represented as the mean + standard error of the average. Symbols at the top of bars indicate significant differences according to Dunnett’s multiple comparison tests. Single symbol (*, ◊, ■), control set for each comparison; two-symbols (**, ◊◊, ■■), P<0.05 and three symbols (***, ◊◊◊, ■■■), P<0.001.
3.2.1.5 Stromule Movement Dynamics Quantification

We divided the types of stromule movement into three categories (Figure 3.7 A). Constant motion or slow movement is highlighted by the green line in the top row. The second type is a sudden or fast movement marked by the yellow line in the middle row. Lastly, magenta dots show stromules tips moved on the sideway to the body of the chloroplast (bottom row). However, I tracked the three types of stromule movement manually. There was a higher percentage of constant or slow movement compared to the sudden or fast movement of stromules in NbGCP4-silenced plants and to the EV control (Figure 3.7 B). This most likely occurred due to stromule dynamicity and stability in the NbGCP4-silenced plants. In other words, the longer a microtubule is, the more it bundles leading to more stability. However, with p50 we see an increase in sudden movement in NbGCP4 silenced lines. To summarize my first Aim A, the results show that specific alterations of MTs can directly change stromule dynamics and further support a direct role for MTs in regulating stromules.
**Figure 3.7 Stromule Movement Dynamics Quantification**

(A) We divided the types of stromule movement into three categories constant, sudden, and side motions. (B) The graph shows a higher percentage of constant movement, slow motion of stromules in *NbGCP4*-silenced plants without p50 compared to the EV control without p50. However, plants with p50 showed an increase in sudden movement in *NbGCP4* silenced lines compared to the EV control. Total of 337, 186, 134, and 127 movements of stromules were recorded for VIGS-EV, VIGS-*NbGCP4*, VIGS-EV with TMV-p50, and VIGS-*NbGCP4* with TMV p50, respectively. Chi-square test was performed to compare. *P<0.05, **P<0.001.*
3.2.2 Sub Aim B: Determine Changes in Stromule Dynamics During Plant Defense:

3.2.2.1 The TMV-p50 Effector Is Required for Stromules to Form and Extend Along MTs

Erickson et al., 1999 claimed that the p50 helicase domain of TMV activates defense signals that lead to HR-PCD in plants (Erickson et al. 1999). During this mechanism, stromules extend from a chloroplast and move along MTs to the nuclei. Since TMV-p50 plays a significant role by enhancing HR-PCD and inducing stromules, I investigated the role of stromule dynamics during plant defense. I infiltrated p50 with TagRFP-MAP-CKL6 as MT marker in NRIIP1-Cerulean transgenic N. benthamiana, and after two days of infiltration, tested the samples. Interestingly, I observed strong induction of stromules from chloroplasts during p50-induced HR-PCD. These results guide me to look to several important factors to understand the changes on stromule dynamics during plant defense such as chloroplasts and stromules count, stromules length, extension and retraction of stromules velocity, and movement types of stromules.
3.2.2.2 The Number of Stromules Per Chloroplasts Do Not How Significant Change During an Active Immune Response

I designed an experiment that had three factors which are mock (infiltration media), citrine (yellow fluorescent protein), and p50-citrine in NRIP1-Cerulean transgenic N. benthamiana plants to visualize the effect on stromules and MTs with p50 and without p50. I used two negative control in this experiment; the first control is mock which is an infiltration media that does not have a factor that may induce defense response. The second negative control is citrine, which indicates stromules due Agrobacterium. Also, I used p50-citrine to look to a contribution of effector tribute immunity in stromules induction. Stromules were manually counted by using ImageJ and by looking to the number of stromules per chloroplast, I found the number of stromules compared to negative control even though there was no significant change (Figure 3.8). Additionally, biological replicate may lead to a conducted to determine if the trend is significant as I saw with Figure 3.4, compare green bars.

3.2.2.3 Stromules Length Increase During an Active Immune Response

Stromules length were manually measured by using ImageJ and results showed a significant increase of stromules length with p50-treated plants compared to mock--treated (infiltration media) and citrine plants citrine (negative control) without p50-treated (Figure 3.9).
3.2.2.4 Extension and Retraction of Stromules Velocities

Previously I examined only the stromules induction which is stromules count and length, but here I wanted to determine if stromules dynamic may change during inducing the immune response by study how fast they extend and interact. Extension and retraction of stromules velocities were manually measured by using ImageJ; as a result, there were no significant changes in the extension and retraction of stromules velocities (Figure 3.10). I think the reason for that may because the effector that I used which is p50-citrine, p50-citrine is not strong as p50-HA that I used with silencing experiment (Figure 3.6). However, I found that stromules retract more than extending with p50-Citrine, the mock, and citrine, but the reason for that is still unknown.

3.2.2.5 TMV-p50 Pathogen Effector Significantly Increased the Movement of Stromules

The average velocity of extension and retraction of stromules seems not change, but when I looked to the different types of stromule movement, there was a significant higher percentage of the sudden type movement compared to the constant and the side movements of stromules (Figure 3.11). The reason here is that p50 pathogen effector in plants may increases the changes dynamic of movement compared to mock and citrine plants. To sum up, of my first Aim, B, the results show that TMV-p50 pathogen effector induces HR-PCD, which leads to changes in stromule dynamics by inducing the structure of stromules and velocity.
Figure 3.8 TMV-p50 Effector in Plants Increase the Number of Stromules

The number of stromules showed an increased by the p50 effector compared to the mock-treated and Citrine. However, there was no significant change in the number of stromules in p50-Citrine plants compared to the mock and citrine. Four images per leaf were generated for quantification from a total of twelve leaves for each condition. The data represented as the mean + standard error of the average, ns P <0.0859 (Kruskal-Wallis statistic).
Figure 3.9 TMV-p50 Effector in Plants Increase the Length of Stromules

The graph shows significant increase in the length of stromules with activation immune response (p50-Citrine) compared to mock-treated and citrine plants without p50-treated. Total of 131, 147, and 144 stromules were measured for p50-Citrine, the mock, and citrine. *P<0.05 and ***P<0.0001.
Figure 3.10 Retraction of Stromules Rate in Plants Increase

Stromules retract faster than extend with p50-Citrine, the mock, and citrine; however, the velocities do not show any significant changes. Data represented as the mean ± standard error of the average, ns, P 0.8126.
Figure 3.11 TMV-p50 Effector in Plants Increase the Changes Dynamic of Stromules

I divided the types of stromule movement into three categories which are a constant motion, a sudden, and a side movement. ImageJ was used to track these different types of stromule movements. However, the graph shows there was a higher percentage of sudden movement of stromules in p50-citrine plants compared to the mock and the citrine. However, without p50 there was an increase in constant movement (mock and citrine). Total of 131, 147, and 144 stromules were measured for p50-Citrine, the mock, and citrine. ****P<0.0001.
3.3 Aim 2: Examine the Function of Kinesins During Stromule Dynamics:

3.3.1 Sub Aim A: Examine the Effect of Overexpression Kinesin 14 on Stromule Movement:

3.3.1.1 Kinesin Superfamily Proteins (KIFs)

Kinesin superfamily proteins (KIFs) are molecular motors that use ATP hydrolysis to power their translocation and move along microtubules (Zhu and Dixit 2012). Kinesin motors play important parts in cell division, cell motility, intracellular trafficking and ciliary function (Zhu and Dixit 2012). Also, kinesins are responsible for most of the microtubule-based anterograde organelle transport (Hirokawa et al. 2009). Since we found that stromules extend and move along MTs, we think that kinesin superfamily proteins can play a role by guiding stromule movement to the nucleus.

Based on phylogenetic analysis, kinesins are classified into 14 families (Verhey and Hammond 2009). Some of these families do not exist in land plants, while others have been shown (Verhey and Hammond 2009). For example, Kinesin-1, kinesin-7, and 14 families have been founded in plants and these families contain half of kinesins that encoded by the Arabidopsis genome (Verhey and Hammond 2009). However, there is some research that supports kinesin family 14 plays an important role in chloroplast movement. For instance, Suetsugu et al. 2010 suggested that kinesin-like KAC proteins (KAC1 and KAC2) act as regulators for chloroplast
movement (Suetsugu et al. 2010). Also, KAC proteins are related to Kinesin-14 family (Zhu and Dixit 2012). For this reason, I chose Kinesin-14 family and tested the most common kinesins in this family; such as KAC1, KAC2, At1g09170, At3g10310, At3g44730, At5g27000, At1g63640 Kinesin for Stromule Extension 1 (KSE1), and At5g41310 Kinesin for Stromule Extension 2 (KSE2) (Table 3.1).
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**Table 3.1 The Most Common Kinesins of Family 14**

These eight kinesin-14s have been shown to be important for spindle organization and chloroplast movement.
3.3.1.2 Kinesins Family 14 That Show Expressions in N. Benthamiana Plants

Testing the most common kinesins in family 14, two appeared to be most interesting when overexposed in NRIP1-Cerulean transgenic N. benthamiana plants (At1g63640 and At5g41310). To simplify, I named the kinesins used KSE 1 and KSE 2 (Kinesin for Stromule Extension) (Table 3.1). When KSE1 and KSE2 were overexpressed, both showed an increase in chloroplasts movement (Figure 3.12 and Figure 3.13). However, the chloroplast movement was much faster with KSE2 compared to the control (Figure 3.13). For this reason, I designed an experiment to quantify the effect on stromule dynamics when overexpressing KSE2 by focusing on three factors, chloroplast and stromule count, stromule length, and chloroplast velocity.
Figure 3.12 Overexpression of KSE1 Shows Increased In Chloroplasts Movement

This figure shows that chloroplasts move faster with overexpression of KSE1. KSE1 was overexposed in NRIP1-Cerulean transgenic *N. benthamiana* plants, KSE 1 was labeled by Citrine (yellow fluorescent protein) and NRIP1-Cerulean (blue fluorescent protein) marks stromules. I took screenshots of a video that shows the movement of chloroplasts with overexpression of KSE1. Scale bar = 10 µm.
Figure 3.13 Overexpression of KSE2 Shows Increased in Chloroplasts Movement

These images show that most of chloroplasts in the control were stable compared to overexpression of KSE2 that showed most of the chloroplasts moved faster. KSE2 was overexposed in NRIP1-Cerulean transgenic *N. benthamiana* plants. I took screenshots of two videos that show these motion of chloroplasts with overexpression of KSE2 or without (control). Scale bar = 10 µm.
3.3.2 Sub Aim B: Quantify the Effect of Overexpressing KSE2 on Stromules:

3.3.2.1 Chloroplasts and Stromules Count

I tested KSE2 in NRIP1-Cerulean transgenic *N. benthamiana* plants using tRFP-sfGFP11 as a control because it is free Tag-RFP in the cytosol. Stromules were manually counted by using ImageJ and by looking to the number of stromules per chloroplast, there was no significant change during overexpression of KSE2 (Figure 3.14). The main reason for this is the experiment did not have a pathogen effector that may induce an immune response in plants, which can increase the number of stromules per chloroplast.

3.3.2.2 Stromules Length

Stromules length were manually measured by using ImageJ and results showed a significant change was observed when KSE2 was overexpressed in NRIP1-Cerulean transgenic *N. benthamiana* plants (Figure 3.15). The included figure shows stromule length was longer during overexpression of KSE2 (top image) compared to the control (bottom image).

3.3.2.3 Chloroplasts Velocity During Overexpressing of KSE2

The most important factor to quantify in this experiment was the velocity of chloroplasts. It is my hypothesis that KSE2 increases the movement of the chloroplasts, and therefore quantifying the velocity at which they move may support this. Chloroplasts were manually quantified using ImageJ (Schindelin et al. 2012). I found that chloroplast velocity
significantly increased in NRIP1-Cerulean transgenic *N. benthamiana* plants during KSE2 overexpression compared to the control (Figure 3.16). The most likely scenario to explain this is the chloroplasts move and jump from line to line of microtubules. In other words, adding more kinesins such as KES2 may permit chloroplasts to move faster compared to a normal plant cell.
Figure 3.14 Stromules and Chloroplasts Counts Do not show Significant Change During Overexpressing KSE2

The graph shows no significant change in the number of stromules during overexpressing of KSE2 plants compared to the control. Four images per leaf were generated for quantification from the total of 32 leaves for each condition. The data represented as the mean + standard error of the average, ns, P 0.7582.
Figure 3.15 The Length of Stromules Shows Significant Change During Overexpressing KSE2

The graph shows significant change in the length of stromules during overexpression of KSE2 plants compared to the control. Four images per leaf were generated for quantification from the total of 32 leaves for each condition. The data represented as the mean ± standard error of the average, ns, P 0.7582. Total of 319 and 319 stromules were measured for KSE2 and the control. ***P 0.0005 (Unpaired t test with Welch’s correction).
Figure 3.16 Chloroplasts Velocity Show Significant Increase of Movement During Overexpressing KSE2

This graph shows the velocity of chloroplasts in NRIP1-Cerulean transgenic *N. benthamiana* plants during KSE2 overexpression compared to the control (free Tag-RFP in the cytosol). Analyzing of chloroplast body movement shows a significant increase in movement velocity when overexpressing KSE2. Data represented as the mean ± standard error of the average. ****P<0.0001 (Unpaired t test with Welch’s correction).
4.1 The Role of Cytoskeletal Elements and Chloroplasts

In this research, chloroplast function and the role of cytoskeletal elements were studied during innate immune responses. Chloroplasts are one of the most important organelles in plant cells because of their role in several cellular functions including photosynthesis, fatty acid synthesis, and the immune response in plants (Alberts et al., 2000). In addition, when pathogens infect plant cells, stromules extend from chloroplasts and deliver defense signals to the nucleus to induce hypersensitive response programmed cell death (HR-PCD). To understand this mechanism more, previous studies examined the role of the two cytoskeletal elements, MTs and AFs, during stromule formation. These cytoskeletal elements play several significant roles that support cellular membranes in plant cells including internal structures, cytoplasmic streaming, cell division, cell elongation, polar growth, and vesicle movement (Cai, Parrotta, and Cresti 2015; Li, Sun, and Ren 2015; Suetsugu et al. 2016). This study shows that stromules extend from chloroplasts and move along MTs and kinesins may be involved in this movement.
4.2 The Role of MTs During Stromule Formation

Previous studies examined the role of MTs during stromule formation and showed that both APM and Oryzalin disruption treatments could affect stromules formation in *Nicotiana* plants. Also, stabilizing MTs using taxol (Kumar et. al., eLife, under revision) resulted in twice the average number of stromules per chloroplast. For this reason, we modified MTs genetically by silencing *GCP4* in *Nicotiana* plants. GCP4 is a subunit of the \( \gamma \)-tubulin complex of MTs. The *GCP4* is not essential for \( \gamma \)-tubulin complex (Vinh et al., 2002), but it plays a significant role in stabilizing the ring compound of MTs (Guillet et al., 2011). The previous study silenced *GCP4* in *Arabidopsis* leaf pavement cells and found that the hyper-parallel of MTs were bundled (Kong et al., 2010). Knockdown of the *GCP4* experiment in *N. benthamiana* leaves showed similar results in MTs organization by SOAX program. *NbGCP4* silencing affected MTs structure which inherently causes stromules to extend from chloroplasts.

*NbGCP4*-silenced plants showed that stromules were longer, and the velocity of stromules was decreased because stromules were more stable and less dynamic. This is most likely due to the disruption balance between MT branching and MT bundling in *NbGCP4* silenced plants. The results support that MT dynamics are an essential regulator of stromule dynamics.
4.3 The Role of MTs During Innate Immunity

The results show that stromules played a significant role in innate immunity and programmed cell death (Caplan et al. 2015). During an immune response, stromules extended from chloroplasts and moved towards the nucleus (Caplan et al. 2015). Also, the results of using TMV-p50 effector-triggered immunity showed the role of MTs during innate immunity and programmed cell death. MTs play a role in inducing stromule formation in addition to increasing their length. Overall, these results show MTs are important during an immune response in plant cells; in other words, during effector-triggered immunity, MTs facilitate stromules to extend and move toward the nucleus to transport defense factors in order to enhance HR-PCD.

KSE2, Kinesin for Stromule Extension 2 showed the role of kinesin during chloroplast movement, which may be guiding the movement of chloroplasts. Results described here show that overexpression of KSE2 increased the movement of chloroplasts. Also, previous studies suggest that kinesin-like KAC proteins (KAC1 and KAC2) play an important role as regulators for chloroplast movement (Suetsugu et al. 2010). Since stromules extend and move along MTs and KES2 increases the movement of chloroplasts, it may be possible that stromules may guide the movement of chloroplasts along MTs using kinesins.
4.4 Future Work

Further research will focus more on chloroplast and stromule movement because there is a motor protein that may guide this movement. Previous studies suggest that myosin, a motor protein of AFs, guides the movement of chloroplasts and stromules to move along actin filaments (Ernest Y. Kwok and Hanson 2003; Ernest Y Kwok and Hanson 2004). However, the results of this current study showed that stromules extend and move along microtubules, and kinesins may guide this movement of chloroplasts. It will be interesting to quantify the role of KSE2 during stromule movement, knockout KSE2 and examine the movement of chloroplasts and stromules with this treatment. Additionally, silencing KSE2 and testing stromule dynamics during plant defense response would yield more information regarding KSE2’s role on stromules.
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