

**CHARACTERIZATION OF THE KINESIN KSE2 IN STROMULE
FORMATION AND FUNCTION**

by

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TABLE OF CONTENTS

LIST OF TABLES	vii
LIST OF FIGURES	viii
LIST OF ABBREVIATIONS	x
ABSTRACT	xi

Chapter

1	CHLOROPLAST, STROMULE AND CYTOSKELETON ROLES IN PLANT INNATE IMMUNITY	1
1.1	Plant Innate Immunity	1
1.2	Chloroplasts Role During Plant Innate Immunity	6
1.3	The Plant Cytoskeleton and its Relation to Chloroplasts and Stromules	10
1.4	Plant Kinesins and Kinesin for Stromule Extension 2 in Stromule Formation and Function	13
1.5	Rationale and Goals.....	17
2	MATERIALS AND METHODS	18
2.1	Plant Line and Visualization of Chloroplasts and Stromules	18
2.2	Plasmids.....	19
2.3	Plant Material and Growth Conditions.....	21
2.4	Agrobacterium Preparation	22
2.4.1	Stock Solutions of Antibiotics.....	22
2.4.2	Protocol to Make Antibiotic Plates.....	24
2.4.3	Growing and Streaking Agrobacterium.....	24
2.5	Agrobacterium Infiltration of the Plant	25
2.5.1	Agrobacterium Prep with Infiltration Media.....	25
2.5.2	Infiltration of the Plant Leaf	25
2.6	Leaf Preparation for Imaging	26
2.7	Laser Scanning Confocal Microscopy.....	26
2.8	Image Processing.....	26
2.9	Manual Quantification of Stromule and Chloroplast Dynamics	27
2.10	Automatic Quantification of Stromule, Chloroplast Dynamics and Cytoskeleton Dynamics using a Deep Learning	27
2.11	Statistical Analysis	31
3	RESULTS.....	32

3.1	Introduction	32
3.2	Aim 1: Characterize Stromule and Chloroplast Dynamics by Overexpressing KSE2 Deletion Constructs:	33
3.2.1	Sub Aim A: KSE2 Full Length Causes Changes in Stromule Dynamics.....	33
3.2.1.1	Overexpression of KSE2 results in increased stromule induction	35
3.2.1.2	Overexpression of KSE2 results in increased branching stromules.....	36
3.2.1.3	Overexpression of KSE2 results in increased stromule kinks	37
3.2.1.4	Overexpression of KSE2 results in increased stromule length	38
3.2.1.5	Overexpression of KSE2 results in increased stromule tip velocity	40
3.2.1.6	Overexpression of KSE2 results in increased chloroplast base velocity	41
3.2.2	Sub Aim B: Overexpression of KSE2 Deletion Constructs Causes Changes in Stromule Morphology	42
3.2.2.1	Overexpressing KSE2 deletion constructs and the effect on stromule induction.....	43
3.2.2.2	Overexpressing KSE2 deletion constructs and the effect on stromule branching.....	44
3.2.2.3	Overexpressing KSE2 deletion constructs and the effect on stromule length	45
3.2.3	Sub Aim C: Overexpression of KSE2 Deletion Constructs Causes Changes in Stromule and Chloroplast Dynamics.....	47
3.2.3.1	Overexpressing KSE2 deletion constructs and the effect on stromule tip velocity	48
3.2.3.2	Overexpressing KSE2 deletion constructs and the effect on chloroplast velocity	50
3.3	Aim 2: Characterize the Interactions Between KSE2 Deletion Constructs and the Microtubule (MT) and Actin (AF) Network:	52
3.3.1	Sub Aim A: Observe KSE2 Deletion Constructs Contact Points with Microtubule (MT) and Actin (AF) Network:	52

3.3.1.1	KSE2 deletion constructs changes contact points with the actin network	54
3.3.1.2	KSE2 deletion constructs changes contact points with the microtubule network.....	55
3.4	Aim 3: Characterize the Effects of KSE2 During Plant Innate Immunity	56
3.4.1	Sub Aim A: Overexpression of KSE2 Deletion Constructs Dynamics Change During PAMP Triggered Immunity	56
3.4.1.1	Application of flg22 while overexpressing KSE2 results in altered dynamics	57
3.4.2	Sub Aim B: Overexpression of KSE2 Deletion Constructs Dynamics Change During Effector Triggered Immunity.....	60
3.4.2.1	Electrolyte leakage analysis observes the effects of KSE2 overexpression on HR-PCD.....	61
4	DISCUSSION	63
4.1	The Role of KSE2 in Stromule Formation in Function.....	63
4.2	How the Loss of KSE2 Domains Effects Contact with the Plant Cytoskeleton.....	67
4.3	KSE2 Effect on the Plant Immune Response	68
4.4	Future Work.....	69
	REFERENCES	70

LIST OF TABLES

Table 2.1 The Plasmids	19
Table 4.1 Table of Aim 1 Means and Significance	66

LIST OF FIGURES

Figure 1.1 Plant Innate Immunity.....	4
Figure 1.2 TMV effector p50 is recognized by an N Immune receptor to trigger ETI..	5
Figure 1.3 Stromules Extend from Chloroplasts to Induce HR-PCD	9
Figure 1.4 Cytoskeleton Elements of Plants and Their Motor Proteins	12
Figure 1.5 Overexpression of KSE2 alters stromule dynamics	15
Figure 1.6 KSE2 Deletion Constructs	16
Figure 2.1 Visualization of Stromules Using Confocal Microscopy	18
Figure 2.2 Leaf-Stage <i>Nicotiana benthamiana</i> Plant	21
Figure 2.3 Visual Representation of the Stromule Analysis Pipeline	29
Figure 2.4 Visual Representation of the Cytoskeleton Analysis Pipeline.....	30
Figure 3.1 Overexpression of KSE2 results in altered stromule dynamics	34
Figure 3.2 Quantification of the increased stromule induction of KSE2	35
Figure 3.3 Quantification of the increased stromule branching of KSE2	36
Figure 3.4 Quantification of the increased stromule kinks.....	37
Figure 3.5 Quantification of the length of extending stromules.....	38
Figure 3.6 Quantification of the length of retracting stromules	39
Figure 3.7 Quantification of stromule tip velocity of extending stromules	40
Figure 3.8 Quantification of chloroplast base velocity of extending stromules.....	41
Figure 3.9 Quantification of the increased stromule induction of KSE2 deletion constructs	43
Figure 3.10 Quantification of the increased stromule branching of KSE2 deletion constructs	44
Figure 3.11 Quantification of the length of extending stromules of KSE2 deletion constructs	45

Figure 3.12 Quantification of the length of retracting stromules of KSE2 deletion constructs	46
Figure 3.13 Quantification of stromule tip velocity of extending stromules of KSE2 deletion constructs	48
Figure 3.14 Quantification of the length of retracting stromules of KSE2 deletion constructs	49
Figure 3.15 Quantification of chloroplast velocities during stromule extension events for the KSE2 deletion constructs	50
Figure 3.16 Quantification of chloroplast velocities during stromule retraction events for the KSE2 deletion constructs	51
Figure 3.17 Stromule endpoint and actin network analysis	54
Figure 3.18 Stromule endpoint and microtubule network analysis	55
Figure 3.19 Overexpressing KSE2 + application of flg-22 results in increased stromule length during stromule extension	57
Figure 3.20 Overexpressing KSE2 + application of flg-22 results in increased stromule tip speed during stromule extension.....	58
Figure 3.21 Overexpressing KSE2 + application of flg-22 results in increased chloroplast base speed during stromule extension.....	59
Figure 3.22 Electrolyte leakage assay using p50-3xHA XVE	61
Figure 3.23 Electrolyte leakage assay using TagRFP-p50 XVE.....	62

LIST OF ABBREVIATIONS

PTI: PAMP Triggered Immunity

ETI: Effector Triggered Immunity

PAMPS: Pathogen Associated Molecular Patterns

PRRs: Pathogen Recognition Receptors

NLRs, NB, LRRs: Nucleotide binding Leucine Rich Repeats

PCD and HR-PCD: Hypersensitive Response-Programed Cell Death

TMV: *Tobacco mosaic virus*

SAR: Systemic Acquired Resistance

CAS: Calcium Sensing Receptor

ROS: Reactive Oxygen Species

GFP, TagRFP, CFP: Green, Red or Cyan Fluorescent Protein

NRIP1: N receptor interacting protein 1

BDM: 2,3 butanedione 2-monoxime

APM: Amiprophos-methyl

KAC1 and KAC2: Kinesin-like proteins 1 and 2

KSE1 and KSE2: Kinesin for Stromule Extension 1 and 2

OE: Overexpression

MT: Microtubule

AF: Actin Microfilament

CytoD: Cytochalasin D

FL: Full Length

ABSTRACT

Chloroplasts play an important role during plant immunity and are the source for many cellular signals during the defense response. During the immune response chloroplasts send out stroma-filled tubular extensions called stromules. Stromules are induced during both PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI). Stromules extend along microtubules, and since kinesins are the only microtubule motors in plants, we hypothesized that a kinesin provides the motive force for stromule extension and stromule-driven movement. Our lab overexpressed 8 different kinesins belonging to kinesin family 14. One of the candidates resulted in increased stromule length and faster chloroplast movement, which we named kinesin for stromule extension 2 (KSE2). My project is to characterize KSE2 to understand its role in stromule formation and function. To do this, I overexpressed 8 deletion constructs in *N. benthamiana* in which we deleted various domains of KSE2. These deletions include the calponin homology domain (Δ CH), the coiled-coiled 1 domain (Δ CC1), both the Δ CH and Δ CC1 (Δ CH+CC1), only the motor (Only Motor), no motor (Δ Motor), the coiled-coiled 2 domain (Δ CC2), the C-terminal tail (Δ tail), both the CC2 and tail (Δ CC2-Tail). By examining and quantifying stromule and chloroplast dynamics of my constructs, I hope to discover the role of KSE2 in stromule formation and function. Additionally, I also look at how the overexpression of the deletion constructs effect cytoskeleton and stromule tip interactions. I also have started to examine the role KSE2 plays during PTI and ETI.

Chapter 1

CHLOROPLAST, STROMULE AND CYTOSKELETON ROLES IN PLANT INNATE IMMUNITY

1.1 Plant Innate Immunity

Plants face a number of environmental challenges to survive, specifically against pathogen attack. To combat the pathogen attacks, plants have developed various ways of defending themselves. Plants lack acquired immunity and thus rely on innate immunity to fight off pathogens. Plant innate immunity is split into two parts, PAMP-Triggered Immunity (PTI) and Effector-Triggered Immunity (ETI). PTI and ETI trigger the generation of signals which lead to the defense against pathogens. These signals can come from various organelles such as the chloroplast and plasma membrane (Jones and Dangl, 2006). When a pathogen invades a plant leaf, the initial immune response involves recognition of conserved pathogen associated molecular patterns (PAMPs). PAMPs are bacterial structures such as flagellin and lipopolysaccharides that are recognized by cell surface pattern recognition receptors (PRRs). PRRs are also involved in the perception of fungi and oomycetes as well (Zipfel and Cyril, 2007). Triggering the PRRs on the plant's surface act as an alarm sending out danger signals triggering the plant innate immune responses. This leads to PAMP-triggered immunity (PTI). PTI is considered a broad defense response and the

first stage of defense against pathogens. Pathogens which manage to elude PTI then deploy effectors which contribute to pathogen virulence (Jones and Dangl, 2006). Pathogenic effectors are then recognized by another form of defense, which is a more robust and specific response, called effector-triggered immunity (ETI). ETI acts mainly inside of the cell using polymorphic NB-LRR protein products (NLR) encoded by R genes. NB-LRR are named after their nucleotide binding (NB) and leucine rich repeat (LRR) domains. These proteins recognize pathogenic effectors which turn on R genes and trigger plant resistance. ETI results in disease resistance and programmed cell death (PCD) at the infection site called the hypersensitive response (HR-PCD). The hypersensitive response restricts pathogen proliferation and prevents the development of disease (Cui et al., 2015). A well-studied example is between TMV and *Nicotiana benthamiana*. During *Tobacco mosaic virus* (TMV) infection, it secretes a specific effector p50 which then gets detected by a NB-LRR which triggers ETI and HR-PCD. The largest group of NB-LRRs contains Toll-interleukin-1 (TIR) homology domain. TIR domains are important for animal innate immunity responses. Specifically, the TIR-NB-LRR indirectly recognizes the 50 kDa helicase (p50) domain of TMV through the TIR domain. This interaction is mediated by NRIP1 which interacts with both the N receptor's TIR domain and p50 (Caplan et al., 2008). Local resistance reactions induce longer lasting systemic immunity which primes uninfected tissues against subsequent pathogen attack. This longer lasting systemic immunity is called systemic acquired resistance (SAR). When SAR is turned on the plant's defense response is more easily and quickly triggered. Since SAR deals with previous

pathogenic encounters, this is a form of plant memory. SAR can last for a few weeks to months (Gao et al., 2015). PTI and ETI are in a constant battle with pathogens who are always trying to get the upper hand. This is known as the zig-zag model, first the pathogen is recognized triggering PTI. In response the pathogen employs effectors to target PTI and effectors are recognized triggering ETI and ETI triggers HR-PCD. Natural selection results in plants with new R specificities and thus pathogens adapt to combat these new specificities resulting in an arms race between plant immunity and plant pathogens (Anderson et al., 2010).

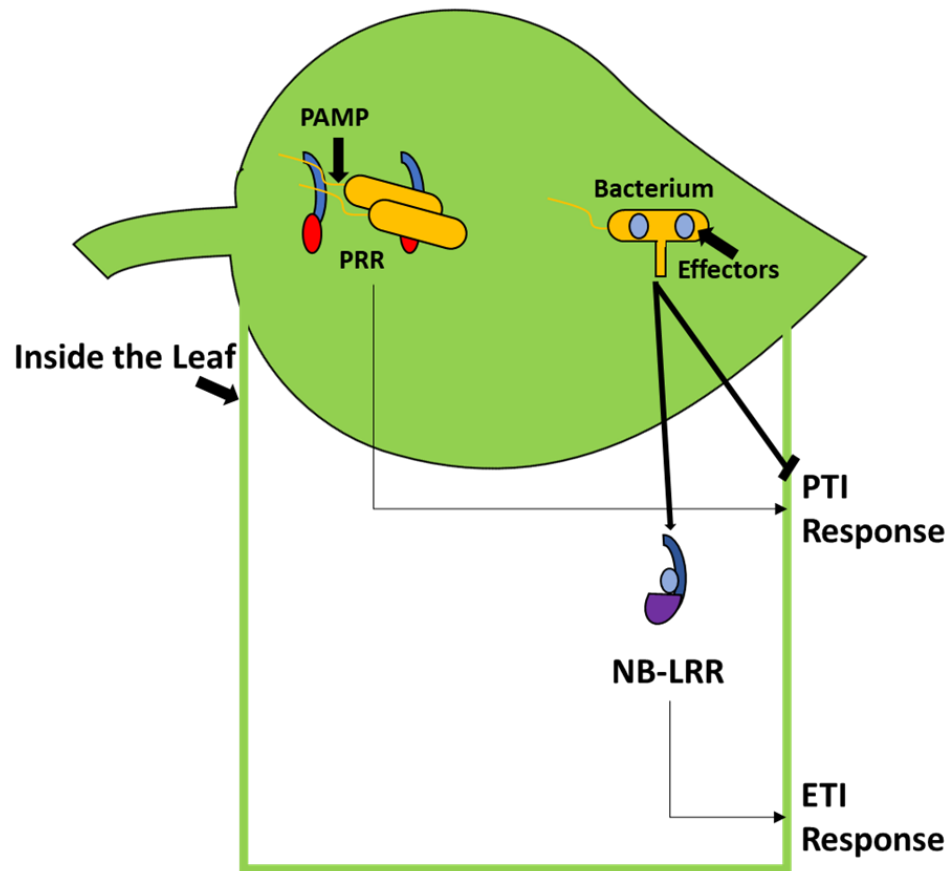


Figure 1.1 Plant Innate Immunity

Pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs) which trigger PAMP-Triggered Immunity (PTI). The pathogen secretes various effectors which targets specific locations within the plant cell to slow and disrupt PTI. However, these effectors are recognized by the plant which trigger a more robust, specific type of immunity called effector triggered immunity (ETI). ETI triggers the hypersensitive response which restricts pathogen proliferation and prevents the development of disease (Chisholm et al., 2006).

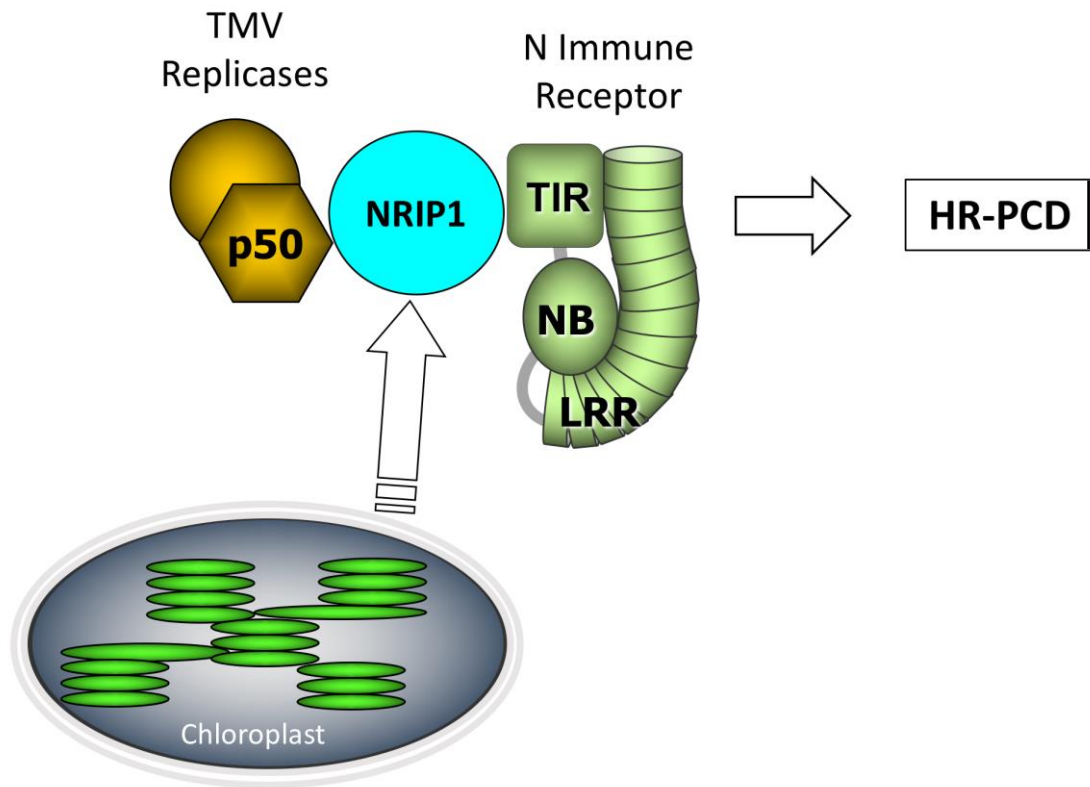


Figure 1.2 TMV effector p50 is recognized by an N Immune receptor to trigger ETI

During *Tobacco mosaic virus* (TMV) infection, the virus gets into the plant cell through existing damage on the leaf exterior. Once inside TMV expresses various effectors such as p50 in the plant cell. These effectors get recognized by a TIR-NB-LRR receptor with an additional defense factor called NRIP1 that will lead to ETI and HR-PCD (Caplan et al., 2008).

1.2 Chloroplasts Role During Plant Innate Immunity

It is common knowledge that chloroplasts play a major role in light harvesting, energy production, redox homeostasis and retrograde signaling. Chloroplast signals can travel long distances within the cell inducing a variety of changes. There is increasing evidence that shows that chloroplasts play an important role during the plant immunity such as during ETI and PTI responses. During infection it has been shown that there is a suppression of photosynthesis reflecting the chloroplast shutting down energy production and carbon availability to possibly limit pathogen growth. This also could indicate the role the chloroplast plays during defense, during infection it shifts from energy production to establishing defense (Padmanabhan and Dinesh-Kumar et al., 2010). The chloroplast is the orchestrator for plant defense as it plays a role as a receiver, an integrator and a transmitter of specific signals that coordinate expression of nuclear and plastid genomes to sustain homeostasis (Maruta et al., 2012). Chloroplasts are known to have a central role in the delivery of defense signals including ROS and calcium. During PTI, there is an increase of Ca²⁺ signaling due to the chloroplast's calcium sensing receptor (CAS). CAS plays an important role in connecting the chloroplast immune responses to the nuclear immune responses which also involves Ca²⁺ to turn on defense. During ETI and PTI, there is a rapid burst of hydrogen peroxide on the plasma membrane, followed by a second burst generated by the chloroplasts, which up regulates defense gene expression through ROS-mediated signaling to the nucleus. The recognition of ROS initiates and promotes cell death during HR-PCD (Yu et al., 2017). Chloroplasts are responsible for other types of ROS

such as superoxide anion radical, hydrogen peroxide and hydroxyl radical.

Chloroplastic ROS is also thought to be responsible for the closure of the plasmodesmata during immunity. Plasmodesmata closure restricts pathogens from easily moving between cells (Lee et al. 2011). Chloroplasts are also involved in the synthesis of important hormones such as salicylic acid, jasmonic acid and abscisic acid. Salicylic acid is synthesized in the chloroplasts and salicylic acid near an infection site promotes HR-PCD (Serrano et al., 2016).

Chloroplasts send out stroma-filled tubular extensions called stromules. Stromules are found during both PTI and ETI plant immune responses. Stromules were first widely recognized during the late 90s when stroma filled plastids were targeted with green fluorescent protein (GFP) (Kohler et al., 1997). Stromules complete function and use is still unclear and up for debate (Hanson and Sattarzadeh, 2011). However, it is known that stromules are induced during defense. They are also thought to increase surface area between the plastid and cytoplasm, and this leads people to think stromules facilitate bi-directional trafficking of proteins and metabolites (Mathur et al. 2012). Stromules are known to extend to make a connection to the nucleus which enhances defense responses which leads to increased HR-PCD. Stromules send out signaling proteins and reactive oxygen species to the nucleus activating and enhancing defense responses. One of those proteins is the N receptor interacting protein 1 (NRIP1). Recognition and defense against tobacco mosaic virus (TMV) requires the chloroplastic protein NRIP1. It is thought that stromules are responsible for importing

NRIP1 to the nucleus. Stromules are induced during all types of bacteria, fungi and virus infections indicating that it may be a general response against pathogens. There also is an increase of stromules near sites of HR and increased levels of ROS. This suggests that H₂O₂ and SA could induce the formation of stromules and that stromules are an important pro-defense molecule against pathogens. Stromules might also play a role in facilitating transport of molecules across organelles and by bringing organelles closer together. Stromules have been shown to move throughout the cell by moving along microtubules and anchoring along actin. Thus, showing chloroplasts play an important role contribute to pro-plant cell death signals and defense in a plant cell. However, the mechanisms in which stromules move and their direct role in plant defense is poorly understood (Caplan et al. 2015).

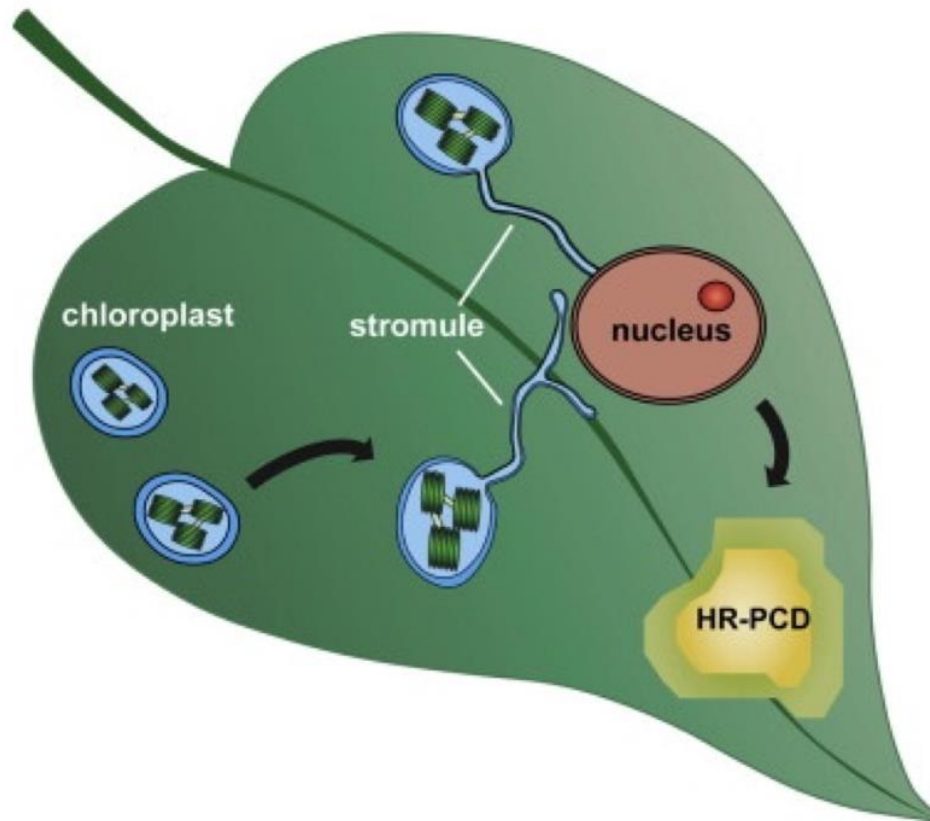


Figure 1.3 Stromules Extend from Chloroplasts to Induce HR-PCD

Chloroplasts use stromules to extend further and make contact with the nucleus in order to transfer signals and molecules from chloroplast to nucleus. This enhancement of signals in the nucleus leads to faster hypersensitive response-programmed cell-death (Caplan et al. 2008).

1.3 The Plant Cytoskeleton and its Relation to Chloroplasts and Stromules

The plant cytoskeleton network is composed of actin and microtubules. In plants, large vacuoles and organelles take up most of the cytoplasmic space leaving the cytoskeleton network to be clustered around the edges of the cell. Some filaments however are in the middle to allow for the transport of signals and molecules to the nucleus. Actin microfilaments in plants provide strength and architecture to the cell and organelle movement. Microtubules are important for intracellular space and cell division (Gunning et al. 2015). The cytoskeleton also plays a role in plant immunity. The actin network has been shown to respond to pathogens attack and disruption of the actin results in enhanced virulence (Henty-Ridilla et al., 2013). Actin during defense response includes transporting antimicrobial compounds and callose to the site of infection (Janda et al., 2014). Bacterial effectors have also been found to target the microtubules directly or indirectly (Lee et al., 2012). Each cytoskeletal element has its own motor protein, actin uses myosin motors and microtubules use kinesin motors. Initial Research supported that stromules move along actin using myosin motors. To support this, researchers used Cytochalasin D and Latrunculin B to inhibit actin microfilaments to observe the effect on stromule formation, movement, and length (Ernest Y. Kwok and Hanson 2003; Ernest Y Kwok and Hanson 2004). Actin inhibitors caused a reduction of stromules however notably some stromule formation did occur (Ernest Y. Kwok and Hanson 2003). Another study by the same group

concluded that stromules extend along actin microfilaments (AF) and the tips of stromules make contact with AFs in *Arabidopsis* epidermal cells (Ernest Y Kwok and Hanson 2004). Additional previous research shows that myosins are the motor responsible for stromule formation (Natesan, Sullivan, and Gray 2005). Myosin motors were treated with an ATPase inhibitor called 2,3 butanedione 2-monoxime (BDM) which was used to observe stromule dynamics. They concluded that a Myosin XI motor protein was involved in stromule movement in *Nicotiana tabacum* (Natesan et al. 2009). However, our lab in collaboration with the Dinesh-Kumar lab found evidence that suggests that microtubules not actin, is required for stromule extension and movement. Stromules were shown to interact and extend along microtubules. This was visualized by confocal microscopy, there was a clear overlap between MTs and stromules. Microscopy also showed that stromules extended along microtubules too. To further support MT are required for stromule formation, the Caplan lab also used amiprophos-methyl (APM) and oryzalin to disrupt MTs to observe the effects on stromule formation and function. They found that stromule induction decreased and movement was drastically hindered. However not all stromules interact with MT suggesting the previous research was not wrong, just incomplete. Actin microfilaments seem to serve as anchor points for stromules rather than being required for stromule formation and function. Interactions between actin and stromules occurred more during tips or kink points. Stromules also interacted with actin more during retraction events (Kumar et. al., 2018).

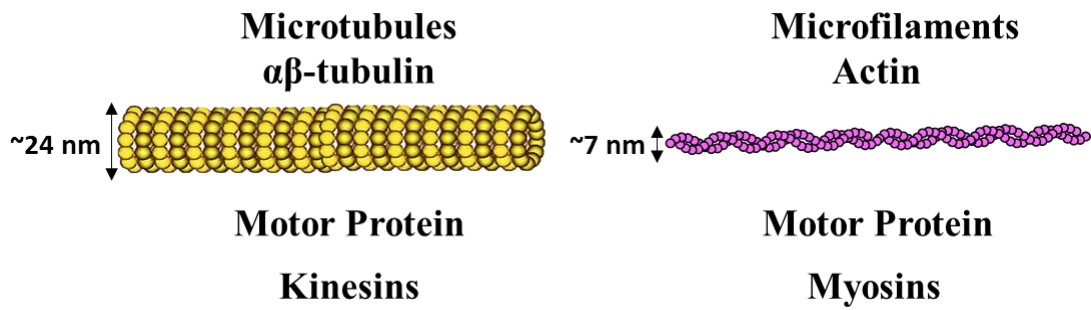


Figure 1.4 Cytoskeleton Elements of Plants and Their Motor Proteins

The two cytoskeleton elements that plants have are actin microfilaments (AF) and microtubules (MT). In addition, each cytoskeleton element has a respective motor protein. Actin microfilaments have myosin motors and microtubule have kinesin motors. The approximate size and their structural subunit are also listed above. (Lodish H, Berk A, Zipursky SL 2008).

1.4 Plant Kinesins and Kinesin for Stromule Extension 2 in Stromule Formation and Function

The kinesin superfamily is one of two molecular motors present in land plants. They use ATP hydrolysis to move along microtubules (Zhu et al., 2012). Kinesins are responsible for most microtubule-based movement within a plant cell (Noda et al., 2009). Kinesins are classified into 14 families. However not all 14 families exist in land plants. The most important families are kinesin-1, kinesin-7, and kinesin-14. These 3 families make up approximately half of all kinesins encoded in the *Arabidopsis* genome (Verhey and Hammond, 2009). Kinesin-14 is the most important family to the Caplan lab due to its role in chloroplast movement. Two Kinesin-like proteins (KAC1 and KAC2) are thought to mediate actin-based movement in *Arabidopsis*. A *kac1* mutant partly disrupted chloroplast movement and a *kac1kac2* double mutant completely stops chloroplast movement (Suetsugu, 2012). KAC has also been tied to Kinesin family 14 (Zhu et al., 2012). This led our lab to believe that one or more kinesins in the kinesin-14 family plays a role in stromule formation and function. A previous lab member tested the 8 most common kinesin-14s by transient overexpression. The 8 chosen were KAC1, KAC2, At1g09170, At3g10310, At3g44730, At5g27000, At1g63640 Kinesin for Stromule Extension 1 (KSE1), and At5g41310 Kinesin for Stromule Extension 2 (KSE2). Among the 8 chosen, KSE1 and KSE2 proved to be the most promising for playing a role in stromule formation and function. When KSE1 and KSE2 were overexpressed, stromule length increased and so did chloroplast velocity. KSE2 had a greater effect on stromule function so we

decided to proceed with only KSE2 in further studies (Alqarni, 2017). The Dinesh-Kumar lab generated eight KSE2 deletion constructs each removing 1 or more domains for a full domain analysis to better understand the role of each domain.

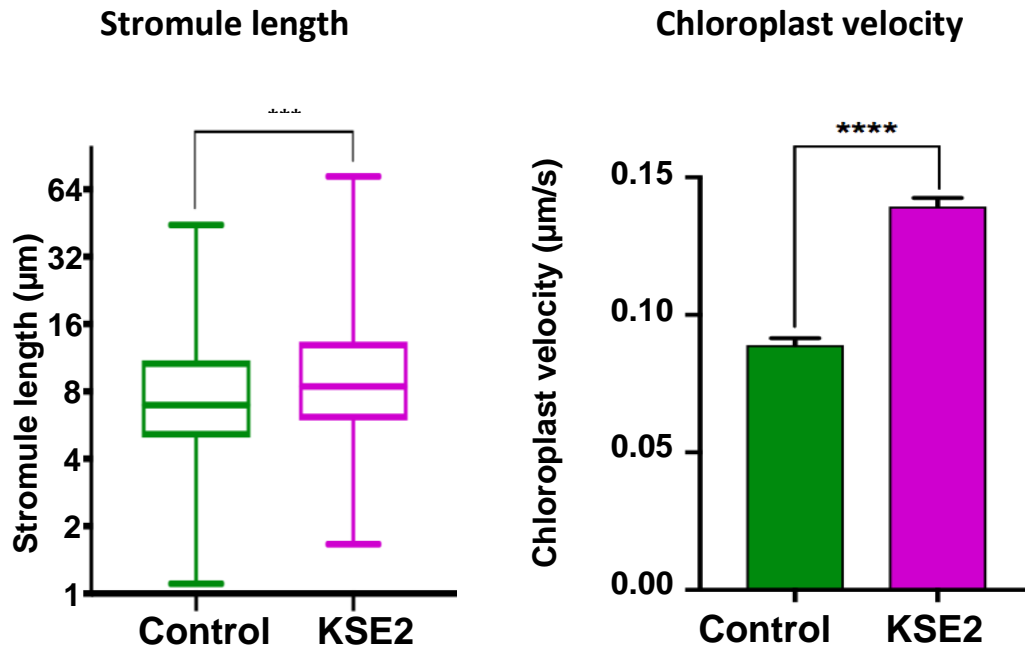


Figure 1.5 Overexpression of KSE2 alters stromules dynamics

A previous screen done by Ali Alqarni in our lab looked at 8 different kinesin-14s to see if overexpression altered stromule and chloroplast dynamics. KSE2 overexpression resulted in an increase in stromule length and chloroplast velocity (Alqarni, 2017).

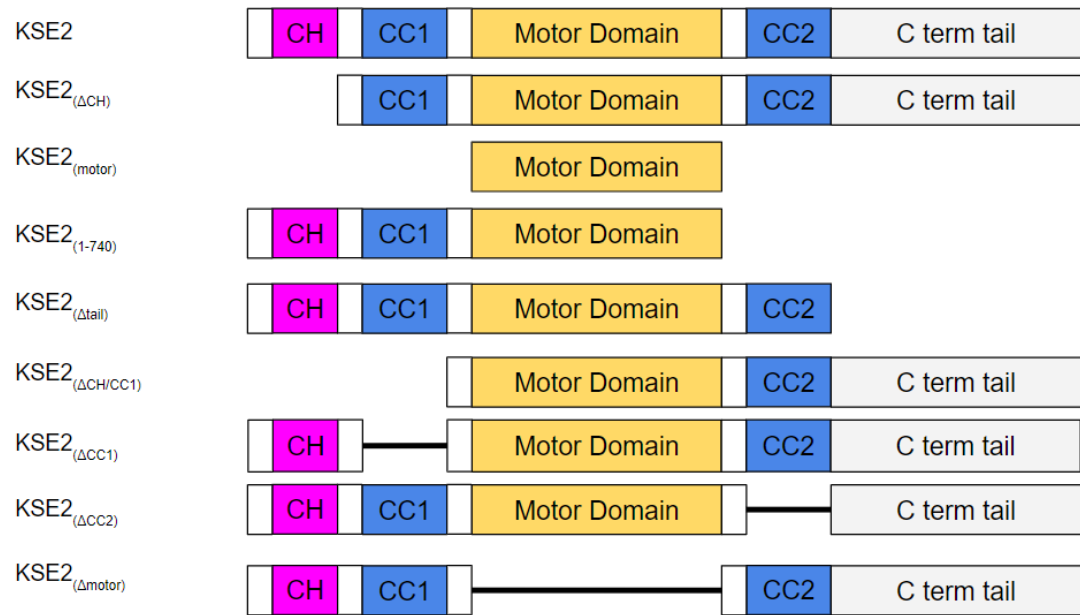


Figure 1.6 KSE2 Deletion Constructs

The previous figure showed that KSE2 altered stromule and chloroplast dynamics. We decided to generate deletion constructs for a full domain analysis. These constructs were generated by the Dinesh-Kumar lab using CRISPR/Cas9. The CH domain stands for the Calponin Homology Domain, or the actin binding domain. The CC1 domain stands for Coiled-Coil 1, or the dimerization domain. The motor domain is the ATP motor. CC2 is the Coiled-Coil 2 domain response for processive movement. Finally, the C terminal tail is responsible for stabilization. The normal functioning KSE2 will be referred to sometimes as the full length (FL).

1.5 Rationale and Goals

Our understanding is that stromules extend along microtubules using kinesins. Our lab used a candidate approach to identify a specific kinesin used for stromule function and movement. This was done by overexpressing 8 different kinesin-14s. The overexpression (OE) screen revealed 2 kinesins, Kinesin for stromule extension 1 (KSE1) and Kinesin for stromule extension 2 (KSE2). They are thought to play a role in stromule function and movement because when overexpressed they resulted in an increase in stromule length and chloroplast velocity (Figure 1.5). We proceeded with KSE2 only because it showed a larger impact on stromule function. Nathan Meier in the Dinesh-Kumar laboratory generated 8 KSE2 deletion constructs each removing 1 or more domains for a full domain analysis to better understand the role of each domain (Figure 1.6). My hypothesis is that KSE2 plays a role in stromule formation, function, and movement. This is accomplished by 1 overall project goal separated into 3 Aims:

1. *Characterize the role of KSE2 in stromule formation and function by*
 - A. *Characterizing stromule and chloroplast dynamics by overexpressing KSE2 Deletion constructs*
 - B. *Characterizing the interactions between KSE2 deletion constructs and the Microtubule (MT) and Actin (AF) network*
 - C. *Characterizing the effects of KSE2 during Plant innate Immunity*

Chapter 2

MATERIALS AND METHODS

2.1 Plant Line and Visualization of Chloroplasts and Stromules

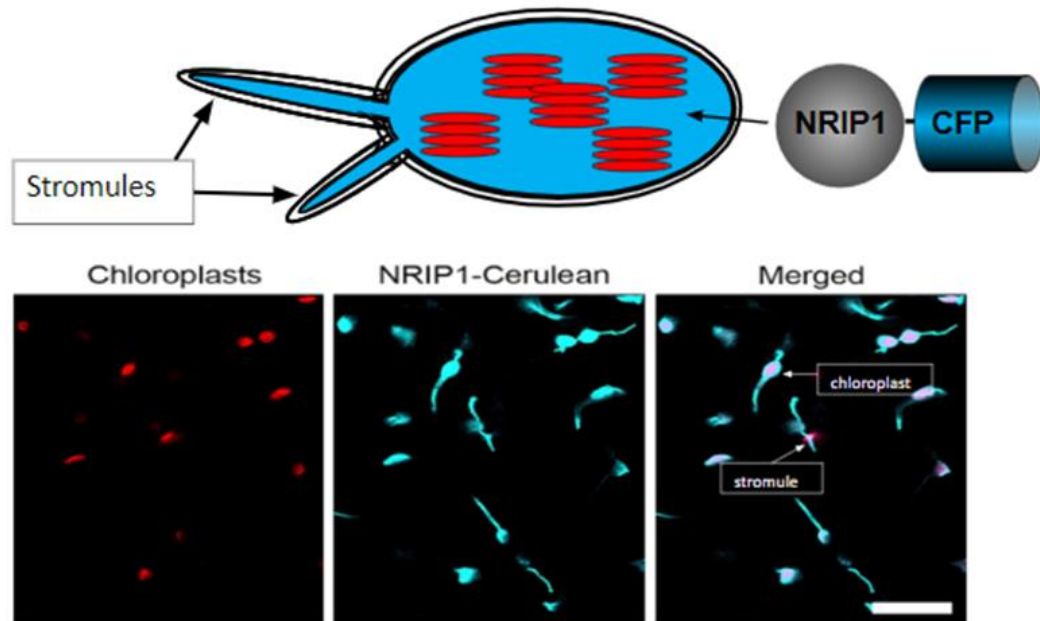


Figure 2.1 Visualization of Stromules Using Confocal Microscopy

Confocal microscopy is a powerful tool to do live cell imaging. Our lab uses confocal microscopy to observe chloroplast and stromule dynamics. The thylakoid stacks inside chloroplasts autofluoresce which allows us to visualize them (red). We use a fluorescent protein marker to view stromules which is the chloroplastic protein NRIP1 fused to a cyan fluorescent protein (NRIP1-CFP) (cyan).

2.2 Plasmids

Table 2.1 The Plasmids

Stock No.	Name	Construct	Selection	Agrobacterium Strain
NDM01	KSE2 Full Length (KSE2 _(FL))	pUBQ-3xMyc-tRFP-At5g41310	KRG	Gv3101
NDM02	KSE2 No Calponin Homology	pUBQ-3xMyc-tRFP-At5g41310	KRG	Gv3101
NDM03	KSE2 Only Motor (KSE2 _(Only Motor))	pUBQ-3xMyc-tRFP-At5g41310	KRG	Gv3101
NDM04	KSE2 No Coil-Coil 2 and C Terminal Tail	pUBQ-3xMyc-tRFP-At5g41310	KRG	Gv3101
NDM05	KSE2 No C Terminal Tail (KSE2 _(ΔTail))	pUBQ-3xMyc-tRFP-At5g41310	KRG	Gv3101
NDM06	KSE2 No Calponin Homology and	pUBQ-3xMyc-tRFP-At5g41310	KRG	Gv3101
NDM07	KSE2 No Coil-Coil 1 (KSE2 _(ΔCC1))	pUBQ-3xMyc-tRFP-At5g41310	KRG	Gv3101
NDM08	KSE2 No Coil-Coil 2 (KSE2 _(ΔCC2))	pUBQ-3xMyc-tRFP-At5g41310	KRG	Gv3101
NDM09	KSE2 No Motor (KSE2 _(ΔMotor))	pUBQ-3xMyc-tRFP-At5g41310	KRG	Gv3101
SPDK2422	TagRFP	tRFP-sfGFP11	SSRC	Gv2260
SPDK2885	Microtubule-Citrine	35S-Citrine-ACK6	SSRC	Gv2260

PAN509	Actin-YFP	Fabd2-eYFP	SSRC	Gv3101
SPDK2464	P50-Citrine	3xHA-P50-Citrine	SSRC	Gv2260
TBS67	Citrine Alone	Citrine	SSRC	Gv2260
SPDK3674	p50-3xHA XVE	XVE:p50-3xHA	SRG	Gv2260
SPDK1690	TagRFP XVE	XVE::tagRFP	SRG	Gv2260
SPDK1700	TagRFP-p50 XVE	XVE::tagRFP-p50	SRG	Gv2260
SPDK3668	Citrine-p50 XVE	XVE::Citrine-p50	SRG	Gv2260

Key:

SPDK: Savithramma P. Dinesh-Kumar

NDM: Nathan Meier

TBS: Tessa Burch-Smith

KRG: Kanamycin, Rifampicin, Gentamicin

SSRC: Spectinomycin, Streptomycin, Rifampicin, Carbenicillin

SRG: Spectinomycin, Rifampicin, Gentamicin

2.3 Plant Material and Growth Conditions

Transgenic *N. benthamiana* plants expressing NRIP1-Cerulean (NN 59.8.3).

Transgenic *N. benthamiana* plants not containing NRIP1-Cerulean were used as well (NN). All plants were grown in a growth chamber at 175 μmol light intensity and 23.0°C for 3-5 weeks.



Figure 2.2 Leaf-Stage *Nicotiana benthamiana* Plant

Pictured above is a properly aged *N. benthamiana* plant used for experimentation.

2.4 Agrobacterium Preparation

Agrobacterium was stored at -80°C in 50% glycerol. Single colonies used to start the frozen stocks were struck on their respective antibiotic plates. Protocol for how we make the antibiotic stock and plates are below. Followed by how we grow agrobacterium from the frozen stock.

2.4.1 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. Pre-wet 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.

- 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 H_2O 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 H_2O .
 3. 0.22 μm syringe filter by drawing through 5-10 ml of sterile H_2O 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 H_2O .

3. 0.22 μm syringe filter by drawing through 5-10 ml of sterile H_2O

and discard water.

5. Sterilize kanamycin stock through the prepared 0.22 μm syringe filter.

2.4.2 Protocol to Make Antibiotic Plates

- This recipe is for 500 mL of LB agar. This makes about 24 plates.

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

2. Allow the flask to cool on a bench.

3. Add antibiotics and swirl the bottle to mix.

4. Using a serological pipette, add approx. 20 mL of liquid agar to each plate

2.4.3 Growing and Streaking Agrobacterium

Agrobacterium is struck from a -80°C frozen stock to the respective antibiotic plate and grown in a 28°C incubator. After proper growth, the agrobacterium is ready for the next step.

2.5 Agrobacterium Infiltration of the Plant

Agrobacterium is mixed with infiltration media then infiltrated into the leaf the next day approximately 48 hours before imaging.

2.5.1 Agrobacterium Prep with Infiltration Media

After proper growth, agrobacterium is mixed into infiltration media (protocol below) and read on a photometer for an OD of 1.0. If two constructs were being overexpressed together, the total OD would be 1.5.

Infiltration Media Contents

50 mL of H₂O

0.1 grams of MgCl₂

0.1 grams of MES

0.1 mL of acetosyringone

The agrobacterium/infiltration media mixture will sit on the benchtop overnight to allow agrobacterium vir gene production required for plasmid integration into the host.

2.5.2 Infiltration of the Plant Leaf

Take a razor blade and poke a small incision on the top surface of the leaf. Then using a 1 mL plastic syringe, fill the syringe with infiltration media and inject the medium from the syringe into the incision hole on the leaf while also holding a finger under the

incision hole to stabilize the leaf. Dry off the leaf with a kimwipe and label the infiltrated regions with a sharpie. Allow the plant to sit 48 hours on the benchtop to allow plasmid integration and expression of your construct.

2.6 Leaf Preparation for Imaging

Using a razor blade, make a right trapezoid from the leaf. Making this shape ensures you know which side is which. Fill a 5 mL syringe halfway with water and drop the leaf inside. Put the plunger into the syringe and push out all the air. Pull the plunger 3 times to push water into the leaf sample. Remove the sample from the syringe and place the sample on a single well Nunc Chamber Coverglass with a drop of water on the slide. Place a glass block onto the Nunc Chamber Coverglass and remove air bubbles from the sample.

2.7 Laser Scanning Confocal Microscopy

N. benthamiana leaf sections were imaged as a Z-stack time series on a Zeiss LSM 710 confocal microscope or LSM 880 confocal microscope fitted with 40X C-Apochromat water immersion objective (Numerical Aperture=1.2) used for live cell imaging. When imaging on the LSM 880, Airyscan was used.

2.8 Image Processing

Image processing was performed using Huygens Professional. Huygens was used to deconvolve the images. Airyscan images were processed by airyscan processing in

Zen. After processing the images were made in maximum intensity projections (MIPS). Fiji, a version of ImageJ was also used to correct drift by a macro to align the videos.

2.9 Manual Quantification of Stromule and Chloroplast Dynamics

Partial quantification was performed by hand in ImageJ. This includes branches of stromules and the total number of stromules and chloroplasts. We counted these by hand because branching was hard for our automatic pipeline to correctly quantify. It had a hard time distinguishing either side of a branch, as well as the branch junctions. For that reasoning it made sense to hand count branches, stromules and chloroplasts in 3 timepoints in all samples.

2.10 Automatic Quantification of Stromule, Chloroplast Dynamics and Cytoskeleton Dynamics using a Deep Learning Pipeline

There are two separate pipelines used to mask our images. The two pipelines are two separate convolutional neural U-networks. Training involves the network running a series of filters over the image learning features and parts of the image. The output of the training are weights that are trained to label features and objects in an image.

Training is done over multiple iterations with the goal to minimize error. We have one pipeline that can mask cytoskeleton such as actin and microtubules and the other masks chloroplasts and stromules. After segmentation of the masks, we want to quantify the labeled features. Our lab is interested in tracking stromule dynamics, we

use active contour method (snakes). Which positions 10 control points over the length of a stromule then tracks the points over the time series. This is done using Matlab. We also use a similar Matlab tracking output to quantify the characteristics of the cytoskeleton too. The characteristics we are interested in quantifying, as well as a visual walkthrough of the pipeline is in Figure 2.3 and Figure 2.4.

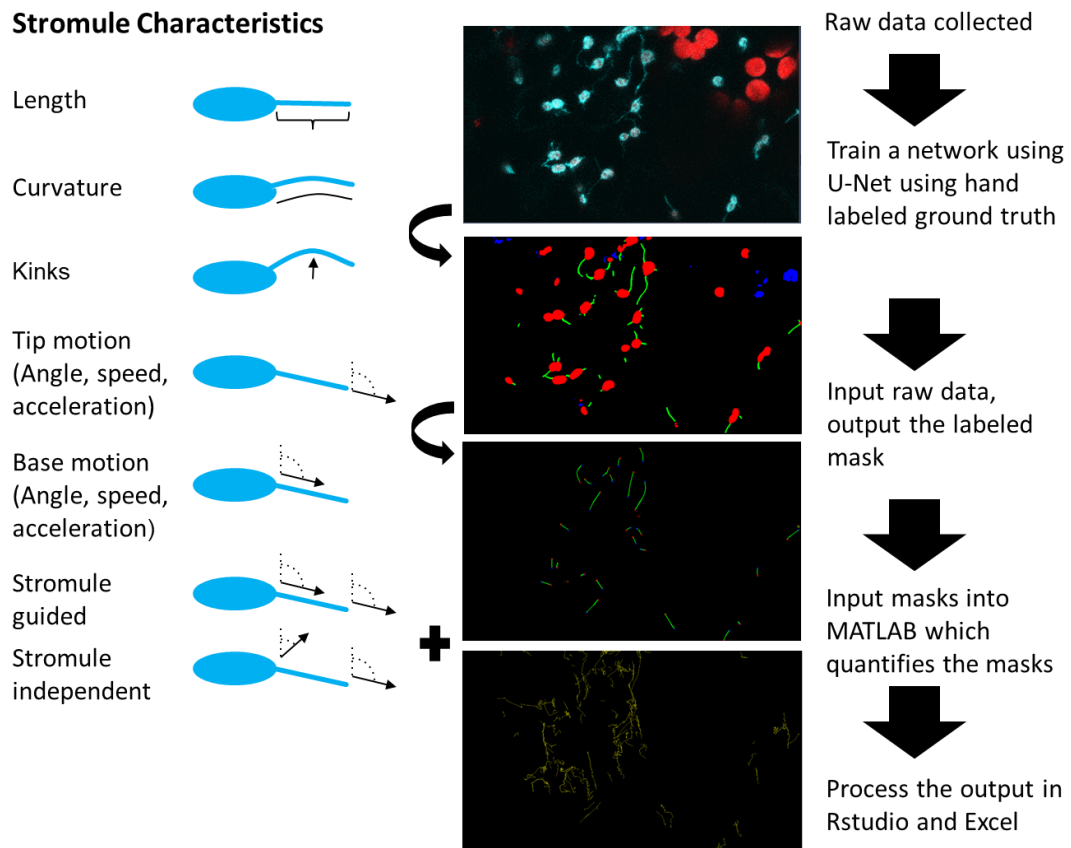


Figure 2.3 Visual Representation of the Stromule Analysis Pipeline

In the figure above, listed are the various characteristics we care about quantifying for both stromules and chloroplasts. The flow chart follows how we go from raw data to the finished output.

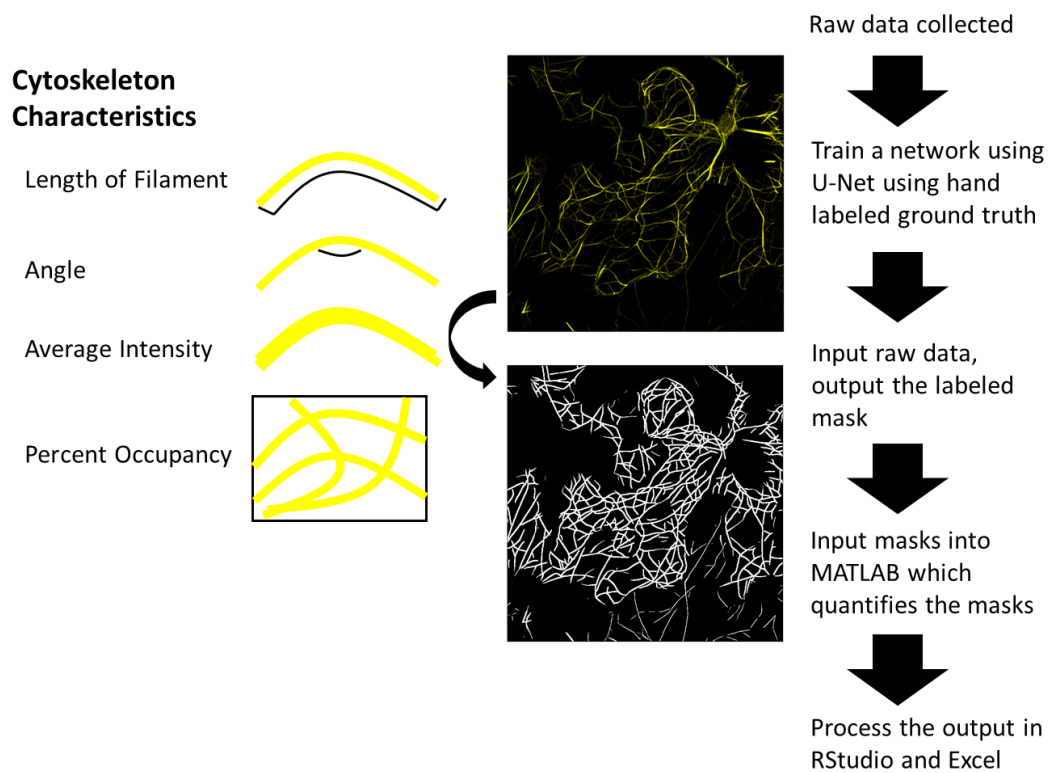


Figure 2.4 Visual Representation of the Cytoskeleton Analysis Pipeline

In the figure above, listed are the various characteristics we care about quantifying for both actin microfilaments and microtubules. The flow chart follows how we go from raw data to the finished output.

2.11 Statistical Analysis

All statistical analysis graphs and tables were performed using Microsoft Excel, RStudio and GraphPad. Specific tests used Dunnett's test to compare multiple overexpression constructs to the control. Two-way Anova was used during electrolyte leakage analysis.

Chapter 3

RESULTS

3.1 Introduction

Chloroplasts play an important role in plant defense by sending various signals to the nucleus to induce the defense response. To assist in this process, chloroplasts induce stroma filled extensions called stromules to better transfer signals and molecules into the nucleus. Previous research has shown to move throughout the cell, chloroplasts interact with both actin and microtubules. Looking specifically at stromules, stromules anchor onto actin while extending along microtubules (Kumar et. al., 2018). Kinesin family 14 has been shown to play a role in chloroplast movement so we hypothesized that a kinesin from kinesin family 14 was responsible for stromule extension and function. A previous lab member tested the 8 most common kinesin-14s by transient overexpression. Among them, KSE1 and KSE2 were the most promising however only KSE2 was taken further due to it having a greater effect on stromules than KSE1. Our lab hypothesized that a kinesin, specifically KSE2 plays a role in stromule formation, function, and movement. The Dinesh-Kumar lab generated 8 KSE2 deletion constructs each removing 1 or more domains for a full domain analysis to better understand the role of each domain.

3.2 Aim 1: Characterize Stromule and Chloroplast Dynamics by Overexpressing KSE2 Deletion Constructs:

3.2.1 Sub Aim A: KSE2 Full Length Causes Changes in Stromule Dynamics

To confirm what was done by a previous graduate student, I first focused on overexpressing KSE2 and expanding the stromule characteristics quantified before moving onto the deletion constructs generated. The characteristics quantified were stromule per chloroplast, branches per stromule, stromule length, stromule tip velocity, chloroplast base velocity (All lengths and velocities were separated by extension or retraction events), percentage of extension vs retraction events, percentage of stromule directed movement and percentage of kinks. These characteristics were chosen to assess and quantify if overexpressing KSE2 alters chloroplast and stromule dynamics. We overexpressed the KSE2-TagRFP construct for two days before collecting time lapse Z-stack images. Part of the data was counted by hand, stromule induction (Figure 3.2), stromule branching (Figure 3.3) and kinks (Figure 3.4). For stromule induction or the total number of stromules per chloroplast we saw a significant increase of stromules compared to the TagRFP control (Figure 3.2). For branching, there was a significant increase in stromule branching when compared to the TagRFP control (Figure 3.3). There was increased number of stromule kinks compared to the TagRFP control as well (Figure 3.4). The other characteristics, stromule length, stromule tip velocity, chloroplast base velocity and stromule directed movement was quantified by our automatic quantification pipeline detailed in Figure 2.3. For these characteristics stromule and chloroplast dynamics were altered compared to the TagRFP control (Figures 3.5 - 3.8).

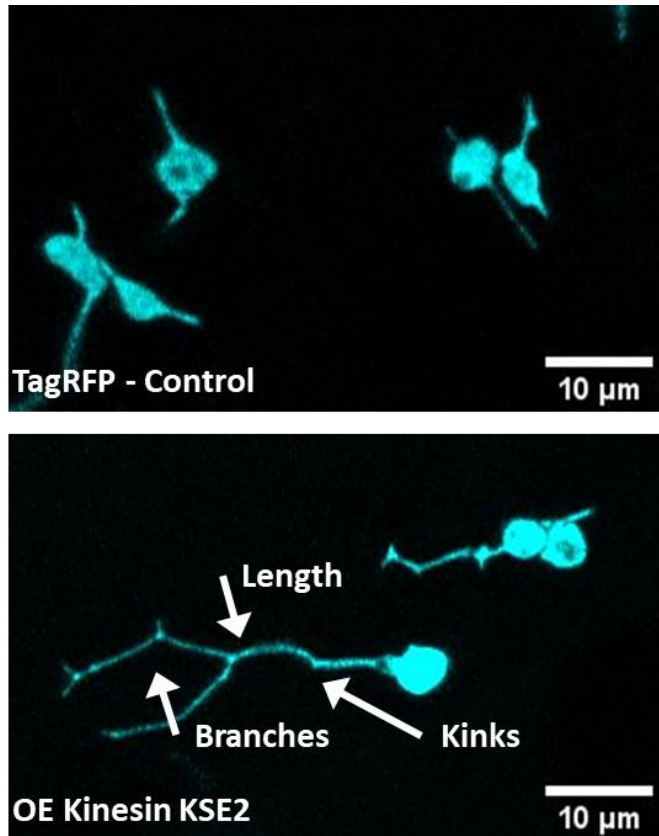


Figure 3.1 Overexpression of KSE2 results in altered stromule dynamics

This figure shows that by overexpressing KSE2 we see altered stromule dynamics. Specifically, we see increased kinks, increased length, and increased branches, as seen above. The arrows point to the altered characteristic. KSE2 was overexpressed in our transgenic NRIP1-Cerulean *N. bethamiana* plants. KSE2 was labelled using TagRFP and NRIP1-CFP labels chloroplasts and stromules.

3.2.1.1 Overexpression of KSE2 results in increased stromule induction

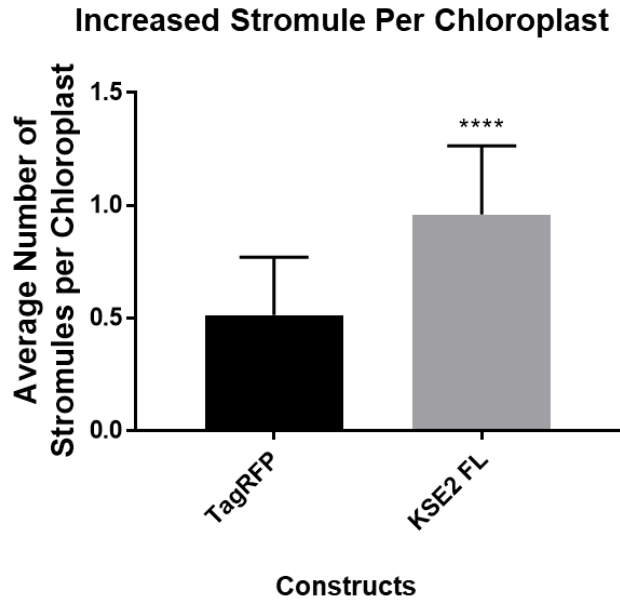


Figure 3.2 Quantification of the increased stromule induction of KSE2

This figure quantifies the KSE2 images taken. Images with good expression were chosen and the number of stromules and chloroplasts were manually counted in three different time points in the series. The chosen time points are 1, 15 and 30. We see that the difference in means is significantly different between KSE2 and the TagRFP control. Overexpressing KSE2 results an increase in the total number of stromules. Mean of TagRFP: 0.5134, Mean of KSE2: 0.9002 with a P-value of <0.0001.

3.2.1.2 Overexpression of KSE2 results in increased branching stromules

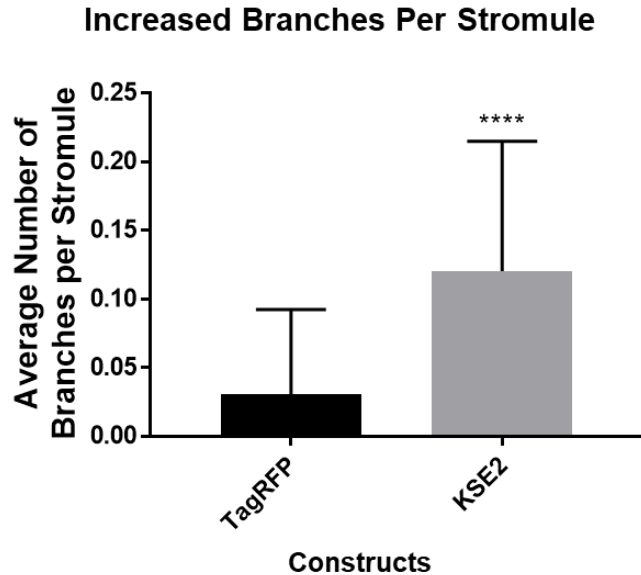


Figure 3.3 Quantification of the increased stromule branching of KSE2

Images with good expression were chosen and the number of branch points and number of stromules were manually counted in three different time points in the time-series. The chosen time points are 1, 15 and 30. We see that the difference in means is significantly different between KSE2 and the TagRFP control. Overexpressing KSE2 results an increase in branching stromules. Mean of TagRFP: 0.03079, Mean of KSE2: 0.1205 with a P-value of <0.0001.

3.2.1.3 Overexpression of KSE2 results in increased stromule kinks

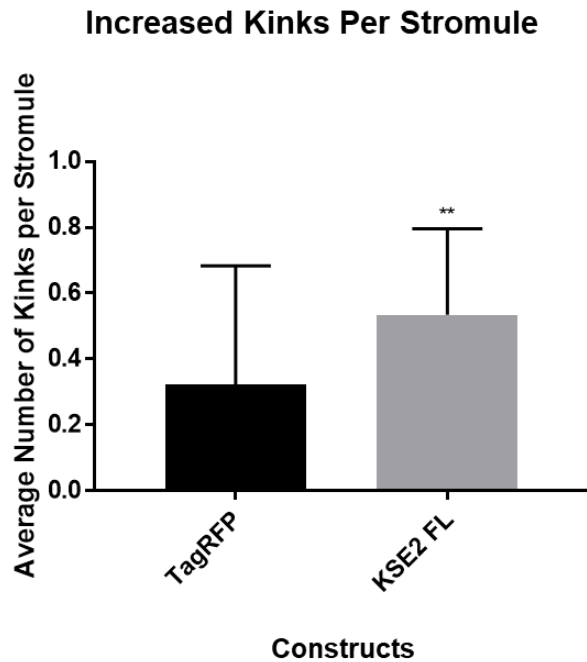


Figure 3.4 Quantification of the increased stromule kinks

Images with good expression were chosen and the number of kinks and number of stromules were manually counted in three different time points in the time-series. The chosen time points are 1, 15 and 30. We see that there is a significant difference between KSE2 and the TagRFP control. Overexpressing KSE2 results an increase in stromule kinks. Mean of TagRFP: 0.3222, Mean of KSE2: 0.5346 with a P-value of 0.0019.

3.2.1.4 Overexpression of KSE2 results in increased stromule length

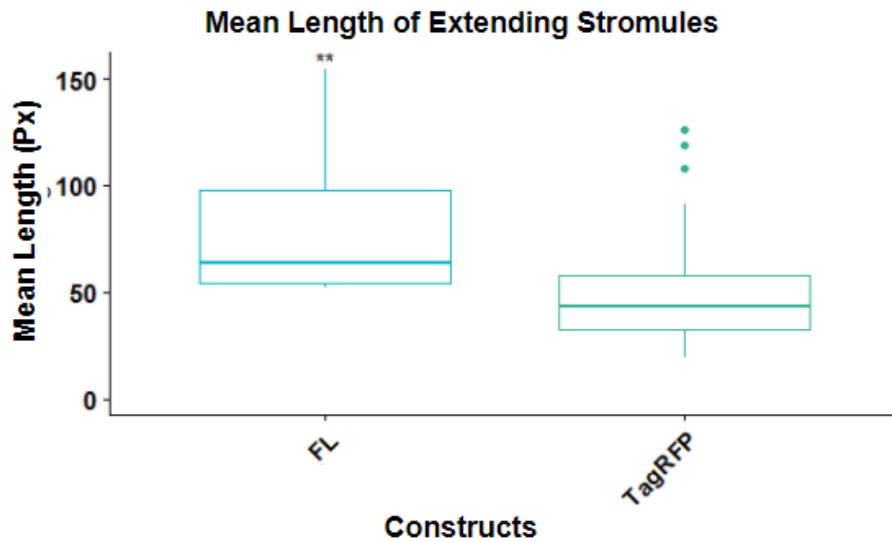


Figure 3.5 Quantification of the length of extending stromules

Images with good expression were masked using the deep learning pipeline then analyzed in MATLAB. The output is the mean length of all stromules in the video. The lengths are then separated by event type, extending, and retracting because stromules are typically longer during extension. This figure looks at extension. Then we can find the mean of that to compare significance. We see that the difference in means is significantly different between KSE2 and the TagRFP control. Overexpressing KSE2 results an increase in stromule length for extending stromules. Mean of TagRFP: 49.386, Mean of KSE2: 80.085 with a P-value of 0.0016.

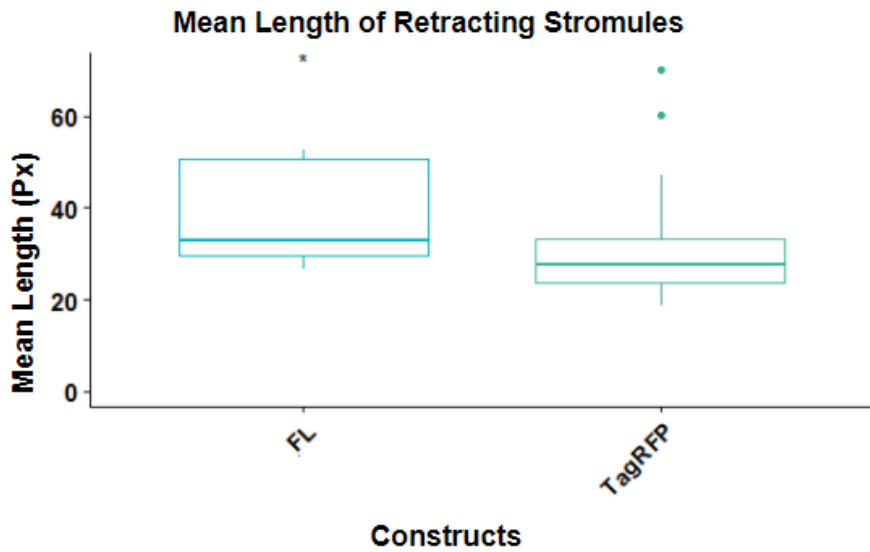


Figure 3.6 Quantification of the length of retracting stromules

Images were masked using the deep learning pipeline then analyzed in MATLAB. The output is the mean length of all stromules in the video. The lengths are then separated by event type, extending, and retracting because stromules are typically longer during extension. This figure looks at retraction. Then we can find the mean of that to compare significance. We see that the difference in means is significantly different between KSE2 and the TagRFP control. Overexpressing KSE2 results an increase in stromule length for retracting stromules. Mean of TagRFP: 30.951, Mean of KSE2: 39.301 with a P-value of 0.013.

3.2.1.5 Overexpression of KSE2 results in increased stromule tip velocity

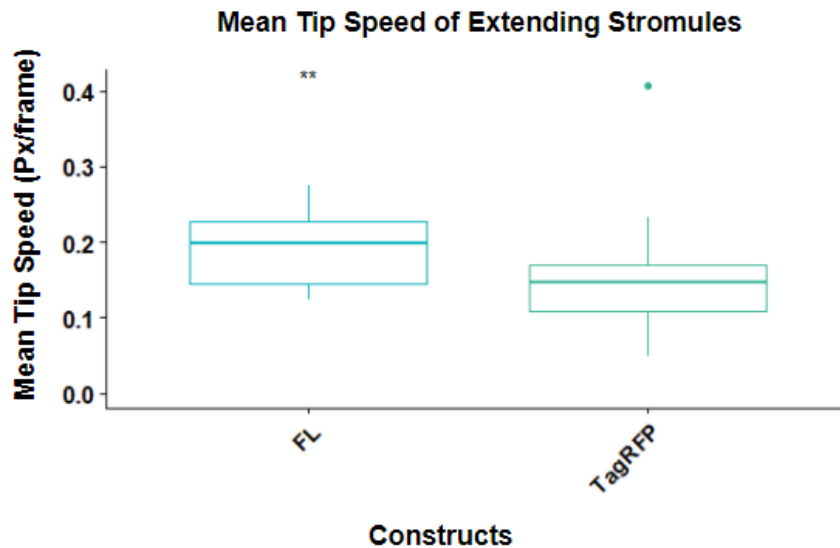


Figure 3.7 Quantification of stromule tip velocity of extending stromules

Images were masked using the deep learning pipeline then analyzed in MATLAB. The output is the mean tip speed of all stromules in the video. The velocities are then separated by event type, extending, and retracting because stromules are typically faster during extension. This figure looks at extension. Then we can find the mean of that to compare significance. We see that the difference in means is significantly different between KSE2 and the TagRFP control. Overexpressing KSE2 results an increase in stromule velocity for extending stromules. Mean of TagRFP: 0.1426, Mean of KSE2: 0.1916 with a P-value of 0.0068.

3.2.1.6 Overexpression of KSE2 results in increased chloroplast base velocity

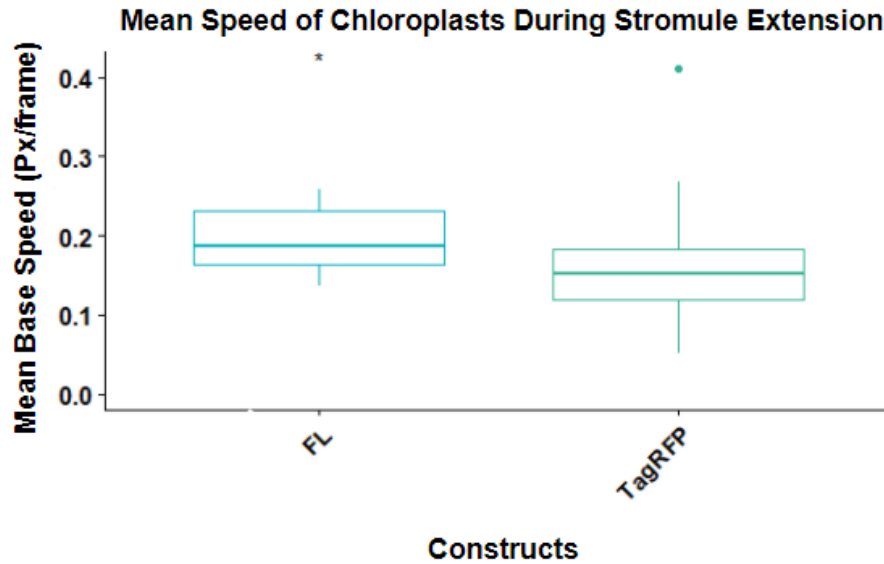


Figure 3.8 Quantification of chloroplast base velocity of extending stromules

Images were masked using the deep learning pipeline then analyzed in MATLAB. The output is the mean base speed of all chloroplasts in the video. The velocities are then separated by event type, extending, and retracting because there is typically more movement during extension. This figure looks at extension. Then we can find the mean of that to compare significance. We see that the difference in means is significantly different between KSE2 and the TagRFP control. Overexpressing KSE2 results an increase in chloroplast velocity during stromule extending events. Mean of TagRFP: 0.1572, Mean of KSE2: 0.1934 with a P-value of 0.0369.

3.2.2 Sub Aim B: Overexpression of KSE2 Deletion Constructs Causes Changes in Stromule Morphology

In addition to expanding the characteristics quantified we wanted to fully characterize KSE2 by analyzing the domains. We achieved this by creating eight deletion constructs with various domains removed to observe the result on stromule and chloroplast dynamics (Figure 1.6). The characteristics quantified were stromule per chloroplast, branches per stromule, stromule length, stromule tip velocity, chloroplast base velocity (All lengths and velocities were separated by extension or retraction events), percentage of extension vs retraction events, percentage of stromule directed movement and percentage of kinks. This will be broken up into two parts, stromule morphology and stromule dynamics. When quantified we wanted to see how the loss of certain domains affected the stromule and chloroplast characteristics chosen. We hypothesized that removing certain domains would result in altered stromule and chloroplast dynamics. We overexpressed the KSE2-TagRFP deletion constructs for two days before collecting time lapse Z-stack images. Part of the data was counted by hand, stromule induction, stromule branching. For stromule induction or the total number of stromules per chloroplast we saw FL, Δ CH, Only Motor and Δ CC1 had a significant increase in the number of stromules compared to the TagRFP control (Figure 3.9). For branching, FL and Δ CH had a significant increase in stromule branching when compared to the TagRFP control (Figure 3.10). Stromule length for extending stromules was increased for FL, Only Motor, Δ CC1 and Δ Motor (Figure

3.11). For retracting stromules we saw a significant difference in stromule length for FL, Δ CH and Only Motor (Figure 3.12).

3.2.2.1 Overexpressing KSE2 deletion constructs and the effect on stromule induction

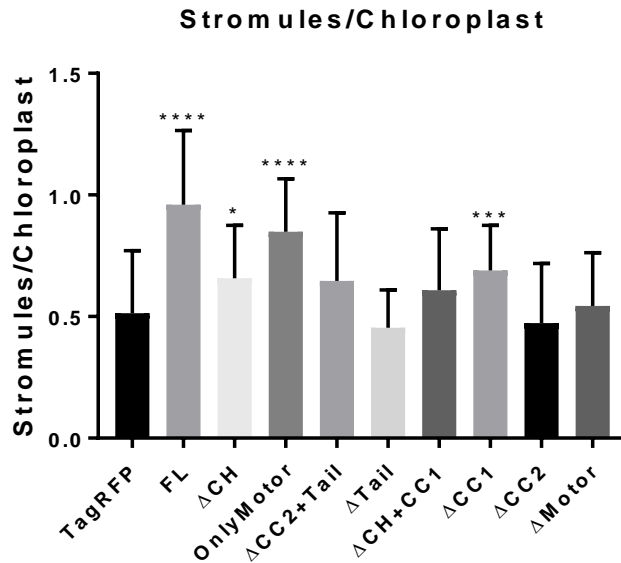


Figure 3.9 Quantification of the increased stromule induction of KSE2 deletion constructs

This figure quantifies the various KSE2 deletions detailed above. Images were chosen and the number of stromules and chloroplasts were manually counted in three different time points in the series. The chosen time points are 1, 15 and 30. We can find the mean of that to compare significance. We compare the mean of TagRFP vs all other constructs. We see that the difference in means is significantly different between FL, Δ CH, OnlyMotor and Δ CC1. Mean of TagRFP: 0.5134, Mean of KSE2: 0.9602 with a P-value of <.0001, Mean of Δ CH: 0.6569 with a P-value of 0.0177, Mean of OnlyMotor 0.8483 with a P-value of <.0001, Mean of Δ CC2+Tail 0.6466, Mean of Δ Tail 0.4545, Mean of Δ CH+CC1 0.6078, Mean of Δ CC1 0.6891 with a P-value of .0007, Mean of Δ CC2 0.4725, Mean of Motor 0.5435.

3.2.2.2 Overexpressing KSE2 deletion constructs and the effect on stromule branching

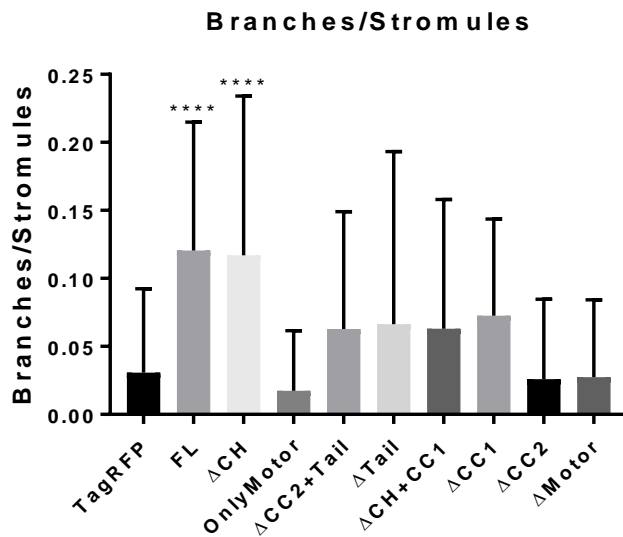


Figure 3.10 Quantification of the increased stromule branching of KSE2 deletion constructs

This figure quantifies the various KSE2 deletions detailed above. Images were chosen and the number of branching stromules were manually counted in three different time points in the series. The chosen time points are 1, 15 and 30. We can find the mean of that to compare significance. We compare the mean of TagRFP vs all other constructs. We see that the difference in means is significantly different for FL and ΔCH compared to the control. Mean of TagRFP: 0.03079, Mean of KSE2: 0.121 with a P-value of <.0001, Mean of ΔCH: 0.117 with a P-value of <.0001, Mean of OnlyMotor 0.0174, Mean of ΔCC2+Tail 0.0628, Mean of ΔTail 0.0664, Mean of ΔCH+CC1 0.0629, Mean of ΔCC1 0.07261, Mean of ΔCC2 0.0260, Mean of Motor 0.0275.

3.2.2.3 Overexpressing KSE2 deletion constructs and the effect on stromule length

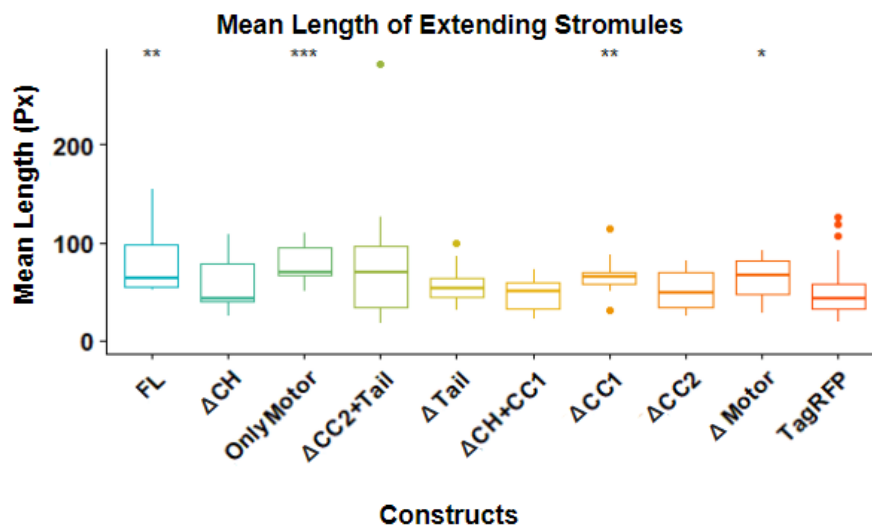


Figure 3.11 Quantification of the length of extending stromules of KSE2 deletion constructs

This figure quantifies the various KSE2 deletions detailed above. Images were masked using the deep learning pipeline then analyzed in MATLAB. The output is the mean length of all stromules in the video. The lengths are then separated by event type, extending, and retracting because stromules are typically longer during extension. This figure looks at extension. Then we can find the mean of that to compare significance. We compare the mean of TagRFP vs all other constructs. We see that the difference in means is significantly different between the TagRFP control and FL, OnlyMotor, ΔCC1 and ΔMotor. Mean of TagRFP: 49.3 , Mean of KSE2: 80.1 with a P-value of 0.013, Mean of ΔCH: 55.6, Mean of OnlyMotor 78.2 with a P-value of .00011, Mean of ΔCC2+Tail 84.3, Mean of ΔTail 57.1, Mean of ΔCH+CC1 47.6, Mean of ΔCC1 64.5 with a P-value of 0.033, Mean of ΔCC2 52.4, Mean of Motor 63.4 with a P-value of 0.19.

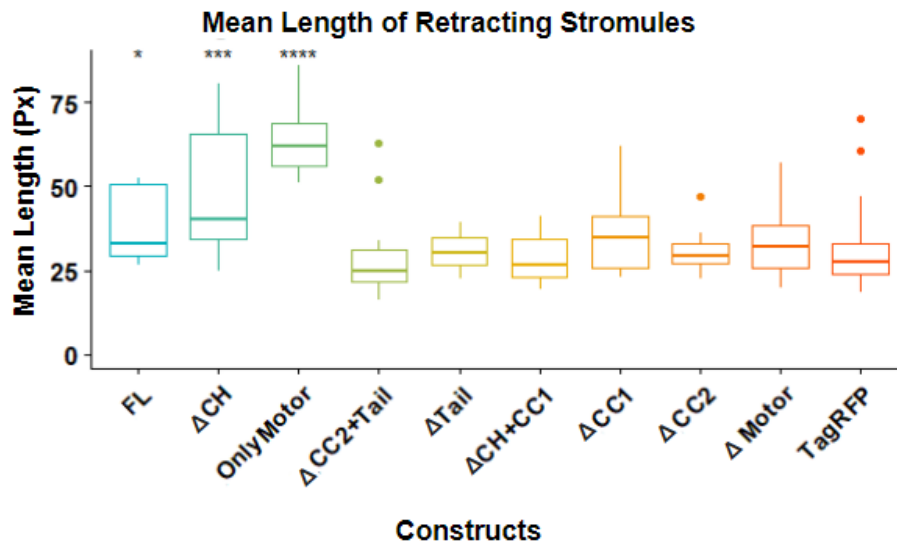


Figure 3.12 Quantification of the length of retracting stromules of KSE2 deletion constructs

This figure quantifies the various KSE2 deletions detailed above. Images were masked using the deep learning pipeline then analyzed in MATLAB. The output is the mean length of all stromules in the video. The lengths are then separated by event type, extending, and retracting because stromules are typically longer during extension. This figure looks at retraction. Then we can find the mean of that to compare significance. We compare the mean of TagRFP vs all other constructs. We see that the difference in means is significantly different between the TagRFP control and FL, ΔCH, OnlyMotor. Mean of TagRFP: 30.9, Mean of KSE2: 39.3 with a P-value of 0.092, Mean of ΔCH 48.8 with a P-value of 0.0031, Mean of OnlyMotor 63.7 with a P-value of 0.00000019, Mean of ΔCC2+Tail 30.1, Mean of ΔTail 30.6, Mean of ΔCH+CC1 28.5, Mean of ΔCC1 35.8, Mean of ΔCC2 30.9, Mean of Motor 33.1.

3.2.3 Sub Aim C: Overexpression of KSE2 Deletion Constructs Causes Changes in Stromule and Chloroplast Dynamics

The other characteristics which focus on stromule and chloroplast movement also were altered when overexpressed. These are stromule tip velocity and chloroplast base velocity. They were quantified by our automatic quantification pipeline detailed in Figure 2.3. For stromule velocity during stromule extension we saw FL, Δ CH, Only Motor, and Δ Tail were significantly different than the TagRFP control (Figure 3.13). For retracting stromules, Δ CC2 had significantly different stromule velocities than the TagRFP control (Figure 3.14). Chloroplast velocities were also altered when overexpressing KSE2. During stromule extension events these constructs had altered chloroplast velocities, FL and Δ Tail (Figure 3.15). During stromule retraction events Only Motor had altered chloroplast velocity (Figure 3.16).

3.2.3.1 Overexpressing KSE2 deletion constructs and the effect on stromule tip velocity

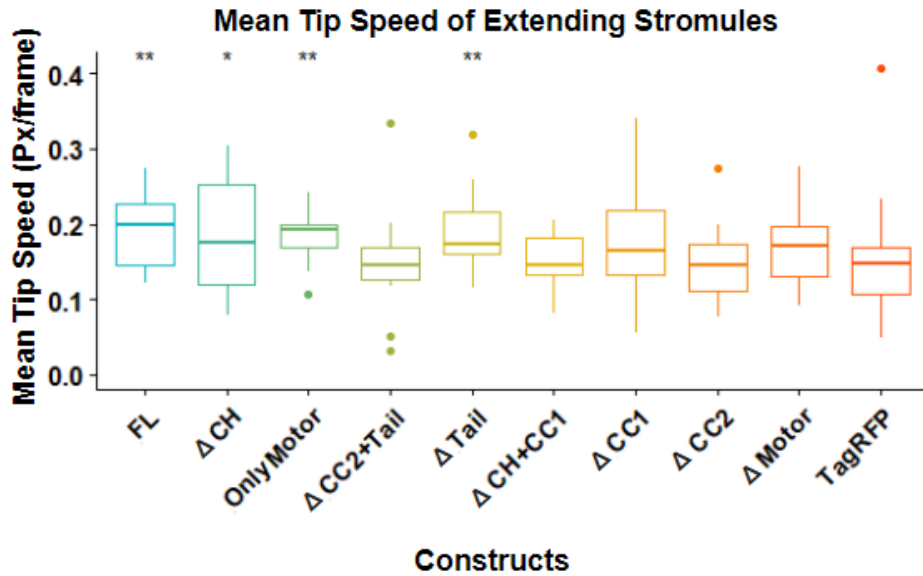


Figure 3.13 Quantification of stromule tip velocity of extending stromules of KSE2 deletion constructs

This figure quantifies the various KSE2 deletions detailed above. Images were masked using the deep learning pipeline then analyzed in MATLAB. The output is the mean stromule tip velocities of all stromules in the video. The tip speeds are then separated by event type, extending, and retracting because stromules are typically faster during extension. This figure looks at extension. Then we can find the mean of that to compare significance. We compare the mean of TagRFP vs all other constructs. We see that the difference in means is significantly different between the TagRFP control and FL, ΔCH, Only Motor and ΔTail. Mean of TagRFP: 0.142, Mean of KSE2: 0.191 with a P-value of 0.048, Mean of ΔCH 0.185 with a P-value of 0.240, Mean of OnlyMotor 0.182 with a P-value of 0.038, Mean of ΔCC2+Tail 0.151, Mean of ΔTail 0.192 with a P-value of 0.038, Mean of ΔCH+CC1 0.151, Mean of ΔCC1 0.178, Mean of ΔCC2 0.151, Mean of Motor 0.167.

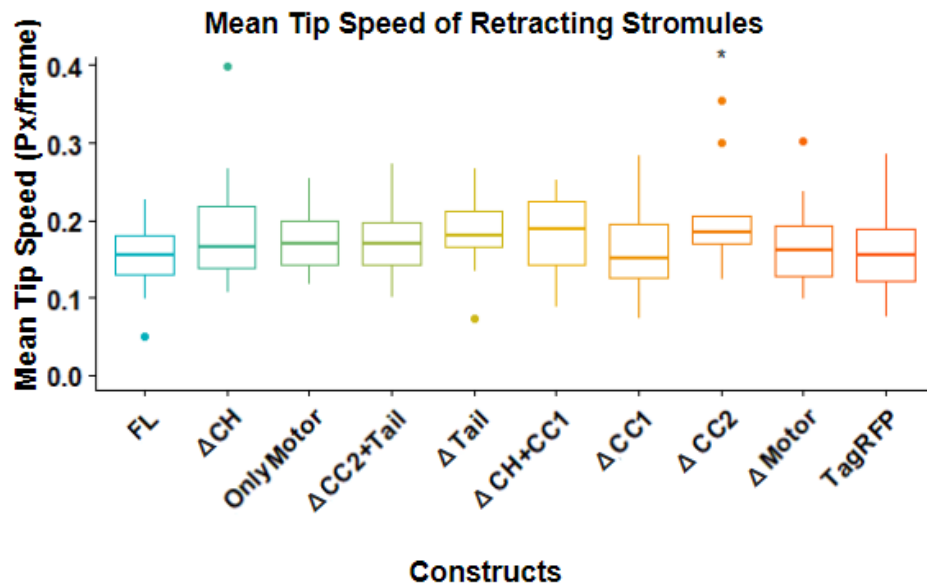


Figure 3.14 Quantification of the length of retracting stromules of KSE2 deletion constructs

This figure quantifies the various KSE2 deletions detailed above. Images were masked using the deep learning pipeline then analyzed in MATLAB. The output is the mean stromule tip velocities of all stromules in the video. The tip speeds are then separated by event type, extending, and retracting because stromules are typically more variable during extension. This figure looks at retraction. Then we can find the mean of that to compare significance. We compare the mean of TagRFP vs all other constructs. We see that the difference in means is significantly different between the TagRFP control and Δ CC2. Mean of TagRFP: 0.153, Mean of KSE2: 0.152, Mean of Δ CH 0.191, Mean of Only Motor 0.172, Mean of Δ CC2+Tail 0.172, Mean of Δ Tail 0.183, Mean of Δ CH+CC1 0.181, Mean of Δ CC1 0.164, Mean of Δ CC2 0.205 with a P-value of 0.17, Mean of Δ Motor 0.169.

3.2.3.2 Overexpressing KSE2 deletion constructs and the effect on chloroplast velocity

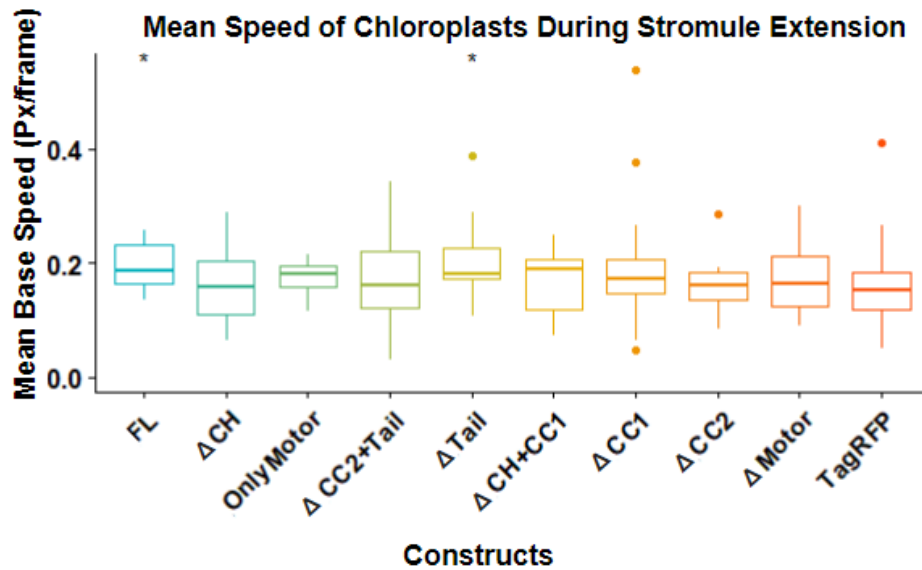


Figure 3.15 Quantification of chloroplast velocities during stromule extension events for the KSE2 deletion constructs

This figure quantifies the various KSE2 deletions detailed above. Images were masked using the deep learning pipeline then analyzed in MATLAB. The output is the mean stromule tip velocities of all stromules in the video. The tip speeds are then separated by event type, extending, and retracting because stromules are typically faster during extension. This figure looks at extension. Then we can find the mean of that to compare significance. We compare the mean of TagRFP vs all other constructs. We see that the difference in means is significantly different between the TagRFP control and FL, ΔTail. Mean of TagRFP: 0.157, Mean of KSE2: 0.193 with a P-value of 0.30, Mean of ΔCH 0.163, Mean of OnlyMotor 0.173, Mean of ΔCC2+Tail 0.171, Mean of ΔTail 0.207 with a P-value of 0.12, Mean of ΔCH+CC1 0.171, Mean of ΔCC1 0.197, Mean of ΔCC2 0.164, Mean of Motor 0.172.

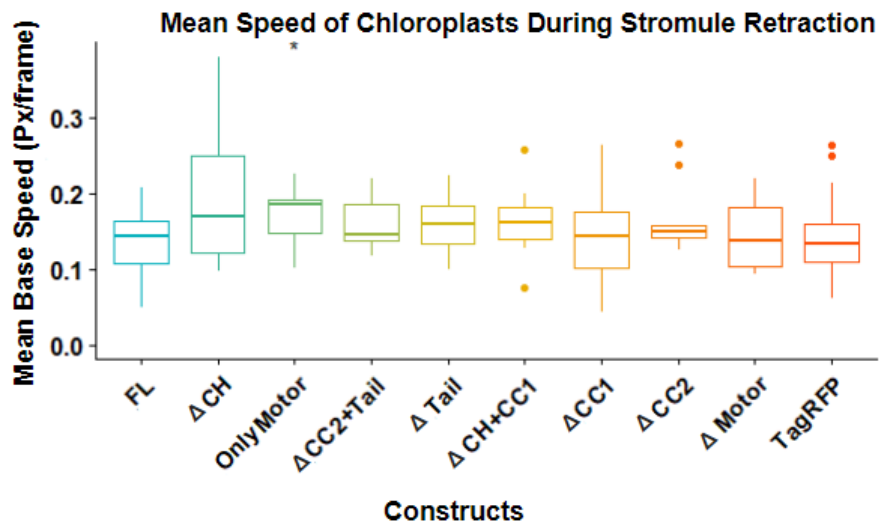


Figure 3.16 Quantification of chloroplast velocities during stromule retraction events for the KSE2 deletion constructs

This figure quantifies the various KSE2 deletions detailed above. Images were masked using the deep learning pipeline then analyzed in MATLAB. The output is the mean stromule tip velocities of all stromules in the video. The tip speeds are then separated by event type, extending, and retracting because stromules are typically faster during extension. This figure looks at retraction. Then we can find the mean of that to compare significance. We compare the mean of TagRFP vs all other constructs. We see that the difference in means is significantly different between the TagRFP control and Only Motor. Mean of TagRFP: 0.138, Mean of KSE2: 0.136, Mean of Δ CH 0.188, Mean of Only Motor 0.171 with a P-value of 0.17, Mean of Δ CC2+Tail 0.162, Mean of Δ Tail 0.159, Mean of Δ CH+CC1 0.162, Mean of Δ CC1 0.143, Mean of Δ CC2 0.166, Mean of Motor 0.147.

3.3 Aim 2: Characterize the Interactions Between KSE2 Deletion Constructs and the Microtubule (MT) and Actin (AF) Network:

3.3.1 Sub Aim A: Observe KSE2 Deletion Constructs Contact Points with Microtubule (MT) and Actin (AF) Network:

In Figure 2.3 and 2.4, the workflow for the analysis pipeline is laid out for stromule and cytoskeleton masking and quantification. Raw images are masked, followed by MATLAB analysis which quantifies the desired characteristics. Aim 1 used the stromule pipeline in isolation to observe changes in stromule and chloroplast dynamics when overexpressing KSE2 and KSE2 deletion constructs. There is also a pipeline that can mask the plant cytoskeleton and quantify the desired characteristics as well. Although observing the two pipelines separately gives us valuable information, combining the two masks together can give us more information to why overexpressing certain deletion constructs results in the way that they do. We do this by overlaying the stromule tracks onto the cytoskeleton mask then by classifying whether the pixels overlap or not we can quantify if a stromule is making contact with the cytoskeleton. This can be broken down into 4 categories. Frame to frame we can categorize stromules as overlapping or not overlapping with the cytoskeleton. Then we can further break down overlapping stromules into two more categories, whether the stromule is anchored to the cytoskeleton or whether the stromule is traversing along the cytoskeleton. This can be done by looking at stromule movement frame to frame, if the stromule has not moved between 3 frames then the stromule is considered

anchored. If the stromule is consistently overlapping with the cytoskeleton between 3 frames, then the stromule is considered traversing.

3.3.1.1 KSE2 deletion constructs changes contact points with the actin network

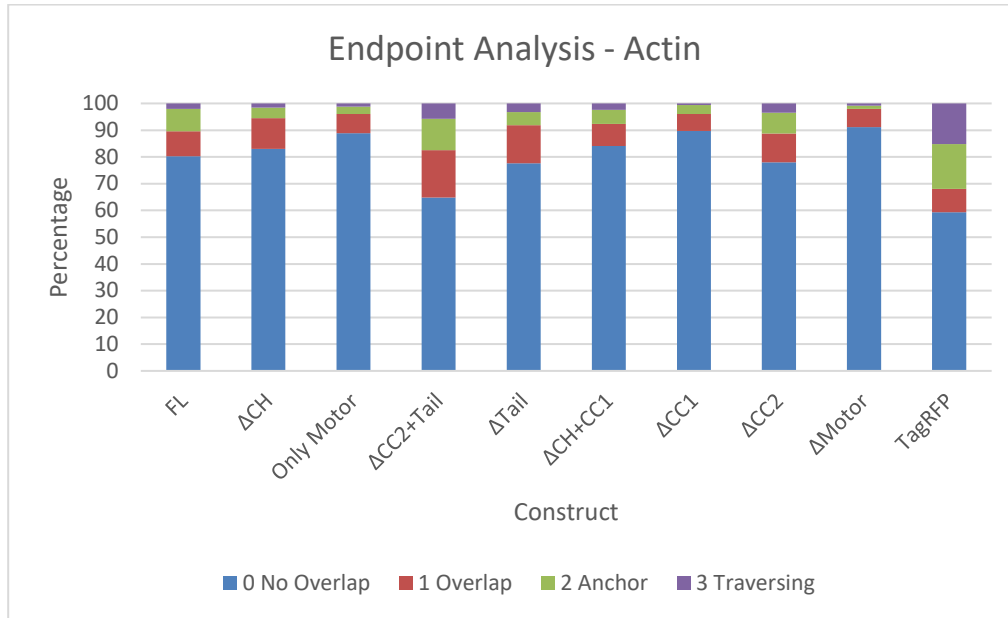


Figure 3.17 Stromule endpoint and actin network analysis

By taking both masks (Cytoskeleton and Stromule) we can overlay the two maximum intensity projections (MIPs) on top of one another to quantify contact points between the stromule tips and the cytoskeleton. Each stromule tip is broken down into 4 categories, not overlapping, overlapping, anchored or traversing. The output categorizes each stromule tip then we sum the total of each category and that is presented as a percentage of total events for the treatment. This figure looks at overexpressing the KSE2 deletion constructs with an actin marker.

3.3.1.2 KSE2 deletion constructs changes contact points with the microtubule network

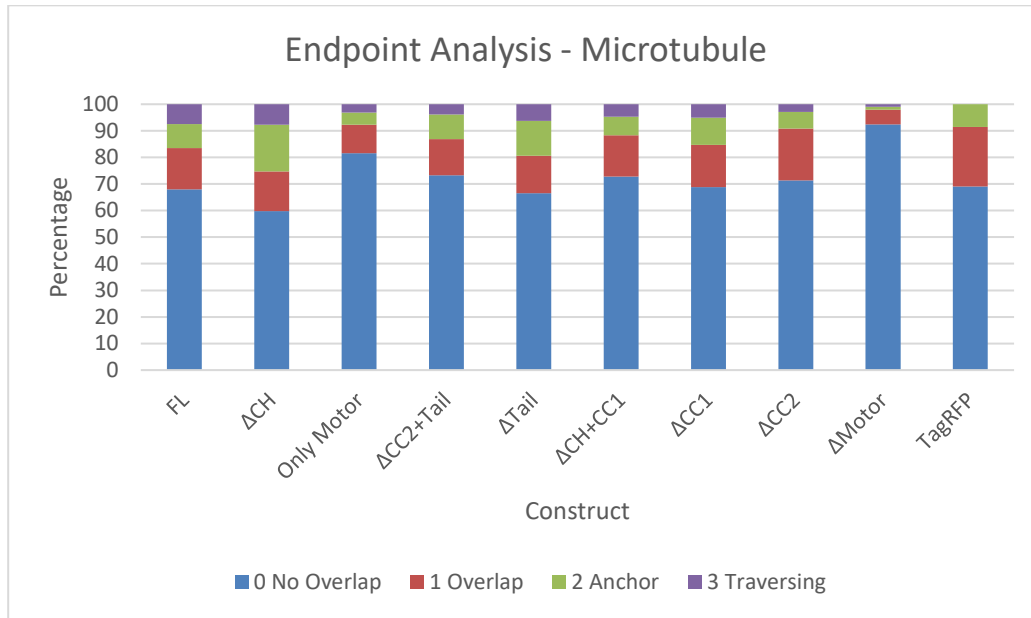


Figure 3.18 Stromule endpoint and microtubule network analysis

By taking both masks (Cytoskeleton and Stromule) we can overlay the two MIPs to quantify contact points between the stromule tips and the cytoskeleton. Each stromule tip is broken down into 4 categories, not overlapping, overlapping, anchored or traversing. The output categories each stromule tip then we sum the total of each category and that is presented as a percentage of total events for the treatment. This figure looks at overexpressing the KSE2 deletion constructs with a microtubule marker.

3.4 Aim 3: Characterize the Effects of KSE2 During Plant Innate Immunity

3.4.1 Sub Aim A: Overexpression of KSE2 Deletion Constructs Dynamics Change During PAMP Triggered Immunity

PAMP triggered immunity is the crucial first step for complete plant immunity against pathogens. It serves as the alarm system for the plant. The signals generated during PTI upregulate specific PTI genes that make ETI possible. Chloroplasts have been shown to be a part of the delivery of these signals. Thus, it is important to observe chloroplast and stromule dynamics during PTI. Application of flg22, a common PAMP used to induce PTI induces stromules and alters chloroplast and stromule dynamics. Additionally, since we know that overexpressing KSE2 alters stromule and chloroplast dynamics it is important to combine both treatments to observe if KSE2 has an effect on the chloroplasts and stromules during PTI. We use flg22 to induce the PTI response in plants currently overexpressing KSE2 to observe the effect on chloroplast and stromule dynamics.

3.4.1.1 Application of flg22 while overexpressing KSE2 results in altered dynamics

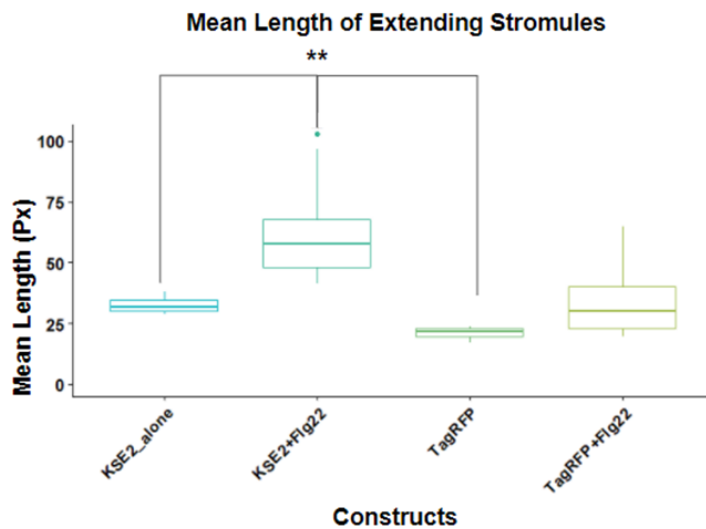


Figure 3.19 Overexpressing KSE2 + Application of flg22 results in increased stromule length during stromule extension

This figure quantifies the videos collected for the treatments stated above. Images were masked using the deep learning pipeline then analyzed in MATLAB. The output is the mean length of all stromules in the video. The lengths are then separated by event type, extending, and retracting because stromules are typically longer during extension. This figure looks at extension. Then we can find the mean of that to compare significance. We see that the difference in means is significantly different between KSE2+flg22, KSE2 Alone and the TagRFP control. Overexpressing KSE2+flg22 results an increase in stromule length for extending stromules during PTI and while overexpressing KSE2. Mean of KSE2: 32.79, Mean of KSE2+flg22: 62.92, Mean of TagRFP: 20.87, Mean of TagRFP+flg22: 34.41.

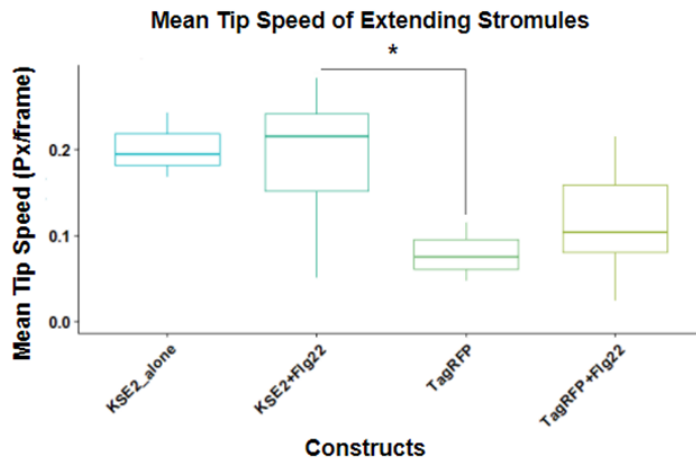


Figure 3.20 Overexpressing KSE2 + application of flg22 results in increased stromule tip speed during stromule extension

Images were masked using the deep learning pipeline then analyzed in MATLAB. The output is the mean tip speed of all stromules in the video. The velocities are then separated by event type, extending, and retracting because stromules are typically faster during extension. This figure looks at extension. Then we can find the mean of that to compare significance. We see that the difference in means is significantly different between KSE2+flg22 and the TagRFP control. However there still is increased stromule tip speed when comparing KSE2 alone vs KSE2+flg22. Mean of KSE2: 0.196, Mean of KSE2+flg22: 0.201, Mean of TagRFP: 0.0789, Mean of TagRFP+flg22: 0.117.

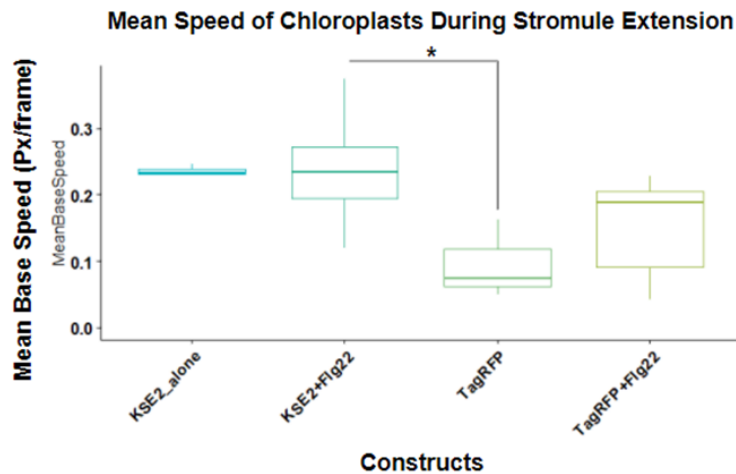


Figure 3.21 Overexpressing KSE2 + application of flg22 results in increased chloroplast base speed during stromule extension

Images were masked using the deep learning pipeline then analyzed in MATLAB. The output is the mean base speed of all chloroplasts in the video. The velocities are then separated by event type, extending, and retracting because stromules are typically faster during extension. This figure looks at extension. Then we can find the mean of that to compare significance. We see that the difference in means is significantly different between KSE2+flg22 and the TagRFP control. However there still is increased chloroplast base speed when comparing KSE2 alone vs KSE2+flg22. Mean of KSE2: 0.236, Mean of KSE2+flg22: 0.239, Mean of TagRFP: 0.095, Mean of TagRFP+flg22: 0.151.

3.4.2 Sub Aim B: Overexpression of KSE2 Deletion Constructs Dynamics Change During Effector Triggered Immunity

Effector Triggered Immunity is the more specific and robust response compared to PTI. The end goal of ETI is to induce programmed cell death and prevent pathogen proliferation. Chloroplasts and stromules have been shown to deliver signals to the nucleus that induce programmed cell death. Thus, it is important to study chloroplasts and stromule dynamics during the ETI response. I hypothesized that since KSE2 increases chloroplast and stromule velocity (meaning they are getting to the nucleus faster) then plants expressing KSE2 should induce programmed cell death quicker. To test this, we performed an electrolyte leakage assay. The electrolyte leakage assay is designed to measure electrolyte leakage from plant membrane tissue being released into solution as a result of programmed cell death. So, we can quantify whether KSE2 induces HR-PCD faster or more robustly compared to normal. We infiltrate our desired construct and 48 hours later we use estradiol to induce P50 expression, this is considered Time point 0. We allow the construct to express for 4 hours before cutting 3 4mm leaf discs and place them into a 12 well plate. Then every two hours we take a conductivity measurement.

3.4.2.1 Electrolyte leakage analysis observes the effects of KSE2 overexpression on HR-PCD

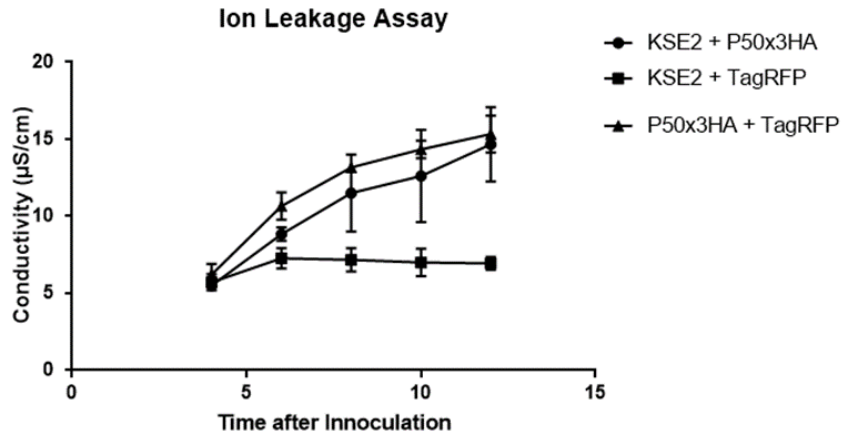


Figure 3.22 Electrolyte leakage assay using p50-3xHA XVE

The electrolyte or ion leakage assay is a measurement of conductivity or the number of positive ions in solution. Conductivity measurements were put into GraphPad and analyzed using a Two-way Anova. KSE2+TagRFP is used as a negative control. There is no significance between KSE2+P50 vs TagRFP+P50. There is significance between KSE2+P50 and TagRFP+P50 vs KSE2+TagRFP but that is to prove that the expression of P50 causes an increase in conductivity (aka cell death).

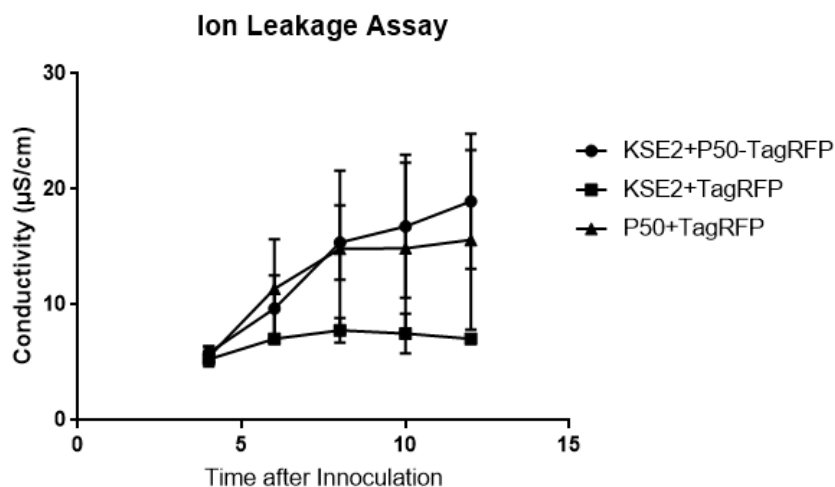


Figure 3.23 electrolyte leakage assay using TagRFP-p50 XVE

The electrolyte or ion leakage assay is a measurement of conductivity or the number of positive ions in solution. Conductivity measurements were put into GraphPad and analyzed using a Two-way Anova. KSE2+TagRFP is used as a negative control. There is no significance between KSE2+P50 vs TagRFP+P50. There is significance between KSE2+P50 and TagRFP+P50 vs KSE2+TagRFP but that is to prove that the expression of P50 causes an increase in conductivity (aka cell death).

Chapter 4

DISCUSSION

4.1 The Role of KSE2 in Stromule Formation in Function

This research was designed to characterize the role of the kinesin for stromule extension (KSE2). KSE2 was uncovered by a previous graduate student who overexpressed 8 different kinesin 14s to see their effect on chloroplast and stromule dynamics. Among the 8 chosen, KSE2 was the most promising for playing a role in stromule formation and function because when overexpressed, stromule length and chloroplast velocity were increased. The characteristics quantified were expanded to see what other effects overexpressing KSE2 had on stromule and chloroplast dynamics. In collaboration with the VIMS lab we were able to use deep learning to mass quantify large data sets (Figure 2.3). After overexpressing KSE2, we saw more effected characteristics such as increased stromule induction, increased branching stromules, increased stromule kinks, increased stromule length, increased stromule velocity and chloroplast velocity. This highly indicates that KSE2 plays a role in stromule formation and function. Specifically, if we split velocity by extension vs retraction events, we can see only extension is increased. This indicates that KSE2 could be one of the motors for stromule extension.

The next step was to characterize the KSE2 domains. We did this by creating 8 different deletion constructs with a combination of one or multiple domains deleted to observe the effect (Figure 1.6). Same as above, we used deep learning to quantify the

same characteristics. When quantified we wanted to see how the loss of certain domains affected stromule and chloroplast dynamics. What we saw was very surprising and interesting. A summary table condensing the graphs is below to better identify patterns of significance (Table 4.1). Looking at the table it seems that the Δ CH domain is the least related to stromule formation and function. When you remove the Δ CH domain you have similar significance and phenotypes as the FL. Meaning that when you remove the Δ CH domain you do not disrupt the overexpression KSE2 phenotype. However, when you also remove the CC1 domain in addition to the CH domain (Δ CH+CC1), you lose all significance. This means that alone the CH domain probably plays a supporting role but removal of Δ CH+CC1 disrupts stromules the most. This makes sense because it is thought that the CH domain is the actin binding domain meaning that actin binding is supportive and not the typical way stromules move. When you delete the CC1 domain alone you see a decrease in tip speed. But stromule induction is still increased. This shows that CC1 seems to be significant for proper movement but not formation. CC1 is the dimerization domain, so stromules must need KSE2 dimers for proper stromule movement. Another domain that seems to be important for KSE2 function is Δ CC2. When you remove the CC2 domain you lose proper extension and retraction events. Combine that with the Tail deletion (Δ CC2+Tail) and you lose all significance. The most odd, is the only motor construct. It lacks all other domains other than the motor however it still acts mostly functional compared to the other domains when you delete one half of KSE2. This is odd because then why do the other domains matter? This

result indicates that when all other domains are deleted, possibly the motors form a special complex which allows movement. Or that there is an alternate pathway for stromule formation and movement and KSE2 is a slower more controlled way to move. So when we remove the domains, making KSE2 near non-functional, stromules can move more freely. This hypothesis will get tested in the knockout lines currently being generated. Then the Δ Motor resulted as we expected, the motor alone is necessary for stromule formation and function. When you delete the motor domain, you get a decrease in all phenotypes except stromule extension length. Which is similar to the CC1 deletion.

Construct	TagRFP	FL	Δ CH	OnlyMotor	Δ CC2+Tail	Δ Tail	Δ CH+CC1	Δ CC1	Δ CC2	Δ Motor
Stromule/Chloroplast	0.513	0.960 ****	0.656*	0.848 ****	0.646	0.454	0.607	0.689 ***	0.472	0.543
Branches/Stromule	0.0307	0.12 ****	0.117 ****	0.017	0.063	0.066	0.063	0.073	0.026	0.027
Stromule Length:Extending	49.3	80.1 **	55.6	78.2 ***	84.3	57.1	47.6	64.5 **	52.4	63.4 *
Stromule Length:Retracting	30.9	39.3 *	48.8 ***	63.7 ****	30.1	30.6	28.5	35.8	30.9	33.1
Stromule Tip Velocity:Extending	0.142	0.191 **	0.185 *	0.182 **	0.151	0.192 **	0.151	0.178	0.151	0.167
Stromule Tip Velocity:Retracting	0.153	0.152	0.191	0.172	0.172	0.183	0.181	0.164	0.205 *	0.169
Chloroplast Base Velocity:Extending	0.157	0.193 *	0.163	0.173	0.171	0.207 *	0.171	0.197	0.164	0.172
Chloroplast Base Velocity:Retracting	0.138	0.136	0.188	0.171 *	0.162	0.159	0.162	0.143	0.166	0.147
Extending Stromule Percentage	41.60%	47.86%	44.69%	56.36%	40.40%	47.07%	44.70%	48.82%	41.62%	51.29%
Average Stromule Directed Movement (%)	26.50%	31.18%	28.07%	29.32%	32.93%	30.63%	30.16%	29.81%	30.05%	31.83%
Kinks (%)	6.09%	5.53%	5.57%	3.88%	6.67%	5.66%	5.92%	3.73%	5.35%	6.27%

Table 4.1 Table of Aim 1 means and significance

This figure takes all the means and significance from Aim 1 for better visualization of patterns and trends. This table also includes percentages of events that do not have graphs or significance. Those are extending stromule percentage, which looks at if that construct prefers extension or retraction. As well as stromule directed movement percentage, which looks at if the stromule is leading the chloroplast in movement. If a number is highlighted red that means it has *** or **** significance. If a number is highlighted orange, then it has * or ** significance.

4.2 How the Loss of KSE2 Domains Effects Contact with the Plant Cytoskeleton

The cytoskeleton is important for providing strength and architecture to the cell. It more importantly is the roadway for many organelles and molecules to move throughout the cell. The most important organelle for my project is the chloroplast which uses the cytoskeleton to move throughout the cell. Chloroplasts also have tubular extensions called stromules which have been seen to interact with the cytoskeleton. My project involves characterizing a kinesin which has both actin and microtubule domains. This raises the question, how do the deletion of various KSE2 domains affect the interactions between stromules and the cytoskeleton? To observe these interactions, we overexpressed our KSE2 deletion constructs with a microtubule or actin marker. When using an actin marker, we see KSE2 Full length has less contact than TagRFP. Could there be another motor that when we overexpress KSE2, KSE2 outcompetes this actin specific motor resulting in less contact in our FL. This data also supports that CC2+Tail is necessary for proper KSE2 function. When we overexpress out Δ CC2+Tail construct, we see much more actin contact than any other construct. On the other side, Only Motor, Δ Motor, Δ CC1 and to a lesser extent Δ CH and Δ CH+CC1 have less contact with the actin cytoskeleton. This data is interesting because we are overexpressing a kinesin which interacts with microtubules. Looking at the microtubule cytoskeleton, KSE2 FL looks to have similar contact percentage as TagRFP. There is the least contact between microtubules and stromules when you remove the Motor domain. This makes sense because this is a microtubule motor, so when removing the Motor domain, you get less contact. Also, interesting, the only

construct with slightly more microtubule contact is Δ CH, which makes sense because if you remove the actin binding domain then it makes sense that you get more microtubule interactions.

4.3 KSE2 Effect on the Plant Immune Response

The plant immune response against pathogens is broken down into two categories, PTI and ETI. PAMP-triggered immunity is the broad non-specific response used as an alert system for the plant. While effector-triggered immunity is the more specific and robust response. The end goal of ETI is to induce programmed cell death to prevent pathogen proliferation. Chloroplasts have been shown to deliver signals during both immune responses. The goal of Aim 3 was to observe the effect of KSE2 during PTI and ETI. To do this we used the application of flg22, a common PAMP used to induce the PTI response. Since Aim 1 showed that KSE2 alters stromule and chloroplast dynamics it is worth looking at the effect of KSE2 during PTI. What we saw was an additive effect when looking at length and tip speed of stromules. Stromules were longer during PTI regardless of whether KSE2 was being overexpressed or not. The same is true for tip speed and chloroplast base speed, but to a lesser extent. To observe the effect of KSE2 during ETI, we performed an electrolyte leakage assay. This was to see if KSE2 sped up or altered the speed that a plant induces programmed cell death due to ETI. What we saw was that there was no significant difference between the application of KSE2 or not. Which means that inner-cellularly chloroplasts and stromules are getting to the nucleus faster however that does not help induce

programmed cell death faster. This indicates that there is slower rate limiting step preventing significantly increased HR-PCD on a macro level.

4.4 Future Work

Future work will focus on expanding the characterization of KSE2 to uncover its role in stromule formation and function. New work related to Aim 2 will include expanding our endpoint analysis pipeline to look at the whole stromule. So, we can further characterize these interactions. It will be interesting to see if the whole stromule interacts differently than just the stromule tip. Related to Aim 3, expanding the flg22 dynamics experiments to all 8 deletion constructs will give insight into the importance of various KSE2 domains. Observing the effect of overexpressing KSE2 during ETI can shed light into why overexpressing KSE2 does not speed up programmed cell death. Other directions include knocking out KSE2 and observing the effect on stromules and chloroplasts. These experiments would expand what we know about KSE2 and its role in stromule formation and function.

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