UNDERSTANDING ACTOMYOSIN-DEPENDENT NETWORKING AND THE ROLE OF GTPASE RHD3 IN THE ENDOPLASMIC RETICULUM

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by

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ABSTRACT

Understanding Actomyosin-Dependent Networking and the Role of GTPase RHD3 in the Endoplasmic Reticulum. (May 2014)

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The endoplasmic reticulum operates in a larger reticular network via membrane fusion of ER tubules. Root Hair Defective 3 (RHD3) is a membrane-bound GTPase, similar to mammalian atlastin GTPases, which may be involved in ER fusion. It is known that an actomyosin system plays a role in ER dynamics, including streaming, networking and tubule activity. Past research has indicated that actin and myosin are contributing factors, and it was hypothesized that in the absence of both, ER tubular activity should diminish entirely. However, in previous trials, tubule growth still persisted even in the absence of myosin and actin. It is possible that RHD3 may act as a motor for ER tubular activity or microtubules could the cause of this continual growth of ER tubules. To observe ER tubular activity in the absence of myosin, actin, and microtubules, myosin triple knockout lines of *Arabidopsis thaliana* were treated with latrunculin B, which is an actin depolymerizer, and oryzalin, which depolymerizes microtubules. In addition, observations of the effects of latrunculin B and oryzalin on the activity of RHD3 mutant tobacco plants can help elucidate the role of RHD3. Furthermore, the treatment with latrunculin B and oryzalin may alter the coherent directionality of ER tubule growth and shrinkage. With this study, more can be understood about ER networking and its specific correlation to mammalian ER activity.

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NOMENCLATURE

GTPase:	Guanosine triphosphatase
RHD3:	Root Hair Defective 3
CFP:	Cyan Fluorescent Protein
YFP:	Yellow Fluorescent Protein
GFP:	Green Fluorescent Protein
TK x1,1,2:	Triple Knockout line of Myosin motors X1-K, MYA1, MYA2 (*Arabidopsis thaliana)*

CHAPTER I

INTRODUCTION

Background and literature

The endoplasmic reticulum (ER), a complex organelle involved in cellular trafficking, plays an important role in higher level plant cells. Organized in a network of polygonal tubules and cisternae, the ER is known to act as a link between various organelles, including chloroplasts and Golgi bodies (Sparkes, 2009). Although the general structure and function of this multi-membrane organelle is well-understood, research regarding the dynamics of ER networking is still in its infancy. The current theory is that ER dynamics and movement is that some independent mechanism acts as a driving factor, and our research is gear towards understanding and identifying this mechanism.

New ER network visualization techniques, such as fluorescence microscopy, confocal microscopy, and GFP-ER labeling, has greatly improved research regarding ER tubule remodeling, or the growth and movement of ER tubules. For example, some studies have analyzed the role of microtubules and Golgi bodies in driving ER networking and remodeling. By treating plant cells with oryzalin, an antimicrotubule chemical, or brefeldin A, an Golgi-deconstructing agent, we have found that ER remodeling in plant cells persists even in the absence of microtubules or Golgi bodies (Sparkes, 2009). This fact implies that there is another factor involved in the active formation and retraction of ER tubules.

One viable mechanism that has been identified is an actomyosin system responsible for ER remodeling. Using confocal microscopy, we have been able visualize ER streaming and identify *Arabidopsis myosin XI-K*, a class of myosin motor in plants, as a driving contributor (Ueda, 2010). In addition, we can use GFP-labelled *Arabidopsis* myosin mutants, with truncated myosin XI-K expression, to observe that ER tubules are less likely to have tubule shrinkage and polygon ring closure. Furthermore, use of *Arabidopsis* triple-knockout myosin mutants, with truncated expression of XI, MYA1 and MYA2, was shown in previous studies to have the greater suppression of ER streaming than other myosin mutants (Ueda, 2010).

In addition, treatment of GFP-labelled plant cells with latrunculin B, an actin depolymerizing agent, is found to inhibit additional movement and growth of ER tubules, suggesting that actin is a significant factor as well. To compare the link between myosin and actin organization, researchers have generated crosses between myosin mutants and plants with F-actin markers and GFP-labeled ER (Ueda, 2010). Analysis of these crosses has shown that myosin XI deficiency is related to significantly diminished ER movement, but no effect in ER networking along actin filament bundles. With evidence that actin and myosin both individually play roles in ER dynamics, we proposed the question: is ER tubule growth entirely dependent on actin and myosin, or are there other factors involved?

Furthermore in *Arabidopsis thaliana*, Root Hair Defective 3 (RHD3) is a protein that binds to GTP. RHD3 mutants have short and kinked root hair mutations. The RHD3 isoforms in *Arabidopsis thaliana* are comparable to mammalian atlastin GTPases, which are involved in shaping ER tubules (Zheng et al., 2011). Previous studies have shown RHD3 as a mediator in the

generation of the tubular network of the ER and may be required for ER morphology and protein transport from the ER to the Golgi apparatus. RHD3 may be another factor involved in ER tubule growth not just in terms of a mediator but also as a motor protein.

Previous research

To specifically determine the roles that actin and myosin play in ER tubule remodeling, we used leaf and stem cells of an *Arabidopsis* cross, expressing ER-localized CFP and the triple-knockout myosin mutation XI, MYA1, and MYA2. Using both confocal and fluorescence microscopy we were able to visualize the ER well and observe significant tubule growth and remodeling. Specifically tubules would spin between planes of the cell, and multiple tubules would form from one initiation site. In stem cells we found more active ER networking than in leaf cells, with several polygonal structures being formed or initiating new tubules. Because these myosin mutants were successfully expressing both CFP and the triple-knockout gene, we transferred them from agar to pots and used the same line in all trials.

One observation in these crosses was that the ER had numerous large cisternae and abnormally shaped ER-bodies, characteristic of the triple-knockout myosin deficiency (Ueda, 2010). Another observation was that while ER tubule growth was mostly normal, there was great impairment in the ability to initiate and fuse these tubules properly. The fact that myosin has been linked to the ability to fuse polygonal rings during ER networking can explain why myosin mutants lack this trait (Griffing, 2010). Multiple videos were recorded of ER tubule activity in this myosin mutant line to use as a baseline comparison.

In addition to the myosin mutants, we also grew actin-YFP labeled *Arabidopsis*. When visualizing these plants' leaf and stem cells, we found very good visualization of actin filaments and bundles, and recorded a video of actin activity. After treating these plants with latrunculin B, a known actin depolymerizing agent, we observed the changes that latrunculin B treatment had on ER networking. Using the actin-YFP line as a control, we found that after approximately 45 minutes, the actin filaments were significantly reduced. Comparing the videos taken before and after latrunculin B treatment at varying times on several different plant cells, we found that almost no actin remained in the cell, and actin filament growth and movement was very rare.

Because we had already found a clear reduction of actin activity in the actin-YFP line, we then used latrunculin B to treat the ER-CFP myosin mutants, to observe how the absence of both myosin and actin would affect ER tubule activity. After approximately 45 minutes, we visualized the treated cells and found a significant reduction of ER tubule growth. However, videos taken of these treated plants show some tubules growing and retracting. Clearly, actin depolymerization did not terminate all ER tubule growth. Even after over an hour under latrunculin B treatment, some tubule activity remained, showing that even in the absence of both myosin and actin, there is some other independent mechanism driving ER networking.

In this study, we sought to identify the roles that the actomyosin system play in driving ER dynamics, including streaming, networking and tubule activity. Past research has indicated that actin and myosin are contributing factors, and we hypothesized that in the absence of both, ER tubule activity should diminish entirely. However, our past results indicated that despite a huge reduction of ER activity, some tubule growth still persisted. This indicates that our previous

hypothesis was not fully accurate, and there must be some other factor that drives ER tubule growth in addition to the actomyosin system.

Objectives

In our current studies, we plan to cross and observe plants expressing actin-YFP, ER-CFP, and the myosin triple knockout gene. Any ER tubule activity that we can visualize following treatment with latrunculin B would have to be attributed to another factor, possibly Root Hair Defective 3 (RHD3). Thus, the objective of the current study is to corroborate that a non-actin driven component of tubule formation may exist with the possibility of RHD3 as a motor affecting tubule growth in *Arabidopsis thaliana*.

It is hypothesized that with the treatment of lat B and oryzalin solutions, ER streaming and tubular activity should persist. If tubular activity persists, it is hypothesized that RHD3 GTPase may act as a motor that is responsible for driving ER activity.

CHAPTER II

METHODS

Sources of plant materials

The plant materials used were *Arabidopsis thaliana* transgenic lines, grown for approximately 6 weeks prior to experimentation, expressing either CFP-labeled ER and triple knockout myosin mutants of XI, MYAI, and MYA2, or YFP-labeled actin filaments. Additionally, tobacco wild type and RHD3 mutant lines were grown for further comparison.

Equipment

The plant were maintained in a Conviron set at room temperature. To visualize fluorescent CFP labeling, various laser microscopes were used such as the Olympus IMT2 Inverted Microscope with 440 nm filter and 63x oil immersion lens and the Leica TCS SP5-RS Confocal Laser Scanning Microscope with 405 and 458 nm lasers and 63x water immersion lens.

Protocols

Identification of properly labeled plant lines

Using a fume hood, 70% ethanol was used to sterilize the bench. Thirty seeds of ER-CFP labeled, triple knockout myosin mutants of XI, MYAI, and MYA2 were isolated in labeled microfuge tubes. Using micropipettes, the plant seeds were then sterilized in 1 mL of 10% bleach for 1 minute and rinsed with 1 mL of autoclaved water four times. Then, the seeds were placed in 0.5 mL of autoclaved water for at least 1 minute and then planted on mannitol salt (MS) agar

with and without Hygromycin. Lines of plants with YFP-labeled actin filaments were grown using the same protocol.

After incubation for seven days in agar, all of the plants with the CFP-labeled ER and the plants with YFP-labeled actin filaments were transferred into moistened peat moss pellets. Five to ten fertilizer pellets were dispersed onto each peat moss pellet. The peat moss pellets were placed in a planting tray filled with distilled water to a depth of one inch and grown at 22 C in constant light in the Conviron for a week.

After a week, samples of each ER-CFP and actin-YFP line were taken and placed onto wet mount slides for screening. Only the samples that expressed the ER-CFP label when observed with the Leica TCS SP5-RS Confocal Laser Scanning Microscope were confirmed to express the triple knockout gene. Likewise, only the samples that properly expressed the actin-YFP label were retained for experimentation. The lines from which all of these properly labeled samples were taken were used to grow in pots, using the protocol described in the next section.

Plant preparation

To grow the properly expressing ER-CFP labeled lines of *Arabidopsis thaliana*, potting soil was thoroughly moistened with distilled water and evenly distributed into five small pots. Approximately five seeds of each ER-CFP labeled line were dispersed onto each pot. Five to ten pellets of fertilizer were also dispersed onto each pot. The pots were placed in a planting tray filled with distilled water to a depth of one inch and then placed in the Conviron for six weeks.

The same protocol was used for growing wild type and RHD3 mutant lines of tobacco. Over the course of six weeks, the plants were watered every other day.

Preparation of latrunculin B, oryzalin, and DMSO solutions

Latrunculin B (lat B) solution (25.28 mM concentration) was prepared using 1 mg dehydrated lat B crystals dissolved in 100 μL of dimethyl sulfoxide (DMSO). Then, 1 μL of lat B solution was diluted with 1 mL of distilled H_2O. Half of the ~1 mL solution of lat B was pipetted into a microfuge tube. The control solution was prepared by diluting 1 μL of DMSO with 1 mL of H_2O, and half was pipetted into another microfuge tube.

Oryzalin solution was prepared using 3.4 mg of dehydrated oryzalin crystals dissolved in 1 mL of DMSO. Then, a 1 to 1000 dilution was prepared by adding 1 μL of concentrated oryzalin solution to 1 mL of distilled H_2O.

Treatment of plant samples with solutions

Wet mount slides of leaves and cotyledons from both ER-CFP and actin-YFP plants were prepared without solution to serve as a baseline comparison. Then the samples were visualized for ER network streaming and tubular activity using both the Olympus IMT2 Inverted Microscope and the Leica TCS SP5-RS Confocal Laser Scanning Microscope.

Following observation of the control, DMSO treatment of plant samples was conducted in order to ensure that the DMSO diluted solution had no significant effect on ER tubular activity. Two

plant samples were submerged in DMSO diluted solution for 45 minutes. Wet mount slides of the samples were then prepared and visualized with the aforementioned microscopes.

After DMSO treatment, two more plant samples were treated with lat B diluted solution for 45 minutes. Wet mount slides of these samples were prepared and visualized using the same microscopes.

During visualization of the control and before and after each of the separate treatments, videos of ER tubular activity within the ER-CFP samples were captured. In addition, videos of actin filament activity within the actin-YFP samples were captured to show the effects of lat B as an actin depolymerizer.

CHAPTER III

RESULTS

Identification of properly labeled plant lines

Seed lines obtained from five myosin triple knockout crosses (6-2, 8-1, 9-20, 3-12, 2-29) from planting pots were analyzed using confocal microscopy for proper ER labeling. In addition, comparisons were made with ER-CFP and actin-YFP control lines.

Initially, finding a properly labeled and viable myosin triple knockout line proved difficult due to irregular growth rates of the myosin triple knockout lines. Perhaps due to the myosin deficiency, the myosin triple knockout plants did not grow as quickly. Lack of adherence to strict maintenance protocols for the myosin triple knockouts may have resulted in unexpected desiccation or fungal contamination of the plants. When crossing to create myosin triple knockouts, time proved to be a limiting factor because only a short time frame exists for plant crossing to produce viable seed lines. Ultimately, the necessary plant lines for experimentation were successfully grown despite minor setbacks.

Qualitative analysis of the myosin triple knockouts indicated that the only properly labeled line was from the 2-29 cross. Observations of heavy cisternalization and slower ER tubule activity, which are characteristic of the myosin triple knockouts, confirmed that the 2-29 cross did, in fact, have the defective myosin proteins MYA1, MYA2, and XI. Table 1 shows full analysis of the myosin triple knockout crosses.

Table 1. Analysis of myosin triple knockouts using confocal microscopy

Myosin triple knockout crosses	Properly expressing ER-CFP label (Y/N)	Characteristics of ER tubule activity
6-2	N	-
8-1	N	-
9-20	N	-
3-12	N	-
2-29	Y	Heavy cisternae, little retraction of tubules, slow activity

Properly expressing ER-CFP and actin-YFP lines were screened using confocal microscopy as well. ER-CFP line (COL Φ A) showed properly labeled ER, which is characterized by normal ER tubule activity, some organelle streaming, and no apparent structural deformations in the ER. This line served as the control for measuring irregularities in ER tubule activity in subsequent experimentation. In addition, actin-YFP (1) showed properly labeled actin filaments in the ER. This line was used later as a baseline to highlight the effects of lat B as an actin depolymerizer.

Treatment with distilled water (control)

Myosin triple knockout mutant lines

A control of 2-29 myosin triple knockout was observed prior to lat B treatment. Multiple plant samples were placed onto wet mount slides and were analyzed using confocal microscopy. ER tubular activity and streaming were recorded and characterized by behavior of tubule extension and retraction. Still images from videos taken with the confocal microscope are shown in Figure 1.

a)

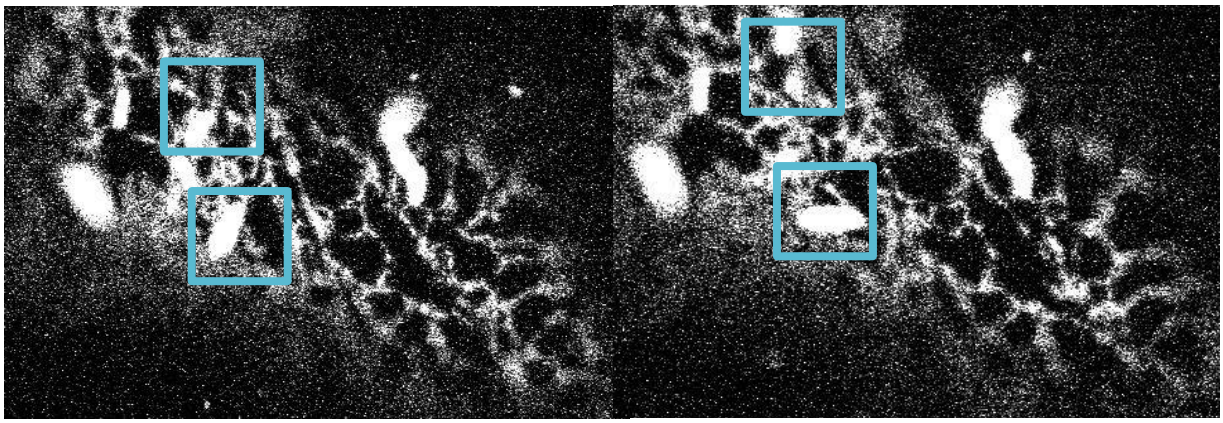

Figure 1. Images of myosin triple knockout in control conditions. a) Image taken at time = 0 sec. Note the ER bodies located within the two boxes. b) Image taken at time = 10 sec. Note the changes in movement of the ER bodies and the presence of newly formed ER tubules.

Over the course of ten seconds, the ER structures, also called ER bodies, showed significant movement. This indicates the presence of substantial ER streaming and tubular activity. The key characteristics of ER tubules in the myosin triple knockout plant lines were more linear extension and retraction of tubules and areas of rapid ER flow. As previous research has shown, this phenotype is normal for the ER of myosin mutant plants.

Using ImageJ, vector analysis of ER flow and streaming was performed to quantify the magnitude and mean speed of ER tubular activity in the myosin mutant lines. Figure 2 shows the same plant cell with a vector overlay, indicating where movement of the ER tubular network occurred.

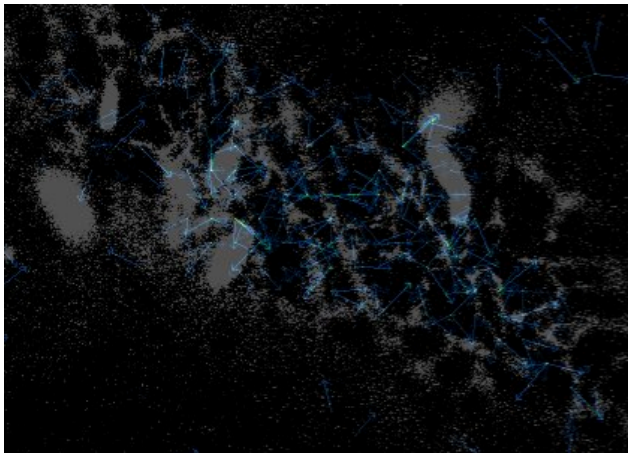

Figure 2. Image of myosin triple knockout in control conditions with vector overlay. Length of vector represents magnitude of ER tubular movement.

The vector overlay indicates exactly which portions of the ER tubular network were exhibiting movement during the recording period. Using these vectors, mean speeds of ER tubular activity were obtained and used as a baseline reference for subsequent treatments.

Actin-YFP labeled plant lines

A control of Col Φ Actin-YFP was observed prior to lat B treatment. Multiple plant samples were placed onto wet mount slides and were analyzed using confocal microscopy. Labeling of actin filaments in the ER was visualized, and still images from videos taken with the confocal microscope are shown in Figure 3.

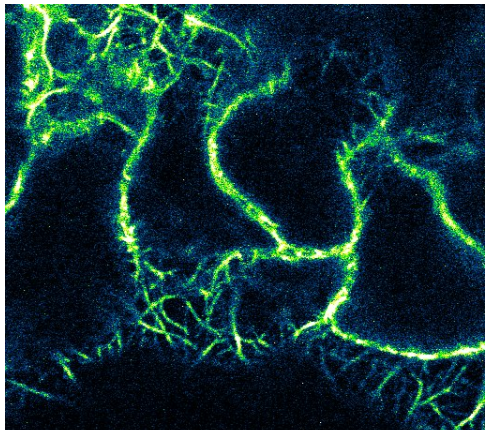

Figure 3. Image of actin-YFP in control conditions. Note the high expression of the YFP label.

The images and videos captured of the actin-YFP control lines showed that the actin filaments of the ER were abundant and interconnected. The intensity of the YFP label was very high in the control lines.

Treatment with latrunculin B solution

Myosin triple knockout mutant plant lines

To confirm that both actin and myosin play a role in tubule activity, lat B solution was applied to both ER-CFP and actin-YFP control lines. For the ER-CFP control line prior to lat B treatment, heavy CFP expression, significant ER streaming, and normal tubule activity were visualized. Following lat B treatment for one hour, contractions of the ER tubular network created small ER bodies. Furthermore, slow movement of tubules, low CFP expression, and little to no tubule retraction were observed. Figure 4 indicates the movement of the ER tubular network over the span of a ten second recording.

a)

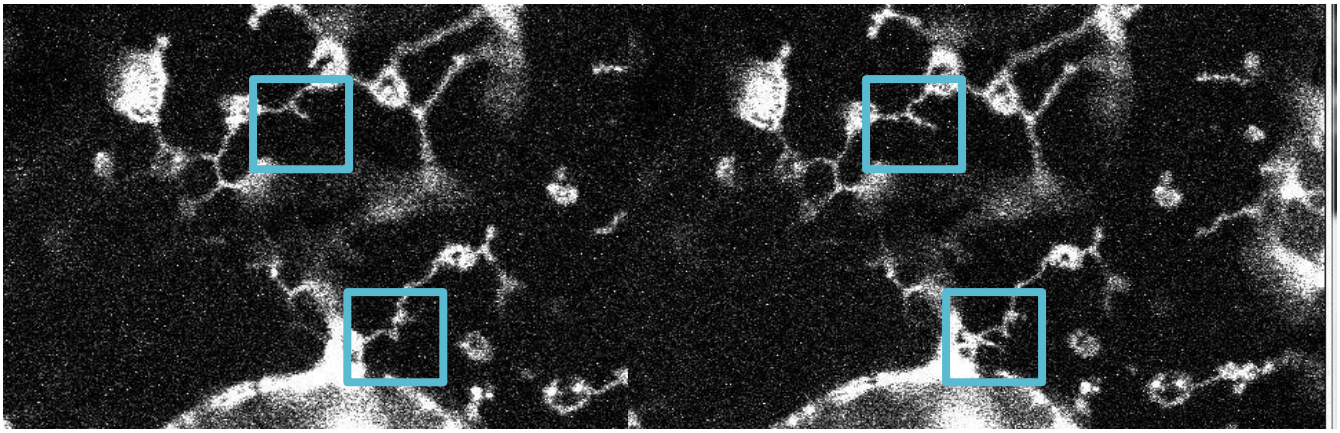

Figure 4. Image of myosin triple knockout with lat B treatment. a) Image taken at time = 0 sec. Note the ER tubules located within the two boxes. b) Image taken at time = 10 sec. Note the formation and extension of new ER tubules and the creation of ring structures with the boxed regions.

Over the course of ten seconds, the ER tubular network exhibited a unique phenotype. In addition to the reduction of ER tubular activity from baseline levels, the lat B treated plant samples showed formation of ring-like structures. However, some ER tubular activity persisted as can be seen in the captured images.

Using ImageJ, vector analysis of ER flow and streaming was performed to quantify the magnitude and mean speed of ER tubular activity in the myosin mutant lines that were treated with lat B. Figure 5 shows the same plant cell with a vector overlay, indicating where movement of the ER tubular network persisted.

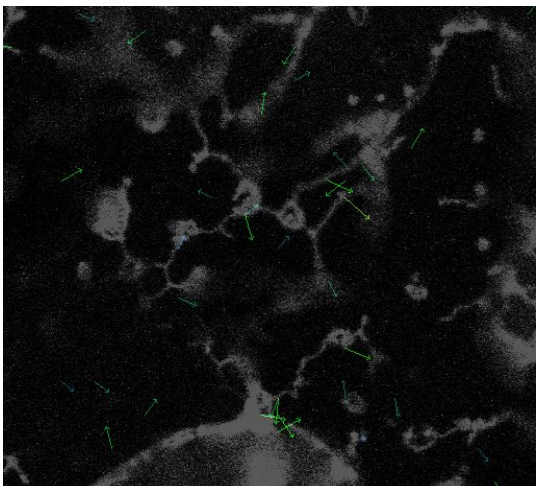

Figure 5. Image of myosin triple knockout in lat B treatment with vector overlay. Length of vector represents magnitude of ER tubular movement.

Actin-YFP labeled plant lines

For the actin-YFP control line prior to lat B treatment, there was a significant amount of well-labeled actin filaments. Since lat B is an actin depolymerizer, lat B treatment of the actin-YFP line confirmed expectations that amount of actin filaments was reduced. This finding of little to no actin filaments presence after lat B treatment can be applied to the myosin triple knockout

lines. In other words, myosin triple knockouts, after lat B treatment, would also have little to no actin filaments present. Figure 6 depicts the actin-YFP plant samples after treatment with lat B.

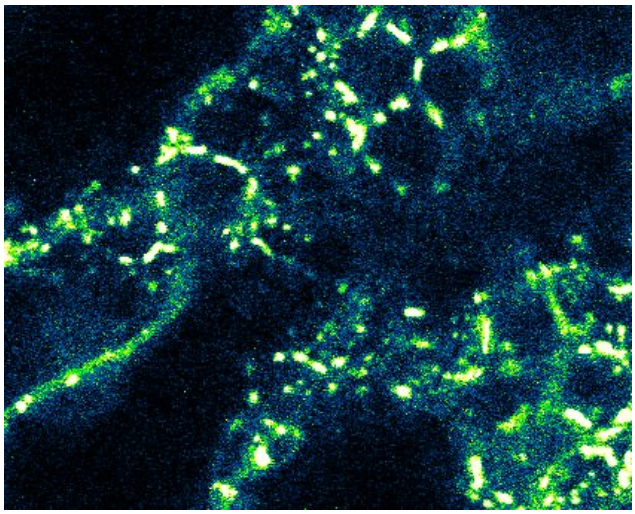

Figure 6. Image of actin-YFP after lat B treatment. Note the disruption of the YFP label and fragmentation of actin filaments.

Treatment with lat B showed a clear depolymerization of actin filaments in the actin-YFP plant samples. The continuous and highly interconnected actin network from the control condition was reduced and fragmented with the treatment of lat B. Based on the images and videos captured, it can be inferred that the same disruption of actin filaments occurs in the myosin triple knockout mutants when they are treated with lat B as well.

Treatment with latrunculin B and oryzalin solution

Myosin triple knockout mutant plant lines

Following treatment of plant samples with lat B solution alone, ER tubular activity was reduced but still persisted. In order to eliminate the contributions of microtubules on movements of the ER tubular network, treatment of plant samples with both lat B solution and oryzalin was used.

Images of myosin triple knockout mutant plant samples were captured from videos taken with the confocal microscope and are shown in Figure 7.

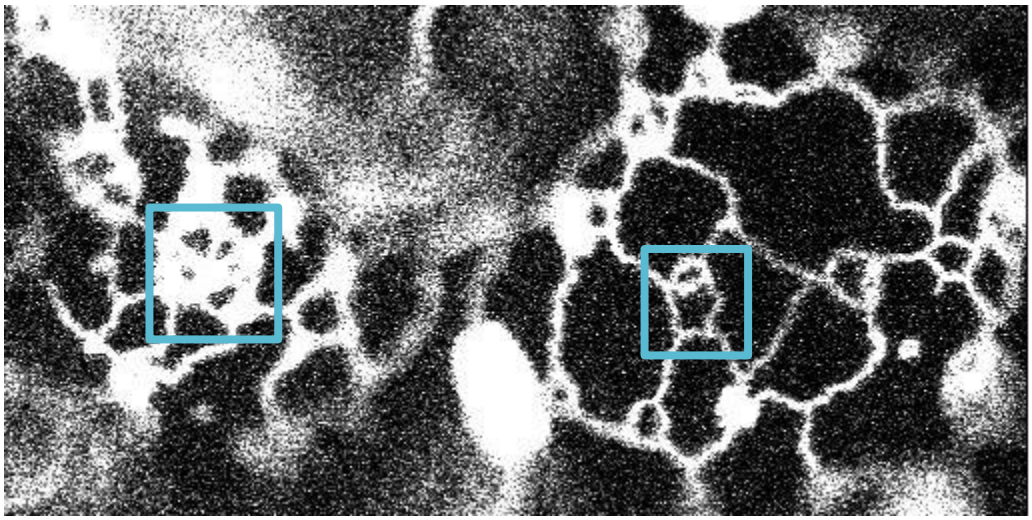

Figure 7. Image of myosin triple knockout mutants after lat B and oryzalin treatment. Note the irregularities of the ring structures formed.

As indicated in the above image, the phenotype of the lat B and oryzalin treated ER differed from that of previous treatments. The addition of oryzalin greatly reduced extension and retraction of ER tubules. However, two unique observations seen were: the ring structures exhibited lateral movement and dynamic fluctuations, and the initiation of ER tubules was seen but no extension or fusion occurred.

Using ImageJ, vector analysis of ER flow and streaming was performed to quantify the magnitude and mean speed of ER tubular activity in the myosin mutant lines that were treated with lat B and oryzalin. Figure 8 shows the same plant cell with a vector overlay, indicating where movement of the ER tubular network persisted.

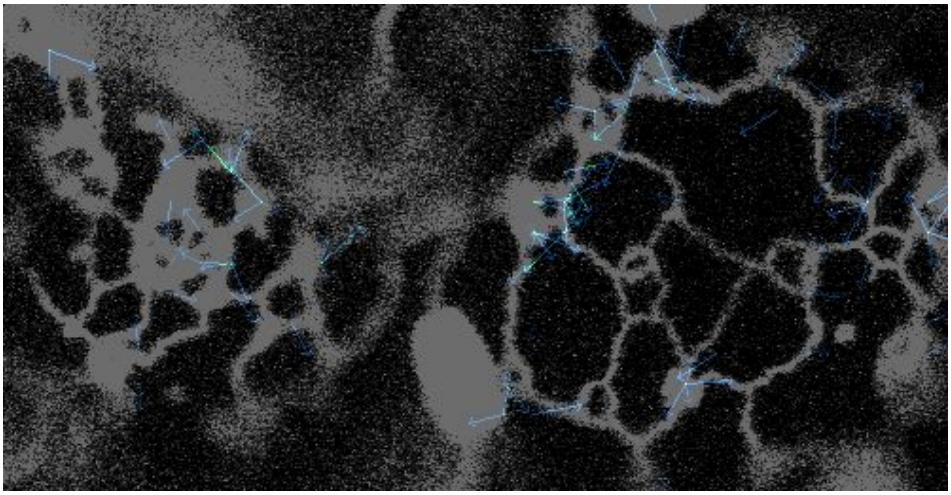

Figure 8. Image of myosin triple knockout mutants after lat B and oryzalin treatment with vector overlay. Note the placement of vectors specifically around ring structures.

As indicated by the vectors, the dynamic fluctuations within the ER tubular network of the lat B and oryzalin treated myosin mutants occurred mainly within the ring structures. Unlike the ring structures seen the phenotypes of plant samples in only lat B treatment, these rings exhibited irregular rotations and rapid interconversions from concave to convex and vice versa.

Quantification of Results

Using vector analysis through ImageJ, mean speeds of vectors were obtained for plant samples in treatment and the control condition. ER tubular activity data was collected over the span of two minutes and is summarized in Table 2.

Table 2. Analysis of myosin triple knockouts using confocal microscopy

Myosin triple knockout crosses	Max speed of ER tubular activity (pixels/frame)	Mean speed of ER tubular activity (pixels/frame)
Control condition	9.582	0.0680
Lat B only	9.214	0.00710
Lat B + Oryzalin	8.630	0.0112

For the myosin triple knockouts in the control condition, the maximum speed of ER tubules observed was 9.582 pixels/frame, and the mean speed was 0.0680 pixel/frame. In the lat B treatment, the maximum speed observed was 9.214 pixels/frame and the mean speed was 0.00710 pixels/frame. In the lat B and oryzalin treatment, the maximum speed observed was 8.630 pixels/frame, and the mean speed was 0.0112 pixels/frame.

CHAPTER IV

DISCUSSION

In this research project, the objective was to corroborate that a non-actin driven component of tubule formation may exist with the possibility of RHD3 as a motor affecting tubule growth in *Arabidopsis thaliana*. Initially it was hypothesized that with the treatment of lat B and oryzalin solutions, ER streaming and tubular activity should persist. If this primary hypothesis is confirmed, then the secondary hypothesis suggests that RHD3 GTPase may act as a motor that is responsible for driving ER activity.

In order to achieve this objective, wild-type and myosin triple knockout mutant lines of Arabidopsis were treated in different conditions: a control water solution, latrunculin B solution, and latrunculin B and oryzalin solution. Confocal microscopy and ImageJ software were used to visualize ER tubular activity of these plants and treated plant samples, and vector analysis was conducted to quantify the amount and speed of tubular activity observed. Our results observed maximum tubule speeds in each of the three conditions were: in the control condition, 9.582 pixels/frame; in the lat B only treatment, 9.214 pixels/frame; in the lat B and oryzalin treatment, 8.630 pixels/frame. There is a clear and apparent decrease in the maximum tubular speed with each successive depolymerization, which indicated that actin and microtubules both played large roles in mediating the ER tubular network.

This fact was also confirmed by qualitative observations of movement in areas of interest in captured images. The differences in phenotype of plant samples from each treatment condition

were significant. The rapid ER streaming and movement of ER bodies in the control myosin mutant condition contrasted with the slower movements in the latrunculin B only treatment, in which ER tubular activity was reduced, involving ring formation and slower tubule extension and retraction. With the lat B and oryzalin treatment, tubular activity diminished even more and was localized in the rings, specifically in fluctuations of the size and shape of these ring structures. In addition, minor initiation of ER tubules was observed but these tubules did not appear to extend or retract. Most importantly however, the fact that ER tubular activity continued even in the lat B and oryzalin conditions supports the hypothesis that tubule activity does persist in the absence of an actomyosin system, which must be due to activity of an unknown motor.

It is possible that this unknown motor may be RHD3, considering recent literature regarding the roles that RHD3 plays in mediating ER fusion. In the near future, this experiment will be expanded to account for RHD3 and test for its potential effects on the ER tubular network. Currently, 6-week old wild-type RHD3 myosin triple knockouts and mutant RHD3 tobacco plants are being grown and will be used with lat B and oryzalin treatments. Analysis of ER tubular activity in these plants should indicate how the ER tubular network performs in the absence of not only the actomyosin network and microtubules, but also in the absence of RHD3.

Based on the findings of this research, the primary hypothesis has been supported. In the presence of lat B and oryzalin, ER tubular activity did persist as was proposed. In the near future, the secondary hypothesis will be tested. If the RHD3 mutants that were previously mentioned are treated with lat B and oryzalin, and ER tubular activity continues, then RHD3 may not be the

responsible motor. However if ER tubular activity can be shown to cease completely, then it is possible that RHD3 is the unknown driving motor for initiation of ER tubules.

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