



**Involvement of Kinesin 4II and 7I in
Chloroplast Motility and Growth in the Moss
*Physcomitrella patens***

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Table of Contents

Signature Page.....	1
Table of Contents.....	2
Abstract.....	3
Acknowledgements.....	5
Introduction.....	6
Chloroplast Movement in Cells.....	6
<i>Physcomitrella patens</i>	7
Kinesins, Microtubules and their Roles in Plants.....	8
RNA Interference and Inducible Gene Expression.....	11
Purpose of Study.....	12
Methods.....	13
Moss Culture.....	13
Growth Assay.....	14
Moss Slide Prep for Microscopy.....	16
Chloroplast Avoidance Assay.....	17
Results.....	19
Reduction of Kinesin 4-IIb or 7-Ib Results in a Growth Phenotype.....	19
Quantification of the Kinesin-RNAi Induced Growth Phenotype.....	22
Kinesin 4-IIb is Necessary for Chloroplast Avoidance.....	24
Visualization of Microtubules during the Chloroplast Avoidance Response.....	27
Discussion.....	30
Bibliography.....	35
Appendix.....	38

Abstract

Understanding how plant cells adapt dynamically to changes in the environment is a fundamental problem of plant biology. Under many conditions, plant cells respond to environmental changes by modifying their intracellular organization. A critical example of intracellular re-organization is chloroplast photo-relocation, which is required for optimal energy harvesting and avoiding photodamage. A key system responsible for the spatial organization of intracellular components is the microtubule cytoskeleton and its associated motor proteins, kinesin 4 and 7. We are testing the hypothesis that kinesin 4 and/or 7 is important for chloroplast photo-relocation. As an alternative, we are using the moss *Physcomitrella patens* as a model system because of its fast growth and the fact that it is easily genetically manipulated. Because mosses have a microtubule dependent pathway for chloroplast positioning, but also an actin dependent system, we have performed control experiments for selectively inhibiting the function of each cytoskeleton. We used a moss line that contained an inducible RNAi system for the different kinesins 4-II and 7-I. With this tool we plan to answer the long-standing question about the participation of kinesins in chloroplast transport. We have also established a reproducible and quantitative assay to evaluate chloroplast photo-relocation. We have quantified chloroplast motility in response to light in inducible RNAi lines for each kinesin and control lines. Loss of microtubule-dependent chloroplast motility with a suppression of either kinesin will support the hypothesis that kinesin 4-II and/or kinesin 7-I participates in the important process of chloroplast photo-relocation.

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1) Introduction:

1.1 Chloroplast Movement in Cells

Chloroplasts are organelles that can be found in most plant and algal cells. They are essential for photosynthesis and therefore the survival of plant and algae species. Photosynthesis is a process by which an organism can turn light energy into chemical energy, which can be used by the organism as a source of free energy to drive biochemical reactions. Chloroplasts perform this function by absorbing photons using the pigments chlorophyll a and chlorophyll b (Davis and Hangarter, 2012). The main types of colors of light that chloroplasts absorb, via chlorophyll are blue and red (Kasahara et al., 2004).

In plant cells, chloroplasts have the ability to move to different locations within the cell depending on the light intensity they are exposed to. In conditions of low light intensity, chloroplasts tend to congregate to the light source in order to gather the most light, thereby increasing photosynthetic activity. (Sato et al., 2001). On the other hand, when faced with conditions of high intensity light, the chloroplasts avert the light source in order to minimize any photodamage that they could receive (Sato et al., 2001). In most plant species these avoidance and accumulation responses are driven by blue light, but mosses, such as *Physcomitrella patens*, have been shown to also respond to red light (Sato et al., 2001). The blue light responses are mediated by the light receptors, phototropins. Phototropins are blue light photoreceptors that control plant phototropism (Christie, 2007) and the chloroplast responses to light (Kasahara et al., 2004). Phototropins are made up of two main parts: a

photosensory N-terminal, and a C-terminal kinase domain (Kasahara et al., 2004). In the model moss, *P. patens* (see below), there are four phototropins that are activated by blue light; photA1, photA2, photB1 and photB2 (Kasahara et al., 2004). Both the photA and photB mediate the chloroplast avoidance response and respond mainly to blue light for chloroplast movement although movement in response to red light has also been seen in *P. patens* (Kasahara et al., 2004). How phototropins regulate chloroplast motility in vascular and nonvascular plants is not well understood. What is clearly established is that in all plants the actin cytoskeleton plays an important role in chloroplast movement (Yamashita et al., 2011), while in bryophytes the microtubule cytoskeleton also participates in chloroplast transport (Shen et al., 2015).

1.2 *Physcomitrella patens*

The moss *Physcomitrella patens* has been extensively used to investigate many aspects of plant cell biological research, including chloroplast photorelocation (Yamashita et al., 2011). Several reasons, important for biological studies, make this moss a valuable model organism. Firstly is the fact that its whole genome has been sequenced, is easily accessible, and is well-annotated (Cove et al., 2009). Secondly, the moss is in a haploid stage for most of its life, meaning that there is only one copy of each chromosome in the nucleus. This makes manipulating the DNA of moss easy and its amenable to rapid genetic screening (Cove et al., 2009). One of the predominant cell types of this haploid stage are chains of filamentous cells called protonemata, which are amenable to a variety of microscopical techniques. *P. patens*

can also undergo high-efficiency mitotic homologous recombination (Kamisugi et al., 2006) in addition to rapid RNA interference (RNAi) (Arif et al., 2013). These techniques are valuable because allow the manipulating the DNA or RNA of certain sequences in the moss to knock out or knock down the expression of certain genes to determine their function. Finally, as any good model organism, *P. patens* is easy to culture in the laboratory as well as having a relatively short life cycle. This allows for multiple experiments to be run in a short period of time.

Protonemata filaments are composed by two different cell types, chloronemata, which have an abundance of chloroplast and have straight cell walls, and caulonemata, which have generally less chloroplasts and can be easily identified because of their oblique cell walls. The protonemal filaments of *P. patens* have been extensively used to characterize chloroplast motility (Kasahara et al., 2004), in particular the participation of cytoskeletal elements in this process.

1.3 Kinesins, Microtubules and their Roles in Plants

In plants, there are two different systems that move vesicles and other organelles around in cells. These are the actin and microtubule cytoskeletons, which act as tracks for the motor proteins myosin and kinesins respectively. In addition to organelle transport, kinesins also participate in the organization of the mitotic spindle (Hirokawa et al., 1998). In plants, as in other eukaryotic organisms, kinesins belong to a large superfamily of molecules with many members conserved in evolution (Richardson et al., 2006). Structurally, kinesins are generally homodimers, each subunit consisting of a head or motor domain, a tail or stalk, and

a flexible linker (Hirokawa et al., 1998). Some kinesins have associated light chains that can act as a cargo-binding region (Hirokawa et al., 1998). The kinesin motor domains bind microtubules and generate the force necessary for cargo transport. Microtubules provide tracks for kinesin-based transport, but they are also important for mitosis and cytokinesis; they participate in aligning chromosomes and separating them during mitosis (Hirokawa et al., 1998), and in plants they form the phragmoplast, which mediates cytokinesis.

In the genome of *P. patens* there are 71 kinesin genes, which can be classified into at least 14 different families (Shen et al., 2012). Many of these kinesins were shown to be associated with the spindle or phragmoplast by experiments involving a fluorescent protein tag to their C-terminus (Miki et al., 2014). Nevertheless, the research of Miki et al. (2014) failed to identify kinesins associated with chloroplasts in moss. This could have been the result of high chloroplast background, or that expression levels were below their detection methods. Because of the microtubule dependent transport of chloroplasts in *P. patens*, it is expected that a subset of kinesins may mediate this transport. Interestingly, kinesins 4II and 7I did not seem to localize to the spindle of the phragmoplast, suggesting a possible participation in chloroplast motility. Furthermore, the phylogenetic analyses of Shen et al. (2012) show kinesin 4II as a monophyletic group with no orthologues in *Arabidopsis thaliana* as seen in **Figure 1**. Similar to other vascular plants, *A. thaliana* uses an actin system for chloroplast motility (Kadota et al., 2006). This suggests that this subfamily is unique for moss and may have a function for chloroplast motility. In contrast, kinesin 7I subfamily has orthologues in *A. thaliana* seen in **Figure 2**, but

some of these molecules have been annotated as associated with mitochondria, suggesting a possible association to chloroplasts in moss (Shen et al., 2012).

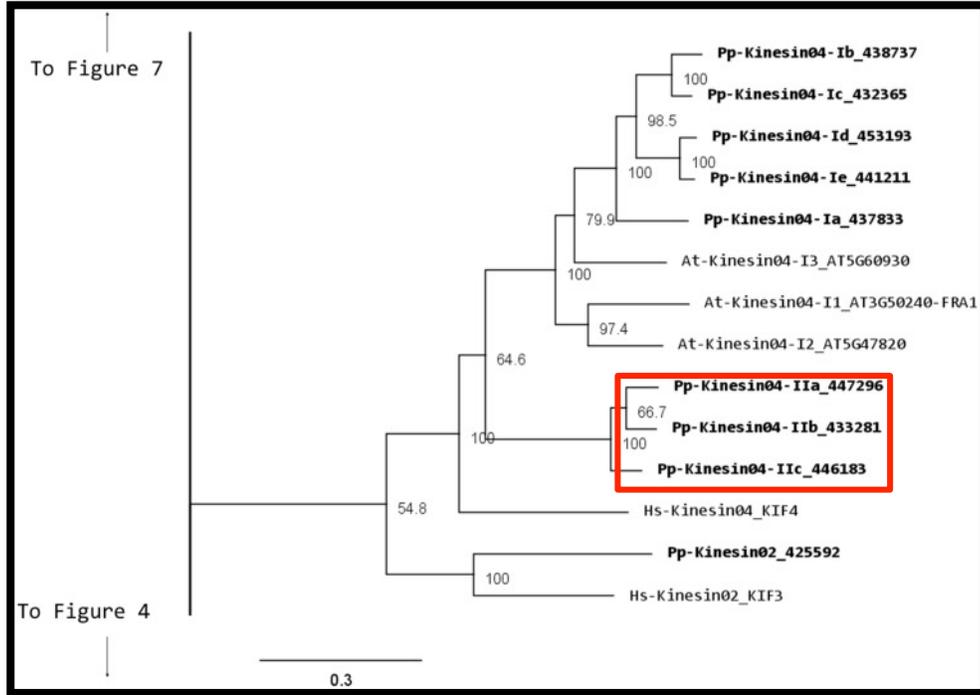


Figure 1: Sub-region of the phylogenetic tree based on their motor domain showing kinesin 2s and kinesin 4s. Focusing on 4-II kinesins for chloroplast motility

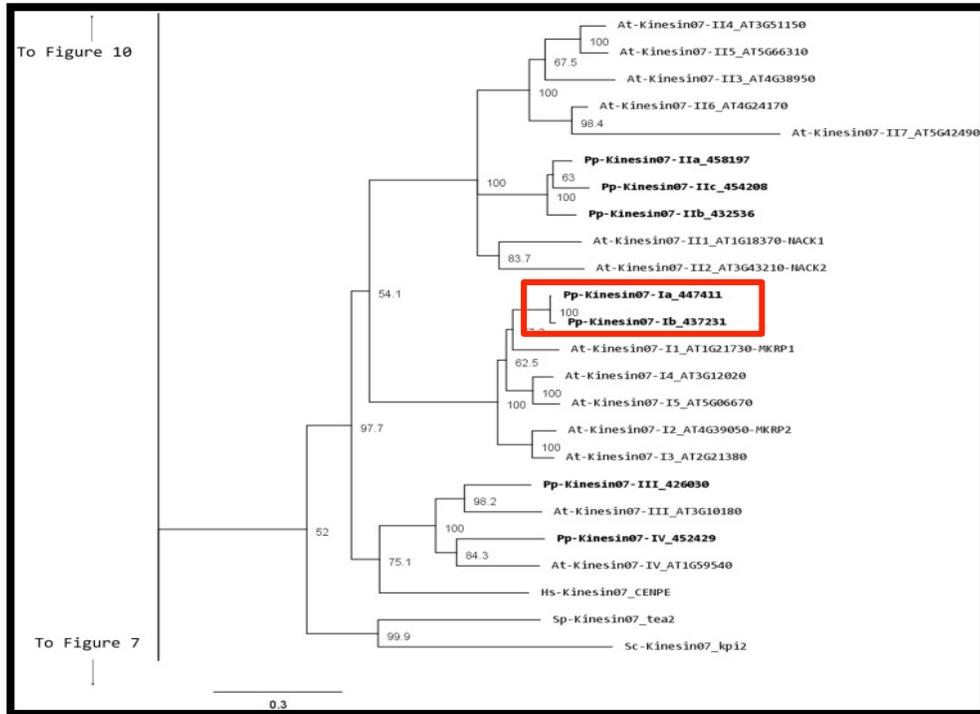


Figure 2: Sub-region of the phylogenetic tree based on their motor domain showing kinesin 7s. Focusing on 7-I kinesins for chloroplast motility

1.4 RNA Interference and Inducible Gene Expression

RNA interference is a mechanism by which a double stranded RNA sequence is expressed so that the internal silencing machinery of the cell can target the endogenous mRNA for destruction (Arif et al., 2013), allowing regulation of mRNA post transcriptionally. This is usually done by the expression of a small interfering RNA expressed from a DNA template complementary to the target mRNA.

There are many ways to control the amount of double stranded RNA produced. One useful strategy is to use an inducible gene system to express the double stranded RNA. In this case expression of the silencing construct will remain

off until the cell is exposed to the presence of the appropriate molecule, or inducer. The inducer will activate the gene silencing construct either directly or indirectly to allow gene expression (Nakaoka et al., 2012). Goshima and co-workers have generated an extensive collection of RNAi lines for *P. patens* kinesins (Miki et al., 2015). We obtained several lines designed for the silencing of Kinesin 4II and Kinesin 7I, with inducible expression of the silencing constructs. **Figure 3** illustrates the inducible promoter that is activated in the presence of beta-estradiol in these RNAi lines (Kubo et al., 2013).



Figure 3: The integration plasmid that allows induction of RNAi of RFP and target genes using the Gateway system. XVE is a chimeric transcription activator that acts as a receptor for β -estradiol. The ccdB regions were replaced with regions that would create RNA to bind RNA of kinesins 4-IIb and 7-Ib.

1.5 Purpose of this Study

The purpose of this project is to study chloroplast motility in *P. patens* and identify which motor protein is responsible for microtubule-dependent chloroplast transport. Based on the evidence described above, for this project, the search for the motor proteins has been narrowed down to kinesin 4IIs and kinesin 7Is. Hence, the hypothesis for this study is that one or both of these kinesins are responsible for chloroplast motility. In order to test this hypothesis, chloroplast avoidance experiments involving an inducible RNAi system for the different kinesin 4II and 7I lines were performed.

2) Methods

2.1 Moss Culture

During experimentation the moss *Physcomitrella patens* was cultured weekly. This allowed for new and healthy moss protonemata for consistent experimental results. The moss was plated on plates of PpNO3 made from PpNO3 packets (Caisson Laboratories Inc. , N. Logan, Utah), 7 grams of plant agar and 1 liter of dH₂O. PpNO3 is used for *P. patens* growth because it promotes the growth of caulonemata cells due to the fact it lacks ammonium, which is an inhibitor of caulonemata growth. All work involving the moss cell culture was done in a hood using sterile technique. A sterile set of forceps was used to add new cellophane to the plate where the moss would grow. The old moss was scraped into a cell culture tube filled with dH₂O (the amount of dH₂O depended on the amount of plates were made). Once inside the tube the moss was ground using a tissue homogenizer (Omni TH), which uses soft tissue plastic tips. After grinding each new plate received a 700 µl mixture of water and moss cells. Grinding moss cells allows for vegetative propagation. PpNO3 plates are cultured at 25 °C and given 18 hr of light a day. For each type of moss, a LB agar plate was inoculated as a control for bacterial contamination. These LB agar plates are checked before bacterial growth before the moss is used again. For the RNAi lines the culturing process was the same with only a couple differences. Initially to make a plate where the kinesin would be knockdown for imaging the RNAi line would be plated on normal PpNO3 plates for four days then switch the cellophane holding the moss to a PpNO3 with 1µM

estradiol plate for three days. This would allow for the RNAi system to be activated preventing new kinesins of the line in question to be made and any kinesin that were left over to degrade. The PpNO3 with 1uM estradiol plates are made by adding 10 µl of 100mM estradiol to the normal mixture of PpNO3 packets (Caisson Laboratories Inc. , N. Logan, Utah),7 grams of plant agar and 1 liter of dH₂O.

For these experiments there were many different types of *P. patens* lines used. **Table 1** below shows the total of 18 different RNAi lines that were initially expanded and were going to be tested for chloroplast avoidance assay described below. As for a control on the growth assay the line that used was the parental line used to make the RNAi lines called GPH002. The GPH002 lines along with the RNAi lines made from it contain coding for GFP tagged tubulin.

Kinesin Targeted	Kin 4IIa RNAi	Kin 4IIb RNAi	Kin 7Ia RNAi	Kin 7Ib RNAi
Clone Number	PMN 414 1-3	PMN 359 1-3	PMN 317 1-3	PMN 369 1-3
Clone Number			PMN 416 1-3	PMN 459 1-3

Table 1: Kinesin RNAi lines expanded and plated with estradiol.

2.2 Growth Assay

In order to determine whether certain conditions affect the growth of *P. patens*, this procedure dictates a means of getting single moss cells to observe plants developing over time. Moss is harvested and mixed with 9mL 8% mannitol and 3mL 2% driselase enzyme obtaining a final concentration of 0.5% of the enzyme. It is left

gently shaking for an hour after which the protoplasts are harvested and sieved on top of a 50mL Falcon tube. The protoplasts are centrifuged in a culture tube at 700rpm/250g for 5 minutes. 10mL of mannitol is gently added to the protoplasts and resuspended. The last two steps are repeated twice more. The number of protoplasts is calculated using a hemocytometer under the microscope.. Protoplasts are plated at a density of 50,000 per plate on PRMB, which helps the moss cells regenerate the cell walls they lose from driselase exposure. The plate is sealed with micropore tape and put in the growth chamber. After 4 days, the cellophane containing the regenerated plants is transferred to a PpNH₄ plate and put back in the chamber for three more days. .

In order to test the effects of knocking down each kinesin some alterations were made to the normal protocol above. The (-,-) is the label for protoplasts that never received any estradiol and followed the normal procedure acted as the control. The (-,+) treatment stood for protoplasts that were originally plated with no estradiol, allowed to regenerate their cell walls, then moved after 4 days to plates with 1μM estradiol. Finally (+,+) is the label for protoplasts that were only plated with 1μM estradiol, meaning the each kinesin was knocked down right after being protoplasted. Seven days after being protoplasted each of the treatments were stained with calcaflour and imaged using fluorescence microscopy.

The images were subsequently analyzed using ImageJ software each treatment for the growth assay consisted of at least an N of 15. The images were thresholded enough to outline the cell by going to the Image then Threshold. This

thresholded plant was then measured for its area, once that value was recorded the plant was rethresholded so that the cell was a solid shape. This block of thresholding was measured and is known as the convex hull. The area is divided against this convex hull and recorded as the solidity. It is possible to think of the convex hull similar to the area but taking a rubber band around the whole plant and taking that area. Therefore plants with lots of branching will have a larger convex hull in relation to the area giving a smaller solidity value. On the other hand a plant with less branching will have a convex hull closer to the area having a higher solidity value closer to one.

2.3 Moss Slide Prep for Microscopy

Slides containing live moss were prepared for microscope viewing by mounting the cells on a thin layer of 200 μ l of PpNO₃ agar. This agar was placed on a slide with parallel strips of tape on either side of the agar. In addition, another slide is placed perpendicular to the original slide covering the agar. The gel is left to set for 1-2 minutes before removing the top slide carefully. The agar pad is trimmed on all sides to achieve even edges that do not fully reach the sides of the slide. Small squares of cellophane from the desired plate of moss are cut and placed face down on the solidified agar. This is left to set for 1-2 minutes before being removed carefully with a tweezer. 20 μ l of either liquid PpNO₃ moss medium or any other liquid mixture is added to the agar pad before covering with a coverslip. The coverslip is then sealed to the slide using melted vaseline, lanolin, and paraffin combined in equal parts.

2.4 Chloroplast Avoidance Assay

Slides prepared as state above with the only difference that for depolymerizing F-actin 25 μM latrunculin B (1 μl of latrunculin B from a 5 mM stock in ethanol, 1 μl of 200 proof ethanol added to 198 μl of the melted agar) was added to a microfuge tube, then 200 μl of this agar was placed on the microscope slide followed by the moss. The top liquid (20 μl) was a mix of 198 μl liquid PpNO₃, 1 μl of 5 mM latrunculin B, and 1 μl of 200 proof ethanol then sealed. The slides were analyzed using a laser scanning confocal system (Leica-SP5) using a 40X oil lens. Moss cells that were being observed were always sub-apical the sub-apical cell also must have enough room for the chloroplasts to move as necessary. **Figure 4** shows that this avoidance response can be recorded and what the avoidance response should look like.

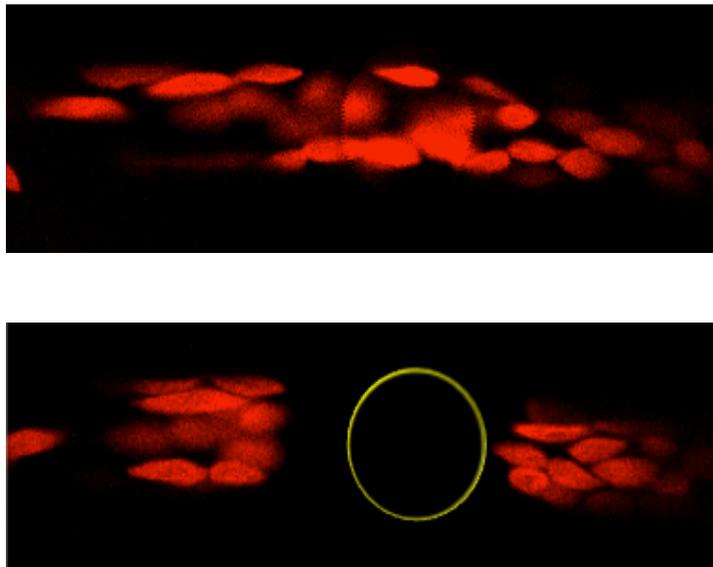


Figure 4: Chloroplast avoidance response induced by high blue light intensity in *Physocmitrella patens* as seen by confocal microscopy (Shen et. al 2014)

Once a specific cell is chosen it is zoomed to a value of three and the laser rotated until the middle of the cell was horizontal on the screen, the pinhole is set to 1 AU. The format was set to 512x185 pixels along with a 30x30 pixel ROI is set up in the center of the cell. Only this ROI portion of the cell was exposed to 488 nm blue light laser at 10% for 20 minutes, this initiates an avoidance response in the chloroplasts. The rest of the cell was illuminated with a 591nm laser at 5%, which illuminates the rest of the cell without causing any response. A double dichroic 488/561nm mirror separates the emission for the excitation laser the emission is then captured by two different detectors. One detector (PMT4) was set at ~700nm for visualizing the chloroplast. The second PMT (Trans detector) produces a brightfield version of the cell. The speed of the scanning was set to 200 Hz, images were taken two seconds apart. In order to accurately compare the results of each test, these time-lapse images were quantified using ImageJ software then underwent statistical analysis. The movies first had their contrast enhanced with ImageJ (enhance contrast function), setting saturated pixels to 0%, and the whole movie was normalized before being reduced twice by 2 to go from 600 images to 150 images per stack. This allowed for easier data plotting for ImageJ and later origin. Recreating the 30x30 pixel ROI region and plotting the Z-stack of that ROI allowed the data of average fluorescence over time to be obtained. Once each movies data had been obtained all of the data per movie was normalized so the starting value was one. This normalized data was then averaged for each treatment and given the standard error using the Origin software. The Origin software was then used to plot this average chloroplast motility of each treatment over time.

3) Results

The goal of this project was to study which motor protein is responsible for chloroplast motility within *Physcomitrella patens*. To investigate this, the strategy selected was inducible RNAi knockdowns. This was considered a viable strategy because *P. patens*' protonemal cells can be vegetatively propagated and it takes seven days in order to get well-developed cells that can be routinely imaged on the confocal microscope. These protonemal cells can be subjected to estradiol induction simply by transferring them on cellophanes to plates with estradiol. This would allow for the RNAi system to be activated preventing new kinesins of the induced line in question to be translated and any kinesin that were left over to degrade over three days. Control plants will be cultured in the same way but without estradiol treatment.

3.1 Reduction of Kinesin 4-IIb or 7-Ib Results in a Growth

Phenotype

Several clones of RNAi lines for each kinesin subfamily that were obtained from the Goshima Laboratory. For this project the focus was on analyzing clones targeting kinesin 4IIa and b, and kinesin 7Ia and b. To test the clones for chloroplast avoidance response, they needed to be expanded, which required approximately one month. Once the cultures were expanded RNAi was induced by estradiol treatment. However during the culture and preparation of these lines and the different treatments, not all of the cells looked the same under the microscope. The

growth that appeared when the RNAi lines for kinesin 4Ib and 7Ib were placed on estradiol was seemingly less branched, with a reduction in caulonemata differentiation and an abundance of shorter chloronemata. Because of the tight packaging of chloroplasts in these cells, this made the chloroplast avoidance response difficult to nearly impossible to conduct. Chloronemata have too many chloroplasts that they cannot move as easily as in caulonemata cells. Another interesting feature of these abnormal growing plants was the fact that tip cells were bloated.

In order to determine if the lack of kinesin 4Ib and 7Ib was resulting in a growth phenotype, a growth assay was designed and conducted. The lines used in this growth assay were kinesin 4Ib 359-1 RNAi , kinesin 7Ib 369-1 RNAi lines as well as GPH002 (GPH). GPH was the parental line of the RNAi lines and did not contain the estradiol promoter as part of its genome. The GPH line acted as a control to confirm that the RNAi lines were not being affected by the estradiol treatment. For **Figures 5 and 6** each column left to right are the same images in DIC, chloroplast autofluorescence, and calcofluor dye. The calcofluor stains the cellulose of the protonemata and give an indication to the difference in growth for each cell. Each row is a different treatment of the same line. These images show the difference between the control RNAi (-,-) lines and induced RNAi (-,+) lines during the growth assay.

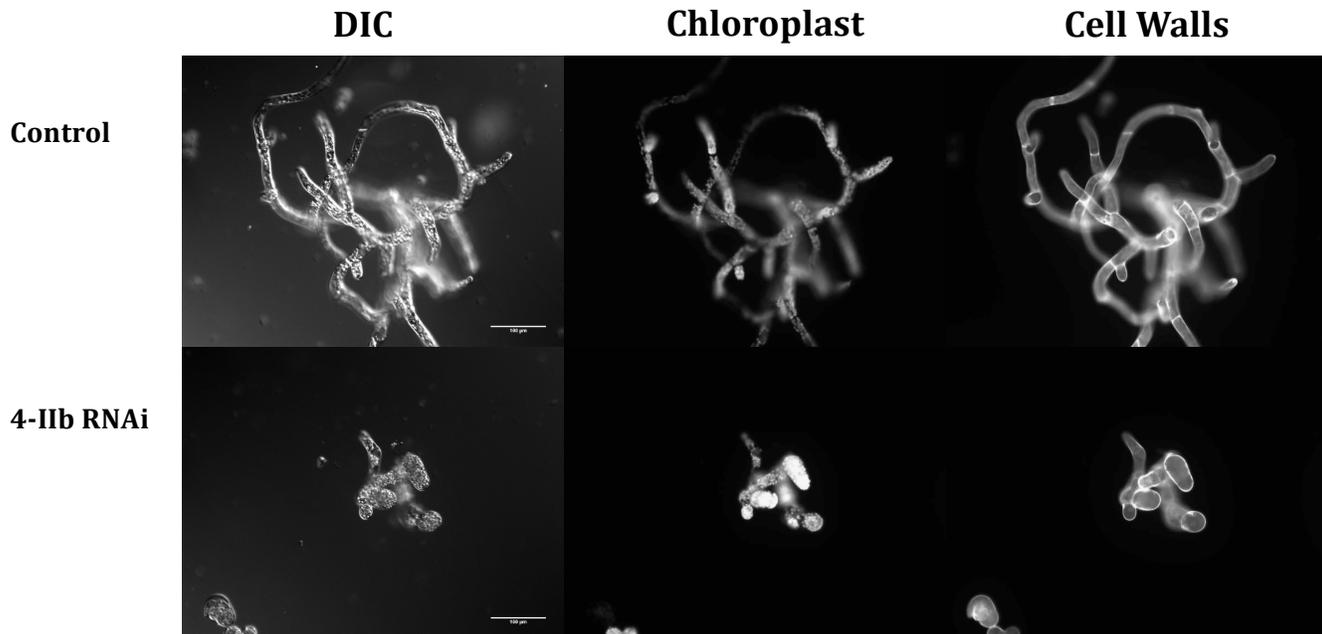


Figure 5: *P. patens* line Kinesin 4-IIb control (top) and 4-IIb induced (bottom). The control cells chloroplast autofluorescence, and calcofluor dye.

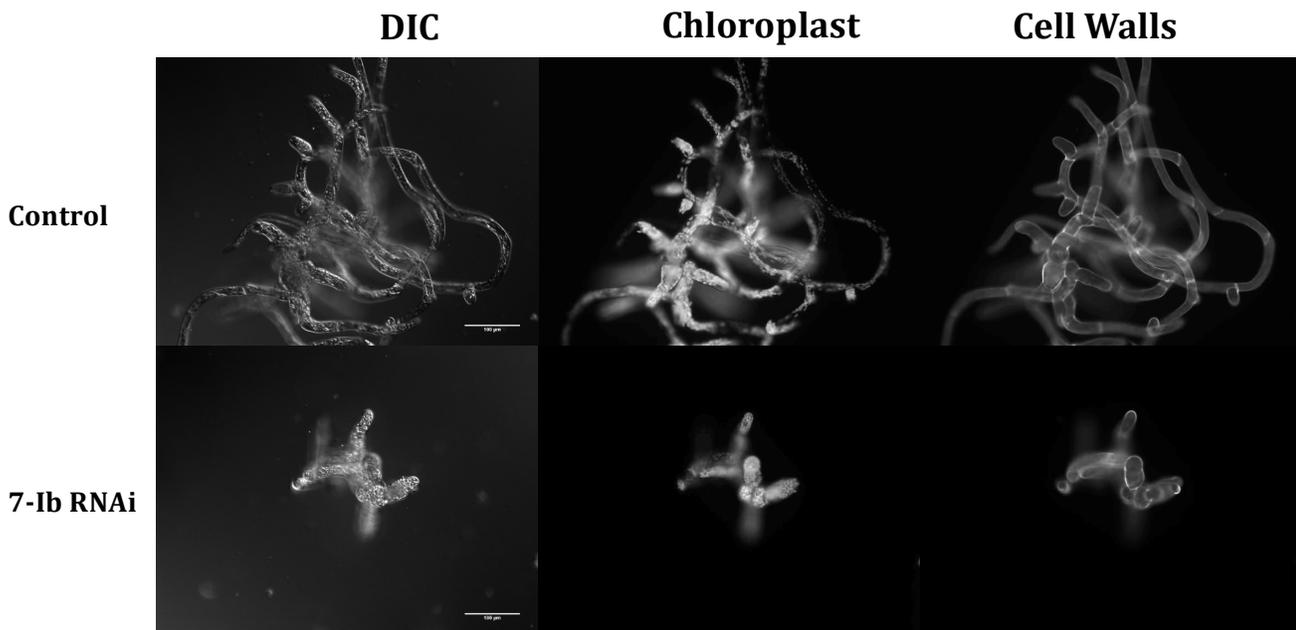


Figure 6: *P. patens* line Kinesin 7-Ib control (top) and 7-Ib induced (bottom). Each column left to right are the same images in DIC, chloroplast autofluorescence, and calcofluor dye.

For each of the RNAi lines shown in **Figures 5 and 6**, when the RNAi system was not activated, the growth was normal and consisted of many branching events. However a stark contrast is seen when the kinesin RNAi line is induced, there is a lot less polarized growth and branching. Looking closely it can be seen that the cell walls of the induced treatment are wider and more rounded compared to the control treatment. (See Appendix to view all of the growth assay images including the parental line, which is identical to the control treatment). The reason there is no (+,+) treatment images for the growth assay is that the protoplasted RNAi lines cannot recover when the RNAi constructs knocked down their respective kinesins.

3.2 Quantification of the Kinesin-RNAi Induced Growth Phenotype

The data for the growth assay was quantified by measuring both the area and the solidity of the different treatments. Using ImageJ it was possible to measure the area and using thresholding calculate the solidity values, the graphs were made on Origin 8.1 software as stated in methods above. **Figure 7** below shows a large and significant difference in growth that was evident between the treated and control plants for both 4Iib-RNAi and 7Ib-RNAi plants. The area disparity was smaller for the kinesin 7Ib RNAi plants compared to the area of the 4Iib-RNAi plants, suggesting a possible difference of phenotype between the two RNAi treatments.

Figure 8 compares solidity, which is a value that takes the area over the convex hull, among the treatments.

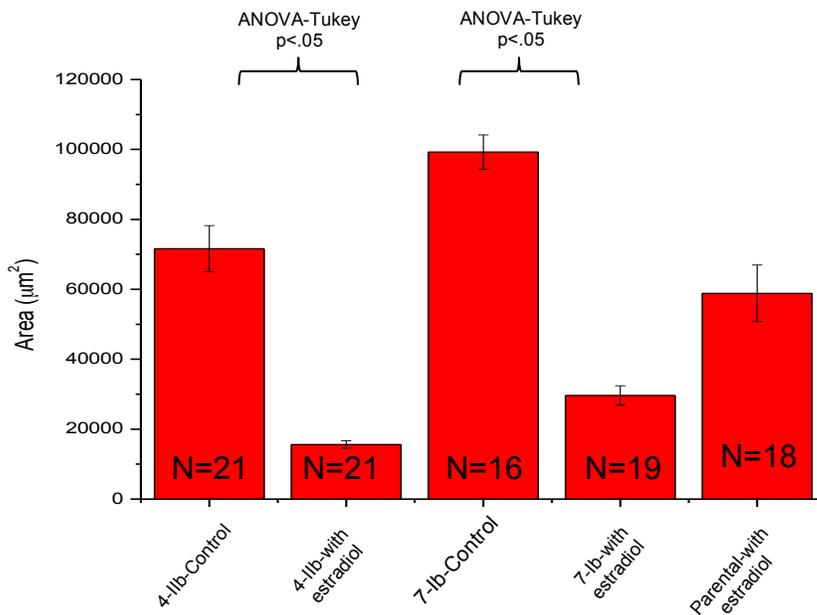


Figure 7: Comparison of the average values of area across different treatments and lines as seen in microns squared. Parental lines treated with estradiol have no significant change to their area. The control shows that estradiol has no direct affect to plant growth. Errors indicate SEM.

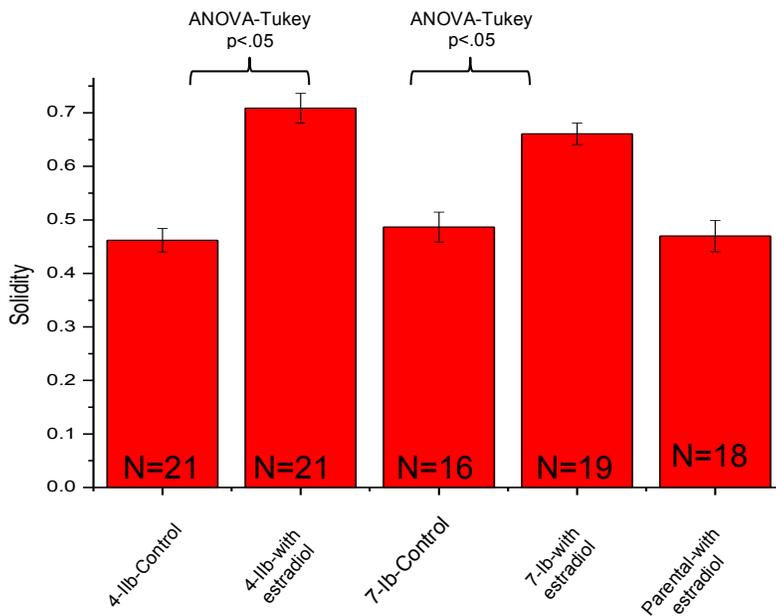


Figure 8: Comparison of the average values of solidity across different treatments and lines. Control values are very similar to parental lines that are treated with estradiol. Errors indicate SEM.

Importantly, **Figure 8** also shows that there is no difference in the overall solidity and therefore polarized growth between the control RNAi lines and the parental lines, when treated with estradiol. This indicated that any growth differences that were being seen were a result of the induced RNAi lines and not the presence of estradiol. Origin software was used to perform statistics to compare the area and solidity data. A statistically significant difference was also observed in the solidity between each of the control lines and their respective induced lines.

3.3 Kinesin 4-IIb is Necessary for Chloroplast Avoidance

Because of the observed growth phenotype in the kinesin 4IIb and 7Ib-RNAi lines, it seemed reasonable to check those RNAi lines first for a chloroplast avoidance phenotype. In order to circumvent the growth phenotype, the induced kinesin 4IIb and 7Ib line were both plated for a full week before then placed on estradiol plates for three days resulting in a ten day culture process before imaging. The control line was just plated on PpNO₃ plates for ten days. Many movies of the chloroplast avoidance response assay were made, each image taken every 2 seconds, creating a movie lasting 20 minutes. Although a reduction of kinesin 7Ib had a growth phenotype there was no apparent effect on chloroplast motility.

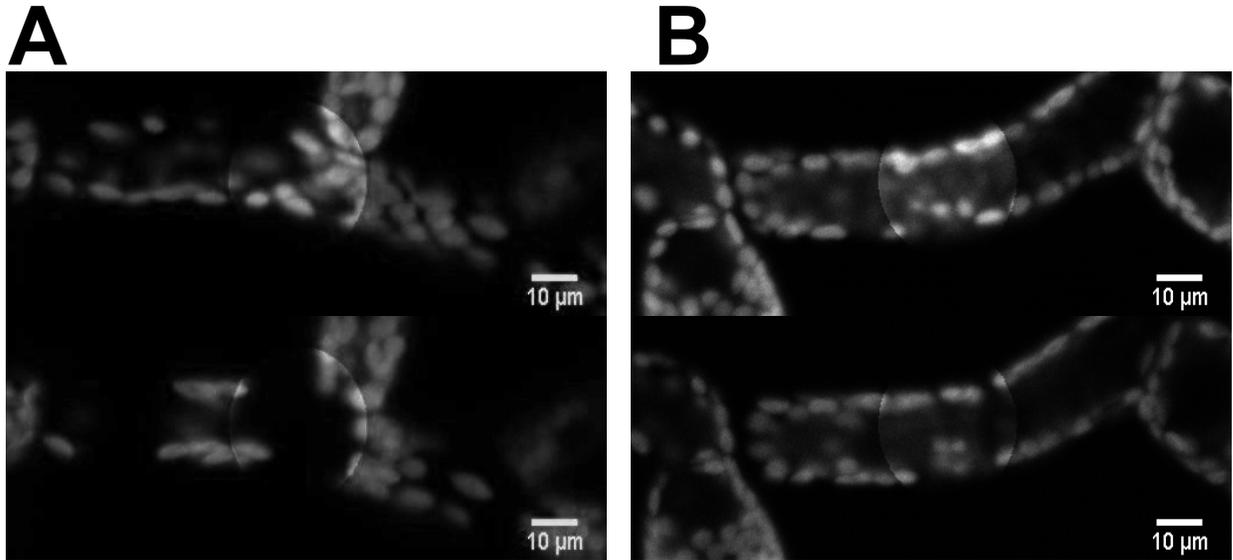


Figure 9: Kinesin 4-IIb chloronemal cells were irradiated within small area near the center to induce an avoidance response from the chloroplasts. **A:** Control cell where the chloroplasts are seen leaving the irradiated area. **B:** Induced cell shows some avoidance signal but most chloroplasts stay in the irradiated area.

In control cells, chloroplast moved out of the highly irradiated zone after 20 minutes of constant illumination (**Figure 9A**). In contrast, in the induced kinesin 4IIb-RNAi the chloroplasts do not seem to move to the same extent after 20 minutes of constant illumination (**Figure 9B**). However, looking at the movies and by quantifying the data (see below) some movement of the chloroplasts can be detected.

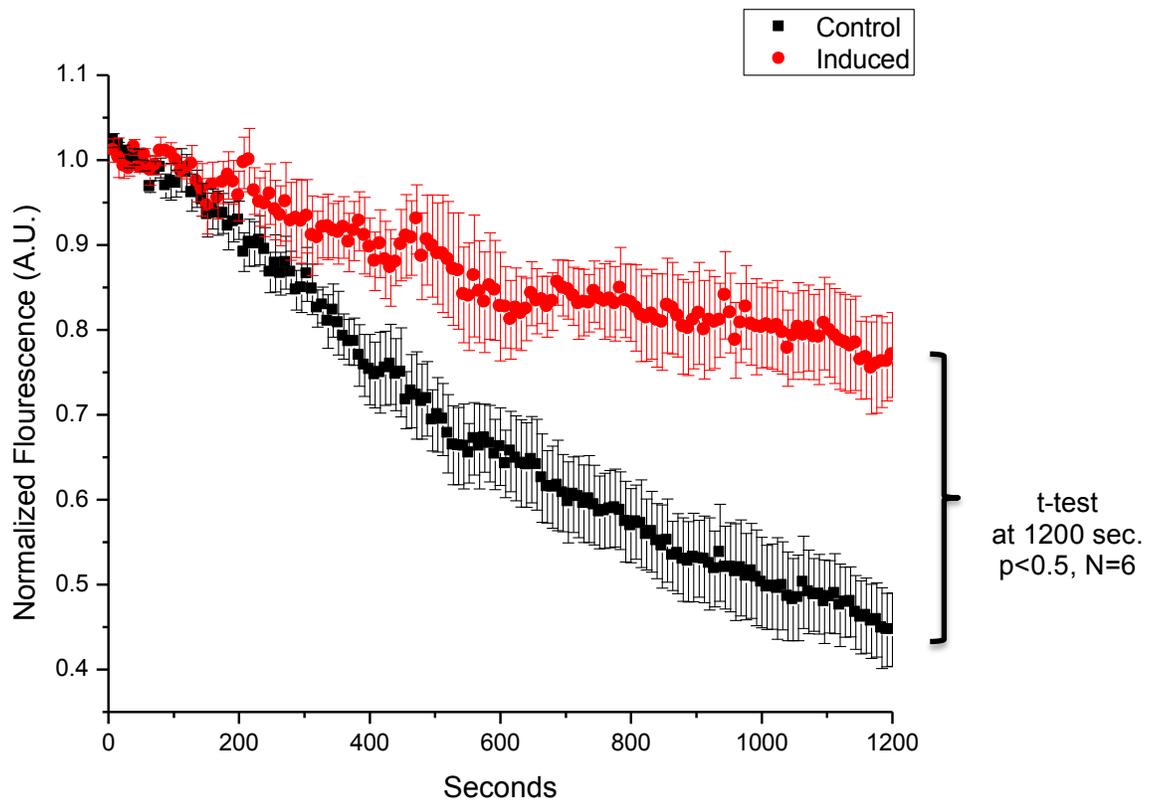


Figure 10: Quantification of the chloroplast avoidance response for the control and induced kinesin 4-IIb plants.

To compare the magnitude and rate of the chloroplast avoidance between the control and induced RNAi lines, the fluorescence signal decay was measured using ImageJ and plotted as a function of time (**Figure 10**). The fluorescence signal shown is the normalized and averaged fluorescence within the ROI illuminated overtime. This gives an overall speed to the chloroplast signal decay as they react to the high intensity blue light and leave the ROI. The average fluorescence was normalized so all the values started at one. The black line in **Figure 10** illustrates the movement of the control treatment. As it can be seen, the chloroplasts move out of the ROI fairly rapidly. The red line illustrates the movement of the chloroplasts

with the induced treatment, it is clear that the chloroplast still move but at a much slower rate. An ANOVA test was done on these data set, which revealed that the difference between the two treatments at the final time point was statistically significant.

3.4 Visualization of Microtubules during the Chloroplast Avoidance Response

Response

To further investigate the mechanism by which kinesin 4IIb participates in the chloroplast avoidance response, an assay was developed to be able to visualize microtubules of cells undergoing the chloroplast avoidance response. This assay allows the quantification of microtubule dynamics while simultaneously imaging chloroplast motility. Some changes were necessary to the chloroplast avoidance assay stated in the methods. First the format of the images was now 512x340 to better fit the cell. The cell chosen was zoomed in to a value of 12 rather than 3 in order to be able to resolve individual microtubules.

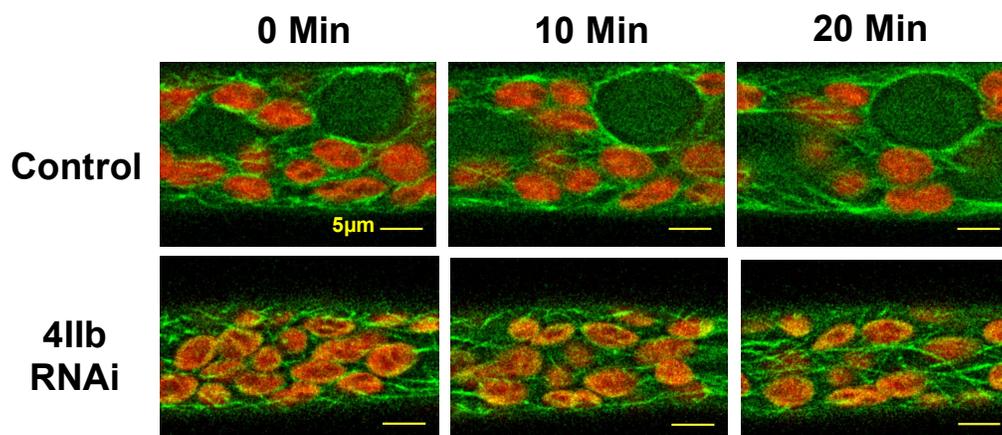


Figure 11: (Top) shows avoidance response for control kinesin 4Iib RNAi line. (Bottom) shows reduced avoidance response for induced kinesin 4Iib RNAi line. Testing effects of microtubule orientation/dynamics and chloroplast motility

Due to these modifications, it was only possible to visualize the middle of the cell, which creating an illuminated region equivalent to the previous ROI. Since only the middle of the cell was being illuminated with the 488nm blue light laser, there was no need for additional red light illumination. The scanning rate was still 200 Hz imaging every 2 seconds for 20 minutes. A triple dichroic 488/561/590 mirror was used in order to separate the emissions of the excited cell. The emission detector used was HyD3 (a high sensitivity hybrid detector), which capture light ~500nm for the GFP-labeled tubulin. PMT4 captured the chloroplasts fluorescence at ~700nm as before, and the PMT Trans detected a brightfield signal. The conditions of illumination necessary to visualize microtubules and the avoidance response had to be optimized, for example the initial attempts to record 3D movies of the microtubules and chloroplast did not result in an avoidance response. One possibility is the lack of consistent illumination in only one plane, which may not induce the chloroplast avoidance response. Alternative illumination regimes will need to be investigated in the future to perform 3D movies. Importantly, in the end, the assay was able to produce an avoidance response for the control and induced treatment if the cell was imaged in one plane rather than in a z-stack as seen in **Figure 11**. No obvious differences between the control and the kinesin 4Iib-RNAi were observed. Future quantitative analyses will be necessary to determine if there

are any differences in dynamics and orientation of microtubules between the control and RNAi lines.

4) Discussion

This project's goal was to investigate which kinesins are involved in chloroplast motility in the moss *Physcomitrella patens*. Through previous research and analysis, the subfamilies of kinesins 4II and kinesin 7I were tested using inducible RNAi and a chloroplast avoidance assay. An unexpected growth phenotype occurred with the kinesin 4IIb and kinesin 7Ia RNAi lines. Conducting a growth assay revealed kinesin 4-IIb and 7-Ib function was important for the growth of *P. patens*. Focusing on these two RNAi lines to evaluate chloroplast avoidance revealed that only kinesin 4IIb was necessary for chloroplast motility. In the end this project identified one motor protein that was important for chloroplast motility in *P. patens*. This research may later be helpful in understanding the molecular mechanisms of growth not only for *Physcomitrella patens* but other plants as well.

Possible effects of a reduction in kinesin 7I and 4II levels in cell growth

Kinesins are motor proteins that have also been known to transport vesicles (Vale, 2003). In cells, vesicles package proteins and polysaccharides for transport and secretion. Because in protonemata cell growth depends on secretory vesicle delivery, it is possible that the bulging of the tip cells observed results from a disruption of secretion. This could be due to changes in regulation of vesicle fusion or in changes of the location of delivery. Further analysis would be needed to ascertain what cellular structures important for cell growth (vesicles, microtubules, etc.) are transported by kinesins 4IIb and 7Ib.

It is important to emphasize that control experiments were performed to determine whether the growth inhibition was occurring as a result of the estradiol treatment or because of the reduction of kinesin levels. No effect of the estradiol alone was detected, either in control lines or in some of the clones expressing RNAi constructs targeted to kinesin 4IIa, 4IIC, or 7Ia. This clearly indicates that estradiol at the concentration used has no effect on growth. The reason for a lack of phenotype in other RNAi constructs could be due to low level of expression of the isoforms and a lack of cross-silencing between constructs.

Preliminary comparison of the morphology of cells undergoing RNAi for kinesin 4IIb plants seemed more detrimental to the growth of *P. patens* than a reduction of kinesin 7Ib (**Figure 7**). Area and solidity quantification suggest that this difference in growth phenotype is present (**Figure 8**), but additional statistical tests will be required to establish a significant difference. Additional comparisons using live cell imaging may also help understand the differences between the two cell types. For example, one kinesin subfamily may be important for tip growth, while the other may be important for the timing of cell division or branching.

Problems in performing chloroplast motility assays in morphological compromised plants

The chloroplast motility assay presented in this project was equivalent to that performed by other groups (Wada, 2013). This is important because for this work a laser scanning confocal microscope was used, this system improves the reproducibility and simplicity of performing this assay. It is evident from looking at

Figure 9A and more importantly from the quantification in **Figure 10**, that the chloroplast avoidance assay was highly reproducible.

The growth phenotype indicated above made imaging of chloroplast motility problematic. To circumvent this problem, RNAi lines were cultured for longer before inducing. Rather than the normal seven days of culturing, the RNAi lines were grown initially for seven days on PpNO₃ plates. Switching the plants to estradiol plates induced RNAi; nevertheless, imaging was never done after ten days as *P. patens* would start growing leaves. This extended growth period also allowed us to identify longer cells, with more dispersed chloroplasts, but it was difficult to unequivocally distinguish caulonemata from chlononemata in all circumstances. The kinesin 71b RNAi line had no correlation between the amount of kinesin and chloroplast motility. This indicates that kinesin 71b has another function most likely something to do with transporting growth proteins as seen in **Figure 6**.

Possible effect of kinesin 4II in chloroplast avoidance response

The hypothesis that kinesin 71 subfamily of proteins participates in chloroplast motility was not supported by the data presented. It is possible that the RNAi constructs did not silence enough of the kinesins to result in a phenotype; nevertheless this is unlikely because of the observed growth phenotype. It is also possible that this kinesin subfamily participates in the chloroplast accumulation response. With the optimization of the chloroplast motility assay presented in this work, this analysis should be possible.

The hypothesis that kinesin 4II subfamily participates in chloroplasts transport was supported by the data presented (**Figure 10**). Nevertheless, chloroplasts in the lines undergoing RNAi still moved to some degree after being illuminated with high intensity blue light. Some possible reasons that can explain why chloroplasts are only slowed and do not stop completely could include the following:

First, it is possible that the inducible RNAi system is not very efficient, meaning that there may still be some amount of kinesin 4IIb left in the cell, that could be moving the chloroplasts. Performing an RT-PCR (Q-PCR) that would show what the expression levels of kinesin 4IIs are in the RNAi lines could test this.

Another possibility is that there may be a functionally redundant kinesin that is performing the function of kinesin 4II in chloroplast motility, but is less efficient. Possible candidates for this alternative kinesin may be one of the other isoforms of kinesin 4II, such as 4IIc or 4IIa. Although knocking down kinesin 4IIa and 4IIc did not show a growth phenotype, which indicates that kinesin 4IIb was important for growth, but kinesin 4IIa or 4IIc may still be involved in chloroplast motility.

Finally, another possibility is that the lack of kinesin 4II may affect the orientation or dynamics of the microtubules in protonemata. To address this, a new assay was developed that allows viewing microtubules while still inducing chloroplast photorelocation as shown in **Figure 11**. Preliminary data indicate that this assay will allow the measurement of microtubule dynamics and orientation, while the chloroplast avoidance response is repeatable. To be able to quantify any

microtubule dynamics and orientation changes, additional experiments would entail conducting more microtubule-chloroplast avoidance assays. Measuring these values will help determine if, as chloroplast move out the high intensity region of light, the microtubules orient themselves more horizontally to allow faster photorelocation. Importantly, quantifying microtubule dynamics would also provide information as to whether there is a preference of microtubule formation going left or right within the cell during chloroplast avoidance.

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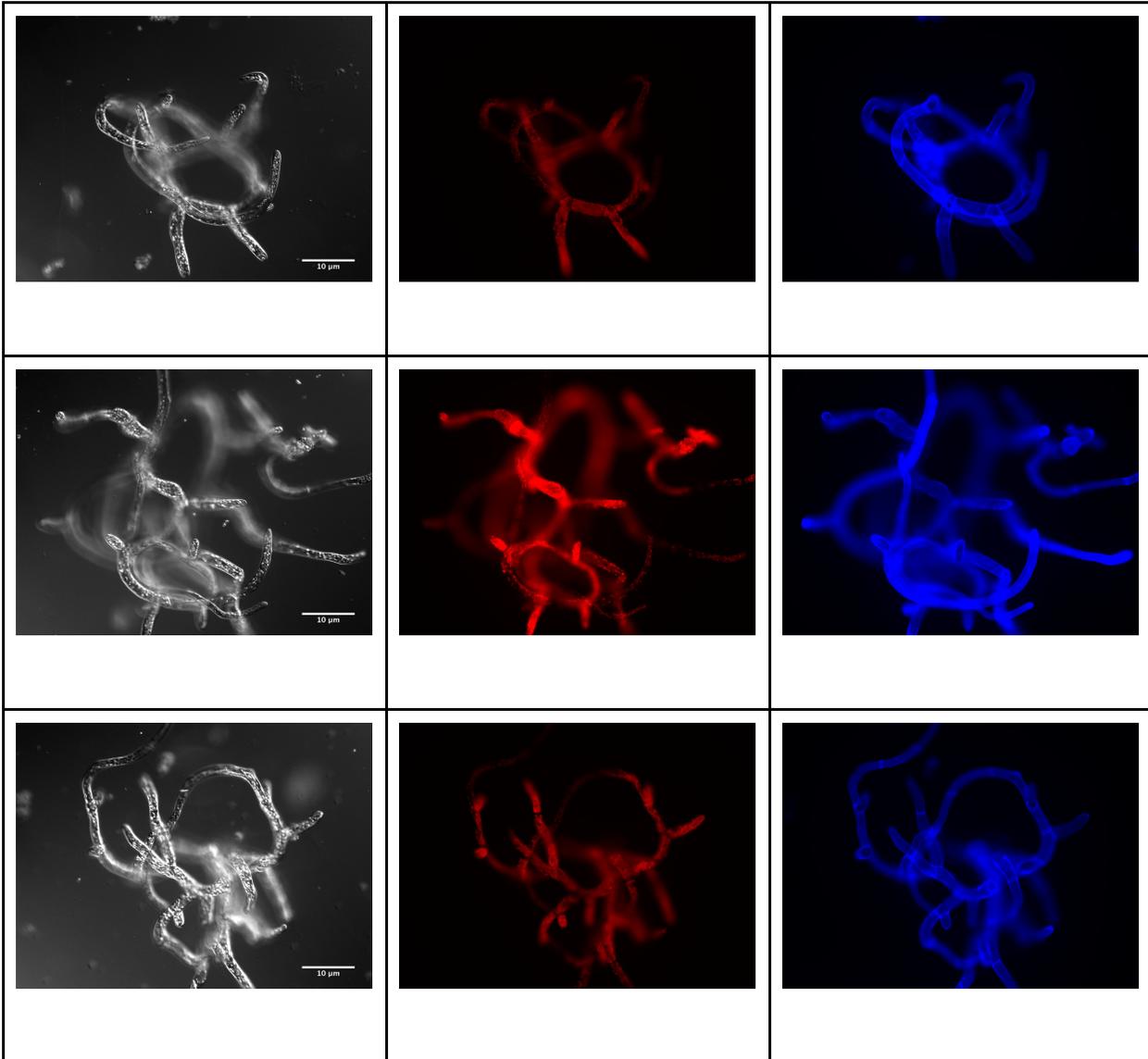
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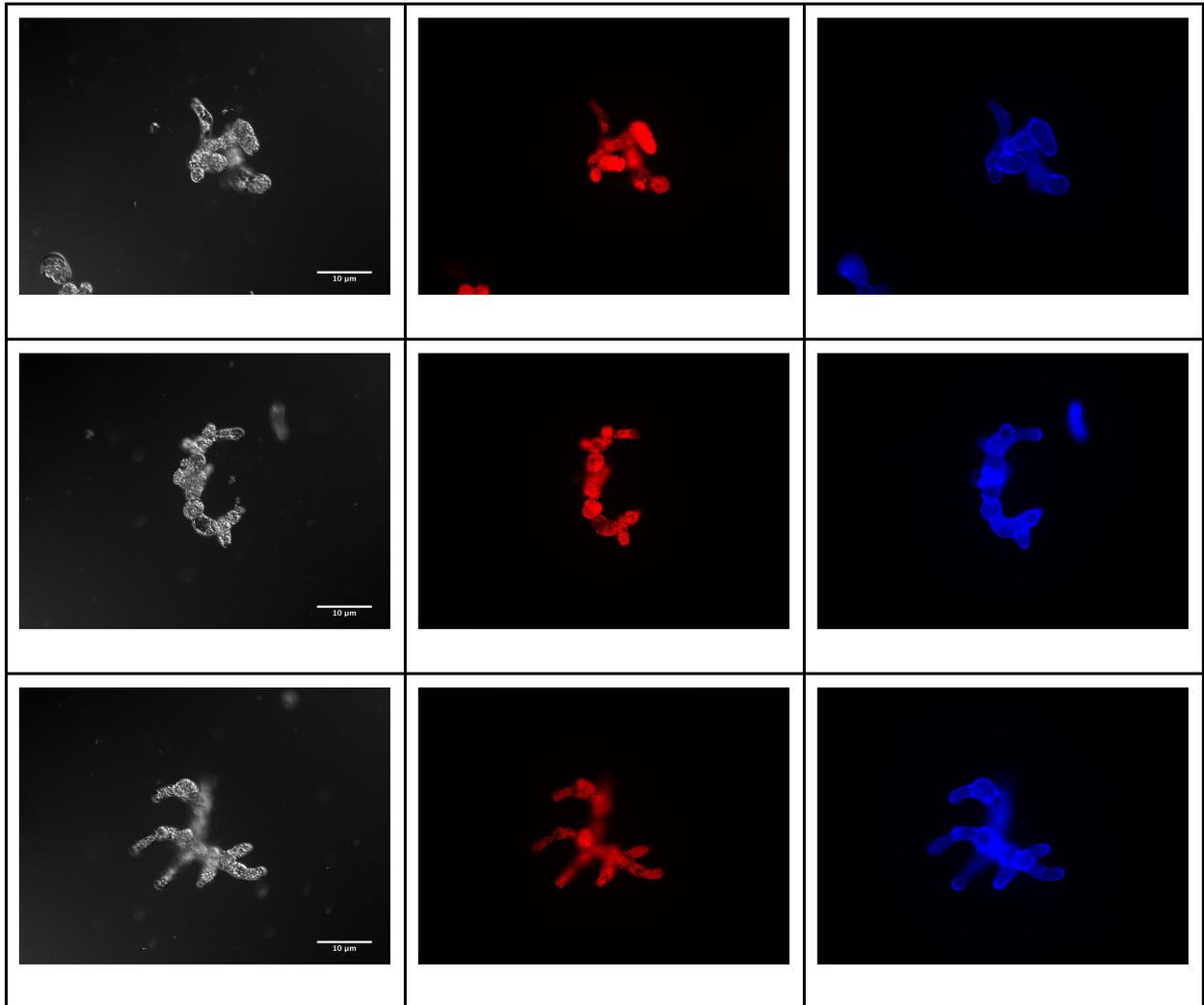
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5) Appendix

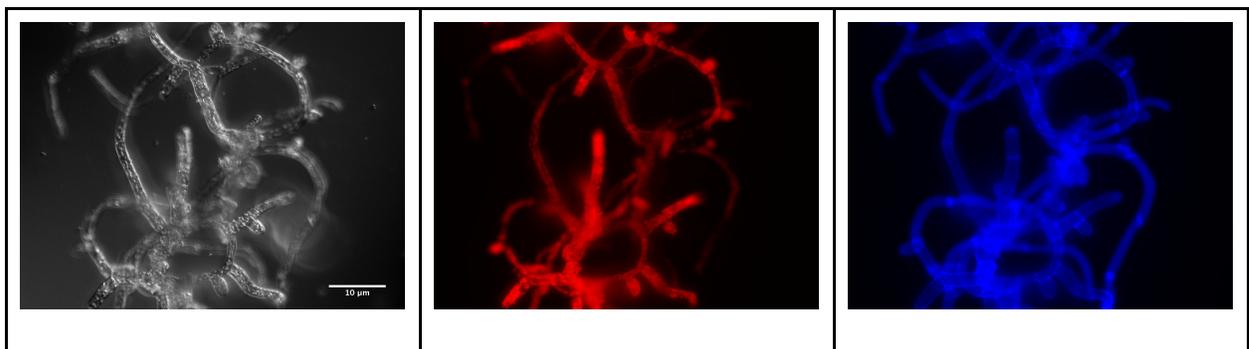
A: Growth Assay

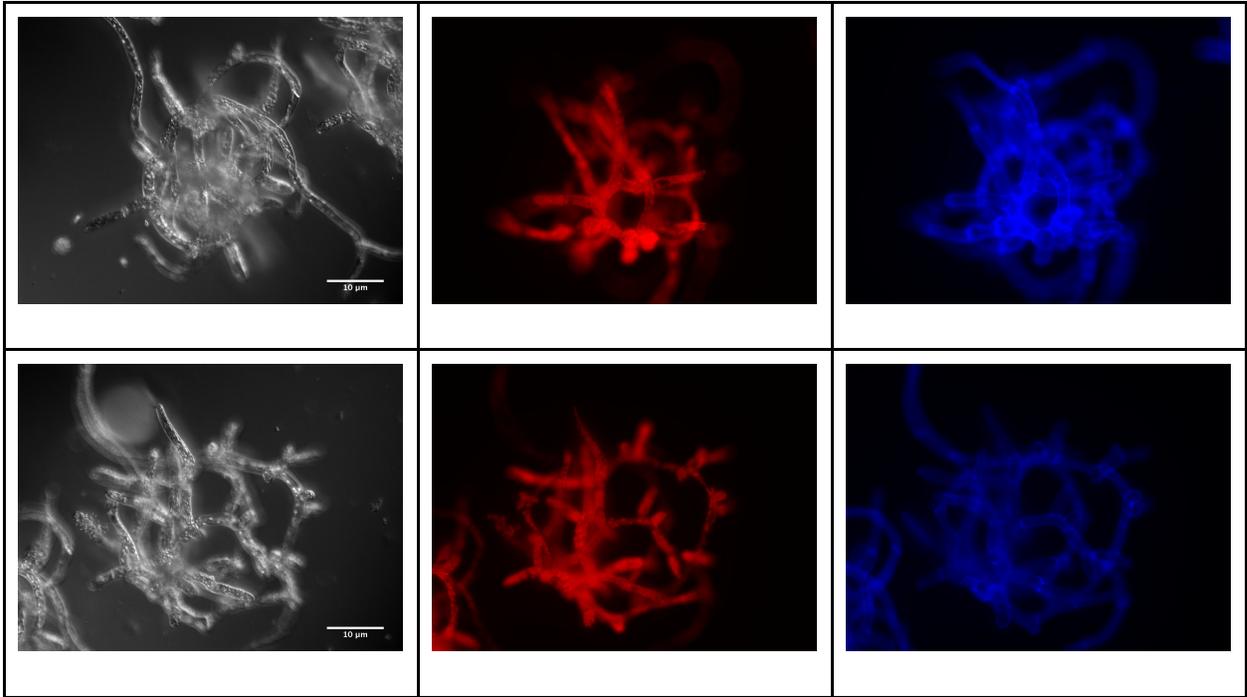


Appendix A-1 a-i: *P. patens* line- Kinesin 4IIb-359-5 (-,-) / Each column left to right are the same images in DIC, chloroplast autofluorescence, and calcofluor dye. Each row is a different cell of the same line.

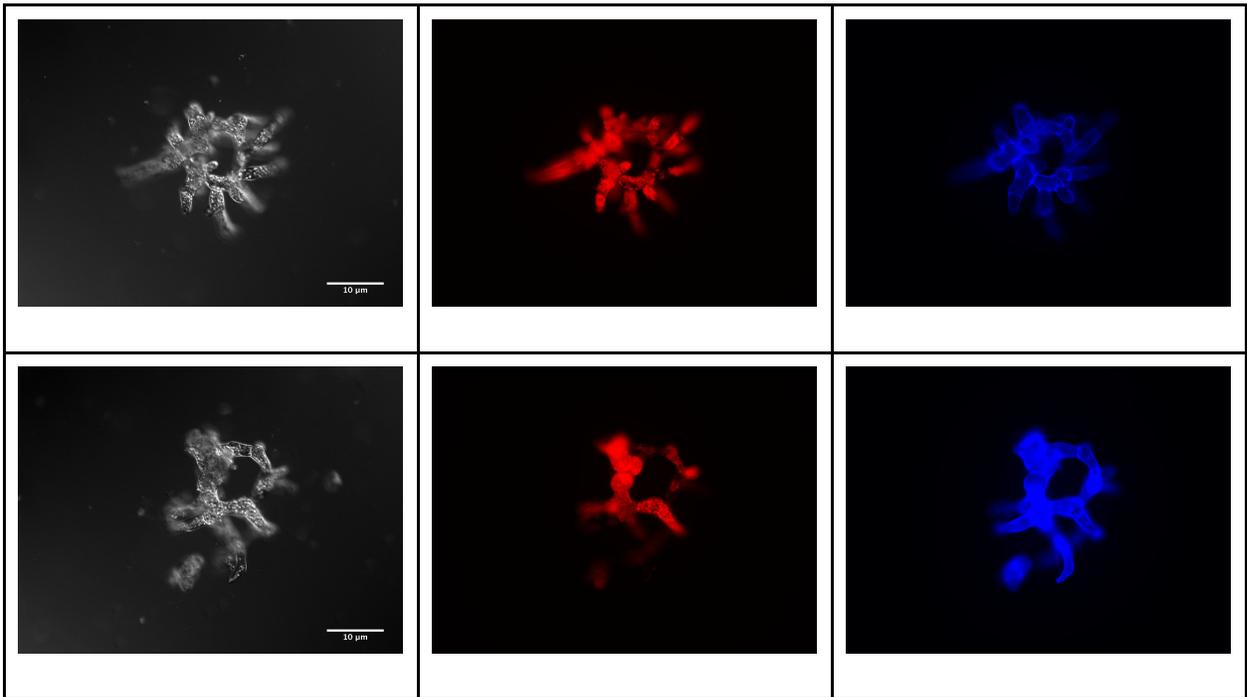


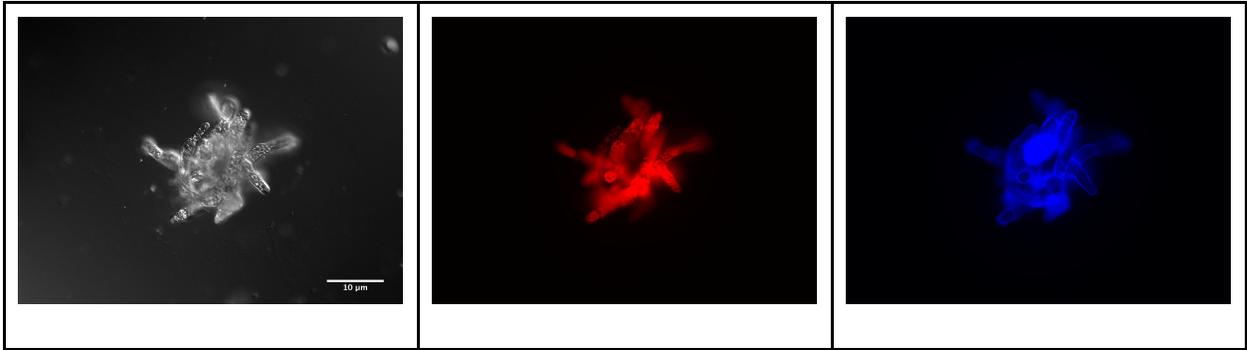
Appendix A-2 a-i: *P. patens* line- Kinesin 4IIb-359-5 (-,+) / Each column left to right are the same images in DIC, chloroplast autofluorescence, and calcofluor dye. Each row is a different cell of the same line.



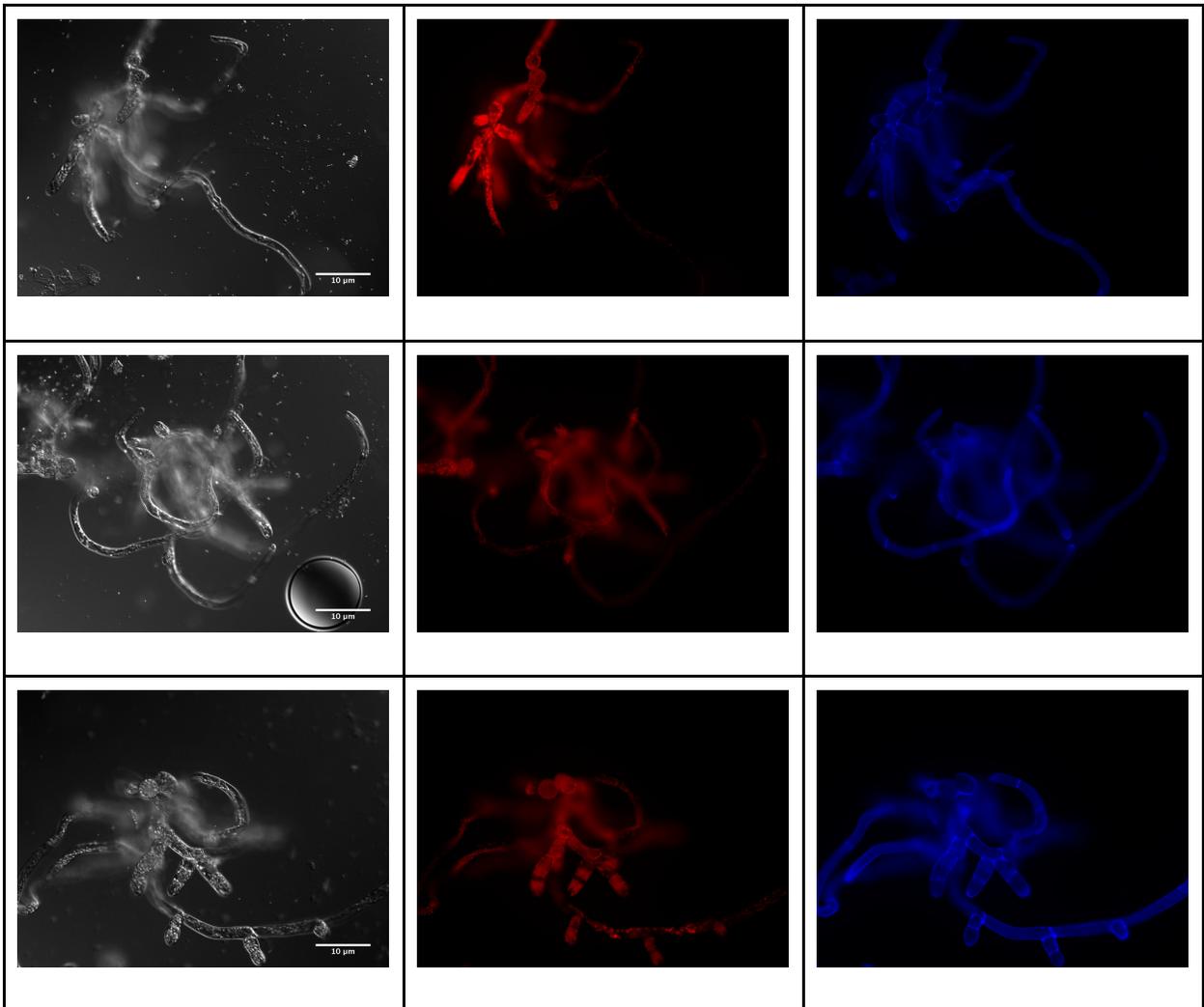


Appendix A-3 a-i: *P. patens* line- Kinesin 7Ib-369-1 (-,-) / Each column left to right are the same images in DIC, chloroplast autofluorescence, and calcofluor dye. Each row is a different cell of the same line.





Appendix A-4 a-i: *P. patens* line- Kinesin 71b-369-1 (-,+) / Each column left to right are the same images in DIC, chloroplast autofluorescence, and calcofluor dye. Each row is a different cell of the same line.



Appendix A-5 a-i: *P. patens* line- GPH-002 (-,+) / Each column left to right are the same images in DIC, chloroplast autofluorescence, and calcofluor dye. Each row is a different cell of the same line