

Microtubule Dependent Motors in the Chloroplast Avoidance Response

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Abstract

Kinesins are an important type of motor that use microtubules to move throughout the cell and are thought to be a factor in regulating the motility of chloroplasts in moss. This project is focused on further understanding the mechanism of microtubule-mediated chloroplast motility by trying to test the chloroplast light avoidance response in moss plants where kinesins 4-II and 7-I have been silenced by RNAi. Latrunculin and oryzalin were used as controls in altering chloroplast movement as they each disrupt the function of actin and microtubules respectively. Under the influence of these drugs the avoidance response should either decrease or disappear altogether. This study and future studies like it will help bring further understanding about the participation of kinesins in chloroplast motility.

Introduction

It is important to understand as much as possible about cell transport systems such as that of kinesins on microtubules. Without these forms of transport it would be very difficult or impossible for a cell to function normally. Much more research on kinesins and microtubules has yet to be done and discovered. This project aims to make a dent in that research by trying to see the importance of the specific kinesins 4II and 7I in the chloroplast avoidance response.

Chloroplast responses to light

Chloroplasts are organelles found only in plant cells that are vital to the survival of the plant. Chloroplasts' main purpose is to conduct photosynthesis. Photosynthesis is the process of converting sunlight into energy for the plant. Chloroplasts perform this function by absorbing light photons using the pigments chlorophyll a and chlorophyll b (Chloroplast, 2014). The light is then converted to energy in the form of ATP and NADPH through the Calvin Benson Cycle so that the plant cell can produce sugars needed for metabolism (Alberts et al., 2002).

Chloroplasts have the ability to move throughout the cell in order to absorb as much light as possible for photosynthesis or avoid damage when the light is too intense. Blue light is the main type of light that the chloroplasts absorb and respond to (Banas et al., 2012). This is the case for *P. patens* in which blue light can induce directional movement of the chloroplasts (Banas et al., 2012). *P. patens* can also be induced to move by red light (Sato et al. 2001); although this type of light was not used in this project. The way chloroplasts move in response to light is similar in all plants and can be classified into an accumulation response and an avoidance response (Sato et al., 2001). When the chloroplasts sense low light intensity they move towards it in order to absorb as much as possible. This is the accumulation response.

However, there is a point where the light is too intense and the chloroplasts can become damaged. In response to this situation, the chloroplasts display a scattering response and move away from the light as much as possible (Sato et al., 2001). This allows for the plant to maximize the amount of light absorbed with a limited energy source while also protecting the chloroplasts from too much light so that they do not get damaged (Sato et al., 2001).

Phototropins

Blue light photoreceptors, phototropins, are what control the chloroplast responses. There are four phototropins in *P. patens* that are activated by the blue light. They have been identified as photA1, photA2, photB1 and photB2 (Kasahara et al., 2004). These genes were further divided into two groups (photA and photB) based upon their deduced amino acid sequences (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).

Phototropins are made up of two parts: a photosensory N-terminal that has two light, oxygen, voltage (LOV) domains, and a C-terminal kinase domain (Banas et al., 2012). The LOV domains (named LOV1 and LOV2) are very similar for the most part but have photochemical properties that differ slightly from each other. LOV1 can reduce the effect of the photoactivation of the kinase, change the photosensitivity of the photoreceptor and act as a dimerization site in vitro (Banas et al., 2012). LOV2 is the domain that is mainly responsible for the photoreceptor activity (Banas et al., 2012). Although both domains have different responsibilities, it has been shown that one LOV domain can make up for the absence of the other. For example when phototropin fragments lack LOV1 they were still observed to go through dimerization (Banas et al., 2012).

Kinesins

Kinesins are motor proteins that use the microtubules in the cell as a means of transport. They generally transport things like organelles and vesicles from the center of the cell outwards towards its edge (Berg et al, 2002). Kinesins move along the microtubules with the help of ATP and use two head groups in order to essentially “walk” along the microtubules (Berg, 2002). Land plants contain many more kinesins than mammals do. *P. patens* have currently had 72 kinesins identified and classified into 15 families (Shen et al., 2012). Of those 72, 43 kinesins have been localized to microtubule-based structures (Miki et al., 2014). It is currently not known exactly why land plants have so many kinesins and the first steps to understanding this are discovering what each kinesin does. Among the kinesins tested by Miki et al. (2014), kinesins 4II and 7I, were not seen to be involved in mitosis particularly in anaphase and cytokinesis (Miki, 2014). Because these two kinesin subfamilies were not associated with microtubules during this process, they most likely have other important functions in the moss such as having a possible participation in chloroplast motility.

Microtubules are also extremely important for other cell processes such as cell division. They are responsible for regulating cytokinesis and lining up the chromosomes and separating them during mitosis (Zhu, 2005). When cell division in plants occurs, the cell plate needs to be formed as it will eventually become the new cell wall (Hiwatashi, 2008). In order for this cell plate to be generated, it needs a phragmoplast which contains two opposing sets of microtubules (called antiparallel microtubules). The plus ends of the phragmoplast microtubules go towards the cell plate while the minus ends of the microtubules go towards the divided nuclei (Hiwatashi, 2008). Then vesicles containing the materials and information needed for the cell plate travel along the microtubules and deliver them to the cell plate formation site (Hiwatashi, 2008). The

role that microtubules play in cell transport is invaluable. Without them it would be very difficult for most plants such as *P. patens* to effectively transport nutrients, materials and organelles around a cell.

RNA interference

Interference RNA (RNAi) is a mechanism for gene expression regulation based on double stranded RNA that can block gene expression (Zamore, 2000). RNAi occurs post-transcription and usually involves the degradation of the mRNA. RNAi can phenocopy mutations that create a loss of gene function (Zamore, 2000). It is also thought that RNAi helps to protect the genome by preventing instability that is caused by transposons (small piece of DNA that inserts itself into a different place on the genome) and repetitive sequences (Zamore, 2000). By blocking expression of the more unstable and therefore dangerous parts of the genome, RNAi has evolved to help instead of harm by blocking gene expression. However, since its gene blocking qualities are so robust, RNAi has become a useful tool in the laboratory. It is commonly used to knock-down gene expression in different organisms to better understand the functions of specific genes and the impact they have on different processes in an organism.

Project Objectives

This project focuses on the kinesin-based chloroplast motility within the cells of moss, more specifically, *Physcomitrella patens* or *P. patens*. This moss is a very good model organism because it shares the fundamental and genetic processes of vascular plants, but has a reasonably short life cycle (8 weeks) and is haploid in its main growth phase, so that results can be obtained in a timely manner. Also, it is one of few multicellular organisms that have extremely efficient homologous recombination, which allows for targeting of specific genes for gene deletion or

precise genetic modifications (Schaefer, 1997). This allows researchers to target specific genes for research with relative ease, thus creating many different lines for analysis and comparison.

This project aims at testing the hypothesis that kinesins are responsible for chloroplast motility within moss cells. Specifically, the project focuses on determining the importance of kinesins 4-II and 7-I in chloroplast movement by targeting the kinesin genes in *P. patens* using RNAi. The initial plan was to test RNAi lines for these kinesins obtained from a collaborator (Miki et al., 2014), but due to technical difficulties and lack of invested time, only a subset of the initial controls were performed.

Hypothesis and Predictions

We hypothesized that the moss with the knock-downs for kinesin 4II and 7I would show a diminished chloroplast avoidance response. In addition, based in published results (Sato et al., 2001), we plan to use as controls moss treated with the drugs (latrunculin and oryzalin) because the treatments should produce a diminished avoidance response as the drugs disable actin and microtubules.

Methods:

In order to ensure accuracy and consistency of results, the moss was cultured weekly and tests were only performed on moss that had been growing for more than a week. This allowed access to fresh young and healthy moss protonemata for consistent results. To plate the moss, a moss-specific media was prepared using a pre-prepared mix from Caisson Labs. The main media used was PpNO₃, although PpNH₄ was also used. However, PpNH₄ exhibited problems, it did not fully dissolve when preparing the media and left behind a precipitate. PpNO₃ was chosen because it promotes the growth of caulonemata cells due to the fact it lacks ammonium which is an inhibitor of caulonemata growth. Therefore, all PpNH₄ plates were phased out of the experiments and only PpNO₃ plates were used.

All work involving the moss cell culture was done in a hood using sterile technique. To further avoid contamination, 99% ethanol was used to sterilize all surfaces present in the hood, instead of the normal 70% ethanol. The moss samples were moved to the hood for culturing and passed onto new plates with fresh PpNO₃ media. A sterile set of forceps was used to scrape a moss off the cellophane covering the gel; about half of each plate was used. Next the moss sample was added to a sterile test tube containing 4 mL of sterile H₂O. Once each sample that was being cultured was added to a separate tube, the samples were homogenized using a Omni TH Tissue Homogenizer equipped with soft tissue plastic tips. Each sample was only ground for a few seconds as to ensure the plant cells were not destroyed thus making the sample unusable. After grinding, the moss and water mixture was then plated on 3 separate PpNO₃ plates and LB Agar plates. Each plate received 1 mL of moss sample. The LB Agar was used as a control for

bacterial contamination and was incubated at 37°C and checked for growth before other moss was used again. PpNO₃ plates were cultured at 25°C and given 18 hours of light a day.

When analyzing the moss cells for chloroplast motility, a small sample of moss was cut from the plate being tested and placed onto a microscope slide with a small area of PpNO₃ gel. Next the cellophane was removed from the moss sample and 40 microliters of a PpNO₃ medium were added. For the moss samples being treated with the drugs, the gel and the buffer were both prepared to have a 0.5% solution of each drug's stock (25 μM latrunculin B final and 10 μM oryzalin final). 200 μL of the melted agar was added to a microfuge tube and mixed with 1 μL of the drug of choice and then 100 μL was placed on the microscope slide for the moss. In the case of the double drug treatment, 1 μL of each drug was added to the 200 μL of agar. The buffer used for the slides was prepared similarly. 200 μL of PpNO₃ was placed in a microfuge tube and then 1 μL of the drug of choice was added and mixed with the PpNO₃. Then 40 μL was put on the slide under the coverslip. In the case of the double drug treatment, 1 μL of each drug was added to the 200 μL of PpNO₃.

The slides were then analyzed under an inverted Zeiss epi-fluorescent microscope. When observing the moss cells under the microscope, a specific set of criteria was used to ensure all the different cells being tested were essentially the same. The cells targeted were sub-apical and were a part of a strand that was healthy and undamaged. The sub-apical cell also must have oblique cell walls, indicative of caulonemata, and enough room for the chloroplasts to move as necessary. Once a specific cell was targeted for testing, it was centered in view and a pinhole diaphragm was used to isolate a small portion of the cell approximately 30 microns in diameter. Only this portion of the cell was exposed to light and thus, initiate an avoidance response in the chloroplasts. Using 488 nm blue light, the cell was exposed to the high intensity light for 20

minutes, while photos were taken every 30 seconds. In order to accurately compare the results of each test, these time-lapse photos were compared side-by-side.

The moss used for this experiment was the kinesin 4IIa strain of moss that has an inducible promoter driving the RNAi for kinesin 4IIa. The RNAi induction takes place when the cells are treated with estrogen. This is the moss that will be used in future experiments so it must also be used as the untreated control. This was to get a baseline result for how the chloroplast avoidance response should look in untreated moss cells when exposed to 488 nm blue light. Once this baseline was established, the moss was treated with two drugs, latrunculin and oryzalin. Latrunculin inhibits the function of actin by binding to the actin monomer and preventing actin from polymerizing. Oryzalin disrupts the function of microtubules by a similar mechanism binding to the plant tubulin with high affinity and preventing polymerization. Since both actin and microtubules are thought to be responsible for chloroplast movement in moss, if only one drug is used to inhibit movement, then it is anticipated that the other mode of transport will still be utilized. However, if both drugs are used at once, then both actin and the microtubules are being inhibited so there should be no chloroplast movement.

Results

In this series of experiments, *P. Patens* was treated with two different drugs to set the baseline of chloroplast motility and determine the involvement of kinesins and microtubules in chloroplast motility. The drugs chosen were latrunculin B and oryzalin. Latrunculin B is an inhibitor of actin function and was chosen to observe control of chloroplast motility. In further experiments, actin will be eliminated in order to observe only kinesin activity so a control must be set. Oryzalin is an inhibitor of microtubules and was chosen because kinesins use the microtubules to travel throughout the cell and without their track, the kinesins should not be able to move. In all trials, the moss was exposed to 488 nm blue light for 20 minutes, while excluding all other light. In the first trial, the moss was not treated with any drugs. As can be seen below in Figure 1, the chloroplasts avoid the lighted area and scatter to the edges of the exposed pinhole. This shows that the untreated moss is behaving normally and acts as a control for the other experiments.

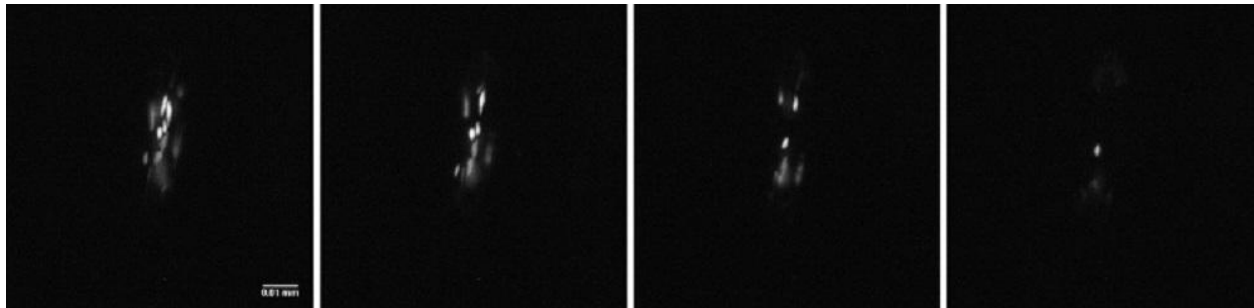


Figure 1: Control Experiment: Untreated Moss

For the next trial, actin was targeted for disruption to eliminate its ability to be involved with chloroplast motility. In order to do this, the moss was treated with latrunculin B, a drug that inhibits actin by binding to the actin monomer near the nucleotide-binding cleft, preventing actin from polymerizing. This drug was expected to slightly disrupt chloroplast motility as the actin is known to provide forces for moving the chloroplasts (Yamashita et al., 2011). As shown in

Figure 2, there is very little motility visible as a small void begins to appear in the center of the cell. However, this trial shows mostly a beaching or damaging of the chloroplasts due to too much exposure to high intensity light.

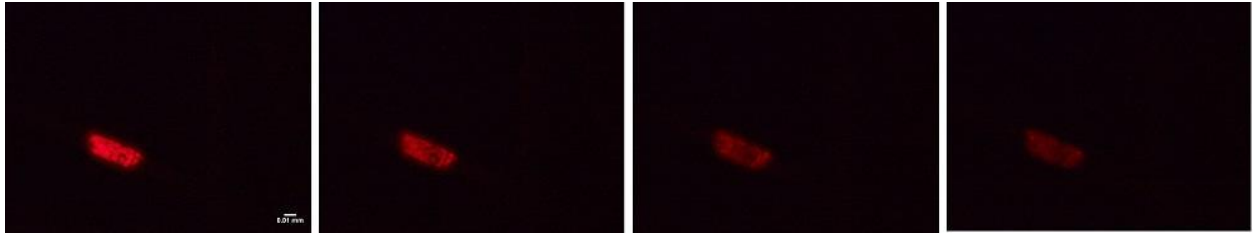


Figure 2: Actin Disruption Experiment: Moss Treated with 25 μ M Latrunculin

The third trial was the moss treated with Oryzalin, a drug that disrupts the cell's microtubules by binding to tubulin, which results in microtubule depolymerization. This treatment was expected to heavily interfere with chloroplast movement due to the disruption of the microtubule pathway even though actin will still be present. As seen in Figure 3, with the microtubules disrupted, it is anticipated that the kinesins, if they are responsible for chloroplast movement, were not able to travel up and down the tracks and as a result the chloroplasts were not able to induce an avoidance response, which supports our hypothesis. The lack of movement suggests that chloroplast motility is dependent on the existence of the microtubules.

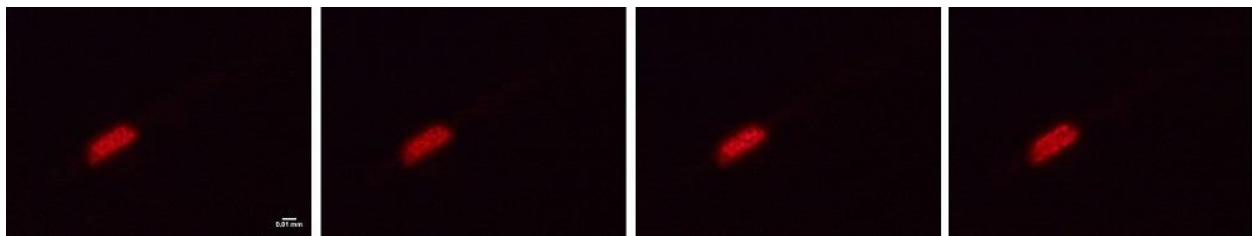


Figure 3: Microtubule Disruption Experiment: Moss treated with 10 μ M Oryzalin

Figure 4 shows the last trial, which was a combined dose of latrunculin B and oryzalin and as expected the chloroplast did not show any motility when the microtubules and actin were

disrupted.

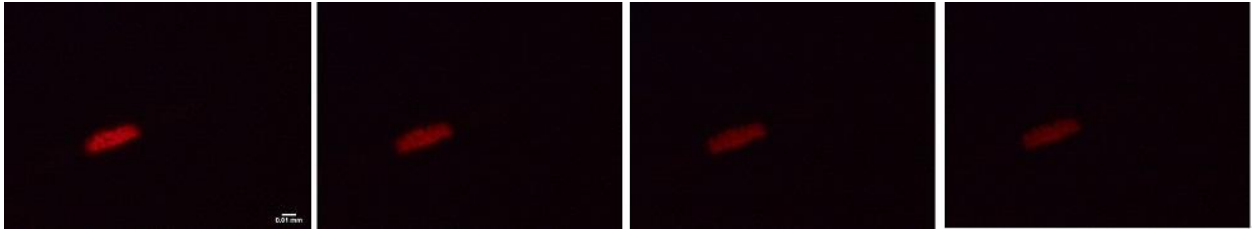


Figure 4: Complete Disruption of Actin and Microtubules: Moss treated with 25 μ M Latrunculin and 10 μ M Oryzalin

Discussion:

This experiment was designed to explore the involvement of kinesins 4 and 7 in chloroplast motility by observing any changes in the avoidance response of chloroplasts under different test conditions. This was done by first maintaining RNAi moss lines. Fresh moss was taken and put onto microscope slides for observation. There were four conditions tested, moss that was untreated, moss treated with just latrunculin, moss treated with just oryzalin and moss treated with both drugs. As seen in Figures 1-4, the untreated moss showed unhindered chloroplast movement while the chloroplasts in moss treated with the drugs showed greatly diminished movement.

From this data it can be concluded that microtubules are very important for chloroplast movement. The drug oryzalin inhibits microtubule movement and when this drug was used by itself and in conjunction with latrunculin, there was no observed chloroplast movement. This means that in the absence of microtubule function, chloroplasts cannot move throughout the cell. In addition, it can be concluded that because microtubules are heavily involved with chloroplast movement, kinesins in general are also important as they use the microtubules to move along in the cell.

Unfortunately, the involvement of the specific kinesins 4 and 7 cannot be determined from these experiments alone and due to time constraints on this project, specific testing for these kinesins was not able to be performed. In order to further understand the involvement of kinesins 4 and 7, both kinesins would need to be separately knocked-out to observe how the chloroplast motility would be affected. Future experiments after this should focus on the specific knock-outs of kinesin 4 and kinesin 7. The RNAi lines that were received from the Goshima lab

in Japan can exhibit the knockouts of kinesins 4 and 7. RNAi is commonly used to disrupt gene expression and is particularly effective in for *P. patens* since the moss is predominantly haploid and contains related genes that can make up for loss of function meaning a known-down in gene expression might not have a visible phenotype (Bezanilla, 2003). RNAi has been proven to be able to disrupt gene expression in *P. patens* despite this and would be an effective tool to knock out the function of kinesins 4 and 7. Observing the actions of the chloroplasts with this knock-out should show diminished chloroplast movement if kinesins 4 and 7 are the main kinesins involved in chloroplast motility. If chloroplast motility is not affected, that would show evidence that chloroplast motility is dependent on other molecular motors besides kinesins 4 and 7.

In order to improve upon this experiment, a few modifications could be made. One setback that was faced in this lab was timing the passing of the moss lines with making the microscope slides. Many times the moss samples grew too old to use under the microscope for observation and the chloroplasts were mostly dead. Similarly, there were times when the moss lines were too young to be able to pass as they hadn't had enough time to grow and so microscope slides could not be made for those samples either. Another problem during this experiment occurred towards the end of experimentation when the moss strains had to be grown on the benchtop instead of in an incubator. The moss grew much more slowly on the benchtop and passing the moss lines and making slides became increasingly more difficult. In addition, there was a few times in which the moss line became contaminated after passing. This resulted in the loss of a few lines that needed to be re-started and set the experiment back. Lastly, there was no experiment that proved the actin and microtubule disruption to confirm that the pathways were truly destroyed. This could be done through staining the actin and microtubules to see whether or not they were intact. Future experiments expanding on this experiment should

consider also including this into their experiment as another way to confirm the disruption of the pathways in the moss.

Further research of kinesins is important for understanding the inner workings of cells and understanding the different types of intracellular functions. Kinesins are important cells transporters and the more that is understood about the different families and how they play a role in cell transport the more research can be done into using their functions to help humans. If transport pathways in the cell are better understood than things like drug usage and how it will affect the body on a cellular level may be better understood. It could also improve the effectiveness of drugs if they are designed with the proper cell transport mechanisms in mind.

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Appendix A:

Specific Materials and Methods for Lab Procedures

Making media

- Packet of PpNO₃ added to 1L of H₂O
- Dissolved with stir bar on hot plate with gentle heat
- Divided into 2 500mL bottles
- 6 g/mL agar added to each 500mL bottle
- Autoclaved for 25 minutes

- Packet of PpNH added to 1L of H₂O
- Tried to dissolve with stir bar over gentle heat in 1L bottle
 - PpNH would not go into solution
- Added 12 g/mL agar to 1L bottle
- Autoclaved for 25 minutes

Pouring Plates

- 40 plates were poured of PpNH media
- Plates were marked with 2 vertical lines 1 black, 1 red

Note: not all media powder dissolved when autoclaved

- 50 plates were poured of PpNO₃ media
- Plates marked with 2 vertical lines 1 blue, 1 black

Passing Lines

Materials:

- PpNO₃ Plates
- Tub 14 moss
- 99% ethanol
- 2 test tubes
- grinder
- 2 grinder tips
- tape
- cellophane
- ethanol candle
- forceps

- LB plate
- sterile H₂O
- pipettes

Procedure:

- Wipe down everything going into the hood
- Put 4 mL of sterile H₂O in each test tube (2 test tubes)
- Remove sample of moss from Tub 14 plates with forceps
- Place moss plate sample in test tube
- Moss plate is re-taped closed after sample is taken
- Cellophane placed on each PpNO₃ plate making it as flat as possible
 - grab and flip cellophane so it doesn't curl
 - Cellophane very curly, dry and wrinkly at first - put on plate and let it sit for a minute and then tried to straighten cellophane
- Grinder tip inserted into grinder
- Moss ground with grinder in each test tube
 - grinder tip changed for each tube
- 1 mL of ground moss and H₂O is plated on each plate
- Plates are taped after moss is plated
- 1 mL from each tube plated on LB plate each
- Moss stored in fridge at 25 degrees C
- LB plates stored in incubator at 37 degrees C

Autoclaving

- grinder tips
- forceps
- cellophane
 - keep wrapped in foil this time after taking it out of the autoclave
 - filter paper between each cellophane sheet
- put grinder tips and forceps into sealed autoclave pouches
- autoclaved at 121 degrees C for 20 minutes