2014

Functional Analysis of Moss Class VIII Myosin and Its Role in Plant Cell Division

Shu-Zon Wu
University of Massachusetts - Amherst

Follow this and additional works at: https://scholarworks.umass.edu/dissertations_2
Part of the Cell Biology Commons, and the Plant Biology Commons

Recommended Citation

This Open Access Dissertation is brought to you for free and open access by the Dissertations and Theses at ScholarWorks@UMass Amherst. It has been accepted for inclusion in Doctoral Dissertations by an authorized administrator of ScholarWorks@UMass Amherst. For more information, please contact scholarworks@library.umass.edu.
FUNCTIONAL ANALYSIS OF MOSS CLASS VIII MYOSIN AND ITS ROLE IN PLANT CELL DIVISION

A Dissertation Presented

by

SHU-ZON WU

Approved as to style and content by:

________________________________________________________________________
Magdalena Bezanilla, Chair

________________________________________________________________________
Jennifer Normanly, Member

________________________________________________________________________
Tobias Baskin, Member

________________________________________________________________________
Patricia Wadsworth, Member

________________________________________________________________________
Elsbeth L. Walker, Program Head
Plant Biology Program
ACKNOWLEDGMENTS

I will never be able to complete this dissertation without the help of many people. My deepest gratitude to my advisor, Magdalena Bezanilla, for her support and guidance over the years. You are the best mentor a person can hope for, and I will never forget the things I have learned from you. I also thank the members of my committee, Jennifer Normanly, Tobias Baskin and Patricia Wadsworth for their helpful comments and suggestions on my dissertation and research project. I also want to thank the members of Bezanilla lab. It is a pleasure to work with such wonderful colleagues. I also thank the members of Hepler lab and Baskin lab, for lively discussion during lab meeting and their help for many experiments.

I want to thank my family and friends from Taiwan, especially my parents and my brother, who have always been extremely supportive, although they don’t like me been so far away from home. I also want to thank my undergrad advisor Dr. Hsou-min Li for introducing me to scientific research.

It has been the most gratifying experience to see what is happening in a cell. I hope I will never forget the excitement and the sense of curiosity it inspired. Also, patience is a virtue, so is perseverance. But most of the time I was just really lucky.
ABSTRACT

FUNCTIONAL ANALYSIS OF MOSS CLASS VIII MYOSIN AND ITS ROLE IN PLANT CELL DIVISION

MAY 2014

SHU-ZON WU, B.S., NATIONAL TAIWAN UNIVERSITY

Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Magdalena Bezanilla

The moss *Physcomitrella patens* is a great model system for studying plant gene function using reverse genetic approaches. It undergoes efficient genotype-targeting by homologous recombination, allowing the generation of specific gene knockout and tagging a gene at its endogenous locus. Additionally, RNAi is quite effective in *P. patens*, providing an effective tool for rapid gene silencing and phenotypic characterization. Taking advantage of these features, this dissertation described the establishment of a system to perform an unbiased gene-by-gene RNAi assay to screen for tip growth phenotypes in *P. patens*. A small set of RNAi constructs were tested, within them one gene was identified to be involved in the vesicle transport between ER and Golgi, which when silenced resulted in severe tip-growth phenotype. This showed that the pilot screen was a successful proof of concept for a large RNAi screen in the future.

A more targeted approach was utilized to study the function of the class VIII myosin gene family. Analysis of the phenotypes of a myosin VIII quintuple knockout line revealed that there were defects in protonemal patterning and cell plate placement. Further, using cellular localization studies I demonstrated the
involvement of actin and myosin VIII in cell division plane specification and phragmoplast guidance.

Overall, this dissertation demonstrates two examples of what moss as a model system can achieve, both in screening for potentially interesting genes and in studying the basic molecular functions of a specific gene family. While the screen provided novel candidates for future studies of polarized growth in moss, the targeted approach has begun to answer a long-standing question in plant cell biology regarding the role of actin and myosin in plant cell division.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>TRANSIENT RNAi ASSAY IN 96-WELL PLATE FORMAT FACILITATES HIGH-THROUGHPUT GENE FUNCTION STUDIES IN PLANTA</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>MYOSIN VIII REGULATES PROTONEMAL PATTERNING AND DEVELOPMENTAL TIMING IN THE MOSS PHYSCOMITRELLA PATENS</td>
<td>23</td>
</tr>
<tr>
<td>4</td>
<td>CELLULAR LOCALIZATION OF MYOSIN VIII AND ITS FUNCTION DURING CELL DIVISION</td>
<td>54</td>
</tr>
<tr>
<td>5</td>
<td>DISCUSSION</td>
<td>93</td>
</tr>
<tr>
<td>6</td>
<td>MATERIAL AND METHODS</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>REFERENCES</td>
<td>130</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1.</td>
<td>Sequence similarity among myosin VIII protein sequences .................. 28</td>
</tr>
<tr>
<td>3.2.</td>
<td>Amount of sequence replaced by the resistance cassette upon correct integration into the myosin locus .................................................. 35</td>
</tr>
<tr>
<td>3.3.</td>
<td>Cell length and branch density measurements on six-day old plants regenerated from protoplasts ................................................................. 43</td>
</tr>
<tr>
<td>6.1.</td>
<td>Primers used in this study ........................................................................ 126</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Transient RNAi assay in <em>Physcomitrella patens</em></td>
<td>9</td>
</tr>
<tr>
<td>2.2</td>
<td>Outline of the semi-automatic transformation of moss protoplast</td>
<td>13</td>
</tr>
<tr>
<td>2.3</td>
<td>Comparison of the original moss transformation protocol and the semi-automated version</td>
<td>14</td>
</tr>
<tr>
<td>2.4</td>
<td>Cloning strategy for generating RNAi constructs</td>
<td>15</td>
</tr>
<tr>
<td>2.5</td>
<td>High-throughput RNAi assay produced consistent results</td>
<td>17</td>
</tr>
<tr>
<td>2.6</td>
<td>Phenotypes of Sec23 RNAi plants</td>
<td>20</td>
</tr>
<tr>
<td>3.1</td>
<td><em>P. patens</em> has five highly similar myosin VIII genes</td>
<td>30</td>
</tr>
<tr>
<td>3.2</td>
<td>Myosin VIII genes are expressed in protonemata and gametophores</td>
<td>33</td>
</tr>
<tr>
<td>3.3</td>
<td>Amino acid sequences of truncated proteins from altered myo8C and myo8D loci</td>
<td>37</td>
</tr>
<tr>
<td>3.4</td>
<td>PCR analysis of insertions in Δmyo8ABCDE</td>
<td>38</td>
</tr>
<tr>
<td>3.5</td>
<td>Myosin VIII transcripts are altered in Δmyo8ABCDE</td>
<td>40</td>
</tr>
<tr>
<td>3.6</td>
<td>Myosin VIII is not required for plant viability</td>
<td>42</td>
</tr>
<tr>
<td>3.7</td>
<td>Myosin VIII is involved in branch formation</td>
<td>46</td>
</tr>
<tr>
<td>3.8</td>
<td>Myosin VIII influences the rate of development</td>
<td>48</td>
</tr>
<tr>
<td>3.9</td>
<td>Myosin VIII is required for normal growth in limiting nutrients</td>
<td>51</td>
</tr>
<tr>
<td>3.10</td>
<td>Auxin partially rescues morphological defects in Δmyo8ABCDE</td>
<td>53</td>
</tr>
<tr>
<td>4.1</td>
<td>Cell plates are improperly positioned in young myosin null plants</td>
<td>58</td>
</tr>
<tr>
<td>4.2</td>
<td>Side branch formation in moss protonemata</td>
<td>60</td>
</tr>
</tbody>
</table>
4.3. Expression of Myo8A-GFP in Δmyo8ABCDE largely restores protonemal branching ................................................................. 62

4.4. Myo8A-GFP localization in apical cells ................................................................. 64

4.5. Myo8A-GFP moves along cortical actin filaments .................................................. 65

4.6. Myo8A-GFP localizes to punctate structures at the cross wall in cell with emerging branch ................................................................. 67

4.7. Myo8A-GFP stays associated with the wall in plasmolyzed cell ................. 68

4.8. Myo8A-GFP stays associated with the wall in plasmolyzed cell which has completed branch ................................................................. 69

4.9. Myo8A-GFP localizes to a spindle-like structure ................................................. 72

4.10. Myo8A-GFP localizes to mitotic spindle and phragmoplast independent of actin ........................................................................ 73

4.11. Myo8A-GFP associates with microtubule plus ends ........................................ 76

4.12. Myo8A-GFP associates with microtubule plus ends in interphase cell ...................................................................................... 77

4.13. Myo8 N-terminus protein sequence contain putative EB1 binding motifs ................................................................................ 78


4.15. Actin is required for proper and efficient contacts between peripheral microtubules and cell cortex ................................................................. 84

4.16. Actin is present between the edge of expanding phragmoplast and cell cortex ...................................................................................... 85

4.17. Myo8A-GFP marks the future site of cell division ........................................ 88

4.18. Myo8A-GFP localizes to dynamic punctate cortical structures on the cell cortex of tobacco BY2 cells ................................................................. 89

4.19. Moss Myo8A-GFP localizes to PPB, CDS and phragmoplast in tobacco BY-2 cell ...................................................................................... 90

4.20. A model for myosin VIII function in phragmoplast guidance .............. 92
6.1. Heating system for top agar

----------------------------------  125
CHAPTER 1

INTRODUCTION

The moss *Physcomitrella patens* is a great model system for studying plant gene function using reverse genetic approaches (Cove et al., 2006). Mosses are non-vascular, multicellular terrestrial plants with simple developmental structures and few cell types. A new moss plant can be generated from a single cell, either a spore or a protoplast. The resulting protomemata tissues are filamentous, and later will differentiate into leafy gametophores. The male and female reproductive organs called antheridia and archegonia form on top of the gametophores, and produce sperms and eggs. After fertilization, the zygotes differentiate into sporophytes, which undergo meiosis to produce spores (Cove et al., 2006). Experimentally, the haploid tissues can be propagated vegetatively by blending in water and culturing on agar medium plates overlaid with a layer of cellophane. The haploid nature of the tissues makes genetic manipulation of the organism and the interpretation of the phenotypes straightforward. Moss protoplasts can be transformed and regenerated into a new plant, allowing us to genetically manipulate the organism at the single cell level and study the resulting phenotypes in a whole plant.

*P. patens* undergoes efficient gene-targeting by homologous recombination, allowing the generation of specific gene knockout and tagging a gene at its endogenous locus (Schaefer and Zrýd, 1997; Schaefer, 2001). With the Cre-lox system (Sauer, 1998), the limited antibiotic selection genes can be recycled and
reused, enabling us to generate multiple-gene knockout lines lacking a whole gene family. Additionally, RNAi is quite effective in *P. patens* (Bezanilla et al., 2005; 2003b), providing an effective tool for rapid gene silencing and phenotypic characterization. Combining all these factors, *P. patens* is an ideal system for studying basic cellular processes in plants. Since moss shares many common processes with vascular plants, the data set generated from studies in moss is potentially useful for many plant systems.

The utility of moss has been taken advantage of to study the actin-mediated molecular processes controlling tip growth and cell division (Augustine et al., 2008; Vidali et al., 2010; 2007; van Gisbergen et al., 2012; Vidali et al., 2009b; Augustine et al., 2011). In tip growing cells, expansion is restricted to the apex of the cell. This type of growth underlies the development of root hairs and pollen tubes in seed plants, and protonemata in mosses. To achieve tip growth, the cell wall must be modeled appropriately to allow turgor-pressure driven expansion to be restricted to the apex of the cell (Rounds and Bezanilla, 2013; Vidali and Bezanilla, 2012). Deposition of new cell wall materials at the site of growth is required and the structures and dynamics of the actin cytoskeleton are also essential (Augustine et al., 2008; Vidali et al., 2010; 2007; van Gisbergen et al., 2012; Vidali et al., 2009b; Augustine et al., 2011). Presumably small GTPase proteins are also involved (Kost, 2008; Eklund et al., 2010a), relaying signals from receptors at the tip. However, many molecular components, such as the receptors and the downstream signaling components, involved in this process remain unidentified, and the detailed mechanism driving tip growth is still unclear.
In moss protonemata, cell division occurs at the apical cells and at the side branches. Most of the mitotic structures found in plants are present in moss protonemata, except for the preprophase band, which marks the future division site before cell division occurs. Despite the lack of a preprophase band (Doonan et al., 1985), moss protonemata divide in an organized pattern, suggesting there is an ancient mechanism controlling division plane specification before preprophase band had evolved in the plant lineage. Several studies investigating moss cell division have focused on the roles of microtubule-associated proteins (Hiwatashi et al., 2008; Spinner et al., 2010; Nakaoka et al., 2012; Kosetsu et al., 2013). Importantly these studies have demonstrated the proteins under investigation have comparable functions in moss and in seed plants, suggesting the molecular mechanism controlling plant cell division might be conserved. The role of actin and actin associating proteins during plant cell division are less understood. During cytokinesis, instead of forming an actin-myosin contractile ring, plant cells form a microtubule-based structure called the phragmoplast, which builds a new cell plate separating the two daughter cells. Actin is known to be present in the phragmoplast (Clayton and Lloyd, 1985; Kakimoto and Shibaoka, 1987). However, actin is not essential for phragmoplast expansion, making its role during plant cell division a puzzle.

In Chapter 2, I describe the establishment of a system to perform an unbiased gene-by-gene RNAi assay to screen for tip growth phenotypes in *P. patens*. Due to the limitation of resources and experimental design, the RNAi screen was not continued. Although only a small set of genes were tested, within them I identified
one gene involved in the vesicle transport between ER and Golgi, which when silenced resulted in severe tip-growth phenotype. This showed that the pilot screen was a successful proof of concept for a large RNAi screen in the future.

In Chapter 3 and 4, I described a more targeted approach to study the function of the class VIII myosin gene family. I analyzed the phenotypes of a myosin VIII quintuple knockout line and revealed that there are defects in protonemal patterning and cell plate placement. Further, using cellular localization studies I demonstrated the involvement of actin and myosin VIII in cell division plane specification and phragmoplast guidance. During the course of these studies I established protocols for studying plant cell division in moss protonemata.

Overall, my thesis demonstrates two examples of what moss as a model system can achieve, both in screening for potentially interesting genes and in studying the basic molecular functions of specific gene family. While the screen provided novel candidates for future studies of polarized growth in moss, the targeted approach has begun to answer a long-standing question in plant cell biology regarding the role of actin and myosin in plant cell division.
CHAPTER 2

TRANSIENT RNAi ASSAY IN 96-WELL PLATE FORMAT FACILITATES HIGH-THROUGHPUT GENE FUNCTION STUDIES IN PLANTA.

Introduction

In recent years, the complete sequences of genomes from many organisms have become available. However, to understand the function of any gene in the genome remains challenging let alone all of them. Experimentally characterizing gene function relies on the analysis of mutant phenotypes. This process is usually limited to the study of a few genes at a time, and as a result the majority of predicted genes do not have experimentally assigned functions (Alonso and Ecker, 2006). One way to address this problem is to systematically alter, either using gain-of-function or loss-of-function approaches, every gene in the genome and analyze resulting phenotypes one-by-one. In animal systems, genome-wide RNA interference (RNAi) screens enable scientists to phenotypically assay hundreds to thousands of genes at a time. These approaches have been applied to whole organisms in Caenorhabditis elegans (Kamath et al., 2003) and to cell-based assays in Drosophila S2 cells (Goshima et al., 2007; Wheeler et al., 2004) and in HeLa cells (Boutros and Ahringer, 2008; Collinet et al., 2010). These studies have proven to be informative, uncovering genes involved in cellular processes and signaling pathways (Boutros and Ahringer, 2008). To date, genome-wide reverse genetic studies have not been carried out in plants.
There are two advantages of developing an RNAi screen in plants. First, traditional genetic screens in plants are mostly based on forward-genetic approaches (Candela and Hake, 2008; Page and Grossniklaus, 2002). The requirement to isolate viable mutants in these screens makes it difficult to identify essential genes. On the other hand, it is easier to identify essential genes in a transient RNAi screen because it is not necessary to propagate mutant lines. Second, so far the annotated arabidopsis T-DNA collections are the closest resource available for knocking out every single gene in the plant genome (Ajjawi et al., 2010). However, the following phenotypic analyses of mutants and deciphering gene function remain challenging at the whole-genome scale (Ajjawi et al., 2010; O’Malley and Ecker, 2010). In contrast, a high-throughput transient RNAi screen will be able to phenotypically analyze large numbers of genes rapidly. Therefore, a reverse-genetic screen by RNAi in plants would complement the limitations of current research tools.

*Physcomitrella patens* is a great model system for conducting an RNAi-mediated loss-of-function study *in planta* for several reasons. First, the genome sequence of *P. patens* is available (Rensing et al., 2008), and there are many cDNA resources available. Second, our lab has developed a rapid transient RNAi assay for studying cell-growth-related phenotypes (Bezanilla et al., 2003b; 2005). This assay enables identification of growth phenotypes within one week of transformation. Third, combined with the semi-automated transformation system I developed in this chapter, the number of genes that can be handled at once is greatly increased, transforming our RNAi assay into a more high-throughput process.
In the Bezanilla lab, gene silencing induced by RNAi in the moss is done by transforming moss protoplasts with a DNA construct that expresses inverted-repeat sequences of the targeted gene (Bezanilla et al., 2005). The RNAi assay is performed in a stable transgenic line expressing a nuclear-localized GFP-GUS fusion protein, NLS-4. The RNAi construct contains a fragment of the target gene fused to 400 bp of GUS in an inverted repeat orientation. The inverted repeats are separated by a ~400 bp linker. Expression of the RNAi construct is driven by the maize ubiquitin promoter, which is a strong constitutive promoter in the moss. Once introduced into cells and transcribed, the mRNA will pair and form double-stranded RNA molecules, which then triggers RNAi-mediated gene silencing of the targeted cDNA and the GFP:GUS fusion protein. The effectiveness of gene silencing is determined by the disappearance of the nuclear GFP:GUS protein. Only transgenic plants lacking nuclear GFP are analyzed (Fig. 2.1). Our lab has successfully used this approach to silence individual genes as well as gene families (Vidali et al., 2007; Augustine et al., 2008; Vidali et al., 2009b).

Traditionally, transformation is mediated by polyethylene glycol (PEG)-accompanying DNA transfer into protoplasts. The simple nature of PEG-mediated transformation makes it possible to be performed in large part by an automated pipetting machine. Furthermore, the reaction volume can be scaled down to fit into a 96-well plate. This adaptation enables the procedure to be performed in a more high-throughput manner.

In this chapter, I described the establishment of a semi-automated moss transformation protocol to increase the number of genes that can be handled in one...
RNAi assay. Once the transformation protocol was established, I performed a pilot screen and successfully identified one gene involved in protein trafficking, which when silenced results in strong tip-growth phenotype. As a proof of concept this pilot study showed that an unbiased gene-by-gene RNAi screen in moss is feasible with sufficient resources and manpower.
Figure 2. 1. Transient RNAi assay in *Physcomitrella patens*.

RNAi assay is performed in the NLS-4 line, which expresses a nuclear-localized GFP:GUS protein. Protoplasts isolated from NLS-4 are transformed with control and RNAi constructs. One week after transformation, phenotypes of regenerated plants lacking nuclear GFP signal are imaged. A control plasmid silences only nuclear-localized GFP:GUS, while the RNAi construct simultaneously silences the nuclear-localized GFP:GUS and the targeted gene.
Results

Developing a high-throughput transformation system in *P. patens*

The standard transformation protocol in Bezanilla lab is as follows. Transformation of *P. patens* is done by mixing 300 μL of protoplasts with 15-30 μg DNA and 300 μL PEG. After incubation at room temperature for 10 min, protoplasts are subjected to a 3 min heat shock in a 45°C water bath, followed by a 10-minute incubation in a room temperature water bath. To wash out the PEG, 5 mL of 8.5% mannitol is added to the protoplasts. The transformed protoplasts recover for an additional 30 minutes at room temperature. The protoplasts are then spun down, resuspended with 1 mL 0.6% top agar (see below), and then plated on two plates containing protoplast regeneration medium. Protoplast regeneration medium is a mannitol-based solution providing the reasonable osmolality for the protoplasts and also containing the ions required for cell regeneration and cell wall synthesis (see chapter 6 for media compositions). The top agar has 0.6% agar in addition to the mannitol-based medium. The agar provides a cushion for the protoplasts and increases regeneration frequency.

I modified the above transformation protocol to scale down the reaction volume and also automated some of the pipetting steps using an Eppendorf epMotion5070. The total reaction volume was reduced to 50 μL and the amount of DNA was reduced to 1 μg, which can be easily obtained from one plasmid mini-prep. Transformation reactions were carried out in a 96-well PCR plate, allowing up to 96 transformations at the same time. Protoplasts resuspended with top agar were
spotted on an 8 X 12 cm² rectangular plate in a 96-well pattern, 6 μL per spot. The protoplasts were plated on top of protoplast regeneration agar medium overlaid with a layer of cellophane (Fig. 2.2). After three days, cellophane was transferred to regular growth medium plates containing antibiotic for selection. One week after transformation, images of transgenic plants were acquired with a fluorescence stereomicroscope equipped with a color CCD camera. Plant area was measured based on the area of chlorophyll auto-fluorescence in the image. This analysis was semi-automated and performed with macros developed for ImageJ, as described in Vidali et al., (2009b). All settings for the Eppendorf epMotion5070 are listed in Materials and Methods. A diagram comparing the traditional and modified transformation protocol is shown in Figure 2.3.

**Building the *P. patens* RNAi constructs**

To generate RNAi constructs, I collaborated with Dr. Hasebe from the National Institute of Basic Biology (Okazaki, Japan) and Dr. Fujita from Hokaido University in Japan, who sent us a collection of ~3500 individual full-length cDNAs isolated from protonemata and young gametophore tissues of *P. patens*. The cDNAs had been cloned into the pTFH22.4 vector (Fujita et al., 2004), which co-expresses GFP, the cDNA, and the nptII gene conferring resistance to G418. The rice actin promoter drives expression of the cDNA, while the 35S promoter drives expression of GFP and nptII. The identities of the cDNAs are available on the public database PHYSCObase (http://moss.nibb.ac.jp/). Therefore, after desirable phenotypes are
found, more detailed gene functional analyses can be carried out. I used recombination-based cloning (Gateway system from Invitrogen) to move the collection of *P. patens* cDNAs from pTFH22.4 into our RNAi vector (Fig. 2.4). As a cost-saving measure, I designed primers that anneal to sequences in the pTFH22.4 vector on either side of the cloned cDNA. I used these primers to amplify individual cDNAs from the collection. The primers also carried attB1 and attB2 sequences required for Gateway cloning. The PCR products, with attB1 and attB2 sites flanking the cDNA, were transferred into a pDONR vector using a BP clonase reaction. The resulting entry clones were recombined in an LR clonase reaction with the destination vector pUGGi to generate the RNAi constructs. Many steps of the cloning process were automated and facilitated by the EpMotion5070 automated pipetting machine (see Materials and Methods).
Figure 2.2 Outline of the semi-automatic transformation of moss protoplast.

Numbers in parentheses correspond to the steps in transformation procedure described in Chapter 6 under subheading “semi-automatic protoplast transformation using EpMotion5070.”
Figure 2.3. Comparison of the original moss transformation protocol (left) and the scaled-down, semi-automated version (right). Steps labeled in blue are performed by epMotion5070.
Figure 2.4. Cloning strategy for generating RNAi constructs from the cDNA library using Gateway system. Collection of moss cDNA were cloned into pTFH22.4 vector. attB1-F and attB2-R are primers used to amplified cDNA from pTH22.4 vector. attB1, attB2, attR, attL denote recombination sequence in Gateway cloning sites. “ccdB” denotes the Gateway cassette, including ccdB gene and chloramphenicol resistant gene. “GUS” denotes 400 bp of GUS sequence. “HygR” and “SpecR” denote hygromycin and spectinomycin resistant cassettes.
Pilot screen

As a pilot study, I first transformed 12 RNAi constructs resulting in known phenotypes into NLS-4 protoplasts using the semi-automated transformation method. These RNAi constructs were previously generated in the lab, containing 200-400 bp of targeting sequence to specific genes. One week after transformation, images of single GFP-off plants were acquired with a stereomicroscope using fluorescence settings for GFP fluorescence. A long pass filter was used, which enables detection of GFP and chlorophyll autofluorescence in one image. A color CCD camera was used to acquire the image to obtain information in both the red and green channels.

Phenotypic analysis of the RNAi plants was performed with macros developed for ImageJ (Vidali et al., 2009b). Young moss plants are composed of filamentous protonemata undergoing polarized growth. Two parameters are chosen to measure plant size and shape. The first is the total area of the plant. Slower-growing plants will have smaller total area. The second is circularity, a morphometric parameter representing the polarity of the plant. Circularity is defined as $4\pi \frac{\text{area}}{\text{perimeter}^2}$. Plants that are more polarized have lower circularity values. Consistent with previous studies (Vidali et al., 2007; Augustine et al., 2008; Vidali et al., 2009b), strong phenotypes, including small plants composed of small spherical cells, were found when genes regulating actin dynamics were silenced (Fig. 2.5). These results demonstrate that the new semi-automated transformation method reproduced phenotypes comparable to the routine RNAi assay performed with the large-volume transformation procedure.
Figure 2.5. High-throughput RNAi assay produced consistent results.

Top, representative images of 1-week-old plants transformed with control and RNAi constructs visualized by epifluorescence stereomicroscopy. The red color is the chlorophyll autofluorescence. The absence of nuclear GFP indicates they are undergoing active gene silencing. Bottom, quantification of RNAi-induced phenotype. (Control, n = 150; ADF, n = 37; Myosin11, n = 69; Profilin, n = 87; Formin, n = 110; Brick, n = 96; Calmodulin D, n = 54; FtsZ, n = 34; CapZ, n = 36; Expansin 1b, n = 34; BimC, n = 133; Villin, n = 40.) Error bars indicate SE.
Next, I transformed 165 RNAi constructs that I had generated from the cDNA collection. Among the 165 RNAi constructs transformed, 37 of them resulted in phenotypes similar to control plants. Of the 128 RNAi constructs that produced phenotypes different from the control, 96 of them resulted in few if any plant alive after antibiotic selection. Living plants with antibiotic resistance had GFP fluorescence, indicating that these were non-silenced plants. In our experience, this phenomenon is often observed when essential genes are silenced. Thus, it is possible that the targeted cDNA is essential or, alternatively, that off-target silencing occurred and multiple genes were silenced simultaneously causing a lethal phenotype. To distinguish between these two possibilities, more specific RNAi constructs using 200-400 bp specific gene targeting sequences are needed. Unfortunately, my strategy of using a single primer set to amplify all the cDNAs from the *Physcomitrella patens* cDNA collection generated constructs with full-length cDNAs. Full-length cDNAs have the potential to silence off-target genes as it is possible that similarities to non-related genes might be found along the coding sequence of the cDNA. Generating specific RNAi constructs would require ordering a primer pair specific for each gene and this is not economically viable at the moment. Because more then half of the phenotypes were hard to interpret, I decided not to pursue the RNAi screen.

While the majority of the RNAi constructs led to uninterpretable phenotypes, 32 of the RNAi constructs did result in smaller plants. However, most of them did not generate enough transformants for reliable phenotypic analysis. Among this group, one was identified that showed an interesting and consistent phenotype of
round and small cells (Figure 2.6). The RNAi construct encoded one of the six Sec23 genes in *P. patens*. Sec23 together with Sec24 forms the inner coat of the COPII complex (Barlowe et al., 1994), which is involved in protein trafficking from the endoplasmic reticulum (ER) to the Golgi (Jensen and Schekman, 2011). Interestingly, no Sec23 mutants or knockouts in plants have apparently never been described in the literature. However, the COP II complex is suspected to be essential for survival since the arabidopsis Sec24A knockout is lethal (Faso et al., 2009).

The RNAi construct yielding the phenotype shown in Figure 2.6 encodes the full-length cDNA of one Sec23 gene. The Sec23 coding sequence has stretches of sequence that are highly similar to four other Sec23 genes. Therefore, this RNAi construct likely silenced five out of the six Sec23 genes. Considering the importance of anterograde trafficking in transporting proteins, lipids and cell wall materials to the cell apex during tip growth, the resulting cell-growth phenotype has biological significance. This result validated that the RNAi screen is able to discover novel genes important for cell growth.
Figure 2.6. Phenotypes of Sec23 RNAi plants.

Top, representative images of 1-week-old plants transformed with control and RNAi constructs. Plants were stained with calcofluor and visualized by epifluorescence stereomicroscopy. The red color is the chlorophyll autofluorescence. The absence of nuclear GFP indicates they are undergoing active gene silencing. Bottom, quantification of RNAi-induced phenotype. (Control, n = 25; Sec23, n = 76)
Discussion

A key point in uncovering unknown gene functions is in observing the phenotypes resulting from the loss of a gene. Traditionally, loss-of-function screens in plants have been performed by random mutagenesis, such as EMS or insertional mutagenesis mediated by Agrobacterium transformation of DNA into the plant. Although these are efficient ways to generate loss-of-function mutants, they are not gene-specific. Once a mutant is isolated, traditional time-consuming genetic approaches, such as chromosome walking, are used to identify the gene associated with the phenotype (Peters et al., 2003). On the other hand, gene-specific targeting mediated by RNAi can specifically target a gene and is a straightforward method to abolish or reduce gene function. However, this process is usually limited to the study of only a few genes at a time.

In this chapter, I described a method to perform a gene-by-gene RNAi screen in *P. patens*. I tested the potency of this assay using 12 specific RNAi constructs with known phenotypes and showed this assay can produce consistent results. A pilot study using 165 unknown RNAi constructs identified the Sec23 gene family as having strong phenotype, demonstrated my approach can successfully identify genes that play essential roles in cell growth. This result also showed the advantage of using an RNAi screen comparing to forward genetic approaches in terms of unmasking gene redundancy. However, results from the pilot study also revealed a flaw in the experimental design. It showed that non-specific RNAi constructs generated from full-length cDNA apparently caused too much off-targeting silencing and lethality, underscoring the importance of using specific RNAi constructs for this
type of approach. Unfortunately, there is no genome-wide RNAi reagent available for moss. To generate that will require designing primer pairs for every single gene in the genome, which is not achievable with our limited resources. In the past few years, genome-wide RNAi reagents have become common and commercially available in many animal systems. In organisms such as *Drosophila melanogaster, Caenorhabditis elegans,* and HeLa cells, RNAi-mediated genome-wide gene functional studies have generated large informative data sets with respect to gene function (Kamath et al., 2003; Wheeler et al., 2004). Establishing a similar set of data in plants will undoubtedly propel the field forward. I believe with the high-throughput RNAi assay protocol I established, we are a step forward in that direction. The next step will be finding an economical way to generate genome-wide RNAi reagents for moss, and improve the efficiency of image acquisition.
CHAPTER 3

MYOSIN VIII REGULATES PROTONEMAL PATTERNING AND DEVELOPMENTAL TIMING IN THE MOSS PHYSCOMITRELLA PATENS

Introduction

Myosins are actin-based molecular motors found in almost all eukaryotes. Myosins are defined by the presence of a highly conserved motor domain, which functions to convert the energy from ATP hydrolysis into force generation along actin filaments. Based on sequence similarity within the motor domain, myosins have been grouped into at least 35 families (Odronitz and Kollmar, 2007). Metazoans, yeast, and amoebae share a number of common myosin families, such as class I, II and V. Interestingly, plants have evolved a distinct subset of myosins that group into two families, class VIII and XI (Bezanilla et al., 2003a; Odronitz and Kollmar, 2007; Peremyslov et al., 2011; Mühlhausen and Kollmar, 2013). In seed plants there are a large number of class XI myosins and a small number of class VIII myosins. For example in Arabidopsis thaliana, four of the 17 myosins are class VIII.

Class XI myosins are most similar in sequence and structure to class V myosins, which play a critical role in vesicle and organelle motility (Reck-Peterson et al., 2000). Structural features shared by both classes include: an N-terminal motor domain, with six light chain binding (IQ) motifs, a coiled-coil, a globular tail and a dilute domain. Recently a number of studies have demonstrated that class XI myosins are essential for organelle motility and structure (Avisar et al., 2008b; Li
and Nebenführ, 2007; Natesan et al., 2009; Sparkes et al., 2008; Yokota et al., 2011; 2009; Tamura et al., 2013), cytoplasmic streaming (Sparkes et al., 2009; Ueda et al., 2010), and tip growth (Vidali et al., 2010).

Although class VIII myosins were the first plant myosins to be cloned (Knight and Kendrick-Jones, 1993) and in seed plants they are the smaller gene family, much less is known about the role of myosin VIII in plants. Class VIII myosins are characterized by an N-terminal motor domain followed by four IQ motifs, a region of coiled-coil and a tail sequence with no obvious conserved motifs.

Functional studies on myosin VIII have been carried out using dominant negative approaches where the C-terminal tail is overexpressed. In one study, overexpression of class VIII, though not class XI, myosin tails specifically affected localization of the viral Hsp70 homolog to the plasmodesmata (Avisar et al., 2008a). Overexpression of the tail of myosin VIII in some cases impaired Golgi motility (Avisar et al., 2009). Furthermore, motility of endosomes containing the ARA6 marker was impaired when these endosomes were associated with truncated ATM1 molecules (Golomb et al., 2008). These data support some of the localization studies and suggest that myosin VIII plays a role in plasmodesmata and endocytosis.

Although these studies provide useful hints of possible roles of myosin VIII in a cell, data interpretation is often unclear due to the presence of multiple myosin VIII genes and possible functional redundancy among them. A detailed analysis of the loss-of-function phenotypes of the myosin VIII family is needed to reveal the fundamental function of class VIII myosin. So far this has not been described
Despite the fact that there are only four class VIII myosins in \textit{A. thaliana}, and the genetic tools required to make a quadruple knockout are available.

Here, I use the moss \textit{P. patens} to study the role of myosin VIII during moss growth and development. \textit{P. patens} has five myosin VIII genes, a similar number to the myosin VIII family in characterized seed plants. Taking advantage of \textit{P. patens’} ability to perform gene targeting, our lab had generated loss-of-function mutants in myosin VIII. I characterized the phenotype of a myosin VIII quintuple knockout through developmental stages and under different conditions. Interestingly, I found that while myosin VIII is not essential for viability, it is involved in patterning of protonemal tissue and control of developmental timing.
**Results**

*P. patens* has five highly similar myosin VIII genes

*P. patens* has five class VIII myosins (Figure 3.1). Based on the predicted gene models from a recent annotation of the *P. patens* genome (v1.6 from http://www.phytozome.net/physcomitrella.php), I isolated full-length transcripts for all five class VIII myosins. Sequence analyses of the full-length transcripts confirm the predicted gene models for myo8A and myo8C. Slight modifications were made to the predicted gene models for myo8B, myo8D and myo8E (annotations submitted to Genbank). Each myosin VIII gene has 23 exons and is very similar both in exon and intron size. Additionally there is a high degree of sequence similarity among the myosin VIII proteins (Table 3.1). All five myosin VIII’s have an N-terminal extension on the motor domain, ranging from 274 to 330 amino acids in length, and four light chain-binding motifs downstream of the motor domain. Two regions of coiled-coil are predicted just downstream of the light chain binding motifs (Figure 3.1).

In contrast to the four IQ motifs and regions of coiled-coil, which are shared among most plant class VIII myosins, the large N-terminal extension on the moss motor domains is not common among class VIII myosins from other plants. However a similar sized extension that contains sequence similarity to the moss myosin N-termini was found on predicted myosin VIIIs from *Oryza sativa* and *Setaria italic*. Thus it is possible that this extension, which was not annotated in the first released *P. patens* genome (v1.1 from http://genome.jgi-
psf.org/Phypa1_1/Phypa1_1.home.html), has not been properly annotated in other sequencing projects or is only found in a subset of class VIII myosins. Since the full-length transcripts contain the sequence encoding for the N-terminal extension and these sequences are similar amongst the five myosin VIII genes, it suggests that the N-terminal extension is a part of the full-length protein.
### Table 3.1 Sequence similarity among myosin VIII protein sequences

Amino acid sequence similarity among myosin VIII protein sequences within the N-terminal extension.

<table>
<thead>
<tr>
<th></th>
<th>myo8B</th>
<th>myo8C</th>
<th>myo8D</th>
<th>myo8E</th>
</tr>
</thead>
<tbody>
<tr>
<td>myo8A</td>
<td>33/49</td>
<td>35/49</td>
<td>70/82</td>
<td>52/66</td>
</tr>
<tr>
<td>myo8B</td>
<td>59/69</td>
<td>34/48</td>
<td>33/49</td>
<td></td>
</tr>
<tr>
<td>myo8C</td>
<td></td>
<td>35/51</td>
<td>32/47</td>
<td></td>
</tr>
<tr>
<td>myo8D</td>
<td></td>
<td></td>
<td>47/61</td>
<td></td>
</tr>
</tbody>
</table>

Numbers indicate % identity/ % similarity.

Amino acid sequence similarity among myosin VIII protein sequences within the motor domain.

<table>
<thead>
<tr>
<th></th>
<th>myo8B</th>
<th>myo8C</th>
<th>myo8D</th>
<th>myo8E</th>
</tr>
</thead>
<tbody>
<tr>
<td>myo8A</td>
<td>75/87</td>
<td>76/86</td>
<td>89/95</td>
<td>85/92</td>
</tr>
<tr>
<td>myo8B</td>
<td>88/94</td>
<td>77/87</td>
<td>77/87</td>
<td></td>
</tr>
<tr>
<td>myo8C</td>
<td></td>
<td>77/88</td>
<td>78/88</td>
<td></td>
</tr>
<tr>
<td>myo8D</td>
<td></td>
<td></td>
<td>87/93</td>
<td></td>
</tr>
</tbody>
</table>
Amino acid sequence similarity among myosin VIII protein sequences within the tail domain.

<table>
<thead>
<tr>
<th></th>
<th>myo8B</th>
<th>myo8C</th>
<th>myo8D</th>
<th>myo8E</th>
</tr>
</thead>
<tbody>
<tr>
<td>myo8A</td>
<td>52/69</td>
<td>48/63</td>
<td>71/80</td>
<td>65/77</td>
</tr>
<tr>
<td>myo8B</td>
<td></td>
<td>71/78</td>
<td>51/66</td>
<td>50/65</td>
</tr>
<tr>
<td>myo8C</td>
<td></td>
<td></td>
<td>48/64</td>
<td>48/63</td>
</tr>
<tr>
<td>myo8D</td>
<td></td>
<td></td>
<td></td>
<td>69/81</td>
</tr>
</tbody>
</table>

Numbers indicate % identity/ % similarity.

Amino acid sequence similarity among myosin VIII protein sequences.

<table>
<thead>
<tr>
<th></th>
<th>myo8B</th>
<th>myo8C</th>
<th>myo8D</th>
<th>myo8E</th>
</tr>
</thead>
<tbody>
<tr>
<td>myo8A</td>
<td>59/73</td>
<td>58/70</td>
<td>79/88</td>
<td>72/82</td>
</tr>
<tr>
<td>myo8B</td>
<td></td>
<td>76/84</td>
<td>59/72</td>
<td>60/72</td>
</tr>
<tr>
<td>myo8C</td>
<td></td>
<td></td>
<td>59/72</td>
<td>58/71</td>
</tr>
<tr>
<td>myo8D</td>
<td></td>
<td></td>
<td></td>
<td>73/83</td>
</tr>
</tbody>
</table>
**Figure 3. 1. *P. patens* has five highly similar myosin VIII genes**

Gene models and conserved protein domain structures of *P. patens* myosin VIII genes are shown. Insertional knockouts were generated by homologous recombination. The knockout constructs are shown below each gene model. Black boxes denote exons that encode for protein, while grey boxes denote untranslated regions. Horizontal arrows above each gene model indicate primers used in RT-PCR for validating altered transcript in Δmyo8ABCDE (see Figure 5). Protein sequences are highly similar among the five myosin VIII genes. All five myosin VIII’s have an N-terminal extension on the motor domain (black), and four IQ motifs (red) downstream of the motor domain. Two regions of coiled-coil (blue) are downstream of the IQ motifs. The vertical arrows above the protein diagrams indicate where protein translation is interrupted in the knockouts.
**Myosin VIII genes are expressed in protonemal and gametophore tissue**

Previous phylogenetic analysis demonstrated that the moss myosin VIIIs group into two clades consisting of: myo8A, myo8D, myo8E and myo8B, myo8C (Peremyslov et al., 2011). To determine which groups were expressed in protonemal and gametophore tissues we analyzed the relative expression level of the five myosin VIII genes using real time quantitative reverse transcription PCR (qRT-PCR). In protonemal tissue, myo8A, myo8B and myo8C have the highest expression and are expressed at similar levels with respect to each other (Figure 3.2). In contrast, myo8E and myo8D transcript levels are 3-fold and 100-fold less, respectively, than myo8A, myo8B and myo8C levels (Figure 3.2). All five myosin VIII genes are also expressed in gametophores (Figure 3.2). However the relative expression level is reduced compared to expression in protonemal tissue, with the exception of myo8D, which appears upregulated in gametophores (Figure 3.2).
Figure 3. 2. Myosin VIII genes are expressed in protonemata and gametophores.

Relative expression levels of myosin VIII genes normalized to ubiquitin 10 as determined by qRT-PCR with RNA isolated from 7 to 9-day-old protonemal tissue (dark grey) or from gametophores isolated from 2-month-old plants. Error bars represent standard error of the mean for at least two biological replicates.
**Myosin VIII quintuple knockout lines**

Our lab previously generated insertional knockouts by homologous recombination for each of the myosin VIII genes. The knockout constructs are shown below each gene model in Figure 3.1. In all cases, at least a small region of the coding sequence toward the 5’ end of the transcript was removed from the genomic locus and replaced with a resistance cassette (Table 3.2). To generate knockouts in multiple myosin VIII genes, knockout constructs were serially transformed into existing and validated knockout lines.
**Table 3.2.** Amount of sequence replaced by the resistance cassette upon correct integration into the myosin locus.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Amount of sequence deleted from locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>myo8A</td>
<td>216 bp</td>
</tr>
<tr>
<td>myo8B</td>
<td>83 bp</td>
</tr>
<tr>
<td>myo8C</td>
<td>121 bp</td>
</tr>
<tr>
<td>myo8D</td>
<td>12 bp</td>
</tr>
<tr>
<td>myo8E</td>
<td>765 bp</td>
</tr>
</tbody>
</table>
Except for myo8B, the resistance cassette that was inserted into the genomic locus was flanked with loxP sites (Figure 3.1). To generate Δmyo8ABCD, Δmyo8BCDE, and Δmyo8ABCDE, the resistance cassettes were removed from the myo8C and myo8D knockout loci in Δmyo8BCD using CRE-mediated recombination (Sauer, 1998). CRE removes all sequences between the two loxP sites, leaving behind a single loxP site. To ensure that myo8C and 8D were disrupted after removal of the resistance cassette, I sequenced these loci from the line treated with CRE recombinase. In both cases, the loxP site was found downstream of the insertion site and sequence analysis confirmed that an in-frame stop codon was introduced into the coding sequences of both myo8C and myo8D (Figure 3.3).

PCR was used to double check proper insertion into the myo8A, myo8B and myo8E loci in Δmyo8ABCDE. Insertion at both the 5’ and 3’ insertions sites was tested (Figure 3.4). PCR genotyping confirmed that a single copy of the knockout construct inserted into the myo8A and myo8E loci (data not shown). PCR analysis of the insertion suggests that these multiple copies had varying orientations (Figure 3.4). However, since no transcript can be detected in the knockout plants (see below), I expect this large insertion has disrupted the gene product.
Altered Myo8C

MFPLKSSGPRSTLEEMDLFRTNTDQEGTSKDNGEEARPPLPARPTSRLPLSSVRARKQVIA
AVKLAPASKIPVDVAKENLADLDAKLPFVIDCLNRDDQQVKPESFRERISDHLSATKAAD
QAPQNGHTVWESTLNTPCTNKSKEEAPWTCPAVSVGRPGSEEAEALDRSNLSDFGGMPN
GSLADEFETELGRDTLALVPEQAPALPHSPSPPAPSRKWKDDGVRLLKKNRINWCLTSDCMWIPGV
IISVEDTEAVRTSDRQERIRVSATKLPLANPAFLERGVDDLKILS SYLNEPSVHLDDLYRSKDIYT
KAGPVLAIVNPFKKIPIYGEDIVQAYQKAAPASSQPHYMYVADSAFGAMKEGINSIIISGESGAGKTE
Altered Myo8D

MLSSNACEGRSTLEEMDLISTGDTEEREVSSDDTPEDLLPLPSRPTSRLPLSSVRRAKALGVCL
DNIVPSNSGAALSKEIVFLGSPIANLTVPSPDLALPKSFASENGTPLAKEDSANNESFASPN
LAYSPTIIPDVLMQSDEVRRSRTLSFGERLNACSTQERSFSFLTAQESSTPHTPLPQNPLVEDTSL
PVTTPSAGKKWDDGILRLKKMYRVWCLSSEYNIAGTIVSAENKDAEAMVRTADHQIIRVNVTRL
KPLANPDELGVDLKLISLYNLEPSVHLHNFRYAHDKIYTRAGPVLIAVNPQKIPYGPDNVQAY
QRRTSESHPHYMTADSAFKAMGRGNSCPAANNFV*

Figure 3.3 Amino acid sequences of truncated proteins expressed from the altered myo8C and myo8D loci are shown. Amino acids in red are introduced by insertion of the knockout construct and the asterisk denotes a stop codon.
Figure 3.4 PCR analysis of insertions in Δmyo8ABCDE knockout.

Models for myo8A, myo8B and myo8E insertions are shown. Above each model horizontal numbered arrows indicate the primers used for PCR. The gel shows PCR products obtained from wild type (WT) and Δmyo8ABCDE (Δ) genomic DNA. Similar results were obtained for all three independent Δmyo8ABCDE lines (data not shown).
To ensure that the myosin VIII transcripts were either missing or altered in the knockout lines, I performed RT-PCR on RNA isolated from wild type and Δmyo8ABCDE plants. I amplified the 5’ end of the transcript from the first exon to an exon downstream of the insertion site (Figure 3.1) using cDNA generated with a pool of myosin VIII reverse primers. For myo8B and myo8E, the transcripts can not be amplified in Δmyo8ABCDE (Figure 3.5), suggesting that the knockout has affected transcription of the 5’ end of these genes. For myo8C and myo8D, transcripts are detected that are 221 and 491bp, respectively, bigger than the wild type products (Figure 3.5). These insertions reflect the presence of the loxP site and sequences from intron 7 for myo8C and intron 4 for myo8D. Due to the high degree of sequence similarity between myo8A and myo8D, we generated myo8A specific cDNA using a single myo8A gene-specific reverse primer. I was only able to detect transcript for myo8A in cDNA generated from wild type RNA, suggesting that the myo8A transcript is missing in Δmyo8ABCDE. Taken together these data show that the myosin VIII genes have been altered and if partial transcripts are present, they code for truncated proteins (Figure 3.1, arrows).
Figure 3.5. Myosin VIII transcripts are altered in Δmyo8ABCDE.

RT-PCR on RNA isolated from wild type and Δmyo8ABCDE protonemal tissue with primers flanking the insertion regions of the resistant cassettes (shown in Figure 1). In Δmyo8ABCDE, no transcript for myo8A, myo8B and myo8E was detected. Myo8C and myo8D transcripts are still present, but are 221 and 491 bp bigger, respectively than the wild type products.
Myosin VIII is not required for viability

Since it is possible to isolate a line containing knockouts of all five myosin VIII genes, myosin VIII is evidently not required for viability. To analyze the phenotype of the myosin VIII knockout lines, I regenerated plants from protoplasts. I measured the size of plants six days after protoplasting. I observed that the single knockouts were slightly smaller than wild type plants (Figure 3.6). The growth defect was dependent on the number of myosin VIII genes that remain. As more myosin VIII genes are removed, the myosin VIII knock out plants became progressively smaller. This suggests that the myosin VIII genes are partially redundant with respect to growth. Not surprisingly, Δmyo8ABCDE was the smallest mutant, with an average plant size 58% the size of wild type plants.

The fact that the myosin VIII knockouts are smaller than wild type is in part due to a reduction in cell size (Table 3.3). However for Δmyo8ABCDE, the reduction in cell size is not as great as the overall reduction in plant size. Thus, it is possible that the plants are either performing expansion or cytokinesis at a reduced rate compared to wild type. To determine whether expansion rates are altered in Δmyo8ABCDE, I acquired time-lapse images of plants growing on solid PpNH4 medium. I found that Δmyo8ABCDE grew at 0.25 ± 0.08 μm/min (n = 32 ± SD), while wild type grew at 0.38 ± 0.06 μm/min (n = 16 ± SD). Using a Student’s t-test I found that this difference is statistically significant (P <0.0001). These data show that there is a reduction in the rate of tip growth, but perhaps not sufficient to account for the overall growth defect.
Figure 3. 6. Myosin VIII is not required for plant viability.

Representative fluorescence images of six-day-old wild type and myosin VIII knockouts plants regenerated from protoplasts. Plants were stained with calcofluor and visualized by epifluorescence stereomicroscopy. Number of plants analyzed is 100 for all lines except: wild type (n = 199), Δmyo8D (n = 98), Δmyo8BD (n = 101), Δmyo8BCDE (n = 99), Δmyo8ABCDE (n = 297). The average area normalized to wild type is shown in the bar chart for each line. Error bars indicate standard error of the mean. Scale bar = 100 μm.
Table 3.3. Cell length and branch density measurements on six-day old plants regenerated from protoplasts.

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>Δmyo8ABCDE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Branch / 100 µm</td>
<td>0.58</td>
<td>1.22</td>
</tr>
<tr>
<td>Apical cell length (µm)</td>
<td>117.47 ± 29.6</td>
<td>75.88 ± 23.1</td>
</tr>
<tr>
<td>Non-apical cell length (µm)</td>
<td>92.61 ± 13.4</td>
<td>62.46 ± 14.6</td>
</tr>
</tbody>
</table>
Myosin VIII is involved in branch formation

As Δmyo8ABCDE had the most profound growth phenotype, I examined its phenotype in more detail to assess how growth is affected. Analysis of 6-day old plants revealed that more side branches were forming in Δmyo8ABCDE compared to wild type (Table 3.3). This phenotype is readily apparent in older plants (Figure 3.7). The edge of wild type plants is composed of highly extended protonemal filaments whose tips are composed of caulonemal cells. The branching pattern is uniform, with the third subapical cell typically containing a single branch. In contrast, Δmyo8ABCDE plants have highly branched protonemal filaments, with substantially smaller caulonemal cells at the apex. The distance between the first branch and the tip of the filament is reduced by 50% in Δmyo8ABCDE. Additionally, there are twice as many branches per unit length in Δmyo8ABCDE compared to wild type (Table 3.3). Interestingly, the increase in branch density is not tightly correlated with cell size, since Δmyo8ABCDE cells are 33% smaller than wild type (Table 3.3).

Instead, the increase in branch density is likely due to multiple branching events from a single cell (Figure 3.7, B). Although this occurred in wild-type plants, it was much more common in Δmyo8ABCDE. The increased branch frequency in Δmyo8ABCDE is readily apparent in the time-lapse movie used to determine tip growth rates. Δmyo8ABCDE branches were also not as uniform as those in wild type. Large and small branches were commonly next to each other (Figure 3.7, B), which is distinct from wild type, where the smallest branches were near the apex of the filament and the longest branches were further from the apex. Additionally, the
\(\Delta\text{myo8ABCDE} \) branches formed secondary branches early in their development (Figure 3.7, A, arrows).
Figure 3.7. Myosin VIII is involved in branch formation.

Wild type and Δmyo8ABCDE plants regenerated from protoplasts were grown on PpNH4 medium. (A) Brightfield images of protonemal filaments of 20-day-old plants. Arrows indicate branches forming on branches in Δmyo8ABCDE plants. Scale bar, 100 μm. (B) 12-day-old plants stained with calcofluor visualized by epifluorescence microscopy. Scale bar, 20 μm.
Myosin VIII influence the rate of development

A 20-day-old wild-type plant grown from a single protoplast has no visible gametophores (Figure 3.8, A). In contrast, the same age Δmyo8ABCDE plant contained fully developed gametophores (Figure 3.8, A), suggesting that development of gametophores is accelerated in the mutant. Of note, the gametophores in Δmyo8ABCDE were indistinguishable from wild type, suggesting that myosin VIII is not required under these conditions to pattern the gametophore. In mosses, cytokinin induces bud formation (Ashton et al., 1979; Bopp, 1963). Interestingly, treatment of plants with 10 µM 6-benzylaminopurine resulted in many more buds in Δmyo8ABCDE compared to wild type (Figure 3.8, B).

Myosin VIII is required for growth in limiting nutrients

Although myosin VIII is not essential for viability under laboratory growth conditions, I investigated whether limiting nutrient availability might enhance the need for myosin VIII function. To test this, wild-type and Δmyo8ABCDE protoplasts were regenerated on protoplast regeneration medium. Protoplasts were either plated in agar or liquid medium. After four days, the regenerating protoplasts were transferred to plates containing only water and agar. When exposed to no nutrient media, moss plants can grow for about three weeks before dying. Long caulonemal filaments are formed at the edge of the plant. These filaments contain very few plastids compared to plants grown in nutrient-rich media. Like wild type, Δmyo8ABCDE plants also grew in the absence of nutrient media. However, as was observed in nutrient media, Δmyo8ABCDE plants had defects in branch formation.
**Figure 3.8.** Myosin VIII influences the rate of development.

(A) 20-day-old wild type and Δmyo8ABCDE plants regenerated from single protoplasts were grown on PpNH4 medium. Δmyo8ABCDE plant contains fully developed gametophores while the same age wild type plant has no visible gametophores. Scale bar, 1 mm. (B) 12-day-old wild type and Δmyo8ABCDE plants grown from single protoplasts. Protoplasts are on PRMB for 4 days, then grown on water containing agar for 4 days before transfer to water agar containing 10 μM 6-BAP for another 4 days. Scale bar, 100 μm.
Interestingly, I observed that the Δmyo8ABCDE phenotype was exacerbated when grown on water containing agar if protoplasts were plated in liquid media as opposed to top agar before regenerating. In particular, the mutant caulonemal filaments were curlier than wildtype in plants regenerated after plating in liquid media (Figure 3.9, A, B). To quantify this, I measured the index of curvature, which is the ratio of the length of a filament to the shortest linear distance between the tip and the filament. Wild type filaments on average have a curvature index of 1.2 and are tightly distributed with 100% of the filaments having a curvature less than 2. In contrast, Δmyo8ABCDE filaments had an average curvature index of 5.6, with a broad distribution and with only 52% having a curvature index less than 2 (Figure 3.9, B).

In addition to the curved filaments, filaments often grew in an undulating pattern (Figure 3.9, C, arrows). Defects in side branch formation were also pronounced. Side branches were aberrantly shaped, often having swollen apical and subapical cells (Figure 3.9, C, D). Branches were shorter than wild type and unevenly distributed along the main filaments (Figure 3.9, C, arrow heads). I also measured the rates of tip growth and found that Δmyo8ABCDE grew 1.8 times slower than wild type on water containing agar (wild type, 0.44 ± 0.05 µm/min; Δmyo8ABCDE, 0.25 ± 0.04 µm/min), indicating that the tip growth defect was enhanced under limiting nutrients. Since these conditions led to an amplification of caulonemal growth and Δmyo8ABCDE had smaller caulonemal cells, the reduction in tip growth rates is consistent and suggests that myosin VIII is preferentially required for caulonemal growth. Taken together, these data demonstrate that
myosin VIII is important for protonemal patterning and growth in low nutrient conditions.
Figure 3.9. Myosin VIII is required for normal growth in limiting nutrients.

Wild type and Δmyo8ABCDE plants regenerated from protoplasts plated in liquid media onto PRMB for four days then transferred to water containing agar. (A) Top: 12-day-old plants. Scale bar, 1 mm. Bottom: The edge of 13-day-old plants. Scale bar, 1 mm. (B) Curvature index was quantified for protonemal filaments from 13-day-old plants. Curvature index for wild type (n = 62) and Δmyo8ABCDE (n = 147) was plotted as a histogram. Inset shows the average curvature index. Error bar represents standard error of the mean. (C) Top: The edge of 13-day-old plants is shown. Filaments grew in an undulating pattern (arrows) in Δmyo8ABCDE. Bottom: The center of 13-day-old plants is shown. Branches were significantly shorter and unevenly distributed (arrow heads) along the main filaments in Δmyo8ABCDE. Scale bar, 100 μm. (D) 12-day-old plants stained with calcofluor visualized by epifluorescence stereomicroscopy. Scale bar, 20 μm.
Auxin partially rescues morphological defects in the myosin VIII mutant

Since Δmyo8ABCDE has shorter caulonemal cells and develops gametophores faster than wild-type plants, I suspected that Δmyo8ABCDE has defects in hormone homeostasis. Therefore, I investigated whether Δmyo8ABCDE mutants could be rescued by increasing the level of auxin in the surrounding media. I regenerated plants from protoplasts plated in liquid media. After four days they were transferred to water containing agar and four days later they were transferred to water with or without 1 µM NAA, a synthetic auxin analog. Δmyo8ABCDE plants grown without auxin exhibited characteristic growth defects, particularly increased undulating growth in protonemal filaments. In the presence of NAA, the protonemal filaments were similar to wild-type plants (Figure 3.10). These data show that auxin can partially alleviate the growth defects resulting from lack of myosin VIII function.
Figure 3.10. Auxin partially rescues morphological defects in Δmyo8ABCDE.

Wild type and Δmyo8ABCDE plants were regenerated from protoplasts and plated in liquid media. Four days after protoplasting plants were transferred to water containing agar. Four days later they were transferred to water with or without 1µM NAA. Scale bar, 100 µm.
CHAPTER 4

CELLULAR LOCALIZATION OF MYOSIN VIII AND ITS FUNCTION DURING CELL DIVISION.

Introduction

To understand the function of myosin VIII, first I wanted to investigate the cellular localization of myosin VIII during protonemal growth and development. Little is known about the localization of class VIII myosins in plants. An antibody raised against the class VIII myosin ATM1 from arabidopsis was used for immunofluorescence and immunogold microscopy (Reichelt et al., 1999). These studies showed that myosin VIII localized to plasmodesmata and new cell plates in the root cells of maize and arabidopsis. Additionally the same antibody was used on maize root apices and found to localize to pit fields (Baluška et al., 2004). A GFP fusion to the full length ATM1 was found to localize to the new cell plate when expressed in tobacco BY-2 cells (Damme et al., 2004). Additionally a GFP fusion to the IQ domain and tail of ATM1 was used to analyze localization in arabidopsis roots, where it was found to localize in different cellular compartments depending on the tissue type. These compartments included the cytoplasm, transverse cell plates and punctate structures that resembled endocytic compartments, some of which were sensitive to brefeldin A (Golomb et al., 2008). Interestingly when this fusion was transiently expressed in Nicotiana benthamiana, it localized to pit fields and the ER (Golomb et al., 2008). A similar GFP fusion to ATM2 expressed in N. benthamiana localized to endocytic compartments (Sattarzadeh et al., 2008). These
Localization studies have implicated a role for myosin VIII in endocytosis, cytokinesis and plasmodesmal function.

Plasmodesmata are channels that connect neighboring cells. The plasma membrane of the adjacent cells sets the outer boundary of the channel, while a structure called desmotubule occupies the center. The desmotubule is composed of compressed endoplasmic reticulum connected to both cells. The space between the plasma membrane and the desmotubule is called the cytoplasmic sleeve, which is the major conduct for cell-to-cell transport. Both myosin VIII and actin have been localized to plasmodesmata (Reichelt et al., 1999; Radford and White, 1998; Ding et al., 1996; White et al., 1994). Actin filaments are hypothesized to link the desmotubule to the plasma membrane lining the channel, and contraction or relaxation of the actin filaments resulting from myosin motility would subsequently alter the aperture of the cytoplasmic sleeve (Overall and Blackman, 1996). In support of this hypothesis, transport through the plasmodesmata is altered by treatment with actin-disrupting agents, a myosin inhibitor, or injection of anti-myosin antibody alter cell-to-cell transport through the plasmodesmata (Radford and White, 2011; Ding et al., 1996).

The involvement of myosin and actin in plant cytokinesis was proposed previously based on drug studies (Molchan et al., 2002). Plant cells divide by building a new cell wall separating the two daughter cells. This is accomplished by a dynamic and complex structure comprising microtubules, actin filaments, endoplasmic reticulum, and Golgi-derived vesicles, known as the phragmoplast. The phragmoplast assembles from the anaphase spindle midzone and directs the motion
of vesicles carrying cell wall precursors to the nascent cell plate. Expansion of the phragmoplast from the center of the cell out towards the parental cell wall requires assembly of new microtubules along the outer edge of the phragmoplast and concomitant removal of microtubules in the center of the phragmoplast as the delivered membranes fuse together building up a new cell wall (Smith, 2001; Jürgens, 2005; McMichael and Bednarek, 2013). Cytokinesis is completed when the phragmoplast reaches and the nascent cell plate fuses with the parental cell wall.

While the phragmoplast is predominately a microtubule-based structure, it has been known for decades that actin filaments are also a component of the phragmoplast (Clayton and Lloyd, 1985; Kakimoto and Shibaoka, 1987). In contrast to microtubules, which are essential for cell plate formation, it remains unclear how actin contributes to phragmoplast function. Primarily based on drug treatments and localization studies, actin has been proposed to stabilize the phragmoplast and link the phragmoplast to the cell cortex (Valster and Hepler, 1997; Molchan et al., 2002; Lloyd and Traas, 1988). However, plant cells still divide in the absence of actin (Baluska et al., 2001; Nishimura, 2003). Furthermore, genetic evidence is lacking for a significant role of actin in phragmoplast function (Jürgens, 2005).

By analyzing the localization of Myo8A-GFP protein, I found myosin VIII appear to link phragmoplast microtubules and actin filaments. This study reveals a role of actin and myosin VIII in steering the phragmoplast from the center of the cell toward the predefined cortical division site at the cell cortex. Lack of either actin or myosin VIII causes defects in new cell plate placement. I also confirmed the plasmodesmal localization of myosin VIII in *P. patens*. Interestingly, myosin VIII at
plasmodesmata is especially enriched throughout the course of branch cell formation. This is apparently the first example of a plasmodesmal protein under developmental regulation.

**Results**

**Myosin VIII quintuple knockout plant has defective cell plate positioning**

In Chapter 3, I showed that plants lacking all five members of the myosin VIII subfamily have multiple, aberrantly positioned side branches. With a closer look, I found that while wild-type plants usually had an organized cell division pattern, myosin VIII null plants positioned the cell plate at branch sites aberrantly (Fig. 4.1 A). Since branch patterning and cell division plane specification are linked, I reasoned that non-branching cells in the myosin VIII null plants might also have a defect in cell division plane specification. In wild type, the apical cells of young plants had cell plates that were usually perpendicular to the long axis of the cell; indeed, more than 84% of apical cell plates were within 15° of the perpendicular plane. In contrast, fewer than 35% of the apical cell plates in myosin VIII null plants were within 15° of the perpendicular axis, and nearly 40% had cell plates with angles greater than 25°, some as high as 45° (Fig. 4.1 B). These data suggest myosin VIII plays a role during cell division in both protonemal cell types.
Figure 4.1. Cell plates are improperly positioned in young myosin VIII null plants. (A) Representative images of 10-day-old wild type and myosin VIII null plants stained with calcofluor. Scale bar, 100 μm. Arrows indicate possibly incomplete cell plates or plates with excess aberrant cell wall deposition. (B) Histograms of cell plate angles of apical cells from 5-day-old plants regenerated from protoplasts. Images of apical cells were acquired as in (A) and cell plate angles were measured manually using ImageJ. Number of cells analyzed: wild type (n = 151), Δmyo8ABCDE (n = 180), Myo8A-GFP in Δmyo8ABCDE (n = 167).
The cell plate positioning defect appears more severe at sites of side branch formation, which possibly reflects the more complex nature of cell division at side branches compared to apical cells. During side branch formation, first a small protrusion forms near the existing cross wall in the parental cell (Fig.4.2 B). The small protrusion grows longer via tip growth while the nucleus slowly migrates toward the junction between the side branch and the parenatal cell (Fig.4.2 C). After mitosis and cytokinesis, a cell plate is built that separates the parental and branch cells (Fig. 4.2 D).

The phenotype observed in myosin VIII null mutants could result from several defects. In some cases, the side branch protrusions formed in the wrong place. In other cases, the nucleus failed to move to the mother cell-branch junction before the cell went into mitosis. Most often, the nucleus moved to the correct position before mitosis, but the cell plate was placed improperly. This implies that myosin VIII might potentially have roles in: deciding when a cell should form a side branch, marking where the side branch should form, forming side branches via tip growth, marking the cell division site, nuclear migration, guiding the phragmoplast to the cortical cell division site, and building new cell plates.
Figure 4.2. Side branch formation in moss protonemata.

(A) Representative graph of moss protonemata. Green circle = nucleus. (B) Usually at the third subapical cell, a small protrusion forms near the existing cross wall in the mother cell. (C) The small protrusion grows longer via tip growth while the nucleus slowly migrates toward the junction between the side branch and the mother cell. (D) After mitosis and cytokinesis, a new cell plate is built and physically separates the mother cell and the branch cell.
MyosinA-GFP is a functional fusion protein

To determine how myosin VIII influences cell plate positioning, I generated a construct encoding Myo8A fused to three tandem copies of monomeric enhanced GFP (hereafter referred to as Myo8A-GFP), expression of which is driven by the strong, constitutive maize ubiquitin promoter. I transformed Myo8A-GFP into the myosin VIII null plants (Δmyo8ABCDE). Myo8A, 8B and 8C are expressed at similar levels and together represent ~90% of the total myosin VIII transcript in protonemal tissue (see Chapter 3, Fig. 3.2). Since myosin VIII’s are partially redundant (see Chapter 3, Fig. 3.6), I reasoned that expression of Myo8A should be sufficient to partially rescue the myosin VIII null phenotype. To test this, I measured cell plate positioning in young plants and found that expression of Myo8A-GFP resulted in plants with 63% of the apical cell plates within 15° of the perpendicular axis. Importantly, cell plates with angles greater than 35° were not observed in the Myo8A-GFP expressing plants (Fig. 4.1, B), indicating that Myo8A-GFP partially restored cell plate positioning in the myosin VIII null plants. Additionally, Myo8A-GFP expression partially rescues a number of other defects in myosin VIII null plants, including apical cell length, protonemal patterning (Fig. 4.3), and timing of gametophore formation (data not shown). Taken together, these data indicate that Myo8A-GFP is a functional fusion protein.
Figure 4. 3. Expression of Myo8A-GFP in Δmyo8ABCDE largely restores protonemal branching (A) and apical cell length (B). (A) 8-day old plants regenerated from protoplasts were imaged with a stereo microscope. Scale bar, 100 µm. (B) Measurements of cell length were made on images of the apical cells from calcofluor stained 5 & 6-day old plants regenerated from protoplasts. Data shown mean ± SD. Scale bar, 50 µm.
Myo8A is an actin-based molecular motor

Using spinning disc confocal microscopy, I found that Myo8A-GFP localized diffusely throughout the cytoplasm, was enriched at the cell cortex in dynamic cortical particles, and in some apical cells was enriched at the apical cell cortex (Fig. 4.4). Using variable angle epifluorescence microscopy (VAEM), I simultaneously imaged Myo8A-GFP and actin labeled with lifeact-mCherry. I observed that a large fraction of Myo8A-GFP cortical particles moved along actin filaments, consistent with actin-based motility (Fig. 4.5 A). Treatment of cells with 25 µM latrunculin B halted motility, as expected for an active, actin-based motor, but did not abolished cortical localization (Fig. 4.5 B).
Figure 4. 4. Myo8A-GFP localization in apical cells.

Two representative images of moss apical cells expressing Myo8A-GFP in myosin VIII null background. Images were maximum projection of z-stacks acquired on a spinning disc confocal microscope. The punctate structures often accumulate near the apex of the growing cell. Large globular structures are chloroplasts, which autofluoresce under these imaging conditions. Scale bar, 10 µm.
**Figure 4.5. Myo8A moves along cortical actin filaments.** (A) Images of Myo8A-GFP and Lifeact-mCherry in moss protonemata were simultaneously acquired with VAEM. In the merge Myo8A-GFP is green and Lifeact-mCherry is red. (B) Moss protonemal cells expressing Myo8A-GFP, were treated with or without latrunculin B (LatB) and imaged with VAEM. In control samples, Myo8A-GFP linear trajectories are apparent in maximum projections of N frames from three seconds of real time, but absent in cells treated with 25 µm LatB. Control, N=21; LatB, N=15. Scale bars, 2 µm.
Myo8A-GFP localizes to plasmodesmata

In sub-apical cells with emerging side branches, I found Myo8A-GFP accumulated near the cross wall of the parental cell (Fig. 4.6). This local accumulation of Myo8A-GFP was temporary; appearing only prior to the completion of cytokinesis in the emerging branch. Compared to the highly dynamic Myo8A-GFP population at the cell cortex, Myo8A-GFP puncta associated near the cross wall were less mobile, suggesting they could be part of a stable structure like plasmodesmata. To confirm Myo8A’s association with plasmodesmata, I plasmolyzed the cells with a 20% sucrose solution. In this solution, the plasma membrane was pulled away from the cell wall, but Myo8A-GFP remained associated with the wall (Fig. 4.7), consistent with the behavior of a plasmodesmal protein. Myo8A-GFP signal was also found associated with the cross wall in cells with completed side branches, although at much lower intensity (Fig. 4.8). This suggests Myo8A-GFP is ubiquitously present at plasmodesmata, but is enriched at a certain developmental stage.
Figure 4. 6. Myo8A-GFP localizes to punctate structures at the cross wall in cell with emerging branch.

Images were maximum projection of z-stacks acquired on a spinning disc confocal microscope. Moss protonemal apical cells expressing Myo8A-GFP in myosin VIII null background. Myo8A-GFP punctate structures accumulate at the cross wall in cell with an emerging branch. Large globular structures are chloroplasts, which autofluoresce under these imaging conditions. Scale bar, 10 µm.
Figure 4. 7 Myo8A-GFP stays associated with the wall in plasmolyzed cell.

Images were maximum projection of z-stacks acquired on a spinning disc confocal microscope. Moss protonemal apical cells expressing Myo8A-GFP (green) and ER-mCherry (red) in myosin VIII null background. Cell was plasmolyzed with 20% sucrose. Myo8A-GFP punctate structures accumulate at the cross wall (white box) in cell with an emerging branch (cell on the left). Large green globular structures are chloroplasts, which autofluoresce under these imaging conditions. Area in the white box is enlarged on the right. Scale bar, 5 µm.
Figure 4.8. Myo8A-GFP stays associated with the wall in plasmolyzed cell which has completed branch.

Images were maximum projection of z-stacks acquired on a spinning disc confocal microscope. Moss protonemal apical cells expressing Myo8A-GFP (green) and ER-mCherry (red) in myosin VIII null background. Cell was plasmolyzed with 20% sucrose. Myo8A-GFP punctate structures were present in the wall, but signal was low. Area in the white box was enlarged on the right, and Myo8A-GFP became visible after enhanced contrast. Large green globular structures are chloroplasts, which autofluoresce under these imaging conditions. Scale bar, 5 µm.
**MyoA-GFP localization in mitotic cells**

In addition to the cortical and plasmodesmal localizations, I was surprised to find that Myo8A-GFP accumulated on a spindle-like structure in dividing cells (Fig. 4.9 A). This Myo8A-GFP signal later transitioned into a cortical ring, within which was filled with FM4-64 labeled membrane, suggesting this structure was likely a phragmoplast (Fig. 4.9 B).

To confirm spindle and phragmoplast localization, I introduced mCherry-α-tubulin into the Myo8A-GFP line. Imaging of this line with a spinning disc confocal microscope revealed that during cell division Myo8A-GFP localized transiently to the mitotic spindle (Fig. 4.10, A). It was enriched in the midzone and to some extent at the poles. Prior to anaphase, a small population of Myo8A-GFP accumulated at the cortex near the midzone of the spindle. During anaphase Myo8A-GFP formed a tight band at the midzone, which transitioned into a ring on the leading edge of the phragmoplast. As the phragmoplast expanded, it joined the cortical Myo8A-GFP population (Fig. 4.10, A). These data suggest that myosin VIII guides the phragmoplast during cytokinesis to a cortical site defined by the presence of Myo8A-GFP.

**Myo8A-GFP recruitment to the spindle and phragmoplast is not actin-dependent**

To test whether actin is involved in recruiting Myo8A-GFP to the mitotic spindle, I first imaged Myo8A-GFP and lifeact-mCherry in dividing cells (Fig. 4.10 B).
When Myo8A-GFP appeared on the spindle, there was little or no accumulation of lifeact-mCherry. At anaphase, when Myo8A-GFP concentrated at the spindle midzone, lifeact-mCherry fluorescence rose above background levels in the vicinity of the spindle. However, when Myo8-GFP fluorescence tightened into a thin band on the phragmoplast leading edge, the lifeact-mCherry fluorescence accumulated significantly around the phragmoplast (Fig. 4.10 B). The timing of the appearance of actin suggests that Myo8A-GFP localization to the mitotic spindle is independent of actin. To test this, I imaged cells entering mitosis in the presence of 25 μM latrunculin B. Strikingly, I found that Myo8A-GFP still accumulated on the mitotic spindle. Similar to control cells, Myo8A-GFP accumulated in the spindle midzone at anaphase and tightened into a thin band at the leading edge of the phragmoplast during cytokinesis (Fig. 4.10 C). Thus, Myo8A-GFP recruitment to the mitotic spindle and behavior during mitosis is independent of actin.
**Figure 4.9. Myo8A-GFP localizes to a spindle-like structure.**

Moss protonemal cell expressing Myo8A-GFP stained with FM4-64. Images are maximum projection of z-stacks acquired with spinning disc confocal microscope. Scale bar, 5 µm. Large globular structures are chloroplast auto-fluorescence. (A) Myo8A-GFP localized to spindle-like structure during cell division. (B) Later, the Myo8A-GFP structure transitioned into a cortical ring, within which were filled with FM4-64 labeled membrane.
Figure 4.10. Myo8A-GFP localizes to mitotic spindle and phragmoplast independent of actin.

Moss protonemal apical cells were imaged during cell division on a spinning disc confocal microscope. All images are maximum projections of z-stacks acquired every 4 minutes. Large globular structures are chloroplasts, as chlorophyll auto-fluorescence is imaged in the GFP channel. (A) Cell expressing Myo8A-GFP and mCherry-tubulin. Myo8A-GFP associates with the mitotic spindle, concentrates in the midzone of the spindle in anaphase, and transitions into a ring on the leading edge of the phragmoplast. Arrows indicate cortical accumulation of Myo8A-GFP. (B) Cell expressing Myo8A-GFP and Lifeact-mCherry. Myo8A-GFP associates with the mitotic spindle before actin starts to accumulate in the region. (C) Cell expressing Myo8A-GFP and mCherry-tubulin treated with 25 µM LatB. In the absence of actin Myo8A-GFP localization is unaffected. Scale bar, 5 µm. In the merge for (A) and (C) Myo8A-GFP is green and mCherry-tubulin is red; in the merge for (B) Myo8A-GFP is green and Lifeact-mCherry is red.
**Myo8A-GFP interacts with microtubule plus ends**

Careful inspection of Myo8A-GFP during mitosis and early cytokinesis revealed that Myo8A-GFP accumulated prefentially on the plus ends of microtubules. This is particularly evident in Figure 4.11, which is a single focal plane imaged through time on a spinning disc confocal microscope. The top half of the early phragmoplast is not in the plane of focus but microtubules that originate from the top half move into the plane of focus. These microtubules often had Myo8A-GFP on the ends (Fig. 4.11, arrows) and join the midzone area that is in focus. Peripheral phragmoplast microtubules also had Myo8-GFP on their ends as they polymerized towards the cell cortex (Fig. 4.11, arrowheads). These data suggest that myosin VIII is recruited to the mitotic spindle and later the phragmoplast by interacting with microtubule plus ends.

The interaction of Myo8A-GFP with microtubule plus ends in mitotic cells is likely cell cycle dependent. However, I also observed a small percentage of Myo8A-GFP in interphase cells transiently interacting with cortical microtubules in the absence of actin (Fig. 4.12). The region of the Myo8A protein that is responsible for this interaction remains to be determined. Interestingly, inspection of the Myo8A protein sequence revealed a predicted EB1 binding motif Ser-x-Ile-Pro (SxIP) in the N-terminus of Myo8A (Fig. 4.13). One possible mechanism of Myo8A recruitment to microtubule plus ends could be through interacting with EB1. This hypothesis needs to be tested in future experiments.
**Figure 4.11** Myo8A-GFP associates with microtubule plus ends. Images of a protonemal apical cell expressing Myo8A-GFP and mCherry-tubulin were acquired with a spinning disc confocal microscope. Images are time points from a single focal plane. The top half of the early phragmoplast is not in the plane of focus but microtubules that originate from the top half move into the plane of focus. These often have Myo8A-GFP on the end of the microtubules (arrows) and join the midzone area that is in the focal plane. Peripheral phragmoplast microtubules also have Myo8-GFP on their ends as they polymerize towards the cell cortex (arrowheads). In the merge Myo8A-GFP is green and mCherry-tubulin is red.
Figure 4. 12 Myo8A-GFP associates with microtubule plus ends in interphase cell. Image of a protonemal apical cell expressing Myo8A-GFP (green) and mCherry-tubulin (red) was acquired with a spinning disc confocal microscope. Cell was treated with 25µM latrunculin B. Images was from a single focal plane. In tip cells there are often long microtubules extending toward the apex of the cell. These microtubules often have Myo8A-GFP on their ends. Scale bar, 5 µm.
Figure 4. 13. Myo8 N-terminus protein sequences contain putative EB1 binding motifs.

Protein sequence alignment of all five moss myosin VII, only the N-terminal part of the protein sequences are shown. Predicted EB1 binding motif Ser-x-Ile-Pro (SxIP) are highlighted in black box.
**Actin is required for Myo8A-mediated phragmoplast guidance**

In the absence of actin, myosin VIII localization is not appreciably affected. Further, mitosis and cytokinesis progress at indistinguishable rates in control and latrunculin B treated cells, demonstrating that actin is not required for phragmoplast expansion or cytokinesis. This raises the possibility that myosin VIII has a role in mitosis and cytokinesis that is independent of actin. Since I initially observed a cell plate positioning defect in the myosin VIII null plants and since myosin VIII accumulates on the leading edge of the phragmoplast, I hypothesized that myosin VIII functions to guide the phragmoplast from the midzone of the mitotic spindle out to the cell cortex where it joins the parental cell wall. While the timing of expansion is not affected in cells lacking actin, actin does accumulate in the phragmoplast coincident with myosin VIII localization to the phragmoplast midzone, suggesting that myosin VIII requires actin to guide the expanding phragmoplast to the cell wall.

To test this, I imaged phragmoplasts labeled with GFP-tubulin and FM4-64. Since actin is essential for growth, it was not possible to perform long-term latrunculin B treatments. Instead, plants were treated with 25 µM latrunculin B for two hours before imaging, ensuring that the observed phragmoplasts had been formed in the absence of actin. Wild-type phragmoplasts had organized microtubules that were mostly perpendicular to the plane of the new membrane. The new membrane was deposited uniformly, appearing as smooth FM4-64 staining in the phragmoplast midzone (Fig. 4.14, top). In contrast, the myosin VIII-null phragmoplasts had slightly disorganized microtubules, with a higher percentage of
microtubules at oblique angles to the plane of the membrane. Concomitantly, the FM4-64 staining was less uniform, suggesting that the membrane was not deposited uniformly (Fig 4.14, middle). Interestingly, treatment with latrunculin resulted in phragmoplasts similar to those observed in the myosin VIII null cells (Fig 4.14, bottom), suggesting that actin is required for myosin VIII-mediated guidance during phragmoplast expansion.
Figure 4. 14. Actin is required for Myo8A-mediated phragmoplast guidance. Phragmoplasts from wild type (WT) and myosin VIII null (Δmyo8) plants expressing GFP-tubulin (GFP-Tub) and stained with FM4-64 were imaged on a spinning disc confocal microscope. For LatB treatment, wild type plants were treated with 25 µm LatB for 2 hours, then stained with FM4-64 and imaged in the presence of 25 µm LatB. In the merge Myo8A-GFP is green and FM4-64 is red. Scale bars, 5 µm.
During phragmoplast expansion, I observed a significant population of peripheral microtubules that briefly touch the cell cortex and the phragmoplast midzone. Myosin VIII localized to the ends of these microtubules (Fig. 4.15). In control cells, the peripheral microtubules were quickly incorporated into the phragmoplast as the phragmoplast expands. However, in the absence of actin the peripheral microtubules with Myo8A-GFP on their plus ends spent significantly more time searching the cell cortex before eventually incorporating into the phragmoplast. Additionally, without actin these peripheral microtubules often grew beyond the midzone (Fig. 4.15, arrow). These data indicate that peripheral microtubules efficiently and accurately incorporate into the expanding phragmoplast in the presence of actin and myosin VIII, suggesting that actin filaments must be present between the edge of the expanding phragmoplast and the cell cortex.

To test this, I simultaneously imaged lifeact-mEGFP and mCherry-tubulin using a laser scanning confocal microscope. I confirmed that actin accumulated at the midzone only at the end of anaphase. As the phragmoplast expanded out from the center of the cell towards the cortex, actin accumulated between the phragmoplast and the cortex (Fig. 4.16). I found that actin very rapidly remodeled. Often one side of the phragmoplast reached the cell cortex before the other (Fig. 4.16, arrows). Once the first side reaches the cortex, actin polymerized at the other edge of the phragmoplast (Fig.4.16, arrowheads). The lifeact-mEGFP fluorescence intensity equalized once both sides reached the cell cortex (Fig. 4.16). These data demonstrate that actin filaments are actively generated between the leading edge of
the phragmoplast and the cell cortex during the time that myosin VIII-mediated motility is required to guide the phragmoplast. Taken together, these data suggest a model whereby myosin VIII on the plus end of the microtubules moves along actin filaments that are generated between the cell cortex and the leading edge of the phragmoplast, ensuring that phragmoplast expansion occurs along a plane defined by actin filaments.
Figure 4. 15. Actin is required for proper and efficient contacts between peripheral microtubules and cell cortex.

Images of protonemal apical cells expressing myo8A-GFP and mCherry-tubulin were acquired with a laser scanning confocal microscope. In control cells, the peripheral microtubules with Myo8A-GFP labeled plus ends are quickly incorporated into the phragmoplast. In the absence of actin (25 μM LatB) the peripheral microtubules spend significantly more time searching the cell cortex before eventually incorporating into the phragmoplast. Additionally, these peripheral microtubules often grow beyond the midzone (arrow). In the merge Myo8A-GFP is green and mCherry-tubulin is red. Scale bar, 5 µm.
**Figure 4. 16. Actin is present between the edge of expanding phragmoplast and cell cortex.**

Images of a protonemal cell expressing Lifeact-mEGFP and mCherry were acquired with a laser scanning confocal microscope. Images are time points from a single focal plane. Actin accumulates between the phragmoplast and the cell cortex. In this cell, the left side of the phragmoplast reaches the cell cortex first (arrow). Once that occurs, more actin accumulates at the right-hand edge (arrow heads). The lifeact-mEGFP fluorescence intensity equalizes once both sides reach the cell cortex (900 s). In the merge lifeact-mEGFP is green and mCherry-tubulin is red. Scale bars, 5µm.
Myo8A-GFP marks future cell division sites in branching cells

A role for phragmoplast guidance is further supported during branch formation. Prior to mitosis in the branching cell, myosin VIII accumulated at the neck region of the emerging cell (Fig. 4.17, top). During cell division, Myo8A-GFP was also present on the mitotic spindle. It accumulated at the spindle midzone (Fig. 4.17, middle) and formed a ring at the leading edge of the phragmoplast, which expanded out to the cell cortex where it joined with the cortical accumulation of Myo8A-GFP that had formed prior to mitosis (Fig. 4.17, bottom). Thus, during branching, myosin VIII appears to mark the future site of cell division, functioning similar to the preprophase band of seed plants.

Myo8A-GFP localizes to the preprophase band, the cortical division site, and the phragmoplast in tobacco BY-2 cells

While the microtubule structures during mitosis and cytokinesis are similar between moss protonemata and seed plant tissues, there is a one significant difference: moss protonemata do not form a preprophase band. The preprophase band in seed plants is essential for defining the plane of cell division. Thus, myosin VIII’s role in phragmoplast guidance may be an ancient mechanism that arose before the evolution of the preprophase band. To test whether myosin VIII might also play a role in cells that have a preprophase band, I generated a tobacco BY-2 cell line stably transformed with moss Myo8A-GFP. Similar to the localization observed in the moss, Myo8A-GFP localized to dynamic cortical particles (Fig. 4.18).
In cells about to enter mitosis, I found that cortical Myo8A-GFP accumulated at the presumptive preprophase band (Fig. 4.19, A). As cells entered mitosis, cortical Myo8A-GFP tightened into a thin band and remained at the cortical division site. In early cytokinesis, Myo8A-GFP was at the cortical division site as well as the phragmoplast midzone (Fig. 4.19, B). As the phragmoplast expanded, the Myo8A-GFP marked the phragmoplast midzone and the phragmoplast expanded out to the cell cortex where Myo8A-GFP had accumulated at the cortical division site (Fig. 4.19, C), demonstrating that myosin VIII also marks the future site of cell division in seed plants.
Figure 4. 17. Myo8-GFP marks the future site of cell division.

Images of a protonemal branching cell expressing Myo8A-GFP and mCherry-tubulin were acquired with a spinning disc confocal microscope. Images are maximum intensity projections of z-stacks from a time-series acquisition. Before mitosis, Myo8A-GFP accumulates at the neck region of the emerging cell (top, arrows). During cell division, Myo8A-GFP is also on the mitotic spindle. It accumulates at the spindle midzone (middle, arrow heads) and forms a ring at the leading edge of the phragmoplast that expands out to the cell cortex where it joins with the cortical accumulation (arrows) of Myo8A-GFP that formed prior to mitosis (bottom). In the merge Myo8A-GFP is green and mCherry-tubulin is red. Scale bar, 10 µm.
Figure 4.18. *Myo8A-GFP localizes to dynamic punctate cortical structures on the cell cortex of BY-2 cells.* VAEM image of a single frame from a time-lapse acquisition is shown on the left. On the right is a maximum projection of frames from five seconds of real time. Linear trajectories are readily apparent in the maximum projection. Scale bar, 5 µm.
Figure 4. 19. Moss Myo8A-GFP localizes to PPB, CDS and phragmoplast in tobacco BY-2 cell. (A) Images of a tobacco BY-2 cell in preprophase expressing Myo8A-GFP. Myo8A-GFP accumulates on the preprophase band (arrow heads). n denotes the position of the nucleus. (B) Images of a tobacco BY-2 cell in cytokinesis expressing Myo8A-GFP. Myo8A-GFP remains at the cortical division site (arrow heads) and is found at the midzone of the phragmoplast (arrow). (C) Images from a time series acquisition of a dividing BY-2 cell expressing Myo8A-GFP and stained with FM4-64. FM4-64 incorporates strongly into the new membrane added to the expanding cell plate (asterisk). Myo8A-GFP labels the phragmoplast midzone as well as the cortical division site (arrow heads). In the merge Myo8A-GFP is green and FM4-64 is red. Images were acquired with a spinning disc confocal. In (C) images are from a single focal plane. Scale bars, 10 μm.
Model for phragmoplast steering mediated by actin-myosin motility

Based on these data, I propose the following model for myosin VIII function in phragmoplast guidance (Fig. 4.20). Myosin VIII localizes to microtubule plus ends recruiting it to the cell division machinery. In early mitosis, as microtubules are dynamically searching to make chromosomal attachments, plus ends are distributed throughout the spindle (Bisgrove et al., 2008; Chan et al., 2005). Consistent with this, Myo8A-GFP decorates the entire spindle in early mitosis. At metaphase, Myo8A-GFP concentrates in the spindle midzone. During anaphase, Myo8A-GFP accumulates at the cortical division site. In branching cells, this accumulation occurs prior to prophase (Fig. 4.17) and in BY-2 cells Myo8A-GFP may be recruited to this site by interacting with the preprophase band (Fig. 4.19). Once the phragmoplast assembles, Myo8A-GFP forms a tight ring at the leading edge of the expanding phragmoplast (Fig. 4.10). At this point, the phragmoplast obtains formins (van Gisbergen et al., 2012) and actin filaments polymerize between the leading edge of the phragmoplast and the cell cortex. Myosin VIII at the cortex can hold onto these actin filaments, aligning them to the cortical division site. I propose that myosin VIII on the plus ends of peripheral phragmoplast microtubules moves along these actin filaments from the cortical division site towards the expanding phragmoplast, thereby translocating microtubules and ensuring that phragmoplast expansion occurs along a plane defined by the cortical division site (Fig. 4.20).
Figure 4.20. A model for myosin VIII function in phragmoplast guidance. In prometaphase myosin VIII localizes to plus ends throughout the mitotic spindle. In metaphase myosin VIII accumulates at the spindle midzone and on the poles. During anaphase, myosin VIII accumulates at the cell cortex and is observed on peripheral microtubules searching the cell cortex. As the phragmoplast forms, the midzone myosin VIII accumulation tightens into a thin band on the very leading edge of the phragmoplast. Actin filaments are generated between the phragmoplast and cortical myosin VIII. Peripheral microtubules with plus-end associated myosin VIII translocate on actin filaments and are incorporated into the expanding phragmoplast.
CHAPTER 5

DISCUSSION

My thesis work has provided significant understanding of the roles of class VIII myosins in cell division and tissue morphogenesis. By analyzing the phenotypes of myosin VIII null plants, I showed that myosin VIII is required for protonemal patterning and development. Myosin VIII null plants are smaller and the protonemal tissue is considerably more branched as compared to wild type. It is interesting to note that the protonemal patterning defect is stronger when protoplasts are plated in liquid media instead of embedded in agar. When protoplasts are plated in agar and allowed to regenerate, they are surrounded on all sides by the agar support. Additionally as the plant begins to grow, it grows against the agar cushion, which slows down growth as compared to growth in liquid media (data not shown). Thus, it is possible that the protonemal patterning defect is more evident at faster growth rates.

When exposed to cytokinin, which induces bud formation in mosses, myosin VIII null plants are induced to form more buds than wild type. The increased number of buds might result from the increased branch density in the mutant or from the fact that the myosin VIII null plants are more advanced in development compared to wild type. Analysis of transcript levels shows that the total amount of myosin VIII transcript is reduced by 33% in gametophores, which suggests that myosin VIII transcripts are developmentally regulated. These data are consistent
with the observation that myosin VIII null plants form gametophores earlier in development as compared to wild type.

Consistent with the hypothesis that myosin VIII null plants develop more rapidly than wild type, I observed that many of the myosin VIII mutant plants exhibited senescence in their protonemal tissue one to two weeks earlier than wild type plants (data not shown). Auxin has been shown to accelerate development in mosses (Eklund et al., 2010b) as well as to induce caulonemal formation (Ashton et al., 1979; Cove, 1992; Johri and Desai, 1973). Additionally, cytokinin induces buds (Ashton et al., 1979; Bopp, 1963). Thus, improper patterning of protonemal branching, smaller caulonemal cells, and early gametophore formation and protonemal senescence suggest that myosin VIII mutant plants have defects in hormone homeostasis.

It is unclear how hormones are transported through the plant body in mosses. Using radio-labelled auxin, it has been shown that while there is polar auxin transport in the sporophyte, it was not detected in the gametophores (Fujita et al., 2008). *P. patens* has four genes with high sequence similarity to the polar auxin transporters from seed plants. Based on transcriptome data sets from GENEVESTIGATOR (https://www.genevestigator.com/gv/) all four genes are expressed in all tissue types throughout different developmental stages. With the expression of PIN proteins in protonemal tissue, it is possible that polar auxin transport occurs in protonemata. However, an alternative is that auxin as well as other hormones and small molecules are transported through plasmodesmata.
Previously, two Arabidopsis class VIII myosins, ATM1 and ATM2, were shown to localize to plasmodesmata by transiently expressing their tail domains as GFP fusions in leaf epidermal cells (Reichelt et al., 1999). An antibody raised against ATM1 was used in localization studies at both light microscopy and EM level on cress roots and maize roots, and has shown labeling of plasmodesmata in both cases (Baluska et al., 2004). Based on these localization data, myosin VIII has been an integral part of the proposed plasmodesmata structure for over a decade. However, its functional roles still remain unclear. By imaging a functional, full-length myosin VIII-GFP fusion (Myo8A-GFP) in live cells, I showed that Myo8A-GFP is greatly enriched at the plasmodesmata in sub-apical cells with an emerging side branch, suggesting that myosin VIII might modulate plasmodesmal transport. This transient enrichment of myosin VIII at the plasmodesmata is interesting because it is apparently the first case showing a plasmodesmal protein under temporal and spatial regulation.

A recent report quantified the movement of molecules through plasmodesmata in moss protonemata (Kitagawa and Fujita, 2013). Using a photo-convertible fluorescent protein, Dendra2, the authors showed that the movement of Dendra2 through plasmodesmata is faster toward the apical side of the filament. Since the apical side of the filament is also the younger part of the tissue, this implies that changes in plasmodesmal properties are linked to developmental stages and the age of the cells. It will be interesting to see whether the differences in Dendra2 movement correlate with the accumulation of Myo8A-GFP at the plasmodesmata. It will also be informative to conduct a similar dye-movement assay.
as was developed for the arabidopsis root meristem, which measures plasmodesmal-mediated permeability of small molecules (Rutschow et al., 2011). I hypothesize that the transient accumulation of myosin VIII alters the size exclusion limit of plasmodesmata causing a change in local hormone concentration, thereby affecting regulation of hormone responsive genes and consequentially promoting side-branch formation and cell division.

A similar hypothesis was proposed for the regulation of AP2-type transcription factors in moss (Aoyama et al., 2012). AP2-type transcription factors control the fate of the side branches, determining whether a cell will become a protonemal apical cell or a gametophore apical cell. A protonemal apical cell becomes a branch. A gametophore apical cell becomes a bud and develops into a leafy gametophore. Plants lacking all AP2-type transcription factors do not form buds. AP2-like transcription factors are expressed in the gametophore apical cell but not in the protonemal apical cell. Interestingly, expression of AP2-like transcription factors is induced by auxin but not cytokinin. The authors propose that a local decrease in auxin concentration reduces AP2-like transcription factor expression in branches and promotes protonemal apical cell formation. Local changes in hormone concentration could result from active transporters or diffusion through plasmodesmata. If myosin VIII-mediated gating of plasmodesmata affects hormone levels in the branching cell, then it is possible that expression of AP-2 like transcription factors is affected in the myosin VIII null plants, since they develop gametophores earlier and form more gametophores than wild-type plants. It will
also be interesting to see whether the change in expression of AP2-like transcription factors coincides with Myo8A-GFP accumulation at the plasmodesmata.

While improper hormone homeostasis may cause many of the phenotypes observed in myosin VIII null plants, it is also possible that these patterning abnormalities stem from other defects. Myosin VIII has been implicated in cell division and endocytosis (Golomb et al., 2008; Sattarzadeh et al., 2008). Smaller plants and cell size might result from slower rates of endocytosis, required for remodeling of the plasma membrane during growth and cell division. I found Myo8A-GFP localized to dynamic spots moving on linear tracks at the cell cortex, in agreement with a potential role in endocytosis. Truncation constructs of ATM1 and ATM2 were shown to colocalize with endosomal compartments, some of which were brefeldin A sensitive (Sattarzadeh et al., 2008). Although I did not notice any intracellular compartments labeled with Myo8A-GFP in the moss, it will be interesting to see whether brefeldin A treatment promotes the formation of Myo8A-GFP positive compartments in moss cells, and determine whether full-length Myo8A-GFP colocalizes with other endosomal markers. If so, it would be worth investigating whether the formation of these compartments is actin dependent, and if these compartments are different in myosin VIII null plants.

Although a role in endocytosis is implied, my study does not demonstrate a functional role of myosin VIII at the cell cortex. Since Myo8A-GFP is often found to be enriched at the apical region of the plasma membrane, another possibility is that myosin VIII is part of the machinery that establishes cell polarity. While myosin VIII is most likely not involved in establishing polarity in apical cells, it may be involved
in determining where a new protonemal filament emerges during branching. In wild
type plants, the new side branches almost always emerge right beside the anterior
cross wall of the mother cell. This suggests there is an established polarity in the
sub-apical cell, despite the lack of active tip growth. However in myosin VIII null
plants, side branches often emerge further away from the apical-most walls of the
mother cells, suggesting the loss of polarity. To test this hypothesis, it would be
informative to compare the localization of cell polarity markers in wild type and in
myosin null plants. A likely candidate is the small GTPase ROP. However, no
functional ROP-GFP fusion is available so far. To investigate this question, ROP will
need to be localized by immunofluorescence, or alternatively another cell polarity
marker will need to be identified. I hypothesize that ROP or the hypothetical cell
polarity marker will mark the sites of side-branch formation before and while the
protrusion is growing, and their localization might be different in myosin null plants
due to improper polarity establishment.

Using variable angle epifluorescence microscopy (VAEM), I observed Myo8A-
GFP moving along actin filaments at the cell cortex, as expected for an actin-based
motor. Surprisingly, the cortical localization of Myo8A-GFP does not exclusively
depend on actin, suggesting that Myo8A is targeted to the cell cortex independent of
actin. Interestingly, I also observed Myo8A-GFP localize to the cortical microtubules.
Whether Myo8A directly interacts with cortical microtubules or is binding to
microtubule-associated proteins remains to be determined. Preliminary data
suggests that in the absence of both actin and microtubule cytoskeletons, Myo8A-
GFP still localizes to the cell cortex, although with greatly reduced density. This
needs to be repeated before a more definite conclusion can be made. However it suggests that Myo8A-GFP can bind the cell cortex independent of either actin or microtubules. To determine how Myo8A-GFP targets the cell cortex, it may be informative to perform a yeast two-hybrid screen or protein pull-down assays to identify Myo8A interactors.

The most striking localization of Myo8A-GFP is found during mitosis and cytokinesis. Myo8A-GFP localizes to the mitotic spindle, often enriched in the midzone and at the poles. Prior to anaphase, a small population of Myo8A-GFP accumulates at the cortex near the midzone of the spindle. During anaphase Myo8A-GFP forms a tight band at the midzone, which transitions into a ring on the leading edge of the phragmoplast. As the phragmoplast expands, it joins the cortical Myo8A-GFP population. Careful inspection of this population of Myo8A-GFP revealed that Myo8A-GFP associates with the plus end of the microtubule. In the phragmoplast midzone area, microtubules with Myo8A-GFP residing on their plus ends were found to join with their counterparts originated from the other half of the phragmoplast, also with Myo8A-GFP on their plus ends. Peripheral phragmoplast microtubules also have Myo8-GFP on their ends as they grow toward the cell cortex. Association of Myo8A-GFP with the microtubules is not actin dependent, but is likely dependent on other microtubule plus-end binding proteins. In support of this, the N-terminus of Myo8A protein sequence has a putative EB1-binding motif, suggesting a possible mechanism for Myo8A recruitment to the plus ends of microtubules.

Myosin VIII localization during mitosis is not actin-dependent. In fact actin filaments don’t even start to accumulate above background until late-anaphase and
become prominent in the phragmoplast. During phragmoplast expansion, I found actin filaments present between the leading edge of the phragmoplast and the cell cortex. Based on these localization studies, I proposed that myosin VIII arrives on the microtubules early on during mitosis, and gradually concentrates on the microtubule plus ends when the spindle turns into a phragmoplast, potentially through its interaction with EB1. At this point, membrane-bound formins arrive at the newly forming cell plate (van Gisbergen et al., 2012) and actin filaments polymerizing around the phragmoplast midzone area. Myosin VIII on the microtubule plus ends and at the cell cortex could hold on to these actin filaments and maintain an actin network across the phragmoplast midzone. Myosin VIII could also translocate microtubules along these actin filaments to guide phragmoplast expansion.

In agreement with this hypothesis, I found that, in myosin VIII null plants, the phragmoplast microtubules often appear less organized and the cell plate membrane appears less smooth. I observed similar defects in wild type cells treated with latrunculin B. This is also consistent with observations in tobacco BY-2 cells where treatment with actin inhibitors results in wrinkled cell plates (Hoshino et al., 2003; Yoneda et al., 2004; Sano et al., 2005; Higaki et al., 2008; Kojo et al., 2013).

Observations of microtubule behaviors in the phragmoplast also support this hypothesis. Free microtubule plus ends at the phragmoplasts periphery often make brief contact with the cell cortex, and are quickly focused at the cell cortex around the phragmoplast midzone region. In the absence of actin filaments, these peripheral microtubules tend to wander at the cell cortex, and microtubule plus
ends often cross over the phragmoplast midzone. Microtubules in the phragmoplasts of myosin VIII null plants show similar behaviors as in latrunculin B treated wild type cells.

There is precedent for myosin-based motility translocating microtubules on actin filaments in other systems. In the budding yeast, \textit{Saccharomyces cerevisiae}, the class V myosin, Myo2p, binds Kar9p, which localizes to cytoplasmic microtubule plus ends by binding the yeast EB1 homolog, Bim1p (Beach et al., 2000). These cytoplasmic microtubules emanate from the spindle pole body embedded in the nuclear envelope and Myo2p mediates their motility along actin cables directed into the bud, moving the nucleus toward the bud neck (Yin et al., 2000; Beach et al., 2000). I imagine a similar mechanism could be at work in plant cells, whereby myosin VIII associates with microtubule plus ends, and subsequently translocates microtubules on actin filaments to guide phragmoplast expansion.

During cytokinesis in seed plants, the phragmoplast expands out to the cortical division site, which is defined by the preprophase band before mitosis. The preprophase band comprises microtubules, actin filaments, and several molecular markers, including TANGLED and RanGAP (Walker et al., 2007; Xu et al., 2008). These microtubules disassemble before mitosis but TANGLED and RanGAP are left behind marking the division site. Moss protonemata do not form a preprophase band but still follow an organized cell division pattern (Doonan et al., 1985), suggesting there is a mechanism for cell division plane specification without forming a preprophase band. Moss protonemata form a two-dimensional filamentous network that is single cell-layer thick. The tissue organization is not
complex, and misoriented cell division planes probably have little consequence in overall development at the protonemal stage. Cell division occurring at the apical cell simply divides the cell in half, with the new cell plate perpendicular to the longitudinal axis in chloronemal cells, or with a 40° angle in caulonemal cells. The cells are narrow, with diameters not much greater than the sizes of the nucleus and the spindle, leaving little room for those two structures to be misplaced. A greater need for cell division plane specification arguably occurs during side-branch formation. Side-branch formation involves an asymmetric cell division in an L-shaped cell. The nucleus migrates toward the junction of the mother cell and the branch and the spindle is oriented along the longitudinal axis of the new emerging cell. The phragmoplast builds the shortest cell plate possible separating the two cells.

In an emerging branch cell, I often observed a dotted ring of Myo8A-GFP accumulated at the cell cortex before mitosis. This ring coincides with the future site of cell division. Myosin VIII remains at the cell cortex throughout mitosis and cytokinesis. Myo8A-GFP at the leading edge of the phragmoplast joins with the ring at the cell cortex. In the absence of myosin VIII, I observed a number of defects in branching cells. Sometimes the nucleus does not migrate close to the branch site and mitosis and cytokinesis proceed in the middle of the subapical cell. More often, the nucleus moves partway to the branch site, but cell plate positioning does not occur at the branch junction. Other times the nucleus migrates to the proper position and cell division proceeds normally. The stochastic nature of these defects suggests that the cortical myosin VIII population contributes to nuclear positioning during
branching but is not required for nuclear movement. If the nucleus moves to the appropriate site and the mitotic spindle is positioned along the long axis of the branching cell at the branch junction, then the cell plate will be positioned properly. However, since there are more possible ways to orient the spindle in a branching cell, in the absence of an active guidance mechanism, it is more likely to observe defects in cell plate positioning. Consistent with this, in the absence of myosin VIII cell plates are often aberrantly positioned in the branching cell.

Positioning the plane of cell division becomes even more important in the moss leafy shoot, or gametophore. Importantly, cells in the gametophore do form preprophase bands. In fact, deletion of TON1, a conserved plant protein critical for formation of the band (Azimzadeh et al., 2008), has severe defects in cell division plane orientation and cell elongation in the gametophore (Spinner et al., 2010). However, protonemal cells, which do not form the band, are not evidently affected in TON1 null plants (Spinner et al., 2010). In comparison, there are few noticeable defects in the gametophores of myosin VIII null plants (Wu et al., 2011), suggesting that myosin VIII is not essential for preprophase band formation. I attempted to image microtubules and myosin VIII in living gametophores. However, identifying dividing cells in gametophores was challenging. It is possible that the imaging conditions prevented cells form entering mitosis as preprophase bands have previously only been imaged in fixed tissue (Spinner et al., 2010; Doonan et al., 1987). To circumvent these technical challenges, I turned to investigating myosin VIII localization in tobacco BY-2 cells, a key model for studies of mitosis and cytokinesis in plants.
Strikingly, in BY-2 cells, I observed that moss Myo8A-GFP localizes to the preprophase band, cortical division site, and the phragmoplast midzone, suggesting that a myosin VIII-mediated phragmoplast-guidance mechanism also exists in seed plants. The discovery that Myo8A-GFP localizes to the cortical division site provides us a portal to connect my observations in moss to the current model of cell division in seed plants (Rasmussen et al., 2011; 2013; Van Damme, 2009). In seed plants, cells with preprophase bands use a microtubule dependent mechanism to position the nucleus and orient the nascent spindle such that it is perpendicular to the future plane of division (Venverloo and Libbenga, 1987; Mineyuki and Furuya, 1986; Katsuta et al., 1990). Once the spindle is properly aligned, subtle defects in phragmoplast guidance would not greatly alter cell plate positioning and subsequent tissue morphogenesis. Thus, I hypothesize that myosin VIII and actin participate in phragmoplast guidance in cells with preprophase bands as well as moss protonemata. Consistent with this, when BY-2 cells are treated with actin inhibitors, phragmoplasts are disorganized generating wrinkled cell plates that are often skewed with respect to the cortical division site (Hoshino et al., 2003; Yoneda et al., 2004; Sano et al., 2005; Higaki et al., 2008; Kojo et al., 2013).

My data from moss and tobacco BY-2 cells suggest that myosin VIII and actin guide phragmoplast expansion during cytokinesis. In fact, myosin VIII is the first protein known to date that physically links phragmoplast microtubules to the cortical division site via actin filaments, and its motor activity along actin provides a molecular mechanism for steering phragmoplast expansion. Based on these findings, I propose that plant cells could use a mechanism reminiscent of myosin V
mediated motility of microtubules in yeast, suggesting that actin-based microtubule translocation is a more universal mechanism possibly having evolved early in the eukaryotic lineage.

If myosin VIII links the microtubule and actin cytoskeletons, it will be interesting to investigate whether myosin VIII motor activity is regulated by microtubules or microtubule associated proteins, and whether its association with microtubules is regulated by the presence or absence of actin filaments.

Interestingly I have observed that microtubule plus-end associated Myo8A-GFP in the phragmoplast is brighter in latrunculin B treated cells. This suggests that in the absence of actin, myosin VIII is unable to dissociate from microtubule plus ends. To investigate the molecular basis of these observations, it will be informative to analyze the biochemical properties of myosin VIII, particularly with respect to its motor activity under different conditions and its binding affinities with a variety of interacting partners. It will also be informative to investigate whether other known proteins that localize to preprophase band and cortical division site interact with Myo8A-GFP. Proteins like TANGLED and RanGFP localize to the preprophase band and cortical division site and are important for cell division plane establishment.

Homologs of TANGLED and RanGAP are present in moss. Data from GENEVESTIGATOR suggest they are expressed in protonemata. It will be interesting to investigate their localization in moss and their relationships with Myo8A-GFP during cell division. One interesting experiment will be to silence TANGLED and RanGAP in moss and see whether Myo8A-GFP mislocalizes, and vice versa.
In conclusion, my study has contributed to our understanding of the function of actin and myosin in plant cell division. Actin has been known to be present in the phragmoplast for over two decades (Clayton and Lloyd, 1985; Kakimoto and Shibaoka, 1987). However, how actin contributes to phragmoplast functions remains unclear. Now with my study of myosin VIII function, I have provided genetic evidence for a significant role of actin and myosin in phragmoplast guidance. Also interesting is the role of myosin VIII as a link between actin and microtubule cytoskeleton, and its function in plamodesmata. I believe this study has been fruitful and has opened the door to many interesting hypothesis and future research.
CHAPTER 6

MATERIAL AND METHODS

Real-Time RT-PCR analysis of myosin VIII relative expression level

Total RNA was extracted from 7 to 9-day-old protonemal tissue or gametophores isolated from 2-month-old plants that had been regenerated from protoplasts using the RNeasy plant mini kit (Qiagen), followed by DNase I treatment according to the manufacturer’s protocol. cDNA was synthesized from total RNA using SuperScript III reverse transcriptase (Invitrogen) and oligo (dT) according to the manufacturer’s protocol. All real-time PCR reactions used 4 ng of cDNA template in a 12.5 μL reaction using the Brilliant II SYBR GREEN QPCR Master Mix (Stratagene). The PCR conditions were as follows: 95°C for 10 min, followed by 50 cycles of 95°C for 30 s, and 60°C for 1 min. The primer sets were designed to have similar amplification efficiency and are listed in Table 6.1. The ubiquitin 10 gene was used as an internal control for all reactions. Real-time PCR reactions were performed in an Eppendorf Mastercycler ep Realplex2 thermal cycler, and data were analyzed with the Realplex software 2.2.

PCR amplification of myosin VIII insertion alleles

To verify that the myo8C and myo8D loci were disrupted after treatment with CRE recombinase, the genomic region surrounding the insertion site was amplified by PCR from genomic DNA isolated from ∆myo8ABCDE using primers as listed in Table 6.1. To determine whether the 5’ and 3’ insertion sites were properly altered in myo8A, myo8B and myo8E loci, PCR amplification of genomic DNA from
Δmyo8ABCDE was performed using a primer outside and a primer inside the knockout construct (See Figure 3.4 and primers listed in Table 6.1).

**RT-PCR amplification of myosin VIII transcripts**

Total RNA was extracted from wild type and Δmyo8ABCDE tissue as described above. cDNA was synthesized with SuperScript III reverse transcriptase using a pool of reverse primers (Table 6.1, reverse primers for expression analysis). For myo8A, cDNA was similarly synthesized but using only the myo8A-specific reverse primer (Table 6.1). 2 μL of cDNA was used as template in a 50 μL PCR reaction. Phusion (NEB) was the polymerase used for the PCR reaction. Transcripts from the region around the insertion sites were amplified using primers listed in Table 6.1. The PCR conditions were as follows: 98°C for 30 s, followed by 35 (25 for myo8A) cycles of 98°C for 10 s, 60°C for 15 s, and 72 °C for 1 min, and then 72 °C for 5 min.

**Moss medium**

**PpNH₄ medium:** 1.03 mM MgSO₄, 1.86 mM KH₂PO₄, 3.3 mM Ca(NO₃)₂, 2.7 mM (NH₄)₂-tartrate, 45 μM FeSO₄, 9.93 μM H₃BO₃, 220 nM CuSO₄, 1.966 μM MnCl₂, 231 nM CoCl₂, 191 nM ZnSO₄, 169 nM KI, 103 nM Na₂MoO₄

**PpNO₃ medium:** 1.03 mM MgSO₄, 1.86 mM KH₂PO₄, 3.3 mM Ca(NO₃)₂, 45 μM FeSO₄, 9.93 μM H₃BO₃, 220 nM CuSO₄, 1.966 μM MnCl₂, 231 nM CoCl₂, 191 nM ZnSO₄, 169 nM KI, 103 nM Na₂MoO₄

**PRMB:** PpNH4 medium supplemented with 6% Mannitol, 10 mM CaCl₂ and 0.8% agar.

108
Plating Medium: PpNH$_4$ medium supplemented with 8.5% Mannitol and 10 mM CaCl$_2$.

Top Agar: PpNH$_4$ medium supplemented with 6% Mannitol, 10 mM CaCl$_2$ and 0.6% agar.

3M solution: 9.1% Mannitol, 15 µM MgCl$_2$·6H$_2$O and 0.1% MES pH 5.6

PEG solution: 8.5% Mannitol, 100mM Ca(NO$_3$)$_2$, 10mM Tris pH 8.0

PEG 8000 (Sigma P-2139)

Plant materials and growth conditions

All moss tissue culture, protoplasting and transformation were performed as described previously (van Gisbergen et al., 2012). Moss protonemal tissues were propagated weekly on PpNH$_4$ containing 0.7% agar overlay with cellophane. For imaging, one-week-old protonemal tissues were protoplasted, plated in plating medium in a density of ~20,000 cells/ 9 cm$^2$ plate, regenerated on protoplast regeneration medium PRMB for four days and transferred to PpNH$_4$ plates. For moss transformation, at least 30 µg of plasmid DNA was linearized, ethanol precipitated, and dissolved in sterile TE buffer. pTKUbi constructs were linearized with Pme I and pTZUbi constructs were linearized with Swa I. Protoplasts were isolated from 7-day-old moss protonemal tissue, and transformed with linearized DNA via PEG-mediated transformation (van Gisbergen et al., 2012; Wu et al., 2011). Protoplasts were plated in top agar, regenerated on PRM for 4 days and transferred to PpNH$_4$ medium containing the appropriate antibiotics (G418, 20 µg/ml; zeocin, 50 µg/ml). To select for stable transformants, transformations were cycled on and
off antibiotic selection for three one-week intervals. Transgenic lines were visually screened on a confocal microscope for expression of the transgene.

pTKUbi-mEGFP-Tub was transformed into WT and Δmyo8ABCDE (Wu et al., 2011) generating the mEGFP-Tub lines. pTKUbi-Myo8A-3mEGFP was transformed into Δmyo8ABCDE (Wu et al., 2011) generating the Myo8A-GFP line. Myo8A-GFP was subsequently transformed with pTZUbi-mCherry-Tubulin, pTZUbi-Lifeact-mCherry and pTZUbi-ER-mCherry to generate Myo8A-GFP/mCherry-tub, Myo8A-GFP/Lifeact-mCherry and Myo8A-GFP/ER-mCherry moss lines.

Growth chamber condition: 16 hours light 8 hours dark at 25°C.

**Tobacco BY-2 tissue culture**

Tobacco BY-2 tissue culture and Agrobacterium-mediated transformation were performed as previously described (Murata et al., 2013). Tobacco BY-2 cell are subculture weekly into new standard BY-2 medium containing 4.3 g/L Maurashige and Skoog basal salts (Sigma, M5524-10L), 30 g/L sucrose, 0.2 g/L KH₂PO₄, 0.1 g/L inositol, 1 mg/L thiamine, and 0.2 mg/L 2,4-D. The pH was adjusted by the addition of KOH to 5.7. Cell culture were grown in 125 mL flask containing 30 mL of medium under constant shaking (120 rpm) at 26 °C.

**Agrobacterium-mediated transformation of tobacco BY-2 cell**

Tobacco BY-2 cells were transformed with pMDC32-Myo8A-3XmEGFP. The GV3101 *Agrobacterium tumefaciens* strain was used for infection.

One day before transformation, inoculate Agrobacterium (GV3101, carrying pMDC32-Myo8A-3XmEGFP plasmid) into 1 mL of LB containing 10µg/mL rifamycin,
30\mu g/mL gentamicin, and 50 \mu g/mL kanamycin. Cultured for 18-24 hours at 30°C with shaking. For transformation, mix 4 mL of 3-day-old BY-2 cell culture with 100 
\mu L of Agrobacterium in an empty petri dish. Spread the mixture well, seal the petri dish with micropore tape. Do not use parafilm. Incubate the mixture at 26°C for 42-48 hours in the dark. Make sure plates do not dry out. Scrape the cells into a 15 mL falcon tube. Add 5 mL of sterilized 3% sucrose solution to the petri dish to recover all the cells. Cetrifuge at 1000 rpm, remove supernatant, resuspend the cell pellet in 5 mL of 3% sucrose solution with pipette blow (10 times). Repeat this step 3 times. Add 4 volumes of 3% sucrose solution to 1 column of BY-2 cell. Spread 1 mL of mixture onto 1 BY-2 medium agar plate containing antibiotics (500 \mu g/mL carbenicilin and 30 \mu g/mL hygromycin). Seal the plates with micropore tape (double layers) and incubate at 26°C in the dark. After two weeks, the transformed cell grow into small colonies. Pick colonies into new medium plates with antibiotic selection.

**Phenotypic analysis, cell wall staining, and imaging.**

All plants used for phenotypic analyses, confocal microscopy and VAEM were regenerated from protoplasts. One-week-old protonemal tissue was protoplasted and regenerated on PRMB for 4 days and transferred to PpNH4 medium. For measurement of cell plate angle and apical cell length, plants regenerated from protoplasts were stained with 0.1 mg ml^{-1} calcofluor solution and visualized by epifluorescence microscopy (Leica MZ16FA) using a UV filter (excitation 360/40 nm, emission 420 long pass) or a Violet filter (excitation 425/40 nm, emission 400
long pass). Cell plate angles and apical cell lengths were measured manually using Image J software.

**Sample preparation for confocal and VAEM imaging**

For imaging cell division, 5- to 8-day-old plants regenerated from protoplasts were placed unto an agar pad in Hoagland’s (4 mM KNO3, 2 mM KH2PO4, 1 mM Ca(NO3)2, 89 µM Fe citrate, 300 µM MgSO4, 9.93 µM H3BO3, 220 nM CuSO4, 1.966 µM MnCl2, 231 nM CoCl2, 191 nM ZnSO4, 169 nM KI, 103 nM Na2MoO4, and 1% sucrose), covered by a glass cover slip and sealed with VALAP (1:1:1 parts of Vaseline, lanoline, and paraffin) (Vidali et al., 2009b). For cell plate staining, 15µM FM4-64 was added in the Hoagland’s solution in the agar pad. For latrunculin B treatment, plants were transferred to PpNH4 medium containing 25 µM latrunculin B for two hours, then transferred to slides containing 25 µM latrunculin B in both the agar pad and the Hoagland’s solution. For plasmolyzed cells, 20 % sucrose instead of Hoagland’s solution was added in the agar pad.

**Variable angle epifluorescence microscopy (VAEM)**

Samples were mounted on an inverted microscope (model Ti-E; Nikon) equipped with a mirror-based T-FL-TIRF illuminator (Nikon) and imaged with a 1.49 NA 100x oil immersion TIRF objective (Nikon). The 1.5x optivar was used for all images to increase magnification. The laser illumination angle was adjusted individually for each sample to achieve the maximum signal-to-noise ratio. Signals were captured with a 1024 × 1024 electron-multiplying CCD camera (iXON3; Andor
Technology) equipped with a dual-view optosplit adaptor. Image acquisition process was controlled by NIS-Elements AR 3.2 software (Nikon).

**Spinning-disc confocal imaging**

The slides were mounted unto an inverted microscope (model Ti-E; Nikon) equipped with a Yokogawa spinning disk head (model CSU-X1) and a 512 × 512 electron multiplying CCD camera (ixON; Andor Technology). Images were collected with a 1.4 NA 100x oil immersion objective (Nikon) at room temperature. Image acquisition process was controlled by MetaMorph software (Molecular Devices).

**Laser scanning confocal microscopy**

Images were acquired on a Nikon A1R confocal microscope system with a 1.4 NA 100x oil immersion objective (Nikon) at room temperature. Image acquisition process was controlled by NIS-Elements software (Nikon).

**Image processing**

All images were minimally processed using Image J software with background subtraction and enhanced contrast. Occasionally we also performed smoothing or applying unsharp mask filter. All settings were the standard Image J settings.

**Plasmid Construction**

All expression constructs were constructed using Multisite Gateway recombination (Invitrogen). All primers used are listed in Table 6.1. Generation of entry clones 3XmEGFP-L5L2 (Vidali et al., 2009b), Lifeact-L1R5 (Vidali et al., 2009a)
and mCherry-L5L2 (van Gisbergen et al., 2012) were described previously. To construct the entry clone Myo8A-L1R5, total RNA was extracted from 7-day-old moss protonemal tissues using RNeasy plant mini kit (Qiagen), followed by DNase I treatment according to the manufacturer’s protocol. cDNA was synthesized from total RNA using SuperScript II reverse transcriptase (Invitrogen) and oligo (dT) according to manufacturer’s protocol. Full length Myo8A coding sequence was amplified from moss cDNA using Myo8A specific primers (P1&P2), and cloned into pGEM-T easy (Promega). The full-length Myo8A coding sequence was then amplified from the pGEM-Myo8A clone using primers (P3 & P4) containing attB1 and attB5r sites, and cloned into pDNOR221-P1-P5r with a BP reaction (Invitrogen). The mCherry and mEGFP coding sequence was amplified using primers (P5 & P6) with attB1 and attB5r sites and cloned into pDNOR221-P1-P5r to generate entry clone mCherry-L1R5 and mEGFP-L1R5. The mCherry coding sequence was amplified using primers (P9&P10) with attB5 and attB2 sites and cloned into pDONR221-P5-P2 to generate entry clone mCherry-KDEL-L5L2. Moss alpha-tubulin coding sequence was amplified from pAct-GFP-TUA1 (Hiwatashi et al., 2008) with primers (P7 & P8) containing attB5 and attB2 sites and cloned into pDNOR221-P5-P2 to generate entry clone Tubulin-L5L2.

Combinations of entry clones were assembled with destination vectors generating constructs for stable expression in moss or tobacco BY-2 cells using LR clonase II plus reactions (Invitrogen) as follows: Myo8A-L1R5 and 3XmEGFP-L5L2 with pTKUbi-gate generating pTKUbi-Myo8A-3mEGFP; Lifeact-L1R5 and mCherry-L5L2 with pTZUbi-gate generating pTZUbi-Lifeact-mCherry; TUA1-L5L2 and
mCherry-L1R5 with pTZUbi-gate generating pTZUbi-mCherry-Tubulin; TUA1-L5L2 and mEGFP-L1R5 with pTKUbi-gate generating pTKUbi-mEGFP-Tubulin; SP-L1R5 and mCherry-KDEL-L5L2 with pTZUbi-gate generating pTZUbi-ER-mCherry; Myo8A-L1R5 and 3XmEGFP-L5L2 with pMDC32 (Karimi et al., 2007) generating pMDC32-Myo8A-3XmEGFP.

The pTKUbi-gate vector has an expression cassette derived from pTHUbi-Gate (Vidali et al., 2007), which contains the maize ubiquitin promoter, Gateway cassette and NOS terminator. Following this expression cassette is a 35S::NptII::ter cassette flanked by lox sites (Wu et al., 2011). The expression and antibiotic resistance cassettes are flanked by moss genomic sequence from the Pp1s249_67V6.1 locus. Nucleotides -2 to -1153 and nucleotides 660 to 1757 are on the 5’ and 3’ ends, respectively, with PmeI sites incorporated such that digestion with PmeI releases the moss genomic DNA targeting arms as well as the expression and resistance cassettes. The pTZUbi-gate vector is similar except that it contains a 35S::Zeo::ter cassette flanked by lox sites (Wu et al., 2011) as the antibiotic resistance cassette, uses moss genomic sequence from the Pp1s141_25V6.1 locus (nucleotides +908 to +2021 and -35 to -1532), and has Swal for release of the moss genomic DNA targeting arms and expression and resistance cassettes.

**Generating RNAi constructs from moss cDNA collection**

Moss cDNA was in pTFH22.4 plasmid. To generate PCR product with attB1 and attB2 site, plasmid containing moss cDNA was diluted 30 times in water and 1 µL was used as PCR template in a 20µL PCR reaction. Primers attB1-pTFH-F and
attB2-pTFH-R were used and Phusion was the DNA polymerase. PCR reactions were set up following the manufacturer's instruction. PCR condition was as followed: 98°C for 30 sec, followed by 35 cycles of 98°C for 10 sec, 58°C for 30 sec and 72°C for 2 min, with final extension at 72°C for 5 min.

After PCR, 2 µL of PCR products were run on a gel to make sure the PCR reactions were successful. Remaining PCR products were diluted by adding 50 µL TE buffer. To set up BP reaction, 1.5 µL diluted PCR product, 0.5 µL pDONR-Spec plasmid (75 ng/µL) and 0.5µL BP clonase II were mixed and incubated at room temperature overnight.

The whole BP reaction was transformed into 20 µL of DH5α competent cell. The mixture was incubated on ice for 30 min, heat-shock at 42°C for 1 min, put back on ice for 1min, and the whole mixture was transferred to a culture tube containing 1 mL of LB. After shaking at 37°C for 1 hour, another 1 mL of LB containing 200 µg/mL of Spectinomycin was added and the culture was incubated in 37°C shaker for overnight. Plasmid was extract from the culture the next day, and digested with Apal to confirm the existence of cDNA fragment. Plasmids were diluted to ~ 50 ng/µL. Proper clones were further cloned into pUGGi plasmid using a LR clonase reaction to generate RNAi constructs.

To set up LR reaction, 1 µL of diluted entry clone, 0.5 µL of pUGGi plasmid (75 ng/µL), 0.5 µL TE buffer, and 0.5 µL LR clonase II enzyme were mixed and incubated at room temperature overnight. The whole LR reaction was transformed into 20 µL of DH5α competent cell. The mixture was incubated on ice for 30 min,
heat-shock at 42°C for 1 min, put back on ice for 1 min, and the whole mixture was transferred to a culture tube containing 3 mL of LB. After shaking at 37°C for 1 hour, another 3 mL of LB containing 200 µg/mL of carbenicillin was added and the culture was incubated in 37°C shaker for overnight. Plasmid was extract from the culture and can be transformed into moss protoplast for RNAi assay.

**Semi-automatic protoplast transformation using EpMotion5070**

**Equipment**

Eppendorf centrifuge 5804 and rotor A-2-DWP

EpMotion 5070 equipped with 1-mL and 50 µL eight-channel dispensing tools, placed inside a tissue culture hood.

Module racks for 30-mL reservoirs connected to a heating element and a thermocouple to measure temperature. The element is connected to a temperature-control unit that measures the temperature of the block. (Figure 6.1)

Thermoadapter for 96-well PCR plate connected to a heating element and a thermocouple to measure temperature. The element is connected to a temperature-control unit that measures the temperature of the block. (Figure 6.1)

Deepwell plate 96/1000 µL (Eppendorf Cat no. 951032603)

Deepwell Mat 96 (1.2 mL) (Eppendorf Cat no. 951030121)

twin-tec PCR plate 96, skirted (Eppendorf Cat no. 951020401)

Reservoir Rack (Eppendorf Cat no. 960002148)

epMotion reservoirs 30 mL, 100 mL (Eppendorf Cat no. 960051009 )
T.I.P.S. Motion 40 μL-1000 μL (Eppendorf Cat no. 960050088)

T.I.P.S. Motion 1 μL-50 μL (Eppendorf Cat no. 960050002)

1 Well Dish Non-Treated Sterile with Lid, 127.8 x 85.5 mm (nunc 267060)

**Preparation before transformation**

1. Autoclave deep-well plates, deep-well mat, PCR plates, and reservoirs.

2. Prepare PRMB plates: Pour 40 mL of PRMB in each 8x12 cm 1-well dish and let the agar solidify. (Note: make sure the surface of the agar plate is level and the height of the agar medium is uniform for each plate.)

3. Cut cellophane into 8x12 cm rectangular shape. Autoclave cellophane in a glass petri-dish containing water.

4. In the tissue culture hood, carefully overlay the surface of each PRMB plate with a piece of wet cellophane. Make sure there is no wrinkle or air bubble. Let the plates dry in the tissue culture hood till there is no visible water flowing on the surface of the cellophane.

**Transformation**

1. Prepare PEG: Weigh out 4 g of PEG 8000 into 50 mL conical tube, melt in microwave, watch carefully while melting. In sterile hood, add 10 mL of PEG solution into the same conical tube, mix well by vortex. Solution can be used after two hours or can be stored at -20°C for long term storage.

2. Aliquot plasmid DNA into 96-well deep-well plate. Each well holds an independent transformation. (Note: The volume of DNA should be less than 15 μL. Each transformation requires 2-3 μg of plasmid DNA. Make mini-prep
from 5 mL of overnight bacterial culture and elute in 40 μL. One mini-prep is generally enough for three transformations.)

3. Generate protoplasts as previously described. Resuspend protoplasts in 3M solution at a density of 2X10^6 cells/mL

**On EpMotion5070:** (Note: All methods described here are based on EpMotion 5070 program setting. All parameters are optimized to handle protoplasts and other transformation reagents.)

4. Put protoplasts in a 30 mL reservoir, aliquot 50 μL into each well in the 96-well deep-well plate with 1-mL eight-channel pipette.

   **EpMotion Command: Reagent Transfer**
   Pipet Tool: TM_1000_8
   Filter Tips: No
   Volume: 50 μl
   Transfer Type: Pipette
   Source: Tubs_1 (30 mL reservoir)
   Destination: DWP 96_1
   Liquid Type: Glycerol
   Dosing Parameter:
   |-- Speed Aspiration: 10000 mm/sec
   |-- Speed Dispense: 20000 mm/sec
   |-- Delay Blow: 150 ms
   |-- Speed Blow: 66000 mm/sec
   |-- Movement Blow: 90% of max movement
   |-- Prewetting: 0 Cycles
   Change Tips before each aspiration
   Change Tips after 0 aspirations
   Mix Before No
   Mix After No
   Special Aspirate from bottom

5. Put PEG solution in a 30 mL reservoir, aliquot 50 μL into each well in the 96-well deep-well plate with 1-mL eight-channel pipette. Mix by pipetting up and down.

   **EpMotion Command: Reagent Transfer**
6. Put the deep-well mat on the deep-well plate. Let the plate stand at room temperature for 10 min.

**Out of the hood: make sure the lid on the deep-well plate is tightly sealed.**

7. Incubate the deep-well plate in 45°C water bath for 3 min. (Note: the deep-well plate float on water easily.)

8. Incubate the deep-well plate in room temperature water bath for 10 min.

**On EpMotion5070:**

9. Put Plating Medium supplemented with 10 mM CaCl$_2$ in a 100-mL reservoir.

Aliquot 900 μL into each well in the 96-well deep-well plate with 1-mL eight-channel pipette. Mix by pipetting up and down.

**EpMotion Command: Reagent Transfer**

Pipet Tool: TM_1000_8
10. Let the plate stand at room temperature for 30 min.

**Out of the hood: make sure the lid on the deep-well plate is tightly sealed.**

11. Spin down protoplasts at 250Xg for 7 min.

**On EpMotion5070:**

12. Take out 900 μL of the supernatant from each well in the 96-well deep-well plate with 1-mL eight-channel pipette.

EpMotion Command: Pool to One Destination
- Pipet Tool: TM_1000_8
- Filter Tips: No
- Volume: 900 μl
- Transfer Type: Pipette
- Source: DWP 96_1
- Destination: Tubs_1 (100 mL reservoir)
- Liquid Type: Speed_xs
- Dosing Parameter: default
- Change Tips before each aspiration
- Change Tips after 0 aspirations
- Mix Before No
Mix After No

13. Resuspend protoplasts and transfer 75 µL to a 96-well PCR plate with 1-mL eight-channel pipette.

   EpMotion Command: Sample Transfer
   Pipet Tool: TM_1000_8
   Filter Tips: No
   Volume: 75 µl
   Transfer Type: Pipette
   Source: DWP 96_1
   Destination: PCR 96_1
   Liquid Type: Glycerol
   Dosing Parameter:
   |-- Speed Aspiration: 20000 mm/sec
   |-- Speed Dispense: 10000 mm/sec
   |-- Delay Blow: 70 ms
   |-- Speed Blow: 66000 mm/sec
   |-- Movement Blow: 90% of max movement
   |-- Prewetting: 0 Cycles
   Change Tips before aspirating a new sample
   Change Tips after 0 aspirations
   Mix Before Yes
   |-- No. of Cycles: 5
   |-- Speed: 5 mm/sec
   |-- Volume: 70 µl
   |-- Fixed Height: No
   |-- Asp./Disp.: 2000 / 5000 mm
   Mix After No
   Special Aspirate from bottom

14. Place the 96-well PCR plate containing protoplasts on a thermoadapter connected to temperature-controlling unit. Keep the PCR plate at 33°C.

15. Put Top Agar in a 30-mL reservoir. Place the reservoir on a holder connected to temperature-controlling unit. Keep the Top Agar at 50°C. (Note: Top Agar contains 1.6% agar.)

16. Aliquot 45 µL of Top Agar into each well in the 96-well PCR plate with 50-µL eight-channel pipette. Mix by pipetting up and down.
17. Dispense protoplast-agar mixture on a PRMB plate overlaid with a cellophane with 6μL spots in a 96-well pattern. Plate even-number columns. See Figure 2.2. (Note: 8X12 cm rectangular PRMB plate is set as a 96-well PCR plate with liquid volume of 8 μL.)
18. Repeat step 17, plate odd-number columns. See Figure 2.2. (Note: 8X12 cm rectangular PRMB plate is set as a 96-well PCR plate with liquid volume of 8 μL.)

19. Replace the PRMB plate with a new PRMB plate.

20. Repeat steps 16 to 18 for each column. (Note: Eight transformations in one column are plated on one PRMB plate. Each transformation is plated to twelve spots in a row. See Figure 2.2 for illustration.)

21. Regenerate protoplasts on PRMB for four days.

22. Four days after transformation, transfer protoplasts by lifting the cellophane and moving them to PpNH₄ plates containing antibiotic selection. (Note: for pUGGi RNAi constructs, use 15 μg/mL hygromycin.)

23. Let the plants grown on PpNH₄ for another three days.

24. One week after transformation, image plants lacking expression of GFP in the nucleus for phenotypic analysis. (Note: With good transformation efficiency, each spot should have at least 10 transformants (antibiotic-resistant plants). Numbers of plants lacking nuclear GFP varies and depends on gene that is being silenced.)

25. For each RNAi construct, at least 25 plants were imaged for growth phenotype by measuring the plant area based on chlorophyll autofluorescence. Plants are imaged with a stereo fluorescence microscope (Leica MZ16FA). Images are acquired with a color camera (Leica DFC 300FX) using the GFP2 filter (excitation 480/40 nm, emission 400 long pass) for
chlorophyll and GFP. The degree of polarization of the plants are estimated using solidity. Solidity is the ratio of the area over the convex hull area, which is the smallest convex polygon that contains the shape under analysis.

Figure 6.1. Heating system for top agar. Module-rack for 30-mL reservoir (A) and thermoadaptor for 96-well PCR plate (B) are attached to heating elements and thermocouples (C), which are each connected to a separate temperature-control unit (D). Heating elements are on the side of the module-rack and beneath the thermoadaptor.
Table 6.1. Primers used in this study. (Continued on next three pages.)

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>8AexpF</td>
<td>AATCCTTCAATCAGTCGTTCG</td>
<td>Expression Analysis</td>
</tr>
<tr>
<td>8AexpR</td>
<td>CGACCATCATCAACAGCATC</td>
<td>Expression Analysis</td>
</tr>
<tr>
<td>8BexpF</td>
<td>TCCTGATCCAATCAGTGGTG</td>
<td>Expression Analysis</td>
</tr>
<tr>
<td>Myo8BRnew</td>
<td>TGTAAGACGGCTTCCGATAACG</td>
<td>Expression Analysis</td>
</tr>
<tr>
<td>8CexpF</td>
<td>AGATAGCAGCCGATGCAAAG</td>
<td>Expression Analysis</td>
</tr>
<tr>
<td>8CexpR</td>
<td>CTTCGTTCTGTTCCACAGTCA</td>
<td>Expression Analysis</td>
</tr>
<tr>
<td>8DexpF</td>
<td>GGCTTCAATCAGTCGTTCG</td>
<td>Expression Analysis</td>
</tr>
<tr>
<td>Myo8DRnew</td>
<td>CCTCCTTTACGCATTCCAAA</td>
<td>Expression Analysis</td>
</tr>
<tr>
<td>8EexpF</td>
<td>CAAGGTTCAAGCAGTCATTCC</td>
<td>Expression Analysis</td>
</tr>
<tr>
<td>8EexpR</td>
<td>GGGCCTCGACTCTTAGCTTC</td>
<td>Expression Analysis</td>
</tr>
<tr>
<td>UbiF2</td>
<td>ACTACCCCTGAGTTGTATAGTTCGG</td>
<td>Expression Analysis</td>
</tr>
<tr>
<td>UbiR</td>
<td>CAAGTCACTATTACTTCGCTTGTCTAG</td>
<td>Expression Analysis</td>
</tr>
<tr>
<td>8Aexon1F</td>
<td>GTGCTTTGAGCAATGATCTGAAGC</td>
<td>Transcript Analysis in</td>
</tr>
<tr>
<td></td>
<td></td>
<td>knockouts</td>
</tr>
<tr>
<td>8Aexon15R</td>
<td>TTGTCTTCAAAATCCACCTCGTTCC</td>
<td>Transcript Analysis in</td>
</tr>
<tr>
<td></td>
<td></td>
<td>knockouts</td>
</tr>
<tr>
<td>8Bexon1F</td>
<td>AATGGTCACATGGGCTTTTGAGACAAATGTG</td>
<td>Transcript Analysis in</td>
</tr>
<tr>
<td></td>
<td></td>
<td>knockouts</td>
</tr>
<tr>
<td>8Bexon6R</td>
<td>CTTTGCCGTTTCTGTTTCCCTGC</td>
<td>Transcript Analysis in</td>
</tr>
<tr>
<td></td>
<td></td>
<td>knockouts</td>
</tr>
<tr>
<td>Exon/Target</td>
<td>Primer Sequence</td>
<td>Function</td>
</tr>
<tr>
<td>-------------</td>
<td>----------------</td>
<td>----------</td>
</tr>
<tr>
<td>8Cexon1F</td>
<td>TGATTTCGGAGGGATGCCAACGG</td>
<td>Transcript Analysis in knockouts</td>
</tr>
<tr>
<td>8Cexon9R</td>
<td>CGTGATATGATCTTTTCGCTTG</td>
<td>Transcript Analysis in knockouts</td>
</tr>
<tr>
<td>8Dexon1F</td>
<td>CAAGAAGGCATTGGGTGTCG</td>
<td>Transcript Analysis in knockouts</td>
</tr>
<tr>
<td>8Dexon6R</td>
<td>CGACTGGAATTGTCATTTCTCAATGTCTTG</td>
<td>Transcript Analysis in knockouts</td>
</tr>
<tr>
<td>8Eexon1F</td>
<td>CAAGATGCCTGTAAAAACCATTGACC</td>
<td>Transcript Analysis in knockouts</td>
</tr>
<tr>
<td>8Eexon7R</td>
<td>TCTATCAAAGTGAGTGCTCAATCAGCTTGCC</td>
<td>Transcript Analysis in knockouts</td>
</tr>
<tr>
<td>8Ad-1</td>
<td>ATGGATTTATCATGCTCTAGCATCAGGG</td>
<td>Genotyping primer (#1)</td>
</tr>
<tr>
<td>8Ad-4</td>
<td>GAAAGGAGTCTAGAGGTGAAGG</td>
<td>Genotyping primer (#2)</td>
</tr>
<tr>
<td>8Ad-3</td>
<td>ACTGCTCGCTGTGCTTTTCG</td>
<td>Genotyping primer (#3)</td>
</tr>
<tr>
<td>8Ad-2</td>
<td>CAATATGTACAGTACAGCATGACG</td>
<td>Genotyping primer (#4)</td>
</tr>
<tr>
<td>PpBRT2</td>
<td>TTGGCTTAGATACCGATACTCCTTG</td>
<td>Genotyping primer (#7)</td>
</tr>
<tr>
<td>HygF</td>
<td>CTGTCGAGAAGTTTCTGATCG</td>
<td>Genotyping primer (#6)</td>
</tr>
<tr>
<td>8E-3</td>
<td>CTCATCCTCATGCGACATTCAC</td>
<td>Genotyping primer (#8)</td>
</tr>
<tr>
<td>8E-6</td>
<td>GTTGCGAATGCTTCCAAAATTAGG</td>
<td>Genotyping primer (#9)</td>
</tr>
<tr>
<td>8E-5</td>
<td>CCCCAGTTCTGATCTGCC</td>
<td>Genotyping primer (#10)</td>
</tr>
<tr>
<td>8E-4</td>
<td>CATTTCAAAAGATTGGCTCTTGCTTTCC</td>
<td>Genotyping primer (#11)</td>
</tr>
<tr>
<td>M8C-Fwd-ForSeq</td>
<td>TATTTAGACAGTGCTCTTCGATAGC</td>
<td>Amplifying altered myo8C locus</td>
</tr>
<tr>
<td>Primer Set</td>
<td>Sequence</td>
<td>Description</td>
</tr>
<tr>
<td>--------------------</td>
<td>---------------------------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>M8C-Rev-ForSeq</td>
<td>GGGTTAATAATGTAATTCTATGCACACTACGAGG</td>
<td>Amplifying altered myo8C locus</td>
</tr>
<tr>
<td>M8D-Fwd-ForSeq</td>
<td>CTCAAGGACAAAGCAAGATACTGG</td>
<td>Amplifying altered myo8D locus</td>
</tr>
<tr>
<td>M8D-Rev-ForSeq</td>
<td>AGCGAGGTCACTAGTAGCG</td>
<td>Amplifying altered myo8D locus</td>
</tr>
<tr>
<td>Myo8A-F (P1)</td>
<td>ATGTATTCTACGAGGCAATTGAGG</td>
<td>Amplifying full-length myo8A</td>
</tr>
<tr>
<td>Myo8A-R (P2)</td>
<td>CTAACCTTGAGGCCTCTTGAGG</td>
<td>Amplifying full-length myo8A</td>
</tr>
<tr>
<td>attB1-my8A-F (P3)</td>
<td>GGGGACAAGTTTTGTATACAAAAAGCAGGGGCTTCATGTATTCTACGAGG</td>
<td>Generating P1P5r-my8A</td>
</tr>
<tr>
<td>attB5r-my8A-R (P4)</td>
<td>GGGGACAAGTTTTGTATACAAAAAGCAGGGGCTTCATGGTGAGCTCCTGAG</td>
<td>Generating P1P5r-my8A</td>
</tr>
<tr>
<td>attB1-mEGFP-F (P5)</td>
<td>GGGGACAAGTTTTGTATACAAAAAGCAGGGGCTTCATGGTGAGCTCCTGAG</td>
<td>Generating P1P5r-mCherry</td>
</tr>
<tr>
<td>attB5r-mEGFP-R (P6)</td>
<td>GGGGACAAGTTTTGTATACAAAAAGCAGGGGCTTCATGGTGAGCTCCTGAG</td>
<td>Generating P1P5r-mCherry</td>
</tr>
<tr>
<td>attB5-Tub-F (P7)</td>
<td>GGGGACAACTTTTTGTATACAAAAAGCAGGGCTTCATGTACAGTACGATGAAGATATTATCAGCATCCAC</td>
<td>Generating P5P2-Tub</td>
</tr>
<tr>
<td>attB2-Tub-R (P8)</td>
<td>GGGGACCACCTTTTTGTATACAAAAGCAGGGCTTCATGTACGATGAAGATATTATCAGCATCCAC</td>
<td>Generating P5P2-Tub</td>
</tr>
<tr>
<td>attB1-pTFH-F</td>
<td>GGGGACAAGTTTTGTATACAAAAAGCAGGGCTTCATGTACGATGAAGATATTATCAGCATCCAC</td>
<td>Generating entry clones for RNAi constructs</td>
</tr>
<tr>
<td>attB2-pTFH-R</td>
<td>GGGGACAAGTTTTGTATACAAAAAGCAGGGCTTCATGTACGATGAAGATATTATCAGCATCCAC</td>
<td>Generating entry clones for RNAi constructs</td>
</tr>
<tr>
<td>attB5-mEGFP-F (P9)</td>
<td>GGGGACAAGTTTTGTATACAAAAGCAGGGCTTCATGTACGATGAAGATATTATCAGCATCCAC</td>
<td>Generating P5P2-mCherry-KDEL</td>
</tr>
<tr>
<td>attB2-GFP-KDEL-R (P10)</td>
<td>GGGGACCACTTTGTAACAAGAAAGCTGGGTA</td>
<td>Generating P5P2-mCherry-KDEL</td>
</tr>
<tr>
<td>------------------------</td>
<td>---------------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td></td>
<td>TCATAGCTCATCTTT CTGTACAGCTCGTCCAT</td>
<td></td>
</tr>
</tbody>
</table>
REFERENCES


