Functions of DFRP and Other DRG Pathway Genes in The Moss Physcomitrella Patens

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GTP binding proteins (G proteins) are molecular switches that regulate many aspects of physiology in all cells. DRG1 and DRG2 (Developmentally Regulated GTP-binding proteins) have been found in all eukaryotes examined. Amino acid identity across eukaryotes is about 65% within an orthologous group and 55% between paralogs of a single species. Although DRGs are likely involved in some aspect of translation regulation, details of their precise cellular functions are not known.

In addition to DRGs there are other genes/proteins that comprise the “DRG pathway.” DFRPs (DRG Family Regulatory Proteins) have been shown to physically and genetically interact with DRGs. Previous work elsewhere and at NIU using the model plant Arabidopsis demonstrated that DFRP and DRG proteins mutually prevent the degradation of their binding partners (DRG1 with DFRP1, and DRG2 with DFRP2).

Current work is focused on the DRG pathway genes in the model moss Physcomitrella patens. Mosses offers several benefits to gene research: 1) most of the moss lifecycle is spent in the haploid phase, which greatly simplifies genetic analyses; 2) mosses can be kept in this phase indefinitely; 3) individual living cells are easy to study with various types of microscopy; 4) unlike virtually all other plants, homologous recombination is very efficient in P. patens,
allowing knockout mutants to be generated quite readily; and 5) the *Physcomitrella* genome sequence and other molecular tools are available.

*Physcomitrella* contains 7 genes in the DRG pathway. The focus of this dissertation is the four *Dfrp* genes (1a, 1b, 2a and 2b). This required production of an extensive set of knockout mutants: 4 singles, 5 doubles, 2 triples and a quadruple mutant (all four *Dfrp* genes removed). Analysis of the different genotypes included detailed phenotype studies. The mRNA and protein levels of all 7 genes were studied using qRT-PCR and Western blotting, respectively. These techniques gave more details about the level of gene/protein regulation in the gene pathway. Some of the genes were expected to take part in the amino acid starvation response. That hypothesis was tested by blocking amino acid production with glyphosate in all knockouts to examine if any of these genes alter the response. The various analyses of all knockouts have led to a better understanding of the gene functions in the DRG pathway.
FUNCTIONS OF DFRP AND OTHER DRG PATHWAY GENES IN THE MOSS

PHYSCOMITRELLA PATENS

BY

ROBERT JAMES SRYGLER
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A DISSERTATION SUBMITTED TO THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOLOGICAL SCIENCES

Doctoral Director:
Joel P. Stafstrom
ACKNOWLEDGMENTS

I would like to thank Dr. Joel Stafstrom for introducing me to the wonderful world of plant genetics. A conversation that a professor started with an undergrad reading in the hallway has led to multiple degrees at NIU. The time and trust that he has given me in his lab is more that I would have ever expected when beginning my first semester here many years ago. His guidance has kept me on the path that has finally led to this dissertation. Thank you to my committee members. Dr. Jozef Bujarski, Dr. Scott Grayburn, and Dr. Mitrick Johns have been supporting my work since the beginning of this project. Dr. Barrie Bode was kind enough to step up as chair of the committee during the last, most stressful stretch of my dissertation. The entire committee has been very helpful with the guidance over these last seven years. The research could not have happened without the help of many people here and outside of the university. Dr. Magdalena Bezanilla and her lab at Dartmouth were instrumental in getting us started with the starting moss culture as well as plasmids and technical advice. Dr. Grayburn has been a great help with technical questions and running some complex qRT-PCR experiments. Dr. Olivier Devergne contributed to the morphology experiments, without his guidance and equipment these results would not be possible.

All of the colleagues that I have met over the years at NIU have made this a successful story. The TAs in the department have made teaching enjoyable. The undergrads who have been in Dr. Stafstrom’s lab, as well as Matt Marcec who completed his Master’s here, all have
given me a great experience. Over the years I have spent a lot of time with Pat McCarthy and Craig Schultz, who both make spending time in the department feel less like work.

Thank you to all my friends and family! There is no way that I would have stood a chance here without the support from everyone. When I first came to DeKalb, knowing that such a supporting group was back home made that move to the unknown much easier to handle. Especially to my parents, this could not have been done without you Mom and Dad! Many bonds have been made during these years that will last a lifetime. The Bio Grad house, MODeeepSouth, was the hub that connected so many of us to so many late nights and kept me going through all of the good and the rough times.

Lastly, thank you to Linda Yates. Your love and support have been more helpful than you know. I lucked out by catching your eye with that kilt! You have been the perfect partner through this stressful time of our lives.
DEDICATION

I dedicate this to Nana and Papa, who always supported their grandkids.
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CHAPTER 1: PHYSCOMITRELLA PATENS AND THE DRG GENE PATHWAY

Introduction

The Model Organism Physcomitrella Patens

This lab has used several plant species to understand the function of the genes in the DRG pathway. “Model organisms” are representative species which are relatively easy to grow and study, and are often used in biological research to investigate complex biological processes. What one learns from studying a simple organism can be applied to other species which are harder to study. For example, fruit flies and the lab rat are used to study processes in mammals. Outcomes from rat studies sometimes benefit human health.

To be chosen as a model organism, a species must present a combination of characteristics that make it both easy to use and beneficial. The ease-of-use can be determined by how much space an organism needs, as well as how much time and money goes into sustaining that organism. If a species can be grown in a small area and does not need much time devoted to its care, the scientist will have more time to devote to the research. To be beneficial, the organism should be easy to study. If many scientists are studying the same species, then valuable resources for that research community will accumulate over time. Lastly, the model organism is only useful if the knowledge gained from studying it can be applied to other species.
that are more valuable to society. The model organism used for this dissertation satisfies all of these requirements.

The moss *Physcomitrella patens* offers several benefits to gene research that make it a good model organism: 1) most of the moss lifecycle is spent in the haploid phase, which greatly simplifies genetic analyses; 2) mosses can be kept in this phase indefinitely; 3) individual living cells are easy to study with various types of microscopy; 4) unlike virtually all other plants, homologous recombination is very efficient in *P. patens*, allowing knockout mutants to be generated quite readily; and 5) the *Physcomitrella* genome sequence and other molecular tools are available.

The moss lifecycle can be completed in as little as 7-8 weeks; a short enough time that multiple generations can be studied in a reasonable period (Engel, 1968). Mosses are simple plants, representative of the first land plants. They do not have true vascular tissue, they are not flowering plants, and they do not produce seeds. Though they are far removed evolutionarily from most common plants, they can still be used to gain useful information that can further our understanding of higher plants. Before much of the genetic work began on *Physcomitrella*, studies were already underway learning how basic physiological processes found in other plants – such as hormone responses – were behaving in mosses (Ashton and Cove 1977).

All plants proceed through an alternation of generations. The gametophyte portion of the lifecycle is a multicellular haploid generation. The remaining portion of the lifecycle, the sporophyte, is a multicellular diploid generation. Most plants have a dominant sporophyte with a highly reduced gametophyte, so the vast majority of the cells are diploid. Mosses however have
a lifecycle that is mostly gametophytic, with only a small portion composed of a sporophyte which grows on the gametophyte. This is very useful for genetic manipulation. Since most of the moss cells are haploid, there is only one copy of a gene present in most cells. Rather than differentiating between a heterozygous versus a homozygous mutant, a moss cell will either have the gene or it will not.

The normal progression of the moss lifecycle begins as the spore germinates into filamentous protonemata. These filamentous cells are shown in Fig.1. Protonemata initially grow by tip growth, performed by apical cell division. Subapical cells eventually start forming branches from the original filament. There are two types of protonema cells. The first, chloronemal cells, have many chloroplasts and form cell plates that are perpendicular to the filament. Eventually the second type, caulonemal cells, begin to form. They have less chloroplasts and the cell plates form at an angle, rather than perpendicular to the filament (Prigge and Bezanilla, 2010). Caulonemal cells divide much quicker than that chloronemal cells (Cove, 2005). Some protonemata cells form into buds, a three-dimensional growth pattern that will eventually form the leafy gametophore (Schaefer and Zrýd, 2001). Reproductive antheridia and archegonia, which produce sperm and egg respectively, develop on the gametophores. When the egg is fertilized, the diploid sporophyte grows on the gametophore. The sporophyte produces spores by meiosis, and the entire cycle starts again. Like development in higher plants, the tissue differentiation for moss is controlled by hormones (Ashton et al., 1979).
Even though the moss lifecycle normally proceeds through a small diploid sporophyte generation, it is easy to keep it in the haploid gametophyte generation indefinitely. The cells revert to produce new protonemata when the plant tissue is mechanically disrupted. The cultures
are renewed every 10-14 days by disrupting the protonemata and spreading aliquots on new plates. These pieces of tissue quickly form a new layer of protonemata on the plates. Protonemata can be regenerated from other tissues, such as the gametaphores, a process termed redifferentiation (Knoop, 1984; Reski, 1998).

The growth patterns described above make *Physcomitrella patens* an easy plant to observe. Most plants have three-dimensional growth, meaning that many of the cells are internal and difficult to directly observe in a living tissue. The filamentous growth of moss produces ample tissue that is one cell thick, making microscopic observations of the living cells much easier. Even the leafy tissues on the gametophore are only one cell thick!

The most important feature of *Physcomitrella* for this dissertation is that it has a high rate of homologous recombination. Gene targeting by homologous recombination is very difficult in higher eukaryotes because there is a low level of homologous recombination observed. Targeting genes in yeast is a common procedure thanks to the higher rate of recombination. *Physcomitrella* is the only plant that is known to have a rate that is high enough to be of use to geneticists. The rate is comparable to *Saccharomyces cerevisiae* (Schaefer and Zryd, 1997).

The research for this dissertation began before gene targeting with CRISPR-Cas9 was very widespread. We chose *Physcomitrella* as our organism because it was the only plant at the time that could be reliably used for gene targeting.

The last beneficial feature of *Physcomitrella patens* is the amount of resources that were available for the species. The genome is fully sequenced and available on the Joint Genome Institute’s Phytozome website (Goodstein et al., 2012). Because of the focus on this species
following the discovery of the efficient transformation, *Physcomitrella* was one of the first two non-flowering plants to begin being sequenced (Cove, 2005). Several versions of the genome have been available as the data has been updated, with v3.3 being the current version (Rensing et al., 2008; Zimmer et al., 2013; Lang et al., 2018). In addition to the benefits of the online resources, another invaluable resource was our collaborator. Dr. Magdalena Bezanilla provided the wildtype *Physcomitrella* tissue that started this project. Her lab also produced a Moss Methods Manual that contained many protocols, some of which were used in this research.

**DRG Gene Pathway**

The research summarized in this dissertation was performed to study DRGs as well as other genes/proteins that comprise the “DRG pathway.” DFRPs (DRG Family Regulatory Proteins) have been shown to physically and genetically interact with DRGs. Previous work elsewhere and at NIU using the model plant *Arabidopsis thaliana* (Srygler, 2012) demonstrated that DFRP and DRG proteins mutually prevent the degradation of their binding partners (DRG1 with DFRP1, and DRG2 with DFRP2). This dissertation focused on the DFRP proteins, while also looking at the DRGs as well as another protein in the DRG pathway, the helicase SLH.
**DRG Family Regulatory Proteins (DFRP)**

A group of proteins that is associated with the DRGs is the DRG family regulatory protein (DFRP) family. There are two orthologous proteins in studied eukaryotes, DFRP1 and DFRP2. These proteins physically interact with DRG1 and DRG2. Knockout mutants missing DFRP1 or DFRP2 show a decrease in protein accumulation of DRGs. The DFRP1 protein interacts with DRG1 (Francis et al., 2012). The interaction was verified by x-ray diffraction which showed the two proteins as a single structure. This interaction appears to be an interaction with the Dfrp domain (Pérez-Arellano et al., 2013). While DFRP2 interacts with both DRG proteins, it has a preference for DRG2 (Ishikawa et al., 2005; Ishikawa et al., 2013; J. Stafstrom, personal communication).

These protein-protein interactions have been studied across multiple kingdoms, including animal, plant, and fungi. Depending on the species, the genes can sometimes have alternative names. Table 1 below lists the alternative names for DRGs and DFRPs. The *Saccharomyces cerevisiae* orthologs of the DRGs (Rbg1 and Rbg2) and DFRPs (Tma46, and Gir2) have similar behaviors. Tma46 and Gir2 (DFRP1 and DFRP2 respectively) have been shown to associate with Rbg1 and Rbg2 (Fleischer et al., 2006; Wout et al., 2009). Rbg1 and Tma46 were found together in complexes in an approximately 1:1 ratio. The same was true for Rbg2 and Gir2 (Daugeron et al. 2011).
Table 1: Alternative names for the genes/proteins in the DRG pathway, used in human and yeast articles

<table>
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<tr>
<th>Genes</th>
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<tr>
<td>Drg1</td>
<td>Rbg1</td>
</tr>
<tr>
<td>Drg2</td>
<td>Rbg2</td>
</tr>
<tr>
<td>Dfrp1</td>
<td>Tma46, Lerepo4</td>
</tr>
<tr>
<td>Dfrp2</td>
<td>Gir2</td>
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</table>

The DFRP1 and DFRP2 proteins are very different from each other, only sharing a small region termed the “DFRP domain” (Ishikawa et al., 2005). The DFRP domain is a short section (~60aa) of each polypeptide. The remainder of each protein is related to other proteins with known functions. DFRP1 has a tandem repeat CCCH zinc finger domain (TZF) that has close similarity to RNA-binding proteins, suggesting a link to RNA metabolism (Ishikawa et al., 2005). DFRP2 has a RWD domain, also known as a GI domain. The domain is similar to eIF2α kinase, which phosphorylates eIF2α to inhibit formation of the ternary complex and inhibit translation of most mRNAs. Different processes initiate the translation of stress-related mRNAs. The GI domain is homologous to the domain found in E2 ubiquitin-conjugating-enzyme, suggesting that poly-ubiquitination may regulate DRGs. It is believed that the DFRP protein may block proteolysis of DRGs by preventing poly-ubiquitylation. DRG proteins are less stable in the absence of DFRP proteins, and vice versa. When DRG is overexpressed, higher DRG protein levels are only sustained if the corresponding DFRP protein is also overexpressed (Ishikawa et al., 2005).

Physcomitrella patens has a total of four Dfrp genes, two Dfrp1 genes and two Dfrp2 genes. Each pair of Physcomitrella Dfrp paralogs are closely related to each other. But the
Dfrp1 and Dfrp2 orthologs are very different from each other, as is true in other studied species. Figure 2 and Table 2 show the comparisons of the amino acid sequence of Arabidopsis and Physcomitrella proteins. The additional Dfrp genes are thought to be the product of a whole genome duplication event. Duplications of genomes in plants have happened often and are thought to allow for the evolution of new traits (Panchy et al., 2016). An earlier analysis suggested that a relatively recent whole genome duplication occurred in the ancestor of Physcomitrella approximately 30-60mya (Rensing et al., 2007). More recently, the newest version of the Physcomitrella patens genome shows that two whole genome duplications occurred 57-70 mya and 38-50 mya. After each duplication there were some chromosome losses and some breaks and fusions (Lang et al., 2018).

Table 2: Percent Identity Matrix comparing sequence identity of Arabidopsis and Physcomitrella DFRP proteins. Matrix created using Clustal2.1.

<table>
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<tr>
<th></th>
<th>AtDFRP1</th>
<th>PpDFRP1a</th>
<th>PpDFRP1b</th>
<th>AtDFRP2</th>
<th>PpDFRP2a</th>
<th>PpDFRP2b</th>
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<tr>
<td>AtDFRP1</td>
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<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>PpDFRP1a</td>
<td>70.7</td>
<td>100.0</td>
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</tr>
<tr>
<td>PpDFRP1b</td>
<td>69.3</td>
<td>91.3</td>
<td>100.0</td>
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<td>--</td>
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<tr>
<td>AtDFRP2</td>
<td>22.1</td>
<td>19.0</td>
<td>18.6</td>
<td>100.0</td>
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<tr>
<td>PpDFRP2a</td>
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<td>19.9</td>
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<tr>
<td>PpDFRP2b</td>
<td>22.3</td>
<td>18.8</td>
<td>18.0</td>
<td>57.6</td>
<td>73.9</td>
<td>100.0</td>
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</table>
GTP-Binding Proteins (G proteins)

GTP-binding proteins (G proteins) are a large and very diverse group that is included with the NTP-binding proteins, all of which have the ability to bind to nucleotides, especially the di- and tri-phosphate nucleotides. The G proteins are believed to have already been present in multiple forms in the last common ancestor to all living organisms. The ancestral G proteins are believed to have been specific for GTP, but as groups have diverged to fulfill many functions, some have lost the ability to bind GTP/GDP and gained the ability to bind ATP/ADP (Leipe et al., 2002).

Figure 2: Bootstrap comparison of amino acid sequence identity between all four *Physcomitrella patens* DFRP proteins and each DFRP protein from *Arabidopsis thaliana*. The DFRP1 proteins and DFRP2 proteins are similar to each other, but the two groups of orthologs are very different. Analysis performed using MEGA multiple sequence alignment.
G proteins generally function in cell signaling pathways, acting like on/off switches. When a G protein is bound to GTP, the protein is usually considered to be in the active state, or “on” state. While bound to GDP, the protein is in the “off” state. The general cycle starts with an inactive G protein that is bound to GDP. This inactive protein is turned on by the action of another protein, termed a guanine nucleotide exchange factor (GEF), which causes the G protein to exchange the GDP for GTP. This exchange activates the G protein. To reverse this process and deactivate it, another protein, called a GTPase-activating protein (GAP), will stimulate the G protein to hydrolyze the bound GTP to GDP. While the G protein is in the active state, it will activate downstream signals (secondary messengers) to propagate a signal within the cell (Bourne et al., 1991).

Heterotrimeric G proteins and monomeric G proteins are two well-characterized groups of the superfamily. The heterotrimeric G proteins have three subunits that are all bound together when the protein is inactive. The first subunit is named $G_\alpha$, and the tightly associated complex of subunits is named $G_{\beta\gamma}$. The activation begins when an effector molecule, such as a hormone, binds to a transmembrane protein. A highly studied transmembrane protein that is involved is the seven-transmembrane protein, or G-Protein-coupled receptor (GPCR). The GPCR spans the cellular membrane and is exposed to both the inside and outside of the cell. An extracellular molecule changes the conformation of the GPCR, which transmits the signal into the cell. It is the transmembrane protein that acts as the GEF and causes the heterotrimeric G protein to lose the GDP in exchange for a GTP molecule. This activated G protein then experiences a separation of the $G_{\beta\gamma}$ subunits from the $G_\alpha$ subunit. While separated, both parts of the heterotrimeric protein can propagate signals. The $G_\alpha$ subunit remains attached to the GTP until a GAP protein causes
hydrolyzation of the $G_\alpha$–bound GTP to GDP. Once this occurs, the $G_\alpha$ subunit and $G_{\beta\gamma}$ subunits join to reform the three-unit heterotrimeric G protein that is bound to GDP in the inactive form (Bourne et al. 1991).

In addition to heterotrimeric G proteins, there is another group of G proteins called the small G proteins. These proteins are comparable to the $G_\alpha$ subunit of the heterotrimeric G proteins. Small G proteins are also active when bound to GTP and inactive when bound to GDP. These proteins function similarly to the heterotrimeric G proteins, except that there is no $G_{\beta\gamma}$ subunit complex. The representative for the small G protein is the Ras GTPase superfamily. Ras and Ras-like proteins have been studied in detail and are understood better than other small G proteins. The superfamily is comprised of five branches based on sequence and function. These five branches are Ras, Rho, Rab, Ran, and Arf (Wennerberg et al., 2005). Like the other G proteins, this superfamily is active when bound to GTP and inactive when bound to GDP. The molecular mechanisms are similar throughout the superfamily, but the highly diverse variations between each group allow these proteins to regulate a wide range of cellular functions.

Mutant Ras proteins are human oncogenes, controlling gene expression and cell proliferation in response to external stimuli (Wennerberg et al., 2005). Rho proteins also use external stimuli to regulate gene expression, as well as actin organization and the cell cycle. The Rab family regulates vesicular transport and protein trafficking. Ran transports proteins, as well as RNA, between the cytoplasm and nucleus. The last family within the Ras superfamily is the Arf proteins. Arf is also a regulator of vesicular transport. Since these small G proteins are involved in so many essential cellular functions, there is much interest in controlling the pathways to treat diseases such as cancer (Prieto-Dominguez et al., 2019).
Many of the G proteins are involved in protein translation. These G proteins function in both the initiation and the elongation steps of protein synthesis (Leipe et al., 2002). Initiation proteins include IF2/eIF5B and eIF2γ/SelB subfamilies. The eIF2γ protein forms a complex that binds to GTP and Met-tRNAi^{Met} to bind Met-tRNAi^{Met} to the ribosome to form the 43S preinitiation complex. Elongation proteins include EF-Tu/EF-1α and EF-G/EF-2 subfamilies. EF-Tu/EF-1α forms a complex with aminoacyl-tRNA and protects against hydrolysis until a codon-anticodon match occurs.

**Developmentally Regulated GTP-Binding (DRG) Proteins**

Developmentally Regulated GTP binding proteins (DRGs) are a highly conserved subfamily among the diverse superfamily of GTP binding proteins. DRGs are in the OBG/DRG family, along with OBGs that are found in bacteria and eukaryotes (Leipe et al., 2002). Bacterial OBGs are thought to be involved in essential cellular processes such as protein synthesis, DNA replication, chromosome segregation, and stress responses (Kint et al., 2014; Shahid et al., 2013). Eukaryotic OBGs are usually targeted to chloroplasts or mitochondria (Hirano et al., 2006). All eukaryotes studied to date have two *Drg* genes, *Drg1*, and *Drg2*. The first genes representing the two *Drg* genes described were from mouse and human, respectively (Li and Trueb, 2000). The DRG1 and DRG2 orthologs have a 65-70% identity between the eukaryotes. Paralogs from a single species have an identity of 55-60% (Li and Trueb, 2000). As shown in figure 3, the two
DRG orthologs have a higher degree of identity, compared to the DFRP orthologs shown in figure 2. This high degree of amino acid conservation among all living eukaryotes suggests that DRGs are critical to a basic function in life.

![Figure 3: Bootstrap comparison of amino acid sequence identity between the two Physcomitrella patens DRG proteins and all three DRG proteins from Arabidopsis thaliana.](image)

Unlike the DFRP1 and DFRP2 orthologs shown in Figure 2, the DRG1 and DRG2 orthologs are much closer in amino sequence identity. *Arabidopsis* has a third DRG, which is from a recent duplication. The AtDRG3 protein is 95% identical to AtDRG2. Analysis performed using MEGA multiple sequence alignment.

DRGs, like other G proteins, have been experimentally shown to bind and hydrolyze GTP (Nelson et al., 2009; O'Connell et al., 2009). However, their rate of GTP/GDP exchange is much higher than the Ras-like proteins (Lin et al., 1999). DRG proteins are highly expressed in growing tissues of Pea (Devitt et al., 1999) and in reproductive organs and growing tissues of *Arabidopsis* (Etheride et al., 1999; Stafstrom, 2008). When human DRG2 is overexpressed, the
cell cycle stops at the G2/M phase (Song et al., 2004). This information suggests that DRGs may be involved in the regulation of cell growth. The difference observed in the phenotype of the wild type, compared to that of the drg1; dru2 double mutant Arabidopsis thaliana, supports the suggestion that DRGs may regulate cell growth. The drg1; dru2 double mutant has a reduced growth compared to the wild-type lines (J. Stafstrom, personal communication; Szygler, 2013).

The Arabidopsis thaliana DRG2 protein has at least three proteolytic forms (Nelson et al., 2009). The full-length polypeptide is 45 kDa, with a slightly shorter 43 kDa band often detected by the same antiserum. A second DRG2 antiserum, which targets a 17 amino acid polypeptide near the C-terminal end of the protein, does not recognize the smaller band. This indicates that the portion of the polypeptide that is removed to produce the 43 kDa band is removed from the C-terminal end. There is a 30 kDa band that is also proposed to be a shorter form of DRG2, which is also the product of proteolysis. The 30 kDa band is the result of loss of part of the N-terminus of the full polypeptide. The different proteolytic forms of DRG2 have different patterns of ribosome association. Cytosolic monosomes co-fractionate with the larger 43 and 45 kDa DRG2 polypeptides. The 30 kDa band is highly enriched in fractions with polysomes. The 43 and 45 kDa bands demonstrated binding capabilities to GTP, whereas the 30 kDa band did not. DRG1 antisera only recognize one band with an M_r of about 43 kDa.

Although DRGs are likely involved in some aspect of translation regulation, details of their precise cellular functions are not known. Attempts to understand their function have studied localization of the proteins in the plant (Stafstrom, 2008) and within in the cell (Suwastika et al., 2014). Research has also studied what structures the proteins are associating
with, such as ribosomes (Nelson et al., 2009) and heat shock granules (Nelson, 2008; Srygler, 2013). In addition to protein translation, DRGs have also been implicated as a proto-oncogene, increasing cell division in tumors when overexpressed (Kiniwa et al., 2015; Lu et al., 2016). This could be explained by the DRG association with microtubule formation control (Schellhaus et al., 2017; Dang et al., 2018). The opposite of uncontrolled growth seen in tumors is the slow growth observed in mutants. The slow growth of *drg* knockouts in *Arabidopsis* was mentioned above. Slow growth is also observed in the pathogenic *Candida albicans*. When DRG1 is removed, the fungus fails to produce filaments capable of penetrating the solid growth media (Chen and Kumamoto, 2006).

DRGs are important to study because they are found in a wide range of different types of organisms, and these genes are similar to each other from one group of organisms to the next. This high degree of similarity indicates that the genes are important for proper function of living organisms. They are also of interest because other better-characterized G proteins have important functions. The goal of this dissertation is to better understand the function of *Drg* as well as other genes which have been linked physically or genetically to the “DRG pathway.” Physical interactions are defined as two proteins physically touching within a cell, which indicates that the proteins are working together as part of a process or pathway. Genetic interactions occur when one gene shares a function with another.
Ski2-Like Helicase (SLH)

Another gene that is part of the DRG pathway is Ski2-Like Helicase (SLH). The protein encoded by this gene is a putative RNA helicase, a completely different type of protein from DRGs and DFRPs. Nevertheless, SLH and DRGs have “redundant functions.” Earlier work using yeast demonstrated a link between SLH and DRG (Daugeron et al., 2011). In yeast, the \textit{drg1;drg2} double mutant did not have a noticeably different phenotype from the wild-type strain. The study used a genetic screen to find that when the \textit{slh} mutant was combined with the two \textit{drg} mutations, the growth was highly reduced. Not only did the growth change, but there was a shift in the ribosome profiles of those cells from having many polysomes to matching profiles of cells with translational defects (Daugeron et al., 2011). As long as the SLH protein, or one of the DRG proteins was functioning, that weak growth was not observed. The functional redundancy between disparate proteins suggests that the protein’s function is integral to the cell’s survival.

There have been some contrasting results coming from the mutant analyses of yeast when compared to the results from \textit{Arabidopsis thaliana} in our lab. Yeast is a single-celled fungus that spends most of its life cycle as a haploid organism (one complete set of chromosomes). \textit{Arabidopsis}, a representative of higher plants, is multicellular and spends most of the lifecycle as a diploid organism (two sets of chromosomes). The phenotype of the yeast double \textit{drg1;drg2} mutant is not noticeably different from that of the wild-type. In contrast, it requires a triple mutant of the \textit{slh} gene in addition to the \textit{drg1;drg2} double mutation to produce a slower-growing phenotype (Daugeron et al., 2011). Conversely, the \textit{drg1;drg2} double mutant in \textit{Arabidopsis} has a slow-growing phenotype compared to the wild-type plant (J. Stafstrom, personal)
communication; Srygler, 2013). Despite extensive efforts, we have not been able to produce a drg1;drg2;slh homozygous triple mutant in a plant (Marcec, 2015). This indicates that a higher plant like Arabidopsis needs at least one of the three genes to survive. This dissertation used the moss Physcomitrella patens, which has a life cycle that is distinct from both yeast and Arabidopsis. Like yeast, moss spends most of the lifecycle as a haploid organism; but like higher plants, moss is a complex multicellular organism. Table 3 lists the genes of Physcomitrella that will be studied here, compared to the orthologous genes in Arabidopsis. Analysis of the knockouts in this “in-between” species was meant to increase the understanding of this genetic pathway which is present in so many lifeforms; with an ultimate goal of having insights about the DRG pathway in humans.

Table 3: Physcomitrella patens genes and the orthologs in Arabidopsis. Each includes the gene name used in this dissertation, as well as the gene name in the Phytozome and TAIR databases. The last column lists the amino acid sequence similarity and identity between the Physcomitrella protein and the corresponding Arabidopsis protein.

<table>
<thead>
<tr>
<th>Arabidopsis</th>
<th>Physcomitrella</th>
<th>Similarity</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRG1 At4g39520</td>
<td>DRG1 Pp3c17_5590</td>
<td>95%</td>
<td>86%</td>
</tr>
<tr>
<td>DRG2 At1g17470</td>
<td>DRG2 Pp3c9_19290</td>
<td>94%</td>
<td>87%</td>
</tr>
<tr>
<td>DRG3 At1g72660</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>DFRP1 At2g20280</td>
<td>DFRP1a Pp3c25_2690</td>
<td>85%</td>
<td>76%</td>
</tr>
<tr>
<td>DFRP1</td>
<td>DFRP1b Pp3c6_18260</td>
<td>83%</td>
<td>75%</td>
</tr>
<tr>
<td>DFRP2 At1g51730</td>
<td>DFRP2a Pp3c5_13990</td>
<td>75%</td>
<td>56%</td>
</tr>
<tr>
<td>DFRP2</td>
<td>DFRP2b Pp3c27_5930</td>
<td>78%</td>
<td>60%</td>
</tr>
<tr>
<td>SLH1 At5g61140</td>
<td>SLH Pp3c2_9850</td>
<td>76%</td>
<td>62%</td>
</tr>
</tbody>
</table>
Materials and Methods

Physcomitrella Propagation

The starting culture of *Physcomitrella patens* was from the Gransden subspecies that was
The cultures are grown in sterile conditions, but they do not require much equipment. The moss
grows under normal fluorescent tube lighting at room temperature. The growth areas are
fluorescent lights suspended approximately 1-1.5 feet above lab benches and shelves. Normal
lighting conditions are 16 hours light, 8 hours dark. However, rate of growth can be increased
slightly by using continuous light (Cove, 2005).

Active cultures must be renewed every 10-14 days. Moss tissue is removed from the
Petri dish, added to 3ml sterile H₂O, and disrupted using a tissue homogenizer. Aliquots of the
disrupted tissue are then spread on two to three new PpNH₄ plates, equivalent to a 1:3 dilution.
The PpNH₄ growth media is 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 (Wu et al., 2011). A sheet of cellophane
is placed over the agar to make removal of the tissue easier. Figure 4 shows the ease of removal
of the moss from on top of the cellophane-covered PpNH₄ plates. After the disrupted moss
tissue is spread on top of the cellophane-covered agar, the plates are sealed with surgical tape
(3M Micropore 1530-0) to decrease contamination while allowing air exchange. Long-term
storage of the moss is done by growing tissue directly on agar without cellophane for one week, and then sealing the plates in parafilm and storing in 4°C with two hours of light per day.

**Figure 4: Moss culture propagation.** Left: The protonemata tissue is removed from the old plate with a sterile spatula. The cellophane that covers the agar gel prevents the filaments from growing into the media. Right: Growth conditions simply require some lab space at room temperature with a pair of fluorescent tube lights.

**Building Knockout Constructs to Target Each Gene of Interest**

To target the genes of interest for removal via homologous recombination, a linearized knockout construct was designed and produced for each of the seven *Physcomitrella* genes. The constructs were built using the Invitrogen BP Clonase II and Invitrogen Gateway LR Clonase II Plus enzyme kits. These enzyme mixes produce DNA recombination between *attB* & *attP* and
attL & attR sites respectively. The BP Clonase enzymes recombine an attB site with an attP site, creating an attL site. The attL site can then recombine with an attR site using the LR Clonase kit. Earlier use of this technology moved single fragments of DNA into plasmid constructs. Advances in this technology added variations of the original att sites to allow for multi-fragment cloning (Petersen and Stowers, 2011). The reactions are specific, so only attB1 will react with attP1, and only attB3 will react with attP3, etc.

To remove a gene by recombination, the construct requires a 1kb homologous region from each side of the targeted gene. Primers were designed to amplify a 1kb region of DNA upstream of the gene which included the ATG start site. Another primer pair was designed for a 1kb region of DNA downstream of the end of the coding region. Table 4 lists the primers designed to amplify a 1kb region up- and downstream of each gene. When the primers were manufactured, the appropriate att site was added to the 5’ end of each primer. Also included in some of the primers were restriction sites for eventual linearization of the finished construct. PCR was used to amplify the 1kb regions with added att sites and restriction sites.
Table 4: Forward and reverse primers to amplify 1kb of genomic DNA flanking each gene.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fragment</th>
<th>att site</th>
<th>genomic sequence (red is rev. compl.)</th>
<th>primer sequence, includes att sites (black) and restriction sites (purple)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRG1</td>
<td>D1upF</td>
<td>attB1</td>
<td>AGGTCTTAGTACTAATCTCTACAGG</td>
<td>GGAGGACAAGTTTGTACAAAAAACAGGCTACCGTACGTTCTAGTACTAATCTCTCAGGCT</td>
</tr>
<tr>
<td></td>
<td>D1upR</td>
<td>attB4</td>
<td>CATGACGTTGACATGTTGATTAC</td>
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</tr>
<tr>
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<td>attB3</td>
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<td>DF1upF</td>
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</tr>
</tbody>
</table>
BP Reaction

The BP Clonase II enzyme mix was used to recombine the \textit{attB} regions at the ends of the PCR product with the \textit{attP} regions of the donor vector as shown in Figure 5. This reaction replaces the \textit{ccdB} gene with the PCR product, forming an entry clone. The BP reaction used 7.5-75 ng \textit{attB}-flanked PCR product, 75 ng pDONR vector (pDONR P1P4 or pDONR P3P2), 1X TE buffer up to 4 µl, and 1 µl BP Clonase II enzyme mix. The reaction was incubated at room temperature for one hour, and then 0.5 µl Proteinase K was added and incubated for 10 minutes at 37°C.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{bp_reaction_diagram.png}
\caption{Two BP reactions to make the entry clones containing the upstream and downstream targeting arms for the DF2a gene. Each PCR product has the \textit{attB} sites and a restriction site (RE) added during PCR amplification. The BP Clonase enzyme kit replaces the \textit{ccdB} gene with the targeting arm. The blue arrows represent the T7 and M13 primers used for sequencing across the att sites to verify sequence of entry clones.}
\end{figure}
The BP reaction product was then transformed into chemicompetent DH5α cells (Sambrook et al., 1989). Chemicompetent cells were transformed by adding the 5.5 µl BP product with 100 µl chemicompetent cells. The cells and the entry clone incubate on ice for 30 minutes, followed by 90 seconds in a 42°C water bath and another 5 minutes on ice. Next, 1 ml LB broth supplemented with 20 mM glucose was added to the mix and incubated for 1 hour in a 37°C shaker (Bertani, 1951). The cells were pelleted for 3 minutes at 16,000xg. The pellet was resuspended in 100 µl LB and spread on an LB + 50 µg/ml Kanamycin agar plate for selection, and incubated overnight at 37°C. The ccdB gene is an inhibitor of most E. coli stains, so growth of transformed E. coli indicates a successful reaction. Glycerol stocks were made, and plasmid minipreps isolated the plasmid from each colony. The proper entry clones were then verified using primers that flank both att sites of the entry clone. A separate entry clone was produced with the upstream targeting region and the downstream targeting region. All 14 entry clones were sequenced across the att sites to ensure that the reactions were successful. The two entry clones for each gene are two of the four plasmids to be used in the next step.

**LR Reaction**

The next step was the 3-fragment LR recombination, shown in Figure 6. The LR Clonase II plus enzyme kit was used to recombine the attL-containing entry clones made above with attR sites in a destination vector, which also contains a ccdB gene for selection. The fourth plasmid contains a selection cassette flanked by attR sites that are different than the sites found in the
destination vector. Selection cassettes were available with Zeocin, Kanamycin, and Hygromycin resistance. The LR reaction places the selection cassette between the two targeting arms. The specificity of the reaction ensures that the three fragments are ligated in the proper order and direction to ensure they line up with the targeted areas on the genome. The reaction includes 12.5-37.5 ng of each entry clone (BP reaction product), 12.5-37.5 ng selection cassette plasmid, 37.5 ng pGEM-Gate destination vector, bring up to 4 µl with TE buffer, and 1 µl LR Clonase enzyme mix. The reaction was incubated at room temperature for several hours, and then incubated at 37°C for 10 minutes. The 5.5 µl LR reaction product was mixed with 100 µl electrocompetent DH5α cells placed in a pre-chilled 1mm gap cuvette and transformed by electroporation. Production of electrocompetent cells followed the cell preparation protocol found in the BTX Electro Cell Manipulator 600 manual. The remaining steps follow the post-heat shock steps listed above in the BP reaction methods.

Each completed knockout construct was verified via PCR with four pairs of primers. Each pair spans one of the four att sites to ensure the correct build of the construct. The att sites for the DF2a knockout construct are shown in Figure 7. All seven gene knockout constructs were also verified by sequencing two amplified regions containing the att sites. The amplified products were made using primers at the locations indicated by red arrows in Figure 7. Once verified, the knockout construct was linearized with restriction enzymes that targeted the restriction sites included 5’ of the upstream targeting arm and 3’ of the downstream targeting arm. The plasmid map in Figure 7 is representative of each of the seven knockout constructs that were built for removing these genes.
Figure 6: LR reaction uses the LR Clonase II Plus enzyme kit. Each of the two targeting arms are ligated into the pGEM-Gate destination vector, with a selection cassette inserted in between the two arms. The 3-fragment reaction replaces the ccdB gene, forming the knockout construct for one gene (DF2a shown here). The final step uses a restriction enzyme to digest the plasmid at the two indicated restriction sites (RE1 and RE2).
Figure 7: Plasmid map of the completed knockout construct made to target the DF2a gene in *Physcomitrella patens*. Each *attB* site is the location where one of the three fragments was ligated to another fragment, or to the destination vector. Each *att* site has a pair of primers to verify that the correct pieces of DNA were ligated in the correct order and direction. Final verification was performed by amplifying the two regions indicated by the red arrows. Each amplified region contained two of the four *att* sites. The amplified regions were purified and sequenced to verify the final knockout construct. The areas labeled RE1 and RE2 are the restriction sites which are later used to linearize the construct prior to moss transformation. Linearization produces a piece of DNA which includes targeting arms for both upstream and downstream of the gene. Those targeting arms flank the antibiotic resistance gene which will be used for selection of transformed plants.
Results

Verification of BP Reaction

Fourteen entry clones were produced using the Invitrogen BP Clonase II kit, one pair for each of the seven genes of interest. Figure 8 shows the plasmid map for the D1up entry clone. This plasmid contains the upstream targeting arm for the *Physcomitrella Drg1* gene. The plasmid collected from individual colonies of transformed DH5α cells were screened by PCR using primers designed to amplify a product only if the proper entry clone was produced. Each pair of primers spans one of the two *att* sites used to produce the plasmid. Figure 9 demonstrates a representative PCR result, verifying the D1up entry clone using both primer pairs to screen four colonies. The selected colony was sequenced across the *att* site for final verification.

Verification of LR Reaction

Seven knockout constructs were produced using the Invitrogen LR Clonase II Plus kit, one for each of the seven genes of interest. Figure 10 shows the plasmid map for the D1 construct. This plasmid contains both targeting arms separated by a selection cassette. Once verified, the construct is linearized to remove the gene-specific region from the remainder of the plasmid. This construct will target the *Physcomitrella Drg1* gene. The purified plasmid from individual colonies of transformed DH5α cells was screened by PCR using primers designed to amplify a product only if the proper knockout construct was produced. Each pair of primers
spans one of the four att sites that was used to produce the plasmid. Figure 11 demonstrates a representative PCR result, verifying the D1 knockout construct using all four primer pairs to screen colonies. The chosen colony was used to amplify two regions, each containing 2 att sites. Each region was then sequenced across the att sites.

**Figure 8: Representative entry clone plasmid map.** This plasmid map shows the D1up entry clone, one of two entry clones used to build the knockout construct to target the Drfl gene for knockout by homologous recombination. Verification of the entry clone was performed using two primer pairs. One pair (M13fwd / D1upR) spans the attL1 site. The other pair (D1upF / T7) spans the attL4 site. If the BP reaction produced the proper entry clone, both primer pairs will amplify a PCR product of the expected size.
Discussion

There were several issues that arose during the building of the knockout constructs. Most of the amplified targeting arms were easily produced. However, one of the fragments required that the primers be redesigned multiple times. The DF1a-up primers were not producing a PCR fragment despite altering the thermocycler conditions. Several different sets of primers were designed in the original region, each with no results. A successful primer was finally produced in a region approximately 600 bp downstream. The failure of multiple primers in the same region suggests that there are sequences in the published *Physcomitrella patens* genome that may not be accurate.

*Figure 9: Representative PCR screen of transformed colonies to verify presence of BP reaction product.* The presence of both bands at the expected size indicates that the BP reaction produced the correct entry clone. The primers correspond to both *attB*-spanning primer pairs shown in Figure 8.
Figure 10: Representative plasmid map of a knockout construct. The final plasmid to target the *Physcomitrella* *Drg1* gene is shown here. The primers indicated in the image show the sites amplified across each of the four *att* sites to verify a proper knockout construct.
The other issue was harder to solve, and used a considerable amount of time and some outside help to figure out. The LR reaction to produce the final knockout constructs used the Invitrogen Gateway LR Clonase II Plus enzyme kit. There are also LR Clonase and LR Clonase II enzyme kits available for similar reactions. The LR Clonase II kit is useful for ligating single fragments into the destination vector, but is not optimized for the multiple-fragment reactions. Plasmids were produced which contained one or multiple fragments in a

Figure 11: Representative PCR screen of transformed colonies to verify presence of LR reaction product. Two transformed colonies are screened for the presence of the complete knockout construct. The presence of all four bands at the expected size indicates that the LR reaction produced the correct gene knockout construct. The primer pairs correspond to those shown in Figure 10.
single piece of DNA, but they were either missing fragments, or the fragments were inserted in the wrong orientations. While the LR Clonase II Plus kit lists the same enzymes, the proprietary formulation is different enough that it is not useful for these purposes.

Fortunately a visit by Dr. Magdalena Bezanilla resolved this issue. As soon as the issues were described, she recognized the problem, having had the same issue in her own lab. She kindly offered to receive shipments of the entry clones to have her lab run the LR reactions using the proper kit, and then return the reaction products. Once the final constructs were received, they were quickly inserted into the competent bacteria and verified as described in the results section.

Much care was taken to verify each step of this process. Since a total of 21 different constructs were produced for this project, seven of which required ligating four pieces of DNA in the correct order and orientation, there were many opportunities for errors to occur. As described above, multiple pairs of primers were used to verify each construct. Every attachment site was flanked by primers to verify every DNA recombination event. After PCR verification, sequencing across the attachment sites was also performed. In the end, confidence was high that every construct was built as expected.
CHAPTER 2: PHYSCOMITRELLA PATENS TRANSFORMATION AND GENE KNOCKOUT

Introduction

Options for Producing Mutations

Reverse genetics is the search for a phenotype caused by a gene or group of genes (as opposed to finding the gene that causes a phenotype). To understand the function of a gene, it is helpful to remove the gene and then observe what change has occurred in the organism. There are now multiple options to choose from when attempting to remove a gene of interest. This section will discuss the pros and cons of the various techniques, including the techniques attempted and used for this dissertation research.

The first option, and one that has previously been used in this lab, is random T-DNA mutations. The lab previously worked with *Arabidopsis thaliana* to study the genes described in this dissertation. Mutated plants are ordered from seed stock centers such as the *Arabidopsis* Biological Resource Center at Ohio State University. These seed stocks are produced from labs that use T-DNA from *Agrobacterium tumefaciens* to make insertional mutations (Sussman et al., 2000; O’Malley et al., 2015). The inserted DNA disrupts the gene (Krysan et al., 1999). Plants are then screened and cataloged by location of the insertion. The benefit of this system is that there are thousands of genes that have already been knocked out, and the plant only needs to be
ordered online for delivery. To make a multiple-mutant plant, two different plants are crossed and then the F2 offspring are screened for homozygous mutants for both genes. The problem with relying on this method of gene knockout is that the insertions are random. If a gene of interest has not been knocked out, the lab is out of luck.

The remaining options are techniques to target specific genes, so a lab does not need to rely on the chance of a random insertion existing in a seed bank. The techniques are similar to the “Search and Delete” option in a computer program. The earlier methods included Zinc finger nucleases (Bibikova et al., 2003; Carroll, 2011) and TALENs (Moscou and Bogdanove, 2009; Boch et al., 2009). Both systems were designed to search for specific sequences and cleave the DNA. These systems were useful but were quickly replaced by CRISPR, which is much easier to design and produce for specific genes (Lander, 2016).

The newer CRISPR-Cas9 system has been quickly adapted for use in many research organisms, and very recently was used in a yet-to-be-published experimental treatment of sickle cell disease in a human (Saey, 2019). CRISPR was first discovered as a prokaryotic acquired immune system that stored genetic information from previous invaders (Barrangou et al., 2007). Once the genetic information was saved in this system, it would be quickly degraded upon recognition during a subsequent infection. This natural defense was quickly converted into a system that could be manipulated to target genes in a wide variety of organisms (Gasiunas et al., 2012; Jinek et al., 2012). This system of gene targeting is now very widespread. Since the technology was still relatively young at the beginning of this project, the homologous recombination method described below was used.
Another option which does not remove the gene, but instead silences or down-regulates a gene is RNAi. RNAi does not alter the DNA but targets the mRNA (Zamore et al., 2000). This is a procedure that can be used in many organisms. It has previously been optimized for use in the organism used for this dissertation, *Physcomitrella patens* (Bezanilla et al., 2003; Bezanilla et al., 2005). One of the benefits of this option is that it can be designed to target homologous sequences in multiple genes within a gene family. This can be useful for removing redundant genes. The drawback is that the gene is still present in the DNA. While the transcript is downregulated, it may still produce some product. The RNAi-produced mutant is also not stable. Since it is a transient mutation, the affected plants must be analyzed while they are still very young (Bezanilla et al., 2003).

The option ultimately used for this project was gene knockout by homologous recombination. Homologous recombination is used in yeast genetics but occurs at low frequency in all plants, except for *Physcomitrella patens*. All other plants studied demonstrate a much higher rate of illegitimate recombination when compared to homologous recombination (Vergunst and Hooykaas, 1999; Schaefer and Zrýd, 2001). For this protocol, a knockout construct was designed specifically for each gene of interest in the organism. The linearized DNA was inserted into a single celled protoplast using PEG-mediated transformation, allowing the DNA construct to locate and remove the entire gene. The benefit of this option is that the entire gene is removed from the chromosome; whereas the T-DNA insertion mutations insert exogenous DNA into a gene, leaving the disrupted gene in place. When CRISPR makes double-stranded breaks and there is no genetic template to allow for homologous recombination, the break is fixed by non-homologous end joining which can lead to frame-shift mutations. Again,
the gene is physically still on the chromosome, just disrupted. The method used in this dissertation completely removed the gene from the genome.

**Single-Celled Protoplasts**

Unlike animal cells, plants have a rigid cell wall that surrounds each cell. Before inserting the gene knockout construct, that cell wall must be degraded. This is accomplished using Driselase, a mix of cell wall-digesting enzymes from *Basidiomycetes sp.* The enzymes are: cellulase, laminarinase, and Xylanase (Sigma-Aldrich, 2019). Plant cells are normally under turgor pressure, which presses the cell membrane against the cell wall. If the cell wall was simply digested in an aqueous solution, the cell would burst. The method used here includes mannitol in growth media and any solution that contains the digested protoplasts. The mannitol, when kept at the proper concentration, provides an isotonic solution that will prevent the protoplasts from shrinking or swelling.

**Materials and Methods**

**Linearization of the Knockout Construct**

Before the knockout constructs can be used for gene targeting, large amounts of the plasmid were produced and then linearized using restriction enzymes. When the primers were
designed to amplify the targeting arms, not only were *att* sites added to each end, but restriction sites were also included on the 5’ end of the upstream targeting arm and on the 3’ end of the downstream targeting arm. As shown above in Figure 7, the use of XhoI and MluI will excise from the plasmid a continuous length of DNA that contains both targeting arms and the selection cassette.

Each transformation procedure described below requires 30 µg of linearized DNA. To purify enough DNA, a plasmid prep was performed using the Promega PureYield Plasmid Maxiprep System. A starting culture was made with either a frozen glycerol stock or a freshly-grown colony of a knockout construct containing *E. coli*. A tube that contained 5 ml LB + 100 µg/ml ampicillin was inoculated from the stock and grown for 8 hours at 37°C in a shaking incubator. The starting culture was then used to inoculate a larger flask; 500 µl of starting culture was added to 250 ml LB + 100 µg/ml ampicillin and grown to O.D.₆₀₀ 2-4 overnight in a shaking 37°C incubator. The culture was centrifuged at 5,000 xg for 10 minutes in a GSA rotor. The pellet was then resuspended, lysed, and neutralized following the kit protocol. The lysed cells were centrifuged at 14,000 xg in an SS-34 rotor. The cleared lysate was then filtered and passed through the binding column using a vacuum manifold. The column was washed with the endotoxin removal wash and then further column washes. Plasmid was then eluted using nuclease-free water. Plasmid concentration was measured using a NanoDrop spectrophotometer.

The purified plasmid was then linearized before transformation. 60 µg DNA was mixed with 30 µl restriction enzyme buffer, brought up to 295 µl with H₂O, and mixed with 5 µl of the appropriate restriction enzyme. The mix was incubation at 37°C for 2-4 hours and then a small
aliquot was run on an agarose gel to check for the appropriately sized band. Lastly, the linearized construct was purified with an EtOH precipitation.

**Transformation**

The linearized constructs were then transformed into *Physcomitrella patens* using PEG-mediated transformation. This procedure is based on the DNA-uptake method used to make the first stable transformations of *Physcomitrella* (Schaefer et al., 1991). To transform the moss, single-celled protoplasts are produced using the enzyme Driselase to digest the cell walls. The protoplasts are made in an isotonic solution of 8.5% mannitol and 0.5% Driselase so that they do not burst when the cell walls are removed. The protoplasts are pelleted three times at 160xg and resuspended each time in an isotonic mannitol solution, as shown in Figure 12. After the last spin, the cells are resuspended at 2 x 10^6 protoplasts/ml in 3M solution (0.5 M mannitol, 15 mM MgCl₂, and 10% MES solution (1% MES, pH5.6)) and then mixed with polyethylene glycol and 30 µg of the linearized knockout construct that has been purified by an ethanol precipitation (Bezanilla, 2012). This mix is heat shocked at 45°C for three minutes to transfer the DNA into the cells. The protoplasts are then grown in a top agar poured on a cellophane-covered bottom agar, shown in Figure 12. Both top agar and bottom agar media is the PpNH₄ media supplemented with mannitol and CaCl₂. The protoplasts regenerate the cell walls for four days, before being transferred to an antibiotic plate. Selection is with 100 µg/ml Zeocin, 30 µg/ml
Geneticin, or 15 µg/ml Hygromycin, depending on the selection cassette used in the knockout construct. The selection lasts for seven days. Then the selection is relaxed for seven days by moving the cells to a regular PpNH4 plate. This is followed by another seven days on a second round of selection. The week between the two rounds of selection is to ensure that any plants that are surviving due to an episomal transformation of the selection cassette will lose it before the next round kills the plant.

**Figure 12: Moss protoplasts.** Left: After digesting the cell walls, filtering, and washing the protoplasts, millions of single cells are suspended in an isotonic solution. Right: Individual protoplast plated in top agar. Note the round shape of the cells compared to the elongated form of the protonemata cells shown in Figure 1.
After the second round of selection, the plants have grown large enough that they can be relocated onto a 12-plant PpNH₄ grid plate, shown in Figure 13. The plants then require another two weeks until they are large enough to gather tissue for genetic testing. Genotyping is performed using sterile toothpicks to excise a small portion of the plant tissue, approximately 2x2mm. The genomic DNA is then purified from the tissue using a modified Edward’s DNA preparation protocol (Edwards et al., 1991).

**Figure 13: Growing plants for genetic testing.** A dozen plants are placed onto each grid plate and grown until large enough that tissue can be harvested. Left: six of the grid plates. Each plant began as a single protoplast. Hundreds of these plants survive selection after transformation with a knockout construct. Each must be screened by PCR to find a true knockout. Right: a closeup of one of these plants. What look like fibers are the protonemata, filamentous growth which occurs before the plant switches to producing gametophores.
Verification of proper insertion of the linearized construct and removal of the gene was performed using up to 10 primer pairs for each gene. Internal primer pairs were designed to test for the presence of the selection cassette and the presence of the gene. Border-spanning primers were used to verify the insertion of the selection cassette at the proper locus, as well as the absence of the gene at that locus. The primer sites in the knockout construct and genomic DNA are shown in Figure 1. A transformation was deemed successful when multiple primer pairs indicated both the proper insertion of the construct as well as the absence of the gene.

**Figure 14: Homologous Recombination.** Linearized knockout construct (1) used to target the genomic gene of interest (2). In this representative example the DF2a gene is removed and replaced with the hygromycin resistance gene (3), which allows for selection of a transformed plant. Arrows represent primers used to verify insertion and gene removal. See figures 16 & 17 for examples of PCR verification at these locations. The hygromycin resistance cassette can then be removed (4) using Cre-mediated recombination to excise the DNA located between the pair of lox sites.
Once the gene knockout was verified, the plant was propagated and used for consecutive gene knockouts. Since there were three different selection cassettes, some of the consecutive knockouts could immediately follow the previous knockout. For knockouts where the new construct contained a selection cassette already present from a previous insertion, the original selection cassette was removed. Removal of the selection cassette was possible because of the lox sites that flanked the selection cassettes, shown in Figure 14. Using Cre-mediated recombination, the selection cassette was removed from the moss genome (Dale and Ow, 1990; Kuhn and Torres, 2002; Schaefer and Zryd, 2001). To accomplish this, protoplasts were produced and then transformed with a plasmid encoding the NLS-Cre enzyme. The NLS portion of the enzyme is to transport the Cre enzyme to the nucleus. Cre locates the lox sites that flank the selection cassette. If the cassette to be excised is in the plastid DNA rather than the nuclear DNA, Cre is also affective (Lutz et al., 2006). The plants were then regenerated and tested with primers specific for the excised cassette. After PCR verification, removal of the selection cassette is verified a second time by placing a piece of tissue on a plate that contained the antibiotic, shown in Figure 15. If the piece on an antibiotic plate died while a piece on the control plate survived, it was deemed ready for transformation with another construct using the same selection cassette.
An alternative to making consecutive knockouts to achieve a multi-gene mutant is to use two different mutants and perform a sexual cross (Perroud et al., 2011). Some of the resulting offspring would include both mutations in a single genome. This type of cross was attempted using a published protocol (Cove et al., 2009). Pieces of protonemata from both plants were placed in a sterile container on a nitrogen-free medium. The plants are grown for several weeks in 24-hour light and 25°C, and then moved to 8-hour light and 15°C for several weeks. The plants alternate between the two growth conditions every couple weeks. This change is supposed to induce the production of reproductive tissues on the gametophores. To make sure that sperm and egg were produced by both mutants at the same time, tissues from plants of

**Figure 15: Follow-up test of selection cassette removal.** Two plants with Hygromycin resistance cassette removed and one control plant (cassette still present). A piece of each plant is grown on a hygromycin plate to verify the results from the PCR; that the cassette has been removed with the Cre enzyme.
different ages were staggered in the container to increase our chance of both being reproductive at the same time. Water was occasionally added to the containers, and they were placed on a slow shaker for a day at a time. The water was required to transfer the sperm to the egg because moss do not produce pollen. The sperm must travel through water to reach the egg. This procedure was attempted several times, but never resulted in the production of a spore-bearing sporophyte.

Results

The plants that survived growing on antibiotic selection were screened by PCR. The first round of testing used a single primer pair to search for the presence of a band indicating the presence of the construct, either using a primer for the selection cassette, or one of the targeting arms. Surprisingly, many of the plants growing on the plates didn’t produce a band with this first screen. For this reason, some of the successful knockouts required screening hundreds of plants before the successful gene knockout was produced. The plants that did succeed in the first round of PCR were then screened with a second pair of primers. This follow-up PCR would usually use one of the primer pairs that would not produce a band in a knockout, such as primers located in the targeted gene.

From a hundred plants, usually there were a few that passed both rounds of PCR screening. Those plants-of-interest were then screened with all available primer pairs to verify with certainty that it was a true gene knockout, and not a false negative. Representative
examples of these multi-primer pair PCR tests are shown below in Figures 16 and 17. Figure 16 shows the eight primer pairs (plus a primer pair for the selection cassette) that was used to screen potential SLH gene knockouts. Any of the primers numbered 2, 4, 6, or 8 would indicate the presence of the gene. If any of those pairs produced a band, the plant was discarded. To be accepted as a true gene knockout, the PCR test would need to lack bands for all even primers, and produce bands with the odd primers, as shown in Figure 17. Primer pairs 5 & 7 will produce bands if the knockout construct is simply present in the cell. If the construct is inserted at the proper locus, then primer pairs 1 & 3 can also produce bands. The gene knockouts were further substantiated during the qRT-PCR and Western experiments described in Chapter 4.

Six of the seven Physcomitrella genes in the DRG gene pathway were successfully knocked out. In addition to single mutants, there were eight multi-gene knockouts obtained. Each multi-gene knockout was produced by using a mutant plant and performing consecutive gene knockouts. Table 5 shows the full list of all 15 mutant Physcomitrella lines produced for this dissertation. As the Methods section described, some of these multi-gene knockouts required the Cre-lox mediated excision of the selection cassette before the consecutive gene knockout could be performed. The original goal was to produce a triple drg1;drg2;slh triple mutant. When it became apparent that the drg1 mutant might not be obtained, the focus shifted to the Dfrp gene knockouts already underway. Crucial to this research was obtaining the quadruple mutant, a plant missing all four DFRP proteins so that any redundant proteins would be absent.
Figure 16: A representative example of the primers that verify presence of the wildtype gene in the genome. If all four even primer pairs produce bands, and the four odd primers do not produce bands, then the gene is still present and the knockout construct is absent.
Figure 17: A representative example of the primers that verify the removal of the wildtype gene from the genome. If the four odd-numbered primer pairs produce bands, and the four even primers do not, then the construct has inserted in the correct locus and removed the gene. This can also be verified by the lack of a band when using the internal primers for the wildtype gene, labeled wt primer.
Producing and testing the quadruple *Dfrp* knockout was the ultimate goal of this dissertation. It was important to produce a mutant plant with all four genes removed to have a functional DFRP deletion mutant. Since some of the knockout constructs used the same antibiotic for selection, two selection cassettes needed to be removed from the genome of a double knockout before the quadruple 1a;1b;2a;2b knockout could be produced. To produce the quadruple knockout, *Dfrp1b* was removed, and then verified. Next, *Dfrp2b* was removed from the 1b single mutant, and that double knockout was verified. Each of the first two knockout constructs used a different selection cassette that was also used in the 3rd and 4th cassettes, so the

Table 5: Successful single- and multi-gene knockouts produced for this dissertation. The focus of this dissertation is on the *Physcomitrella Dfrp* genes. *Drg* and *Slh* physically or genetically interact, so are all included in the “DRG pathway.”

<table>
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<tr>
<th>Single DFRP knockouts</th>
<th>Triple DFRP knockout</th>
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<tr>
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</tr>
<tr>
<td>DF1b</td>
<td>✓</td>
</tr>
<tr>
<td>DF2a</td>
<td>✓</td>
</tr>
<tr>
<td>DF2b</td>
<td>✓</td>
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<table>
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<tr>
<th>Double DFRP knockouts</th>
<th>Quadruple DFRP knockout</th>
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</thead>
<tbody>
<tr>
<td>1a;1b</td>
<td>✓ 1a;1b;2a;2b</td>
</tr>
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<td>✓</td>
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<tr>
<td>2a;1b</td>
<td>✓</td>
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<table>
<thead>
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<th>Other Genes of Interest</th>
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<tbody>
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<tr>
<td></td>
<td>DRG2</td>
</tr>
<tr>
<td></td>
<td>SLH</td>
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Producing and testing the quadruple *Dfrp* knockout was the ultimate goal of this dissertation. It was important to produce a mutant plant with all four genes removed to have a functional DFRP deletion mutant. Since some of the knockout constructs used the same antibiotic for selection, two selection cassettes needed to be removed from the genome of a double knockout before the quadruple 1a;1b;2a;2b knockout could be produced. To produce the quadruple knockout, *Dfrp1b* was removed, and then verified. Next, *Dfrp2b* was removed from the 1b single mutant, and that double knockout was verified. Each of the first two knockout constructs used a different selection cassette that was also used in the 3rd and 4th cassettes, so the
Cre-lox system was used to remove both cassettes. Then the removal was verified as described in the Materials and Methods section. After removal of the selection cassettes was verified, the double knockout was used to produce a triple, and then the quadruple *Dfrp* mutant.

Figures 18 and 19 show the multiple PCR tests that were performed to verify with high certainty that this was a true quadruple *dfrp* knockout. Figure 18a gives the primer sites in the genomic DNA and the knockout construct. Figure 18b shows the screen of 48 of the plants which survived selection on the antibiotic plates. From 48 plants, only one was missing the *Df2a* gene. Figure 19a used two primer pairs. Primer pair 3 indicates the insertion of the knockout construct at the correct locus. Primer pair 4 indicates the presence of the *Df2a* gene. The sample (#B2) has the proper insertion, as it matches the 2a control and the 1a;2a control. Both controls are DNA samples from two other unique *df2a* knockouts. Figure 19b shows the original DNA sample from the quadruple knockout, and a “new” DNA sample made from the same plant to verify the first sample. The quadruple knockout was compared to wildtype DNA, all four single *dfrp* knockouts and two doubles. Each sample was screened with primers for all four *Dfrp* genes and a control. The gel in figure 19b gave confidence that this plant was a true quadruple *dfrp* knockout.
Figure 18: Primers for verifying the last of the four knockouts in the quadruple DFRP mutant.  
A: All primers used to test for the Df2a gene and knockout by insertion.  
B: Screen of 48 plants which survived selection. Primers 15-251/15-252 indicated in the diagram were used to screen for the absence of the Df2a gene. The missing lane marked “plant B2” was then tested further, results shown in Figure 19.
Figure 19: Additional PCR tests of the Quadruple Dfrp knockout.  

A: The quadruple knockout was verified by testing for both the presence of the insertion at the proper locus (primer 3) and the absence of the Df2a gene (primer 4). Primer pairs 3 & 4 are highlighted in figure 18a.  

B: Two DNA preps from the quadruple knockout compared to seven control DNA samples. Each sample was tested with primer pairs for the four different Dfrp genes and SLH (positive control). Note: the first two lanes with the 2b primers are false negatives.
Discussion

The transformation process was fairly simple in design. The plant tissue was reduced to protoplasts, individual plant cells, using an enzyme mix that digests the cell wall. The cell suspension was then mixed with the linearized knockout construct and treated with conditions that caused some cells to take-up the construct. Once inside the cell, the targeting arms of the construct recombine with the homologous region of the genomic DNA and replace the gene with an antibiotic resistance gene. When the cells have regenerated the cell walls after a few days, they begin a few rounds of selection on antibiotic plates. Any cells that survive selection and grow into larger plants should be doing so because of the presence of the selection cassette. From those plants, some should contain the selection cassette in place of the targeted gene.

Many False Positives

Unfortunately, there were many setbacks that caused the knockout portion of this project to take much longer than expected. The first issue was that the first couple rounds of transformation had used a linearized knockout construct that contained some reagents that were harmful to the protoplasts. After linearization using restriction enzymes, the restriction mix was promptly used for the transformation. The result was that the cells quickly died. There were few or no surviving protoplasts at the point when the cells were moved to the first round of selection. This issue was recognized and remedied by performing an ethanol precipitation following the restriction enzyme digest.
The next problem, as mentioned in the Results, was that there were many plants that were surviving the antibiotic selection process that were not true knockouts. A lot of time was dedicated to testing many plants that were eventually discarded. The Appendix shows the number of plants tested for this dissertation. The first reason was that some of the surviving plants did not have the selection cassette present. When the initial PCR screening used the selection cassette primers, such as 14-442/14-443 in Figure 17, approximately half of the growing plants were negative for the cassette. The antibiotic dose was questioned. The selection media used 100 µg/ml Zeocin, 15 µg/ml Hygromycin, or 30 µg/ml Geneticin (Wu et al., 2011). These antibiotic doses were verified as the same used in other Physcomitrella research as well. It didn’t seem to be a problem with the specific batches of antibiotics used for this research, as they were lethal as shown above in Figure 15.

The plants that did have the selection cassette present were often not true knockouts. This can happen when the cassette does not properly insert into the correct locus as shown in Figure 20. The figure shows an example of a follow-up PCR test of a plant that was positive for a screen for presence of the knockout construct. Primer pairs 5 and 7 indicate that the construct is present in the plant. However, the presence of bands for primer pairs 2, 4, 6, and 8 show that the gene is still present. As the absence of primer pairs 1 and 3 show, the construct is present, but not at the correct locus. Figure 20 is in contrast to the positive and negative results shown in figures 16 and 17 above. The knockout construct can be present, but without inserting into the chromosome. Episomal expression in Physcomitrella has been demonstrated previously (Schaefer et al., 1991; Ashton et al., 2000; Murén et al., 2009). Another related result was insertion of the construct without removal of the gene. An example of this is shown in Figure
21. The positive result with primer pairs 1 & 4 and the negative result with primer pairs 2 & 3 show that the construct recombined with the upstream region, but not the downstream region. This probably led to the insertion of the construct upstream of the gene.

**Figure 20**: A representative example of the primers that verify presence of the knockout construct without the removal of the wildtype gene from the genome. The knockout construct is present in the plant, as indicated by the positive results with primer pairs 5 & 7. However, the positive results with primer pairs 2, 4, 6, and 8 show that the gene is still present. The absence of bands with both primers 1 & 2 indicate that the construct did not recombine with either targeted region.
Figure 21: A representative example of the primers that verify improper insertion of the knockout construct into the genome. The knockout construct inserted into the chromosome at the proper upstream site, as show by primer pairs 1 & 2, but primers 3 & 4 show that the targeted gene is still present in the chromosome at the downstream region. Results like this demonstrate the reason that so many primer pairs were used to verify each gene knockout. Odd insertions have produced some bands, that by themselves would indicate a successful knockout.
Mixed Colony Plants

One last problem that arose when verifying mutant plants was “mixed colony” plants. Each plant that is tested should have started from an individual transformed cell. The plant is the equivalent of a colony of bacteria that contains many clones of a single cell. Some of the 8-primer PCR test would show both the presence of the construct in the correct locus as well as the presence of the gene in the correct locus. The hypothesis was that the plant being tested had resulted from two surviving protoplasts growing very close together. One protoplast was a knockout, while the other was not. This was the correct assumption in at least one case. The 1a;2b double DFRP mutant was a mixed colony plant. The protonemata tissue was picked apart and many small pieces were grown to find the one that was a true gene knockout.

Physcomitrella Sexual Crosses

When it became apparent that the knockouts through homologous recombination were taking a long time to produce, an alternate approach was taken. As described in the Materials and Methods section, Physcomitrella can be brought to sexual maturity and then crossed with another individual. This was attempted, using combinations of two different double Dfrp mutants, or a single and a triple Dfrp mutant. This was attempted before the quadruple Dfrp mutant was produced through homologous recombination. Unfortunately, despite following published protocols, the moss never produced sporophytes. That indicated that the plants were not fertile despite using the protocol described above. A note in the protocol stated that strains
that were vegetatively propagated for long periods may not be fertile (Cove et al., 2009). The Gransden subspecies that was used for this research has been maintained vegetatively for decades.

**Unobtained Drg1 Knockout**

The original goal of this dissertation was to produce a *drg1;drg2;slh* triple mutant in *Physcomitrella patens*. This was important since the triple mutant *Arabidopsis thaliana* was not able to be produced by the lab. The *Arabidopsis drg1;drg2* double mutant had an observable phenotype, including slower growth of both root and aerial tissues. As described in the Results section, the *Physcomitrella drg1* mutant was not produced. This should have been possible; the sequence upstream and downstream of the *Drg1* gene were both verified by PCR and sequenced once the entry clones were built. After the LR reaction, the D1 knockout construct was also verified by PCR and sequencing. It is possible that with more time for additional transformations and PCR screening, that a *drg1* knockout would eventually be obtained. Since the *Dfrp* genes were producing better knockout results, the remaining time focused on getting to the *dfrp* quadruple knockout. As will be described in the next chapter, the *dfrp* knockouts should function as knockdowns of the *Drg* genes.
CHAPTER 3: MORPHOLOGY AND AMINO ACID STARVATION

Introduction

Morphology of Gene Knockouts in *Arabidopsis thaliana*

Previous work in this lab has studied the phenotypic effects of the single and double *drg* and *dfrp* mutants in *Arabidopsis thaliana* (Nelson, 2008; Srygler, 2012; J. Stafstrom, personal communication). The *drg* double mutant has the most obvious change in phenotype. The plant generally looks weaker; it grows smaller, and it is a lighter shade of green. Figure 22 shows an example of the *drg1;drg2* double mutant compared to the *dfrp1;dfrp2* double mutant in *Arabidopsis* (modified from Srygler, 2012). The phenotype is more pronounced in the double compared to either single *drg* mutant. The double *dfrp* mutant had a much more subtle change in growth.

**Genotype Effect on Phenotype: Arabidopsis vs. Saccharomyces**

The change in phenotype of the *drg1;drg2* double knockout in *Arabidopsis* was not observed in the yeast, *Saccharomyces cerevisiae*. In the yeast, the growth of the double mutant closely resembled the growth of the wildtype yeast (Daugeron et al., 2011). As described in the Chapter 1 Introduction, a change in growth was not observed until they made a triple knockout with the addition of a mutation in the *Slh* gene. Since *Arabidopsis* and *Saccharomyces* are two
very different organisms, and show different reactions to a double \textit{drg1;drg2} mutation, this dissertation was aiming to study the effects on an intermediate species. Like the yeast, the lifecycle of \textit{Physcomitrella} is mostly as a haploid organism. But like \textit{Arabidopsis}, the growth pattern of \textit{Physcomitrella} is as a multicellular organism. The interest was to experimentally observe whether the \textit{drg1;drg2} double mutant moss would behave more like the haploid yeast or the multicellular \textit{Arabidopsis}. As described at the end of Chapter 2, the lack of a \textit{drg1} mutant had somewhat quashed the ability to test the question. While there is no \textit{drg1 Physcomitrella} knockout, there may be a knockdown mutant as described in the next section.

\begin{figure}[h]
\centering
\includegraphics[width=0.7\textwidth]{figure22}
\caption{An example of a phenotype change in \textit{Arabidopsis drg} and \textit{dfrp} double mutants. Root growth assays measured the length of the root after seven days of growth on vertical agar plates. The plants in the bottom half of the image are two different wildtype strains. The upper-left quadrant contains the \textit{drg1;drg2} double mutant, and the upper-right contains the \textit{dfrp1;dfrp2} double mutant. The root lengths are displayed on the graph. Growth under normal conditions is recorded by the blue bars. The \textit{drg} double mutant (L3) has much shorter roots compared to the two wildtype groups (L16, L17). The \textit{dfrp} double (L29) is slightly shorter, but not nearly to the degree observed in the \textit{drg} double. (Image modified from Srygler, 2012).}
\end{figure}
DFRP-Induced Knockdown of DRG

As explained in the Introduction of Chapter 1, the DRG and DFRP proteins physically interact. It is believed that this interaction stabilizes each protein and prevents protein degradation. Previous research in this lab has shown that when *Arabidopsis Dfrp1* or *Dfrp2* are knocked out, the corresponding DRG1 or DRG2 proteins are downregulated (Srygler, 2012; J. Stafstrom, personal communication). The small change in the phenotype of the *Arabidopsis dfrp1;dfrp2* mutant may be because there is less DRG protein accumulation. It is possible that the *Physcomitrella dfrp* quadruple knockout produced here will have less DRG protein. Therefore, by removing the *Dfrp* genes, there may be enough of a downregulation of DRG protein that a small phenotype change would be observed. As will be demonstrated in Chapter 4, this knockdown of DRG proteins is observed in *Physcomitrella dfrp* mutants.

Amino Acid Starvation and the DRG Gene Pathway

The yeast DRG2 ortholog (RBG2) and DFRP2 ortholog (GIR2) are thought to be involved in the amino acid starvation response in *Saccharomyces* (Ishikawa et al., 2013; Wout et al., 2009). When amino acid levels are low, uncharged tRNA molecules bind to the A-site of ribosomes and begin a cellular signal to adjust the metabolism. The uncharged tRNAs are detected by GCN1, which then signals GCN2. GCN2 phosphorylates eIF2, preventing eIF2B from acting as a GEF and converting eIF2-GDP back to eIF2-GTP. As described in Chapter 1, a
GEF is a protein that activates a GTP-binding protein by exchanging the bound GDP for GTP. Low levels of eIF2-GTP in the cell reduces the available ternary complexes. Depleted levels of ternary complexes make it more difficult for the two subunits of the ribosome to assemble.

Under normal conditions, translation of GCN4 is minimal because ribosomes dissociate after encountering some upstream ORFs on the mRNA. However, when the amino acid levels are low, the depleted ternary complexes act as a regulatory process by causing more of the scanning ribosomes on the mRNA to bypass the inhibitory uORFs upstream of GCN4. More of the scanning ribosomes are complete 80S ribosomal complexes and are ready to translate the GCN4 protein when they reach the start site. In summary, less ternary complexes caused by uncharged tRNAs will increase the translation of GCN4. This upregulating of GCN4 lowers translation rates and increases production of amino acid biosynthesis enzymes (Hinnebusch, 2005).

GIR2 (yeast DFRP2) has been shown to interact with GCN1, possibly acting as a competitive inhibitor of the interaction between GCN1 and GCN2. This could be due to the GI domain which is shared between GCN2 and GIR2. When GIR2 was increased, the cells behaved as they would under amino acid starvation. When both GIR2 and GCN2 were increased the cells behaved as they would under normal conditions, again indicating that they competitively bind with GCN1 (Wout et al., 2009). GIR2 and its binding partner RBG2 (DFRP2 and DRG2 orthologs) have also been shown to have increased stability under amino acid starvation, when in complex (Ishikawa et al., 2013). That study showed that the two bound proteins were also binding to GCN1 as amino acid starvation increased. When the binding was disrupted through a mutation, stress-induced cell growth of the yeast was disrupted. This suggests that DRG2 may
also be involved in the general amino acid control pathway. In the studies described here, the morphological differences of each mutant produced in Chapter 2 were analyzed, including the \textit{drg2} mutant and the \textit{dfrp2a}, \textit{dfrp2b}, and \textit{dfrp2a;2b} mutants.

DFRP2 is not the only protein in the DRG pathway that shares a domain with proteins known to be involved in amino acid starvation control. One of the domains found in both DRG1 and DRG2 is the TGS domain. This domain is also found in the prokaryotic SpoT and RelA proteins (Wout et al., 2009). SpoT and RelA have synthetase and hydrolase activity to control the amount of alarmone (p)ppGpp in a cell (Hernandez and Bremer, 1991; Xiao et al., 1991). The ppGpp molecule is a regulator of the stringent control, the bacterial response to amino acid starvation (Artsimovich et al., 2004). The bacterial OBG protein is in the same class of proteins as eukaryotic DRGs (Leipe et al., 2002). OBG proteins physically interact with SpoT (Raskin et al., 2007). It is possible that DRGs are involved in a similar amino acid starvation response in plants.

During nutrient starvation, a major setback that all cells face is the lack of amino acids for protein assembly. Plants not only lack amino acids for protein assembly, but they also use amino acid biosynthetic pathways in the production of secondary products such as auxin (Zhao et al., 1998). Loss of these metabolites may affect the growth and development of the plant. The Shikimate pathway, which was blocked in the present studies using glyphosate, is one of those biosynthetic pathways (Mobin et al., 2015).
Materials and Methods

**Morphological Analysis Using Chlorophyll Autofluorescence**

Morphological analysis of *Physcomitrella* was based off protocols used previously with the moss (Vidali et al., 2007). Each genetic line was grown on cellophane-covered PpNH₄ plates for nine days, and then used to generate protoplasts. Details of the protoplast generation protocol are described in Chapter 2. Protoplasts are single cells, with the cell wall removed. Starting from protoplasts ensures that each plant that will be analyzed has started from the same point. Prior to this experiment, serial dilutions of protoplast were grown to determine the optimal concentration of protoplasts that would allow enough plant growth without overcrowding the plates.

The protoplasts were grown on mannitol-containing PRMB plates for five days under 24-hour light. The cellophane, with the protoplasts, was then transferred to thin PpNH₄ plates. The thin plates were required so that the plants were in the depth of field of the microscope. They were then grown on the PpNH₄ plates for another five days under 24-hour light. On the tenth day, the plants were photographed using the EVOS M5000 Imaging System. All images were obtained using the 10X objective, and the Texas Red (585/29 Ex; 624/40 Em) light cube for fluorescence. This wavelength causes excitation of chlorophyll. Chlorophyll autofluorescence was recorded and used as an indicator for plant morphology.

Z-stacks were obtained using the EVOS M5000 software, so that different sections along the entire z-axis of the plant were in focus. Those z-stacks were then merged into a single image.
using Helicon Focus to obtain a single clear image of the plant. The merged image was then processed and analyzed using the open source ImageJ software (Rasband, 1997-2018). Images were cropped to 1500x1500 pixel squares. The auto threshold feature of ImageJ was used to view the outcome of all 16 thresholding methods, and the most accurate method for each image was chosen. Thresholding converts the image into a black and white image. Black pixels are measured as “plant” and the white pixels are measured as “not plant”. The image was then cleaned up by removing any background noise using the Despeckle feature as well as by manually removing spots. Lastly, any gaps in the plant caused by low fluorescence were manually filled in.

The analysis feature of ImageJ was used to measure morphological parameters of each group of plants. Measurements were assessed of basic features such as area and perimeter. Other values that were measured were circularity and solidity, which can describe the shape of the plant (Vidali et al., 2007). Circularity is the ratio of area to perimeter. A round plant will have a circularity value of 1. Lower values represent a linear shape. Solidity is a way to measure the amount of branching on the plant. A value of 1 for solidity will be a plant with no branches. As more branches produce more spaces within the plant, the value will decrease towards zero. For example, a mutant which decreases cell elongation will have a more globular shape. The small cells will have very little space between them because they are not spreading over the surface. This plant will have a solidity value near 1. Another plant which has normal cell growth will more quickly spread outwards, leaving empty areas between the branches. This plant will have a lower solidity value. The measurements used here are analyzing the total area of the plant as well as the growth patterns of the protonemata. If the plants were measured again
at a later stage, features such as number of buds and gametophores, and shapes of the phyllids could be analyzed as well (Schween et al., 2005; Demko et al., 2014).

Amino Acid Stress Response Induction and Analysis

The herbicide glyphosate was used to induce amino acid starvation in *Physcomitrella*. Under normal conditions plants produce their own amino acids. Biosynthesis of the aromatic amino acids is facilitated by the enzyme 5-enolpyruvylshikimate 3-phosphate, or EPSP (Schönbrunn et al., 2000). EPSP is the target of glyphosate. Exposing *Physcomitrella* to this herbicide blocks the enzymatic pathway to starve the plant of aromatic amino acids, and is used to trigger the amino acid response in the moss. There are very few auxotroph mutants available in plants. To allow scientists to produce phenocopies of such mutants in plants, treatments like glyphosate can allow the study of growth in the absence of nutrients.

There are no known publications of glyphosate application with this moss, so several pilot experiments were performed to develop and optimize a protocol. The first decision was whether to include glyphosate in the media or to apply topically to the plant. Topical application is how the herbicide is used in common farming practices, but it would be hard to quantify each dose accurately for this experiment. The concern with adding glyphosate into the growth media was that it may not be effective; that it could possibly bind to the agar. That concern was put to rest, as it was reported that glyphosate had previously been used in agar plates for testing of
uptake of the molecule into bacteria cells induced by phosphate starvation (Fitzgibbon & Braymer, 1988).

Next was the question of glyphosate dosage for *Physcomitrella*. No publications were found listing glyphosate dosage with this moss, so preliminary tests started with a range used in other plants. Glyphosate for other species include: 1.58-6.32 mM foliar spray on multiple species of turfgrass (Su et al., 2009; Unver et al., 2010); 15 mM spread on top of agar for *Arabidopsis* (Zhao et al., 1998); and 0, 0.5, 1, and 1.5 mM added to cultured *Echinacea* tissue in liquid media (Mobin et al., 2015).

Wild-type *Physcomitrella* protonemata were disrupted and grown for five days on PpNH₄ plates following the standard tissue propagation protocol described in Chapter 1. The plant tissue was then moved to PpNH₄ plates supplemented with a range of glyphosate levels. PpNH₄ glyphosate plates were made by adding filter-sterilized 0.05 M glyphosate to the autoclaved media before pouring the plates. The dosages were 0, 0.1, 1, 2, 3, 6 and 10 mM glyphosate final concentration. Several sections of each plate were labeled and photographed nearly every day. This exposure to glyphosate proceeded for approximately two weeks, until the 0 mM glyphosate plates had become overgrown with protonemata.

Two concentrations of glyphosate were then chosen based on the preliminary tests. The higher dose is 6 mM, high enough to kill the tissue in less than a week. The lower dose of 3 mM prevents growth but does not kill the plant. On 3 mM glyphosate, the protonemata retain the green color but don’t appear to have any cell division. Each mutant line of *Physcomitrella* and the wild-type tissue were disrupted and grown on PpNH₄ plates for five days. On the fifth day,
they were moved to new PpNH₄ plates supplemented with 0 mM, 3 mM or 6 mM glyphosate. Three sections of each plate were labeled and photographed using a dissecting microscope nearly every day, starting with Day 0 when they were first moved onto glyphosate. Images were compiled, tracking the growth of wild-type and mutant *Physcomitrella* over a week to compare how each mutant responded to amino acid starvation.

Each image was then processed and analyzed with ImageJ software. The photograph was converted to a binary image, where black pixels represented plant tissue. The Analyze Particles setting of ImageJ was then used to measure total area of the black pixels. The total area represented the amount of plant tissue in the field of view. The average of the triplicate measurements for each plate was recorded each day to track growth on each plate. The analysis compared the change in percentage of area covered in plant from day 0 up to day 6.

**Results**

**Morphological Analysis**

Each mutant was grown from protoplasts and then imaged on the EVOS M5000 fluorescent microscope. Figure 23 shows a representative example of the original images and the processed image used for morphological analysis. A series of z-stacks were taken of each plant using the fluorescent microscope. The z-stacks were then merged into a single photo showing the entire plant in focus. Using ImageJ, the merged photograph was converted to a
binary image and then the background noise was removed. Any low-fluorescence gaps in the plant which were not fixed by the program were manually adjusted.

Figure 23: Chlorophyll autofluorescence as an indicator of plant size and shape. Upper left: One of eight images in the z-stack showing chlorophyll autofluorescence. Upper right: Merged z-stacks, showing a single view containing the clearest areas of each of the eight images in the z-stack. Lower left: Binary image produced with ImageJ. The background noise was removed, and some of the gaps in the plant were automatically filled in. Lower right: All of the gaps must be manually adjusted so the analysis program makes the correct estimate of measurements such as area.
An average of 24 plants were imaged and processed from each of the 15 genetic lines. The values for area, perimeter, circularity, and solidity are shown in Figures 24 and 25. Circularity and solidity measurements are described in the Materials and Methods above.

Figure 24: Average area and perimeter measurements for *Physcomitrella* mutants. Measurements processed with ImageJ software.
Figure 25: Average circularity and solidity measurements for *Physcomitrella* mutants. Measurements processed with ImageJ software.
Follow-up Morphological Analysis

As described in the Discussion below, the morphological experiment was repeated, to focus on only the wildtype and *dfrp* quadruple mutant. Testing fewer plants allowed the plates to be centered below the light source. The results of this follow-up showed that, when light intensity is equivalent, there is a morphological difference between the two plants when grown from protoplasts. Figure 26 shows representative images of the wildtype compared to two quadruple mutants. Figures 27 and 28 show the measurements of the two populations of plants.

Figure 26: Representative example of wildtype and quadruple *dfrp* mutant. The wildtype Physcomitrella (left) has a much larger growth pattern compared to the Quad mutant (two plants, right). Scale bar is 100µm.
Figure 27: Area and Perimeter of wildtype and quadruple dfrp mutant. When light conditions are controlled, the measurements of the wildtype (n=26) and quadruple dfrp mutants (n=29) become noticeably different.
Figure 28: Circularity and Solidity of wildtype and quadruple *df rp* mutant. When light conditions are controlled, the measurements of the wildtype (n=26) and quadruple *df rp* mutants (n=29) become noticeably different.
Growth Following Amino Acid Starvation

All 15 *Physcomitrella* genetic lines were placed on PpNH₄ plates supplemented with 0 mM, 3 mM, and 6 mM glyphosate. Photographs were taken of three areas on each plate starting the day that the glyphosate exposure began. The daily photographs were analyzed by ImageJ. For each plate, the average change in percentage of plant tissue was calculated. The results for all 15 lines on 0, 3, and 6 mM glyphosate is shown in Figure 29. Figures 30-32 show a representative set of images showing the plant’s normal growth on a control plate. On 3 mM glyphosate, the plant is alive, but with growth nearly halted. The 6 mM glyphosate plates kill the tissue within days.

![Effect of glyphosate on each mutant](image)

**Figure 29: Effects of 3 mM and 6 mM glyphosate on each *Physcomitrella* mutant compared to control.** The change in average percentage of coverage used the day 6 percentage of area minus the percentage of area covered on day 0. Measurements were processed with ImageJ software.
**Figure 30:** Representative growth on no-glyphosate plate. Control showing regular growth. Day 0 image is taken when the plant tissue is moved onto the control plate.

**Figure 31:** Representative growth on 3 mM glyphosate. The plant tissue appears to live but not grow on 3 mM glyphosate.
Discussion

Morphology of Plants Grown from Protoplasts

The results of the morphology experiment were surprising. If a change in any of the measurements shown in figures 24 and 25 were caused by changes in genetics, one would expect that the wildtype plants and the quadruple mutant plants would have the largest differences; and then the single, double, and triple mutants would fall on a spectrum between the two. That is not

Figure 32: Representative growth on 6 mM glyphosate. The plant tissue dies very quickly on 6 mM glyphosate.
observed here. Instead, the wildtype and quadruple mutants have similar appearances, and some of the other plants vary unexpectedly.

It was believed that the measurement variations are instead due to unequal lighting conditions for the plants. Figure 33a is an image of the 45 plates of moss grown for this experiment. To ensure that all mutants experienced equal growth conditions, all genetic lines were produced and grown at the same time. The crowded conditions of this large experiment may have inadvertently caused a change in conditions. All the plates were grown together on a lab bench with the fluorescent lighting hung 1.5 feet above the bench. There may have been enough of a difference in light intensity between the center of the bench, which is directly below the middle of the fluorescent light tubes, and the edges of the bench. It was hypothesized that the plates closest to the center of the bench were the plates with the most growth. The follow-up experiment used fewer plates, and only compared the wildtype and quadruple mutants. To ensure equal growth, all plates were placed directly under the middle of the fluorescent tubes, shown in Figure 33b. The results of the follow-up test (Figures 26-28) indicate much more variation between the wildtype and the quadruple mutant than what was observed in the original results. Any future experiments must control for light intensity when comparing one plant to another.
Figure 33: Lighting conditions for morphology experiment. **A:** The crowded bench. Growing 45 plates concurrently may have affected the results by placing some plates towards the edge of the bench, with slightly lower light intensities. **B:** Adjusted growing conditions. The follow-up experiment (currently being analyzed) used fewer plates to ensure all were exposed to the same light intensity.
**Analysis of Amino Acid Starvation**

The comparison of all 14 mutants and the wildtype *Physcomitrella* after induced amino acid starvation did not demonstrate a difference between the different genotypes. The results quantified in Figure 29 demonstrate the little-to-no growth of plants growing on 3 mM glyphosate, when compared to the control group. The plants growing on 6 mM glyphosate die very quickly, and the dead tissue appears to slightly decrease in area compared to when it is first placed on the glyphosate plates. Unexpectedly, there was not a noticeable difference in the mutants compared to the wildtype. The hypothesis was that the removal of the DRG2 protein, or the DFRP2 proteins, would decrease the plant’s ability to sense amino acid starvation. To demonstrate that, the expected result would be that the functional knockout would initially have more growth on 3 mM glyphosate compared to the wildtype moss. Since the knockout would not respond to amino acid starvation, and not conserve amino acids during starvation, it would die sooner compared to the control.

It is possible that there is a difference in response to amino acid starvation, but that the difference will need to be observed with a change in experimental design. The experiment reported here had grown the moss on normal PpNH$_4$ plates for five days, and then tracked growth for the next six days after the plants were moved onto glyphosate. One alteration to the experiment could include moving the plants back to PpNH$_4$ after exposure to glyphosate. This would measure how quickly each mutant recovered from 3 mM glyphosate. If the mutants continue normal protein production while under amino acid starvation, and use the amino acid reserves during that period, they might take longer to recover after the glyphosate is removed.
CHAPTER 4: GENE EXPRESSION: RNA AND PROTEIN ACCUMULATION

Introduction

As described previously in chapters 1 and 3, *drg* mutations affect the accumulation of DFRP proteins. Also, *dfrp* mutations affect the accumulation of DRG proteins. The experiments in this chapter used the *drg2* mutant as well as the twelve different *dfrp* mutants produced in chapter 2 to analyze how each gene knockout affects the accumulation of the other genes/proteins of interest. *Physcomitrella patens* is unique compared to the other studied organisms, in that the moss has two copies of each *Dfrp1* and *Dfrp2* ortholog. This presents an opportunity to see if the variation between each of the two pairs of genes is enough to produce different results in one mutation compared to the other.

**Drg Affects Dfrp and Vice Versa**

What is known of the DRG effects on DFRP proteins and vice versa is described in Chapter 1. The research in this chapter is meant to further that understanding. This lab has previously observed the described effects in *Arabidopsis* using Western Blots. Figure 34 shows a Western analysis of two different wildtype *Arabidopsis* lines compared to a *drg* double mutant and a *dfrp* double mutant (Srygler, 2012). The *drg1;drg2* mutant shows very little accumulation of DFRP2, and surprisingly an increase in accumulation of DFRP1. The *dfrp1;dfrp2* mutant has
less DRG1 compared to the wildtype, and very little accumulation of DRG2. As described in the Chapter 2 Discussion, it might be this knockdown of DRG that makes the dfrp double mutant have a phenotype that is a less-drastic version of the drg double mutant. The experiments in this chapter analyzed both the protein and mRNA accumulation to study the effects of each knockout on the remaining genes/proteins.

Figure 34: Example of effects of Drg and Dfrp mutants on protein accumulation. The protein samples in the first two lanes are two different wildtype ecotypes of Arabidopsis. The third lane is the drg double mutant, followed by the dfrp double mutant in the fourth lane. There is no protein accumulation from the corresponding mutated genes. There is also less accumulation of DRG proteins in the dfrp mutant, and less DFRP protein accumulation in the drg mutant. Image from Srygler M.S. thesis, 2012.
In addition to affecting *Drg* expression, the mutation of each *Dfrp* gene may also affect the expression of the other three *Dfrp* genes. If one of the genes is knocked out, one could assume that expression of the other gene in the family would be upregulated to compensate. A recent article looked at a family of four genes. When one gene was knocked out, the remaining genes in that family were actually downregulated (Burkart et al., 2015). The mutants with different combinations of the four *Dfrp* genes present in *Physcomitrella* offer an opportunity to study how each knockout affects the other genes. Since each pair of *Dfrp1* and *Dfrp2* genes are much more similar to each other than to the other pair of genes, the effect that each had on the other three was important to analyze. The transcription of all four *Dfrp* genes were measured by qRT-PCR across all of the mutant *Physcomitrella* plants.

**Materials and Methods**

**Drg Gene Pathway Transcription Comparisons**

Transcription of each gene of interest was measured by qRT-PCR. Primers were designed to target each of the seven genes, amplifying 100-200 bp of cDNA each. Table 6 lists each primer sequence and the target gene. Two of the primer pairs are intron-spanning, to verify that the amplicon is from cDNA, not genomic DNA. To verify that all primer pairs were amplifying cDNA, a negative control reaction with RNA treated with DNase1 was used with
each primer pair. As expected, Ct values from each negative control reaction were determined to be sufficiently higher than the cDNA template reactions. Each primer pair was also tested with end-point PCR to amplify the cDNA. The product was run on an agarose gel to verify the correct size of each amplicon.

Table 6: List of primers used for qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1F</td>
<td>CAAAATTGGCTAAGTTGCGACG</td>
</tr>
<tr>
<td>D1R</td>
<td>CAGAAAAGGTTCTCTGTGAGCTTG</td>
</tr>
<tr>
<td>D2F</td>
<td>CTTGATGCCTCTAAGAGTGAAGG</td>
</tr>
<tr>
<td>D2R</td>
<td>TGATAGCAGAGCTCTCCTCATCCAC</td>
</tr>
<tr>
<td>DF1aF</td>
<td>AACCCAAGGTTCCCGTG</td>
</tr>
<tr>
<td>DF1aR</td>
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</tr>
<tr>
<td>DF1bF</td>
<td>GAGGACAAGACCTCGGATTG</td>
</tr>
<tr>
<td>DF1bR</td>
<td>GATCATCAACTCCACGCTTG</td>
</tr>
<tr>
<td>DF2aF</td>
<td>GTAATTGAGAAGCATGAGGGAG</td>
</tr>
<tr>
<td>DF2aR</td>
<td>GTCGTCGCAATGCTCTCG</td>
</tr>
<tr>
<td>DF2bF</td>
<td>GACGACATGCAGGAGTTTACG</td>
</tr>
<tr>
<td>DF2bR</td>
<td>GATGTTGCGCAAATTGACC</td>
</tr>
<tr>
<td>S1F</td>
<td>GTGATGACGATGAAGACGATTGG</td>
</tr>
<tr>
<td>S1R</td>
<td>CGGAAAATTGACCATGACTGTGCT</td>
</tr>
<tr>
<td>Ade PRTf</td>
<td>AGTATAGTCTAGAGTTGTTCCG</td>
</tr>
<tr>
<td>Ade PRTr</td>
<td>TAGCAATTTGATGGCAGCTC</td>
</tr>
</tbody>
</table>
The first set of primers to target a reference gene was taken from a sequence used in a published qRT-PCR experiment that used *Physcomitrella*. The published primer sequence had a single base mismatch when compared to the gene sequence on the Phytozome database. However, the same sequence was ordered for this experiment because it proved reliable in a previously published study. Preliminary tests did not produce an amplicon with that primer sequence, so that primer pair was discarded. The reference gene ultimately used was the AdePRT gene sequence listed at the bottom of Table 6. This sequence came from a study that compiled a list of *Physcomitrella* reference genes that each produced comparable Ct values across different types of tissues and treatments (LeBail et al., 2013). Three of the primer pairs were ordered and tested with the samples used in this experiment. The reference gene chosen for the main experiment produced a low Ct value in preliminary tests, and was deemed trustworthy because it was used in another published paper (Sanchez-Vera et al., 2017).

*Physcomitrella* protonemata from each of the 15 genetic lines were disrupted and then grown on PpNH₄ plates as described in Chapter 2. The moss was grown for twelve days and then collected for transcription analysis. Plant tissue from two plates of each line were pressed between paper towels to remove water, then weighed and frozen at -80°C. Each sample produced 125-300 mg of plant tissue. The tissue was ground under liquid nitrogen in a chilled mortar and pestle. Approximately 25 mg of glass beads were added to each mortar to assist in tissue homogenization.

RNA was purified with the Ambion RNAqueous 4PCR kit, with the optional Plant RNA Isolation Aid addition. Each sample was treated with DNase1. Quantification of RNA was performed with a NanoDrop spectrophotometer. The Ambion RETROscript kit was used for
reverse transcription to produce cDNA from 2 µg RNA. A Stratagene Mx3000P real-time PCR system was used to perform the qRT-PCR and to analyze the results. The primer mix for each reaction included 12.5 ng of each oligonucleotide, and Sigma reference dye R4526. The qRT-PCR reaction included 3 µl of 1:10 dilution cDNA, 9.5 µl primer mix, and 12.5 µl Sigma S4438 SYBR Green JumpStart Taq ReadyMix. The reaction used 1-minute extension times at 72°C and 55°C annealing temperature for 30 seconds. Ct values were calculated and compared for each gene across each cDNA sample. Each primer pair also tested a water sample as a negative control. Following the MIQE guidelines, the Ct values were converted to delta delta Ct compared to the reference gene (Bustin et al., 2009).

**DRG Gene Pathway Protein Accumulation**

*Physcomitrella* protonemata from each of the 15 genetic lines were disrupted and then grown on PpNH₄ plates as described in Chapter 2. The moss was grown for twelve days and then collected for Western analysis. Plant tissue from three plates of each line were pressed between paper towels to remove water, then weighed and frozen at -80°C. Each sample produced 150-300 mg of plant tissue. The tissue was ground under liquid nitrogen in a chilled mortar and pestle. Approximately 25 mg of glass beads were added to each mortar to assist in tissue homogenization. The frozen homogenate was added to a grinding buffer at 5 ml/gm. The grinding buffer was modified from the Moss Manual from Dr. Magdalena Bezanilla (Bezanilla, 2012). The buffer was 100 mM Sodium Phosphate, 10 mM Dithiothreitol, and 20% Glycerol. Total protein concentrations were measured using the Bio-Rad protein assay (Bradford, 1976).
Samples were added to an SDS loading buffer and heated at 95°C for ten minutes. The samples were then loaded (100 µg total protein per lane) onto a 12% PAGE gel and electrophoretically separated. The protein was then transferred to a PVDF membrane and blocked with 5% instant non-fat powdered milk (“Blotto”). The blot was probed with the primary antisera overnight at room temperature. After a rinse and three 10-minute washes in TBS-T (Tris buffered saline with 0.05% Tween-20), the membrane was incubated in the secondary antibody ECL Donkey anti-rabbit IgG conjugated to horseradish peroxidase (1:8000) (GE Healthcare UK Limited NA934V) for approximately 2 hours. After another three washes in TBS-T, the membrane was mixed with LumiGLO and recorded using the Syngene G:BOX iChemi Chemiluminescence Imager. Primary antibodies used in these experiments are listed in Table 7.

### Table 7: Primary antisera used for Westerns

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Preparation</th>
<th>Dilution</th>
<th>Raised against</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRG1 #29</td>
<td>(NH₄)₂SO₄ precipitated</td>
<td>1:2,000</td>
<td>full polypeptide</td>
</tr>
<tr>
<td>DFRP1 #80</td>
<td>Crude antisera</td>
<td>1:3,000</td>
<td>full polypeptide</td>
</tr>
</tbody>
</table>
Results

**Drg Gene Pathway Transcription Comparisons**

Figures 35 - 37 display the results of the qRT-PCR experiments. Delta Ct was calculated by comparing all Ct values for a particular cDNA template against the Ct value of the reference gene from that template. Delta delta Ct was then calculated by comparing each delta Ct of a gene in each mutant to the Ct value of that gene in the wildtype plant. The delta delta Ct value was then converted to the fold change. Figure 35 shows individual graphs with the relative expression of both Drg genes and the Slh gene in each of the 14 mutants. Figure 36 shows individual graphs with the relative expression of Dfrp1a and Dfrp1b in each of the 14 mutants. Figure 37 shows individual graphs with the relative expression of Dfrp2a and Dfrp2b in each of the 14 mutants.

There were not many differences from one genotype to the next. All of the fold-changes were less than a two-fold difference. The results shown in Figures 35-37 come from a single replicate. This same experiment was replicated one time prior, but there were a couple unexplained results. The unexplained results were probably due to experimental error, so they were not included here.
Figure 35: mRNA relative expression for Drg1, Drg2, and Slh in all 14 mutants. Expression of each gene was compared to the reference gene, adenine phosphoribosyl transferase. Delta delta Ct values were calculated and converted to fold change for Drg1 (D1), Drg2 (D2), and Slh (S1).
Figure 36: mRNA relative expression for \( Dfrp1a \) and \( Dfrp1b \) in all 14 mutants. Expression of each gene was compared to the reference gene, adenine phosphoribosyl transferase. Delta delta Ct values were calculated and converted to fold change for \( Dfrp1a \) (1a) and \( Dfrp1b \) (1b). Note: The 1b;2b;1a triple mutant has expression levels of \( Dfrp1a \) comparable to the genetic lines with a functioning 1a gene. This qRT-PCR and the Western blot in Figure 39 below revealed that one of the triple knockouts was only a double \( dfrp \) knockout.
Figure 37: mRNA relative expression for $Dfrp2a$ and $Dfrp2b$ in all 14 mutants. Expression of each gene was compared to the reference gene, adenine phosphoribosyl transferase. Delta delta Ct values were calculated and converted to fold change for $Dfrp2a$ (2a) and $Dfrp2b$ (2b).
**DRG Gene Pathway Protein Accumulation**

The 15 genetic lines of *Physcomitrella* were analyzed with antisera targeting the DRG1 protein and the DFRP1 proteins. Figure 38 shows the protein accumulation levels of DRG1 in each of the mutants compared to the wildtype plant. Total protein loading was equal in each lane, so the difference in band intensity is indicative of the relative protein accumulation for DRG1. As hypothesized, the knockout of each *Dfrp1* gene produces a DRG1 protein knockdown. Each of the single *Dfrp1* mutations has less accumulation that the wildtype plant. The double 1a;1b knockout has no detectable DRG1 protein. The same is true for the quadruple *dfrp* knockout, which contains the 1a;1b mutations in addition to the 2a;2b mutations.

**Figure 38: DRG1 protein accumulation in all 15 Physcomitrella lines.** The D1 knockout was never obtained, but the accumulation of DRG1 protein in these Western blots show that when the two *Dfrp1* genes are knocked out, it acts as a DRG1 knockdown.
Figure 39 shows the protein accumulation levels of DFRP1 in each of the mutants compared to the wildtype plant. Total protein loading was equal in each lane, so the difference in band intensity is indicative of the relative protein accumulation for the DFRP1a and DFRP1b proteins. This Western verifies what was shown in qRT-PCR in Figure 36, that the plant thought to be a 1b;2b;1a triple knockout is indeed producing detectable levels of DFRP1a protein.

**Figure 39:** DFRP1 protein accumulation in all 15 Physcomitrella lines. The plant believed to be a 1b;2b;1a triple has detectable levels of a DFRP1 protein. This verifies what was shown in Figure 36. The important takeaway from this blot (and the qRT-PCR in Figure 36) is that the quadruple dfip mutant is a true knockout.
Discussion

The results of the qRT-PCR did not vary as much as expected. It appears that there is very little change in gene transcription from one mutant to the next. As stated in the Results section, this experiment used a single template for each measurement. There are several follow-up experiments that will need to be performed to determine if these qRT-PCR results are significant and reproducible. A technical replicate of the qRT-PCR will be performed, to reanalyze the same template. After that, a biological replicate can use new cDNA produced from a unique set of tissue samples. With more replicates, the graphs shown in Figures 35-37 will increase from single measurements, to averages with standard deviations for more-precise results that can be used for statistical analysis.

The Western blots and the qRT-PCR results pair together nicely to show that the mutants produced for this project are actual knockouts of each protein, with the exception of one of the triple knockouts described in the Results section. An important observation with these specific genes/proteins, is that the downregulation of the DRG1 protein is occurring at the protein level, not the transcription level. The mRNA levels and protein levels are often not correlated when the proteins encoded by those genes are part of a multi-protein complex (Schmidt et al., 2007). These results show that the complex between the DRG and DFRP proteins is allowing increased protein accumulation despite relatively equal levels of gene transcription in each mutant.
CONCLUSION

The goal of this dissertation was to produce gene knockouts in the *Drg* gene pathway, and then to study the effects of the gene mutations to better understand the function of these highly conserved genes. While the original focus was on the two *Drg* genes and *Slh*, the project shifted to concentrate on the four *Dfrp* genes. Since the *Dfrp* gene knockouts were more successful than *Drg* and *Slh*, the primary goal became to produce the *Dfrp* quadruple knockout and study the loss of the four DFRP proteins. A lot of time and effort went into producing the mutants for this project. Due to the time required, there was not much time to analyze these valuable tools. While some experiments are shown here, there is further valuable research that can be performed now that these tools are in hand.

The mutants were tested for the effects of each on the transcription of the *Drg* pathway genes. The results included in Chapter 4 were from a single replicate. It appears that none of the produced gene mutations had a noticeable effect on the mRNA levels of the tested genes. The qRT-PCR experiments will be repeated in the immediate future to include biological replicates. Repeating the gene expression testing will increase the statistical reliability of these results.

Since the change in expression does not seem to be occurring at the transcription level, it is probable that the effects of DRG knockouts on DFRP accumulation, and vice versa, is occurring at the translation level. The Western analyses in Chapter 4 show the effect of each gene knockout on the protein accumulation of DRG1 and DFRP1. Importantly, it demonstrated
that the two knockout combinations that lack DFRP1 (1a;1b and the quadruple dfrrp knockout) both produce no detectible levels of DRG1 protein. The only gene that was not able to be mutated was the Drg1 gene. Since the 1a;1b mutation leads to a DRG1 functional knockdown, these plants can be used to indirectly test the effect of DRG1 removal. There are more antibodies available in the lab that will be used to analyze the accumulation of DRG2 and the DFRP2 proteins. These additional experiments will be of value to understand the Drg gene pathway.

The morphological effects of each mutation were also tested. The first attempt tested many individual plants grown from single-celled protoplasts. All fourteen mutants and the wildtype Physcomitrella were analyzed and compared using several parameters. The results from this large-scale experiment are believed to be unreliable due to the light intensity variation across the 45 plates used for the experiment. As shown with the second set of morphological analyses, when conditions are properly controlled, there are noticeable differences between the growth of the wildtype and the quadruple dfrrp mutant. Now that this is understood, it will be helpful to replicate the full morphological experiment with equal lighting conditions to discover if any of the single, double, or triple mutants also show a change in growth compared to the wildtype Physcomitrella.

Another experiment that will be good to repeat with slightly altered conditions is the amino acid starvation analysis. The results in Chapter 3 showed that each of the Physcomitrella mutants reacted similarly to the sub-lethal and lethal doses of glyphosate. Not only will this experiment be repeated with carefully-controlled lighting conditions, but there are several variables that can be changed to tease out the slight variations between the different mutants
under nutrient stress. As the Chapter 3 discussion describes, slight variations in the glyphosate dosage will be examined with each mutant, as well as how quickly each plant can recover after amino acid starvation conditions are removed.

These genetic mutants were produced using homologous recombination to completely remove each respective gene from the genome. Unlike some mutations which are transient, these plants are stable gene knockouts. As long as the plants are preserved, they are valuable tools to study the function of each of the genes in the Drg gene pathway. It is the hope of the author that the work that has gone into producing these Physcomitrella lines will allow continued research into the Drg gene pathway.
REFERENCES


Number of plants tested for the dissertation. A major portion of the time required to complete this project was needed to produce the gene knockouts. Multi-gene knockouts were performed consecutively. Each successful knockout was verified before the plant was used for the next knockout. Three of the mutant plants had the selection cassette(s) removed so the same antibiotic(s) could be used for subsequent gene knockouts. Unfortunately, some of the knockouts required the PCR screening of many plants. As described in chapter 2, many plants that were not true knockouts were surviving the antibiotic selection. The table above lists the number of plants that were individually tested for each knockout. Each knockout that eventually produced a mutant is indicated with a checkmark. The labels for multi-gene knockout attempts include a plus sign. The genes before the plus sign indicate the plant used for the transformation. The gene after is the gene being targeted. For example, the 2a + 1a transformation targeted the dfrp1a gene in a 2a single mutant. Some of the mutants above included multiple attempts. For example, the S1 gene knockout was produced after testing 755 plants from at least ten different transformation attempts. Overall, DNA was prepared from nearly 4500 individual plants.

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