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INVESTIGATING THE LEAF CUTICLE OF THE MOSS
PHYSCOMITRELLA PATENS

By Eric John Ricci, B.A.

A Thesis Submitted in Partial Fulfillment of the
Requirements for the Master of Arts in the Department of
Biology

The Faculty of Arts and Sciences

Rhode Island College

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ABSTRACT

To minimize water loss, the aerial parts of vascular land plants are covered with a hydrophobic layer called a cuticle. The cuticle typically consists of two major components: cutin and waxes. In contrast to vascular plants, research reports indicate that some primitive nonvascular plants, such as mosses, lack a cuticle. This study attempts to reconcile contradictory literature reports about the presence of a waxy cuticle on the leaves of the moss *Physcomitrella patens*.

Histochemical staining was utilized to investigate the presence of a cuticle on *Physcomitrella* leaves. Experiments employing dyes that stain hydrophobic substances were generally consistent with the presence of a cuticle. Hydrophilic dyes were also used to infer the presence of a cuticle through an absence of staining. Histochemical results were supported by observations made using freeze fracture electron microscopy. Leaves consistently fractured through an extracellular layer having a rippled texture, which resembling cuticles known to occur in vascular plants. Attempts to isolate a cuticle from *Physcomitrella* leaves by partial digestion with chromic acid or treatment with polysaccharide-degrading enzymes were also successful.

The existence of a cuticle on the leaves of *Physcomitrella* is further supported by genetic observations. The *P. patens* genome contains several genes that are homologous genes known to participate in cuticle synthesis in vascular plants. One of these, the homolog of the *Solanum lycopersicum* *CDI* gene (*SlCDI*), may participate in cutin biosynthesis and was selected for further characterization. Using homologous recombination, knockouts of *Physcomitrella* samples deficient in the homolog *CDI* were generated (*PpCD1KO*). Unfortunately, knockouts showed no changes in phenotype and may indicate the presence of a cuticle, supported by other genes with similar catalytic functions.

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DEDICATION

This thesis is dedicated to all of my deceased relatives, particularly:

Mr. & Mrs. Altieri Di Censo

Dr. Benjamin Ricci, Ph.D.

Dr. Lino Tiberi, D.M.D.

Mr. & Mrs. Rudolph Ricci

Mr. & Mrs. George Mollo

“So wait and pray each night for me, till we meet again!” – Bing Crosby

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I love you with all of my heart!

CHAPTER I: INTRODUCTION & LITERATURE REVIEW

Origin and Significance of the Plant Cuticle

The plant cuticle is an insoluble hydrophobic “skin” that covers the aerial portion of land plants (Riederer, 2006). The primary function of plant cuticles is to minimize water loss from cells; however, cuticles play many other roles in maintaining homeostasis (Holloway, 1971). The typical plant cuticle typically consists of waxes and a hydrophobic polyester called cutin (Jeffree, 1996). Despite its relatively simple appearance, the plant cuticle is complex in both its physical structure and chemical composition. Recently, there has been renewed interest in the science of plant surfaces, particularly the cuticle (Riederer, 2006). These new efforts to decipher the chemical composition, mechanisms of biosynthesis, and evolutionary importance of the plant cuticle have benefitted from the rise of new technologies, including molecular genetics.

The term “cuticle” is derived from the Latin word *cuticula*, which is literally translated as “thin skin” (Riederer, 2006). Writing in French in 1827, the Swiss botanist Augustin Pyramus de Candolle was first to use the term—‘cuticule’—in its modern sense. Another European scientist, Alexandre Brongniart, was among the first to visualize the cuticle using hydrophobic stains, such as Sudan dyes, which selectively partition with hydrophobic substances (Gahan, 1984; Riederer, 2006).

The evolutionary significance of the cuticle is substantial. As photosynthetic organisms made the transition from water to land, the ability to resist water loss through evapotranspiration was a crucial adaptation (Pollard et al., 2008). The aerial parts of all terrestrial vascular plants protect themselves with the thin, waxy waterproofing layer known as the cuticle. While this structure of cutin and wax is not found in algae (Cook & Graham, 1998), there are contradictory reports about whether a cuticle occurs in the most primitive land plants, the mosses. In particular, this uncertainty extends to the moss *Physcomitrella patens*. This thesis seeks to address this question.

Functional Overview of the Plant Cuticle

The typical plant cuticle is composed of several different layers (Fig. 1-1). Cutin, a hydrophobic polyester, typically is the most abundant component of the cuticle and makes up the inner part of this covering (Pollard et al., 2008). Epicuticular waxes cover the cutin layer and can form three-dimensional crystals that interact with the external aerial environments. The layer where the wax and cutin interdigitate is called the intracuticular wax layer (Buschhaus & Reinhard, 2011), or the “cuticle proper” (CP). The CP lacks cellulosic (cell wall) elements (Jeffree, 1996). In contrast, the cuticular layer (CL) is made up of both cutin and cellulosic components (Jeffree, 1996).

Figure 1-1: Anatomy of the plant cuticle. The cuticle is located outside of the plasma membrane and cell wall, and is composed mainly of cutin and waxes. The cuticle proper (CP) is a layer that consists primarily of waxes and cutin, devoid of cell wall components. In contrast, the cuticular layer (CL) has cutin, wax, and cell wall polysaccharides. The red lines are representative of hydrophilic channels, possibly due to the presence of hydrophilic wall components.

Because cuticles form a significant physical barrier to the movement of many materials (Martin & Juniper, 1970), the aerial surfaces of higher plants have evolved modifications to allow for exchange of materials. For example, to facilitate the exchange of gasses, such as CO₂, the epidermal surfaces of vascular plants must be covered with stomata. In contrast to vascular plants, however, the “leaves” of moss gametophytes are a single cell layer thick and thus lack stomata, although these may occur on moss sporophytes (Zeiger, 2004).

Surprisingly, although cuticles are effective at reducing water loss, they do not completely prevent passage of hydrophilic materials. The crystalline waxes and cutin polymers that make up the cuticle are not perfectly ordered, and hydrophilic polysaccharide fibrils embedded within the cuticular layer (CL) can occasionally reach the outer surface. This interdigitation of cutin and cellulose microfibrils may allow some water and other hydrophilic materials to enter the plant cells (Burghardt & Riederer, 2006). In fact, about 21% of the average cuticle is composed of non-hydrophobic material (mainly carbohydrates) (Schreiber & Schönherr, 1990; Riederer & Schönherr, 1984). Environmental conditions can modify the effect of the cuticle on water movement. The size and shape of aqueous polar channels are partially controlled by relative humidity (Buchholz, Baur, & Schönherr, 1998). At low humidity, these pathways constrict and water is conserved. Elevated humidity causes the aqueous polar pores to increase in size, and sometimes form new aqueous pathways. Work by Kerstiens et al. (1989) shows that water transport (absorption, etc.) through the cuticle is mainly due to the polysaccharide components. The existence of polar components within the hydrophobic cuticle needs to be considered when using histochemical methods to identify cuticles. The same aqueous pores that allow limited water movement through the cuticle, also allow polar ionic compounds and solutes to enter the cell through the cuticle, the so-called polar path of diffusion (Burghardt & Riederer, 2006).

As a physical barrier between cells and the external environment, an additional function of the cuticle is to protect the plant. Protecting DNA from damage by UV light is a vital

function today, and was likely even more critical for early land plants, due to the relatively high UV levels that occurred at that time (Rozema, et al., 2002). Domínguez et al. (2011) state that the phenolic compounds, which are found in many plant cuticles, absorb various wavelengths of ultraviolet radiation (UV). Therefore, non-absorbed wavelengths are reflected producing specific patterns of UV reflectance that protect the cellular DNA from UV light (Domínguez, 2011). This is supported by the ultraviolet reflectance studies by Bornman & Vogelmann (1988) who found that when cuticular wax was removed from conifer leaves by chloroform treatment, UV reflectance was reduced, and absorption of harmful radiation between 240-290 nm was increased.

A final benefit of the plant cuticle is that it inhibits water from forming a film on the leaf surface. These aqueous films can harm plants by promoting microbial growth and blocking stomata used for gas exchange (Neinhuis & Barthlott, 1997).

Evolutionary Aspects of the Cuticle

Because of their hydrophobic polymeric nature, plant cuticles can be preserved against decay for extended periods of time. In fact, fossilized cuticles provide important physical evidence of the evolutionary history of land plants. The oldest remains of an intact plant cuticle, from the Devonian period approximately 400 million years ago, show the presence of accessory cells that surround openings in the leaves (Kolattukudy, 2001). These fossils resemble modern stomata and are physically preserved within the ancient cuticle (Riederer, 2006). Plants that possess cuticles often have stomata (or precursors to them), and longer root systems, all serving as evolutionary and physiological adaptations to arid environments (Riederer, 2006).

Green algae are thought to be the evolutionary ancestors of land plants because these groups share many evolutionary similarities, including the presence of cellulosic cell walls, formation of a cell plate during cytokinesis (Allen, 1968), and ability to synthesis long chain fatty acids (Kenrick & Cane, 1997). Because they are aquatic organisms, algae

do not need a cuticle to retain water (Edwards et al., 1996). However, there is evidence that some algae produce long carbon chains, including cutin (Kenrick & Cane, 1997). Research by Derenne et al. (1992) indicated that the alga *Nannochloris* is capable of synthesizing hydrophobic polymethylenic chains. Previous research discovered non-hydrolysable polymers surrounding cell walls in some members of the family Chlorophyceae, but neither this layer, nor the glycoprotein coverings that surround some algal cells, are equivalent to the cuticle of higher plants (de Leeuw & Largeau, 1993). Regardless of the fact that algae lack a true cuticle, the machinery for cuticle formation, such as long chain fatty acid synthesis, are present in these primitive organisms.

Bryophytes, a group that include mosses, liverworts and hornworts, are nonvascular plants that are considered the next evolutionary step forward from algae, and are known to exhibit more adaptations to terrestrial life, including components of cuticles (Kenrick & Crane, 1997). Despite successfully making the transition from water to land, most mosses still require a moist environment, in part because they lack an organized vascular system. A few mosses, such as the desert bryophyte *Syntrichia caninervis* (Bowker et al., 2000) can survive in very dry habitats by being able to withstand prolonged desiccation, sometimes in a metabolically inactive state. Yet, even these mosses still require surface water for successful sexual reproduction (Mishler, 1988). Because of their relatively primitive evolutionary position and the fact that they typically grow in moist habitats, it is not obvious if a cuticle covering would be necessary in bryophyte mosses. The published literature disagrees about the occurrence of moss cuticles. However, there are several documented examples of specific mosses that have a cuticle. For example, the desert moss (referenced above), *Syntrichia caninervis*, and *Polytrichum commune*, are two bryophyte mosses that possess waxy cuticles (Xu et al., 2009; Potter et al., 1996). In addition, a cuticle was identified on the calyptra (a cap covering the reproductive sporophyte structure) and leaves of the moss *Funaria hygrometrica* (Budke, Goffinet, & Jones, 2011). Though relatively uncommon, certain bryophytes do possess thin cuticle layers. This thesis focuses on the contradictory claims that have been made regarding the presence of a cuticle on leaves of the moss *Physcomitrella patens*.

Cuticle Structure and Biosynthesis

The cuticle of higher plants is not a homogenous single layer. Its chemical composition is heterogeneous, as suggested by its multilayered structure, with wax found predominantly on the outer portion of the cuticle and cutin found on the inner portion.

Epicuticular waxes, which come into direct contact with the environment (Heredia, 2003), often form crystals that differ in size and shape from one cell to another. In many species, a more uniform waxy film is found beneath the wax crystals (see Fig. 1-1). The major components of cuticular waxes are very long chain aliphatic lipids, although they may also contain aromatic lipids such as triterpenoids, sterols, and flavonoids (Kunst & Samuels, 2003). Note that epicuticular waxes can have a degree of variety, meaning that different crystalline wax shapes, which protrude from an underlying simple waxy film (Bargel et al., 2006).

Biosynthesis of cuticular wax is a complex multistep process. Synthesis occurs in the epidermal cells that will ultimately be coated with the cuticle (Pollard et al., 2008). Carbon building blocks, derived from acetyl coenzyme A (acetyl-CoA), are organized and connected into aliphatic very long chain fatty acids (VLCFAs) ranging in length from 24 to 34 carbons (Kunst & Samuels, 2003). Wax synthesis occurs in three phases: *de novo* assembly, endoplasmic reticulum elongation, and final wax modification. Generally, lipids of the cuticle (for both waxes and cutin) are synthesized in leucoplasts within epidermal cells. Here, C₁₆ and C₁₈ fatty acids are assembled by condensation reactions between carbon atoms and acetyl CoA. Fatty acid chains are extended to VLCFAs in the endoplasmic reticulum by specialized enzymes, known as fatty acid elongases (Kunst & Samuels, 2003; Samuels, Kunst, & Jetter, 2008).

How epicuticular wax is exported from the epidermal cell cytoplasm, through the cell wall, and then through the cutin layer is not fully understood (Nawrath, 2006). After processing in the endoplasmic reticulum, waxy components move to the plasma membrane. Jenks et al. (1994) reported that large numbers of vesicles appear transiently

at sites of wax secretion. Transport of cuticle monomers through the plasma membrane is accomplished by ATP-binding cassette (ABC) transporters, which hydrolyze ATP to accomplish this energetically unfavorable reaction (Kunst & Samuels, 2003). Wax-polyester monomer transport through the cell wall may be accomplished by various lipid transfer proteins (Suh, et al., 2005) that are thought to exist in the apoplast (Heredia, 2003).

Beneath the cuticular wax layer is the single largest component of the cuticle: cutin. In many plants, cutin can make up 40-80% of the entire cuticular mass (Heredia, 2003). It is found proximal to the primary wall of epidermal cells. Cutin monomers are polymerized to form a hydrophobic layer that interdigitates with wax and the cell wall (see Fig. 1-1). The monomers that form cutin are often composed of C₁₆ or C₁₈ oxygenated fatty acid carbon chains that differ only in the functional groups attached to the chains (Domínguez, et al., 2011). The most common C₁₆ monomers include 10,16- dihydroxyhexadecanoic acid, 9- dihydroxyhexadecanoic acid, and 16-hydroxyhexadecanoic acid (Kolattukudy, 2001). Common C₁₈ monomers include 9,10,18-trihydroxyoctadecanoic acid, and 18-hydroxy-9,10-epoxyoctadecanoic acid (Domínguez, et al., 2011). Understanding the most abundant monomers in cutin is important because the cutin biosynthesis homologous gene in *Physcomitrella patens* (*PpCD1*) that I chose to knock out (Chapter III) controls relative polymerization of the cutin monomer 2-mono(10,16-dihydroxyhexadecanoyl)glycerol (2-MHG). Reducing the synthesis of C₁₆ monomers significantly reduces the amount of cutin deposited on the plant surface (Yeats, 2012).

These fatty acid monomers of cutin secreted into the cell wall space are linked to form a polymer via ester bonds (Heredia, 2003). Throughout the carbon chain, secondary hydroxyl groups contribute to polymer branching, again via ester bonds (Pollard, et al., 2008). Additional bonding comes from various secondary hydroxyl groups, which contain cross-linking ester bonds (Heredia, 2003; Baker et al., 1982). Once polymerized, the cutin polyester matrix is quite durable and provides the majority of the physical strength of the plant cuticle (Baker et al., 1982). Although cutin resists dissolution in

common organic solvents, it can be depolymerized by various methods such as alkaline hydrolysis (Wyatt et al., 2008).

The mechanism of cutin polymerization is still incompletely understood. Early research on cutin polymerization in the mid 1970s focused on pulse labeling experiments to determine the location monomers throughout the cutin biosynthesis pathway. For example, Walton and Kolattukudy (1972) radioactively labeled 16-hydroxy C₁₆ acids in *Vicia faba* leaves and observed that the monomer was directly polymerized into a cutin polyester. When examined over time, the radiolabeled monomers were incorporated into the polymer shortly after their synthesis (Kolattukudy, 2001).

There are two theories of how cutin polymerization occurs in plants: chemical polymerization and or enzymatic polymerization. A classical view of polymerization is that large families of enzymes catalyze the ester bond formation between monomers (Girard et al., 2012). Examples of these enzyme families are the glycerol-3-phosphate acyltransferase (GPAT) and the glycine–aspartic acid–serine–leucine motif lipase/hydrolase (GDSL) families of proteins. The *CD1* gene of *Solanum lycopersicum* encodes an acyltransferase that has been identified as identical to GDSL1 (Girard et al., 2012). Both GPATs and GDSLs are two specific examples, from different gene and enzyme families, which demonstrate the different and complex acyltransferase enzymes that catalyze cutin polymerization. Genes that encode for acyltransferases, specifically, a homologous gene to *Solanum* CD1 peptide in *Physcomitrella patens* will be examined in Chapter III of this thesis.

In contrast to the classical view of enzymatic polymerization, a newer theory has been presented to complement enzymatic polyesterification. In chemical polymerization (Domínguez, et al., 2011), cutin monomers spontaneously self-assemble by forming ester linkages amongst themselves. Spontaneous chemical polymerization may be facilitated by orientation of monomers by weak bonding interactions such as hydrogen bonds.

Regardless of how polymerization occurs, the structure of cutin polymers is very important. The majority of primary hydroxyl groups on the cutin monomers are ester cross-linked with other side chains of other cutin monomers (Wen, 2009). However, there are few non-esterified carbons on functional groups present in the polymer (Kolattukudy, 1996).

Although the monomeric structure and ester linkages of cutin are well documented, less is known about the polymeric structure of the cutin layer within the cuticle. Is the polymer anchored to the cell wall at several individual sites or continuously? Does the cutin layer exist as numerous polymer molecules or as a single continuously ester cross-linked layer? Domínguez et al. (2011) notes that the different chemical functional groups on cutin monomers, such as various carboxyl and hydroxyl groups, will generate an amorphous, polyesterified, nonlinear polymer. Pollard et al. (2008) states that the dominant monomers in cutin polyester are omega hydroxyl fatty acids. Clearly, determining the molecular organization of cutin monomers into a polymer will require further study. If *Physcomitrella* can be shown to possess a cuticle, it may serve as a useful model organism for understanding this process.

In addition to cutin and wax, plant cuticles may also contain other components such as cutan and suberin. Cutin can be replaced in whole or part by cutan (Domínguez, 2011). In contrast to the ester bonds that link together cutin monomers, cutan monomers are linked together by ether bonds (Villena et al., 1999; Pollard, et al., 2008). In addition to the different linkage, cutan is made of different monomeric building blocks than cutin (Villena, et al., 1999). The presence of cutan, with its distinct composition (methylene chains and aromatic carbons) and unique bonding pattern (ether bonds instead of cutin ester bonds), may explain why some cuticles are not fully depolymerized when exposed to alkaline solvents (Villena, et al., 1999; Heredia, 2003; Sachleben et al., 2004). Some cuticles of higher plants may also contain another hydrophobic component called suberin. Although suberin resembles cutin and cutan, it is distinguished from these polymers by its saturated aliphatic carbon chains and by the fact that its monomers are less than

twenty carbons long (Pollard, et al., 2008). Glycerol also plays an important role in suberin integrity by producing a crosslinked matrix (Moire et al., 1999). Overall, relatively little is known about cutan and suberin (Walton, 1990; Pollard, et al., 2008), because they are not as prevalent as cutin and epicuticular waxes.

Generation of *Physcomitrella* Knockout (KO) Mutants

Genetic manipulations, including gene knockouts (KO), can contribute to investigating the *Physcomitrella* cuticle. For example, to investigate whether *Physcomitrella patens* produces a leaf cuticle, a gene thought to be involved in cuticle biosynthesis could be eliminated and the resulting mutant examined for evidence of an altered surface layer. After performing an extensive literature review of genes involved in cutin synthesis, a gene known as *PpCDI* was selected as a candidate for the knockout. In tomato (*Solanum lycopersicum*) the *SlCDI* gene encodes an acyltransferase that acts upon 2-mono (10,16-dihydroxyhexadecanoyl) glycerol to produce cutin polymers (Yeats et al., 2012). In a recent paper on a higher plant *CDI* gene, a knockout mutant resulted in only 5% of normal cutin deposition (Isaacson et al., 2009). Genetic experiments such as this can provide evidence to determine if leaves of *Physcomitrella* do, indeed, have a cuticle.

Introduction to Mosses

Mosses, the largest and most recognizable class of bryophytes, are small nonvascular, non-woody plants that generally grow low to the ground in moist environments; an example of this is *Physcomitrella patens* (Fig. 1-2). The “leaves” of mosses are one cell layer thick and are sometimes referred to as gametophores. As the name implies, gametophores are leafy structures that will bear haploid gametes. While in a strict botanical sense only vascular plants have leaves, this thesis will refer to the leaf-like organs borne on the gametophore as “leaves” for simplicity.

Figure 1-2: A light microscopy image of the moss *Physcomitrella patens*. The gametophores of this monoecious bryophyte consist of leafy structures that are a single cell thick. Because of this and other reasons, *P. patens* is often utilized as a model organism in regards to biological and chemical testing. The genome is fully sequenced and searchable online through various databases. No scale bars available.

Bryophytes exhibit a dominant gametophyte life history, which is detailed in Figure 1-3. Moss gametophytes are larger and live longer than the diploid moss sporophyte (Zobell, 2005). When a haploid spore germinates, filamentous protonemata (Latin for “first threads”) are produced by mitosis (Zobell, 2005). There are two types of protonemal cells: chloronemata and caulonemata (Glime, 2007). Chloronemal cells are characterized by perpendicular crosswalls, colorless cell walls, and many green chloroplasts (Reski, 1998). In contrast, caulonemal cells have oblique elongated cells that consist of significantly fewer chloroplasts (Girod et al., 1999). The first cell type to form from the

germinating spore is chloronema (Reski, 1998). Chloronemal cells can develop into either a bud (the initial stage of the gametophore), caulonemal cells, or remain chloronemal cells with increased branching (Olsson, 2005). This process of differentiation is controlled by plant hormones such as cytokinin and auxin (Girod et al., 1999).

Figure 1-3: The life cycle of *Physcomitrella patens*. A sporophyte (2n) forms spores (1n) via meiosis. Germinating spores will develop into haploid protonemal cells that can generate buds, which will ultimately turn into gametophores bearing leaves. Gametophores produce gametes by mitosis. Fertilization forms a 2n zygote that develops into sporophytes (2n) and begin the life cycle anew. Schematic is modified slightly from Frank, Decker & Reski (2005).

The protonema will continue to grow and produce ancillary buds, from which leaves (gametophores) will ultimately grow (Zobell, 2005). Single-celled root-like structures called rhizoids extend from the base of the gametophores helping to anchor the plant and to absorb water and nutrients (Sakakibara et al., 2003). Because of this activity, rhizoids are generally believed to lack a cuticle (Wada & Staehelin, 1981). Gametophore leaves are a single cell layer thick. The leaves are characteristic of the gametophyte stage, which, like the majority of the moss life cycle, is haploid. The gametophores bear the gametangia (sexual organs) that will divide by mitosis to produce male and female gametes. Male gametophytes produce antheridia that make sperm (Cove et al., 2006). Female gametes are formed in the archegonia of gametophores (Girod et al., 1999). When adequate moisture is present, sperm released from antheridia swim to an egg within an archegonium (Schaefer, 2002). Fertilization within an archegonium produces a zygote, the first cell of the diploid sporophyte phase of the life cycle. The zygote divides mitotically to produce a diploid embryo and eventually a green stalk called a seta (Cove et al., 2006). As maturation continues, a capsule (sporangium) grows at the top of the seta. Cells within the sporangium, which is covered by the calyptra, undergo meiosis to produce over 4000 haploid spores, completing the life cycle (Zobell, 2005; Cove, 1992). The complete life cycle can be completed in just over three months (Cove, 1992).

***Physcomitrella patens*, a Model Bryophyte**

The moss *Physcomitrella patens* has been used as a model organism for studying many aspects of plant biology. As a monoecious organism, *Physcomitrella* possesses both antheridia and archegonia (Frank, Decker, & Reski, 2005). The relatively small size of this species makes it well suited for research in the confined spaces of a laboratory (Cove, Knight & Lamparter, 1997). Despite its relatively simple structure, there are differentiated cell types including rhizoids, seta and sporangia (Zobell, 2005).

The life cycle of *Physcomitrella* has a dominant haploid gametophyte generation that is larger than the 2n sporophyte stage, which is physically connected to the haploid

gametophyte leaf (Frank, Decker, & Reski, 2005). A diagram of the *P. patens* life cycle is shown in Figure 1-3.

Another reason why *P. patens* is a popular model organism is because it is one of the few practical plant systems for targeted gene knockouts via homologous recombination (HR; Koprivova et al., 2004; Frank, Decker, & Reski, 2005; Schween et al., 2003). This makes *Physcomitrella* a useful model organism for studying the function of genes in plants, through the process of reverse genetics (Schaefer, 2002). Note that *Physcomitrella* is not unique in this capability, as another moss, *Ceratodon purpureus*, has been reported to exhibit similarly high rates of HR (Brücker et al., 2004). Reverse genetics involves observing the phenotype of a specific gene, after that gene is either altered or completely removed (Sessions et al., 2002).

Because the dominant life stage of mosses is haploid and has only a single copy of each chromosome, any mutants formed by homologous recombination are expressed (Schaefer, 2002). As described more completely in Chapter III, homologous recombination can be employed to replace, alter, or knock out genes of interest using constructs that contain complimentary flanking sequences to the target gene (Kamisugi et al., 2006).

An additional advantage of *Physcomitrella* as a model system is that its genome has been fully sequenced (Quatrano et al., 2007). This makes it possible to search *P. patens* for homologs of genes whose function is known in higher plant species (Reski, 1998). As a basal land plant, *Physcomitrella* is positioned as a transitional organism between aquatic algae and land-adapted vascular plants (Rensing et al., 2008). As such, it can provide an insight into both the evolution and function of genes. The investigation of a cuticle on *P. patens* leaves will not only be useful on a practical level, but could also provide information about the evolution of cuticle biosynthesis.

Are Leaves of *Physcomitrella* Covered with a Cuticle?

As primitive land plants that often grow in wet environments, it is not clear that mosses require a cuticle. This is illustrated by the contradictory literature reports regarding the presence (or lack thereof) of a cuticle on the leaves of *P. patens*. Four sources that specifically addressed whether the leaves of *Physcomitrella patens* possess a cuticle have been identified. Without citing any references or new research, two sources explicitly dismiss the possibility of a cuticle existing on the leaves of *Physcomitrella*. Liénard et al., (2008) simply states, “The leaves [of *Physcomitrella*] have no cuticle or stomata and are one-cell thick”. Sakata et al. (2009) also dismisses the possibility of a cuticle in *Physcomitrella* “because mosses do not possess the vascular bundle system, and the gametophore leaves consist of single layer cells without cuticle...” (Sakata et al., 2009). Despite these statements, it is noteworthy that this idea is put forth as common knowledge, without real evidence, perhaps because *P. patens* is viewed as an evolutionarily primitive organism.

In contrast to Sakata & Liénard, several other studies have indirectly and directly investigated the question of the leaf cuticle, including Wyatt et al. (2008) & Buda (2012, personal communication). Wyatt et al. (2008) focused on characterizing the protonema of *P. patens*, but also provided histochemical observations that were consistent with the presence of a cuticle associated with the leaves. They state that, “Conventional staining and light microscopy (LM) were used to provide the first direct evidence that protonemal cells of *P. patens* lack a cuticle” (Wyatt et al., 2008). It is important to note that this statement is made regarding protonemal cells, which are the progenitors to the gametophores themselves. However, this paper further states that the leaves, when exposed to “a protracted (7 h) pretreatment with 10% NaOH” would develop a stain, which was “consistent with the presence of a leaf cuticle” (Wyatt et al., 2008). In addition, in personal communications with Dr. Eric Roberts, Dr. Jocelyn Rose and his student Gregory Buda of Cornell University have indicated that they have obtained

structural, chemical and genetic data consistent with the presence of a waxy cuticle on *Physcomitrella* leaves.

Concluding Thoughts

The goal of the research project described in this thesis is to test the hypothesis that leaves of *Physcomitrella patens* are covered with a cuticle. Specifically, two questions will be addressed: (1) do the leaves of *Physcomitrella patens* have a cuticle? and (2) if so, what genes contribute to the formation of a cuticle?

For this thesis project, I examined the structure of *Physcomitrella* leaves using both light microscopy and freeze fracture electron microscopy. For light microscopy (LM), evidence supporting the presence of a cuticle was obtained from experiments using histochemical stains, enzymes and chemical pretreatments, to achieve a more complete understanding of the exterior of the leaves. Evidence of a leaf cuticle was also discovered through freeze fracture electron microscopy, where samples revealed a morphologically similar layer to that of fractured cuticles from higher plants. To substantiate the conclusions from microscopic observations, mutants lacking a gene thought to be involved in cutin biosynthetic pathway were created through homologous recombination.

In addition to all of the background information presented in this chapter, one additional point, expressed by Riederer (2006) is worth thinking about: “Does it make sense, and is it fun at all, to spend so much time with the outermost micrometer of a plant?” For me, studying the plant cuticle is a worthwhile endeavor for at least three reasons: (1) the cuticle has played an evolutionary vital role in allowing plants to adapt and thrive in the relatively dry land environment. (2) The water conservation function of the cuticle is perhaps even more critical today as the Earth’s temperature continues to rise; due largely to anthropogenic production of greenhouse gases (Marcott et al., 2013). (3) Finally, I believe that contributions, such as this thesis, which characterize the structure, composition and genetics of the plant cuticle, are valuable in part because they provide

the observations required to for a more complete understanding of the basic biology and evolution of the cuticle. In addition, while the focus of this research was academic, the results obtained may help to predict how plants will respond to environmental changes, such as global climate change, and may provide rational targets for genetic manipulations of agricultural species to modify cuticle biosynthesis in ways that make crops more resistant to drought.

CHAPTER II:

HISTOCHEMISTRY & ULTRASTRUCTURE EVIDENCE OF THE *PHYSCOMITRELLA* CUTICLE

Introduction

There are many ways to demonstrate the presence of a cuticle on the leaves of *Physcomitrella patens*. One of these is to determine if the surface of leaf cells are coated with a continuous layer that can be physically isolated by selectively digesting the cell wall polysaccharides and cytoplasm that lie beneath it. A second classical method to demonstrate the presence of a cuticle is by using histochemical stains. A third approach is to visually examine the ultrastructure of the leaf. In this thesis, the electron microscopic features of *P. patens* leaves were examined by a technique known as freeze fracture electron microscopy. Chemical and biochemical analysis, as well as molecular genetic studies (described in Chapter III) can also contribute to detection and subsequent characterization of the cuticle. While none of these methods alone is necessarily adequate to prove the hypothesis that *Physcomitrella* leaves are covered with a cuticle, combining the results of several of these techniques can provide sufficient evidence to confidently accept or reject this proposition.

The selective isolation of cuticle layers in higher plants has been practiced for years (e.g., Orgell, 1955; Lendzian et al., 1986; Franke et al., 2005). This approach is based on the principle that the properties of the polysaccharide portion of the cell wall differ significantly from the cuticle. In one approach, samples can be treated with chromic acid (a mixture of the strong oxidizing agent sodium dichromate in sulfuric acid). Because the polysaccharide portion of the wall, which includes cellulose and other hemicelluloses, and the cytoplasm are primarily hydrophilic, chromic acid hydrolyzes with them readily. The hydrophobic cuticle associates less with the chromic acid and is digested more slowly. If application of acid is timed properly, the cytoplasm and polysaccharide part of the cell wall can be digested, leaving a thin translucent cuticle in the outline of the leaf.

Similar results can be obtained by treating leaf cells with specific enzymes that hydrolyze polysaccharides, but do not recognize waxes or cutin. While neither of these digestion methods alone can prove the existence of a cuticle, they can provide evidence supporting its existence. This thesis describes results of treating leaves with both chromic acid and the cell wall digesting enzyme mixture known as Driselase.

The structure that Augustin Pyramus de Candolle observed when he first coined the term ‘cuticle’ was detected, in part, on the basis of its distinctive staining pattern (Candolle, 1840), and thus it seems reasonable to examine *Physcomitrella* leaves with similar techniques. Wyatt et al. (2008) examined the protonemata and (indirectly) the leaves of *Physcomitrella* by using a series of dyes. In many studies, hydrophobic dyes have been used to “selectively” label the cuticle. In principle, hydrophobic stains should partition into the layers of the hydrophobic cuticle. In practice, it is important to remember that other cell components may show affinity for hydrophobic dyes and stains, and that the nature of the interaction between the stains and their targets can be influenced by many other factors. In addition, many stains are useful markers only if their targets are relatively abundant. Even if *Physcomitrella* leaves are covered with a cuticle, it is likely that the layer would be exceedingly thin compared to those found on vascular plants. This difference in cuticle thickness could reduce or eliminate staining.

While it is somewhat counterintuitive, hydrophilic dyes can also be used to identify the cuticle. The principle underlying this method is that if the epidermal cell wall is covered with a hydrophobic layer (the cuticle), this will prevent the dyes from reaching and binding the hydrophilic polysaccharides of the cell wall. For example, researchers have employed the hydrophilic stain toluidine blue in this method to screen large numbers of *Arabidopsis* plants for mutations that made the cuticle defective (Tanaka et al., 2004).

While classical histochemical staining can be used to identify the cuticle, the resolution of these light microscope methods is limited. Electron microscopy offers the possibility of higher resolution observations. The most common method for examining samples by electron microscopy is called thin sectioning. In this method, chemically fixed samples

are dehydrated, embedded in epoxy resin, and then cut into ultrathin sections. After staining with uranium and lead salts, structures such as the cuticle, can be detected. In part because of limitations on equipment, thin sectioning was not employed in this research. However, an alternative form of sample preparation was available. Dr. Eric Roberts prepared *Physcomitrella* using a technique known as freeze fracture. (Note that Dr. Roberts prepared the samples used for this thesis, but all were examined and photographed by myself.) While freeze fracture is less common than thin sectioning, the technique is straightforward (for a review, see Severs, 2007). Samples are rapidly frozen, then transferred to a cold stage held at high vacuum. The samples are then broken open (“fractured”), which exposes cell surfaces. These exposed surfaces are coated (“replicated”) with a thin layer of platinum and subsequently stabilized with an additional layer of carbon. Finally, the replicated specimen is removed from the vacuum, and all biological material digested with a strong acid, leaving only the resistant platinum-carbon replica of the sample. It is this replica that is examined in the electron microscope, and not the actual organism. While the freeze fracture technique is most often used to study biological membranes, published studies have also successfully visualized the plant cuticle (Willison & Brown, 1977; Willison, 1980; Wada & Staehelin, 1981).

This chapter will show evidence that supports the hypothesis that *Physcomitrella* leaves are covered by a cuticle. Digesting the leaves in either chromic acid or the enzyme Driselase can be used to isolate a surface layer from leaves that is consistent with the presence of a cuticle. Results from light microscopy employing hydrophobic stains provided some evidence of the presence of a cuticle on the leaves of *P. patens*, although one stain was not effective. When hydrophilic stains were applied, leaves generally resisted staining as would be predicted with the protective presence of a hydrophobic cuticle. Finally, freeze fracture transmission electron microscopy of leaves revealed a rippled texture on leaf surfaces that resembles the cuticle known to occur in other vascular plants (e.g., Wada & Staehelin, 1981).

CHAPTER II: MATERIALS AND METHODS

***Physcomitrella* Strains and Growth Medium**

Physcomitrella patens strains GD-11 or GD-4 were used for all observations. The media described by Knight et al. (2002) for *Physcomitrella patens* were used for all routine culturing. All samples of *P. patens* were grown on either BCD or BCDAT medium. Mosses cultured on BCD medium (see below) will preferentially form leafy gametophores, while BCDAT medium prolongs the protonemal stage and delays appearance of leaves.

When preparing the culturing medium, dry ingredients shown in Table 2-1 were added to 1 L purified water with stirring. The solution was then autoclaved for 25 minutes and tempered until cool to the touch. One mL of filter sterilized 55.5 g/L CaCl₂ was added aseptically to the autoclaved solution. The trace elements solution used was stored at room temperature.

Wild-type *Physcomitrella* strains were grown on cellophane disks placed over the agar medium in plastic Petri dishes, which allowed easier removal of individual mosses for experiments (Knight et al., 2002). Note that the transformed *PpCDIKO* strains were grown without cellophane. Plants were cultured in a plant growth chamber, under constant light, at 24°C. Tufts of the “leafy” gametophores were removed from the cultures using forceps that had been sterilized by dipping in 95% ethanol and then flamed multiple times. Culture manipulations were typically carried out under the “BIO Guard” containment hood at Rhode Island College, when transfer to new culture medium was required.

Table 2-1: Growth Medium

	BCD (per L)	BCDAT (per L)
MgSO ₄ heptahydrate	0.25 g	0.25 g
KH ₂ PO ₄ ,	0.25 g	0.25 g
KNO ₃	1.00 g	1.00 g
FeSO ₄ septahydrate	12.5 mg	12.5 mg
Diammonium tartrate	-	0.92 g
Trace Elements Solution*	1.00 mL	1.00 mL
Agar	7.00 g	7.00 g
CaCl ₂ (after autoclaving)	1.00 mL	1.00 mL

* Trace elements per liter = 55 mg CuSO₄.5H₂O; 55 mg ZnSO₄.7H₂O; 614 mg H₃BO₃;
 389 mg MnCl₂.4H₂O; 55 mg/l CoCl₂ 6H₂O; 28 mg KI; 25 mg Na₂MoO₄ 2H₂O.

Imaging

Most light micrographs used in this study were recorded on a Nikon Eclipse E600 (Japan) microscope at Rhode Island College. A few light micrographs were captured on an Olympus IX-71 (Japan) inverted microscope, also at Rhode Island College. *NIS Elements BR*, *QCapture* and *Picture Frame* software was used to capture images from the CCD cameras attached to each microscope.

For all experiments that required direct comparison of intensity or color, image pairs were recorded using identical magnification, light intensity, aperture setting and exposure time, unless otherwise noted. For other images, brightness, contrast and sharpness were adjusted using global digital filters using Adobe Photoshop or ImageJ.

All freeze fracture samples were viewed and images captured using transmission electron microscopy (TEM). Images were recorded in .TIF format at Connecticut College using a Morgagni 268 TEM Digital Transmission Electron Microscope.

Cuticle Degradation and Isolation

Two chemical agents were used to target the cuticle by removing the epicuticular waxes and or cutin polymers. Treatment with chloroform extracts epicuticular waxes but not cutin (Bewick et al., 1993). Sodium hydroxide (NaOH) is a cutin depolymerizing agent that removes both cutin (Thomasson, 1978) and cuticular waxes (Mueller, Carr & Loomis, 1954) of the plant cuticle. Cuticle degradation procedures involving chloroform and NaOH were not carried out together, but performed separately on separate samples. For histochemical reactions, samples of *P. patens* were treated with chloroform for 2 hours or more. 10% w/v NaOH caustic solution was prepared from a 10N (40%) stock solution of NaOH.

Two agents were used to selectively isolate cuticles. Enzymatic isolation of the cuticle was accomplished using Driselase, a commercially available polysaccharide digesting enzyme mixture derived from *Basidiomycetes* sp. culture supernatant (Sigma Aldrich Company, CAS # 85186-71-6). A 2% w/v solution of Driselase was prepared using distilled water and used without pH adjustments. Leaf samples were treated from 3-5 days at 37°C.

Chemical isolation of the cuticle utilized a 50% chromic acid solution. Leaf incubation with chromic acid was performed at room temperature, for times ranging from as little as 5 minutes and or up to an hour. Complete leaf digestion, including the cuticle, is easily possible at 1 hour or more of chromic acid exposure.

Light Microscopy (LM) Stain Preparations and Procedures

A 0.05% w/v aqueous solution of toluidine blue O (Eastman Company, C.I. # 52040) was prepared according to Tanaka et al. (2004) in distilled water. *P. patens* leaves were pretreated with a 10% NaOH (20-28 hr; Wyatt et al., 2008), washed four times in distilled water, checked against pH paper to match the pH of distilled water (pH 6-7), and then stained with toluidine blue for two minutes and rinsed with distilled water.

Two different Sudan III stains were used for histochemical observations. The first, Sudan III fat stain, was obtained as a premixed solution from the Carolina Biological Supply Company (cat. #89-2963). A second Sudan III stain was prepared according to Gahan (1984). A saturated solution on Sudan III in 70% aqueous ethanol was prepared and any insoluble material was removed by centrifugation for five minutes at 8000 rpm.

Sudan IV stain was prepared in ethanol according to Schneider (1981), filtered, and added to an equal volume of glycerin. Difficulties staining the moss cuticle with Sudan IV using the protocol of Chiffelle & Putt (1951) were rectified using the procedure described by Lee & Priestly (1924). In Lee and Priestly's method, the sample is

immersed in stain solution and heated in a boiling water bath for up to 5 minutes. Samples were rinsed in distilled water and wet mounted on microscope slides.

All stains (toluidine blue O, Tinopal, Sudan III and Sudan IV) were centrifuged at 8000 rpm for 5 minutes before use to remove any insoluble particulate material.

Light Microscopy Fluorescent Dye Preparations and Procedures

Tinopal fluorescent dye was made as a 0.01% w/v stock solution in distilled water by dissolving 0.01 g of dye in 100 mL of distilled water. Tinopal was obtained from Dr. Alison Roberts; a similar product, Fluorescent Brightener 28, is commercially available from Sigma-Aldrich, St. Louis, MO, catalog number F3543. The stock solution was dispensed into microcentrifuge tubes and frozen at -20°C. Tinopal fluorescence was viewed using a Nikon UV/DAPI filter set (Davey & Kell, 1997).

Plasmolysis Medium

Some leaf samples were plasmolyzed for 2-4 days, in either 10% w/v NaCl, or 8.5% w/v mannitol (Michel, 1971). Some samples were plasmolyzed to avoid confusion from chloroplast autofluorescence or other autofluorescent cytoplasmic contents, when using fluorescing dyes.

Freeze Fracture Sample Preparation for Transmission Electron Microscopy (TEM)

Freeze fracture replicas of leaves from strain GD-11 (prepared by Dr. Eric Roberts) were examined. Freeze fracture requires four basic steps: 1) freezing, 2) fracturing, 3) shadowing, and 4) cleaning. Double replica freeze fracturing breaks a sample into two halves, and preserves surface details by shadowing the fractured sample with platinum-carbon.

Freshly excised *Physcomitrella* leaves were first mounted in yeast paste (which improved fracture properties) between two copper planchettes (supports) and subsequently frozen in liquid nitrogen-cooled propane to approximately -196°C . Freezing of the sample, which must be accomplished quickly to avoid ice crystal formation, was performed using a commercial freezing device (EMS-002 Rapid Immersion Freezer, Electron Microscopy Sciences, Hatfield, PA). The frozen specimen is then transferred to the stage of the freeze fracture device held between -140°C to -100°C under high vacuum ($< 1 \times 10^{-7}$ mbar). Sample fracturing must be performed under vacuum to avoid vapor condensation on the newly exposed surfaces that would obscure specimen detail when viewed under transmission electron microscopy (TEM). The samples were fractured by separating the two copper planchettes, much like separating the two halves of an Oreo cookie. A replica of the surface of the fractured specimen is then formed by shadowing with a layer of platinum and carbon (ca. 1-2 nm) at an angle of 45° . To stabilize the newly formed replica, the specimen is coated with a film of carbon from 85° angle while being rotated. The replica is removed from the freeze fracture device and all of the organic material is digested using chromic acid. After rinsing by floating on water, the cleaned replica is picked up on Formvar-coated copper grids (for review, see Severs, 2007).

CHAPTER II: LIGHT MICROSCOPY RESULTS

Light Microscope Histochemistry: Cuticle Isolation

After 50% chromic acid was applied to leaf samples of *Physcomitrella* at room temperature, most cytoplasmic contents were rapidly dissolved. The result was a thin, colorless sheet of putative cuticular material. Fig. 2-1A shows a partially digested leaf treated in chromic acid for 5 minutes. A uniformly gray region, devoid of cytosolic contents, shows the cuticle of the leaf, while other regions, presumably not completely digested, show cuticle together with remnants of the cytoplasm (Fig. 2-1A). Acid insoluble cuticular material stained lightly with toluidine blue (TB; Fig. 2-1B). The regions of darker TB staining, accentuated in this phase contrast micrograph, are due to folding of the thin cuticle onto itself. While the putative cuticle is more resistant to chromic acid than other leaf components, it is also ultimately susceptible to hydrolysis. The extent of digestion is directly related to how long leaves are exposed to chromic acid; complete leaf digestion, including the cuticle, occurs after one-hour treatment with chromic acid at room temperature (results not shown).

Figure 2-1: *Physcomitrella* leaves were treated with chromic acid for < 25 minutes. A partially digested leaf (A) shows a thin, slightly translucent layer, which is consistent with a separating cuticle. A putative isolated cuticle (B) is lightly stained by toluidine blue. Scale bar = 100 μm .

An alternative method of cuticle isolation from leaves was hydrolysis using the enzyme Driselase. Driselase is a mixture of fungal enzymes that hydrolyze cell wall polysaccharides and does not act upon the cuticle (Martin, 1960). After treating *Physcomitrella* leaf samples in 2% w/v Driselase for 3-5 days at 37°C, the majority of the cytoplasm and cell wall components had been digested leaving an evanescent sheet of material that appeared to be derived from the leaf surface (Fig. 2-2A&B). In some cases, the remaining thin, delicate, translucent layer maintained the shape of the leaf (Fig. 2-2A). Because the material was difficult to see using bright field light microscopy, it was examined and photographed using phase contrast microscopy. The dark parallel lines seen in phase contrast micrographs are areas where the cuticle has folded. Even after extensive enzymatic digestion, light microscopic observation show that small amounts of undigested cytoplasm remain as evidenced by small, dark dots visible in both Figs. 2-2A, and 2-2B.

Figure 2-2: Treatment of *Physcomitrella* leaves with the polysaccharide-degrading enzyme mixture Driselase results in a thin undigested leaf covering, presumably a cuticle. Images are unstained leaves viewed using phase contrast microscopy. Scale bars = 50 µm.

Staining Driselase-resistant cuticles with toluidine blue occasionally resulted in light purple staining, occasionally accompanied by regions of intense staining (Fig. 2-3A). The darkly stained areas generally occurred only in compact regions where enzyme accessibility was presumably minimal, such as tightly curled or folded leaves. Most Driselase-resistant regions had more cytoplasmic remnants. In these cases, the undigested material was often in the form of dark spots (Fig. 2-3B). Even in relatively clean areas devoid of obvious cytoplasmic contaminants, the putative cuticle region stained lightly with toluidine blue (Fig. 2-3B).

Figure 2-3: Phase contrast images of Driselase-treated *Physcomitrella patens* leaves stained with toluidine blue (TB). (A) Partially degraded leaf treated with Driselase. (B) A putative isolated cuticle with a small amount of undigested cytoplasmic material exhibits light staining after TB treatment. Cells of the leaf tip are visible at the bottom of the image. Scale information not recorded. Compare to control leaves in Fig. 2-4A.

Light Microscope Histochemistry: Hydrophilic Staining

When intact leaves and rhizoids were exposed to toluidine blue, leaves were largely unstained (Fig. 2-4A) even after incubating the leaves in dye for an hour. In a few cases, small patches or regions of leaves can take up the stain. This could signify wounding of the tissue because it appears near cut ends of leaves or in places where the leaf has been damaged. Rhizoids (which would be unlikely to have a cuticle because of their absorptive function) stain intensely. In contrast to untreated cells, *Physcomitrella* leaves stained vibrantly with TB when a cuticle was removed by 10% w/v NaOH (Fig. 2-4B). Treatment with NaOH does not affect the staining of rhizoids, which appear purple in all experiments.

Figure 2-4: Representative LM images of *P. patens* leaves, and rhizoids treated with toluidine blue (TB) stain. (A) Leaves generally remain unstained with only a few areas of TB stain. Rhizoids (presumably not covered by a cuticle) stained intensely after TB treatment. (B) Intense TB staining of leaves occurs if the samples are pretreated with NaOH, which would degrade cuticles. Scale bars = 100 µm.

TB staining experiments were also performed with *Physcomitrella* leaves that had been extracted with chloroform. The results were similar to NaOH extraction (Fig. 2-4), with control leaves largely unstained while leaves treated with chloroform showed uniform staining (Fig. 2-5). In a few cases, small regions of control leaves took up some stain, possibly at wound sites caused from handling. As previously observed, rhizoids stain intensely in both control cells and chloroform treated cells.

Figure 2-5: Chloroform treatment of *P. patens* leaves removes the cuticle leading to staining by toluidine blue. (A) In control samples (not extracted with chloroform) leaves resist TB staining. Rhizoids, presumably not covered by a cuticle, stain intensely with TB regardless of chloroform application. (B) After treatment with chloroform, intense toluidine blue staining of leaves occurs, presumably because the cuticle waxes are removed. Scale bars not recorded.

When intact leaves of *Physcomitrella*, such as those shown in Fig 2-6A, are exposed to Tinopal (a hydrophilic dye that binds β -glucans, including cellulose and other cell wall polysaccharides), very little cell wall fluorescence is observed (Fig. 2-6C; note that Fig. 2-6B&D show the same field, but examined with different types of microscopy). As with toluidine blue, the absence of staining is presumably due to the presence of a leaf cuticle. After pretreatment with 10% w/v NaOH to remove the cuticle (Fig. 2-6B), application of Tinopal produced strong fluorescence in the leaf cell walls (Fig. 2-6D). Note, as before, that Figs. 2-6B&D show the same field. The exposure times for the fluorescence micrographs in Figs. 2-6C&D are identical. Treatment with NaOH also destroys the chloroplasts of cells (compare Figs. 2-6A&B). Unlike leaf cells, rhizoids and protonemal filaments stained positively with Tinopal in both NaOH-extracted and control samples (data not shown).

Figure 2-6: Representative LM images of *P. patens* leaves and rhizoids treated with Tinopal, a hydrophilic fluorescent dye that binds β -glucans, such as cellulose of the plant cell wall. When intact leaves seen in bright field light microscopy in (A) are exposed to Tinopal and examined by fluorescence microscopy (C), essentially no observable fluorescence is visible. This is likely because an intact cuticle blocked infiltration of Tinopal. In samples pretreated with NaOH (B & D), which removes the cuticle, leaves stained strongly when examined using fluorescence microscopy (D). Images A & B (bright field microscopy) and images C & D (fluorescence microscopy) were recorded under identical conditions and show the same fields. Scale information not recorded.

Light Microscope Histochemistry: Hydrophobic Staining

Sections of *Malus domestica* (apple) were used as controls for staining reactions with hydrophobic Sudan III dye following the protocol of Gahan (1984). In unstained sections (Fig. 2-7A) a clearly defined, non-cellular cuticle is readily visible above the epidermis. Beneath the cuticle, reddish cells, containing anthocyanins, were observed. Following staining with Sudan III (Fig. 2-7B), the thick apple cuticle stained a vibrant reddish-orange color. (Both images were recorded under identical exposure conditions). The thickness of the cuticle is approximately 6.3 μm . In addition to the obvious cuticle, red flocculent material was sometimes observed above and below the cuticular layer. This material seems to be precipitated dye and does not represent cuticle staining.

Figure 2-7: Sections of *Malus domestica* (apple) cuticle before and after staining with Sudan III. This sample was used to validate Sudan staining procedures. (A) An unstained cuticle of apple demonstrates that a thick cuticle can be detected without staining. (B) A stained cuticle of apple serves as a positive control for Sudan III labeling of a cuticle. Some flocculent red material (presumably precipitated dye) is occasionally associated found on Sudan III-stained specimens. Scale bars not available.

When leaves of *Physcomitrella* (Fig. 2-8) were treated with Carolina Biological Sudan III stain solution following the staining protocol of Gahan (1984) no obvious staining of the wall was noted (Fig. 2-8B). This result is superficially inconsistent with the presence of a cuticle. The red flocculent material above leaf cells seen in Fig. 2-8B after staining is presumably a precipitate of the Sudan III stain, similar to that observed in stained apple sections, and is not derived from the moss.

Figure 2-8: *Physcomitrella* before and after staining with Sudan III. (A) Unstained (control) leaves of *P. patens* viewed with bright field light microscopy. (B) Staining with Sudan III produced some red flocculent material, but, unexpectedly, the surface of leaves *P. patens* did not stain noticeably. Scale bars = 100 μm .

Because Sudan III staining failed to demonstrate the presence of a cuticle on *Physcomitrella* leaves, a different Sudan dye (Sudan IV) and staining procedure were used. Using the Sudan IV protocol described by Wyatt *et al.* (2008), control leaves (Fig. 2-9A) treated with 10% w/v NaOH and Sudan IV dye. The result was the leaf samples treated with NaOH, which is known to remove cuticles, were pale (Fig. 2-9A). However, Sudan IV-stained leaves appeared reddish (Fig. 2-9B) when observed under identical conditions. A single leaf folding over onto itself under the coverslip causes the different intensities of red coloration in Fig. 2-9B.

Figure 2-9: *Physcomitrella* leaves stained with Sudan IV. (A) Leaves treated with NaOH to remove cuticular materials did not show positive staining when treated with Sudan IV. (B) Leaves that were not subjected to NaOH extraction stained positively with Sudan IV. No chloroplasts area visible in this sample because the Sudan IV protocol involves boiling in an alcoholic solution of the stain for several minutes. Both images were recorded using the same illumination and exposure conditions. Scale bars not available.

After leaves have been extracted with 10% w/v NaOH, which would remove any cuticular material, no histochemical differences were observed between leaves stained with Sudan IV (Fig. 2-10A) and control (unstained) leaves (Fig. 2-10B). Unlike Fig. 2-9B, the folding over of the leaves in this figure does not reveal more vibrant Sudan IV staining. For valid comparison, Figures. 2-9, 2-10 were recorded under identical exposure conditions.

Figure 2-10: Leaves of *Physcomitrella patens* pretreated with 10% w/v NaOH (28h). (A) Leaves from sample are stained with Sudan IV hydrophilic dye and did not illustrate positive staining. Leaves from sample (B) were unstained. Both of these samples demonstrate negative staining for Sudan IV. No scale information available.

CHAPTER II: TRANSMISSION ELECTRON MICROSCOPY RESULTS

Transmission Electron Microscopy – Freeze Fracture

Histochemical evidence for the presence of cuticle on *Physcomitrella* leaves was substantiated by observations using freeze fracture electron microscopy. Although freeze fracture most commonly separates the two leaflets of lipid bilayer that make up biological membranes, no membrane fractures were observed in replicas of *Physcomitrella* leaves. Instead, the fracture plane appeared to follow a path across the surface of the leaf (Fig. 2-11). In many cases, the leaf was fractured in its entirety (Fig. 2-11; a single *Physcomitrella* leaf is highlighted in false color). Black bars are part of the copper grid that supports the replica. The outlines of individual cells are visible. The tears present in Fig. 2-11 are not part of the living cell, but are caused by sample preparation. The process of thawing the fractured replica sample and subsequent cleaning with acid, stressed the metal replica and formed visible cracks.

Figure 2-11: Freeze-fracture replicas of *Physcomitrella* leaves. At low magnification, the replica of a nearly intact single leaf (colorized green) and a copper support grid (black bars) are visible. At this magnification, outlines of leaf cells are evident, but the boundaries between cells are indistinct. No plasma membrane surfaces are present. Scale bar = 100 μm.

Figure 2-12A is a series of images of the same area, shown at increasing magnification. (The white arrow indicates the same structure as the magnification is increased.) Individual cells can be distinguished, although boundaries are indistinct (Figs. 2-12A&B). Figure 2-12B focuses on the junction between both cells, where a tear is also visible. The dark diagonal line visible in Figs. 2-12C&D are due to undulation in the replica itself. While some of this undulation may be due to deformation of replica during processing, part of it is due to the three-dimensional structure of the cells underlying these areas. Fig. 2-12D shows the rippled surface that is likely part of the cuticle.

Figure 2-12: A series of images that show a *Physcomitrella* leaf cuticle at increasing magnification. At lower magnifications (A & B) the outlines of individual cells are visible. At these relatively low magnifications, the surface appears almost featureless. As magnification increases (C & D) a finely textured surface morphology starts to become visible. The white arrows in A-C point to a common landmark (a rip in the replica).

At higher magnification (e.g., Fig. 2-13), the fractured surface shows striations, much like ripples on a pond. The striations sometimes appear to be arranged in lamellae. While the pattern shows evidence of long-range order, fine scale variation in the pattern of the lamellae is also seen. The edges of the lamellae are shallow ridges that are highlighted by electron dense shadows, which result from unidirectional shadowing with platinum and carbon. The rippling pattern demonstrated in Figure 2-13 is common in appearance to freeze fracture visualized cuticles in vascular plants.

Figure 2-13: Freeze fracture replica showing the surface of a single leaf cell showing a rippling pattern of undulating twirls and twists that is typical of many freeze fractured cuticles. This chevron pattern of lamellae was common in the majority of *P. patens* freeze fracture samples.

Some areas of the fractured *Physcomitrella* leaf cuticle show regions where the fracture plane changes layers (Fig. 2-14). In addition to this shingle like appearance, some regions had rounded regions that may show both the cuticle and cell wall layers. In the circular hole in the middle of the micrograph, possible fibrils from the cell wall layer are present.

Figure 2-14: *Physcomitrella patens* leaf cuticle showing a surface typical of most of the leaves examined. The surface is rippled, almost as if covered with shingles. Some regions of the fractured cuticle showed rounded regions where the cuticular material had either not been deposited or had been ripped away in the fracture process. Fibrillar material, possible cellulose microfibrils are seen within the circular region.

Some samples had blister-like structures. These protrusions represent a change in fracture plane. Often, the various blisters appear to be filled with material that shows different fracture characteristics. Note that the blister-like structures are surrounded by the common chevron-like undulating lamellae, which are the most common phenotype of the fracture samples.

Figure 2-15: Blister-like structures visualized in cuticle fractures. (A) Some cuticle regions show numerous “blisters” that may result from heterogeneity of the cuticle or from interdigitation of cell wall polysaccharides with components of the cuticle. (B-D) These images show a range of structural features in the region of the blisters at higher magnification. In (D) the lamellae common in other regions of the leaf surface are visible.

Another series of images (Fig. 2-16) shows details of the blisters and lamellar striations visible in fractured samples of *P. patens* leaves. Lamellar structures, characteristic of plant cuticles, are interspersed with wall “blisters”.

Figure 2-16: Lamellar structures interspersed with wall “blisters”. (A) Some regions of the wall consist primarily of lamella interrupted by occasional rounded blisters. (B) The blisters sometimes appear to be filled with material with different fracture characteristics than the surrounding lamellae.

Distinct from the warty-blisters detailed in the previous micrographs, Figure 2-17 shows the distinct layering of the fractured *P. patens* cuticle. The lamellar structures sometimes appear to lie beneath granular material. The granular material could be epicuticular wax, while the lamellae are consistent with cutin.

Figure 2-17: Freeze fracture image of *P. patens* cuticular layer showing distinct layering. (A) Differentiated zones are sometimes visible within the cuticle. (B) At higher magnification, layers of shingle-like structures are overlaid by granular material. It is possible that the granular material is closer to the surface of the wall and may represent a chemically distinct region of the cuticle.

Figure 2-18 shows layers present in the fractured cuticle. The fracture plane appears to “jump” from layer to layer. The patch visible in the upper right corner of the micrograph shows granular material that appears to coat the lamellar strips.

Figure 2-18: Lamellae and granular components of the cuticular layer. Stair-step layers of organized material are sometimes seen in cuticle replicas. The patch visible in the upper right hand corner of the micrograph, possibly wax, lies above the lamellar strips in the remainder of the image.

CHAPTER II: LIGHT MICROSCOPY DISCUSSION

Historically, the presence of a cuticle has been established by using histochemical stains that interact with hydrophobic components such as waxes, cutin, and cutan (Lee & Priestly, 1924, Chiffelle & Putt, 1951; Schneider, 1981; Gahan, 1984; Wyatt, et al., 2008). A wide variety of stains and protocols have been utilized for this purpose (e.g., Tanaka et al., 2004; Wyatt et al., 2008). While some researchers have claimed that *Physcomitrella* lacks a cuticle (Liénard et al., 2008; Sakata et al., 2009), the histochemical results of one research group have suggested that, like higher plants, *Physcomitrella* does possess a cuticle (Wyatt, et al., 2008). The Wyatt paper focused on the claim that the protonemata of *P. patens* lacked a cuticle, however, the histochemical staining utilized for these experiments suggested the presence of a leaf cuticle. In addition, in personal communication Dr. Gregory Buda (Cornell University) has also indicated that he has evidence for the presence of a leaf cuticle.

Due in part to the weak staining reaction seen in the published micrographs, and in part to the contradictory literature reports regarding the presence of cuticle on *Physcomitrella* leaves, new histochemical and microscopic observations seemed warranted. These new experiments, described in this chapter, included attempts to (1) isolate cuticles from cells using enzymes or acids, (2) to stain the cuticle directly using hydrophobic dyes, (3) to demonstrate the presence of a cuticle through the absence of staining by hydrophilic dyes, and (4) to observe the ultrastructural morphology using freeze fracture electron microscopy.

Cuticle Isolation

One direct method to demonstrate the presence of a cuticle on *Physcomitrella* leaves is to physically isolate it. As shown in the Results section of this chapter, this has been accomplished using both chemical and enzymatic methods.

Chromic acid degradation is an established technique to isolate plant cuticles (Lendzian et al., 1986). Lendzian warns, however, that although “chromic acid treatment may preserve morphological features of cuticles if the concentration is chosen properly”, that the structure (i.e., the highly organized lipid layer) is gradually degraded. Despite these problems, chromic acid treatment still provides a useful means to demonstrate the existence of a superficial wall layer with different chemical resistance than the polysaccharide portion of the wall.

Digestion of *Physcomitrella* leaf tissue with chromic acid begins to change the leaf color and produce bubbles rapidly after exposure to the acid. After brief digestion, much of the thin cuticular “skin” that remains appears devoid of cellular material (Fig. 2-1B), although undigested granular cytosolic contents may remain (Figs. 2-1A). These isolated sheets are delicate and easily folded or damaged, so they must be transferred to microscope slides with great care. Unsurprisingly, different exposure times to chromic acid will yield significantly different degradation results. Chromic acid pretreatment for 25 minutes resulted in isolation of cuticle layers that were quite clean (Fig. 2-1B). In contrast to Lendzian et al. (1986), thicker angiosperm and conifer cuticles required two-day treatment with 40% w/v chromic acid for adequate digestion. In *Physcomitrella*, exposure to 50% chromic acid for one hour completely hydrolyzes the entire leaf, including the putative cuticle layer. This difference is probably because of the *P. patens* cuticle is much thinner than that of higher plants.

In contrast to chromic acid digestion, enzyme preparations such as Driselase provide a gentler and more selective means to separate the cuticle from the surface of *Physcomitrella* leaves. Enzymatic isolation of cuticles is a known method of cuticle visualization (Orgell, 1955; Martin, 1960; Franke et al., 2005). Driselase is a commercial enzyme preparation isolated from basidiomycete fungi and contains a mixture of fungal carbohydrases (Sigma Aldrich, 2011). Its effectiveness in degrading most components of the *Physcomitrella* cell wall is demonstrated by the fact that it is commonly used to isolate protoplasts from moss protonema (Roberts et al., 2011). While it efficiently

hydrolyzes a variety of polysaccharides, including cellulose, Driselase does not attack the hydrophobic cuticle. While the minimum digestion time was not determined, treatment of *Physcomitrella* leaf samples in Driselase for 3-5 days removed most of the cell, leaving only a thin clear layer with contours matching the original leaf sample (Figs. 2-2 & 2-3). Much like the products of acid hydrolysis, these thin sheets of material, are consistent with the presence of a cuticle.

Figure 2-3A&B also shows regions of cellular debris that were not digested by Driselase. This may occur when portions of the leaf are protected from direct contact with the enzyme. This possibility is supported by the observation that cell debris was commonly found in tightly rolled samples (Fig. 2-3A). It is also possible that the enzyme mixture became inactive before hydrolysis was complete. (No time course experiments were conducted to determine how rapidly cellular material was digested.)

One curious result was obtained when staining the sheets isolated by either acid or enzyme hydrolysis. If the isolated sheet was a hydrophobic plant cuticle, then it would not be expected to stain with hydrophilic dyes such as toluidine blue. While the densest toluidine blue staining clearly occurs in regions containing abundant cell wall polysaccharide and cytoplasm (Fig. 2-3A), the putative cuticle layer also exhibits a light purple staining (Fig. 2-3B). Some of the staining of the putative cuticle layer is accentuated by folding of the sheet (similar to the increased red coloration demonstrated by leaf folding, after Sudan IV staining shown in Fig. 2-9B) and from viewing the sample using phase contrast microscopy (which produces intrinsically darker images based on optical path length rather than actual absorption). It is also possible that some minor toluidine blue staining results from trace amounts of hydrophilic wall materials that are either not targets of the enzymes in Driselase or are partially protected by coating with hydrophobic cuticular materials. These materials may be part of the hydrophilic transport system of the cuticle as described in the Introduction of this chapter.

Overall, both chromic acid and enzymatic extractions are effective methods to physically isolate thin sheets that were consistent with the presence of a cuticle on the surface of the *Physcomitrella* leaf. The cuticular sheets isolated by these two methods may be useful for future electron microscope or biochemical studies.

Light Microscope Histochemistry

The hypothesis that the *Physcomitrella* leaf material that resisted acid and enzyme hydrolysis is a cuticle can be further tested using histochemical stains. As outlined in the Introduction to this chapter, stains can be broadly categorized into two groups: hydrophobic and hydrophilic. Stains that are largely hydrophobic, such as Sudan III or Sudan IV, are generally expected to function as “positive stains”, that is, they impart color by dissolving into the hydrophobic components of the cuticle. Stains that are largely hydrophilic, exemplified by toluidine blue and Tinopal, generally act as “negative stains”, that is, reagents that demonstrate the presence of cuticle through a lack of staining. While hydrophilic stains would bind avidly to the polysaccharide components of the moss cell wall, the presence of a hydrophobic cuticle coating the leaf surface would prevent such staining. It should be noted that absolute categorizations of stains as hydrophilic or hydrophobic are not possible.

Light Microscope Histochemistry: Hydrophilic Staining

Many hydrophilic dyes have been used to label cell wall components (Anderson et al., 2010; Hoch et al., 2005). The chemical structures of two of these molecules, toluidine blue (Tanaka et al., 2004; Wyatt, et al., 2008) and Tinopal (Shin & Brown, 1995) are shown in Fig. 2-19. Despite containing aromatic rings, side groups attached to these molecules make them relatively hydrophilic (Gokul, & Shankar, 2012). These stains tend to interact with hydrophilic cellular constituents, including components of the cell wall (O'Brien, et al., 1964; Shin & Brown, 1995). When using hydrophilic dyes, evidence for

the existence of a leaf cuticle occurs when these stains do *not* label the cell walls and cytosolic materials of the target. Like hydrophobic stains, hydrophilic dyes are not absolutely specific, so results should always be considered in light of other supporting evidence. For example, despite being readily water soluble, toluidine blue can also label the hydrophobic wall polymer lignin (O'Brien, et al., 1964). When this occurs, the color of toluidine blue is usually shifted to a greenish-blue color, a phenomenon known as metachromasia. Despite such ambiguities, toluidine blue has been recognized as useful and convenient method to characterize cuticles (Tanaka et al., 2004). In contrast to toluidine blue, the fluorochrome Tinopal is somewhat more specific as it binds to β -glucans, a class of polysaccharides that includes cellulose and hemicellulose (Shin & Brown, 1995).

Figure 2-19: Toluidine blue (A) and Tinopal (B) are hydrophilic dyes used to test for the presence of a cuticle. The presence of a cuticle will reduce the ability of these dyes to bind to hydrophilic components of the cell wall. Only when a cuticle is absent (or removed) do they show significant labeling. Image sources: Toluidine blue: <http://www.sigmaaldrich.com/catalog/product/sial/198161?lang=en®ion=US#> Tinopal: <http://www.sigmaaldrich.com/catalog/product/sigma/f3397?lang=en®ion=US>

With both toluidine blue and Tinopal there are some cases (e.g., Figs. 2-1B, 2-3, 2-4B, 2-5B, & 2-6D) when unexpected staining occurs in cells. This seems to happen primarily when the leaf surface is damaged, for example near cut surfaces or by forceps when attempting to transfer leaves to slide. Occasional regions that stain anomalously could also be due to irregularities of cutin biosynthesis on certain portions of the leaf.

Toluidine blue staining

Toluidine blue staining is a recognized method of identifying a cuticle and epidermal differentiation (O'Brien et al., 1964; Tanaka, et al., 2004; Wyatt, et al., 2008; Gokul, & Shankar, 2012). The staining of intact leaves with toluidine blue for two minutes following the protocol of Tanaka et al. (2004) gives a result similar to Tinopal (e.g., Figs. 2-4A & 2-5A). Application of the dye to intact leaves does not result in significant staining except in cases where the leaf is damaged. As with Tinopal, this result is consistent with the presence of a leaf cuticle. In contrast to leaf cells, rhizoids (Figs. 2-4 & 2-5) and protonemal filaments (data not shown) stained intensely, since these cell types, like roots of vascular plants, readily absorb water and therefore lack a cuticle (Wyatt et al, 2008).

If a leaf cuticle is truly responsible for blocking toluidine blue staining of *Physcomitrella* leaves, then treatments that remove this layer should lead to strong labeling of the cell. In fact, this prediction is exactly what is observed. Extraction of leaves with 10% w/v NaOH for 20-28 hours is known to degrade the cuticle in part due to alkaline hydrolysis of cutin polymers (Thomasson, 1978). NaOH treatment led to extensive labeling with toluidine blue (Fig. 2-4B), while not changing the positive staining pattern of rhizoids and protonema. Similar results are obtained when leaves are extracted in chloroform, which should dissolve waxes (data not shown).

In contrast to intact leaves, weak staining with toluidine blue occurs in cuticular layers isolated by chemical or enzymatic digestion (e.g., Figs. 2-1 & 2-3). There are several

reasons why this may occur even though the cuticle is a known hydrophobic structure. First, the staining is very weak and may simply be negligible nonspecific labeling that occurs with many histochemical reagents. While barely detectable in bright field images, the presence of the label is enhanced when examined using phase contrast. A second possibility is that the weak labeling seen in these samples occurs by the same mechanism that allows toluidine blue to stain hydrophobic lignins as described by O'Brien et al. (1964). While O'Brien and colleagues did not report cuticle staining, lignin and the cuticle are similar enough that some limited interaction with toluidine blue would not be surprising. Third, the cuticle is a structural network that contains many layers, one of which is the cuticle proper (CP). The CP is underlain with cell wall material that is "progressively impregnated with cutin" (Jeffree, 2007). These hydrophilic cuticle components may allow for the passage of hydrophilic materials. For example, 21% of the average cuticle is constructed with non-hydrophilic material, which can be various carbohydrates (Schreiber & Schönherr, 1990; Riederer & Schönherr, 1984). Minor staining of a cuticle with toluidine blue could occur due to small amounts of cell wall polysaccharides that are accessible to the dye.

Tinopal staining

Tinopal is a hydrophilic fluorescence dye that binds to β -glucans and other cellulose-like components found in the plant cell wall (Shin & Brown, 1995). The staining results using Tinopal were nearly identical to those observed in using toluidine blue. Absence of Tinopal labeling of walls in *Physcomitrella* leaf cells (Fig. 2-6C) is consistent with the occurrence of a cuticle on the leaf surface. Unlike the leaf cells, rhizoids and protonemal filaments fluoresce brightly when exposed to Tinopal, presumably because they lack a cuticle.

In contrast to non-extracted leaves, *Physcomitrella patens* leaves treated with NaOH to digest waxes and cutin, leaf cells stain vibrantly (compare Figs. 2-6C&D). Pretreatment

with NaOH had non-noticeable effects on the staining of rhizoids or protonemal filaments; this result was exactly replicated in the toluidine blue experiments (Fig. 2-4).

Since Tinopal, like toluidine blue binds to hydrophilic elements in the leaves, applying these stains to isolated cuticles that lack the hydrophilic polysaccharides of the cell wall would not be expected to produce significant staining. In the case of Tinopal fluorescence, this expectation is largely realized. As seen in Figure 2-6, the walls of cells in intact leaves (A&C) show essentially no fluorescence when compared to cells that have treated with NaOH to remove cuticular materials (B&D). This is impressive because Tinopal is a fluorescent molecule and, as such, is a more sensitive tool than non-fluorescent stains such as Sudan IV or toluidine blue that are used for bright field microscopy observations. In regions where the leaf is damaged (and the cuticle is therefore compromised), limited cell wall fluorescence can be seen (data not shown).

In sum, negative staining with toluidine blue and Tinopal both provide similar results that are generally consistent with the hypothesis that a cuticle covers the leaves of *Physcomitrella*.

Light Microscope Histochemistry: Hydrophobic Staining

Positive staining of the *Physcomitrella* leaves with hydrophobic dyes would be predicted if the leaves are covered in a cuticle similar to those found in vascular plants. Hydrophobic stains that have been used to label cuticles include Sudan III (Gahan, 1984) and Sudan IV (Lee & Priestly, 1924; Chiffelle & Putt, 1951; Schneider, 1981; Wyatt, et al., 2008). These molecules all have extensive conjugated aromatic rings that contribute both to their bright color and high hydrophobicity (Fig. 2-20). The hydrophobic nature of these compounds presumably allows them to dissolve in the hydrophobic components of the cuticle, including waxes and cutin. The nature of this interaction is nonspecific, meaning any hydrophobic substance has the potential to take up the stains. In addition,

stains are typically applied in alcoholic solutions, which have the potential to dissolve some of their intended targets.

Figure 2-20: Sudan III (A) and Sudan IV (B) are hydrophobic stains that have been used to detect plant cuticles. The additional methyl groups on Sudan IV may contribute to greater overall hydrophobicity of the molecule. Image sources: Toronto Research Chemicals, <http://www.trc-canada.com>.

In order to verify the efficacy of one of these hydrophobic dyes (Sudan III), staining reactions were first carried out using hand cut sections of apple fruit as positive controls. Apples have previously been shown to have a thick cuticular waxy layer (e.g., Kamimura, 1964). In unstained sections, a relatively thick, colorless cuticle was visible overlaying the epidermis. The red color in cells beneath the cuticle (shown in Fig. 2-7) is due to anthocyanins in the vacuoles of epidermal and hypodermal cells responsible for the apple's famous red color. Following application of Sudan III, the cuticle layer stained intensely red, consistent with the hydrophobic nature of this covering. In apple fruit, the cuticle is quite thick (approximately 6.3 μm for the sections shown in Fig. 2-7). The cuticle in other plants, including *Physcomitrella*, is presumably substantially thinner with corresponding reduction of staining intensity. In addition to the obvious staining of the apple cuticle (Fig. 2-7B), treatment with Sudan III also produced a flocculent (fluffy) material that appears loosely associated with section. This material was also visible 20 – 30 minutes after initial staining, even in areas of slide without a specimen (data not shown). This artifact may be a result of precipitation that occurs when Sudan III (which is applied as an alcoholic solution) comes in contact with water during wash steps.

The Sudan III and protocol utilized for the identifying the apple cuticle (Fig. 2-7) was applied to leaves of *Physcomitrella*. While previous experimental evidence suggested the presence of a cuticle on its leaves, *P. patens* leaves stained with Sudan III did not show significant staining. There are at least four reasons why the Sudan III might fail to label leaf cells. First, the *Physcomitrella* cuticle may be present, but could be chemically different from the cuticle in the apple (for example, composed of different waxes or a modified form of cutin). Second, a moss cuticle might be so thin that any staining that does occur is not easily discernable. Third, differences in staining protocols used with Sudan III and Sudan IV may yield different results. Of course, a final possibility is that the leaf lacks a cuticle. The latter possibility seems unlikely simply because it disagrees with several other lines of evidence described in this thesis.

Sudan III is only one of several related dyes used to label cuticles in vascular plants. For example, Wyatt et al. (2008) has utilized a different stain, Sudan IV. When Sudan IV was applied to samples of untreated leaves and to leaves extracted with 10% NaOH a pattern of staining consistent with the presence of a cuticle was obtained. Sudan IV staining of non-extracted leaves resulted in light red coloring (Fig. 2-8B). The more intense coloration seen in a sector of the leaf in Figure 2-8B is due to the leaf folding back upon itself, thus doubling the relative intensity. This is consistent with the observations of Wyatt et al. (2008).

When *Physcomitrella* leaves that have been pretreated with NaOH to remove the cuticle (Thomasson, 1978) are exposed to Sudan IV (Figs. 2-9A & 2-10), they appear essentially identical to NaOH extract leaves that have not been exposed to Sudan IV (Fig. 2-10B). The absence of staining in the extracted leaves is again consistent with the presence of a cuticle. Note that hydrophobic dyes were not used to stain the isolated cuticle because the treatments used by the Sudan IV protocol (ethanol, boiling, etc.) would have destroyed this thin layer.

CHAPTER II: TRANSMISSION ELECTRON MICROSCOPY DISCUSSION

The original impetus for the asking whether *Physcomitrella* produces a leaf cuticle came from observing an unusual morphology in freeze fracture replicas of moss leaves. Dr. Eric Roberts had initially hoped to examine protein complexes in the plasma membrane of leaf cells involved in cellulose synthesis. Unexpectedly, however, no plasma membrane fractures were observed. Instead, only shingle-like structures, which occasionally had blister-like regions were found. It is ironic that this unsuccessful effort to view the plasma membrane of *Physcomitrella* leaves, serendipitously provided the initial evidence for the presence of a leaf cuticle, and subsequently laid the framework for this graduate thesis. Dr. Eric Roberts prepared all of the replicas examined for this thesis while working in the laboratory of Dr. Candace Haigler in the summer of 2012.

In the freeze fracture technique, the fracture plane that develops when a specimen is cleaved follows the path of lowest energy (often separating the leaflets of biological membranes). While many fracture paths are possible, they often run through hydrophobic regions. The reason that hydrophobic samples fracture is because the hydrophobic tails of wax and cutin monomers will form hydrophobic interactions that are substantially weaker than many other chemical interactions. Layers of hydrophobic material represent planes of weakness at which fracturing readily occurs (Willison & Brown, 1977).

When freeze fracture separates the two leaflets of biological membranes, embedded membrane proteins (intramembranous particles or IMPs) are invariably observed. This behavior has been exploited in *Physcomitrella* and other plants to identify distinctive clusters of plasma membrane particles known as cellulose synthesizing complexes (CSCs) that synthesize cellulose microfibrils (e.g., Giddings, 1980). Although fracture planes often split membrane bilayers, they have also been documented to fracture plant cuticles (Willison & Brown, 1977; Willison, 1980; Wada & Staehelin, 1981).

Knowing that both hydrophobic cuticles and plasma membranes can be split in half,

Physcomitrella freeze fracture replicas were examined by transmission electron microscopy (TEM). The fact that the fractured surfaces of *Physcomitrella* leaves lacked intramembranous particles demonstrated that the exposed structures were not membranes. These fractured surfaces are almost certainly the cuticular layer corresponding to the layer previously obtained by enzyme and acid digestion and observed by light microscope histochemistry.

At relatively low magnification (Fig. 2-11) the boundaries of individual cells are also easily detected. At low magnification, a seemingly featureless, continuous layer covers the surfaces. The fact that no discontinuities are seen between cells indicates that the layer exposed by fracturing cannot be plasma membrane, because the membranes of each cell would have to be separated by cell walls. In addition, this sample and every other sample examined lack intramembranous particles (IMPs), showing conclusively that the fractured layer is not plasma membrane.

The lamellate structure of the cuticle, including both the outermost “cuticle proper” layer and the subtending cuticular layer, is often more apparent in electron microscope thin sections (reviewed by Jeffree, 2006). The cuticle proper layer of most species is “of lamellate construction” exhibiting alternating light and dark layers in thin sections (Jeffree, 2006). The lamellar orientation in the cuticle proper layer of most, but not all, species of plants is “strictly parallel to the epidermal cell surface” (Jeffree, 2006). The cuticular plates are not always oriented in a single direction; some have a whorled appearance. This appearance is fully consistent with the structures seen in *Physcomitrella*.

Figures 2-12D & 2-13 detail the lamellar structures that are common to every fractured *Physcomitrella* sample investigated. As the magnification is increased (Fig. 2-13), the surface appears to be composed of plate-like pieces that sometimes show evidence of lamellation (layering). This surface morphology is completely consistent with the several reported cuticles from vascular plants that have been examined by freeze fracture, most similarly to the work of Wada and Staehelin (1981).

It has been established that plant cuticles are lamellar in nature (Willison & Brown, 1977; Willison, 1980; Wada & Staehelin, 1981; Krüger, et al., 1995). However, the lamellae observed in *Physcomitrella* leaves most strikingly resemble the cuticle reported in protonema of *Adiantum capitlus veneris* by Wada and Staehelin (1981; see comparison in Fig. 2-21A). In both samples, a layer of plate-like pieces resembling shingles covers the entire plant surface. Wada and Staehelin (1981) state that the layered structure is a waxy cuticle and did not characterize its structure or physical makeup any further. Note that the *Adiantum* “protonema” referred to by Wada and Staehelin is quite different from the *Physcomitrella* protonema. While the *Physcomitrella* protonema resemble filamentous algae and almost certainly lack a cuticle, the protonema of *Adiantum* is a relatively large organized structure that clearly has a cuticular layer associated on the outer surface of its cell walls.

Figure 2-21: Freeze-fracture micrographs of the protonemata of *Adiantum capitlus veneris* (A), and the leaf of *Physcomitrella patens* (B). Figure 2-21A, taken from the paper by Wada and Staehelin (1981), shows the cuticle of *Adiantum*, exhibiting a multilayered waxy surface coat (WC), a fibrillar (cell wall) layer (FL) and a small piece of the protoplasmic face of the plasma membrane (PM). A morphologically similar layer, shown at the same magnification, is seen on fractured leaf cells of *Physcomitrella* (B).

Fractured *Physcomitrella* leaf samples appear structurally similar to cuticles observed in freeze fracture by other researchers (e.g., Willison, 1980; Willison & Brown, 1977). Willison and Brown (1977) note that both the ovule and fibers of *Gossypium hirsutum* L. (cotton) exhibit a characteristic fracturing pattern with layers similar to those found in fractured *Physcomitrella* leaves. For example, the surface shown in Figure 2-15A-D is visually similar to their observations. They indicated that “the stratified lamellae revealed by freeze fracture...must represent the waxy cuticle” (Willison & Brown, 1977). Although cotton fibers often fracture through the cuticle layer, they noted that freeze fractured samples of cotton root hairs, which lack a cuticle, typically occur through the plasma membrane. This situation is analogous to the difference between the leaves and rhizoid filaments of *Physcomitrella*. Though rhizoids of *Physcomitrella* were never fractured for this experiment, histochemical experiments indicate that rhizoids of *P. patens* lack a cuticle.

Figure 2-15A-D is a series of images that shows increasing magnification of the blister-like protrusions that are similar to the structures also evident in the micrographs of cotton fibers (Willison & Brown, 1977) and tobacco leaves (Willison, 1980). A minority of the *P. patens* leaf micrographs contained these structures. The majority of the images had the lamellar composition that Wada and Staehelin’s (1981) micrographs had. These structures sometimes appeared to be filled with material with different morphology (Figs. 2-16). This spotty, warty, and blister-like morphology could be indicative of cell wall structures protruding through the cuticle. This could indicate a fracture through the Cuticle Layer (CL) layer of the cuticle, because this layer is made up of both cell wall and cutin.

Other research by Willison (1980) identified a cuticle on the leaves of *Nicotiana tabacum* (tobacco) that also shows a lamellate cuticle after freeze fracturing. Granular structures are visible underneath the cuticle; this could be similar to the blister-like structures in Figures 2-15 & 2-16, that have not broken through into the cuticle. Similar to *Physcomitrella* leaves, tobacco leaves showed preferential fracturing through the

cuticular plane to reveal lamellae, although the tobacco cuticle has a somewhat different morphology the cuticular layers or either cotton fibers or *Physcomitrella*.

Figures 2-17 and 2-18 show multiple fracture planes within the cuticle of *P. patens*. The distinct layering evident in the Willison (1980) micrographs is sometimes visible within fractured *P. patens* samples. Most importantly, these micrographs show that the shingle-like structures are overlaid by granular material. This is not only evidence of cuticular layering within the fractured sample, but may indicate different chemical compositions between cuticular layers. For example, it is possible that granular material may be made up of wax crystal, and that the granules visualized here may begin to represent that layer. Visualizing the distinct layering is consistent with the anatomy of the plant cuticle.

The reason that cuticles in vascular plants form lamella is not completely understood. This pattern may result from alternating layers of fatty acids in cutin, wax, or an interdigitated combination of the two (Jeffree, 2006). It is possible that *Physcomitrella* leaves could serve as a useful model to study such questions.

In sum, the results drawn from the freeze fracture replicas *Physcomitrella* leaves suggest that the leaf surface is covered with distinct lamellate cuticle. Which layer of that cuticle, the cuticle proper (CP), the cuticular layer (CL), is fractured remains uncertain. Judging from the micrographs, both the CP and CL fracture at different points of the sample. Regardless of the identification of these specific layers, the fracture samples provide evidence that is consistent with the presence of a cuticle.

CHAPTER III:

CUTIN BIOSYNTHESIS GENES IN *PHYSCOMITRELLA PATENS*

Molecular Genetics of the Cuticle

Data presented in this thesis provides several types of evidence for the existence of a cuticle on the leaves of *Physcomitrella*: isolation of a resistant surface layer, histochemical reactions, and ultrastructural evidence using freeze fracture electron microscopy. These results support and expand upon other research reports, such as Wyatt et al. (2008), and Buda (personal communication). However, these experiments only serve as “skin deep” evidence for the presence of a leaf cuticle. The visualization of the *Physcomitrella* cuticle described in the previous chapter raises many more questions. Does the cuticle consist of both cutin and wax? Is the cuticle chemically identical to those of vascular plants? Is there genetic evidence for the presence of a cuticle? There are many more questions that cannot be answered by structural and histochemical observations alone.

An alternative way to investigate the *P. patens* cuticle is to study it genetically. If the leaves of *Physcomitrella* are covered with a cuticle, the genome should contain genes homologous to cuticle biosynthetic genes of higher plants. Identifying such genes would make it possible to genetically manipulate *P. patens*, including generating knockout lines. This research could provide further support for the presence of leaf cuticle, while also extending our understanding of both cuticle function and evolution.

Creating gene knockouts in *Physcomitrella patens* involves several steps. First, a list of genes from vascular plants that are involved in cuticle biosynthesis is created. Next, these genes are used as queries to search for homologs within the *P. patens* genome. After a candidate *Physcomitrella* gene is identified, a “knockout vector” is constructed and the cuticle gene deleted by homologous recombination.

The identification and selection of target genes is obviously critical. Ideally, the targeted genes would be specific to cuticle synthesis and function near the end of the cuticle biosynthetic pathways. For example, synthesis of hydrophobic cuticle monomers would likely utilize lipid synthesis enzymes. However, because lipid synthesis is central to many metabolic pathways, inhibiting a gene product that catalyzes an early step in fatty acid synthesis pathway would affect not only cuticular lipids, but also many other cellular processes. A better possible target would be a gene involved in cuticle polymerization. The glycine–aspartic acid–serine–leucine motif lipase/hydrolase (GDSL) family of proteins is involved in cutin polymerization and functions near the end of cuticle biosynthesis pathway (Girard et al., 2012). In vascular plants, one specific *GDSL* gene is *cutin deficient 1* (*CDI*).

In this thesis, a *Physcomitrella* mutant lacking the *CDI* homolog (the *Ppcd1KO* knockout line) was produced through the process of homologous recombination. Knockouts and targeted gene replacement are classic methods used to study the function of specific genes. The gene that is deleted is typically replaced by a selection cassette that includes antibiotic resistance to facilitate recovery of mutant lines. Once mutants are recovered, the function of the targeted gene can often be inferred by examining the phenotype produced.

Searching the *Physcomitrella patens* Genome for Cuticle Synthesis Genes

The scientific literature has described a number of genes involved in cuticle biosynthesis. For this thesis, the coding sequences (cds) of several known cuticle genes from other plants species were BLASTed against the *P. patens* genome using the Cosmoss website (<http://www.cosmoss.org/>). BLAST searching attempts to align the known cuticle genes (the query) to genes in the database (in this case *Physcomitrella*) referred to as the “subject”. Alignment is achieved by inserting gaps into the query or subject sequences in order to produce optimized alignments between the nucleotides or amino acids.

Because BLAST will align sequences regardless of whether they are truly homologous, it is important to have some way to evaluate which results are biologically meaningful. BLAST searches provide several pieces of useful data for potential matches: E-values, bit-scores, and percent identity. The E-value (“expected value”) is the likelihood of two sequences showing alignment by chance alone. A lower E-value signifies a more significant match of the query within the searched genome. In contrast, a higher bit-score indicates a better statistical match within the selected size of the genome search. Finally, a higher percent identity result is preferential. “Identity” is defined as an exact match between the query sequence of the known gene and the subject sequence (*i.e.*, the *Physcomitrella* genome) after computer alignment.

Representative samples of higher plant genes involved in cuticle biosynthesis were selected to use in a search of the *P. patens* genome. Several research reports have already identified genes within the *P. patens* genome that could participate in cuticle biosynthesis (Rensing, 2006; Yang et al., 2010), thus it seems reasonable to examine the putative *Physcomitrella* cuticle through the same techniques. In one study, a *Physcomitrella* homolog of the *Zea mays crinkly4 (cr4)* gene, which encodes a kinase required for cuticle synthesis, was identified (Tanaka et al., 2004). Significantly, Yeats et al. (2012) has identified a tomato *CDI* gene that is essential for cutin biosynthesis. My research has also identified several possible cuticle homologs in *Physcomitrella* (Table 3-1), including a cutin synthase gene that is homologous to the tomato *CDI* gene, yet distinct from that that described by Yeats et al. (2012).

Table 3-1: Putative *Physcomitrella* Homologs of Known Cuticle Synthesis Genes

Gene	Organism	Function
<i>fiddlehead</i> (<i>FDH</i>)	<i>Arabidopsis</i> <i>thaliana</i>	Elongation fatty acids for cuticle monomers (Nawrath, 2006)
<i>bodyguard</i> (<i>BDG</i>)	<i>Arabidopsis</i> <i>thaliana</i>	Epidermis-specific extracellular protein involved in the synthesis of the cuticle (Pollard et al., 2008)
<i>Eceriferum</i> (<i>cer</i>)	<i>Arabidopsis</i> <i>thaliana</i>	Epicuticular wax production (Tanaka et al., 2004)
<i>cutin deficient 1</i> (<i>CDI</i>) GI: XP_001764401.1	<i>Solanum</i> <i>lycopersicum</i>	Extracellular acyltransferase that catalyzes the polymerization of cutin monomers (described by Yeats et al., 2012)
<i>cutin deficient 1</i> (<i>CDI</i>) GI: XP_001758743.1	<i>Solanum</i> <i>lycopersicum</i>	Extracellular acyltransferase that catalyzes the polymerization of cutin monomers (identified in this thesis)

The fact that multiple homologous genes known to participate in cuticle biosynthesis in vascular plants are also present within the *Physcomitrella* genome is consistent with other types of evidence presented in this thesis and support the hypothesis that *Physcomitrella* leaves possess a cuticle.

My thesis research concentrates on genes involved in the synthesis of cutin rather than waxes. This decision was made for practical reasons. In personal communication with Dr. Roberts, Dr. Gregory Buda indicated that he and his lab had already made substantial progress on identifying, knocking out, and characterizing cuticle wax genes in *Physcomitrella*. Therefore I sought to further characterize genes that affect the cutin component of the *Physcomitrella* cuticle.

Cutin Properties, Biosynthesis and *CDI* Activity

Typical plant cuticles are layered structures composed primarily of cutin and epicuticular waxes. Cutin is a polymer—more technically a polyester of glycerol, hydroxyl- and epoxy-omega fatty acids—that interdigitates with both the wax layer above and cell wall polysaccharides below summarized in Figure 1-1 (Graca et al., 2002).

The formation of a cutin layer occurs in three distinct stages: 1) synthesis of cutin monomers, 2) transport of cutin monomers out of the cell, 3) polymerization of monomers into a continuous polymer of cutin. Cutin monomers are made in leucoplasts or smooth endoplasmic reticulum of epidermal cells (Samuels Kunst, & Jetter, 2008; Pollard et al., 2008). According to Kolattukudy (2001), typical C₁₆ monomers of cutin are 10,16- dihydroxyhexadecanoic acid, 9- dihydroxyhexadecanoic acid, and 16- hydroxyhexadecanoic acid. In contrast to cuticular waxes, which are very-long-chained carbon molecules (C₂₄- C₃₄), the hydrocarbon chains of cutin monomers are much shorter (Pollard et al., 2008). Monomers are transported from the cytoplasm, across the plasma membrane and into the cell wall space (the apoplast). Both cutin monomers and waxes

are moved across the plasma membrane by ATP-binding cassette (ABC) transporters (Girard et al., 2012). Lipid transfer proteins (LTPs) may carry cutin monomers through the cell wall, towards the site of polymerization (Pollard et al., 2008). Polymerization of cutin monomers occurs in the wall (Kolattukudy, 2001).

While the biosynthesis of cutin monomers is well documented, the mechanisms of extracellular polymerization are not well understood (Pollard et al., 2008; Girard et al., 2012). Monomer ester linkages occur most often at the primary hydroxyl groups (Fig. 3-1) and result in the formation of a polymer. Multiple ester bonds can be formed between monomers by multiple types of polyester synthases (PS), also known as cutin synthases. The activity of cutin synthases results in a branching polymer structure (Pollard et al., 2008). Overall, the plant cutin polymer is strengthened and supported by primary alcohol ester linkages, catalyzed by cutin synthases. Note that additional bonding comes from various secondary hydroxyl groups, which contain cross-linking ester bonds (Heredia, 2003). Branching of cutin polymers occurs when carboxyl groups bond to hydroxyl groups of another monomer or to glycerol. This branching, involving multiple ester linkages and often is termed “crosslinking” (Girard et al., 2012). Because cutin polymers are connected to each other by multiple, branching ester bonds, they are relatively strong and resistant to depolymerization, but can be depolymerized by alkaline hydrolysis (Thomasson, 1978).

Figure 3-1: An example of a cutin oligomer, formed by several cutin monomers, made of omega-hydroxy fatty acids. This diagram is available at AOCS Lipid Library (<http://lipidlibrary.aocs.org/plantbio/polyesters/index.htm>).

Enzyme families such as glycerol-3-phosphate acyltransferase (GPAT) and glycine–aspartic acid–serine-leucine motif lipase/hydrolase (GDSL) are known to be involved in cutin polymerization. GPAT enzymes have been demonstrated in *Arabidopsis thaliana* to have cutin polyester synthase catalytic activity in glycerol related cutin components (Li et al., 2007). Members of the GDSL family of enzymes are extracellular, and are involved in cutin polymerization reactions (Yeats et al., 2010; Girard et al., 2012).

In 2009, the CD1 protein of *Solanum lycopersicum* (encoded by the *Solanum lycopersicum* gene *cutin deficient 1* or *SlCD1*; GI#: 339717982) was recognized as an acyltransferase (Isaacson et al., 2009; Figure 3-2). The CD1 protein functions as a cutin synthase, transferring a single cutin monomer (2-MHG) to another 2-MHG monomer (Beisson & Ohlrogge, 2012, & Yeats et al., 2012). If the *GDSL1* (=CD1) gene is knocked out, significantly less cutin is polymerized on the plant surface (Yeats, 2012). Isaacson et al. (2009) showed that a mutant *CD1* line had a 95% reduction in cutin deposition on the surface of *Solanum* fruit. In tomato, the CD1 protein is crucial to the polymerization of cutin (Girard et al., 2012). CD1 is the first extracellular cutin synthase enzyme identified to form polyester bonds involved in cutin biosynthesis (Beisson & Ohlrogge, 2012). The fact that the CD1 protein has been identified as a cutin synthase that functions near the end of the cutin biosynthesis pathway is a significant reason that the *Physcomitrella* homolog of *CD1* was selected for gene knockout experiments for this thesis. There are at least two genes in the *Physcomitrella* genome that show high similarity (and therefore presumed homology) to the tomato *CD1* gene. One of these, GI XP_001764401.1, was described by Yeats et al. (2012) and the second, GI XP_001758743.1, was identified as part of this thesis research project.

Figure 3-2: In higher plants, the *CDI* gene encodes for an acyltransferase enzyme that transfers a hydroxyacyl group from 2-MHG to the growing cutin polymer. Polymerization is accomplished extracellular and results in increased deposition of cutin on the plant surface. (Image taken from Yeats et al., 2012).

The simple presence of a *Physcomitrella* homolog to the *Solanum CDI* cutin synthase gene does not guarantee that the *PpCDI* has the same function as *SlCDI*. One way to verify its function is to create a *PpCDI* knockout. In *Physcomitrella*, knockout mutants lacking a specific gene can be generated through homologous recombination. In tomato, the phenotype of a *CDI* knockout was a dramatic reduction (95%) of surface cutin levels on tomato fruits (Issacson et al., 2009; Girard et al., 2012; Yeats et al., 2012). The phenotype of a knockout mutant of the homologous *CDI* gene in *Physcomitrella patens* (*PpCDI*KO) will clearly be different (since mosses do not produce fruit), but will be instructive.

Because there are at least two *Physcomitrella* homologs of the *Solanum CDI* gene, it may eventually be necessary to make a double knockout. Even if double knockouts are required, it is necessary to first construct a single knockout and characterize its phenotype before the second mutation can be induced.

This chapter will present genetic evidence that supports the hypothesis that *Physcomitrella* leaves have a cuticle. As part of this thesis, knockouts of the *Physcomitrella CDI* gene (GI XP_001758743.1) have been successfully created. The initial phenotypes are not dramatic—itsself perhaps a significant observation. Further characterization of this mutant by other researchers may identify cuticular alterations. Perhaps the function of *CDI* is duplicated by other *Physcomitrella* genes, such the *CDI* homolog identified by Yeats et al. (2012). Alternatively, it is possible that even if cutin synthesis is eliminated the wax portion of the cuticle is still intact and functioning as a structural barrier. Of course, it is also possible that *Physcomitrella CDI* plays some metabolic role other than cuticle polymerization. In any case, the availability of a *CDI*-deficient mutant line now allows these larger questions to be addressed.

CHAPTER III: MATERIALS AND METHODS

Overview of Knockout Methods

The basic methods for creating *Physcomitrella* knockouts are outlined in Roberts et al. (2011). In essence, a construct is prepared that includes 5' and 3' intergenic sequences flanking the gene targeted for deletion. The knockout target gene is then replaced by a selectable marker, such as the antibiotic hygromycin. Most cloning steps involved in the creation of the knockout vector are accomplished using the Invitrogen Multisite Gateway system. When the knockout vector is transformed into wild-type *Physcomitrella* protoplasts, some undergo homologous recombination with the wild-type gene locus. Knockout mutants are selected by treating cells regenerated from the protoplasts with the antibiotic hygromycin. To ensure that homologous recombination has occurred in the appropriate location, the cells are genotyped using PCR.

General information on Gateway cloning is described in the Gateway Multisite Manual (http://tools.invitrogen.com/content/sfs/manuals/multisitegatewaypro_man.pdf), accessed January, 2013. An overview of the generation of moss knockout mutants is given by Roberts et al. (2011).

Isolation of *Physcomitrella* Genomic DNA

Physcomitrella patens DNA used for PCR amplification of intergenic regions flanking the *PpCD1* gene was isolated using the method of Ravit Eshed (personal communication). Colonies (ca. 190 mg) of moss gametophytes were homogenized in liquid nitrogen with a mortar and pestle. The frozen powder was placed in 1.9 mL extraction buffer (1 mL of DNA extraction buffer stock, 1 mL nuclei lysis buffer, 0.4 mL 5% sarkosyl and 3.8 mg sodium bisulfite) for 20 minutes at 65°C. The composition of DNA extraction buffer stock and nuclei lysis buffer are given below.

84 μ L of chloroform:octanol (24:1) was added to the tubes, which were inverted 6 times and then centrifuged at 13,000 rpm for 5 minutes. The upper aqueous layer (containing the DNA) was transferred to a new tube and DNA was precipitated by adding 0.9 mL of solution containing 0.2M sodium acetate (pH 7) and 80% ethanol. The tube was inverted 6 times and placed at 4°C for 15 minutes. After centrifugation at 13,000 RPM for 5 minutes, the DNA pellet was washed with 1.5 mL of 70% ethanol. The sample was centrifuged again and the supernatant discarded. After another round of centrifugation, any residual liquid was discarded and the tubes were left open for 10 minutes to allow the ethanol to evaporate (ethanol will interfere with PCR). The remaining genomic DNA pellets were dissolved in 100 μ L of ultrapure water and samples stored frozen at -20°C.

DNA Extraction Buffer Stock

1) To 80 mL of ultrapure water add:

sorbitol	6.36 g
Tris base	1.20 g
EDTA (disodium)	0.17 g

2) pH to 7.5 with hydrochloric acid.

3) Adjust volume to 100 mL with ultrapure water.

Nuclei Lysis Buffer

1) To 40 mL of ultrapure water add:

1 M Tris (pH 7.5)	20 mL
0.25 M EDTA (pH 8.0)	20 mL
CTAB	2.00 g
sodium chloride	11.7 g

2) pH to 7.5 with hydrochloric acid.

3) Adjust volume to 100mL with ultrapure water.

4) Sterilize by autoclaving.

DNA Quantitation

DNA samples were quantified by using a NanoDrop 8000 Spectrophotometer (Thermo Scientific, USA) using 2 μ L samples.

Plasmid Minipreps

A QIAprep Spin Miniprep Kit (cat. no. 27104, Qiagen) was used for plasmid DNA isolations. All steps were performed in accordance with Qiagen Miniprep instructions.

Agarose Gel Electrophoresis

All gel electrophoresis of nucleic acids was performed using 1% agarose gels in TAE buffer containing ethidium bromide. Gels were run at 100V (constant). An NEB 2LOG Ladder (New England Biolabs 1KB) was used to determine the approximate size of DNA fragments.

Gene Selection

A *Physcomitrella* homolog of the *Solanum lycopersicum* *CDI* gene (GenBank accession JF968592.1, GI number is 339717982; see <http://www.ncbi.nlm.nih.gov/nuccore/JF968592>) was selected for knockout experiments. In this thesis, this gene is referred to *SlCDI*. The coding sequence (CDS; GI 33971798) and deduced amino acid sequence (GI 339717983) of the *Solanum lycopersicum* *CDI* gene used for experiments are listed below.

```
>(gi|339717982:59-287, 1214-1344, 1431-1673, 1931-2183, 2358-2590) Solanum
lycopersicum cutin-deficient 1 protein gene, complete cds
ACAAACCAATTTATAATATTTTTGTAAACAACACACTTTTTTTATTTTTTTTGCCAAAATGGCCACACCTACTATTATT
TTGAGCTTCTTGTTGATTTTTGGAGTGGCTATTTGTCAAAGTGAAGCTAGGGCATTTTTTGTGTTTGGTGATTCACTTG
TTGATAGTGGAATAATAATTATTTGGCTACTACTGCAAGGGCTGATTCACCACCTTATGGTATTGATTATCCAACACG
TAGAGCAACTGGTCGTTTCTCTAATGGCTACAACATTCCTGACATTATCAGTTGAGTCCATACATCTCTTTTTTTAGTA
TTACATTTTCGTATGACTTATAGGTCACGAGGTTACTGATGTTTGCATCAGGATAGTATACGGTGATTAAGTCATGTTT
TTTATCATAGAATAGTGGTGGTCTTTTCTAAATTTTTGTGTGAATGTGAGATGTATGGTATGTACATTATATCAGATT
CATATGAACCTAATAATTTTCGATGTAGAATGTGAAGTTATGAAAAATAATAAATATGAATTTTTTAAGTTAATGCTAAGAA
CACATAGAAAGTTTGAATCTCGGATATACCTTTGATATTTGGAGAATTGTGGAGATATTGTAATAATTGTCTCTGTAAGT
CACGAGTTCGAGCTGTGAAGTCGGATACTGATGCTTGTGCTAGGATAGATGACCTACATTACGTCCTCTTAGGGTGTA
TCCTTTTATCGAACACTAGATGAACGTGAAATATTTGTGTATTAACTGTTTTTGTCTGATATTTAATTATGTGTG
TTTTTGCCTCTTTTATGAAAGGTACTTATGAACACTAAAATTATAATGTTAACTTATTATAGATGATAATCTGTCTTAT
TTTTTAGATTACGTGTTCTACTTTATTTTTTAAGACAATTATCTATAGTTTTCTTTTATAAGTTTAATTTCTATATACA
TCGATGATATAAAGAATCTTTTAATTATAAGCTAATTTAAAAATACTAAACTAATCGTTTATAAAAAATTAAATCTAAA
ATTAACATAGACATAAGACAAGTTACATGTTATAAGCAATTATTTTTCAGTAAATATACACAAGGTAATCCTTCTTC
AAGGTTGACCTAATATTAATATAATATTTTTTTTTTACATTTTCAGTATATATAAGTTAAATTTGTGATCGATCGATCAA
CTTCGATTATTTTACTCTTTTTTTTTCAGGTCAACAAATTGGTTTCATCAGAGTCACCACTACCTTACTTAGATCCAGCTC
TTACTGGACAAAGACTTCTTGTTGGTGCTAACTTTGCATCTGCTGGAATTGGAATACTAAATGACACTGGAATCCAATT
```

TGTACGTACAAAAAACTTTAGCCTAAAATTCAAATTATTTCAAACATTTTAATAATTAATTTGTTCTGTTTAATGATA
 ATATGCAGATTAATATTTATTCGAATGCCACAACAATTGGCCTATTTTAGACAATATCAAAGTAGAGTAAGTGGCCTTAT
 TGGTGAAGCAAATACTCAAAGACTTGTAATCAAGCTCTTGTTCTTATGACTCTTGGAGGCAATGATTTTGTCAACAAC
 TATTATCTTGTGCCCAATTCTGCGCGATCAGCCAATTTTCTATTCAAGATTATGTCCCTTATTTGATAAGAGAATATC
 GTAAAACTTTGATGGTACGTATTGAGTTTAGGCGATCTGTTAGTTGATAATTTTCAGAGATTTCCCTTTCTGACATAAC
 ATTCATTCAATACAAACATTTATAACAAATTTAAGTTTTTCACCAAACATAATTGGTTGTCAATTGCTAGCTGTTTTTA
 ATTAAGGTGACAAAGATTGAGAAAAACATTGAATGCACATTGAAGTTATTATGTTACTTTTTGTATAGGTAGATAATA
 ATACAGAAGTTTTATTAATATACATTTGTTGCAGAATGTGTATAATCTTGGAGCTCGTCGTGTAATTGTAAGTGAAGT
 GGACCGTTAGGTTGTGTTCCAGCAGAACTAGCTCAACGTAGCAGGAACGGGGAATGTTACCCGAGTTGCAACGAGCTG
 CAGGCCTGTTTAACCCCCAGCTTACGCAAATGTTGCAGGGGTTAAATAGTGAAC TAGGCAGCGATGTTTTTATTGCTGC
 AAATACACAACAAATGCATACGAATTCATTACTAATCCACAAGCATATGGTAACATCTTTTTTACTTTTCGTAAAAATC
 AATATAATGCTTTTAAATTTACATCCCTGTTTCATGTTTCAGTGGCAGAGCCAGGATTTTCATTATGCTGGCTCTGGGTTT
 TGGGCTCTGGCCTGTGGTTGACTAGTGTTATTAATATTGACAATGGATTCCATTTTTTTGTGACAGGATTTATAACATC
 AAAGGTAGCATGTTGTGGACAAGGACCATATAACGGTCTTGGTCTATGTACACCGCTCTCTAATTTGTGCCCGAATAGA
 GATGTTTACGCGTTTTTGGGACCCGTTCCATCCATCTGAGAGGGCAAATAAGATCATGTGTCAGCAATCATGTCTGGTA
 CAACGGAGCTTATGAATCCAATGAATCTCAGTACGATTCTGGCTATGGATTACATGCATAAGACATATCTAAGATATC
 TGGAACTCGATTCACTTGTACCTTTTTTGTGCTAATTTTTGGCTATAAATAAGATGTATGCAACACTTCATGTTGTGC
 TACTTTTAAATTTACAAAAAAGTTGGTTGTGTGCTATGTTTTTATTCACATAATTCAGTAATCTAATTTGTAGGGTGG
 AGTGTGATATTGTTGAAGATGTAAACCAAAGTGTTTTTATTAATTTATATAGTAATATATTTTTCAGTGTTAA

>gi|339717983|gb|AEJ88779.1| cutin-deficient 1 protein [Solanum lycopersicum]

MATPTIILSFLLI FGVAICQSEARAFFVFGDSLVD SGNNYLATTARADSPPYGIDYPTRRATGRFSNGYNIPDIISQQ
 IGSSSEPLPYLDPAL TGQRLLVGANFASAGIGILNDTGIQFINIIRMPQQLAYFRQYQSRVSGLIGEANTQRLVNQALV
 LMTLGGNDFVNYYLVPNSARSRQFSIQDYVPYLIREYRKILMNVYNLGARRVIVTGTGPLGCVPAELAQRSRNGECSP
 ELQRAAGLFNPLTQMLQGLNSELGSDVFI AANTQQMHTNFITNPQAYGFITSKVACCGQGPYNGLGLCTPLSNLCPNR
 DVYAFWDFPHPSERANKIIVQQIMSGTTELMNPMNLSTILAMDSHA

Identification of a *CD1* Homolog in *Physcomitrella* using BLAST

In order to identify a *Physcomitrella* homolog of the tomato *CD1* gene (*SlCD1*), the deduced amino acid sequence of the SlCD1 protein (shown above) was BLASTed against the *Physcomitrella patens* genome using the Cosmoss website (<http://www.cosmoss.org/>).

The Cosmoss database queried was “*P.patens.V1.6_proteins* (38,587 proteins from V1.6 gene models)”. Default settings were used for all search parameters, except for the matrix, which was set to BLOSUM80.

Physcomitrella sequence Pp1s34_98V6.1 was selected as a candidate gene for knockout because it had an excellent E-value and bit score. (Note that a second sequence, Pp1s34_98V6.2, was identical to Pp1s34_98V6.1.) In order to identify 5' and 3' genomic sequences flanking the candidate gene (*PpCD1*), the sequence was opened in the

Cosmos Genome Browser. The 5' flanking sequence was approximately 1250 nucleotides. The 3' flanking sequence was approximately 950 nucleotides and included a short portion of the *PpCD1* coding region.

The genomic *PpCD1* sequence used was:

```
ATGTTTCATTTTTTCGAAGAGAGTTTCATGTTTCATGCTTGCCGCCGATCTTCTGCTGCTGAGTGGCAGTCAAGTTGGTCG
GGGGAAGTGGACTGTGTATTAGCATCTCAGCTCAGGGGATTGCCGCTGAATATTTTTGATGAAGTTGCGAAACTCCGCG
TGACATGACTCGACTGTCTTACTGGATTCTTCGTCATGGATTTCCCTGATAGTTGCTGCTATGCGTGAAGTATGTG
ATCACTTGGCCATGTCGAGCATGTTGCAGACTATGTTGTGATTGTCGATGGTGAACCTCCAAATGGCGCAGATCCCCAGT
TGCCCTCATAGCTAATGAAAAATACTTGGCTTGTGTTTGAAGGATTGGAAGTGAGAGGCGCGTCCAGTGCAATG
TTTGGACTCTTCTGTTATCGTAACCTGCCCCCTGCCAATGTTGTGCTCCTGCTTTCGTTTGGCGAGTAGTGTAAATTTG
AGGTTGTCACCTATGCTCAGGTTTACAGAGAGCTTCCAGTCAATATCACTTTGTAGAAGTTCTGACTTAATTTGAAGT
TGAAGCTATTTTCGGAACCCCTTCAGCAGGTTTAAATCGACGCTCACTAAAATAACTATCTTAGAGGGTGCAGGTGCTTAT
TTAATCCGTGTTTTAGAGATTTTTCTTACGCGATTAATTGATGTTTGTCTCAGATTACTACAGCTTCAATTTAGAGA
AGCTGCAAGGCTCTGCGACCTTGGTGTGACTACGGCGACCGCGGAGAGACAAAATGGCAAGTTTAGAAAATCAGCAATG
GCTAGTGATCTTGGTCATTGGTGTAGTCATGGCAATAACATTGAGCGCCACAGGTGTGGAGGCGCAGGGAAAGAAGACT
CCCGCGACCTTTATCTTTGGAGACTCACTCGTGGACGTGCGCAACAACAACCTACATCTTACCTTGGCCGTCGCAGATC
ACAAGCCCTACGGGATTGACAGGGCCGACAAGGTTCCCACTGGCAGATTCTGCAACGGGAAAATCATCCAGATCTCGT
CAGTAAGTGTCTCACTTCTCTCTCTCACAATTACAATTCTCATTTCTGATTAGCAAATCAACCTATTACTGACGAA
TGTCGATTCCGTTTGCAGTGTGCATTTGGTTCGGTGACATTGAAAGACGAGTGACAAGATTTGATGTGCAGATGATTAT
CTGGGTACTCCATATCCTCTGCCAGTGCTAGCCCCGAAGCGACGGGAGCTAACCTTCTTACGGAGTCAACTACGCCT
CGGCTGGTGTGCTGGCATCTGGAAGACAGGGCTCAATCTTTGTACGTAGTAAGAAACAATTAATGGCGCAGGAATTGT
TGAGATGTGGCGTCTGAGCCGTTTCGTTTGTGACTGCGCAATTACCACATGTGCAGATTGGGCGTGTAACAATATCA
CAGCAATTTGGCTACTTCCAGAAAACGAAACAGCAGATTGAGCTGATCATCGGTCAACCGGCGGCTGACGAGCTGATCC
ACAACGCTATCTACTCTTTCACAGTCGGGGGGAACGACTTCGTCAATAACTATATGGCGGTCAACACGAGCACCAGCCG
AAAATATACACCCTCGCAGTATCAAGATCTCCTAATCAACAACCTTTCATGGCCAGCTCAAGGTATATTCAGCTACCTCT
TACTCCTTTGATCGAGAACATGTTTCATCATATCTGACCAGGAGTTTACGGTGCCTGTTTTTAATAAACTTATATGACC
GCTTGCTTGATTGACGCGCTATGGTCTGGGAATGAGGAAGTTTCATGCTCTTAACATGGGCCCATCGGATGCGCCC
CGTCCGTGCTTTTCGTGAAGAGCCAAGCGGGGAGTGCGTCCAGGAGGTGAACAATTACGCCCTGGGGTTCAACGCTGC
CTTGAAACCATGTTGACAGCCTGCAAGCGGAGCTTCCGGGCTCCATCTTCATCTACGCGAACGCTTTCGACATTGTG
AGGGGCATCATTGCCGACCCCTTGAAGTATGGATTACGGAACCCGTGACCACGGCCTGCTGCGGCGCGGGGCAATACA
ACGGCATCGACGGATCGTGCCGACCATCGGCCATCTGTCCAGACAGAACCAAGTCCGTTTTCTGGGATGCCTTCCA
TCCCACGGAGAAGGTGAACAAGATCTGCAACGATCAGTTCTCCACGGCGGCTTGACGCAATCAGCCCCATGAATGTG
GCCCAGTTGCTAGCCATGTAA
```

Design of Primers Used for Gateway Cloning

Four PCR primers (Eurofins MWG|Operon, Ebersberg, Germany) were designed to amplify to the intergenic flanking sequences of the *PpCD1* gene. These primers also incorporated *att* sequences that are required for subsequent Gateway cloning. In general, these primers should have a GC content of at least 50%, and length of 18 to 28 base pairs. The primers are listed in Table 3-2, and shown diagrammatically in Figure 3-3.

Table 3-2: *PpCD1* Primer Sequences

Forward Primer: 5' - 3'	Reverse Primer: 5' - 3'
<u><i>Attb1KOcd1 (FWD)</i></u> GGGGACAAGTTTGTACAAAAAAG CAG GCTTAGTTCAGGGCTGACAAATCC TCT	<u><i>Attb4KOcd1 (REV)</i></u> GGGGACAAC TTTGTATAGAAAA GTT GGGTGCTCCTTGTACCCATCTCC ATC
<u><i>Attb3KOcd1 (FWD)</i></u> GGGGACAAC TTTGTATAATAAAGT T GTACCACGGAGAAGGTGAACAAG AT	<u><i>Attb2KOcd1 (REV)</i></u> GGGGACCACTTTGTACAAGAAA GCTG GGTTTGTTGCTCACATGAAACTC TGC

Table 3-2: Text sequences of the both sets of forward and reverse primers for the *PpCD1* gene. The *att* sequences and primer binding sites are visualized in Figure 3-3.

Forward Primers: 5' - 3'

Attb1KOcd1

Reverse Primers: 5' - 3'

Attb4KOcd1

Forward Primers: 5' - 3'

Attb3KOcd1

Reverse Primers: 5' - 3'

Attb2KOcd1

Figure 3-3: Primer sequences designed to amplify intergenic sequences flanking the *PpCD1* gene. Gateway *att* sequences engineered into these primers are shown in blue; primer binding sites are shown in green.

The relative position of the primers with respect to the *PpCD1* genomic sequence is shown in Figure 3-4. The 5' flanking region of *PpCD1* was amplified using the forward *Attb1KOcd1* primer, and the reverse *Attb4KOcd1* primer. The 3' flanking region was amplified using the forward *Attb3KOcd1* primer and the reverse *Attb2KOcd1* primer.

Figure 3-4: Diagram of the *Physcomitrella* genomic region (>scaffold_34:518965..528964, using nomenclature of CosMoss.org) showing *PpCD1* (long green bar in center of the figure). The 5' intergenic region flanking *PpCD1* (upper gray bar) was amplified using primer pair *Attb1KOcd1* (forward) and *Attb4KOcd1* (reverse). The 3' flanking region (lower gray bar) was amplified using primer pairs *Attb3KOcd1* (forward) and *Attb2KOcd1* (reverse). The 3' amplicon also included a small, insignificant coding portion of the *PpCD1* gene.

PCR Amplification of *PpCD1* Flanking Sequences

All PCR reactions were run in accordance with the manufacturer's recommendations. Hot-start amplification conditions are shown in Table 3-3. A total of 35 cycles were performed.

Table 3-3: PCR Conditions

	Temperature	Duration
Initial denaturation	95°C	3 min
Denaturation	92°C	8 s
Annealing	60°C	30 s
Elongation	72°C	45 s
Final Extension	72°C	5 min
HOLD	4°C	Infinite

Generation of Gateway Entry Vectors

The Gateway donor plasmids (pDONR), into which the PCR products were to be inserted, were pDONR 221-P1P4 (Fig. 3-5) and pDONR 221-P2P3 (Fig. 3-6). These vectors were obtained from Invitrogen (CA, USA) via Dr. Alison Roberts at the University of Rhode Island.

Both pDONR plasmids contain a kanamycin selection cassette, a pUC origin of replication, and a *ccdB* “death gene” used to ensure that only cells that have taken up recombinant Gateway destination plasmids will survive.

Figure 3-5: pDONR221-P1P4 Plasmid. The PCR product containing the 5' flanking region of the *PpCDI* gene will replace the *ccdB/Cm^R* region using a Gateway BP reaction. Figure taken from Invitrogen “Multi Site Gateway Pro” manual on 1/29/13.

Figure 3-6: pDONR221-P3P2 Plasmid. The PCR product containing the 3' flanking region of the *PpCD1* gene will replace the *ccdB/Cm^R* region using a Gateway BP reaction. Figure taken from Invitrogen "Multi Site Gateway Pro" manual on 3/13/13.

Each PCR amplicon was inserted into the appropriate pDONR 221 vector using a Gateway BP reaction as described in the Gateway Multisite Manual (http://tools.invitrogen.com/content/sfs/manuals/multisitegatewaypro_man.pdf).

These BP recombination reactions exchange the PCR amplicons (including *att* recombination sites) with the *ccdB* death gene. This reaction also converts the pDONR *attP* sites to *attL* sites. This process is summarized in Figure 3-7.

The products of each BP reaction (i.e., two pDONR vectors, p221-L1L4 and p221-L3L2, each containing one of the PCR-amplified *Physcomitrella* intergenic regions) were transformed into chemically competent *E. coli* as described in by the Gateway Multisite Manual. Cells incorporating recombinant plasmids were selected by plating on LB medium containing 50 µg/mL kanamycin (kan). Individual kanamycin-resistant colonies were selected and grown in liquid LB-kan medium overnight for plasmid minipreps.

Plasmid sequencing was performed on p221-L1L4 and p221-L3L2 to ensure that the plasmids contained the intended amplicons. Sequencing was performed by the Rhode Island Genomics and Sequencing Center, at the University of Rhode Island (Kingston, RI).

Figure 3-7: A schematic interpretation of the BP reaction, and the subsequent BP plasmid produced. Note: there were two different BP reactions each incorporating a different PCR amplicon (one containing 5' intergenic sequence and the other 3' intergenic sequence flanking the *Physcomitrella CD1* gene). For clarity, only the PCR product containing the 5' amplicon is shown in the diagram above.

Multisite Gateway LR Reaction to Create Destination Vector for Homologous Recombination

The knockout (KO) vector used to generate *PpCDI* deficient mutants was created using a MultiSite Gateway Cloning Kit (Invitrogen Life Technologies, CA, USA). The final Gateway destination vector was formed from an LR reaction involving three plasmids. The first plasmid (p221-L1L4) contained a 5' region flanking the *PpCDI* gene, and the second (p221-L3L2) contained a 3' region flanking the *PpCDI* gene. The third plasmid (pBHSNRG, obtained from Dr. Alison Roberts, University of Rhode Island) contained a hygromycin resistance cassette and other sequences required for selection, plasmid propagation, and recombination.

As shown in Figure 3-8, the two *Physcomitrella* intergenic sequences flanking of the *PpCDI* gene incorporated into pDONR 221-L1L4 and pDONR 221-L3L2 were combined with pBHSNRG using a Gateway LR reaction as described by Roberts et al. (2011). The products of the LR reaction were transformed into *E. coli*. A miniprep was performed to isolate the recombinant LR plasmid from the *E. coli* cells, so they could eventually be inserted into the protoplasts, to begin the transformation procedure.

Plasmid sequencing was performed on the recombinant LR plasmid to ensure that it contained the intended amplicons and hygromycin resistance cassette.

Figure 3-8: Summary of Multisite Gateway LR reaction that generated the plasmid used to knockout *PpCD1* in *Physcomitrella*. pDONR plasmids p221-L1L4 and p221-L3L2 (top line) contained the 5' and 3' intergenic sequences flanking *PpCD1* needed for homologous recombination to occur. pBHSNRG contains a hygromycin resistance cassette. The LR recombination reaction created a new plasmid in which the two *Physcomitrella* intergenic sequences surround a hygromycin resistance gene. Insertion of this plasmid into the *Physcomitrella* genome by homologous recombination resulted in the creation of a *CD1* knockout line of *Physcomitrella patens*.

Figure 3-9: Recombinant Plasmid - LR Product. This is the final product, after the LR reaction had combined hygromycin resistance cassette, and both 5' and 3' amplicons into a single LR recombinant plasmid. To increase the rate of homologous recombination, this recombinant plasmid was cut with both the HindIII / SalI restriction enzymes (restriction sites shown above).

Transformation of *Physcomitrella* for Homologous Recombination

To increase the efficiency of homologous recombination, the final recombinant plasmid (Fig. 3-9) was double digested with HindIII / SalI to separate the part of the plasmid that will undergo homologous recombination from the plasmid “backbone”. Preparation and transformation of protoplasts was performed as described by Roberts et al. (2011).

Following transforming, *Physcomitrella* protoplasts were placed on regeneration medium (Roberts et al., 2011). After five days, transformed cells were selected by plating with 15µg/mL hygromycin BCDAT plates for one week. Because some cells can take up the hygromycin resistance cassette without incorporating it into the genome (and thus show antibiotic resistance) it is necessary to perform a second selection. Protonemata that survive the first round of hygromycin selection are transferred to BCDAT medium lacking hygromycin antibiotics for one week. During this time, antibiotic resistance cassettes that were not stably integrated into the genome will be lost from the plant cells. The protonemata are then exposed to a second round of hygromycin selection. This time, only the cells that have stably incorporated the LR plasmid into the genome by homologous recombination will survive.

Genotyping of *Physcomitrella* Knockout Mutants

To ensure that homologous recombination had inserted the knockout construct into the correct position in the genome (*i.e.*, replacing *PpCDI* gene), putative transformants were genotyped by PCR. Genomic DNA from sixteen individual colonies (each derived from a single protoplast) was isolated using the protocol of Li and Chory (1998). The brownish pellets that were obtained were dissolved in 200 µL of molecular grade water to serve as templates for PCR genotyping. PCR genotyping reactions were performed as shown in Tables 3-5 and 3-6. A total of 30 cycles were performed.

DNA from each transformant was amplified in two separate PCR reactions, one to verify proper insertion of the 5' portion of PpCD1KO vector (using primers CD1flankF and BHRRR) and the second to verify proper insertion of the 3' region of the vector (using primers CD1flankR and BHRRF). Only samples in which both corresponding PCR reactions successfully amplified a product were used for further study.

Figure 3-10: Strategy for genotyping putative *PpCD1* knockout lines (PpCD1KO). Present are the 5' and 3' amplicons, as well as hygromycin resistance cassette.

Table 3-4: Genotyping Primer Sequences

Forward Primer: 5' - 3'	Reverse Primer: 5' - 3'
<u>CD1flankF (FWD)</u> ATTACTGGTGAAGGCGCTGT	<u>BHRRR (REV)</u> TCTATTTCTTTGCCCTCGGA
<u>BHRRF (FWD)</u> TGACAGATAGCTGGGCAATG	<u>CD1flankR (REV)</u> GATGACCAAATACAGACCCACA

Table 3-5: Genotyping Master Mix

Ultrapure H ₂ O	13.75 µl
Paq Buffer (Stratagene)	2.50 µl
Forward Primer (25µM)	0.50 µl
Reverse Primer (25µM)	0.50 µl
dNTP (10mM each)	0.25 µl
Paq DNA Polymerase	0.25 µl
Shorty Prep DNA	4.00 µl

Table 3-6: Genotyping PCR Conditions

	Temperature	Duration
Initial denaturation	95°C	3 min
Denaturation	95°C	45 s
Annealing	57°C	30 s
Elongation	72°C	90 s
Final Extension	72°C	5 min
HOLD	4°C	Infinite

Phenotypic Analysis of *PpCD1* Knockout Mutants

Leaves of mutant strains and wild-type controls were compared using a stereomicroscope and stained for cuticle using the hydrophilic dyes toluidine blue and Tinopal as described in Chapter II.

CHAPTER III: RESULTS – MOLECULAR GENETICS

The results of a Cosmos BLAST using the *S. lycopersicum* CD1 peptide sequence as a query against protein models derived from the *Physcomitrella* genome is given below. Subsequent homologs of *SICD1* identified by BLAST searches of the *Physcomitrella* genome have E-values of e^{-72} or poorer.

Table 3-7: BLASTp Results

Sequences producing significant alignments:	Score (bits)	E-value
Pp1s34_98V6.2 Phypa_430773 protein	680	1e-80
Pp1s34_98V6.1 Phypa_430772 protein	680	1e-80
Pp1s68_64V6.1 Phypa_437001 protein	680	1e-80

The alignment of sequence the *Solanum* CD1 peptide and the *Physcomitrella* peptide Pp1s34_98V6.1 Phypa_430773 is shown below.

Pp1s34_98V6.1 Phypa_430772 protein sequence for anther-specific proline-rich protein
 apg Length = 370
 Score = 297 bits (680), Expect = 1e-80
 Identities = 152/346 (43%), Positives = 204/346 (58%), Gaps = 4/346 (1%)

Query: 15 GVAICQSEARAFFVFGDSLVD SGNNNYLATTARADSPPYGIDYPTRRATGRFSNGYNIPD 74
 GV + A F+FGDSLVD GNNNY+ T A AD PYGID + TGRF NG IPD
 Sbjct: 27 GVEAQGKKTPATFIFGDSLVDVGNNNYIFTLAVADHKPYGIDRADKVPTGRFCNGKIIPD 86

Query: 75 IISQQIGSSESPLPYLDPALTGQRLLVGANFASAGIGILNDTGIQFINIIRMPQQLAYFR 134
 +++ +G+ PLP L P TG LL G N+ASAG GIL DTG FI + + QQ YF+
 Sbjct: 87 LVNDYLGTPYPLPVLAPEATGANLLHGVNYASAGAGILEDTGSIFIGRVTTISQQFGYFQ 145

Query: 135 QYQSRVSGLIGEANTQRLVNQALVMTLGGNDFVNYYLVPNSARSRQFSIQDYVPYLIR 194
 + + ++ +IG+ L++ A+ T+GGNDFVNYY V S SR+++ Y LI
 Sbjct: 146 KTKQQIELIIGQPAADELIHNAIYSFTVGGNDFVNYYMAVTTST-SRKYTPSQYQDLLIN 204

Query: 195 EYRKILMNVYNLGARRVIVTGTGPLGCVPAELAQRSRNGECSPQLQRAAGLFNPQLTQML 254
 + L Y LG R+ IV+ GP+GC P+ L+ +S+ GEC E+ A FN L ML
 Sbjct: 205 NFHQQLKTAYGLGMRKFIVSNMGPICAPSVLSSKSQAGECVQEVNYYALGFNAALKPML 264

Query: 255 QGLNSELGSDVFIAANTQOMHTNFITNPQAYGFITS-KVACCGQGPYNGL-GLCTPLSNL 312
 Q L +EL +FI AN + I +P YGF ACCG G YNG+ G C + +L
 Sbjct: 265 QSLQAELPGSIFIYANAFDIVRGIADPLKYGFTEPVTTACCGAGQYNGIDGSCRTIGHL 324

Query: 313 CPNRDVYAFWDPFHPSEERANKIIVQQIMSGTTELMNPMNLSTILAM 358
 CP+R FWD FHP+E+ NKI Q + G + ++PMN++ +LAM
 Sbjct: 325 CPDRTKSVFWDAPFHPTEKVNKICNDQFLHGGLDAISPMNVAQLLAM 370

The peptide and coding sequences, together with GenBank ID numbers of the *PpCDI* gene used for knockout studies in this thesis are given below.

```
>ID Pp1s34_98V6.1; SV 1; linear; unassigned DNA; STD; PLN; 1694 BP.
tcatacaaccgccttcacactgcttgcgacctcatggtgctcatcaatctcgccttgactctttcaggtggcattttcag
cttctcgtggttgcgcgacgagctcacattcagctgtggttcagcagctgctacttgccgggatcgctaccgcaatttt
ctgcgacgtttctcgtgattggaagtgaagggcgctgctccagtgcaatggttgactcttctgattactacagcttca
atctcagagaagctgcaaggctctgcgaccttggtgtgactacggcgaccgaggagagacaaaatggcaagtttagaaa
atcagcaatggctagtgtatcttggctcattgggtgtagtcattggcaataacattgagcgccacaggtgtggaggcgagg
aaagaagactcccgcgacctttatcttggagactcactcgtggagctcggaacaacaactacatcttcaccttggcc
gtcgcagatcacaaagccctacgggattgacagggccgacaaggttcccactggcagattctgcaacgggaaaatcatcc
cagatctcgtcaatgattatctgggtactccatctcctctgccagtgtctagccccgaagcgacgggagctaaccttct
tcacggagtcaactacgcctcggctggtgctggcatcctggaagacacgggctcaatctttattggcggtgtaacaata
tcacagcaatttggctacttccagaaaacgaaacagcagattgagctgatcatcggtcaaccggcggtgacgagctga
tcacaaacgctatctactctttcacagtcggggggaacgacttctgcaataactatatggcggtcaccacgagcaccag
ccgaaaataacacccctcgcagtatcaagatctcctaatacaaaactttcatggccagctcaagacggcggtatggtctg
ggaaatgaggaagttcatcgctcttaacatgggccccatcggtatgcgcccgctcgtgctttctcgtgaagagccaagcg
ggaggtgcgtccaggaggtgaacaattacgccttgggttcaacgctgccttgaacccatgttgagagcctgcaagc
ggagcttccgggctccatcttcatctacgcgaacgctttcgacattgtgaggggcatcattgcccgaacccctgaagat
ggattacggaaccgtgaccacggcctgctgcggcgcggggcaatacaacggcatcgacggatcgtgccgcaccatcg
gccatctctgtccagacagaaccaagtcggttttctgggatgccttccatcccacggagaaggtgaacaagatctgcaa
cgatcagttcctccacggcggccttgacgcaatcagccccatgaatgtggcccagttgctagccatgtaattctccct
ttattcttcaccatcctcacactcgttaataagtagagtttcaattacacacagaatcaatgctaagcaatatcgagtc
cactattagctcctttctgaaggatgtgagcgattgtactcatccactaattcattctcttggccaaaaactcttccc
ccctgctctcgctaccaatgccatttgctacttctggtctaagctcacaacactcgagccaagcatactcacatcc
acctcgacactgacgaccgctggagagatgttgta
```

```
> Pp1s34_98V6.1; SV 1; linear; AA; STD; PLN; 370 BP.
maslenqqwlvilvigvmaitsatgveaqqkktpatfifgdsldvgnnnyiftlavadhkpygidradkvptgrfc
ngkiipdlvndylgtppyplpvlapeatganllhgvnyasagagiledtgsifigrvtisqqfgyfqtkkqieligqp
aadelihnaiysftvgndfvnnymavttstsrkytpsqqdlnnfhgqlktayglmrkfivsnmgpigcapsvls
sksqagecvqevnnyalgfnaalkpmlqslqaelpgsifiyanafdivrgliadplkygftepvttaccgagqyngidg
scrtighlcpdrtksvfwdafhptekvnkicndqfihggldaispmnvaqlam
```

The gene includes six exons (four of which encode amino acids and two contain the 5' and 3' untranslated regions) and five introns (Fig. 3-11).

Figure 3-11: Structure of the *PpCDI* gene used for knockout studies. The gene includes six exons, four of which (shown in red) encode amino acids. Two alternative Cosmoss gene assemblies (Pp1s34_98V6.1 and Pp1s34_98V6.2) are shown, but the amino acids encoded by these are identical. Four of the six exons encode amino acids (red) and two of them contain the 5' and 3' untranslated regions (pink).

Amplification of 5' and 3' intergenic sequences flanking *PpCDI*

Wild-type DNA from *Physcomitrella* was used as a template for amplification of sequences on the 5' and 3' sides of the *PpCDI* gene. As shown in Fig. 3-12, the products amplified from these reactions were reasonably specific and of the expected size. The expected sequences of the 5' and 3' amplicons are shown schematically in Fig. 3-13 and 3-14, while the actual results of the PCR reactions are shown in Fig. 3-12.

Figure 3-12: Agarose gel electrophoresis showing successful PCR amplification of intergenic sequences flanking the *PpCDI* gene. Both the 5' and 3' flanking sequences were successfully amplified. Lanes 1 and 3 show the 5' *PpCDI* flanking sequence, while lanes 2 and 4 are PCR amplification of the 3' flanking sequence. Lane 5 is a 2-log molecular weight ladder from NEB (New England Biolabs). An empty lane between lanes 1 and 2 has been digitally removed in this image. Note that since there were two PCR amplifications of the same flanking sequence, only the tube with the brightest lane was selected.

Figure 3-13: Expected 5' amplicon following PCR of genomic *Physcomitrella* DNA using primer pair *Attb1KOcd1* (forward) and *Attb4KOcd1* (reverse). Note that this product includes non-genomic sequences (such as *attB1* and *attB4*, shown as blue bars) that were engineered into the primers in order to facilitate downstream cloning reactions.

Figure 3-14: Expected 3' amplicon following PCR of genomic *Physcomitrella* DNA using primer pair *Attb3KOcd1* (forward) and *Attb2KOcd1* (reverse). Note that this product includes non-genomic sequences (such as *attB3* and *attB2*, shown as blue bars) that were engineered into the primers in order to facilitate downstream cloning reactions. Note that an insignificant portion of the *PpCD1* gene is found inside the 3' amplicon.

With the successful generation of amplicons for both the 5' and 3' intergenic sequences flanking *PpCDI* (both incorporating *att* sites for subsequent Gateway cloning), the next step was to insert them into a plasmid. Two Gateway BP reactions resulted in the creation of two separate pDONR plasmids: pDONR221-P1P4, and pDONR221-P3P2. One plasmid contained the 5' modified *PpCDI* amplicon, and the other contained the 3' modified *PpCDI* amplicon. Sequencing of these samples gave the predicted results.

A Multisite Gateway LR reaction was used to create the homologous recombination vector containing the pBHSNRG hygromycin resistance cassette flanked by 5' and 3' amplicons required for recognition during homologous recombination. Sequencing of these samples gave the predicted results, as did a restriction digest using BsrGI (Fig. 3-15). While BsrGI was useful for diagnostic purposes, it could not be used to “trim” the vector for transformation due to the presence of the BsrGI site within the region designed for homologous recombination.

Figure 3-15: Agarose gel electrophoresis of BsrGI restriction digests of six clones of the LR recombinant plasmids. The three bands observed were consistent with the fragments predicted, suggesting that the clones had the expected sequences. The MW lane shows a 2-log molecular weight ladder from NEB (New England Biolabs).

In order to maximize the efficiency of the final recombinant LR vector used for homologous recombination, the vector was “trimmed” with restriction enzymes HindIII / SalI at locations outside of the coding region. Agarose gel electrophoresis (Fig. 3-16) showed that the restriction digest produced two fragments of the expected size. While all of these fragments were present in the transformation mixture, only the largest, which contained the 5′ and 3′ intergenic region surrounding the hygromycin resistance cassette, was functionally significant.

Figure 3-16: Agarose gel electrophoresis of the LR recombinant plasmid cut with the restriction enzymes HindIII and SalI. This double digest was used to reduce the size of the vector used for homologous recombination in order to increase the efficiency of the transformation.

Restriction fragments of the recombinant plasmid derived from the *PpCDIKO* vector were used to transform *Physcomitrella* protoplasts. The spherical protoplasts, formed by treating protonema with the cell wall degrading enzyme Driselase, appeared healthy and contained numerous green chloroplasts (Fig. 3-17).

The protoplasts were allowed to recover and regenerate walls on the PRMT medium without antibiotic as described by Roberts et al. (2011). After several days, the regenerating cells were transferred onto BCDAT medium that contained hygromycin for selection. Cells were kept on the selection medium for a week, then transferred off for another week, and finally back onto the selection medium. Colonies that survived the both rounds of hygromycin selections were used for genotyping. (Fig. 3-18)

Figure 3-17: Protoplasts are plant cells that lack a cell wall and are, therefore, spherical. (A) Individual protoplasts contain numerous chloroplasts. (B) When protoplasts were mixed with LR recombinant plasmid, a small percentage underwent homologous recombination. These recombinants were selected by plating on antibiotic plates.

Figure 3-18: Hygromycin antibiotic selection plate used to identify *Physcomitrella patens* *CDI* knockout strains. Cells transformed by homologous recombination should contain a resistance cassette that allows them to grow on BCDAT medium containing hygromycin. Putatively transformed cells remain green (one example is circled), while cells that are not transformed turn brown and eventually die. Due to anomalous recombination events, not all resistant colonies necessarily contain the resistance cassette in the appropriate location. Subsequent genotyping results identified four transformants that had incorporated the vector in the correct place within its genome.

Genomic DNA from sixteen individual colonies (each derived from a single protoplast) was isolated. Putative *PpCDI*KO DNA was prepared for PCR genotyping. One of the primer pairs recognized a region within the selection cassette, and the other recognized a portion of the genomic DNA (Fig. 3-10). Out of 16 putative knockout lines, genotyping results showed that four knockouts had physically replaced the *PpCDI* gene with the hygromycin resistance cassette (Fig. 3-19).

Figure 3-19: Gel electrophoresis results of *PpCD1KO* genotyping. Lanes 1-16 contain DNA derived from separate transformants. The top and bottom gels are PCR products from the same template amplified using different primers. The 16 lanes on top were amplified using a 5'-genome specific primer and a 3' primer from the hygromycin resistance cassette. The corresponding lanes on the bottom of the gel were amplified using a 3'-genome specific primer and a 5' primer from the hygromycin resistance cassette. The four lane pairs marked with an asterisk (*) indicate successful knockout lines, that is, transformants where the resistance cassette has been inserted in place of the *PpCD1* gene. The image of this gel was digitally rearranged in order to make the paired PCR genotyping reactions easier to interpret. The MW lane shows a 2-log molecular weight ladder from NEB (New England Biolabs).

CHAPTER III: RESULTS-MORPHOLOGY *PpCDI*-DEFICIENT MUTANTS

All mutants were capable of producing leaves, although one line, *PpCDIKO* #15, produced fewer leaves than other mutants and controls. In addition, line *PpCDIKO* #15 produced a larger number of upright protonemata. Leaves of mutant lines were examined using bright field dissecting microscope. No obvious morphological differences were in leaf structure were observed (Fig. 3-20 and 3-21).

Figure 3-20: Low magnification micrographs of separate knockout lines (and wild-type) of *Physcomitrella patens* showing colonies. No significant gross, distinct phenotype was apparent when comparing the knockout mutants to the wild-type sample, except for *PpCDIKO* #15, which showed a reduced number of leaves and a larger number of upright protonema. (A) *PpCDIKO* #4. (B) *PpCDIKO* #7. (C) *PpCDIKO* #14. (D) *PpCDIKO* #15. (E) Wild-type.

Figure 3-21: Higher magnification micrographs of leaves from all transgenic knockout lines and wild-type samples of *Physcomitrella patens*. No significant gross leaf phenotype was visible. (A) *PpCD1KO* #4. (B) *PpCD1KO* #7. (C) *PpCD1KO* #14. (D) *PpCD1KO* #15. (E) Wild-type.

As described in Chapter II, toluidine blue is a non-specific hydrophilic dye used for histochemical staining. Wild-type leaves that have an intact cuticle take up very little of the stain (Figs. 2-4A, 3-22, 3-23). Both the wild-type and all four transgenic *PpCDIKO* samples were treated with toluidine blue under identical conditions and images were recoded using the same exposure settings. Both wild-type and all four knock out lines stain similarly. In a few cases, small regions of both the wild-type and *PpCDIKO* leaves in proximity to wounded tissue take up a limited amount of stain. In contrast to leaves, rhizoids (which would be unlikely to have a cuticle because of their absorptive function) stain intensely in both wild-type and knock-out lines.

Figure 3-22: Representative light micrographs of both wild-type and *PpCDIKO* leaves after treatment with toluidine blue. (A) *PpCDIKO* #4. (B) *PpCDIKO* #7. (C) *PpCDIKO* #15. (D) *PpCDIKO* #14. (E) Wild-type. Minor toluidine staining of leaves occurs often at wounded sites. Scale information not recorded.

Tinopal is a fluorescent hydrophilic dye that binds β -glucans, including cellulose and other cell wall polysaccharides. As with toluidine blue, the lack of staining is consistent with the presence of a leaf cuticle. For simplicity, only one *PpCDIKO* sample was compared to the wild-type. Strong Tinopal fluorescence in the leaf cell walls was missing from both the wild-type and *PpCDIKO* sample. (Fig. 3-23 C & D). The exposure times for the fluorescence micrographs in Fig. 3-23 are identical. Note that both rhizoid and protonemal filaments, which lack a cuticle, stained positively with Tinopal in both wild-type and knockout samples.

Figure 3-23: *P. patens* leaves and rhizoids treated with Tinopal. Leaves seen in brightfield light microscopy in (A & B) are exposed to Tinopal and examined by fluorescence microscopy (C & D) show no observable fluorescence, perhaps indicating an intact cuticle remains and blocks Tinopal infiltration. In contrast, rhizoids, which are thought to lack a cuticle, fluoresce brightly. All images were recorded under identical conditions and show the same fields viewed by brightfield and fluorescence microscopies. Scale information not recorded.

CHAPTER III: DISCUSSION

Selection, Background, and Genotyping of *CDI*

In this study, a *Physcomitrella* mutant lacking the homolog of the tomato *CDI* was produced through the process of homologous recombination. There are several reasons why the *PpCDI* gene was chosen. First, the tomato *CDI* gene is clearly involved in cutin polymerization (Girard et al., 2012). Second, *CDI* activity occurs at the terminal step of cutin biosynthesis and therefore deleting it is less likely to interfere with other cellular processes. Third, because *Physcomitrella* is an evolutionary primitive organism, analysis of a *PpCDI*KO could provide insight as to whether the *CDI* gene is functionally conserved. Finally, knockout experiments of the *CDI* gene in *Arabidopsis thaliana*, and *Solanum lycopersicum* have demonstrated a significant reduction in cutin deposition (Shi et al., 2011; Isaacson et al., 2009; Girard et al., 2012). Such dramatic decreases in cutin have not been previously observed in cutin gene knockouts.

PCR genotyping validated four *PpCDI*KO mutants in which the *PpCDI* gene had been successfully replaced with a hygromycin resistance cassette in the correct genomic context. While these results show conclusively that homologous recombination had occurred to create the knockout, future research should include an additional genotyping step. A PCR reaction using *PpCDI* specific primers should be performed using (1) wild-type genomic DNA and (2) genomic DNA from the *PpCDI* knockouts. Amplification of *PpCDI* gene would be expected from wild-type DNA, but should not occur in genomic DNA isolated from mutants. Amplification in mutant lines might occur if diploid cells formed via protoplast fusion during the transformation step or if a secondary homologous recombination event occurred during transformation; however, both of these possibilities are relatively unlikely to have occurred in each of the four *PpCDI*KO lines.

Analysis of *PpCDI* KO Gross Morphology

Initial analysis of the phenotypes of the four *PpCDI* knockout lines did not show dramatic results—itsself perhaps a significant observation. In higher plants, a common (but not universal) morphological phenotype of cutin deficient mutants is organ fusion, especially leaves (e.g. Heredia, 2003). Shi et al. (2011) has described an *Arabidopsis* mutant in which two *CDI* homologs were silenced resulting in fusion of floral organs and petals (themselves modified leaves). Because cuticle deficient mutants often exhibit organ fusion as a phenotype (Sieber et al., 2000; Samuels, Kunst, & Jetter, 2008), one expected morphology of *PpCDI* KO mutants could be fused leaves. However, at the gross morphological level, leaves of mutant lines were essentially indistinguishable from wild-type plants (Figs. 3-20, 3-21, 3-22, 3-23). All mutants formed protonemata that were capable of differentiating into gametophores with leaves that were morphologically equivalent to controls.

There are several reasons why leaves of the mutants might not show a gross leaf phenotype. First, not all cutin deficient mutants of higher plants display leaf fusion (e.g., Shi et al., 2011; Isaacson et al., 2009). Second, because the photosynthetic organs of *Physcomitrella* lack vascular tissue they are not strictly analogous to the leaves (or flowers) of higher plants, and thus may not behave in the same way even if the cuticle is disrupted. Indeed, in higher plants, non-leafy organs such as tomato fruit that lack the *CDI* gene did not display any gross morphological alterations (Girard et al., 2012). Third, wax is presumably still secreted in *PpCDI* knockouts and may form a surface layer that can substitute for a complete cuticle. Note that Isaacson et al. (2009) and Girard et al. (2012) reported slight modifications to wax components, but no significant change in wax load on the plant surface (except for one mutant line from Girard). Fourth, and perhaps most likely, the leaves *PpCDI* knockouts may not show altered morphologies because they still produce a small amount of cutin. As discussed in more detail below, this could result if *Physcomitrella* contains more than one *CDI* homolog, and or other cuticle related proteins (GPATs, P450s).

Despite the similarity of leaves, one knockout line (*PpCD1KO* #15) did show a distinctive morphology: a reduced number of leaves, a larger number of upright protonemata, and slower growth (Figs. 3-20D, 3-21D). Because all of the knockout lines should be genetically identical, the different protonemal morphology of (*PpCD1KO* #15) is at first surprising. However, aberrant morphologies can occur in clones for a variety of reasons including anomalous secondary homologous recombination events or perhaps epigenetic changes. The fact that this mutant line is unlike the three other mutants (as well as the fact that its leaves stain in a similar fashion to wild-type) makes it seem unlikely that the unusual appearance is a direct consequence of deletion of the *PpCD1* gene.

Analysis of *PpCD1KO* Histochemistry

In addition to the genotyping experiments, and gross morphology observations, histochemical experiments were performed to analyze the knockout samples. When treated with hydrophilic stains, such as toluidine blue and Tinopal, *PpCD1* knockouts demonstrated staining patterns similar to wild-type plants. In mutants of tomato in which the *CDI* gene was silenced using RNAi technology, fruits displayed a mosaic of toluidine blue staining on the surfaces of different mutant lines (Girard et al., 2012). In this thesis, however, toluidine blue staining of moss leaves from all transgenic lines was essentially identical to the wild-type. This suggests that a functional cuticle is still present on the leaves of *PpCD1* mutants.

If *PpCD1* knockouts lack a cuticle, they would be expected to stain more strongly than wild-types, so what can explain these results? One possibility is that the production of epicuticular waxes was not affected by knockout of a cutin gene and this wax may inhibit staining; this is consistent with the literature review of other *CDI* homologs (Isaacson et al., 2009; Girard et al., 2012).

An alternative explanation is that mutants still produce a small amount of cutin. Isaacson et al. (2009) and Girard et al. (2012) both noted that preventing expression of the *CD1* gene in tomato reduced cutin levels in fruits by 95% or more, without affecting the gross morphology. By analogy, perhaps *PpCD1* knockouts still produce 2-5% of the cutin. This residual production would obviously be outside of the control of the *PpCD1* gene, and either activity supported by other genes, and or the newer theory of self cutin assembly by chemical polymerization (Dominguez et al., 2011), discussed in Chapter I. Regardless, this 2-5% production of cutin may be sufficient to serve as a functional barrier to staining.

Another explanation for the absence of toluidine blue and Tinopal staining is that *Physcomitrella* may contain other genes with catalytic activity similar to *PpCD1*. Several *Physcomitrella* genes may encode enzymes that promote polymerization of cutin monomers including GPATs (glycerol-3-phosphate acetyltransferases; Yang et al., 2010), the cytochrome P450 family member CYP86 (Rensing et al. (2008), and the CD1 homologous GDSL protein (Yeats et al., 2012).

Yang et al. (2010) states that several genes that encode enzymes that process extracellular lipids, such as GPATs, are present within the *Physcomitrella* genome. The function of GPATs (G3P acyltransferases) has been shown in *Arabidopsis* to be essential for the construction and polymerization of cutin monomers (Li et al., 2007). The function of GPATs was characterized by the generation of *A. thaliana* knockout lines that produced significantly reduced levels of cutin (Li et al., 2007). Yang et al. (2010) notes that homologous cuticle genes were absent in algae, but present in *Physcomitrella*. The existence of these homologs in mosses, and not in algae, is consistent with the idea that the evolution of GPATs that synthesize and polymerize extracellular polyesters were critical for the ability of plants to adapt to a dry land environment.

Other enzymes also play roles in cutin synthesis. As shown in *Arabidopsis* (Kandel, 2006), one cytochrome P450 enzyme in the CYP86 family is able to catalyze cutin synthesis reactions by adding the omega hydroxyl group (via hydroxylation) that is active

in the ester bonds between two monomers of cutin. Rensing et al. (2008) noted the presence of CYP86 enzymes in the *Physcomitrella* genome, and like Yang et al. (2010), stated that the genes encoding these enzymes are not present in green algae. The fact that Rensing et al. (2008), Yang et al. (2010) and others have found evidence for cuticle genes in *Physcomitrella* support the hypothesis that this moss is likely to possess a cuticle. The presence of genes that may play a role in cutin synthesis is also consistent with the light microscopy isolation, histochemical and freeze fracture findings detailed in Chapter II of this thesis.

Yeats et al. (2012) identified a *Physcomitrella* homolog of *CDI* that is very similar, but not identical, to the *PpCDI* knocked out for the experiments described in this thesis. If any of these genes participate in cutin biosynthesis, then perhaps they can functionally replace *PpCDI* in knockout mutants. In the future, researchers will be able to use the *PpCDI* knockouts I have generated to prepare double or triple knockouts to further investigate this possibility.

Finally, although the role for the *Solanum CDI* gene in cutin biosynthesis is proven, the *P. patens CDI* homolog is evolutionarily primitive and may play no direct role in cutin polymerization. A far more likely hypothesis of a dramatic lack of phenotype when stained with toluidine blue is that there could be multiple compensatory cutin polymerization elements and pathways that compensate for the *PpCDI KO*.

Concluding Thoughts: *Physcomitrella* Has a Leaf Cuticle

The fact that the *Physcomitrella* genome contains homologs to higher plant genes involved in the synthesis of both waxes and cutin is evidence for the presence of a leaf cuticle. The existence of multiple genes within *P. patens* genome not only provides evidence for a leaf cuticle, but may also explain why gross morphological and histochemical phenotypes of the knockout were not observed. In addition to this, there could be other (known and unknown) compensatory acyltransferase pathways that

compensate for the *PpCDIKO*.

Further characterization of the *PpCDIKO* mutants might include the following. First, *Physcomitrella* knockout lines could be examined through transmission electron microscopy (TEM), and scanning electron microscopy (SEM). Both of these types of microscopies were used by Isaacson et al. (2009) to demonstrate a significant reduction in cuticle thickness in the *Solanum CDI* mutants and visualize substantial physical differences when compared to the M82 (wild-type) line. Second, freeze fracture experiments could be performed on the *PpCDIKO* leaves, and fracture patterns could be compared to the wild-type images shown in Chapter II of this thesis. Third, the *PpCDIKO* transgenic line could be further altered to become a “double knockout”, which would remove the other *CDI* homolog gene identified by Yeats et al. (2012).

This thesis sought to determine whether the leaves of *Physcomitrella patens* are covered with a waxy cuticle. The data presented here are all consistent with this hypothesis and extend our knowledge of its structure. A putative cuticle was physically isolated from leaves by separately using both chromic acid and Driselase. The leaf cuticle was demonstrated through histochemical staining (although one hydrophobic stain did not react as anticipated). Also, samples consistently fractured through a weak hydrophobic layer, which was not the plasma membrane, revealing morphological similarities to fractured cuticles of higher plants. Genetic manipulation created a knockout, transgenic line that lacked a homologous cutin synthase gene. Though an obvious phenotype was not observed with the *PpCDIKO* lines, there are several reasons that explain this, which are consistent with the literature review and genomic evidence of cutin genes within the *P. patens* genome. In sum, the evidence indicates the leaves of *Physcomitrella patens* are covered with a hydrophobic cuticle. The evidence presented here will resolve the ambiguity within the literature review about the *P. patens* cuticle (Wyatt et al., 2008; Liénard, 2008; Sakata et al., 2009). It is evident that through the work presented in this thesis, that *Physcomitrella* can now be utilized to further investigate the function, structure, and biosynthetic pathways of cutin.

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