Exploration of Plasma Membrane Domains and Pectin Targeting in
Arabidopsis thaliana Mucilage Secretory Cells

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Preface

The work presented in this thesis is entirely my own, for which I take full responsibility. This research was conducted under the supervision of Dr. Tamara Western.
Abstract

The primary cell wall of dicotyledonous plants consists largely of cellulose and hemicelluloses embedded in a gel of pectins. Pectins are a heterogeneous group of polysaccharides that are synthesized in the Golgi apparatus and transported to the cell surface via secretory vesicles. Antibody studies have shown that there is a non-uniform and dynamic occurrence of different pectins across cell and tissue types, demonstrating the complexity of cell wall structure. How pectins are targeted to distinct cell wall domains, however, is unclear. To investigate the establishment of plasma membrane domains involved in pectin targeting, *Arabidopsis thaliana* mucilage secretory cells (MSCs) were used as a model. MSCs synthesize large quantities of pectins that exhibit polarized targeting to a distinct ring-shaped domain for secretion to the apoplast. Review of the literature and microarray data suggested the ROP1 small GTPase as a likely candidate in establishing plasma membrane domains in MSCs. *ROP1* and five other genes were chosen as candidates for further investigation. Qualitative and quantitative analyses were carried out with T-DNA mutants and fluorophore-fusion lines for these candidate genes. Phenotypic analysis revealed a moderate decrease in adherent mucilage in a *kinesin-13a* mutant, and a drastic decrease in a *sec8* mutant. During pectin secretion, confocal imaging revealed that ECFP:ROP1 appears to localize to nonsecretory domains at the plasma membrane. GFP:KINESIN-13A and GFP:SEC8 appear to localize to secretory domains. This study identifies the potential molecular pathways involved in establishing plasma membrane domains and pectin targeting in MSCs.
Résumé

La paroi primaire des dicotylédones est composée de la cellulose et de la hémicellulose encastré dans la pectine. Les pectines sont des polyosides synthétisées dans le corps Golgi qui sont après transportés à la surface de la paroi par des vésicules sécrétoires. C’était démontré avec des anticorps que les pectines ne sont pas distribuées uniformément dans la paroi et qu’il existe des différences de distribution entre les parois de tissus dans les plantes. Ceci démontre la complexité de la structure de la paroi. Le mécanisme qui dirige les pectines vers des endroits spécifiques dans la paroi n’est pas encore très bien connu. Un modèle qui peut être utilisé pour étudier le mécanisme comprend les cellules sécrétoires de mucilage (CSM) de la plante *Arabidopsis thaliana*. Les CSMs synthétisent de grosses quantités de pectine et aussi démontrent le transport polarisé de pectine. Dans les CSMs, la pectine accumule dans des formes d’anneaux avant d’être sécrété dans l’apoplast. Une revue littéraire et des données de microréseau suggèrent que ROP1, un GTPase, est un candidat possible pour l’établissement des domaines de membranes dans les CSMs. ROP1 et cinq autres gènes étaient choisis pour examiner leur rôle par rapport à l’établissement des domaines de membranes dans les CSMs. Des mutants comprenant des lignes de T-DNA et aussi des lignes de fusion-fluorescente a été utilisé pour des analyses quantitatives et qualitatives. Une analyse phénotypique a démontré une réduction dans le mucilage adhérant du mutant *kinesin-13a*, et une réduction sévère dans le mutant *sec8*. L’imagerie confocale a démontré que ECFP:ROP1 se localise dans des domaines non-sécrétoires à la membrane de la paroi pendant le stage de sécrétion de pectine dans les CSMs. Par contre, GFP:KINESIN-13A et GFP:SEC8 se localisent à des domaines sécrétoires pendant le stage de sécrétion de pectine dans les CSMs.
Ces données aident à identifier les cascades moléculaires impliquées dans l’établissement des domaines pectineuses à la membrane des CSMs et comment les pectines sont dirigées dans les CSMs.
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Chapter 1

Literature Review and Objectives
1.1 Introduction

The focus of my research project is to investigate how pectins are targeted to a specific plasma membrane domain for secretion in the seed coat mucilage secretory cells (MSCs) of the model plant *Arabidopsis thaliana* (*Arabidopsis*). This literature review will cover the basics of pectin structures, as well as their synthesis, trafficking, and general knowledge of vesicle secretion. The establishment of plasma membrane secretory domains in plants is also reviewed. While findings across different species and tissues will be touched upon, the focus will be on what is known for *Arabidopsis* MSCs.

1.1.1 Plant Cell Walls

All plant cells are surrounded by a highly dynamic extracellular structure called the cell wall. Plant cell walls are comprised of a complex assortment of polysaccharides, including cellulose, hemicelluloses, pectins, and a minor amount of proteins (Braidwood et al. 2013; Keegstra 2010). Cellulose is synthesized at the plasma membrane and directly deposited into the apoplast (Cosgrove 2014), whereas hemicelluloses and pectins are synthesized within the Golgi apparatus and targeted in vesicles to the plasma membrane for secretion to the apoplast (Ridley et al. 2001). The study of the cell wall and each of its components is an important area of research, as cell walls play a vital role in plant development by determining cell shape, mechanical properties of tissues, defense against pests and pathogens, and cell-cell adhesion and communication. The study of cell walls is also important from an economic standpoint as agriculture, silviculture, and biofuel industries can benefit from modifying cell wall biosynthetic pathways in order to develop improved plants for different industrial
applications (Levy et al. 2002; Loqué et al. 2015). The roles of different cell wall components and their associated biosynthetic genes and proteins can be studied using various techniques, including mutant analysis, live cell microscopy for protein localization, sectioning and staining, immunological assays, and carbohydrate extraction and analysis.

1.1.2 Mucilage Secretory Cells

A number of plant species develop seeds with specialized seed coat epidermal cells known as mucilage secretory cells (MSCs) (Western 2012; Yang et al. 2012). The genetic model plant Arabidopsis (Western et al. 2000; Arsovski et al. 2010; Francoz et al. 2015), as well as the food crops flax (Linum usitatissimum) (Mazza and Biliaderis 1989) and chia (Salvia hispanica) (Capitani et al. 2013), are just some of many species that produce seed coat mucilage. As the outer integument of the developing seed coat matures, the outermost layer of epidermal cells differentiates to give rise to MSCs. During the differentiation of these specialized cells, MSCs synthesize and secrete to the apoplast an abundance of complex polysaccharides, including pectins, cellulose, and hemicelluloses that together form a gel known as mucilage. Upon seed maturation and hydration, mucilage is extruded from the MSCs and envelops the seed. The role that mucilage plays in the organism’s life cycle appears to vary between species and may include preventing seed desiccation during germination, providing a source of nutrients to the growing seedling, and acting as an adhesive to stick to vectors for dispersal (Western 2012; Yang et al. 2012).

The best studied seed coat MSCs are those of Arabidopsis. Indeed, the Arabidopsis MSCs have become a model system for studying cell wall synthesis.
(Haughn and Western 2012). Fertilization marks the beginning of MSC differentiation, which can be broken down into distinct stages (Figure 1). Firstly, cell size increases 3.5- to 4.5-fold within the first four days post anthesis (DPA), which correlates with increased vacuole volume (Western et al. 2000). Amyloplasts and starch granules then accumulate at the outer tangential cell wall at 3 DPA (Western et al. 2000, Windsor et al. 2000). At 7 DPA, the number of Golgi stacks doubles and vesicle abundance and size increase (Western et al. 2000, McFarlane et al. 2008; Young et al. 2008), which correlates with the onset of a high degree of mucilage production. At this point, an abundance of pectins is synthesized, targeted to the plasma membrane and secreted to a distinct donut-shaped apical domain in the apoplast (Western et al. 2000). As mucilage synthesis and secretion occur, the vacuole shrinks and localizes to the basolateral domain of the cell and the cytoplasm and amyloplasts localize to form a central volcano-shaped cytoplasmic column that spans to the radial cell wall (Western et al. 2000; Windsor et al. 2000). A dense array of cortical microtubules is laid down, lining the cytosolic face of the plasma membrane in the specific domain where the mucilage pocket forms (McFarlane et al. 2008). Whether the cortical microtubules are involved in targeting and/or secreting pectin or other mucilage components to the apoplast remains unclear. From 10 to 13 DPA, a secondary cell wall is synthesized at the junction between the mucilage pocket and cytoplasm, stretching from the radial cell wall to the outer tangential cell wall, eventually filling the cytoplasmic column and creating a solid structure called the columella (Western et al. 2000). Apoptosis and desiccation then takes place, causing the mucilage and primary cell wall to shrink, and giving the MSC its
characteristic raised central columella surrounded by a recessed, donut-shaped mucilage pocket.

Upon seed hydration, the highly hydrophilic mucilage rapidly swells, rupturing the primary cell wall and enveloping the seed (Western et al. 2000). The extruded mucilage is comprised of two layers: an inner adherent layer, and an outer non-adherent layer which can be easily shaken off. Cellulosic rays extend from the columellae and into the inner adherent mucilage layer. These rays are composed of uncoiled cellulose and ruptured cell wall remnants (presumably bound to pectins and hemicellulose) responsible for adhering the inner mucilage layer to the seed (Harpaz-Saad et al. 2011; Griffiths et al. 2015). Recent findings suggest that cellulose can bind directly to pectin (Wang et al. 2015), which could explain why mutants with cellulosic ray reduction show decreased mucilage adherence to the seed coat (Griffiths et al. 2015).

1.1.3 Mucilage Composition and Pectin Synthesis

Pectins are a diverse group of complex acidic polysaccharides that form the matrix of the plant primary cell wall, and are characterized by 1,4-linked α-D-galacturonic acid (GalA) residues (Ridley et al. 2001). Pectins are grouped into three main types: rhamnogalacturonan I (RG-I), rhamnogalacturonan II (RG-II), and homogalacturonan (HG) (Atmodjo et al. 2013). HG is comprised of unbranched α-1,4-linked GalA residues that may be methyl-esterified or acetylated. RG-I is comprised of alternating galacturonic acid and rhamnose (Rha) residues in the conformation α-1,2-L-Rha-α-1,4-D-GalA. RG-II consists of an HG backbone containing varying combinations of linkages to four different side chains, including two disaccharides, an octasaccharide, and a nonasaccharide (Atmodjo et al. 2013; Western 2006). Pectins are the main
component of mucilage in *Arabidopsis* seed coat (Voiniciuc et al. 2015). Linkage analysis, enzymatic digestion, and carbohydrate-specific antibody assays have demonstrated that non-adherent mucilage is mainly comprised of RG-I and small amounts of HG, whereas adherent mucilage is also primarily comprised of RG-I, but also includes arabinans, galactans, cellulose, xylans, and galactoglucomannan (Haughn and Western 2012; Macquet et al. 2007; Voiniciuc et al. 2015a; Voiniciuc et al. 2015b; Willats et al. 2001).

Pectins are synthesized within the Golgi apparatus via a complex assortment of enzymes. In the cytosol, nucleotide sugar synthases synthesize the nucleotide sugars UDP-\(\alpha\)-GalA and UDP-\(\alpha\)-Rha which act as substrates for pectin backbones (Seifert 2004; Atmodjo et al. 2013). UDP-\(\alpha\)-Rha synthesis is catalyzed by the RHM family of UDP-\(\alpha\)-Rha synthases as well as the related protein UER1, while UDP-\(\alpha\)-GalA is synthesized by UDP-\(\alpha\)-glucuronate 4-epimerases (Usadel et al. 2004). Nucleotide sugar transporters (NSTs) inserted in the Golgi membrane act in transporting nucleotide sugars from the cytosol to the Golgi lumen (Atmodjo et al. 2013). Inside the Golgi lumen, nucleotide sugars are linked into long chains by glycosyltransferases (GTs), such as GalAT which is responsible for catalyzing the synthesis of HG (Sterling et al. 2001). At least 53 glycotransferases are required for pectin synthesis within the Golgi (Ridley et al. 2001). Within the Golgi lumen, pectins then can undergo methylesterification or acetylation by pectin methyltransferases and pectin acetylesterases, respectively (Atmodjo et al. 2013). Following synthesis and modification, pectins are bundled into vesicles and targeted to the plasma membrane for secretion to the apoplast, where further restructuring can occur by enzymes such as
pectin methylesterases which facilitate the removal of methyl groups from HG (Pelloux et al. 2007).

Various genes involved in pectin biosynthesis affect mucilage production. Genetic analysis has revealed that, while all three Arabidopsis RHM genes are expressed in differentiating MSCs, RHM2/MUM4 is specifically upregulated at the time of mucilage synthesis to provide additional substrate for RG-I synthesis (Western et al. 2004). Mutations in MUM4 cause a drastic reduction in mucilage production, as well as flattened columellae and impaired mucilage extrusion upon seed hydration (Usadel et al. 2004; Western et al. 2004). Conversely, no GalA synthase (UGlcAE family) has yet been identified as having a specific role in Arabidopsis mucilage production. The URGT family of NSTs have been shown to be involved in mucilage production, as urgt2 mutants display significantly reduced mucilage containing fewer or shorter RG-I chains, indicating its involvement in transporting UDP-L-Rha from the cytosol to the Golgi lumen for RG-I biosynthesis (Rautengarten et al. 2014). Additionally, the GalA NST UUAT1 has been shown to affect mucilage production, as mutants exhibit reduced mucilage synthesis with decreased GalA in adherent and non-adherent mucilage (Saez-Aguayo et al. 2017). Finally, two GTs, GATL5 and GAUT11, have been shown to affect mucilage production (Caffal et al. 2009; Kong et al. 2013). gatl5 mutants produce mucilage with approximately 40% less GalA and Rha compared to WT; however, the molecular weight of mucilage increases 63%, suggesting that GATL5 affects the size of RG-I molecules (Kong et al. 2013). A gaut11 mutant displays impaired mucilage extrusion, and reductions in Rha and GalA levels compared to WT (Caffal et al. 2009).
1.1.4 Pectin Trafficking

To date, there is no direct evidence of how pectin-laden vesicles are trafficked to specific plasma membrane domains, whether it is along actin filaments, microtubules, or by other means (Kim and Brandizzi 2014). Cells that exhibit polarized growth, such as pollen tubes or root hairs, are often used as models to study vesicular trafficking, as there is a high rate of vesicle secretion of proteins and cell wall components at the growing tip (Cole and Fowler 2006). A fine network of filamentous actin lines the growing tip of root hairs (Ketelaar et al. 2003). It was found that actin disruption impedes vesicle delivery to the plasma membrane, although actin is not needed for fusing vesicles to the plasma membrane (Ketelaar et al. 2003). Additionally, a knockout mutant of the microtubule-associated kinesin FRAGILE FIBER1 causes a reduction in secretion of fucose-alkyne-labeled pectin (Zhu et al. 2015). These results indicate that both actin and microtubules may be associated with pectin secretory pathways.

The SECRETORY CARRIER MEMBRANE PROTEIN (SCAMP) family of proteins may play a role in trafficking pectins to the plasma membrane. SCAMPs are transmembrane proteins that localize to the TGN, plasma membrane, developing cell plate, and early endosome (Law et al. 2012). A study in Nicotiana tabacum revealed that NtSCAMP2 is localized to the TGN, and associates with the TGN-derived linked secretory vesicle clusters (SVCs) which are targeted to the plasma membrane for fusion and secretion (Toyooka et al. 2009). Immunogold labeling of Nicotiana tabacum BY-2 cells with the monoclonal antibody JIM7 (specific for homogalacturonan) shows localization to anti-NtSCAMP2-labelled SVCs, which suggests the vesicles contain pectins (Toyooka et al. 2009). In mitotic cells, NtSCAMP2-labelled SVCs do not traffic to
the plasma membrane, but instead to the developing cell plate, which further suggests NtSCAMP2-labelled SVCs carry cell wall materials (Toyooka et al. 2009). Five SCAMP proteins have been identified in *Arabidopsis* (Law et al. 2012), and SVCs have been observed in expanding *Arabidopsis* cotyledons (Toyooka et al. 2009); however, SCAMP proteins in *Arabidopsis* have not been well-characterized to date. The online gene expression microarray database “*Arabidopsis* Seed Coat eFP Browser” (Dean et al. 2011) indicates that all SCAMP proteins are expressed at varying degrees in the developing seed coat. These data may suggest that SCAMP proteins are worth investigating in relation to the trafficking of pectin-laden vesicles in MSCs from Golgi stacks to the mucilage pocket plasma membrane domain.

Few studies have investigated pectin trafficking in MSCs. It has been established that pectins are bundled into Golgi-derived vesicles, which are trafficked to the plasma membrane for secretion to the mucilage pocket (Western 2012). Immunogold labelling of cryofixed MSCs at 7 DPA, using the RG-l-specific monoclonal antibody CCRC-M36, show pectins localized to Golgi cisternae and the *trans*-Golgi network (Young et al. 2008), where dense secretory vesicles are formed and trafficked to the plasma membrane (McFarlane et al. 2008). Interestingly, the number of Golgi stacks increases by nearly 100% specifically at 7 DPA (during pectin synthesis and secretion), and their localization is evenly spread throughout the apical and basolateral cytoplasm domains, as opposed to being localized along the mucilage pocket pectin secretion domain (Young et al. 2008). This even distribution of Golgi stacks that exhibit high vesicle production differs from what is observed during mitosis, when many Golgi stacks aggregate to the phragmoplast as polysaccharide-laden vesicles are targeted to the
growing cell plate (Dupree and Sherrier 1998; Nebenführ et al. 2000). Additionally, approximately 70% of Golgi stacks throughout the MSC are labelled with CCRC-M36 (Young et al. 2008), which indicates that a trafficking and targeting mechanism exists for long-distance vesicle shuttling to the mucilage pocket plasma membrane domain to accommodate for the variable spatial origin of vesicles.

Additionally, the trans-Golgi network-localized protein ECHIDNA has been shown to affect pectin trafficking in MSCs (McFarlane et al. 2013). While studying an echidna mutant, McFarlane et al. (2013) observed a drastic decrease in mucilage extrusion when seeds were imbibed in water and stained with ruthenium red (RR). The authors used stained sections, transmission electron microscopy, and the mucilage-specific monoclonal antibody CCRC-M36 to reveal a high reduction in mucilage accumulation within the mucilage pocket. Additionally, they noted that pectin-laden vesicles are mistargeted in echidna, as they observed vesicle accumulation in the central lytic vacuole and endoplasmic reticulum bodies. A subsequent study identified the RAB GTPase interactors YIP4a and YIP4b as forming a complex with ECHIDNA at the trans-Golgi network (Gendre et al. 2013). The double mutant yip4ayip4b displayed a phenotype similar to echidna, where mucilage production was greatly reduced and pectin-laden vesicles were mislocalized to the center of the cell (Gendre et al. 2013). The authors concluded that the ECHIDNA-YIP4 complex appears to be involved in targeting cell wall polysaccharides to appropriate secretory domains, due to the drastic decrease in polysaccharide secretion to the mucilage pocket, as well as the mislocalization of pectin-laden vesicles.
Interestingly, a dense array of cortical microtubules lines the cytosolic face of the plasma membrane specifically along the mucilage pocket domain of MSCs (McFarlane et al. 2008). Griffiths et al. (2015) concluded that cellulose synthase complexes embedded in the plasma membrane are trafficked linearly along these cortical microtubules, where they synthesize long strands of cellulose chains that are directly inserted into the mucilage pocket parallel to the seed’s surface. Disruption of cortical microtubule arrangement in the heat-shock inducible mor1-1 mutant causes a reduction in pectin accumulation and impaired extrusion from the mucilage pocket (McFarlane et al. 2008); however, pectin secretion persists despite microtubule disruption, suggesting that microtubules are not solely responsible for trafficking vesicles to the mucilage pocket plasma membrane domain.

1.1.5 Vesicle Secretion

Following trafficking from the Golgi apparatus, secretory vesicles undergo tethering and fusion to the plasma membrane for secretion to the apoplast. One of the pathways responsible for targeting and tethering secretory vesicles is through the exocyst complex (Žárský et al. 2013). The exocyst is an octameric complex comprised of eight subunits localized to the plasma membrane (Fendrych et al. 2012). The subunits and their paralogs (total number of Arabidopsis genes in parentheses) that comprise the exocyst include: SEC3 (2), SEC5 (2), SEC6 (1), SEC8 (1), SEC10 (1), SEC15 (2), EXO70 (23), and EXO84 (3) (Cvrčková et al. 2012). Microarray expression analysis of all EXO70 genes across 11 different Arabidopsis tissues reveals that many EXO70 paralogs display varied expression across tissue types, and are only active during cell differentiation, suggesting tissue-specific roles for exocyst subunit paralogs.
during times of growth and secretion (Synek et al. 2006). The exact mechanism by which the exocyst targets and fuses vesicles remains unknown; however, mutant studies of different subunits have established that the exocyst plays an important role in polarized secretion and growth (Hála et al. 2008; Žárský et al. 2013). Live cell imaging of exocyst subunits fused to fluorophores shows subunit colocalization and localization of the complex at areas of high vesicle secretion, including SEC6, SEC8, SEC15b, EXO70A1, and EXO84b at the growing cell plate (Fendrych et al. 2010), and SEC3a, SEC8, and SEC10 at the pollen tube growing tip (Bloch et al. 2016; Synek et al. 2017). Exocyst subunit mutants typically result in dwarf phenotypes and disruption of polarized growth due to perturbation of vesicle secretion. For example, an exo84b mutant causes a dwarfed phenotype, accumulation of vesicles in Arabidopsis leaf epidermal cells, and disrupted division of guard cells and leaf epidermal cells (Fendrych et al. 2010). An exo70a1 mutant results in dwarfed plants which display abnormal secondary cell wall thickening patterns in tracheary elements and an accumulation of membrane-bound compartments in the cytosol (Li et al. 2013). Mutants for the SEC8 subunit show a varying degree of phenotypes, ranging from reduced pollen tube growth to severe reductions in pollen germination and reduced competition against wild type (WT) pollen during pollination (Cole et al. 2005).

Research on the plant exocyst complex is a relatively new field (Žárský et al. 2013). To date, only a single study has investigated the role of the exocyst in Arabidopsis MSC development. Kulich et al. (2010) found that knocking out exo70A1 results in small mucilage pockets and a significant reduction in mucilage production, as well as rectangular-shaped MSCs with larger, flattened columellae compared to Col-0.
sec8 mutant seeds showed more severe phenotypes, including significant to complete loss of mucilage release, and flattened MSCs that are mostly devoid of columellae. This study was limited to using scanning electron microscopy (SEM) and basic light microscopy for RR staining and sections stained with toluidine blue. Although the localization of EXO70A1 and SEC8 was not investigated, the mutant phenotypes indicate that the exocyst complex plays a clear role in the secretion of mucilage polysaccharides to the mucilage pocket secretory domain. More investigation of the exocyst and these subunits should be carried out to characterize their role in pectin secretion in MSCs.

1.1.6 ROPs and Plasma Membrane Domains

The RHO OF PLANTS (ROP) family of small GTPases has been identified to encode master regulators for the establishment of plasma membrane domains involved in polarized growth and secretion (Berken 2006; Burkart et al. 2015; Nagawa et al. 2010; Nibau et al. 2006). ROPs function similarly to Rho-family GTPases found in animals and fungi; however, they are specific to the plant kingdom, and 11 ROPs have been identified in Arabidopsis (Nagawa et al. 2010). ROPs (and GTPases in general) act as molecular switches, where they exist in either an active or inactive state based on conformational changes mediated by ROP regulators. Guanine nucleotide exchange factors (GEFs) activate ROPs by facilitating the removal of GDP and the binding of GTP, whereas GTPase-activating proteins (GAPs) mediate hydrolysis of GTP to GDP, thereby inactivating ROPs (Nagawa et al. 2010). A third regulator type, guanine nucleotide dissociation inhibitors (GDIs), functions by inhibiting inactive ROPs from returning to an active state by locking them in an inactive state (Nibau et al. 2006).
Active ROPs are anchored to the plasma membrane and function by binding to downstream effector proteins, which triggers signalling cascades involved in secondary cell wall patterning (Oda and Fukuda 2013a), cytoskeletal organization (Mucha et al. 2011), and vesicular trafficking (Lavy et al. 2007), as well as pathways related to hormone, disease, and environmental signalling (Berken 2006). Various effectors and downstream proteins involved in ROP-mediated pathways have been discovered through studying cells that experience anisotropic growth, such as pollen tubes, root hairs, and pavement cells, as well as xylem, which displays secondary cell wall patterning (Berken 2006; Fu et al. 2009; Lavy et al. 2007; Mucha et al. 2011).

The role of ROPs in controlling cytoskeletal organization has been well-established in a number of different studies (Mucha et al. 2011). For example, *Arabidopsis* leaf epidermal pavement cells exhibit areas of polarized growth, leading to interdigitation with neighbouring cells. It was found that activated ROP2 promotes the formation of localized cortical actin microfilaments needed in areas of polarized growth by activating the effector ROP-INTERACTIVE CRIB MOTIF-CONTAINING PROTEIN 4 (RIC4) (Fu et al. 2005). Conversely, ROP6 activity restricts local polarized growth by promoting the formation of well-ordered cortical microtubules by activating the microtubule-associated protein RIC1 effector (Fu et al. 2009). Therefore, in pavement cells, ROP2 causes lobation, whereas ROP6 cause indentation. In xylem cells, activated ROP11 recruits the ROP effector MICROTUBULE DEPLETION DOMAIN 1 (MIDD1)/ROP INTERACTIVE PARTNER 3 (RIP3) which, in turn, binds to both microtubules and the microtubule-associated protein KINESIN-13A (Oda and Fukuda 2013b). When bound to RIP3, KINESIN-13A depolymerizes microtubules, which inhibits
local secondary cell wall deposition, leading to the characteristic cell wall pits present in mature xylem cells (Oda and Fukuda 2013b). Additionally, KINESIN-13A associates with the Golgi apparatus, and kinesin-13a mutants cause Golgi clustering and increased trichome branching, which suggests KINESIN-13A is involved in secretory vesicle trafficking and/or targeting to the plasma membrane (Lu et al. 2005).

In addition to regulating the cytoskeleton, ROPs are also involved in establishing secretory domains at the plasma membrane. Lavy et al. (2007) investigated protein-protein interactions with the ROP effector INTERACTOR OF CONSTITUTIVE ACTIVE ROP1 (ICR1)/RIP1. Using a pull-down assay for RIP1, they found interaction with the SEC3 exocyst subunit. Additionally, bimolecular fluorescence assays show that RIP1, ROP6, and ROP10 localize at the plasma membrane in pavement cells. Knocking out rip1 causes swollen root tips, irregular cell division planes, and pollen sterility (Lavy et al. 2007). Taken together, these data suggest a pathway between ROPs, their effectors, and the exocyst, where RIP1 acts as a scaffold between ROPs and downstream proteins. To further validate this, Li et al. (2008) found that fluorescence resonance energy transfer between ROP1 and RIP1 shows localization and interaction at areas of high secretion (interaction further confirmed with co-immunoprecipitation), including the pollen germination site as well as the tip of growing pollen tubes. Additionally, it has been found that overexpressing ROP1 in pollen tubes leads to pollen tube swelling and loss of polarized growth (Li et al. 1999; Gu et al. 2003; Hwang et al. 2005; Li et al. 2008), and dominant negative rop1 mutants display inhibited pollen tube elongation (Gu et al. 2003; Hwang et al. 2005; Li et al. 1999). Similar results were found with ROP2 in root hairs, where GFP-ROP2 localizes to the future site of polarized root hair outgrowth.
and remains expressed at the growing tip (Jones et al. 2002). Constitutively active (CA) rop2 results in a loss of polarized root hair growth and dominant negative (DN) rop2 reduces growth rates and causes wavy root development (Jones et al. 2002).

To date, ROP activity has not been studied in MSCs. Their role in establishing domains of polarized secretion and regulating cytoskeletal organization makes them a promising area of study in MSC development where polarized secretion is exhibited and cortical microtubules are present along the mucilage pocket.

1.1.7 Candidate Genes

The ROP family of small GTPases was chosen to screen for potential candidates in establishing the pectin secretory domain in MSCs as they are known to establish plasma membrane domains (described in the previous section). To judge the candidacy of ROPs, publicly available and private microarray datasets for gene expression in WT versus mucilage mutant *Arabidopsis* seed coats were screened for ROP expression (TL Western, personal communication; Dean et al. 2011; Kong et al. 2013; Schmid et al. 2005). Of the 11 ROP genes, only ROP1 showed specific and significant upregulation at 7 DPA in wild type ecotypes, which coincides with the period of pectin synthesis and secretion to the mucilage pocket. Additionally, ROP1 expression is reduced in the seed coat of plants carrying mutations in *APETALA2* (*AP2*), a master regulator of outer integument (i.e., seed coat) differentiation (Jofuku et al. 1994; Ohto et al. 2009; Western 2001). The decrease in ROP1 expression in *ap2* mutants suggests that it is specifically expressed during MSC development and differentiation. This may indicate that ROP1 is involved in triggering a signalling cascade involving pathways that may be similar to
what has been observed in other tissues for \textit{ROP1} and other \textit{ROP}s. For these reasons, \textit{ROP1} was chosen as the primary candidate gene for this study.

Further candidate genes (Table 1) were chosen based both on their expression patterns in seed coat microarray datasets as well as on their association with \textit{ROP} function. Genes screened included the \textit{RIP} and \textit{RIC} families of \textit{ROP} effectors, as well as all predicted \textit{GEFs}, \textit{GAPs} and \textit{GDIs}. Of these direct interactors with \textit{ROP}s, only members of the \textit{RIP} family of effectors had expression patterns that correlated with a potential specific role in seed coat mucilage production. \textit{MIDD1/RIP3} and \textit{RIP5} were chosen because they are recognized as \textit{ROP} effectors. Unlike \textit{RIP3}, which has been linked to polarized growth domains studied by various groups, little is known about \textit{RIP5} aside from a yeast two-hybrid screen which showed interaction with \textit{DN-rop4} (Mucha et al. 2010). \textit{ICR1/RIP1} and \textit{ICR2/RIP2} were also chosen as candidates as they are known \textit{ROP} effectors; however, it was not possible to isolate suitable T-DNA insertional mutants for these genes. \textit{KINESIN-13A} was chosen because it is a known target in \textit{ROP} signalling pathways, and directly interacts with RIP3. \textit{KINESIN-13A} appears to be involved in secretory vesicle trafficking, as it associates with the Golgi apparatus, and knockout mutants lead to mislocalized Golgi stacks and increased trichome branding (Lu et al. 2005). Furthermore, a dense array of cortical microtubules lines the secretory domain adjacent to the mucilage pocket and \textit{KINESIN-13A} is a known microtubule-associated protein, which raises the question of whether \textit{KINESIN-13A} may be involved in establishing this microtubule domain. Finally, two exocyst subunits, \textit{EXO70A1} and \textit{SEC8}, were chosen as candidates. The exocyst is involved in targeting and tethering secretory vesicles at the plasma membrane and it is a known downstream target in
different ROP pathways. Additionally, a single limited study of mutants for exo70a1 and sec8 in Arabidopsis MSCs revealed phenotypes of varying degrees of severity, ranging from reduced mucilage secretion and extrusion and misshapen MSCs, to a complete loss of mucilage secretion and significantly flattened MSCs (Kulich et al. 2010). The exocyst appears to be clearly involved in secretion to the mucilage pocket, and more information can be gathered to elucidate the interactions between the exocyst and other members of the pectin targeting pathway.

1.1.8 Hypothesis and Research Goals

The overall goal of this study is to identify and characterize components of the molecular pathway responsible for targeting pectins to the distinct ring-shaped secretory domain at the plasma membrane that gives rise to the mucilage pocket in Arabidopsis MSCs.

A review of the literature of targeting to plasma membrane domains and expression patterns of related gene families leads to the hypothesis of this research project that ROP1 and other associated candidate genes found to be expressed in differentiating seed coats play roles in setting up the plasma membrane domain in MSCs to which pectins are targeted for secretion.

The specific objectives of this study are twofold:

(1) Identification and/or establishment knockout, knockdown, and/or overexpressing lines for each candidate gene, and characterizing the resulting phenotypes using various qualitative and quantitative techniques.
(2) Obtaining and/or constructing candidate protein fluorophore fusion lines to observe protein localization \textit{in vivo} in MSCs during the period of pectin synthesis and secretion.
Figure 1. Arabidopsis thaliana seed coat mucilage secretory cell development. Following fertilization, cell size increases and amyloplasts (A) accumulate in the cytoplasm at four days post anthesis (DPA). A large vacuole (V) is present in the cell at this stage. At 7-8 DPA, mucilage (M) is synthesized and secreted to the apoplast between the plasma membrane and primary cell wall (PW). From 10 to 13 DPA, a secondary cell wall is synthesized (dark blue), creating a central volcano-shaped columella (C). Apoptosis occurs, resulting in a mature cell which is shrunken and desiccated. When exposed to water, the hydrophilic mucilage rapidly swells and bursts through the primary cell wall, releasing adherent and non-adherent mucilage which envelops the seed. Cellulosic rays (R) extend into the adherent mucilage. Figure from Voiniciuc et al. (2015c).
Chapter 2

Experimental Methods
2.1 Experimental Methods

2.1.1 Plant Growth Conditions

Agar plant growth medium lacking sucrose (AT medium) was prepared as per Haughn and Somerville (1986) and used for seed germination. Seeds were sown directly onto prepared AT plates and each plate was sealed with micropore tape to allow gas exchange while creating a barrier against airborne contaminants. The plates were wrapped in plastic wrap to prevent desiccation during cold stratification at 4°C from four to seven days. After stratification, the plates were removed from the plastic wrap and placed under continuous light in a plant tissue culture chamber (Percival Scientific) at 22°C. Following development of true leaves, four to seven seedlings were transplanted to a 12.7 cm pot, containing moistened PROMIX-PGX soil (Premier Tech, Rivière-du-Loup, Canada) and 80 mL of standard 20-20-20 NPK nutrient solution. The planted pots were wrapped in plastic wrap for two to three days to maintain humidity and moved to a plant growth chamber (Conviron, Winnipeg, Canada) at 20°C and 50% humidity, under constant light. Seeds were collected from desiccated siliques, processed through a fine steel mesh strainer (approximately size 40 sieve) to remove non-seed matter, and stored in 1.5mL centrifuge tubes. It should be noted that whenever possible, all mutant lines, wild-type lines, and control lines were grown simultaneously to control for potential variation in growth conditions during different times, which could lead to changes in observed phenotypes.
2.1.2 Genetic Analysis of T-DNA Lines

Putative homozygous lines and segregating populations of T-DNA insertional mutants (Alonso et al. 2003) were obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University, USA) (Table 1, Figure 2). Plants were grown and leaves removed for DNA extraction as per Edwards et al. (1991), and DNA was stored at 4°C in 1.5 mL centrifuge tubes. To determine the genotype of each plant for homozygous mutant isolation, 2 μL of DNA was used in a 20 μL PCR reaction using 0.2 μL of standard Taq polymerase (New England Biolabs (NEB), Massachusetts, USA) and amplified using 1 μL each of gene-specific primers (10 μM) for each T-DNA line (Table 2, Table 3). Water, 2 μL of 10x buffer solution, and 2 μL of dNTPs (10mM) were added to the reaction, which ran for 30 cycles. Forward and reverse primers (also referred to as left and right primers by the Salk Institute Genomic Analysis Laboratory, California, USA) were used to amplify across the predicted insertion site, and a T-DNA-specific border primer and left primer were used in a separate reaction to screen for the presence of an insertion (Table 2, Table 3). Heterozygous and WT plants were removed from pots, leaving homozygous populations to grow. Subsequent generations were also genotyped to confirm isolation of homozygous lines.

To confirm the predicted location of the T-DNA insertion in each line, a T-DNA border primer and a gene-specific primer were used to amplify extracted DNA from each line. The amplified fragments were run in a 0.5% agarose gel, extracted using the column-based Monarch DNA Gel Extraction Kit (NEB), and ligated into the pCR2.1 vector (Invitrogen, California, USA) using a TOPO-TA reaction (Invitrogen). The ligated vectors were then transformed into chemically competent DH5α cells and selected by
streaking onto standard LB plates containing kanamycin (50μg/mL). Individual colonies were grown in overnight cultures, and plasmids isolated using a QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). Plasmids were confirmed as containing the amplified fragment via PCR and/or restriction enzyme digestion and sent to Genome Quebec (Montreal, Canada) for sequencing. Sequencing reads and chromatograms were analyzed and aligned to respective genomic DNA sequences using the online software Benchling (www.benchling.com) to determine the location of T-DNA insertion (Figure 2).

Qualitative real-time PCR (RT-PCR) was used to characterize how T-DNA insertions affected respective gene transcription in each mutant line. To carry out RNA extraction, a modified protocol was used with an RNeasy Plant Mini Kit (Qiagen). For each line, 12-14 inflorescences were removed across four homozygous plants per line and immediately placed in a sterile 50 mL conical centrifuge tube containing liquid nitrogen (LN$_2$). A clean and sterile magnetic stir bar (12mm x 8mm) was added to the centrifuge tube and placed in a rack on top of a magnetic stir plate. The plate was turned on, which caused the stir bar to move quickly and erratically around in the centrifuge tube at high speeds. Instead of using only the RLT buffer, 60μL of 10% polyvinylpyrrolidone 40 (PVP40) (w/v) and 6μL of 2-mercaptoethanol (BME) were added to 540 μL of RLT. PVP40 was added to eliminate phenolic compounds (Wan and Wilkins 1994), and BME was added to inhibit RNase activity (Li and Trick 2005). After preparing the RLT-PVP40-BME solution, 598.5 μL was added drop by drop to the slurry of ground tissue and LN$_2$ while the plate remained turned on. The level of LN$_2$ was maintained at approximately 20 mL for at least 10 minutes, and until the plant tissue was reduced to a fine powder. The centrifuge tube was then placed on ice for
approximately 30 minutes to allow the LN$_2$ to evaporate, and the slurry to melt. Immediately upon melting, the slurry was added to a QIAshredder column. The standard RNA extraction protocol was then completed as per Qiagen’s RNeasy Mini Handbook. RNA was eluted in 40 µL of DNase/RNase-free UltraPure water (Thermo Fisher Scientific, Massachusetts, USA) warmed to 45°C, and 1.5 µL of eluate was used to quantify RNA concentration and purity using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). Standard yields ranged from 200-1000ng/µL, with a 260/280 value above 2.09.

A Quantitect Reverse Transcription Kit (Qiagen) was used to synthesize cDNA from 1 µg RNA using oligo-dT and random primers. A DNase treatment was also carried out to reduce the potential of genomic DNA contamination. To analyze gene transcripts in T-DNA lines, gene-specific RT-PCR primers were designed using Primer3Plus (Untergasser et al. 2007) and Benchling (Table 4, Table 5). For each T-DNA line, primer pairs were targeted to amplify across the insert to identify if WT-length transcripts were present (Figure 3), as well as upstream and downstream of the insert (data not shown). PCR amplification was carried out over 27 cycles. As a control, the same primer pairs were used with Col-0 (WT) cDNA in simultaneous reactions. Additionally, the housekeeping gene GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (GAPC) was amplified as a loading control. All products were separated on a 1% agarose gel, and the resulting bands were compared between T-DNA and WT lanes to analyze transcripts based on band intensity and size.
2.1.3 Ruthenium Red Staining and Imaging

Ruthenium red (RR) (Sigma-Aldrich, Missouri, USA) was used to stain acidic polysaccharides (pectins) in seed coat mucilage to screen for mutant phenotypes in homozygous T-DNA lines. The first RR staining method involved shaking seeds directly in RR solution to analyze adherent mucilage (Figure 5). Approximately 80-100 seeds were placed in a 1.5 mL centrifuge tube and filled with 1200 μL of 0.01% aqueous RR solution. The tube was then placed on an orbital shaker at the highest speed for 40 minutes. The RR solution was removed, the seeds rinsed twice with UltraPure water, and resuspended in 500 μL of 0.1% Tween 20 (Thermo Fisher Scientific). Tween 20 was added to reduce water surface tension, which drastically improved imaging quality by eliminating a bubbling/dome-shaped effect when the solution was pipetted onto the slide, allowing seeds to be evenly spread. Roughly 200 μL of seeds suspended in solution was pipetted onto a glass slide and imaged using a Leica MZ16F stereomicroscope (Leica Microsystems, Wetzlar, Germany) with a QImaging Micropublisher 3.3 RTV camera (Q Imaging, Surrey, Canada) at 12.5x magnification for quantification, and 40x magnification for single seed imaging. Nikon Elements software (Nikon Instruments, New York, USA) was used to capture images. The slide was bottom-illuminated under full intensity light. Care was taken to image seeds of all lines under the same parameters to quantify differences in RR staining.

The second RR staining method followed the same protocol described in the previous paragraph, except for the addition of a pretreatment step using ethylenediaminetetraacetic acid (EDTA) (Figure 7). EDTA is a metal chelator which is thought to disrupt calcium bridges in pectin chains, thus promoting pectin hydration.
and/or primary cell wall weakening (Arsovski et al. 2009a). In *mum4-1* mutants, which show a severe reduction in mucilage synthesis and lack of mucilage extrusion upon hydration in water, pretreating the seeds with an EDTA solution causes a significant increase in mucilage extrusion. To screen for potential extrusion-related phenotypes, seeds were first shaken in 1200 μL of 50 mM EDTA (pH 8.0) for 30 minutes, the solution was removed, and RR staining was then initiated as described in the previous protocol.

The third method of RR staining allowed analysis of the non-adherent and adherent layers of mucilage by dropping seeds in RR without shaking (Figure 4). Each well of a 24 well tissue culture plate was filled with 400μL of 0.1% RR. One seed was dropped in each well, with care taken to avoid shaking. After one hour, each well of the plate was imaged at 4x magnification using the Leica stereomicroscope.

### 2.1.4 Image Analysis using FiJi

Following RR imaging using specific acquisition parameters (Appendix 1), the total area of adherent mucilage per seed was quantified using the image analysis software FiJi (Schindelin et al. 2012) (Figure 6). A high throughput measurement technique was created by writing a macro code to screen through folders of images to output measurements of the area (in pixels) of each seed’s adherent mucilage (Appendix 2). The analysis is optimized by changing the image to 8-bit and adjusting the threshold so that only the mucilage is “visible” and measured by the software. Additionally, particle sizes above and below an optimized threshold are omitted, and particle shape above and below an optimized degree of circularity are also omitted. The
measured outputs for each *Arabidopsis* line were compiled in Microsoft Excel spreadsheets for statistical analysis.

### 2.1.5 Calcofluor-White Staining

To screen for phenotypes caused by perturbation of cellulose synthesis or deposition, seeds were hydrated and stained with calcofluor-white (CFW, Sigma-Aldrich) (Figure 8). CFW is a fluorescent dye that mainly binds to β-glycans in cellulose (Macquet et al. 2007; Willats et al. 2001). A 20 mg/mL stock solution of CFW was prepared in 40 mM sodium hydroxide, and 800 μL aliquots of aqueous 1mg/mL CFW were added to 1.5 mL microcentrifuge tubes. Approximately 20-30 seeds were added to each tube, covered with foil, and vortexed periodically for four minutes. The solution was removed from each tube, and seeds were washed three times and resuspended in 800 μL of UltraPure water. Seeds were analyzed with a Leica DM6000B epifluorescence microscope using UV light and images were captured using a QImaging Retiga EXi FAST camera at 400x magnification. All lines were compared to Col-0 images using a qualitative visual analysis to discern obvious, distinct differences.

### 2.1.6 Total Sugar Analysis

To screen for phenotypes associated with increased or decreased mucilage production, a standard phenol-sulfuric acid assay (DuBois et al. 1956) was used to quantify the concentration of total carbohydrates present in the adherent and non-adherent layers of mucilage for each line (Figure 10). The assay works by adding concentrated sulfuric acid to a carbohydrate sample, causing a catabolic reaction, reducing all polysaccharides to monosaccharides. Phenol is added to the reaction and
acts as a colorimetric agent. Increasing concentrations of carbohydrates causes the solution to develop a deeper yellow/orange colour.

For each line, a total of 20 mg of seeds was added to a 1.5 mL microcentrifuge tube and filled with 1200 μL of UltraPure water. The tube was quickly vortexed to suspend the seeds in solution, and was shaken on an orbital shaker at high speed for 30 minutes. After shaking, 400 μL of solution was removed and placed in a new tube. This was used as the non-adherent mucilage sample. Ultrapure water was added and removed four times to remove the remainder of non-adherent mucilage from the tube containing the seeds, and seeds were resuspended in 1200 μL of UltraPure water. To separate the adherent mucilage from the seeds, the tube was subjected to probe sonication for 30 seconds at 30% amplitude using a Branson 102C probe sonicator (Branson Ultrasonics Corporation, Connecticut, USA). Removal of adherent mucilage using sonication is a relatively recent and accepted technique (Zhao et al. 2017). Following sonication, 400 μL of solution was removed and used as the adherent mucilage sample. The phenol-sulfuric acid assay for total sugars was carried out as per DuBois et al. (1956), using disposable glass culture tubes (13x100 mm) for each reaction. A DU800 spectrophotometer (Beckman Coulter, California, USA) was used to measure absorbance, and concentrations were calculated based off absorbance readings from a glucose standard subjected to the same assay. As a control to confirm the efficiency of sonication for removing adherent mucilage, multiple samples of Col-0 seeds were stained with RR following sonication, which revealed all mucilage was removed (data not shown).
2.1.7 Cloning and Transformation

Multiple fluorophore fusion lines were constructed or modified from existing vectors and transformed into Arabidopsis Col-0 to characterize protein localization in MSCs (Table 7). Genes were cloned from cDNA or vectors as templates, and primers designed to amplify from the start codon to stop codon were used for amplification using Q5 proofreading polymerase (NEB) (Table 8, Table 9). Amplicons were run in a 0.5% agarose gel, purified using a Monarch Gel Extraction kit (NEB), and dATP overhangs were added using an A-tailing reaction with standard Taq polymerase (NEB). The fragments were then ligated into the pcR8 Gateway entry vector (Invitrogen) and transformed into ccdB-resistant chemically competent DB3.1 cells which were prepared in lab as per Das and Dash (2014) using TSS buffer. Plasmids were extracted from overnight cultures using a QIAprep Spin Miniprep Kit (Qiagen), and the constructs confirmed through digestions and sequencing. Binary plant transformation Gateway destination vectors (Curtis and Grossniklaus 2003) were modified to contain the seed coat specific DIRIGENT PROTEIN1 (DP1) promoter (Esfandiari et al. 2013). proDP1 was chosen to express constructs in an effort to avoid causing severe overall plant phenotypes that would preclude making seeds, and all candidate genes are expressed in the seed coat, which suggests proDP1 is a biologically relevant promoter. LR reactions (Invitrogen) were used to move cloned genes into the destination vectors. Freeze-thaw transformation (Höfgen and Willmitzer 1988) was used to move binary vectors into Agrobacterium tumefaciens strain GV3101 competent cells. Agrobacterium-mediated floral dipping (Clough and Bent 1998) was then carried out to transform constructs into bolting Arabidopsis plants. Positive transformants were isolated by
germinating seeds on AT plates containing either hygromycin (15 mg/mL) or kanamycin (50 mg/mL). Fluorophore DNA sequences were amplified by extracting DNA and using PCR with fluorophore-specific primers to further confirm presence of the construct.

Seeds for proKINESIN-13A:GFP-KINESIN-13A were a gift from Dr. Yoshida Oda (National Institute of Genetics, Japan), and for proSEC8:GFP-SEC8 from Dr. John Fowler (Oregon State University, USA). Plasmids containing fusion sequences for ECFP-ROP1, mCherry-CA-rop1, and mCherry-DN-rop1 were a gift from Dr. Zhenbiao Yang (University of California, Riverside, USA). A plasmid containing EXO70A1-GFP was a gift from Dr. Liwen Jiang (The Chinese University of Hong Kong), and a plasmid containing EYFP-RIP3 was a gift from Dr. Antje Berken (Max Planck Institute for Brain Research, Germany).

2.1.8 Confocal Microscopy

Plants were confirmed to carry a copy of the fusion protein by growth on selective medium and by using PCR with primers that target the fluorophore sequence. To acquire proper seed staging, flowers at the initiation of pollination were marked as 0 DPA by painting their pedicels with diluted, nontoxic, water-based paint. When siliques reached the stage of interest, pots containing growing plants were moved to the confocal microscopy room for imaging. A silique was removed, placed on wet filter paper, and viewed under a dissecting microscope. Using fine forceps, the valves were peeled off to reveal seeds attached to the septum. A press-to-seal silicone spacer with adhesive sides (Invitrogen) was attached to a standard glass slide and 38 μL of molten 0.5% agarose was pipetted into the spacer. Once the agarose cooled, the septum with attached seeds was trimmed to size and placed on the agarose. Approximately 7.5 μL
of perfluoroperhydrophenanthrene (PP11, Sigma-Aldrich) was pipetted onto the agarose as a mounting medium. PP11 has been identified as an effective nontoxic, nonfluorescent medium for use with plant samples (Littlejohn et al. 2014). It has a much lower surface tension than water, which reduces air bubble accumulation between seeds and the septum, which can negatively impact imaging (personal observation). The top layer of plastic on the spacer was peeled off to expose the adhesive layer, and a #1.5 coverslip was pressed onto the spacer, creating a seal. The slide was then imaged using a Leica SP8 confocal microscope using LAS X software (Leica Microsystems) to capture z-stacks both in laser and DIC channels (Figure 11, Figure 12, Figure 13). Z-stacks were further analyzed by creating 3D projections using the image analysis software Dragonfly (Object Research Systems Inc., Montreal, Canada).

It is important to note that slides were prepared singly, immediately before imaging, as autofluorescence appeared to increase over time following sample preparation. Additionally, possibly due to the nature of imaging membrane-bound proteins and/or GTPases, it was noted that direct manipulation of seeds caused a decrease in fluorophore signal and, in many cases, a significant increase in autofluorescence. For this reason, seeds were left attached to the septum during slide preparation and imaging. Each fluorophore was imaged using the same magnification, excitation spectrum, and laser intensity to allow comparison between WT samples and fluorophore fusion lines.

2.1.9 Scanning Electron Microscopy

Mutations that perturb pectin secretion have been shown to cause morphological changes to MSCs (Kulich et al 2012). As a preliminary screen to detect obvious mutant
MSC phenotypes, mature dry seeds were imaged using a TM3030Plus tabletop SEM (Hitachi High-Technologies, Ontario, Canada) (Figure 9). Conductive double-sided adhesive tabs (Electron Microscopy Science, Pennsylvania, USA) were used to mount seeds to aluminum pin stubs. Seeds were not sputter coated prior to imaging. Images were captured using the backscatter electron detector under 15kV accelerating voltage.

2.1.10 Statistical Analysis

ANOVA tests were used to compare mucilage area size and total sugar concentrations between mutant lines and Col-0. The software SPSS (IBM Analytics) was used to analyze datasets using one-way ANOVA tests. The Student-Newman-Keuls post hoc test was used to identify significant difference between groups with a cutoff of p=0.05.
Chapter 3

Results
3.1 Results

3.1.1 Isolating Homozygous T-DNA Lines

To determine if the candidate genes **ROP1**, **RIP3**, **RIP5**, **KINESIN-13A**, **EXO70A1**, and **SEC8** are involved in pectin targeting and secretion in MSCs, T-DNA insertional lines were identified for each gene which could create knockdown or knockout mutations resulting in an observable phenotype. Putative homozygous T-DNA lines and segregating seed stocks were obtained from ABRC (Table 1, Figure 2). T-DNA lines with predicted insertions in exons were selected where possible in order to increase the likelihood of gene perturbation; however, some intron and UTR lines were selected when they were the only option. In addition, several lines were abandoned due to issues with PCR genotyping and when ABRC released new sequencing data suggesting certain lines had multiple insertions or insertions in different loci (Appendix 3). Even though **RIP1** and **RIP2** were also chosen as candidates, they were not investigated in this study, as suitable T-DNA lines could not be isolated.

Plants were grown and DNA was extracted from leaves for PCR genotyping using gene-specific primers and a T-DNA border primer to identify homozygous mutants (Table 2, Table 3). To confirm T-DNA insertion location within the locus, the amplicon resulting from the border primer and gene-specific primer was sequenced by Genome Quebec (Montreal, Quebec) and aligned to the respective gene using Benchling. The majority of alignments matched the predicted insertion sites, although Salk_124488C (**ROP1**) and CS850979 (**RIP3**) revealed insertions in the 3’ UTR instead of predicted exons. These two lines were retained for further analysis, as insertions in the 3’ UTR have been shown to yield mutant phenotypes, albeit at a low degree, by putatively
affecting mRNA stability and/or localization on polysomes (Mysore et al. 2000; Xiong et al. 2009; Xu and Chen 2013). Ten T-DNA lines were isolated to investigate the six candidate genes in this study (Table 1, Figure 2).

3.1.2 RT-PCR Analysis of T-DNA Lines

Following isolation of homozygous T-DNA mutants, qualitative RT-PCR was performed to test if WT transcripts were being expressed in respective mutants (Figure 3). Inflorescences were chosen for ease of RNA extraction as expression data from literature and online tools, such as the Arabidopsis eFP browser (Schmid et al. 2005), indicated that all candidate genes are expressed in inflorescences in addition to seed coats. Gene-specific primers were used to amplify across the point of T-DNA insertion for each mutant using WT cDNA as a control (Table 4, Table 5).

There were no WT transcripts observed when primers amplified across the T-DNA insertion points of all mutants except for exo70A1 Salk_022286, which had a 3’ UTR insertion. It was not possible to design a primer downstream of the insert site for this line. Therefore, primers were used to amplify in two areas upstream of the insert, which yielded bands comparable to those found when amplifying WT cDNA. This indicates that there may be only limited gene perturbation in this line. In the remaining lines, the lack of WT transcript indicates a potential knockout, although intron insertion lines have a higher potential of exhibiting knockdown mutations as the T-DNA insert may be spliced out, which could reconstitute a WT transcript (Wang 2008). Additionally, when primers targeted sequences upstream of the insert, every line displayed WT banding (typically at lower intensity, data not shown), which indicates a reduced rate of
transcription and a potential for the formation of a truncated or altered protein that could retain some degree of native function.

3.1.3 Mucilage Phenotyping with the Polysaccharide Stains Ruthenium Red and Calcofluor White

As mucilage is mainly composed of pectins, RR dye was used to screen for mutant phenotypes (McFarlane et al. 2014) by staining the pectic polysaccharides present in mucilage of each T-DNA line. All screened seeds were harvested and pooled from four plants per line that were grown at the same time under the same growth conditions. Three types of tests were performed using RR, including: (1) RR drop test (no agitation), which allows for observation of adherent and non-adherent mucilage; (2) RR shake test, for observing adherent mucilage (non-adherent mucilage removed by shaking); and (3) RR EDTA test, which is used to weaken the primary cell wall, allowing even small amounts of mucilage to extrude from MSCs for adherent mucilage observation. Three control lines were used with each test, including: (1) Columbia-0, displaying WT mucilage; (2) mum4-1, displaying very limited mucilage extrusion due to the disruption of a Rha synthase, resulting in highly reduced mucilage synthesis (Western et al. 2004; Oka et al. 2007); and (3) cesa5-2, displaying increased non-adherent mucilage and decreased adherent mucilage due to the disruption of a cellulose synthase subunit (Harpaz-Saad 2011, Mendu et al. 2011). In addition, calcofluor white stain, which binds to β-glycans, was used to visualize potential cellulose phenotypes (Willats et al. 2001).
3.1.3.1 Ruthenium Red Drop Test

After testing over 20 seeds per line (seeds pooled from four plants per line grown at the same time) by dropping them in RR without shaking, a qualitative visual analysis found that the shape, size, density, and colour of non-adherent mucilage showed variance within seeds of the same line, which made it difficult to screen for potentially minor changes in mucilage between lines (Figure 4). Due to the observed variability, only lines showing consistently drastic changes in mucilage compared to Col-0 were characterized as displaying mutant phenotypes.

Col-0 (WT) seeds displayed a thick layer of intensely-staining adherent mucilage directly around the seed and an even release of cloudy non-adherent mucilage surrounding the entire seed. *cesa5-2* (control line) exhibits reduced cellulose synthesis, resulting in a thinner layer of dark-stained adherent mucilage combined with increased non-adherent mucilage which appears denser and more diffuse than Col-0. *mum4-1* (control line) exhibits decreased pectin synthesis, resulting in impaired mucilage extrusion. The mutant lines for *rop1, rip3, rip5, kinesin13a,* and *exo70a1* all displayed a WT phenotype. A clear mutant phenotype was observed in *sec8 Salk_147751C*. This line displayed a range of phenotypes, varying from highly reduced adherent and non-adherent mucilage, to patchy extrusion and, in some cases, a complete lack of extrusion. When mucilage extrusion occurred, there was always extrusion present at the funicular end of the seed.

3.1.3.2 Ruthenium Red Shake Test

To analyze the adherent layer of mucilage, seeds (pooled from four plants per line grown at the same time) were shaken in RR to remove the non-adherent layer, and
then imaged using the same acquisition parameters to allow comparison (Figure 5). The images were processed with Fiji to quantify the area of adherent mucilage (Figure 6) and compared to Col-0 using a one-way ANOVA test (Table 6). Col-0 (WT) seeds extruded an even thickness of mucilage around the whole seed. The control line cesa5-2 exhibits decreased cellulose synthesis, resulting in a thinner adherent layer of mucilage compared to Col-0. The control line mum4-1 exhibits reduced pectin synthesis which impairs mucilage extrusion, resulting in a lack of mucilage phenotype. The mutant lines for rop1, rip3, rip5, and exo70a1 all displayed visual WT phenotypes (Figure 5). When quantified, the lines that showed a small but significant increase in mucilage compared to Col-0 include rip3 Salk_022078C and rip5 Salk_038458, whereas exo70a1 Salk_022286 showed a small but significant decrease in adherent mucilage (Figure 6, Table 6). Upon close inspection, kinesin-13a appeared to have thinner, slightly translucent adherent mucilage compared to Col-0 (Figure 5), which was further confirmed when the mucilage area was measured (Figure 6, Table 6), indicating a moderate reduction in mucilage. sec8 displayed a severe phenotype compared to Col-0. The majority of sec8 seeds did not release mucilage, or released a limited quantity in patchy areas typically at the funicular end of the seed (Figure 5). A small number of sec8 seeds displayed a total release of mucilage surrounding the seed; however, the mucilage was thinner and with a wavy edge compared to Col-0. Additionally, sec8 mucilage tended to stain darker than Col-0, with few seeds displaying a highly translucent, thin layer of mucilage surrounding the seed. Furthermore, quantification of the area of mucilage revealed a striking decrease in mucilage compared to Col-0 (Figure 6, Table 6).
In summary, at least one mutant allele of each candidate gene except *rop1* displayed a significant difference in adherent mucilage area compared to Col-0, albeit in varying degrees of severity. Small significant changes in mucilage area require careful interpretation due to variability in mucilage production from plant to plant related to sensitivity in growth conditions (e.g. location in growth chamber, distance between plants in pot, etc.). This could be tested by repeating the experiment with more biological replicates. *kinesin-13a* appears to display a moderate reduction in mucilage, and *sec8* displayed a drastic reduction in mucilage. The control mutants, *mum4-1* and *cesa5-2*, displayed significant mucilage reduction as predicted, which validates this method of quantification. Using FiJi to quantify adherent mucilage area appears to be a highly precise technique and potentially capable of identifying/confirming subtle phenotypes.

3.1.3.3 Ruthenium Red EDTA Test

Some mutants that synthesize reduced amounts of mucilage or chemically altered mucilage are incapable of extruding their mucilage upon hydration. A low abundance of hydrophilic pectins do not swell enough to rupture the primary cell (Western 2012), and alteration in polysaccharide composition can disrupt normal swelling (Arsovski et al. 2009a), inhibiting mucilage release. To facilitate mucilage extrusion, seeds were first hydrated in a solution of EDTA, which is a metal chelator that reduces pectin cross-linking, thus weakening the primary cell wall and/or increasing the ability of the mucilage to swell in response to hydration (Arsovski et al. 2009b)

Each mutant line was subjected to EDTA treatment prior to RR staining (Figure 7). Col-0 (WT) displayed an even extrusion of mucilage surrounding the seed. The
control line cesa5-2 displays a phenotype similar to Col-0, but with lightly stained mucilage due to a decrease in adherent mucilage caused by perturbed cellulose synthesis. The control line mum4-1 exhibits reduced pectin synthesis, and requires EDTA treatment to extrude a small layer of thin, lightly stained mucilage devoid of obvious cellulosic rays. The lines for rop1, rip3, rip5, kinesin-13a, and exo70a1 displayed a WT phenotype. sec8 displayed an entire release of mucilage surrounding each seed, in contrast to the patchy release or lack of release observed in the RR shake tests (Figure 5). None of the sec8 seeds displayed Col-0 levels of mucilage, and although some seeds showed thick and entire mucilage extrusion, the majority of seeds extruded very thin, sheet-like mucilage with few rays. This phenotype was similar to the mum4-1 phenotype, but the sec8 mucilage stained darker red, and the funicular end of the seed extruded thicker mucilage with rays, which was not observed in mum4-1. These results indicate a decreased amount of mucilage accumulation within the sec8 mucilage pockets, or altered mucilage structure causing a release defect, as EDTA pretreatment is required to release all mucilage from MSCs.

3.1.3.4 Calcofluor-White Staining

As mucilage contains small amounts of cellulose, and cellulosic rays are important in binding adherent mucilage to the seed (Griffiths et al. 2015), each T-DNA line was screened for cellulose phenotypes. This was carried out by hydrating and staining seeds with CFW, a fluorescent dye that binds to β-glycans that are present in cellulose (Willats et al. 2001) (Figure 8). Stained seeds were imaged using the same acquisition parameters under an epifluorescence microscope and visually compared to the Col-0 phenotype. It was difficult to obtain consistently high-resolution images of
individual rays with epifluorescence microscopy; therefore, a qualitative analysis was carried out based on MSC patterning, columella morphology, and general morphology of clustered rays along the edge of each seed. Future imaging could be improved by using confocal microscopy.

Col-0 (WT) columellae displayed a ring of fluorescence around their edges, surrounded by a void which coincides with the mucilage pocket. Rays lined the entire seed coat, displaying an intense signal which tapered off as the rays extended away from the seed. The control line cesa5-2 displayed shortened rays of low signal intensity due to a decrease in cellulose synthesis. The control line mum4-1 differed greatly from Col-0, as the whole MSC displayed fluorescence, and a thin, disordered low-intensity signal was observed in place of typical rays. The lines for rop1, rip3, rip5, kinesin-13a, and exo70a1 displayed WT phenotypes. sec8 seeds displayed patchy MSC fluorescence patterning and extrusion. Ruptured MSCs occurred in clusters across the seed coat, where the majority of ruptured MSCs were localized to the funicular end of the seed. MSCs in sec8 appeared to largely retain the WT hexagonal shape phenotype, and cellulosic rays were present in ruptured cells; however, a number of rays exhibited a bent shape, especially when localized to low-density regions of ruptured MSCs. Bent rays may be caused by reduced support from neighbouring rays, as single MSCs appeared to exhibit more bent rays than MSCs localized in more densely populated regions of the seed coat.

3.1.4 Scanning Electron Microscopy

As a preliminary screen for obvious changes in mature MSC morphology, the seed coats of mature dried seeds were imaged using an SEM and compared to the Col-
0 (WT) phenotype using a qualitative analysis (Figure 9). The Col-0 seed coat is patterned with mostly hexagonal-shaped MSCs. Each MSC is outlined by a radial cell wall and contains a central raised columella which is surrounded by a depressed ring-shaped mucilage pocket domain. The *cesa5-2* control line exhibits reduced cellulose synthesis, but MSCs appear to display WT morphology. The *mum4-1* control line displays severely flattened columellae due to highly reduced pectin synthesis and deposition in the mucilage pocket (Western et al. 2004). The mutant lines for *rop1*, *rip3*, *rip5*, and *exo70a1* appear to develop MSCs with WT morphology. *kinesin-13a* appears to develop many misshaped MSCs with elongated, flattened columellae, potentially due to decreased pectin deposition in the mucilage pocket. The majority of *sec8* seeds develop very few MSCs, which appear misshapen and randomly inserted across the seed coat. Upon close inspection, a thin web-like hexagonal-shaped pattern decorates the seed coat surface of *sec8* seeds (Fig 8, arrows). The shapes appear to match the radial cell wall outline of WT MSCs, but their origin is unclear. Together, these observations suggest possible involvement of KINESIN-13A in mucilage secretion, and a role for SEC8 in proper MSC development. Observed phenotypes should be confirmed with measurements and statistical analysis.

### 3.1.5 Total Sugar Analysis

To determine if mutant phenotypes were caused by an increase or decrease in total sugar concentration of the mucilage due to changes in polysaccharide synthesis, a total sugar analysis of mucilage was performed for each line across four replicates. Compared to Col-0, the control line *cesa5-2* displayed 37.8% adherent mucilage and 138.1% non-adherent mucilage, which is consistent with the *cesa5-2* mucilage
phenotype (Figure 10). The control line *mum4-1* displayed 59.1% adherent mucilage and 0% non-adherent mucilage compared to Col-0, which is consistent with the *mum4-1* mucilage phenotype. The lines for *rop1, rip3, rip5, kinesin-13a*, and *exo70a1* all displayed WT sugar concentrations for adherent and non-adherent mucilage. *sec8* displayed a significant reduction in total non-adherent sugar concentration (12.7%, p<0.05) whereas there was no significant change in adherent mucilage (91.5%, p>0.05); however, *sec8* showed a significant reduction in total mucilage (48.7%, p<0.05). It was surprising to find that *sec8* contains a WT concentration of adherent mucilage, as the mucilage phenotype appears to be drastically reduced, even when seeds are pretreated with EDTA. This may indicate a compositional change in *sec8* mucilage, or it might suggest that sonication weakens the primary cell wall, which is supported by a similar observation in *mum4-1* demonstrating only adherent mucilage in these sugar concentration assays.

3.1.6 Localization of ROP1, KINESIN-13A, and SEC8 in MSCs

Fluorophore-fusion lines were either created or obtained for all candidate genes as a means of observing protein localization during MSC development (Table 7). As there is an upregulation of pectin synthesis and secretion at 7 DPA, it was hypothesized that the candidate proteins would be expressed during this period and that they may be localized to the mucilage pocket to set up the donut-shaped secretion domain. *GFP-KINESIN-13A* and *GFP-SEC8* were expressed under their native promoters, and were obtained as seed stock from Dr. Yoshihisa Oda and Dr. John Fowler, respectively. The remaining lines were constructed by cloning from cDNA or by modifying pre-existing plasmids, which were then expressed under the *DP1* seed coat specific promoter.
(Esfandiari et al. 2013) (Table 8, Table 9). Additionally, plasmid templates for CA-rop1 and DN-rop1 mutants fused to mCherry were obtained from Dr. Zhenbiao Yang and modified for expression under the DP1 promoter. All constructs were transformed into Col-0 via Agrobacterium-mediated floral dipping, and selected by germination on selective growth media. Eighteen to 22 independent transformant lines were isolated, selfed, and harvested for each line. Five independent transformants each for proDP1:mCherry-CA-rop1 and proDP1:mCherry-DN-rop1 were analyzed; however, mCherry signal was not detected using confocal microscopy with seeds at 4 DPA, 7 DPA, and 10 DPA. More independent transformant lines should be analyzed to reach a conclusion. Only GFP-KINESIN-13A, GFP-SEC8, and ECFP-ROP1 were further analyzed for expression in MSCs at 7DPA using confocal microscopy with multiple plants per line.

Protein localization was described based on the published morphology of MSCs across different stages of development as described in numerous publications (e.g. Western 2006; McFarlane et al. 2008; North et al. 2014). Additionally, DIC images were taken in conjunction with z-slices to confirm the location of cell walls, cytoplasmic column, starch granules, and other clearly observed morphological structures. Z-stacks were analyzed using 3D projections to visualize an improved spatial resolution of fluorescence. Col-0 was imaged under the GFP and ECFP channels using the same acquisition parameters as a control to measure autofluorescence. Autofluorescence was weak in both channels, and there was a very low and diffuse signal in the cell wall under ECFP excitation; however, it was not nearly as intense or pronounced as
observed in fluorophore lines. A nonfluorescent void was clearly observed in all imaged samples, which aligned with the area of the developing mucilage pocket.

3.1.6.1 KINESIN-13A Localization in MSCs

Multiple plants from a non-segregating GFP-KINESIN-13A line were imaged and consistent signal localization was observed. GFP-KINESIN-13A displayed a clear signal in MSCs at 7 DPA (Figure 11). The signal appeared to be localized to the plasma membrane from the radial cell wall extending across the cytoplasmic column and ending at the primary cell wall at the apex of the MSC. Fluorescence at the plasma membrane showed higher intensity along the cytoplasmic column, with the signal becoming weaker between the base of the column and the radial cell wall. GFP intensity was relatively homogenous across the cytoplasmic column, and diffuse fluorescence was observed within the cytosol. There was no localization to the basal face of the cell below the cytoplasm (data not shown). McFarlane et al. (2008) observed a dense array of cortical microtubules that line the cytosolic face of the plasma membrane along the cytoplasmic column within the same domain where KINESIN-13A was localized; therefore, KINESIN-13A may be interacting with microtubules in this domain, as it known to be a microtubule-associated protein.

3.1.6.2 SEC8 Localization in MSCs

Multiple plants from a non-segregating GFP-SEC8 line were imaged and consistent signal localization was observed. GFP-SEC8 displayed a distinct increased signal compared to Col-0 (Figure 12). Intense GFP signal appeared to be localized to the plasma membrane along the cytoplasmic column. This fluorescence was relatively
homogenous, and the signal quickly tapered off at the base of the cytoplasmic column where the plasma membrane curled across the bottom of the mucilage pocket toward the radial cell wall. Low intensity, diffuse fluorescence was observed within the cytosol, and there was no localization to the basal face of the cell below the cytoplasm (data not shown). As the exocyst complex is involved in tethering and fusing vesicles for secretion at the plasma membrane (Žárský et al. 2013), these observations may indicate that SEC8 is involved in mucilage secretion along the interface of the mucilage pocket and cytoplasmic column.

3.1.6.3 ROP1 Localization in MSCs

Four independent ECFP-ROP1 insertion lines showed varying degrees of fluorescence with consistently similar signal localization; however, the lines ECFP-ROP1-3 and ECFP-ROP1-4 were chosen for further imaging because they exhibited the highest signal intensity. Similar to GFP-KINESIN-13A, ECFP-ROP1 signal appears to be localized to the plasma membrane, spanning from the radial cell wall to the primary cell wall at the apex of the cytoplasmic column (Figure 13). ECFP signal was homogenous across the plasma membrane, and lower intensity, diffuse fluorescence was observed throughout the cytosol. Unlike GFP-SEC8 and GFP-KINESIN-13A, ECFP-ROP1 displayed high intensity localization along the radial cell wall and at the cytoplasmic column apex, which are nonsecretory domains. While imaging focused on the region of the mucilage pocket, it appears that ECFP-ROP1 is also localized along the basal face of the cell below the cytoplasm (Figure 13 F, G). This region of the cell was not thoroughly imaged; therefore, further imaging is required to confirm the
localization at this domain. Together, these data suggest that *ROP1* may be involved in setting up the nonsecretory domains of the MSC.
Table 1. T-DNA insertional mutant lines.

<table>
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<th>Gene</th>
<th>AGI Code</th>
<th>T-DNA Line</th>
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<td>RIP3</td>
<td>At3g37080</td>
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<td>3' UTR</td>
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<td>Salk_022078C</td>
<td>Exon 3</td>
</tr>
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<td>At5g60210</td>
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<td>Intron 2</td>
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<td>Salk_104697</td>
<td>Exon 10</td>
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Note: RT-PCR analysis revealed absence of full length transcript in all lines except EXO70A1 Salk_022286 (see Figure 3).
Table 2. Genotyping primer pairings.

<table>
<thead>
<tr>
<th>Gene</th>
<th>T-DNA Line</th>
<th>Left Primer</th>
<th>Right Primer</th>
<th>Border Primer</th>
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<td>CS850979</td>
<td>salk102807 LP</td>
<td>RIP3-RT-2 RP</td>
<td>WiscDs-1 LB</td>
<td>745</td>
</tr>
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Note: All primers anneal at 58°C.
### Table 3. Genotyping primers used for T-DNA lines.

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<tr>
<th>Gene</th>
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<th>Primer Sequence (5' to 3')</th>
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<td>TACCGTGGACAGATGTTTTT</td>
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## Table 4. RT-PCR primer pairings.

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<td>GAPC-RT-1 LP</td>
<td>GAPC-RT-1 RP</td>
<td>N/A</td>
<td>245</td>
<td>334</td>
</tr>
</tbody>
</table>

Note: All primers anneal at 58°C. Amplification targets are in relation to the T-DNA insertion site.
### Table 5. RT-PCR primer sequences used with T-DNA lines.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Name</th>
<th>Primer Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROP1</td>
<td>salk075913 RP</td>
<td>TACCGTGGAGCAGATGTTTTC</td>
</tr>
<tr>
<td>ROP1</td>
<td>salk075913-3 LP</td>
<td>TCATAGAATGGGACATGCCCTTCTG</td>
</tr>
<tr>
<td>RIP3</td>
<td>RIP3-RT-6 RV</td>
<td>TTCATCAACAAATGTGGGCAACG</td>
</tr>
<tr>
<td>RIP3</td>
<td>RIP3-RT-7 FW</td>
<td>GACTCCAAAATCAAGGCCAGG</td>
</tr>
<tr>
<td>RIP5</td>
<td>S_124841-1 LP</td>
<td>CAGCGCTAGAGCCTGAATCT</td>
</tr>
<tr>
<td>RIP5</td>
<td>S_124841-1 RP</td>
<td>GCTGCGAGACTAGCAACTCT</td>
</tr>
<tr>
<td>RIP5</td>
<td>S_038458 &quot;RP&quot;</td>
<td>GCCTCTAGTTGCTCGGTGAC</td>
</tr>
<tr>
<td>EXO70A1</td>
<td>EXOA1-RT-1 LP</td>
<td>AGAGCTGTGTGGATGAGAGCA</td>
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<tr>
<td>EXO70A1</td>
<td>EXOA1-RT-1 RP</td>
<td>TCTGCGACGGAGGAGATC</td>
</tr>
<tr>
<td>EXO70A1</td>
<td>EXOA1-RT-2 LP</td>
<td>AGAGTTGCCCTGGGACAAAGATACT</td>
</tr>
<tr>
<td>EXO70A1</td>
<td>EXOA1-RT-2 RP</td>
<td>AGGCCCAAGCGTTGAGG</td>
</tr>
<tr>
<td>SEC8</td>
<td>SEC8-RT-1 FW</td>
<td>TGAAGAGCGCGGACGACTTTTG</td>
</tr>
<tr>
<td>SEC8</td>
<td>SEC8-RT-4 RV</td>
<td>AAGCGGGGATTGAGCATCTG</td>
</tr>
<tr>
<td>KINESIN-13A</td>
<td>S_104697 RP</td>
<td>ATCAAACTGCGAATTCAATGC</td>
</tr>
<tr>
<td>KINESIN-13A</td>
<td>KIN13A-RT-2 RP</td>
<td>TCCTTGAGACGGTGGAGG</td>
</tr>
<tr>
<td>GAPC (CTRL)</td>
<td>GAPC-RT-1 LP</td>
<td>TCAGACTCGAGAAAGCTGCTAC</td>
</tr>
<tr>
<td>GAPC (CTRL)</td>
<td>GAPC-RT-1 RP</td>
<td>GATCAAGTCGACCACACCGG</td>
</tr>
</tbody>
</table>
Table 6. Area of adherent mucilage (pixels) between T-DNA lines and Col-0 (WT). Asterisk denotes significant difference compared to WT following one-way ANOVA test, where the critical F value is 1.93 for a cutoff of p=0.05.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Mean Area ± SE</th>
<th>%WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-0</td>
<td>244</td>
<td>8361.5 ± 58.8</td>
<td>-</td>
</tr>
<tr>
<td>cesa5-2</td>
<td>277</td>
<td>3949.0 ± 46.8*</td>
<td>47.2</td>
</tr>
<tr>
<td>rop1 s075913C</td>
<td>192</td>
<td>8325.2 ± 61.8</td>
<td>99.6</td>
</tr>
<tr>
<td>rop1 s124488C</td>
<td>229</td>
<td>8170.6 ± 70.9</td>
<td>97.7</td>
</tr>
<tr>
<td>rip3 s022078C</td>
<td>255</td>
<td>8766.0 ± 51.4*</td>
<td>104.8</td>
</tr>
<tr>
<td>rip3 cs805979</td>
<td>281</td>
<td>8461.9 ± 58.0</td>
<td>101.2</td>
</tr>
<tr>
<td>rip5 s124841C</td>
<td>189</td>
<td>8846.9 ± 68.9</td>
<td>105.8</td>
</tr>
<tr>
<td>rip5 s038458</td>
<td>243</td>
<td>8458.3 ± 151.7*</td>
<td>101.2</td>
</tr>
<tr>
<td>kin13a s104697</td>
<td>256</td>
<td>7247.0 ± 59.3*</td>
<td>86.7</td>
</tr>
<tr>
<td>exo70a1 s022286</td>
<td>245</td>
<td>7980.8 ± 62.0*</td>
<td>95.4</td>
</tr>
<tr>
<td>sec8 s147751C</td>
<td>53</td>
<td>2163.2 ± 51.5*</td>
<td>25.9</td>
</tr>
</tbody>
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Table 7. Fluorophore fusion lines created or obtained.

<table>
<thead>
<tr>
<th>Fusion Line</th>
<th>Promoter</th>
<th>Vector</th>
<th>Ecotype</th>
<th>Note</th>
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</thead>
<tbody>
<tr>
<td>ECFP-ROP1</td>
<td>proDP1</td>
<td>pMDC100</td>
<td>Col-0</td>
<td>Plasmid template from Dr. Zhenbiao Yang</td>
</tr>
<tr>
<td>mCherry-CA-rop1</td>
<td>proDP1</td>
<td>pMDC100</td>
<td>Col-0</td>
<td>Plasmid template from Dr. Zhenbiao Yang</td>
</tr>
<tr>
<td>mCherry-DN-rop1</td>
<td>proDP1</td>
<td>pMDC100</td>
<td>Col-0</td>
<td>Plasmid template from Dr. Zhenbiao Yang</td>
</tr>
<tr>
<td>EYFP-RIP3</td>
<td>proDP1</td>
<td>pMDC100</td>
<td>Col-0</td>
<td>Plasmid template from Dr. Antje Berken</td>
</tr>
<tr>
<td>GFP6-RIP5</td>
<td>proDP1</td>
<td>pMDC43</td>
<td>Col-0</td>
<td>Cloned from cDNA template</td>
</tr>
<tr>
<td>GFP6-KATANIN1</td>
<td>proDP1</td>
<td>pMDC43</td>
<td>Col-0</td>
<td>Cloned from cDNA template</td>
</tr>
<tr>
<td>EXO70A1-GFP</td>
<td>proDP1</td>
<td>pMDC100</td>
<td>Col-0</td>
<td>Plasmid template from Dr. Liwen Jiang</td>
</tr>
<tr>
<td>GFP-SEC8</td>
<td>proSEC8</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Seeds from Dr. John Fowler</td>
</tr>
<tr>
<td>GFP-KINESIN-13A</td>
<td>proKIN-13A</td>
<td>pGWB501</td>
<td>Unknown</td>
<td>Seeds from Dr. Yoshihisa Oda</td>
</tr>
</tbody>
</table>
Table 8. Cloning primers used to create fluorophore fusion constructs.

<table>
<thead>
<tr>
<th>Fusion Line</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECFP-ROP1</td>
<td>RIP3-N-YFP-4 FW</td>
<td>ROP1-CADN-2 RV</td>
<td></td>
</tr>
<tr>
<td>mCherry-CA-rop1</td>
<td>ROP1-CADN-1 FW</td>
<td>ROP1-CADN-2 RV</td>
<td></td>
</tr>
<tr>
<td>mCherry-DN-rop1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EYFP-RIP3</td>
<td>RIP3-N-YFP-4 FW</td>
<td>RIP3-N-YFP-5 RV</td>
<td></td>
</tr>
<tr>
<td>GFP6-RIP5</td>
<td>RIP5-1 FW</td>
<td>RIP5-2 RV</td>
<td></td>
</tr>
<tr>
<td>GFP6-KATANIN1</td>
<td>KTN-1 FW</td>
<td>KTN-2 RV</td>
<td></td>
</tr>
<tr>
<td>EXO70A1-GFP</td>
<td>EXO-GFP-1 FW</td>
<td>EXO-GFP-6 RV</td>
<td></td>
</tr>
<tr>
<td>proDP1</td>
<td>DP1-5 FW</td>
<td>DP1-6 RV</td>
<td>5' <em>Pmel</em> and 3' <em>Ascl</em> to ligate into pMDC100</td>
</tr>
<tr>
<td>proDP1</td>
<td>DP1-5 FW</td>
<td>DP1-7 RV</td>
<td>5' <em>Pmel</em> and 3' <em>KpnI</em> to ligate into pMDC43</td>
</tr>
</tbody>
</table>
Table 9. Cloning primer sequences used to create fluorophore fusion constructs.

<table>
<thead>
<tr>
<th>Fusion Line</th>
<th>Primer Name</th>
<th>Primer Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECFP-ROP1</td>
<td>RIP3-N-YFP-4 FW</td>
<td>ATGGTGAGCAAGGGCGAGGAG</td>
</tr>
<tr>
<td>EYFP-RIP3</td>
<td>RIP3-N-YFP-5 RV</td>
<td>TTAATCAGAAGGAGCTGAGAAGAC</td>
</tr>
<tr>
<td>mCherry-CA-rop1</td>
<td>ROP1-CADN-1 FW</td>
<td>ATGGTGAGCAAGGGCGAGGAGGATAACATGG</td>
</tr>
<tr>
<td>mCherry-DN-rop1</td>
<td>ROP1-CADN-2 RV</td>
<td>TCATAGAATGGAGCATGCCTTCTGCAGGAGGAGGATAACATGG</td>
</tr>
<tr>
<td>ECFP-ROP1</td>
<td>ROP1-CADN-1 FW</td>
<td>ATGGTGAGCAAGGGCGAGGAGGATAACATGG</td>
</tr>
<tr>
<td>mCherry-CA-rop1</td>
<td>ROP1-CADN-2 RV</td>
<td>TCATAGAATGGAGCATGCCTTCTGCAGGAGGAGGATAACATGG</td>
</tr>
<tr>
<td>mCherry-DN-rop1</td>
<td>ROP1-CADN-2 RV</td>
<td>TCATAGAATGGAGCATGCCTTCTGCAGGAGGAGGATAACATGG</td>
</tr>
<tr>
<td>GFP6-RIP5</td>
<td>RIP5-1 FW</td>
<td>ATGCAGACCCAAAAAGGCAAGAAATGGATCTCC</td>
</tr>
<tr>
<td>GFP6-RIP5</td>
<td>RIP5-2 RV</td>
<td>CTATTTCTGAGGCTTCTCCACAAAAACTCC</td>
</tr>
<tr>
<td>EXO70A1-GFP</td>
<td>EXO-GFP-1 FW</td>
<td>ATGGCTGTGGATAGCAAGGATGCTGCGAGGAGGATAACATGG</td>
</tr>
<tr>
<td>ECO70A1-GFP</td>
<td>EXO-GFP-6 RV</td>
<td>TCACTTGATCGTCCACAAAAACTCC</td>
</tr>
<tr>
<td>GFP6-KATANIN1</td>
<td>KTN-1 FW</td>
<td>ATGGTGAGCAAGGGCGAGGAGGATAACATGG</td>
</tr>
<tr>
<td>GFP6-KATANIN1</td>
<td>KTN-2 RV</td>
<td>TTAAGCAGATCCCAAACTCAGAGAGC</td>
</tr>
<tr>
<td>proDP1 (5' Pmel)</td>
<td>DP1-5 FW</td>
<td>GCGGGAATTAAACGCTTCTGGGAAGCTC</td>
</tr>
<tr>
<td>proDP1 (3' Ascl)</td>
<td>DP1-6 RV</td>
<td>TGGATAGGGCGCCCTGTTAGAGTGTAGGTAG</td>
</tr>
<tr>
<td>proDP1 (3' KpnI)</td>
<td>DP1-7 RV</td>
<td>GCGGGAATTAAACGCTTCTGGGAAGCTC</td>
</tr>
</tbody>
</table>
Figure 2. Diagrams of genomic DNA sequences for six candidate genes. White boxes represent untranslated regions, black boxes represent exons, and lines between boxes represent introns. T-DNA insertion points are indicated with black triangles, and have been confirmed by sequencing. Bar at bottom right corner of each diagram indicates length of 100 base pairs. Gene diagrams created using Exon-Intron Graphic Maker (www.wormweb.org/exonintron).
Figure 3. RT-PCR results of amplification across the T-DNA insertion point in homozygous mutant lines. Col-0 (wild type) is the ecotype of each T-DNA line. The respective T-DNA mutants of *ROP1*, *RIP5*, *KINESIN-13A*, *RIP3*, and *SEC8* did not show amplification, which suggests full length transcripts are not present in these lines. The *EXO70A1* mutant salk022286 contains an insertion in the 3’ UTR, which prevents the use of a reverse primer downstream of the insertion site. Therefore, two areas of the gene upstream of the insertion point (primer pair (1) and primer pair (2)) were amplified that revealed wild type banding, indicating transcription rate is not perturbed. Black line above lanes indicates the same primer pair used to amplify cDNA in each line. The housekeeping gene *GAPC* was amplified as a loading control.
Figure 4. Seeds dropped in ruthenium red stain without agitation to observe adherent and non-adherent mucilage. Col-0 (WT) displays radiating non-adherent mucilage surrounding the seed. cesa5-2 (control line) exhibits reduced cellulose synthesis, resulting in a thinner layer of adherent mucilage combined with increased non-adherent mucilage which appears denser and more diffuse than Col-0. mum4-1 (control line) is a mutant exhibiting decreased pectin synthesis resulting in impaired mucilage extrusion. The lines for rop1, rip3, rip5, kinesin-13a, and exo70a1 appear to display a WT phenotype. sec8 displays patchy extrusion of a highly reduced amount of adherent and non-adherent mucilage, although a small percentage of seeds exhibit either a patchy extrusion of mucilage across the entire seed coat, or no release at all. Seeds imaged at 40x magnification.
Figure 5. Seeds shaken in ruthenium red stain to observe adherent mucilage. Col-0 (WT) displays an even extrusion of mucilage surrounding the seed. cesa5-2 (control line) is a mutant exhibiting decreased cellulose synthesis, resulting in reduced adherent mucilage. mum4-1 (control line) is a mutant exhibiting decreased pectin synthesis resulting in impaired mucilage extrusion. All lines appear to display WT mucilage phenotypes except for kinesin-13a and sec8. kinesin-13a adherent mucilage consistently appears to be thinner and more lightly stained/translucent compared to Col-0. Most sec8 seeds display a lack of stained adherent mucilage, or patchy extruded mucilage; however, some seeds display adherent mucilage surrounding the whole seed, appearing as having a rough edge compared to the smooth edge of Col-0 adherent mucilage. sec8 mucilage typically stains very dark red, but a thin layer of clear mucilage is observed in some seeds. Seeds imaged at 40x magnification.
Figure 6. Quantification of the area of adherent mucilage following ruthenium red staining using Fiji software. cesa5-2 (control line) is a mutant exhibiting decreased cellulose synthesis, resulting in decreased adherent mucilage. mum4-1 (control line) is a mutant exhibiting decreased pectin synthesis resulting in impaired mucilage extrusion and decreased total sugar accumulation in MSCs. rip3 salk022078C and rip5 salk038458 show a slight but significant increase in adherent mucilage compared to Col-0. kinesin-13a shows a moderate reduction in adherent mucilage, whereas sec8 shows a drastic reduction in adherent mucilage compared to Col-0, which indicates their potential role in the mucilage secretory pathway. The remaining lines display a limited variance in mucilage area compared to Col-0. A significant difference is observed (p<0.05) between groups with different letters.
Figure 7. Seeds treated with the metal chelator EDTA to facilitate total mucilage extrusion from MSCs followed by staining with ruthenium red to observe adherent mucilage. Col-0 (WT) extrudes an even layer of mucilage surrounding the entire seed. *cesa5-2* (control line) is a mutant exhibiting decreased cellulose synthesis, resulting in reduced adherent mucilage with a lighter stain compared to Col-0. *mum4-1* (control line) is a mutant exhibiting decreased pectin synthesis, which requires chelator treatment to facilitate extrusion of the small amount of mucilage. The mutant lines for *rop1*, *rip3*, *rip5*, *kinesin-13a*, and *exo70a1* appear to display a WT phenotype. All *sec8* seeds display adherent mucilage extrusion, indicating the need for cell wall weakening to facilitate release. Most *sec8* seeds display a thin layer of lightly-stained adherent mucilage, lacking obvious rays; however, a small number of seeds exhibit a darker-staining, thicker adherent mucilage which fully envelopes the seed. Seeds imaged at 40x magnification.
Figure 8. Calcofluor white staining to observe cellulose in mucilage and seed coat under UV excitation with an epifluorescence microscope. Col-0 (WT) displays MSCs with fluorescent columellae and bright cellulosic rays. cesa5-2 (control line) is a mutant exhibiting decreased cellulose synthesis resulting in short rays. mum4-1 (control line) is a mutant exhibiting decreased pectin synthesis resulting in flattened MSCs that are evenly fluorescent, and thin wispy fluorescence in place of rays. The mutant lines for rop1, rip3, rip5, kinesin-13a, and exo70a1 display ray and MSC phenotypes resembling Col-0, where signal intensity, ray density, and MSC morphology showed variation between seeds of the same line (including in Col-0), but obvious and consistent mutant phenotypes were not observed. sec8 exhibits patchy MSC patterning, as well as a drastic reduction mucilage extrusion and shortened, bent cellulosic rays. The rays present in sec8 appear to contain less cellulose due to lower fluorescence compared to Col-0. Seeds imaged at 400x magnification.
Figure 9. Seed coat MSCs of mature dry seeds imaged using a scanning electron microscope. Col-0 (WT) displays many hexagonal-shaped MSCs, each outlined by a radial cell wall and containing a central raised columella surrounded by a depressed ring-shaped mucilage pocket domain. The control line cesa5-2 exhibits reduced cellulose synthesis but appears to display WT MSC morphology. The mum4-1 control line displays severely flattened columellae due to highly reduced pectin synthesis and secretion to the mucilage pocket. Using a qualitative visual analysis for obvious mutant phenotypes compared to Col-0, the mutant lines for rop1, rip3, rip5, and exo70a1 appear to develop MSCs with WT morphology. kinesin-13a appears to develop many misshaped MSCs with elongated, flattened columellae. The majority of sec8 seeds are largely devoid of MSCs, containing few misshapen and randomly inserted MSCs across the seed coat. Although difficult to image, the sec8 seed coat is patterned by a thin “web” of hexagonal shapes (arrows). The shapes appear to match the radial cell wall outline of WT MSCs, but their origin is unclear. Bar is equal to 40 μm.
Figure 10. Total sugar concentration of adherent, non-adherent, and total mucilage, measured using a phenol-sulfuric acid colorimetric assay. Col-0 (WT) contains a slightly higher concentration of sugar in non-adherent mucilage compared to adherent mucilage (A, B). *cesa5-2* (control line) is a mutant exhibiting impaired cellulose synthesis which causes increased non-adherent mucilage (B) and decreased adherent mucilage (A). *mum4-1* (control line) is a mutant exhibiting decreased pectin synthesis resulting in impaired mucilage extrusion and decreased total sugar accumulation in MSCs (C). The lines for *rop1*, *rip3*, *rip5*, *kinesin-13a*, and *exo70a1* contain WT sugar concentrations in adherent (A), nonadherent (B), and total mucilage (C). When compared to Col-0, *sec8* displays significantly less non-adherent (B) and total mucilage (C); however, *sec8* does not display a significant difference in adherent mucilage (A). A significant difference is observed (p<0.05) between groups with different letters.
Figure 11. Confocal imaging of proKINESIN-13A:GFP-KINESIN-13A in MSCs at 7 DPA under GFP excitation. (A) Maximum intensity projection of Col-0 (wild type) autofluorescence indicates low signal in the cytoplasmic column and cell wall. (B) Maximum intensity projection of GFP-KINESIN-13A indicates areas of localization and higher intensity compared to Col-0. The PET look up table was used to visualize fluorescence based on signal intensity (0=low, 255=high) in C, D, and E. Orthogonal projection (C) and z-slice (D) through middle of MSC shows KINESIN-13A localization to the plasma membrane along the mucilage pocket, with highest intensity present along the cytoplasmic column, and diffuse fluorescence throughout cytosol. Note the absence of signal at the radial cell wall (D) and cytoplasmic column apex (E), where ECFP-ROP1 is highly localized (Figure 12). Circular voids within the columnella (D) represent the location of starch granules. (E) 3D projection of z-stack shows spatial distribution of KINESIN-13A and consistent patterning across multiple MSCs.
Figure 12. Confocal imaging of proSEC8:GFP-SEC8 in MSCs at 7 DPA under GFP excitation. (A) Maximum intensity projection of Col-0 (wild type) autofluorescence indicates low signal in the cytoplasmic column and cell wall. (B) Maximum intensity projection of GFP-SEC8 indicates areas of localization and higher intensity compared to Col-0. The PET look up table was used to visualize fluorescence based on signal intensity (0=low, 255=high) in C, D, and E. Orthogonal projection (C) and z-slice (D) through middle of MSC shows SEC8 localization to the plasma membrane but restricted to the cytoplasmic column, and low-intensity diffuse fluorescence throughout cytosol. (E) 3D projection of z-stack shows spatial distribution of SEC8 and consistent patterning across multiple MSCs.
Figure 13. Confocal imaging of proDP1:ECFP-ROP1 in MSCs at 7 DPA under CFP excitation. (A) Maximum intensity projection of Col-0 (WT) autofluorescence indicates low signal in the cytoplasmic column and cell wall. (B) Maximum intensity projection of ECFP-ROP1 indicates areas of localization and higher intensity compared to Col-0. The PET look up table was used to visualize fluorescence based on signal intensity (0=low, 255=high) in C, D, E, F, and G. Orthogonal projection (C) and z-slice (D) through middle of MSC shows ROP1 localization to the plasma membrane along the mucilage pocket, with highest intensity at the radial cell wall and cytoplasmic column apex, and diffuse fluorescence throughout cytosol. (E) 3D projection of z-stack shows spatial distribution of ROP1 and consistent patterning across multiple MSCs. Z-slice near basal face of cell (F) and associated 3D projection (G) suggests ROP1 localization along cell base.
Chapter 4

Discussion and Future Experiments
4.1 Discussion

4.1.1 ROP1 Appears to Localize to Nonsecretory Domains in MSCs

ROP proteins have been well characterized for roles in modifying the cytoskeleton and both establishing and inhibiting domains of polarized growth and secretion at the plasma membrane (Berken 2006; Burkart et al. 2015; Nagawa et al. 2010; Nibau et al. 2006). To investigate whether ROP1 is involved in pectin targeting and secretion in MSCs, proDP1:ECFP-ROP1 was constructed and transformed into wild type Arabidopsis Col-0. MSCs were imaged at 7 DPA, which coincides with a period upregulated pectin synthesis and targeted secretion to the mucilage pocket.

ECFP-ROP1 appeared to localize to the plasma membrane, spanning from the radial cell walls and along the mucilage pocket to the cytoplasmic column apex (Figure 12). ECFP signal was significantly higher at the radial cell wall and high at the cytoplasmic column apex compared to the plasma membrane domain adjacent to the mucilage pocket. Additionally, preliminary data suggests that ECFP-ROP1 appears to localize below the cytosol, at the basal face of the cell (Figure 13 F, G). Additional imaging needs to be carried out to confirm localization at the basal domain. This localization pattern is opposite of where the mucilage pocket secretory domain lies, suggesting that ROP1 may be involved in establishing the nonsecretory domain, spanning the entire base of the cell and wrapping up along the radial cell wall as well as the columella apex. This type of activity is not surprising, as ROPs have been identified in establishing nonsecretory domains in xylem cells (Oda and Fukuda 2013b) and pavement cells (Fu et al. 2009); therefore, it is possible that ROP1 may be acting in a similar manner in MSCs.
To further investigate the role of ROP1 in MSC, the T-DNA lines rop1 Salk_075913C and Salk_124488C were analyzed to screen for phenotypes. Qualitative analysis of mucilage stained with RR revealed WT phenotypes in drop tests (Figure 4) as well as when the seeds were pretreated with EDTA (Figure 7). WT phenotypes were also observed in mucilage stained with CFW (Figure 8). Quantification of the area of adherent mucilage stained with RR indicated that both rop1 lines display WT phenotypes (Figure 6, Table 6). The total sugar concentration of adherent and non-adherent mucilage appeared WT in both lines (Figure 10), and SEM analysis did not indicate drastic changes in MSC morphology or seed coat patterning (Figure 9).

Additionally, under the DP1 promoter, independent transformant lines for CA-rop1 and DN-rop1 did not appear to cause a mucilage phenotype (data not shown).

Overall, ROP1 appeared to localize to nonsecretory domains, and both rop1 mutant lines lacked mutant phenotypes. The lack of mutant phenotype may not be surprising, as ROP1 localization does not suggest involvement in pectin targeting and secretion at the mucilage pocket, therefore perturbing ROP1 function may leave pectin secretory functions fully functional. It should be noted that the T-DNA insertion in Salk_075913C is located in an intron, and the insertion in Salk_124488C is located in the 3' UTR (Figure 2). These are not optimal locations, as insertions in introns can be spliced out, and insertions in the 3'UTR have a highly reduced potential to affect the translated protein, as it does not interrupt the coding sequence (Wang 2008).

Additionally, ROP1, ROP3, and ROP5 are typically treated as functionally redundant due to their high sequence homology (Li et al. 1999; Hwang and Yang 2006). Although microarray data indicates that only ROP1 is specifically and significantly upregulated at
7 DPA, *ROP3* and *ROP5* show high expression in the seed coat throughout MSC differentiation (*Arabidopsis* eFP Browser). Knocking out multiple ROP proteins may aid in observing a mucilage phenotype, if a phenotype can arise from perturbing ROP1 function.

To further investigate ROP1 activity during MSC differentiation, ECFP-ROP1 localization should be observed before and after 7 DPA, to characterize the change in localization from the onset of MSC differentiation through to maturation. Although it is likely that ECFP-ROP1 is localized to the plasma membrane, this should be confirmed by crossing *ECFP-ROP1* to a line with a membrane-bound fluorophore marker, or with a membrane stain, such as FM4-64 (Griffiths et al. 2016).

4.1.2 KINESIN-13A Localizes to Mucilage Pocket Secretory Domain

KINESIN-13A is recognized as a downstream target of ROP signalling. In xylem cells, KINESIN-13A binds directly to RIP3 and microtubules to establish nonsecretory domains (Oda and Fukuda 2013b). Lu et al. (2015) observed KINESIN-13A localizing with the Golgi apparatus, and *kinesin-13a* caused Golgi mislocalization and increased cell outgrowth and branching in trichomes, indicating a role in cytoskeletal organization and Golgi trafficking. To observe the localization of KINESIN-13A in differentiating MSCs, seeds of the stable transformant *proKINESIN-13A:GFP-KINESIN-13A* were obtained for analysis. Confocal microscopy was used to visualize GFP-KINESIN-13A expression in MSCs at 7 DPA, during the period of pectin upregulation and secretion (Figure 11).
GFP-KINESIN-13A appeared to localize to the plasma membrane, and displayed low intensity diffuse fluorescence in the cytoplasm. At the plasma membrane, KINESIN-13A displayed the highest signal along the cytoplasmic column adjacent to the mucilage pocket, where pectin vesicles are targeted for secretion. This domain is known to contain a dense array of cortical microtubules (McFarlane et al. 2008) which cellulose synthase complexes move along to deposit cellulose into the mucilage pocket (Griffiths et al. 2015). GFP-KINESIN-13A localization indicates it may be involved in cortical microtubule organization in this domain, or potentially involved in trafficking cellulose synthase complexes along the microtubules.

KINESIN-13A and ROP1 appeared to localize to opposite plasma membrane domains in MSCs. KINESIN-13A is absent at the radial cell wall (Figure 11D), whereas ROP1 shows strong localization to the same domain (Figure 13D). The same lack of overlapping localization is observed at the cytoplasmic column apex where ROP1 is highly localized (Figure 13C, 12E) and KINESIN-13A is not (Figure 11C, 10E). ROP1 shows weak localization along the cytoplasmic column (13D) compared to the high localization of KINESIN-13A (11D). These observations suggest that each protein is interacting in a different pathway, where KINESIN-13A may influence the establishment of the secretory domain, and ROP1 appears to establish the nonsecretory domain. Similar antagonistic ROP pathways have been observed in Arabidopsis pavement cells, where ROP2 establishes secretory domains, and ROP6 establishes nonsecretory domains (Fu et al. 2005). As KINESIN-13A is a known target in ROP signalling, another ROP protein may be responsible for establishing the plasma membrane secretory domain at the interface between the cytoplasmic column and mucilage pocket.
To further analyze the role of *KINESIN-13A* in MSC development, the T-DNA mutant *kinesin-13a1 Salk_104697* was isolated for analysis. This mutant line has been published and verified as a knockout mutant by Oda and Fukuda (2013b). RT-PCR confirmed lack of WT transcript when amplified across the T-DNA insertion point (Figure 3). This mutant line is further confirmed by observing the published phenotype of increased trichome branching (Oda and Fukuda 2013b).

It was found that *kinesin-13a1 Salk_104697* displayed somewhat reduced adherent mucilage. Through imaging adherent mucilage, visual analysis demonstrated thinner, more translucent mucilage compared to Col-0 (Figure 5). Adherent mucilage measured using Fiji analysis revealed a moderate but significant reduction of mucilage area, equaling 86.7% WT (Figure 6, Table 6). Total sugar concentrations of adherent and nonadherent mucilage measured WT levels; therefore, additional tests should be performed to confirm mucilage phenotypes and investigate mucilage composition.

Cellulose was imaged in hydrated *kinesin-13a* seeds using CFW; however, this analysis proved inconclusive due to limited resolution using an epifluorescence microscope (Figure 8). Repeating CFW imaging using a confocal microscope may prove beneficial in imaging individual rays to compare their length with WT rays. Finally, the suggestion that *kinesin-13a* causes MSC development perturbation is supported by preliminary SEM imaging (Figure 9). The *kinesin-13a* seed coats appear to contain an abundance of misshapen MSCs with elongated and flattened columellae, indicative of reduced mucilage secretion. Measurements and statistical analysis should be carried out to confirm these observations.
4.1.3 SEC8 Localizes to Mucilage Pocket Secretory Domain

Kulich et al. (2010) revealed that the SEC8 exocyst subunit is involved in pectin secretion in MSCs. The authors noted drastic reductions in pectin secretion in sec8 mutants, resulting in impaired extrusion and the release of a limited amount of mucilage after acid-alkali treatment. To further characterize the role of SEC8 in pectin secretion, seeds of the stable transformant proSEC8:GFP-SEC8 were obtained for analysis. Confocal microscopy was used to visualize GFP-SEC8 expression in MSCs at 7 DPA, during the period of pectin upregulation and secretion (Figure 12).

GFP-SEC8 appeared to localize to the plasma membrane along the cytoplasmic column. Unlike ECFP-ROP1 and GFP-KINESIN13A, which showed distinct domains of increased localization along the plasma membrane, GFP-SEC8 appears to be restricted to the interface along the mucilage pocket and cytoplasmic column. This localization pattern correlates with the secretory domain where pectin-laden vesicles are targeted for secretion to the mucilage pocket. Because SEC8 (and the whole exocyst) is involved in targeting and tethering secretory vesicles for exocytosis, this localization supports the involvement of SEC8 in pectin targeting in MSCs. Exocyst subunits are known downstream interactors in ROP signalling pathways (Lavy et al. 2007); therefore, ROP activity may be involved in targeting the exocyst to the observed secretory domain.

To further analyze the role of SEC8 in pectin targeting and secretion, the T-DNA mutant Salk_147751C was isolated for analysis. Qualitative analysis revealed a mutant phenotype using RR staining tests. RR drop tests indicated varying degrees of reduced adherent and non-adherent mucilage (Figure 4). A range of extrusion phenotypes was displayed, from entire extrusion, to patchy extrusion or lack of mucilage; however,
mucilage quantity appeared consistently reduced compared to WT. EDTA treatment followed by RR staining indicated that there is a small amount of mucilage in MSCs across the seed coat, as all seeds showed at least a thin layer of mucilage surrounding the seed, with rays localized to the funicular end (Figure 7). CFW staining revealed that most seeds exhibit a seed coat with patchy groupings of fully-developed MSCs in the middle of the seed and funicular end that extrude mucilage containing rays (Figure 8). A drastic reduction in adherent mucilage was observed when seeds were shaken in RR (Figure 5), equaling 25.9% WT (Figure 6, Table 6). The reduced mucilage phenotype was supported by analysis of total sugar concentration, revealing a significant reduction in total (48.7% WT) and non-adherent (12.7% WT) sugar, but it was surprising to find no significant difference in adherent mucilage sugar concentration (91.5% WT), as RR staining clearly shows a drastic reduction. This may be explained by primary cell wall weakening facilitated by the sonication treatment, causing mucilage release similarly observed with EDTA treatment (Figure 7).

Finally, preliminary SEM imaging of mature seeds revealed a drastic change in MSC patterning (Figure 9). Most seed coats exhibited few randomly inserted, misshapen MSCs; however, their morphology was distinctly different from the mum4-1 phenotype, which arises from reduced pectin synthesis. Unlike mum4-1 MSCs that display severely flattened columellae and lack visible mucilage pockets, sec8 MSCs contain distinct flattened columellae and mucilage pockets. Upon close inspection, a web-like hexagonal-shaped pattern decorated the surface of seed coats. The pattern appeared to match the outline of WT radial cell walls, but their origin is unclear. EDTA treatment with RR staining suggests that all MSCs across the seed coat contain a small
amount of mucilage, as there is an even extrusion of thin mucilage surrounding treated seeds.

Together, these analyses, along with published phenotypes, implicate the involvement of SEC8 in pectin secretion. Sectioning and staining should be carried out at different developmental stages to track MSC differentiation in sec8 seed coats. Do pectin-laden vesicles localize to the secretory domain but fail to fuse at the plasma membrane? Do they accumulate within the central lytic vacuole and endoplasmic reticulum bodies, similar to echidna mutants (McFarlane et al. 2013)? It would be interesting to observe vesicle trafficking using fluorophore tags or transmission electron microscopy to characterize their activity in sec8 MSCs.

EXO70A1 was another exocyst subunit investigated by analyzing the T-DNA mutant Salk_022286. Qualitative analysis using RR staining tests, CFW staining, and SEM imaging revealed WT phenotypes. Total sugar present in adherent and non-adherent mucilage measured WT levels (Figure 10). Quantified mucilage area revealed a slight, but significant reduction compared to Col-0 (Figure 6, Table 6); however, at 95.4% WT, the result was discounted due to the potential for small variation in mucilage production between plants. These results are not surprising, as sequencing revealed a suboptimal T-DNA insertion in the 3’ UTR (Figure 2), and RT-PCR revealed WT expression (Figure 3), which suggests Salk_022286 does not likely perturb gene expression and/or function. Kulich et al. (2010) observed drastic changes in mucilage production and MSC morphology in a mutant exo70a1 allele. Because the published phenotype of strong exo70a1 alleles implicates the exocyst in pectin secretion during MSC differentiation, additional T-DNA mutants should be isolated to continue
characterizing the role of EXO70A1 in this pathway. Additionally, proDP1:GFP-EXO70A1 was constructed and transformed into Col-0 and 22 independent transformant lines were harvested for future analysis.

4.1.4 Phenotypic Analysis of RIP3 and RIP5 Mutant Alleles

Members of the RIP family of ROP effectors are known to bind directly to active ROPs, acting as scaffolds for binding to downstream proteins (Lavy et al. 2007). Mutant phenotypes have been observed by manipulating RIP expression. For example, RIP1 overexpression causes a loss of polarized growth in pollen tubes (Li et al. 2008), and RIP3 knockdown inhibits downstream KINESIN-13A from binding to microtubules (Oda and Fukuda 2013b). Therefore, as an indirect method of characterizing potential ROP activity in MSCs, two T-DNA lines each for RIP3 and RIP5 were analyzed.

Qualitative analysis of mucilage was assessed using RR staining and CFW staining. CFW staining revealed WT phenotypes, as did RR drop tests and preliminary SEM imaging. WT phenotypes were also observed when seeds were treated with EDTA prior to RR staining. Quantitative analysis of adherent mucilage area indicated a small but significant increase in adherent mucilage (compared to WT) in the lines rip3 Salk_022078C (104.8% WT) and rip5 Salk_038458 (105.8% WT), whereas rip3 CS850979 and rip5 Salk_124841C did not show a significant difference (Figure 6, Table 6). As noted in previous sections, this may be due to natural variation in mucilage production between plants and, thus, not biologically relevant. Total sugar concentrations of adherent and non-adherent mucilage in each line were not significantly different than WT.
No WT transcripts were found when using RT-PCR to amplify across T-DNA insertion location (Figure 3); however, T-DNA insert location varied among lines (Figure 2). *rip3* Salk_022078C and *rip5* Salk_038458 contain insertions in exons, whereas *rip3* CS850979 and *rip5* Salk_124841C contain insertions in the 3'UTR and second intron, respectively. As explained with the *rop1* and *exo70a1* mutant alleles, insertions in these areas of the gene are not ideal and may not always cause gene perturbations. Additionally, Mucha et al. (2010) found that *RIP2* and *RIP3* share high sequence similarity, and both are capable of binding to KINESIN-13A in yeast two-hybrid assays, which suggests potential redundancy and masking of *rip3* mutant phenotypes. To rule out this possibility, all *rip3* and *rip5* mutant allele lines were crossed to create double mutants for future analysis.

Based off phenotypic analysis of these T-DNA mutants, there is no conclusive evidence which indicates *RIP3* or *RIP5* are involved pectin targeting and secretion when a single gene is perturbed. To further investigate *RIP3* and *RIP5* activity, fluorophore fusion lines were created and transformed into Col-0 (Table 7). Over 20 independent transformants each for *proDP1:EYFP-RIP3* and *proDP1:GFP6-RIP5* were selected and harvested for future investigation. Confocal microscopy analysis of differentiating MSCs may reveal whether RIP3 and/or RIP5 localization is indicative of involvement in pectin targeting and secretion.

4.2 Future Directions

The results of this study provide the framework for future research with this project. Additional mutant alleles should be obtained or created for each candidate
gene, as many of the mutant alleles investigated appeared to be weak mutants due to suboptimal T-DNA insertion points.

Fluorophore fusion lines were crossed with each other to observe dual localization within the same MSC. Additionally, each fluorophore fusion line was crossed to the microtubule marker lines mCherry-MAP4 and/or GFP-TUA6, to characterize potential microtubule colocalization. All crossed lines were grown and harvested for future analysis.

It will be important to visualize vesicle localization and cell ultrastructure to investigate whether mutant genes affect trafficking, targeting, or ultrastructure organization. Transmission electron microscopy would work well for this type of analysis, and would be specifically interesting to see if cortical microtubule arrangement if affected by kinesin-13a, which is known to bind to microtubules.

As ROP1, KINESIN-13A, and SEC8 appear to localize to distinct plasma membrane domains in MSCs, it will be important to confirm membrane localization by using dyes or by crossing lines with appropriate membrane marker lines. Additionally, in order to further understand the molecular pathways involved in the activity of each candidate, coimmunoprecipitation assays could be carried out. This would be greatly beneficial in identifying interacting proteins, to further elucidate each pathway. The use of anti-fluorophore columns would ease this process by reducing protocol time and enhancing antibody specificity to the tagged gene of interest.

Finally, all mutant lines of interest should be subjected to molecular complementation with WT gene copies. This should rescue mutant phenotypes to confirm the gene of interest is indeed responsible for perturbations.
Chapter 5
References
5.1 References


Arsovski AA, Villota MM, Rowland O, Subramaniam R, & Western TL. 2009b. MUM ENHANCERS are important for seed coat mucilage production and mucilage secretory cell differentiation in Arabidopsis thaliana. Journal of Experimental Botany 60(9):2601-2612.


Haughn G, & Western T. 2012. *Arabidopsis* seed coat mucilage is a specialized cell wall that can be used as a model for genetic analysis of plant cell wall structure and function. *Frontiers in Plant Science* 3(64):1-5.


Appendix 1: Image Acquisition Settings for Ruthenium Red Stained Seeds to be Measured with FiJi.

The following parameters were used to capture images for RR stained seeds using a stereomicroscope (Leica MZ16F) running Nikon Elements software: Red=1.3, Green=1.0, Blue=2.2, Magnification=1.25x, Exposure=3.8ms. Bottom illumination was used at maximum light intensity.

Appendix 2: Macro Code Used for FiJi Measurement of Mucilage Area.

The following macro code was used with FiJi to measure the area of adherent mucilage for RR stained seeds:

```java
run("8-bit");
setAutoThreshold("Default");
run("Threshold...");
setThreshold(30, 90);
setOption("BlackBackground", false);
run("Convert to Mask");
run("Set Measurements...", "area perimeter shape display redirect=None decimal=3");
run("Analyze Particles...", "size=1730-18000 circularity=0.09-1.00 show=[Overlay Masks] display exclude summarize");
```
Appendix 3: List of Abandoned T-DNA Lines.

Table A1. T-DNA mutant lines abandoned in this study due to issues with genotyping or insert location.

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<tr>
<th>Gene</th>
<th>AGI</th>
<th>T-DNA Line</th>
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<td>Salk_038460</td>
</tr>
<tr>
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<td>CS815291</td>
</tr>
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<td>At2g37080</td>
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