Trafficking Dynamics in *Arabidopsis* Pollen-Stigma Interactions

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Reproduction in the Brassicaceae family depends on self-recognition, where cross-compatible pollen is distinguished from self-incompatible pollen prior to acceptance by the stigma. The stigma only gives compatible pollen the resources required for hydration and germination of a pollen tube for fertilization. These resources are sequestered away from self-incompatible pollen, inhibiting inbreeding. Many questions remain surrounding the specific signalling events that govern this self-recognition system. This research investigates how resource delivery to self-incompatible pollen is prevented in *Arabidopsis*. Topics explored include the localization of Sec5a, an exocyst complex member that delivers secretory vesicles to compatible pollen; the localization of ARC1, an E3 ubiquitin ligase which prevents the exocyst from delivering resources; and autophagy’s degradation of the sequestered resources.
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Chapter 1

1 Introduction

1.1 Reproduction in Flowering Plants

The production of offspring is central to the continuation of life through subsequent generations. Many plants use sexual reproduction to this effect. The seed bearing plants (spermatophytes) are an example of sexual higher plants and comprise two clades. The angiosperms are a clade that produces flowers as sexual structures. The gymnosperms are a clade that do not produce flowers, and are believed to have contained the progenitor of the angiosperms (Augusto, Davies et al. 2014). The arrival of flowers as reproductive structures provide many advantages for angiosperms, including that flowers are able to be vector pollinated, which provides greater control over pollen dissemination, and the development of a fruit in which seeds are encased and protected. These factors may contribute to the fact that most plants are angiosperms (Armbruster 2014).

This thesis undertakes research in the sexual reproduction of *Arabidopsis* species within the Brassicaceae. *Arabidopsis* flowers are produced at the top of cauline bolts. Once the plant is developmentally ready to undergo reproduction the shoot apical meristem ceases to produce rosette leaves and initiates a cauline bolt, where the shoot apical meristem differentiates into an inflorescence meristem. Secondary meristems arise in the axils of both rosette and cauline leaves, which also become inflorescence meristems (Smyth, Bowman et al. 1990, Kwiatkowska 2008). Flowers are produced in a spiral phylotaxis. These flowers are comprised of organs arranged in concentric circles denoted at whorls. In *Arabidopsis*, the first and outermost whorl contains four carpels, followed by the second whorl containing four petals, the third having six
stamens, and the final and innermost whorl containing two fused carpels containing ovaries at
the base of the style. The top of the style contains a stigma which has small finger-like
projections known as papillae, which interact with pollen grains (Figure 1) (Irish 2010).

*Arabidopsis* species have a dry stigma, lacking surface secretion (Dickinson 1995). This
gives the female control over which pollen grains are accepted. Pollen grains are produced in
anthers, which are the topmost organ of the stamen, pedestaled by filaments. Once mature, the
anther dehisces, releasing desiccated pollen grains. In order for pollen grains to contribute their
male gametes (sperm cells) for sexual reproduction, they first must become rehydrated, allowing
for metabolic activity and the development of a pollen tube. Pollen tubes pierce the stigmatic
papillae and are guided by female factors along the transmitting tract of the pistil to the ovary.
Ovaries contain ovules suspended by funiculae attached to the ovarian wall. Pollen tubes extend
along the funiculus to the micropylar opening of the ovule where a single pollen tube ruptures
inside of a synergid cell releasing two sperm cells. One sperm cell fertilizes the egg cell and the
other the central cell of the ovule. The ovule then develops into a seed with the fertilized egg cell
becoming the embryo and the fertilized central cell forming the endosperm. (Sprunck 2010)

Within the angiosperms, flowers of an individual plant may contain either a single
complement of gametes each (monecious plants), or both male and female gametes within the
same flower (diecious plants). The presence of both gametes within the same flower lends itself
to the potential for self-fertilization (Eckardt 2011). Under many situations this is unfavourable
as it leads to inbreeding depression and offspring of a lower genetic diversity (Sletvold, Mousset
et al. 2013). Dioecious plants have evolved multiple barriers controlling the acceptance of pollen
to restrict inbreeding. Specific barriers may exclude the acceptance of self-pollen by means of
spatial and temporal separation of gametes, as well as self-incompatibility systems. Spatial
separation includes herkogamy, where the male reproductive organs (pollen) are located at a different plane, along the z-axis, from the female reproductive organ (pistil), rendering gamete sharing in the absence of a vector unlikely (Luo and Widmer 2013). Temporal separation includes dichogamy, where male and female reproductive structures mature at different times (Indriolo, Safavian et al. 2014). Additionally, and often in conjunction with these barriers are self-incompatibility systems. de Nettancourt provided a unified definition of self-incompatibility in 1977, which described the process as “the inability of a fertile hermaphroditic seed plant to produce zygotes after self-pollination.” The nature of this SI process is variable across taxa and includes different signalling pathways, which ultimately result in a pre-zygotic reproductive barrier for selfing (De Nettancourt 1977). An important feature of this aforementioned signalling process is that it occurs once pollen interacts with the stigma, whereas the other methods are in an attempt to prevent self-pollen from initially interacting with the stigma.

Two main types of self-incompatibility pathways exist and are delineated by the reproductive control of either the gametophyte or sporophyte, each of which result in the blocked pollen acceptance at different stages. Gametophytic self-incompatibility is controlled by the pollen’s identity and usually results in destroyed pollen tubes as they enter the pistil, before they reach an ovule (Wilkins, Poulter et al. 2014), whereas sporophytic self-incompatibility is dependent on the genotype of the plant, and inhibits pollen grains from initially being hydrating, producing a pollen tube, and penetrating the stigma (Chapman and Goring 2010). Many Brassicaceae species contain sporophytic self-incompatibility pathways, including *Arabidopsis lyrata* (Figure 2) (Kusaba, Dwyer et al. 2001, Schierup, Mable et al. 2001, Indriolo, Tharmapalan et al. 2012). This pathway is able to be reconstituted in self-compatible *Arabidopsis* species such as *Arabidopsis thaliana* by transforming key regulatory genes back into this species (Indriolo,
Safavian et al. 2014). Within the Brassicaceae, SI is the ancestral state, but has been lost within some species such as *Arabidopsis thaliana* (Foxe, Slotte et al. 2009). This breakdown is likely the result of evolutionary pressure from a lack of cross-compatible pollen availability by features such as population bottlenecks. A rarity of mating partners increases the desirability of self-fertilization.
Figure 1. Arabidopsis thaliana Flower. This flower has been dissected to make inner features visible. Pollen, the male gamete is produced within anthers. The stigma, which is the top portion of the pistil contains finger like projections called papillae, which are the pollen contact interface for reproduction.
Figure 2. *Arabidopsis lyrata* Self-incompatibility. DIC (A,C) and aniline blue stained fluorescent (B,D) images of an *Arabidopsis lyrata* stigma after self-incompatible (A,B) or compatible (C,D) pollination. When *Arabidopsis lyrata* is self-pollinated, the pollen isn’t accepted and after one hour it is easily washed away (A,B). One hour after a compatible pollination, pollen grains have adhered to the stigma and germinate a pollen tube (C,D). One of the pollen tubes is indicated by an arrow (D).
1.2 Reproductive Signalling in the Brassicaceae

Mature pollen exists in a desiccated, metabolically quiescent state, and the metabolism commencement is required to initiate pollen tube growth and is dependent on external resources. When pollen lands on a self-incompatible *Arabidopsis* species’ stigma, the stigma will assess its compatibility, and will only allow compatible pollen to hydrate and germinate a pollen tube for fertilization (Dickinson 1995) (Figure 3). This dry stigma ensures that only compatible pollen receives resources to allow for germination. Following pollen landing on a stigma, a miscible ‘foot’ interface is formed between the coat of the pollen grain and the waxy cuticle of stigmatic papillae. This foot provides connectivity for the pollen grain to secrete factors which serve as a means for the plant to assess if pollen is self-incompatible pollen, or compatible cross-pollen (Zinkl, Zwiebel et al. 1999).
Figure 3. Pollen Acceptance is Dependent on Female Supplied Resources. Upon recognition of a pollen grain’s compatibility by a dry stigma, pollen acceptance begins with hydration. Pollen hydration is mainly controlled through resource delivery from stigmatic papillae. Pollen is desiccated and in a metabolically quiescent state as it initially contacts an *Arabidopsis thaliana* stigma (A) 10 minutes after compatible pollination, pollen grains have become hydrated and expanded with stigma supplied water (B). They then may produce a pollen tube to deliver sperm cells to an ovule.
1.3 Compatibility Signalling

When compatible pollen lands on a stigmatic papilla, little is known about the compatibility signalling pathway, apart from the observation that resource-filled secretory vesicles are delivered to the papillar plasma membrane under the pollen contact site (Safavian and Goring 2013). Eukaryotic cells are highly compartmentalized and an important feature of this is the ability to relocalize specific cellular components amongst compartments. In the *Arabidopsis* dry stigma, the acceptance of pollen requires the delivery of compatibility factors to the papillar plasma membrane (Dickinson 1995, Safavian and Goring 2013). These resources are delivered within vesicles, which are membrane enclosed cargo bundles (Safavian and Goring 2013, Safavian, Zayed et al. 2015). Classic vesicle dynamics denote the movement of vesicles in a secretory or retrograde fashion. Secretory vesicles move from the endoplasm through the trans golgi network to a final destination where their membrane fuses with an accepting membrane to release the vesicular constituents. Retrograde vesicle movement occurs in a somewhat opposite direction as a means to redistribute or recycle cellular components (Jürgens 2004). Two features of vesicle dynamics is the ability to direct vesicles to close proximity of their destination, a process known as tethering or docking (Vukasinovic and Zarsky 2016), and the final fusion of the vesicle membrane to the destination membrane through the action of SNARE (Soluble NSF Attachment Protein Receptor) proteins (Jürgens and Geldner 2002).

Within the *Arabidopsis* stigma, the exocyst complex is responsible for tethering the secretory vesicles towards the papillar plasma membrane (Safavian, Zayed et al. 2015). The exocyst complex is composed of the Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 subunits. *Arabidopsis thaliana* has two copies each of Sec3, Sec5 and Sec15 genes; three copies of the Exo84 gene, and 23 of the Exo70 gene (Chong, Gidda et al. 2010, Vukašinović, Cvrčková
et al. 2014). In the *Arabidopsis* stigmatic papilla, the Exo70A1 exocyst subunit moves from the Golgi and sits at the papillar plasma membrane in stage 13 flowers, as demonstrated by confocal analysis (Samuel, Chong et al. 2009). With compatible pollination, Exo70A1 is proposed to direct the remaining exocyst subunits associated with resource-rich secretory vesicles to the papillar plasma membrane. When each of the exocyst subunits, that are most highly expressed in stigmas, are disrupted by T-DNA insertions or stigma-specific RNAi silencing, there is a marked reduction in pollen acceptance, hydration, and seed set (Safavian, Zayed et al. 2015). Apart from Exo70A1, there has been no *in planta* investigation into the localization of exocyst components during pollen-stigma interactions. Additionally our understanding of the secretory vesicle dynamics that occur during this process is limited. It has been reported that secretory vesicles fuse to the papillar plasma membrane upon compatible pollination as visualized by transmission electron microscopy, but the origin of these secretory vesicles and docking complex involved are unknown (Safavian and Goring 2013). Finally, an increase in extracellular Ca\(^{2+}\) delivered by the ACA13 transporter has also been observed in pollen acceptance (Iwano, Igarashi et al. 2014) (Figure 4).
Figure 4. Model for *Arabidopsis* Pollen Compatibility. In cross-compatibility, an unknown recognition event signals for the female release of resources to the pollen grain. Exo70A1 directs the secretory vesicle partitioned exocyst to deliver compatibility factors to the papillar plasmalemma, such as water and putatively the ACA13 calcium pump.
1.4 Self-Incompatibility Signalling

Following self-incompatible pollinations, the pollen S Locus Cysteine Rich (SCR) peptide ligand is sensed by the stigma’s S Locus Receptor Kinase (SRK) (Schopfer, Nasrallah et al. 1999, Takasaki, Hatakeyama et al. 2000, Takayama, Shiba et al. 2000, Silva, Stone et al. 2001). The genes encoding SCR and SRK are tightly-linked, multi-allelic genes, segregating as one unit during meiosis (Stein, Howlett et al. 1991, Boyes and Nasrallah 1993). If these proteins interact in a allele-specific manner, self-incompatibility is signalled. If the SCR ligand does not match SRK, the pollen is then compatible. Thus, when self-pollen lands on a stigmatic papillae, the SI signalling pathway is initiated by the allelic-binding of SCR and SRK which results in the activation of SRK. It is believed that an activated SRK in combination with the lipid anchored M Locus Protein Kinase (MLPK) phosphorylate and activate the Arm Repeat Containing 1 (ARC1) E3 ubiquitin ligase (Murase, Shiba et al. 2004, Samuel, Mudgil et al. 2008). ARC1, in combination with an E1 ubiquitin activating enzyme and an E2 ubiquitin conjugating enzyme, is believed to ubiquitinate and deactivate the exocyst protein Exo70A1 (Samuel, Chong et al. 2009). When Exo70A1 is deactivated by ubiquitination from ARC1, secretory vesicles are not able to be directed to the papillar plasma membrane and pollen grains do not hydrate and germinate (Samuel, Mudgil et al. 2008, Safavian and Goring 2013) (Figure 5).
Figure 5. Model for *Arabidopsis* Self-Incompatibility Signalling. Self-incompatible pollen is not accepted by *Arabidopsis* stigmas. It releases SCR that is allelic to, and thereby activates the stigma’s SRK. Active SRK autophosphorylates itself then phosphorylates ARC1, thereby activating it. ARC1 then ubiquitinates Exo70A1, which deactivates it and prevents the exocyst complex from tethering secretory vesicles to the papillar plasma membrane. Vesicles are not secreted, and instead are degraded in the vacuole through autophagy.
1.5 Ubiquitin Deactivates Exo70A1

During the self-incompatibility response, Exo70A1 may be deactivated by either mono or poly ubiquitination. Ubiquitin is a small regulatory peptide found ubiquitously throughout eukaryotic organisms. Ubiquitin may be added to proteins as a single unit, or as a chain of multiple peptides, allowing for dynamic function depending on stoichiometry (Hershko and Ciechanover 1998). Polyubiquitin is a tag responsible for sending cellular components to the 26S proteasome for degradation and recycling (Glickman and Ciechanover 2002). Exo70A1 being polyubiquitinated would result in its removal from the papillar plasma membrane and subsequent degradation. This degradation would successfully prevent spurious pollen hydration. Alternatively monoubiquitination is involved in protein trafficking (Ramanathan and Ye 2012). Having Exo70A1 sorted away from the papillar plasma membrane to components such as vesicles and potentially autophagic bodies would prevent unintended pollen acceptance.

1.6 Activation of Autophagy in Arabidopsis Self-Incompatibility

With Exo70A1 being deactivated by ARC1, vesicles are no longer delivered to the papillar plasma membrane with SI. It has been recently shown that upon SI signalling, secretory vesicles and portions of cytoplasm appear to be degraded by a macroautophagic response (Safavian and Goring 2013). With macroautophagy, large cellular components are invaginated into a phagophore, forming a double membrane autophagosome, which then fuses to the central vacuole releasing a single membrane autophagic body into the vacuole. The presence of vacuolar hydrolases and low pH result in the degradation of the autophagic body’s cargo (Yoshimoto 2012). As a result, the resources required for pollen acceptance are degraded through autophagy. Within A. lyrata, 10 minutes after a self-incompatible pollination event, TEM analysis show no visible secretory vesicles fusing to the papillar plasma membrane, but shows structures within
the central vacuole consistent with degraded autophagic bodies. When pistils were incubated with E-64, a cysteine protease inhibitor which arrests autophagy in plants, 10 minutes after self-incompatible pollination, intact autophagic bodies apparently containing secretory vesicles and portions of cytoplasm were observed within the central vacuole (Safavian and Goring 2013).

Autophagy is an evolutionarily conserved process for degrading unwanted cytoplasmic components in the vacuole. Autophagy is important in plants during a myriad of processes, such as growth and development, stress response, and senescence. Cytoplasmic components, including organelles, are degraded in the vacuole to recycle their raw materials if they are no longer needed or damaged, and/or cytotoxic (Lv, Pu et al. 2014). Two important types of autophagy that occur in plants are macroautophagy and microautophagy. Macroautophagy is the process where bulk cytosolic constituents are sequestered into a double membrane autophagosome, The outer membrane fuses to the tonoplast, releasing the inner membrane bound autophagic body into the vacuole. In animal cells, an autophagosome may fuse with a lysosome, which is acidic and contains hydrolytic enzymes, generating a autolysosome. It is not clear if this occurs in plants as well. Microautophagy is the process where the tonoplast protrudes and envaginates to engulf portions of the cytoplasm. This forms intra-vacuolar vesicles. Autophagic bodies and intra-vacuolar vesicles are digested by resident vacuolar hydrolases aided by the low pH of the environment (Mitou, Budak et al. 2009).

The first genes associated with autophagy (ATGs) were discovered in yeast mutant screens for sensitivity to nutrient deficiency. Because cells largely cannot recycle cytoplasmic components in the absence of autophagy, atg mutants are more sensitive to nutrient deficiency (Tsukada and Ohsumi). More than 30 yeast ATG genes have been identified, 18 of which are central to autophagy function in yeast. There are about 24 Arabidopsis homologs. No
Arabidopsis homologs have been identified for ATG14, 17, 29 and 31 (Thompson and Vierstra 2005). It's believed that the core autophagy processes are probably largely homologous between yeast and plants however, ATG gene families have expended in plants (Avin-Wittenberg, Honig et al. 2012).

The function and regulation of ATG proteins can be divided into 4 classes: the ATG1 protein kinase complex, the phosphatidylinositol 3-kinase (PI3K) complex specific to autophagy, the ATG9 complex, and two ATG8-lipidation and ATG12-conjugation ubiquitin like complexes (Figure 6). The ATG1 protein kinase complex, which in yeast consists of ATG1, 13, 17, 29, and 31, has been described as a master regulator, because of its induction of autophagy within multiple processes. However, its own regulation is dependent on the target of rapamycin (TOR) complex. (Diaz-Troya, Perez-Perez et al. 2008, Shin and Huh 2011). Under optimal growth conditions, TOR’s hyperphosphorylation of ATG13 stops it from interacting with ATG1. The lack of ATG1-ATG13 interaction prevents autophagy induction. Under stressful growing conditions, TOR does not hyperphosphorylate ATG13, which is free to form a complex with autophosphorylated ATG1 and initiate autophagy (Shin and Huh 2011, Suttangkakul, Li et al. 2011).

With the ATG1 complex’s initiation of autophagy, the PI3K and ATG9 complexes are activated. The PI3K complex, containing VPS34 and 25, and ATG6 and 24, is responsible for producing phosphatidylinositol-3-phosphate (PI3P) lipids at the site of autophagosome nucleation, denoted as the phagophore assembly site (PAS), and recruit PI3P interacting proteins such as the ATG18-ATG2 complex (Takatsuka, Inoue et al. 2004). ATG9 is the only transmembrane ATG protein, and potentially delivers lipids to the PAS, but little is known about its function (Rao, Perna et al. 2016).
Following nucleation, the ATG8 and ATG12 ubiquitin-like conjugation systems are required for membrane elongation and completion of the autophagosome. With the first system, ATG12 is conjugated to ATG5 in a manner mechanistically similar to ubiquitination. ATG12 is activated by ATG7, which acts like an E1 enzyme, then with ATG10 as an E2-like enzyme, and a yet unidentified E3 enzyme, conjugated to ATG5 (Shintani, Mizushima et al. 1999). The ATG12-ATG5 conjugate then complexes with ATG16, which collectively oligomerize (Kuma, Mizushima et al. 2002). This complex may function as a redundant E3 enzyme for the subsequent ATG8 ubiquitin like system (Hanada, Noda et al. 2007), and is required for recruiting ATG8 to autophagic membranes (Suzuki, Kubota et al. 2007). With this localization to autophagic membranes, ATG8 is widely used as a FP-tagged marker for autophagosome detection.

The second ubiquitin-like system results in the conjugation of phosphatidylethanolamine (PE) to ATG8. This is initiated by the C-terminal cleavage of ATG8 by ATG4, the residuum is thereupon activated by ATG7 as an E1-like enzyme, and PE is conjugated to the cleavage site with ATG3 as an E2-like enzyme (Ichimura, Kirisako et al. 2000). The E3 enzyme component is poorly understood, but the ATG12-ATG5-ATG16 complex is involved (Hanada, Noda et al. 2007).
Figure 6. Autophagy Induction and Function in *Arabidopsis thaliana*. Autophagy is involved in many biological processes. Autophagy induction is regulated by the ATG1 complex, with lipids supplied and directed by the ATG9 and PI3K complexes. Autophagosome enclosure is dependent on the ATG12 and ATG8 conjugation systems. Reprinted by permission from Macmillan Publishers Ltd: Cell Death and Differentiation (Hofius, Munch et al. 2011), copyright 2011.
1.7 Potential for Additional Self-Incompatibility Pathways

The SCR/SP11 and SRK genes naturally found in A. thaliana are non-functional (Kusaba, Dwyer et al. 2001, Tang, Toomajian et al. 2007, Tsuchimatsu, Suwabe et al. 2010). Additionally, ARC1 has been deleted in A. thaliana (Indriolo, Tharmapalan et al. 2012). Recent work from our research group showed that a strong SI response in A. thaliana ecotype Columbia-0 requires the addition of SCR/SP11, SRK and ARC1 transgenes, but within the A. thaliana ecotype, Shahdara, a moderate SI response is observable with only the addition of SCR/SP11 and SRK. Additionally, the strong SI response observed in the Shahdara ecotype with the addition of SCR/SP11, SRK and ARC1 is stronger than that observed within Columbia-0 (Indriolo, Safavian et al. 2014). This raises interesting questions about the role of ARC1 and the potential of peripheral components of the SI signalling pathway. Within Brassica napus W1, no ARC1 knockout mutants are available; however, RNAi was employed to knockdown ARC1 expression (Samuel, Mudgil et al. 2008). A complete breakdown in SI was never observed, and the limited expression of ARC1 might have been sufficient to allow for a limited SI response, or a peripheral branch of the SI pathway may exist. The knockout of ARC1 by using techniques such as CRISPR/Cas9 would be invaluable in determining the extent of ARC1’s role and the potential for peripheral pathways. Additionally, approach herkogamy naturally occurs within the self-incompatible Arabidopsis lyrata (Indriolo, Safavian et al. 2014). When self-incompatibility was reconstituted in the self-compatible A. thaliana, the addition of ARC1 causes approach herkogamy, which does not normally occur in this species. RNAi knockdown of ARC1 in Arabidopsis lyrata did not remove approach herkogamy in this species.
1.8 Thesis Objectives

The overarching goal of this research is to contribute further insights into *Arabidopsis* pollen-stigma interactions. Questions remain surrounding the localization of signalling components and vesicle trafficking in the compatibility and self-incompatibility pathways. Additionally, questions remain surrounding the type of ubiquitination system used to deactivate Exo70A1, Exo70A1’s fate during SI, and the extent of ARC1’s role during SI.

I hypothesize that secretory vesicles associated with the exocyst complex deliver resources to compatible pollen, and that post translational modification regulates ARC1 and Exo70A1’s subcellular localization.

To this end, the objectives of this thesis are to contribute to:

1) the characterization of vesicle trafficking using GFP-tagged Sec5a following compatible pollination by confocal microscopy.
2) the investigation of autophagy’s requirement in SI by confocal analyses through the expression of *SCR*, *SRK*, and *ARC1* transgenes with an autophagy marker in Col-0.
3) further defining the role of ARC1 in *A. lyrata* by cloning CRISPR/Cas9 ARC1 KO constructs.
4) characterizing the subcellular localization of FP-tagged ARC1 and Exo70A1 within stigmatic papillae in response to compatible and self-incompatible pollination by cloning FP-tagged ARC1 and Exo70A1 constructs.
Chapter 2

2 Materials and Methods

2.1 Plant Materials and Growth Conditions

*Arabidopsis thaliana* ecotypes Columbia -0 (Col-0) (CS22625) and Shahdara (Sha) (CS22652) were used in this study, both obtained from the Nordborg Collection (Nordborg, Hu et al. 2005) maintained by the ABRC.

Apart from otherwise noted, *Arabidopsis thaliana* seeds were surface sterilized using dilute bleach, and surfactant solution under agitation for 15 minutes then cold stratified in sterile water at 4°C for 4 days to sync germination. Stratified seeds were sown on 0.4% agar petri plates containing 1/2 strength MS growth media, and incubated under 24 hour light for two weeks. Seedlings were transplanted to Sunshine® Sphagnum based potting substrate (Sun Gro) and grown under a 16 hour light cycle at 23°C with 80 microeinsteins of light within growth chambers. Primary cauline bolts were excised after reaching at least 4 cm to allowing multiple cauline bolts to present per plant with greater silique uniformity between cauline branches.

2.2 Plant transformation

Plant expression vectors were transformed into *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell 1986) via electroporation. Approximately 100ng of expression vector was added to 50ul of electro-competent GV3101 cells in a 2mm electroporation cuvette and treated to a pulse of 2kV, 100k ohms, and 25 uF. Transformed cells were selected on petri plates with appropriate antibiotics, in addition to 100 ug/mL Rifamycin SV, appropriate for selecting GV3101 cells. *Arabidopsis thaliana* was then transfected with expression vectors within Agrobacterium by the floral dip method (Clough and Bent 1998). Stocks were inoculated
into 5 ml of LB medium and cultured with appropriate antibiotics at 30°C for one or two days until stationary phase. One ml of culture was then inoculated into 200 ml of LB medium and cultured into log phase overnight with appropriate antibiotics. Cultures were centrifuged at 5000 RPM for 20 minutes at room temperature and resuspended in 100 ml of a 50g/1 sucrose solution supplemented with 500 ul/L Silwet L22 surfactant. More than one vector may be transformed into Arabidopsis at the same time by mixing equal portions of Agrobacterium inoculum containing the desired vectors at this time. 80 plants with cauline bolts approximately 10 cm long after primary bolts were excised were used for dipping each set of transgene combinations. The bolts were gently rocked in Agrobacterium inoculum for 5 seconds and plants were returned to regular growth conditions within a plastic bag to maintain high humidity overnight. The bag was subsequently removed. One week after primary dipping the process was repeated for a second time. T0 seeds were then harvested and transformants selected using appropriate selectable or screenable markers.

2.3 Plant DNA extraction

Genomic DNA was obtained from fresh leaves using a liquid/liquid extraction, modified from Murray and Thompson’s CTAB protocol (Murray and Thompson 1980). Leaves with a diameter of approximately half a centimetre were ground in 250ul of CTAB buffer. This buffer contains 1M NaCl, 0.02M EDTA, 0.1M Tris-Cl pH 8 with the addition of 0.4% v/v 2-mercaptoethanol added before use. Leaf homogenates were incubated at 65°C for 15 minutes followed by the addition of an equal volume of chloroform, gently mixed and then centrifuged for 5 minutes with a hard spin at room temperature. 200ul of the aqueous phase was separated and mixed with 150ul of 2-propanol. DNA was then allowed to precipitate for at least half an hour at room temperature. Samples were then treated to a hard spin for 20 minutes and the
supernatant was discarded. Pellets were washed with ice cold 70% ethanol and left to air dry to completion under a gentle air stream. The DNA samples were then resuspended in deionized and sterile water and stored at -20°C.

2.4 ARC1 plasmid construction

The pORE-O3 expression vectors containing the coding sequences of TagRFP:ARC1, EGFP:ARC1, and FLAG:ARC1 were generated by restriction enzyme cloning. Early attempts to generate expression vectors containing these coding sequences by Gateway® cloning were unsuccessful. The coding region of ARC1 was amplified off of a pGEM:ARC1 plasmid obtained from Dr. Emily Indriolo. Cassettes containing TagRFP:ARC1 and EGFP:ARC1 were generated by overlap extension PCR (Heckman and Pease 2007) with the addition of 5’ EcoR1 and 3’ PstI restriction enzyme recognition sequences. FLAG:ARC1 was generated by PCR with the 5’-3’ primer containing a NotI restriction enzyme recognition sequence followed by the FLAG sequence as an adaptor. A PstI cut site was added to the 3’ end of the cassette. Each of these cassettes were generated with tags added, in frame, to the 5’ terminus of ARC1. Cassettes were subcloned into pGEM® T-Easy plasmids (Promega) which were subsequently digested. TagRFP:ARC1 and EGFP:ARC1 subclones were digested with EcoR1. The FLAG:ARC1 subclone was double digested with NotI and PstI. Digest fragments were cloned into the pORE-O3 plasmid (Coutu, Brandle et al. 2007) that previously contained the SLR1 promoter (Chapman 2010). Plasmids were analyzed by restriction enzyme digestion once cassettes were subcloned and also once expression vectors were generated. Additionally, subcloned cassettes were sequenced.
2.5 Generating TagRFP:ARC1 lines

p548 (pBIN-AISCRIb-AlSRKb) (Nasrallah, Liu et al. 2004, Boggs, Nasrallah et al. 2009) obtained from the Nasrallah research group and the pORE:TagRFP:ARC1 vectors were co-transformed into Arabidopsis thaliana ecotypes Shahdara and Columbia-0 as previously described. T0 seeds were sown directly onto ProMix® (Premier Tech) sphagnum potting substrate and pORE:TagRFP:ARC1 transformants were selected for by 0.1% w/v BASTA treatment of seedlings once the first true rosette leaves reached approximately half a centimetre in diameter. BASTA treatment was repeated every three days for a total of three times. Chemical selection was performed for only one resistance marker, as double selection is stressful and results in false negative transformant identification. 100 surviving seedlings were then transplanted into pots containing ProMix medium and genotyped for the presence of the Kanamycin resistance selectable marker located within the SCRb-SR Kb construct. This resulted in the identification of co-transformants. T0 transformants were then phenotyped for self-incompatibility traits by the presence of reduced silique size, and self-pollen rejection assayed by DIC microscopy of analine blue stained stigma preparations.

2.6 TagRFP:ARC1 confocal localization

Transgenic SRCb:SRKb TagRFP:ARC1 flowers and Col-0 flowers were emasculated at stage 12, the remaining pistils were covered with plastic film and plants returned to growth conditions for development into stage 13 overnight. Pistils with fully elongated stigmatic papillae were visualized in the absence of pollen, and 10 minutes after pollination. After pollination treatment half pistils were excised and mounted in Vectashield (Vector Laboratories). Slid preparations were then observed using a TCSP8 (Leica) confocal with 20X and 40X oil emersion objectives. A 561 nm laser excited RFP and emission spectra were observed between
600 and 650 nm by a HyD detector. Laser power and sensor sensitivity were assigned values that resulted in minimal signal within the 600-650 nm range when wild type Col-0 lacking TagRFP was imaged.

2.7 Exo70A1 plasmid construction

The pORE-E3 expression vectors containing coding sequences of TagRFP:Exo70A1 and FLAG:Exo70A1 were also generated by restriction enzyme cloning, as Gateway® Cloning proved recalcitrant. The coding sequence of Exo70A1 was amplified off of a pGEM:YFP:Exo70A1 plasmid obtained from Dr Emily Indriolo. The cassette containing the N-terminal EGFP:Al-Exo70A1 coding sequence was generated by overlap extension PCR (Heckman and Pease 2007) with the addition of a 5’ NotI and 3’ PstI restriction enzyme recognition sequences. The cassette containing N-terminal FLAG:Al-Exo70A1 coding sequence was generated by PCR using a 5’ oligo containing a NotI restriction enzyme recognition sequence followed by a FLAG as an adaptor. A PstI restriction enzyme recognition sequence was added to the 3’ end of the cassette. Cassettes were subcloned into the pGEM® T-Easy plasmid (Promega) and subsequently digested with NotI and PstI. Digest fragments were cloned into the pORE-E3 expression vector (Coutu, Brandle et al. 2007) containing the ENTCUP2 constitutively active promoter (Malik, Wu et al. 2002). Plasmids were analyzed by restriction enzyme digestion once cassettes were subcloned and also once expression vectors were generated. Additionally subcloned cassettes were sequenced.

2.8 ARC1 CRISPR/Cas9 KO plasmid construction

I generated two vectors to knock out Arabidopsis lyrata ARC1 using the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-Associated nuclease 9 (Cas9) system. These constructs were based on the pCAMBIA-1302 vector obtained from the
Weeks research group (Jiang, Zhou et al. 2013). This vector contains the user designed 20 nt spacer RNA upstream of a Protospacer Adjacent Motif (PAM), the guide RNA scaffold for the Cas9 nuclease, and the coding region for the Cas9 nuclease. The constructs were designed to disrupt *Arabidopsis lyrata* ARC1 near its 5’ terminus and were chosen as they had low off target sequence identity according to E-Crisp modeling (Heigwer, Kerr et al. 2014). This was verified by BLASTn (NCBI) (Zhang, Schwartz et al. 2000) queries of the guide RNAs against the *Arabidopsis lyrata* genome (taxid:59689). These guide RNAs also contained a unique restriction enzyme recognition site within 3 nt upstream of the 5’ terminus. This is the region where Cas9 cleaves the target gene. Disruption of this region may be genotyped by the ability of these restriction enzymes to digest amplified fragments.

The spacer sequence for pCRISPR:ARC1.1 is **GTGCTCAGTCCAGTAAACTATGG**, which is 20 nucleotides of the ARC1 coding sequence between nucleotides 323 to 342 of 2106 and contains the *Hpy*166II restriction enzyme recognition sequence GTNNAC. The spacer sequence for pCRISPR:ARC1.2 is **GATTCGCATCCTTACGACGG**, which is 19 nucleotides of the ARC1 coding sequence between nucleotides 18 and 36 of 2106 and contains the *Hpy*99I restriction enzyme recognition sequence CGWCG. There is a 5’ guanine residue added which is not part of the ARC1 coding sequence as required for the U6 promoter sequence of the pCAMBIA plasmid.

The pCRISPR:ARC1 vectors were generated by restriction enzyme cloning. Cassettes containing pCAMBIA-3102 sequence between the *SalI* and *KpnI* restriction enzyme recognition sequences were amplified using overlap extension PCR (Heckman and Pease 2007). The internal primers contained the ARC1 guide RNA sequences to replace the original sequence. Cassettes were subcloned into pGEM® T-Easy, restriction enzyme digested and ligated into pCAMBIA-
plasmids with the SalI - KpnI region removed. The generated constructs were assayed by restriction enzyme digests after being subcloned and once the expression vector was generated. Additionally, the subcloned plasmids were sequenced.

2.9 Generating GFP:ATG8a lines

The pGFP:ATG8a (Thompson, Doelling et al. 2005) plasmid obtained from the Vierstra research group, p548 (pBIN-\textit{AlSCRb-AlSRKb}) (Nasrallah, Liu et al. 2004, Boggs, Nasrallah et al. 2009) from the Nasrallah group, and pORE:SLR1\textsuperscript{pro}:ARC1 obtained from Dr Emily Indriolo were triple transformed into Col-0 by the floral dip method previously described. T0 seeds underwent BASTA selection as previously described to select for ARC1 transformation and then PCR genotyped for the presence of the \textit{GFP:ATG8a} and \textit{SCRb-SRKb} constructs. Transformants were phenotyped for self-incompatibility by observing reduced silique size. Two lines were selected as self-incompatible by observing reduced silique size.

2.10 Confocal visualization of autophagy with self-incompatibility

Transgenic \textit{EGFP:ATG8a} flowers and Col-0 flowers were emasculated at stage 12, the remaining pistils were covered with plastic film and plants returned to growth conditions for development into stage 13 overnight. Pistils with fully elongated stigmatic papillae were visualized in the absence of pollen, 5 minutes after pollination, and 10 minutes after pollination. After pollination treatment half pistils were excised and mounted in Vectashield (Vector Laboratories). Slid preparations were then observed using a TCSP8 (Leica) confocal with a 40X oil emersion objective. A 488nm laser excited GFP and emission spectra was observed between 505 and 530 nm by a HyD detector. Laser power and sensor sensitivity were assigned values that resulted in minimal signal within the 505-530 nm range when wild type Col-0 lacking GFP was imaged.
2.11 Generating GFP:Sec5a lines

The pEGFP:Sec5a (pGWB606:pro35S:EGFP:Sec5a) expression vector containing N-terminal tagged EGFP:Sec5a made by Dr. Darya Safavian, and a pMRFP:Vamp721 (pGWB1:proVamp721:mRFP:Vamp721) expression vector containing the mRFP:Vamp721 vesicle marker (Asaoka, Uemura et al. 2013) obtained by the Sato research group, were co-transformed into Col-0 by the floral dip method as previously described, completed by Dr Darya Safavian. T0 seeds were screened by BASTA selection as previously described for EGFP:Sec5a transformation, and PCR genotyped for co-transformants (by Eli Jany). 13 preliminary lines have been generated.

2.12 GFP:Sec5a Confocal visualization

Col-0 or transgenic GFP:Sec5a flowers at the beginning of stage 13 with fully elongated stigmatic papillae were emasculate and and visualized in the absence of pollen, 5 minutes after pollination, and 10 minutes after pollination. After pollination treatment half pistils were excised and mounted in Vectashied (Vector Laboratories). Slide preparations were then observed using a TCSP8 (Leica) confocal with a 40X oil emersion objective. A 488nm laser excited GFP and emission spectra were observed between 505 and 530nm by a HyD detector. Laser power and sensor sensitivity were assigned values that resulted in minimal signal within the 505-530nm range when wild type Col-0 lacking GFP was imaged.
Chapter 3

3 Results

3.1 TagRFP:ARC1 localization

The model of ARC1 deactivating Exo70A1 in self-incompatibility would predict that at some point during the SI response ARC1 would localize to the papillar plasma membrane, where Exo70A1 is located in the resting state of mature stigmatic papillae (Samuel, Chong et al. 2009). Additionally, ARC1 may move away from Exo70A1 under resting or compatible conditions, so that Exo70A1 is not spuriously deactivated. Since ARC1’s sub-cellular localization has not previously been explored. I generated two fluorescent protein tagged constructs with either TagRFP or EGFP added to ARC1’s N-terminus.

Three self-incompatible lines were generated with the double transformation of SCRb-SRKh, and RFP:ARC1 constructs into Arabidopsis thaliana Col-0 Each of these lines showed moderate self-incompatibility; thus indicating that the tagged ARC1 protein was functional (Figure 7). Pollination assays with aniline blue staining showed that cross-compatible Col-0 pollen was fully accepted by stigmas, and the pollen of the transgenic lines was fully accepted by Col-0. However, there was a reduction in self-pollen acceptance of these transgenic lines. Silique size in all three lines was reduced in comparison to Col-0, indicating a lower yield of seed set. Arabidopsis thaliana is naturally self-pollinating and a reduction in seed set as visualized by smaller silique sizes indicates self-incompatibility. The potential of stigma or pollen defects contributing to pollen rejection was ruled out after demonstrating that the transgenic pollen was fully accepted by Col-0, and that the transgenic stigmas fully accepted Col-0 pollen. Although these lines showed a self-incompatibility response, it was variable amongst flowers of individual plants. The reduction in silique size was not uniform, as there was
a greater range of silique sizes in the transgenic lines than what naturally occur in wild-type Col-0 (Figure 7).

Preliminary confocal analysis was undertaken to determine the localization of ARC1 in unpollinated papillae, and in response to self-incompatible pollination. One line demonstrated ARC1 localized to the papillar plasma membrane and in diffuse punctate structures in freshly open, unpollinated stigmas (Figure 8). The other two lines did not demonstrate a detectable TagRFP:ARC1 signal. After a 10 minute self-incompatible pollination, this signal appeared to dissipate around the pollen contact site (Figure 9). A refined observation was not possible in this line, as the self-incompatibility phenotype was lost in the subsequent generation. This was likely the result of transgene silencing, as all plants were PCR genotyped. Additional primary transformants have been generated with the co-transformation of either TagRFP:ARC1 or EGFP:ARC1 with SCRb-SRKb in Col-0 and Sha for future screening of new lines.
Figure 7. TagRFP:ARC1 is Functional in Arabidopsis thaliana. Confocal localization of RFP:ARC1 is specific to the stigma, as it’s expression is driven by the stigma-specific SLR1 promoter (A). Three representative siliques sizes of line 56 compared to a representative Col-0 silique. The siliques are smaller, meaning pollen is being rejected, however the penetrance of the self-incompatibility phenotype isn’t uniform (B) Upon a 2 hour pollination, line 56 shows cross-compatible pollen acceptance from wild-type Col-0 (C,D) and self-pollen rejection (E,F). Self-incompatibility was reconstituted with the transformation of SCRb:SRKb and TagRFP:ARC1.
Figure 8. TagRFP:ARC1 Localizes to the Plasma Membrane and Punctate Structures in Unpollinated Stigmas TagRFP:ARC1 is seen at the plasma membrane and in a diffuse and irregular punctate structure (A,B,C,D). Col-0 autofluorescence is visible at the cell wall (E,F).
Figure 9. TagRFP:ARC1 Fluorescence Dissipates After Self Incompatible Pollination. Background auto-fluorescence as seen in untransformed Col-0, 10 minutes after self-pollination (A,B). 10 minutes after a self-incompatible pollination, TagRFP:ARC1 fluorescence is visible around the periphery of the papillae, but has diminished at the pollen contact site (C,D). Pollen contact site indicated by arrow (C,D).
3.2 CRISPR/CAS9 ARC1 knockout

The simplest way to determine a gene’s function is to make observations with its absence. To determine the precise requirement of *ARC1* in self-incompatibility, a complete knockout mutant is required as previous studies on *ARC1* were based on knockdown constructs (Stone, Arnoldo et al. 1999, Indriolo, Tharmapalan et al. 2012). To accomplish this reverse genetics approach in *Arabidopsis lyrata*, I generated two CRISPR/Cas9 *ARC1* knockout constructs (Figure 10). CRISPR/Cas9 is a system to introduce mutations in a target gene by generating a targeted double-stranded DNA break, followed by the host non-homologous end-joining repair system which frequently introduces small insertions or deletions (Luo, Gilbert et al. 2016).

A preliminary workflow has been established to transform and screen these constructs in *Arabidopsis lyrata*, in future experiments. Each of the two CRISPR:ARC1 constructs together with *GFP:ATG8a* (autophagy marker) were individually co-transformed into *Arabidopsis lyrata* by the previously described floral dip method, supplemented with vacuum infiltration of the Agrobacterium solution into the plant tissue. Because *Arabidopsis lyrata* is self-incompatible, T0 seeds were produced by crossing lines with distinct *S*-locus haplotypes. Each of the two CRISPR:ARC1 and GFP:ATG8a construct pairs were transformed into and crossed between two different *S*-locus haplotype combinations each. Approximately 100 seeds were produced, however no lines were generated, resulting from technical difficulties surrounding seed germination. The petri plates and imbibing seeds dried out before germination occurred. Future transformant screening in *Arabidopsis lyrata* will benefit from seed germination in sterile tissue culture boxes instead of petri plates. The increased volume of agar substrate maintains
appropriate humidity longer, allowing for a longer germination time. The pCRISPR:ARC1 constructs are available for future *Arabidopsis lyrata* transformation.
Figure 10. *ARC1* CRISPR/Cas9 Knockout. Transfer DNA between right and left boarders of pCRISPR:ARC1 constructs (A). Location of the spacer DNA targets to be disrupted in ARC1 by the pCRISPR:ARC1 constructs (B).
3.2 Autophagy induced by self-incompatibility

The involvement of autophagy within self-incompatibility is a recent discovery, and preliminary evidence suggests that autophagy results in the removal of secretory vesicles to prevent resources from being delivered to self-incompatible pollen in Arabidopsis lyrata (Safavian and Goring 2013). This process has not been explored in Arabidopsis thaliana, and the requirement of autophagy in the self-incompatibility pathway has not been established. To this end, autophagy induction during self-incompatibility was assessed in Arabidopsis thaliana by confocal visualization of the GFP:ATG8a autophagy marker under differential pollination conditions. Two self-incompatible lines were recovered after a Col-0 triple-transformation with the SCRb-SRKb, ARC1 and GFP:ATG8a constructs. These lines showed a moderate reduction in silique size, which was assumed to represent self-incompatibility. Confocal experiments in the most self-incompatible line showed GFP puncta 5 minutes after a self-incompatible pollination that were never observed within its un-pollinated stigma, upon a compatible pollination or within an untransformed Col-0 stigma (Figure 11). These puncta appeared to be located within the cytoplasm of the papillae cells. This visualization of GFP:ATG8a accumulating into puncta shows the sites of autophagosome formation and indicates that macroautophagy is occurring. This evidence indicates that self-incompatibility triggers autophagy in Arabidopsis thaliana, and is in agreement with what has been seen in Arabidopsis lyrata (Safavian and Goring 2013). Subsequent visualization of this line did not occur. The self-incompatibility phenotype was lost in the next generation of both lines. All plants were PCR genotyped; the loss of self-incompatibility was likely the result of transgene silencing. An additional transformation was completed with these transgenes for future screening.
After the preliminary determination that autophagy is involved in *Arabidopsis thaliana* self-incompatibility, the requirement of autophagy for self-incompatibility was addressed. Reconstitution of self-incompatibility was attempted in *atg5*-1 and *atg7*-2 null mutant backgrounds with the co-transformation of the *SCRb-SRKb*, and *ARC1* constructs. Each of these mutant backgrounds lack autophagy, as autophagosomes are not able to form in the absence of ATG5 or ATG7. None of the primary transformants in either the *atg5*-1 or *atg7*-2 backgrounds clearly showed self-incompatibility as assayed by significantly reduced silique size. Of the transformants, one line in the *atg5*-1 background and two lines in the *atg7*-2 background were selected because they had slightly smaller siliques. The near-wildtype silique sizes of these lines may indicate that the *SCRb-SRKb* and *ARC1* transgenes are functional in the reconstitution of the self-incompatibility pathway, but the lack of autophagy disrupts the SI response to the extent that most pollen grains receive sufficient resources to be accepted. None of these lines showed a discernible self-incompatibility, but to determine if the lack of autophagy is responsible for the lack of self-incompatibility these lines should be backcrossed to wild-type Col-0. This generates heterozygous *atg5*-1/ATG5 and *atg7*-2/ATG7 genotypes in the F1 generation. Because the mutations are recessive, the wild-type allele will be functional, reconstituting autophagy. The presence of self-incompatibility in any of these heterozygous lines would demonstrate that autophagy is required for self-incompatibility. The potential absence of self-incompatibility in the F1 generation would indicate that the transgenes in the primary transformants were not functional. This is an important control so that the lack of self-incompatibility is not erroneously attributed to disrupted autophagy. As a positive control in this respect, potential F1 self-incompatibility can be compared to the same transgene combinations made in a wild-type Col-0 background that was backcrossed to Col-0. Preliminarily, the *SCRb-SRKb* and *ARC1 atg5*-1 line
was backcrossed to Col-0 in the T0 generation. The resulting *SCRb-SRKb* and *ARC1 atg5-1/ATG5* F1 seeds and the two *atg7-2* lines have been saved for future analysis. As well, since the initial co-transformation frequency of *SCRb-SRKb* and *ARC1* in the *atg5-1* and *atg7-2* backgrounds was poor, additional transformations with *SCRb-SRKb*, *ARC1*, and *GFP:ATG8a* have been completed in these backgrounds and T0 seeds saved for future screening.
Figure 11. Self-incompatible Pollination Induces Autophagy in Arabidopsis thaliana. SCRb:SRKb ARCl GFP:ATG8a line 3, 5 minutes after Col-0 pollination (A) or self-incompatible pollination (B) GFP foci upon self-incompatible pollination shows ATG8a associated with autophagosomes.
3.3 Sec5a Associated Secretory Vesicle Dynamics

The strongest evidence of the exocyst complex’s involvement in tethering secretory vesicles to the papillar plasma membrane for pollen acceptance has been shown in transgenic lines with the knockout or stigma-specific knockdown of each exocyst subunit (Safavian, Zayed et al. 2015). All lines showed a reduction in the acceptance of wild-type compatible pollen. This demonstrates that the pollen grains are not able to receive the resources they require when the exocyst complex does not deliver vesicles to them. However, visualizing the vesicle dynamics in this process had not been undertaken. While Exo70A1 is plasma membrane localized (Samuel, Chong et al. 2009), the Sec5a exocyst subunit is one of the exocyst members associated with secretory vesicles, and so predicted to travel to the plasma membrane with the induction of exocytosis. Thus, confocal microscopy of GFP:Sec5a transgenic lines was carried out to explore these vesicle dynamics.

Confocal observations were made with two GFP:Sec5a lines in Col-0. One line showed GFP fluorescence in the cytoplasm 5 minutes after compatible pollination, and an increase in fluorescence 10 minutes after pollination. No significant GFP fluorescence was observed in unpollinated stigmas or wild type stigmas lacking GFP (Figure 12). This increase in fluorescence was observed throughout the cytoplasm of the papillae and did not appear to be specific to a particular location, such as underneath the pollen contact site.

The second line showed much less GFP fluorescence with compatible pollinations, even after 10 minutes. The strong increase of cytoplasmic GFP signal of line 1 was not observed. However, the weaker response allowed for the visualization of GFP:SEC5a in the absence of a saturating signal. At 15 minutes post pollination, an accumulation of signal was observed at the papillar plasma membrane directly underneath a compatible pollen grain (Figure 13). This is
indicative of exocyst associated secretory vesicles being tethered in a directed manner to the site of pollen grain adhesion, providing the resources for pollen hydration and acceptance specifically where they are required. Additional investigations into these vesicle dynamics can be made, as seeds from 13 independent transgenic lines have been saved.
Figure 12. Compatible Pollination Upregulates GFP:Sec5a Protein Abundance. For GFP:Sec5a line 14, when compared to an unpollinated stigma (A,B), there is an increase in GFP:SEC5a fluorescence five minutes after a compatible pollination (C,D). Ten minutes after a compatible pollination the GFP:Sec5a fluorescence is brighter (E,F). This indicates that compatible pollen are sending acceptance factors to induce exocyst tethering for vesicle secretion.
Figure 13. GFP:Sec5a Accumulates at Pollen Contact Site. For GFP:Sec5a line 4, when compared to resting stigmatic (A,B), a compatible pollination results in GFP:Sec5a accumulation at the papillar plasma membrane of the pollen contact site (C,D).
Chapter 4

4 Discussion

4.1 ARC1 localization

ARC1’s involvement in self-incompatibility was initially identified in Brassica, through an interaction screen with SRK (Gu, Mazzurco et al. 1998). It has been shown to have E3 ubiquitin ligase activity in vitro and to interact with Exo70A1 (Samuel, Chong et al. 2009). ARC1 interactions with SRK and Exo70A1 have also been demonstrated in Arabidopsis lyrata. Knocking down ARC1 expression with RNAi has shown a breakdown in the self-incompatibility response in both Brassica and Arabidopsis (Stone, Arnoldo et al. 1999, Indriolo, Thar mapalan et al. 2012). It has also been shown to be required for reconstituting a strong self-incompatibility response when the self-incompatibility pathway is reconstituted in Arabidopsis thaliana (Indriolo, Safavian et al. 2014). All of this evidence strongly supports its involvement in self-incompatibility, however, there is not currently any direct in planta evidence that ARC1 interacts with and ubiquitinates Exo70A1, and that this deactivation of Exo70A1 is causal to self-incompatibility. An important step in addressing these questions is to look at ARC1’s subcellular localization patterns under different pollination conditions. Using Arabidopsis thaliana for this is ideal, as its stigmatic papillae are thin and easily visualized with microscopy, the plants are easily transformed, and there is a large library of mutants available.

ARC1 was seen to localize close to the periphery of the stigmatic papillae under resting conditions. This is consistent with its need to interact with both SRK and Exo70A1, which are also plasma membrane localized (Samuel, Chong et al. 2009, Rea and Nasrallah 2015). Additionally, TagRFP:ARC1’s fluorescence diminishing after a self-incompatible pollination may be the result of its use, after which it is either degraded or re-localized. These findings
remain preliminary, as ARC1 localization has been challenging to view, which may be the result of ARC1 protein stability. As an E3 ubiquitin ligase, it would not be surprising if ARC1 auto-regulates itself at the post-transcriptional level. Pharmacological agents that block protein degradation such as MG132 may be required in order to see a fluorescent protein tagged ARC1 signal. This approach was required to visualize the related *Arabidopsis* PUB10 E3 ligase (Jung, Zhao et al. 2015). Additionally, the *EGFP:ARC1* construct was generated as a backup to the TagRFP version, in case one construct is not functional. Having two fluorophores to choose from is also convenient if one wishes to visualize ARC1 localization in combination with an additional fluorescent protein-tagged gene of interest. Finally, the putative plasma membrane localization will need to be confirmed using a plasma membrane marker. As well, the putative structures will need to be further investigated using different markers in the endomembrane system.

### 4.2 ARC1 involvement in Self-Incompatibility

Previous research has found it challenging to determine the extent of ARC1’s involvement in self-incompatibility with the use of knockdown constructs (Stone, Arnoldo et al. 1999, Indriolo, Tharmapalan et al. 2012). ARC1 involvement in *Brassica* self-incompatibility is well accepted within the field, however, ARC1’s requirement in reconstituting self-incompatibility in the naturally self-compatible species *Arabidopsis thaliana* is poorly understood and ecotype-specific (Reviewed in Goring and Indriolo 2014). The partial disruption of *ARC1* expression in the naturally self-incompatible species *Brassica napus* and *Arabidopsis lyrata* has been investigated with RNAi. A reduction in the self-incompatibility response is seen in these RNAi lines, but in the absence of an *ARC1* knockout in a naturally self-incompatible species, its precise requirement cannot be completely understood. With the proposed role of ARC1 in deactivating Exo70A1 to inhibit self-incompatible pollen acceptance (Samuel, Chong et al. 2009), and the
putative induction of autophagy as a result of self-incompatibility (Safavian and Goring 2013), deletion of *ARC1* will not only assay its requirement in self-incompatibility, but also assess if it causes autophagy induction. Autophagy may be monitored after self-pollination in *ARC1*’s absence.

Non-model organisms lack the convenience of the large array of tools afforded to model organisms. Unfortunately T-DNA insertion mutants have not been produced in self-incompatible Brassicaceae species, and no naturally occurring or induced mutations in *ARC1* have been identified. Generating a targeted mutation in ARC1 is possible with using gene-editing methods such as the CRISPR/Cas9 system (Jiang, Zhou et al. 2013). The CRISPR/Cas9 system is based off of a bacterial immunity to bacteriophage infection system. The Cas9 nuclease will cleave double stranded DNA when provided with a small stretch of homologous RNA conjoined to a guide RNA scaffold. This process is functional when the components are transformed into eukaryotes. The targeted double strand break in genomic DNA is repaired by non-homologous end joining, which often results in frame shift mutations of the targeted coding region rendering the gene non-functional (Perez, Wang et al. 2008).

4.3 Intersection of Self-Incompatibility and Autophagy

The involvement of autophagy in the self-incompatibility response has only recently been identified, and is poorly understood. It has been reported that after self-incompatible pollination in *Arabidopsis lyrata*, putative autophagic bodies have been visualized by TEM studies, and autophagosomes have been seen by confocal studies (Safavian and Goring 2013). The aforementioned observations of autophagy when self-incompatibility is disrupted with ARC1 KO will provide additional insight into autophagy’s involvement in *Arabidopsis lyrata*. My
observation of GFP:ATG8a puncta after self-incompatibility demonstrates that autophagy is also involved in *Arabidopsis thaliana* SI.

After testing if autophagy is involved in *Arabidopsis thaliana* self-incompatibility, an important subsequent question to answer is if autophagy is required for self-incompatibility. Even though self-incompatibility was not observed in any of the *SRCb-SRKb* and *ARC1* transformed *atg5-1* or *atg7-2* mutants, this is not yet sufficient evidence to imply causality. If this lack of self-incompatibility is caused by the lack of autophagy, then backcrossed lines possessing heterozygous *ATG/atg* alleles should demonstrate self-incompatibility. Additionally, the autophagy marker GFP:ATG8a was included as a third transgene along with *SRCb-SRKb* and *ARC1* constructs in the additional *atg5-1* and *atg7-2* transformations. This provides a second line of inquiry into the connection of autophagy and self-incompatibility apart from aforementioned genetic dissection. Autophagosome-associated GFP:ATG8a signals indicate that autophagy is occurring, and its exclusion to the presence of self-incompatibility would demonstrate a linkage of these two processes.

### 4.4 Sec5a associated vesicle dynamics

Exo70A1 was the first identified exocyst component involved in Brassicaceae pollen acceptance (Samuel, Chong et al. 2009). Confocal studies have shown RFP:Exo70A1 moves from the Golgi to the plasma membrane of stigmatic papillae once they are ready to accept pollen (Samuel, Chong et al. 2009). Exo70 proteins are known to act as a landmark for directing exocyst tethered vesicles, and RFP:Exo70A1’s observed localization pattern is in agreement with the exocyst complex delivering secretory vesicles to the papillary plasma membrane upon compatible pollinations. After pollen acceptance, RFP:Exo70A1 fluorescence was no longer observed at the papillary plasma membrane, showing the expenditure of the protein after the
completion of vesicle tethering. The release of female resources of a dry stigma for compatible pollen acceptance in the Brassicaceae is well established, and TEM analysis has demonstrated secretory vesicle fusion to the papillar plasma membrane upon compatible pollination.

GFP:Sec5a’s increase in fluorescence upon compatible pollination is the first direct in planta evidence of exocyst mediated vesicle transport caused by compatible pollination. Line 14’s increase in GFP:Sec5a signal is indicative of the stigmatic papillae undergoing a large amount of exocyst mediated vesicle transport. Although this indicates that an increase in SEC5a-associated vesicles after compatible pollination are distributed throughout the entire cytoplasm, it does not indicate the direction of their transport. The accumulation of GFP:Sec5a signal localized to the region of pollen contact in weaker expressing line 4 potentially shows polar transport of these vesicles. Additional observation of lines is required to provide a definitive picture of these vesicle dynamics.

4.5 Conclusions

Self vs. non-self recognition in Brassicaceae reproduction is an interesting example of how plants, being non-sentient, distinguish reproductive advantage. This ability to distinguish between one’s self and other individuals is mediated through signaling pathways that result in either the release of pollen acceptance factors for permitting fertilization, or the sequestration and degradation of aforementioned resources. Despite all that is currently known, additional questions remain in the involvement of ARC1 to deactivate Exo70A1 in a manner that induces autophagy to block required resources for pollen hydration. My thesis has addressed this by contributing work to investigate these processes at many levels.

Contributions this work has made include establishing resting ARC1’s plasma membrane
localization, autophagy’s involvement in *Arabidopsis thaliana* self-incompatibility, and visualizing the delivery of secretory vesicles in compatible pollination. A major component of this thesis has been in developing systems that have yet to be fully implemented. Continuation of this work will be instrumental in exploring Brassicaceae reproduction, an economically important crop family.
Bibliography


Tsukada, M. and Y. Ohsumi "Isolation and characterization of autophagy-defective mutants of Saccharomyces cerevisiae." (0014-5793 (Print)).


