AN INVESTIGATION OF THE EXOCYST COMPLEX AND ITS ROLE IN COMPATIBLE POLLEN-PISTIL INTERACTIONS IN ARABIDOPSIS

BY

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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ABSTRACT

Compatible interactions between male gametophytes (pollen) and the female reproductive organ (pistil) are essential for fertilization in flowering plants. Recognition at a molecular level allows “compatible” pollen grains to adhere/germinate on the stigma while pollen grains from unrelated plant species are largely ignored. The exocyst is a large eight subunit complex that is primarily involved in polarized secretion or regulated exocytosis in eukaryotic cells where it functions to tether vesicles to the plasma membrane. Recent research has implicated one of the Exo70 family members, Exo70A1, in compatible pollen-pistil interactions in Arabidopsis and Brassica. The loss of Exo70A1 in Arabidopsis Col-0 stigmas leads to the rejection of compatible pollen producing a “female sterile” phenotype. Through my research I have demonstrated that, driven by a stigma-specific promoter, an RFP:Exo70A1 fusion protein rescues this defect in exo70A1-1 mutant and Exo70A1 is found to be localized to the plasma membrane at flower opening.
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<th>Definition</th>
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<tbody>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>ABRC</td>
<td>Arabidopsis Biological Resource Centre</td>
</tr>
<tr>
<td>AGI</td>
<td>Arabidopsis Genome Initiative</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>ARC1</td>
<td>arm repeat containing 1</td>
</tr>
<tr>
<td>Arf</td>
<td>ADP-ribosylation factor</td>
</tr>
<tr>
<td>ARM</td>
<td>armadillo</td>
</tr>
<tr>
<td>AtExo70A1</td>
<td>Arabidopsis thaliana Exo70A1</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine phosphate</td>
</tr>
<tr>
<td>BAR</td>
<td>Bio-Array Resource</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>BnExo70A1</td>
<td>Brassica napus Exo70A1</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BY-2</td>
<td>bright yellow-2 (tobacco cells)</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>cer</td>
<td>eceriferum mutant</td>
</tr>
<tr>
<td>Col-0</td>
<td>Columbia (Arabidopsis)</td>
</tr>
<tr>
<td>CTAB</td>
<td>hexadecyltrimethyl-ammonium bromide</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>double distilled water</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DIC</td>
<td>differential interference contrast</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli (bacteria)</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>FRAP</td>
<td>fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>gDNA</td>
<td>genomic DNA</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Type 4 glucose transporter</td>
</tr>
<tr>
<td>GTPases</td>
<td>small guanosine triphosphatases</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>ICR1</td>
<td>adaptor protein</td>
</tr>
<tr>
<td>Kan</td>
<td>kanamycin</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani broth</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madine-Darby Canine Kidney cells</td>
</tr>
<tr>
<td>MLPK</td>
<td>M locus protein kinase</td>
</tr>
</tbody>
</table>
GFP  green fluorescent protein
MgSO₄  magnesium phosphate
mRNA  messenger RNA
MS  murashige skoog salt
MTOC  microtubule organizing center
N-  amino
NaCl  sodium chloride
NaOH  sodium hydroxide
ORF  open reading frame
P  phosphates
PCP  pollen coat protein
PCR  polymerase chain reaction
PIP  plasma intrinsic protein
PM  plasma membrane
Rab  Ras-like proteins (brain)
RFP  red fluorescent protein
Rho  Ras homologous
RNA  ribonucleic acid
RNAi  RNA interference
Rops  Rho of plants
rpm  rotations per minute
RT  reverse transcription
SCR/SP11  S cysteine-rich protein/S protein 11
sec  temperature sensitive secretory mutants
SI  self-incompatibility
SLG  S locus glycoprotein
SLR1  S locus related 1
SNARE  soluble N-ethyl-malemide-sensitive fusion protein attachment protein receptor
SRK  S receptor kinase
ST  sialyl transferase
TAIR  The Arabidopsis Information Resource
T-DNA  transferred DNA
TEM  transmission electron microscope
TIP  tonoplast intrinsic protein
TGN  trans-Golgi network
THL  thioredoxin h protein
Ub  ubiquitin
W1  Self-Incompatible cultivar of Brassica
Westar  Self-Compatibe cultivar of Brassica
Y2H  yeast two-hybrid
Chapter 1 : LITERATURE REVIEW

For my thesis, I have investigated the role of the exocyst complex in the *Arabidopsis* stigma, including its localization at various stages of a compatible pollen-pistil interaction. The exocyst is a multimeric complex involved in vesicle trafficking and tethering of vesicles to the plasma membrane in eukaryotic cells. In order to understand my research in an appropriate context, this literature review will highlight cellular processes such as; vesicle trafficking, the role of various docking complexes (focusing on the exocyst in other systems), and pollen-pistil interactions in members of the *Brassicaceae*.

1.1 Vesicle Trafficking in the Eukaryotic Cell

In a eukaryotic cell, vesicle trafficking is essential and has been implicated in a number of processes such as exocytosis and cell growth. Because the eukaryotic cell has a compartmentalized endomembrane system, the process of vesicle trafficking allows for cargo to be transported between different cellular organelles and compartments as well as exported to/imported from its surroundings. Cellular trafficking involves vesicle formation, vesicle movement throughout the cell, and fusion of the vesicle with an acceptor membrane. Vesicle trafficking occurs in the cell through two main pathways; endocytic and secretory.

The secretory (or anterograde) pathway directs newly synthesized proteins first to the endoplasmic reticulum (ER), through the Golgi apparatus, and finally to various locations in the cell including the plasma membrane (PM), endosomes, and vacuoles.
The endocytic pathway is the counterflow of cargo originating from the PM and deposited in the vacuole. In addition, this retrograde pathway provides a recycling mechanism within the cell in that it can be a means to retrieve and redirect proteins back to their appropriate compartments for reuse (Alberts et al. 2002). Vesicle trafficking in plants is involved in numerous processes such as cell growth, cell expansion, pollen tube elongation, and the plants immune response to pathogens (reviewed in Samaj et al. 2005; Robatzek 2007)

1.1.1 **Exocytosis**

Exocytosis is a highly ordered and specialized form of anterograde transport by which secretory vesicles are targeted specifically to the plasma membrane and contents are secreted to the cells’ outer surroundings (Figure 1). For example, following synthesis on the endoplasmic reticulum and transport through the Golgi apparatus, extracellular proteins cross the plasma membrane by exocytosis. Synthesized membrane components and integral membrane proteins necessary for plasma membrane growth and function during the life of the cell are also incorporated from the secretory vesicle membrane during exocytosis. In eukaryotes, the process of exocytosis is essential for intercellular communication, secretion of enzymes and defence compounds, addition of cell wall material, and maintenance of the plasma membrane.

1.1.2 **Polarized Membrane Traffic**

The concept of “polarity” is important to most eukaryotic cells. Specifically, polarization of the plasma membrane plays an important role in establishing cell polarity and varying the membrane composition can be a key determinant of this. Secretory proteins
Figure 1.  Exocytosis and Vesicle Trafficking

Anterograde trafficking in the cell consists of newly synthesized material moving through the ER to the Golgi/TGN and then to multiple destinations within the cell. Exocytosis is a specialized form of anterograde trafficking and involves the delivery of secretory vesicles from within the cell to the plasma membrane surface for fusion and release of cargo. Docking complexes at the plasma membrane facilitate tethering of vesicles to the membrane prior to SNARE-mediated fusion.
Figure 1

Docking complexes

Plasma Membrane

Endosome

Vesicles

Trans-Golgi

Vacuole

Vesicles

ER

Nucleus
are delivered to specific regions of the plasma membrane, thereby defining and maintaining a unique identity to a particular region. Polarized delivery and incorporation of proteins into specific regions of the plasma membrane are fundamental to a wide range of biological processes such as neuronal synaptogenesis (Hsu et al. 1998) and epithelial cell polarization in mammals (reviewed in Gibson and Perrimon 2003).

In plants, tip growth processes, such as growth in root hairs of Arabidopsis, is an excellent example of polarized membrane trafficking for the purpose of cell expansion. The root hair is a single elongated cell present along the roots of plants that aid in maximizing absorption of nutrients from the surrounding environment (Carol and Dolan 2002). Root hairs are cylindrical, tubular outgrowths oriented perpendicular to the main cell axis in the root. At the tip of the hair, adjacent to the plasma membrane is a dense cytoplasm filled with clathrin-coated secretory vesicles derived from the Golgi apparatus and endoplasmic reticulum. These vesicles lie primarily along microtubule and actin networks and their purpose is delivery of cellular cargo (such as components needed for synthesis of new membranes) to specifically targeted areas within the cell (Galway et al. 1994, 1999). Upon maturation of the root hair, this vesicle-rich zone at the tip disappears as a secondary cellulose layer is laid down in the cell wall (Galway et al. 1999). Root hairs are also highly vacuolated with the central vacuole occupying most of the diameter of the cell, but stopping short of the apical zone (Galway et al. 1997, 1999).

The concept of tip growth will be important to understand as it can also be applied to the development of stigmatic papillae in the Arabidopsis flower. Papillae are also derived from a single elongated cell and therefore, this polarized tip growth process will be
relevant when discussing the development of papillae and its contribution to compatible pollen-pistil interactions.

1.1.3 **GTPases and Vesicular Movement Throughout the Cell**

Small guanosine triphosphatases (GTPases) are “molecular switches” found throughout the cell and are required in many cellular processes. Each step of the cellular trafficking process is facilitated by GTPases which have two conformational forms: a GDP-bound inactive form and a GTP-bound active form (Schmidt and Hall 2002). Transformation to its active conformation from the inactive state is regulated by guanine nucleotide exchange factors (GEFs) and only in its active state are GTPases able to recruit downstream effectors for different roles within a cell. Control of cycling within the cell and inactivation of GTP-bound GTPases is regulated by GTPase-activating proteins (GAPs) (Bernard and Settleman 2004).

There are five major subfamilies in the Ras superfamily of GTPases (Ras, Rho, Rab, Ran and Arf) and a conserved G-nucleotide domain required for GTP/GDP binding is the common motif between family members (Paduch et al. 2001). While Ras GTPases are not found in the plants (Leipe et al. 2002), in the mammalian system they are thought to be involved in cell proliferation and control of gene expression (Repasky et al. 2004). Rho GTPases are regulators of polarization, membrane traffic, cell growth and development. Rho GTPases are also not found in plants and have been implicated in the yeast system to aid in cell cycle progression as well as actin organization (Olsen et al. 1995; Ridley 2006). A well-studied Rho GTPase, Cdc42, is an essential regulator in establishing polarity in yeast (Zhang et al. 2001; Park and Bi 2007) and it is also thought to play a role in the recruitment of exocyst subunits (Munson and Novick 2006).
While Rho GTPases are not officially present in plants, there is a class of “Rho of plants” GTPases called the Rops. Rops have been found to be involved in polarized tip growth in pollen tubes (Li et al. 1998).

Rab GTPases represent the largest sub-family of Ras-like GTPases that play key roles in the secretory and endocytic pathways. Rab GTPases are present in animal systems as well as plants and there are 57 Rab GTPase members in *Arabidopsis* (reviewed in Vernoud et al. 2003). Rabs are membrane-bound, localized on distinct compartments of the cell and are important for compartment recognition of membrane-bound compartments targets (Novick and Brennwald 1993; Novick and Zerial 1997). Rab GTPases are also known to regulate intracellular transport of vesicles in secretory pathways (Zerial and McBride 2001). Interestingly, mutating Rab proteins can result in a breakdown of protein transport in addition to changes in the sizes of entire organelles (Pfeffer 1999). Clearly, Rabs play many crucial regulatory roles in membrane trafficking, in fact, Rabs are used as markers for a specific compartment within the cell. For example, the Golgi is marked by the RabH group (Latijnhouwers et al. 2007). In plants, the function of numerous Rabs has been characterized. In *Arabidopsis*, *rabA4b* mutants displayed defects in the regulation of vesicle trafficking at the root tip resulting in diminished root hair growth (Preuss et al. 2004). Recent studies have also found that mutant pollen in which the *AtRabA4d* gene was disrupted displayed bulged pollen tubes with a reduced rate of growth *in vitro* as well as showed altered deposition of some cell wall components (Szumlanski and Neilson 2009). As well, *AtRabG3b* has been identified as a modulator for cell death progression during pathogen response and senescence process in plants (Kwon et al. 2009).
Ran GTPases are found in the plant system and are likely involved in and crucial for establishing nucleocytoplasmic transport directionality (Moore and Blobel 2003). In addition, Ran GTPases have also been implicated in playing an important role in cellular mitosis acting at the end of the process and inducing nuclear envelope reassembly (Kalab et al. 1999). The ADP-ribosylation factor (Arf) family of GTPases is involved in recruiting factors contributing to vesicle formation and coat formation at the endoplasmic reticulum membrane and in vesicle transport in the cell (Pasqualato et al. 2002). Arfs are found in both the animal and plant systems with 21 reported Arf GTPases in *Arabidopsis* (Memon 2004). Overall, GTPases play an important role in signal transduction and vesicle transport within the cell, both are process important during cell polarization.

Within the cell, vesicles and other motor proteins are transported on the cell’s cytoskeletal network composed of actin filaments or microtubules. In the dynamic actin network, microfilaments are oriented with their plus-ends facing the plasma membrane of the cell (Cramer 1999), and in yeast, exocytotic vesicles (cargo) are transported along the actin cytoskeleton using plus-end directed myosin motor proteins (Finger and Novick 1998). In animals, short-term transport is thought to occur along the actin network where, alternatively, in plants, long actin filaments are thought to facilitate and provide a route for long-term transport within the cell (Langford 2002).

Microtubules also form a highly organized system within the cell. Motor proteins that are associated with microtubules include kinesins and dyneins (Reddy and Day 2001). In animals, microtubules also have directionality in that their minus-ends are anchored to the microtubule organizing center (MTOC) and their plus-ends reach out to the
periphery of the cell. Kinesin motor proteins have the ability to traverse the microtubules in either direction (plus- or minus-end directed) (Kull et al. 1996). It is interesting that plants appear to lack a classic MTOC but it has been suggested that it is this very feature that has allowed plants the flexibility to be highly adaptive to their changing environments (Wasteneys 2002). Protein and vesicle motility is often actin-dependant (Langford 2002) and this will become important when looking at exocytotic processes in the stigma of Arabidopsis.

1.1.4 Docking/Tethering Complexes

When a Rab GTPase is recruited to a membrane, the GDP is exchanged for a GTP and the Rab becomes associated with the target membrane. The now activated Rab can begin recruitment of other effectors (for the purpose of vesicle membrane fusion) to the site such as docking complexes. One of the effectors recruited are SNAREs (soluble N-ethyl-maleimide–sensitive attachment protein receptors). Overall the vesicle fusion machinery appears highly conserved (Sanderfoot et al. 2000) and SNARE protein families are generally large (reviewed in Jahn and Scheller 2006) with 25 in yeast and 36 in humans. The Arabidopsis genome contains a remarkable number of SNAREs with 68 members, and they play a role in (but are not sole determinants of) membrane specificity of docking vesicles and are absolutely essential for membrane fusion events (reviewed in Chen and Scheller 2001). In order to facilitate a membrane fusion event, four SNARE motifs are required to form a stable complex including the SNAREs on opposing membranes (Jahn and Scheller 2006). The result of their coiled-coiled domains interacting is a helical bundle that essentially brings the two membranes into close enough proximity for the lipid bilayers to fuse.
Prior to the action of SNAREs, other docking/tethering complexes are involved in assisting vesicle docking at the membrane site. These complexes are often multimeric structures that also play a role in conferring specificity of the docking vesicle to the target membrane. The term *tethering* refers to the initial recognition and binding to bring two membrane compartments in close proximity of each other and essentially acting as a “molecular bridge” (Wiederkehr et al. 2004). The tethering complex of interest for this study is the exocyst complex which has been documented to be involved in docking of exocytotic vesicles (reviewed in Cai et al. 2007).

1.2 **The Exocyst Complex**

The exocyst is a large eight subunit multimeric complex that is primarily involved in polarized (targeted) exocytosis in eukaryotic cells where it functions in the tethering and docking of exocytotic vesicles to the plasma membrane. The Exo70 subunit is an essential component of the complex and is believed to serve as a spatial landmark, along with Sec3 on the plasma membrane to indicate where the exocyst complex is to assemble (Finger and Novick 1998). Previous research has found the exocyst complex to be involved in many processes including yeast budding and the trafficking of the Glut4 transporter to the plasma membrane in adipocyte cells (as discussed below) (Inuoe et al. 2003).

1.2.1 **Features Common to All Systems**

Orthologs of the exocyst have been identified in yeast, animal and more recently, plant systems. The exocyst has eight subunits: Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p and Exo84p. However, the subunit interactions and complex assembly
conformation are not entirely conserved between each system. A feature common across all systems appears to be the exocyst’s role in directing and tethering vesicles to areas of the plasma membrane undergoing polarized or targeted growth (ie. tip growth and budding sites).

1.2.2 The Yeast Exocyst
The exocyst complex was originally identified in budding yeast (Saccharomyces cerevisiae) through the isolation of 23 temperature-sensitive secretory (sec) mutants (Novick et al. 1980). Six of the Sec proteins were found to co-immunoprecipitate with newly identified Exo70p and the complex was named the exocyst (Terbush et al. 1996). The eight exocyst subunits include Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p and Exo84p (TerBush et al. 1996; Guo et al. 1999a) (Figure 2; Table 1). The yeast Sec genes are all single copy (Novick et al. 1980), and the total size of the yeast exocyst complex is approximately 845kDa (Hsu et al. 1998). Initial characterization studies found that mutations in the Sec genes led to an accumulation of transport vesicles in the cytoplasm of the cell (Finger and Novick 1997) and halted formation of the growing polarized buds in yeast (TerBush et al. 1996). From subcellular immunofluorescence localization studies as well as molecular association analysis, it has been proposed that the yeast exocyst assembles at sites of exocytotic vesicle fusion at the budding site and is responsible for establishing the initial connection to the plasma membrane. Vesicle fusion is most active at the tips of small buds, and considering its function, it is no coincidence that this is also the site of exocyst localization in budding yeast (Guo et al. 1999b).
The yeast exocyst acts as a tether to dock post-Golgi secretory vesicles wherein their membrane fusion is promoted by SNAREs (Novick et al. 1980; Hsu et al. 2004) and is often referred to as a Rab effector complex. Subcellular localization of the exocyst is regulated by both Rab and Rho GTPases (Guo et al. 1999a) and vesicle tethering to the exocyst is thought to be regulated by the Rab GTPase, Sec4p, which is present on the secretory vesicle (Goud et al. 1988). Activated (GTP-bound) Sec4p interacts with the Sec15p subunit of the exocyst (Roth et al. 1998; Guo et al. 1999a) and this interaction is exclusive as Sec15p did not interact with any other Rab GTPases when tested (Guo et al. 1999a). Cdc42, a member of the Rho family of small GTPases, appears to control the localization of the exocyst and similarly, the rho1 mutant resulted in mislocalization of all subunits (Zhang et al. 2001; Guo et al. 2001).

In yeast, the fusion protein Sec3p-GFP was found to localize at sites of polarized exocytosis (new bud sites and the mother-bud neck) and localization of Sec3p is independent of the actin cytoskeleton (Finger et al. 1998). This suggests that Sec3p serves as the spatial landmark on the plasma membrane arriving independent of the cytoskeletal network, recruiting the remaining exocyst subunits for assembly into the functional complex (Finger and Novick 1998). In fact, fluorescence recovery after photobleaching (FRAP) analysis has recently demonstrated that Sec3p and Exo70p arrive at the plasma membrane in an independent manner prior to exocyst assembly and vesicle fusion (Boyd et al. 2004) suggesting that perhaps both subunits are acting together to landmark exocyst assembly.
Figure 2. Yeast exocyst subunit interaction map

Protein:protein interactions between yeast exocyst subunits and small GTPases as compiled from an extensive literature review (Table 1).

[ — ] These protein:protein interactions were determined by co-immunoprecipitation assays

[ . . . ] These protein:protein interactions were determined by using purified recombinant proteins in pull-down assays

[ - - - ] These protein:protein interactions were determined by yeast two-hybrid assays

[ ▯ ▯ ] These protein:protein interactions were determined by two or more assays (co-immunoprecipitation, yeast two-hybrid or pull down assays)

PM = plasma membrane

○ = exocyst subunit

□ = GTPase

ষ = secretory vesicle
Figure 2

YEAST EXOCYST

PM


EXO70p — EXO84p

Rho1p — Rho3p

Cdc42p

Bem1p

RalA

SEC4p GTPase

Vesicle
<table>
<thead>
<tr>
<th>Subunit</th>
<th>Interactor</th>
<th>Proof</th>
<th>Source</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEC3p</td>
<td>Sec5p</td>
<td>Y2H, Co-IP</td>
<td>Guo et al. 1999a</td>
<td></td>
</tr>
<tr>
<td>SEC5p</td>
<td>Sec3p</td>
<td>Co-IP, Y2H</td>
<td>Guo et al. 1999a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sec6p</td>
<td>Co-IP</td>
<td>Guo et al. 1999a</td>
<td></td>
</tr>
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<td>Sec10p</td>
<td>Y2H</td>
<td>Guo et al. 1999a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exo70p</td>
<td>Co-IP, pull-down</td>
<td>Guo et al. 1999a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exo84p</td>
<td>Y2H</td>
<td>Guo et al. 1999b</td>
<td>weak interaction</td>
</tr>
<tr>
<td>SEC6p</td>
<td>Sec5p</td>
<td>Co-IP</td>
<td>Guo et al. 1999a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sec8p</td>
<td>Co-IP, pull-down</td>
<td>Guo et al. 1999a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sec10p</td>
<td>pull-down</td>
<td>Sivaram et al. 2006</td>
<td></td>
</tr>
<tr>
<td></td>
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<td>Dong et al. 2005</td>
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<td>pull-down</td>
<td>Guo et al. 1999</td>
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<td>SEC10p</td>
<td>Sec5p</td>
<td>Y2H</td>
<td>Guo et al. 1999a</td>
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<td>pull-down</td>
<td>Sivaram et al. 2006</td>
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<tr>
<td></td>
<td>Sec15p</td>
<td>Y2H, Co-IP</td>
<td>Guo et al. 1999a</td>
<td>Nterm interaction</td>
</tr>
<tr>
<td></td>
<td>Exo70p</td>
<td>pull-down</td>
<td>Dong et al. 2005</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exo84p</td>
<td>Y2H</td>
<td>Guo et al. 1999b</td>
<td>bridging effect?</td>
</tr>
<tr>
<td>SEC15p</td>
<td>Sec10p</td>
<td>Y2H, Co-IP</td>
<td>Guo et al. 1999a</td>
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</tr>
<tr>
<td>Exo70</td>
<td>Sec5p</td>
<td>Co-IP, pull-down</td>
<td>Guo et al. 1999a</td>
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</tr>
<tr>
<td></td>
<td>Sec6p</td>
<td>pull-down</td>
<td>Dong et al. 2005</td>
<td>strong interaction</td>
</tr>
<tr>
<td></td>
<td>Sec8p</td>
<td>pull-down</td>
<td>Dong et al. 2005</td>
<td>strong interaction</td>
</tr>
<tr>
<td></td>
<td>Sec10p</td>
<td>pull-down</td>
<td>Dong et al. 2005</td>
<td>strong interaction</td>
</tr>
<tr>
<td>Exo84p</td>
<td>Sec5p</td>
<td>Y2H</td>
<td>Guo et al. 1999b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sec10p</td>
<td>Y2H</td>
<td>Guo et al. 1999b</td>
<td></td>
</tr>
</tbody>
</table>

Y2H = Yeast two-hybrid  
Co-IP = Co-immunoprecipitation  
Cterm = Cterminus  
Nterm = Nterminus
The localization of the “landmark” is dynamic and has been shown to be cell-cycle dependent because the sites of exocytotic secretion shifts throughout the cycle (Boyd et al. 2004).

The remaining six subunits are transported to the sites of exocyst assembly via secretory vesicles and therefore act in an actin-dependent manner (Finger and Novick 1998; Boyd et al. 2004). Proof of exocyst assembly started to surface when Exo84p was also shown to localize to the bud tip of the mother/daughter connection which are known regions of polarized secretion in yeast. Interestingly, evidence that Exo84p plays a key role in spatial organization came when Zhang et al. (2005) reported that mutations in Exo84p resulted in mislocalization of Exo70p, as well as Sec10p and Sec15p suggesting that exocyst is a dynamic signaling process as many of the subunits seem to have a function in determining location of complex assembly. The other Sec genes were also found to be localized in areas known for exocytotic activity. For example, immunofluorescence microscopy analysis showed high concentrations of Sec8p at the tip of small buds in yeast (TerBush and Novick 1995) further strengthening the theory that indeed the exocyst was assembled at areas of increased polarized exocytosis. Additionally, the presence of SNAREs at these areas of increased exocytotic activity has been shown in that a SNARE-binding component (Sec1p) can be co-precipitated with the exocyst indicating that Sec1p likely links exocyst-mediated vesicle tethering with SNARE-mediated vesicle docking and fusion (Wiederkehr et al. 2004).

1.2.3 The Mammalian Exocyst

The mammalian exocyst complex (originally termed the Sec6/Sec8 complex) has been characterized based on sequence homology to the yeast subunits and was found to be
approximately 736kDa (Kee et al. 1997). Highly conserved mammalian homologs of all eight yeast exocyst subunits have been identified (Hsu et al. 1998). Isolated from rat brain, rSec6 and rSec8 were the first subunits to be identified in the mammalian system (Ting et al. 1995). Yeast two-hybrid, co-immunoprecipitation and pull-down assay studies revealed that the same subunits of the exocyst complex in the mammalian system exist in a slightly different configuration than its yeast counterpart (Figure 3; Table 2). For example, in the mammalian system, Exo70 serves as the spatial landmark and is responsible for exocyst assembly on the plasma membrane for outbound vesicles, although Sec3 has not been shown to act as a spatial landmark in the mammalian system (Matern et al. 2001; Li and Chin 2003). While the configuration of the complex is not identical between systems, similarities include regulation through GTPases. For example, Rho GTPase (TC10) is required for Exo70 subunit (and thus, exocyst) localization to the plasma membrane in mammalian cells (Inoue et al. 2003).

Similar to its proposed function in yeast, the mammalian exocyst has proven to be an essential player in the exocytotic pathway and crucial for mammalian development. For example, Vega and Hsu (2001) have demonstrated that the exocyst is present at the tip of the growing neurite in neuronal cells. As well, mammalian epithelial cells maintain polarity by delivering specific proteins to their rightful place in the basolateral or apical regions of the plasma membrane, therefore allowing distinction between these membrane regions (Mostov et al. 1992). In fact, most polarized cells have distinct plasma membrane properties which are the result of polarized trafficking of proteins and lipids to targeted areas of the cell and do not display a ubiquitous distribution. Inhibition
Figure 3. Mammalian exocyst subunit interaction map

Protein:protein interactions between mammalian exocyst subunits and small GTPases. Interaction maps compiled from extensive literature review (Table 2).

[ — ] These protein:protein interactions were determined by co-immunoprecipitation assays

[ . . . ] These protein:protein interactions were determined by using purified recombinant proteins in pull-down assays

[ - - - ] These protein:protein interactions were determined by yeast two-hybrid assays

[ — ] These protein:protein interactions were determined by two or more assays (co-immunoprecipitation, yeast two-hybrid and pull down assays)

PM = plasma membrane

○ = exocyst subunit

□ = GTPase

□ = secretory vesicle
Figure 3

MAMMALIAN EXOCYST

PM

EXO70

SEC15

SEC10

EXO84

SEC8

SEC3

SEC5

SEC6

Rab11

TC10

Arf6

RalA/B

Vesicle

Rab1A
<table>
<thead>
<tr>
<th>Subunit</th>
<th>Interactor</th>
<th>Proof</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>SEC3</td>
<td>Sec5</td>
<td>Y2H</td>
<td>Matern et al.</td>
</tr>
<tr>
<td></td>
<td>Sec8</td>
<td>Y2H</td>
<td>2001</td>
</tr>
<tr>
<td>SEC5</td>
<td>Sec3</td>
<td>Y2H</td>
<td>Matern et al.</td>
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<td></td>
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<td>SEC6</td>
<td>Sec5</td>
<td>Y2H</td>
<td>Matern et al.</td>
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<td>Sec8</td>
<td>Y2H</td>
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<tr>
<td></td>
<td>Sec10</td>
<td>pull-down</td>
<td>Vega et al.</td>
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<td>Vega et al.</td>
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<td>SEC8</td>
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<td>Y2H</td>
<td>Matern et al.</td>
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<td>Sec6</td>
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<td>Vega et al.</td>
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<td>Vega et al.</td>
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<td>Exo70</td>
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<td>2001</td>
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</table>
of polarized plasma membrane protein transport was first demonstrated by introducing antibodies against Sec8 into Madin-Darby canine kidney (MDCK) cells (Grindstaff et al. 1998).

It was also shown that Sec8 is essential for the biogenesis of epithelial cell polarity (Grindstaff et al. 1998). In fully polarized epithelial cells, the mammalian exocyst appears responsible for targeting proteins to the basolateral membrane (Yeaman et al. 2004). Furthermore, over-expression of exocyst subunits Sec6 and Sec10 resulted in an increase in the synthesis of basolateral plasma membrane proteins (Lipshutz et al. 2000), suggesting that components of the exocyst complex also contribute to the regulation of exocytosis at the protein synthesis level.

Perhaps the most convincing example of exocyst involvement in polarized transport in mammalian cells is the Type-4-Glucose Transporter (GLUT4) system in adipocyte cells. For insulin-stimulated glucose uptake to occur there is a highly regulated process of exocyst complex tethering of preloaded GLUT4 glucose transporter-containing vesicles to the plasma membrane (Inoue et al., 2003). Insulin perception results in the activation of the TC10 GTPase which in turn leads to the re-localization of Exo70 to lipid raft domains in the plasma membrane. The exocyst complex is then assembled and tethers GLUT4 vesicles to the plasma membrane, and thus, delivering GLUT4 transporters for glucose transporters (Inoue et al. 2003, 2006). A decreased level of GLUT4 at the plasma membrane was seen when subunits Sec6, Sec8 and Exo70 were knocked-down (Inoue et al. 2006). Thus, many studies have concluded that the mammalian exocyst, like its yeast counterpart, has a direct role in regulating vesicle trafficking by
acting as a tethering method to anchor exocytotic secretory vesicles to specific plasma membrane domains for vesicle fusion (Wang and Hsu 2006).

1.2.4 Plant Exocyst

While all eight subunit orthologs have been identified in *Arabidopsis thaliana*, there is very little information available on the function of each subunit in plant systems. A molecular mass of about 900kDa has been reported for the plant exocyst (Hala et al. 2008) which is larger then the expected 760kDa, and it is speculated this increase is due to proteins, such as adaptor proteins, interacting with the complex. Studies have reported that *Arabidopsis* has a single copy of Sec6, Sec8, and Sec10, two copies each of Sec3, Sec5 and Sec15, three copies of Exo84, and a 23 member gene family of Exo70 (Elias et al. 2003). Having 23 genes encoding the Exo70 subunit in *Arabidopsis* is in contrast to the one copy of Exo70 in yeast and mammals and indicates that the Exo70 family may have expanded to adapt to some specialized features. These specializations could include such plant-specific functions as vesicular trafficking in pollen tip growth, root hair growth and stigmatic papillae elongation (Synek et al. 2006; Chong et al. 2009).

Original analysis of *Arabidopsis* plants with insertional mutations in the highly expressed Exo70 gene family members - *AtExo70B2, AtExo70D3, AtExo70F1, AtExo70G1,* and *AtExo70H7* - did not result in any immediately detectable phenotypes, indicating a possible functional redundancy between these family members (Synek et al. 2006). This is in a sharp contrast to the distinct phenotypes seen in single Exo70A1 mutants (*exo70A1-1*) in plants (Synek et al. 2006; Samuel et al. 2009). It has been suggested that perhaps only a selected sub-group of the Exo70 proteins function in the exocyst
complex and others have evolved to perform new regulatory roles within the plant, independent of the exocyst complex (Synek et al. 2006; Chong et al. 2009).

The first reports of predicted exocyst function in plants came when Wen et al. (2005) identified a previously studied maize root hair mutant, defective in root hair elongation, to be the putative maize Sec3. The roothairless1 (rht1) mutation was the result of a transposon insertion into the Sec3 gene causing improper elongation of root hairs. This mutant phenotype could be due to a disruption in the tip growth process which relies heavily on polarized exocytosis of plasma membrane building components at the tip of root hairs resulting in lengthwise elongation of the cell (as reviewed in Carol and Dolan 2002). Further confirming the involvement of predicted exocyst proteins in exocytotic processes are studies on Arabidopsis thaliana plants with mutations in Sec5a/b, Sec6, Sec8 and Sec15a which displayed reduced pollen tube growth rates causing a partial transmission defect (Cole et al. 2005; Hala et al. 2008).

Synek et al. (2006) have documented an array of phenotypic defects in insertional mutations in the Exo70A1 gene (exo70A1-1 and exo70A1-2). Both polar growth of root hairs and stigmatic papillae were disrupted. Morphologically, the plant organs were smaller and the plants showed a loss of apical dominance, a dwarf stature, and a dramatically reduced fertility (Synek et al. 2006). Overall analysis of the available data suggests that the putative exocyst subunit Exo70A1 plays a role in cell expansion and organ morphogenesis. As previously mentioned, a pre-requisite for the exocyst to carry out its proposed function, its components must be shown to interact and co-localize in the region of the cell where the exocyst would be active, for example, polarized sites of secretion. In the plant system (using Arabidopsis), yeast two-hybrid analysis revealed
strong interactions between Sec3a/Exo70A1, Sec15b/Sec10, and Sec6/Sec8 (Hala et al. 2008). As well, it has been demonstrated that Sec6, Sec8 and Exo70 all co-localize to the tobacco pollen tube tip which is a site of secretion and polarized exocytosis (Hala et al. 2008). Similar to yeast and mammalian systems, the Sec3 has been shown to interact with Rho GTPases, specifically through the adaptor protein ICR1 in vivo (Lavy et al. 2007).

Recently, it has been proposed that the Arabidopsis exocyst complex may have a role in vesicular trafficking within “recycling domains” thought to reside at the trans-Golgi network (TGN) and early endosome cellular compartments (Zarsky et al. 2009) and this prediction is consistent with results obtained using transiently transformed bright yellow-2 (BY-2) tobacco cells and various markers for the exocyst subunits and subcellular organelles (Chong et al. 2009). While BY-2 cells are undifferentiated do not undergo polarized secretion, these cells nonetheless provided a number of interesting patterns of localization of exocyst subunits (Chong et al. 2009). The localization of various exocyst components were compared to markers in the endocytic pathway, namely, SNARE proteins, Syp21, Syp42, and Syp52 and co-localization results revealed that exocyst subunits localized to globular-like structures in the perinuclear region of the cell. This perinuclear region is likely to include early and late endomembrane/TGN, as assessed by overlapping localization of exocyst subunits with components of the endosome system. This pattern is also supported by research in other systems, notably the mammalian system wherein the exocyst was reported to facilitate docking of vesicles at the plasma membrane originally derived from the endomembrane system (Prigent et al. 2003; Zhang et al. 2004).
Taken together, the data collected on the plant exocyst complex reveals strong evidence that the function of the exocyst in plants mimics that demonstrated in other systems, indicating a conservation of function at the cellular level.

### 1.2.5 Conserved Interactions between Exocyst Subunits

An extensive literature search and review was undertaken to determine a comprehensive interaction map for the animal and yeast exocyst subunits and interacting GTPases. Looking at the yeast exocyst (Figure 2, Table 1) and the animal exocyst (Figure 3, Table 2), interactions between the subunits has been shown using different assays including co-immunoprecipitation, yeast two-hybrid and pull-down assays. After compiling the data from the literature, it became apparent that there are a significant number of interactions conserved between yeast and mammalian exocyst subunits (Table 3) but there are also some notable differences. For example, in the yeast exocyst, Sec3p and Sec8p do not seem to have a direct interaction as is seen in the animal exocyst. Conversely, Sec5p in yeast interactions with Sec10p and Exo70p and this relationship is not seen in the animal system. It is important to note, however, that an absence of a reported interaction does not necessarily mean that the two subunits do not interact \textit{in vivo}, but rather it may not have been detected yet.

More recently, a yeast two-hybrid study was published discussing pair-wise interactions in the plant exocyst (Hala et al. 2008). The authors found two sets of subunits that seem to have conserved interaction across the three systems. Sec6 and Sec8 subunits have been shown to interact in yeast, mammals and now plants along with Sec15b and Sec10. What was interesting was that a new subunit relationship has been detected
solely in plants (Hala et al. 2008) and while part of the same multimeric complex, this potential direct interaction between Sec3 and Exo70 has not been previously recorded.

The plant exocyst has been implicated in a number of cellular processes including a role in compatible pollen-pistil interactions through its role in the tip-growing papillae cells in the stigma and this connection will be discussed in the following sections.

1.3 Compatible Pollen-Pistil Interactions in Brassicaceae

In Brassicaceae, the central position of the flower is occupied by the female reproductive organ, the pistil, which is the pollen-accepting organ composed of two fused ovule-bearing carpals. At maturation of the flower, the pistil consists of the stigma, style, and the ovary (Figure 4). There are two types of stigmas: wet and dry. Wet stigmas indiscriminately capture pollen due to a rich exudate covering the stigma (Luu et al. 1999). Whereas, dry stigmas have papillae that are absent of surface secretions and are covered in a cuticle/pellicle which together act as a barrier to pollination (Swanson et al. 2004). On the male side, it is important to note that pollen grains have two wall layers. Specifically, the intine (cellulose-based) and the exine (sporopollenin-based), the latter of which is where the pollen coat is deposited (Edlund et al. 2004). Upon pollen landing on the papillae cells of the stigma, there is a cascade of ordered events leading to a successful pollination.
Table 3. Interactions conserved between subunits in the yeast and mammalian exocyst complex

Interactions between exocyst subunits have been found to be conserved in both the yeast exocyst and mammalian exocyst based on the available literature (see Figures 2, 3 and Tables 1, 2). These interacting pairs are excellent candidates for exploration of interacting subunits in the plant exocyst complex, wherein subunit orientation is currently unknown.
### Table 3: Common Interacting Subunits

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Successful reproduction in plants can be an extremely selective process. If a random pollen grain lands on a stigma of another plant species, it is not able to complete fertilization because an active recognition system is in place to allow only successful fertilization following related-species compatible pollinations. In a compatible pollen-pistil interaction, the pollen hydrates on the stigmatic papillae and germinates with a pollen tube emerging from the pollen grain. The successive events that lead to a compatible pollination can be divided into four stages; pollen capture/adhesion and “foot” formation, pollen hydration, pollen germination/tube emergence and pollen tube penetration (reviewed in Edlund et al. 2004). In plant species with “dry” stigmas, such as Brassica and Arabidopsis, this recognition process starts at the earliest stages of pollen capture and adhesion (Roberts et al. 1980; Dickinson 1995; Swanson et al. 2004).

My research looked at pollen-pistil interactions in species with a dry stigma (namely Arabidopsis) which requires stigmatic papillae and components from the pollen for adhesion and will be focused on the cascade of events occurring in the papillae at the initial point of pollen-pistil contact and the molecular events that follow in the stigma proceeding after the recognition of compatible pollen.
**Figure 4. Arabidopsis pistil (female reproductive organs)**

DIC image taken of a Col-0 pistil using an epifluorescence microscope. Structural features of the pistil include the stigmatic papillae, stigma, style, and ovary.

The stigma is the upper portion of the pistil that receives the pollen and the stigmatic papillae are the finger-like projections on top of the stigma that first come in contact with the pollen.

The style is the stalk-like conduit underneath the stigma, yet above the ovary which allows the pollen tubes to grow down to the ovules for fertilization.

The ovary contains the ovules (female gametophyte) and is the large lower portion of the pistil.

Scale bar = 100µm
Figure 4

DIC

Papillae

Stigma

Pistil

Style

Ovary
1.3.1 **Stages of Stigma Development**

It is critical that the stigma be at the correct stage of development in order to be receptive to pollen. Stigma receptivity refers to its ability to capture pollen and provide the hydration and factors necessary for the pollen to germinate a pollen tube (Heslop-Harrison 2000). Flower development in *Arabidopsis* has been divided into twelve stages using various landmark events during maturation (Smyth et al. 1990). It is essentially the final stages of flower development that are crucial for the stigma’s ability to be receptive to pollen. Stage 11 of flower development is the tight bud stage and begins when the epidermis of the stigma differentiates to form specialized papillae cells which look like small finger-like projections on top of the pistil covering the entire stigmatic surface. These papillae cells are immature at this point, with their stature bearing a short and stumpy appearance. Stage 12 is the final bud stage and during which, the upper part of the stigma becomes differentiated into a short, 100μm to 120μm long style and a clear boundary separates it from a cap of stigmatic papillae (Smyth et al. 1990) (Figure 5A). Upon bud maturity at the end of stage 12, the densely packed papillae cells are 20μm to 35μm long (Smyth et al. 1990) and the papillae continue to rapidly elongate and the flower bud is mature and ready to open. Stage 13 commences upon flower opening as the papillae are fully elongated and the stigma is appropriately receptive as anthesis of the anthers (male organ) occurs, releasing pollen ready to pollinate the stigma and complete the fertilization process (Smyth et al. 1990) (Figure 5A).

1.3.2 **Pollen Capture, Adhesion and Formation of the Pollen “Foot”**

Studies have shown that pollen adhesion on a dry stigma depends on pollen components. For example, multiple pollen mutants in *Arabidopsis* were found to be specifically defective in the initial pollen adhesion step (Edlund et al. 2004). The pollen
grain has a highly sculptured exine outer layer with a pollen coat composed of lipids and proteins filling in the cavities of the exine (Mayfield et al. 2001; Murphy 2006). This stage of pollen capture and initial adhesion is mediated by the exine and does not appear to be dependent on the pollen coat since this initial stage is not affected when the pollen coat is absent (Zinkl et al. 1999). Interestingly, mutants defective in adhesion, such as less adherent pollen1 (lap1), still appear to be able to hydrate and germinate even in spite of its abnormal exine properties (Zinkl and Preuss 2000).

Upon contact and initial adhesion with the stigmatic papillae, the pollen forms a more robust connection composed of adhesive elements important for pollen-stigma recognition (Roberts et al. 1980; Stead et al. 1980; Zinkl et al. 1999). This step is now mediated by the “glue-like” surface properties of both the pollen grain and stigma (Clarke et al. 1979; Roberts et al. 1980; Zinkl et al. 1999). In the Brassicaceae, the surfaces of the stigmatic papillae are coated with a continuous waxy cuticle and a thin overlaying proteinaceous pellicle. It is the pellicle which has the adhesive properties and has been proposed to be essential for pollen-stigma recognition events, specifically during foot formation, since removal of the pellicle disrupts these events (Mattsson et al. 1974; Stead et al. 1980). To achieve this robust connection, there is a cross-linking between the pollen grain and the stigmatic papillae surfaces (Elleman et al. 1992; Zinkl et al. 1999) and as the contents of both the pollen and stigmatic surfaces mix, a meniscus-shaped “foot” forms at the interface (Elleman and Dickinson 1990; Preuss et al. 1993) (Figure 5B).
Figure 5. Stages of *Arabidopsis* stigma development and early events following a compatible pollen-pistil interaction

(A) At flower development stage 12, the flower bud is still tightly closed and the stigmatic papillae are not yet elongated and bear a short and stumpy appearance. Once flower development has moved into stage 13, the papillae are fully elongated (at about 75-100μm) and are now receptive to pollen. Red arrows indicate relative size of stigmatic papillae at each stage. Scale bar = 100μm

(B) Early events following compatible pollen contact with a stigmatic papilla. Pollen capture is followed by a “foot” formation allowing a strong adhesion to the papilla surface so hydration of the pollen can proceed. Once the pollen is hydrated and thus, metabolically active, the pollen tube emerges and penetrates into the papilla surface making its way down to the ovules for fertilization.
Figure 5

A

Stage 12
Immature papillae from closed bud

Stage 13
Mature elongated papillae from open flower

B

Pollen Capture and “Foot” Formation

Pollen Hydration

Pollen tube penetration
Two stigma-specific proteins, the S locus glycoprotein (SLG) and the S locus related-1 (SLR1) protein have been proposed to be required for pollen recognition and foot formation with the pollen in Brassica (Luu et al. 1997, 1999). Decreased pollen adhesion was observed in Brassica transgenic lines with reduced SLR1 expression through transgenic suppression, and when Brassica stigmas were pre-treated with antibodies to SLR1 and SLG (Luu et al. 1997, 1999). Both SLG and SLR1 have been found to bind to pollen coat proteins (PCPs) and are thought to mediate pollen adhesion and foot formation through these interactions (Doughty et al. 1993; Hiscock et al. 1995; Takayama et al. 2000).

1.3.3 Hydration of the Pollen

Pollen grains are quiescent and desiccated upon maturation, and can only become metabolically active and germinate following rehydration on the stigma (Heslop-Harrison 1979; Zuberi and Dickinson 1985). The degree of pollen desiccation at the time of anther dehiscence will determine how rapidly the pollen is hydrated on the papillae cell and can take from a few minutes up to an hour depending on the species (Heslop-Harrison 2000). In Brassicaceae, the passage of water to the pollen during hydration must occur from an internal stigmatic source due to their “dry” stigma (Heslop-Harrison 1979; Roberts et al. 1984; Elleman et al. 1992) (Figure 5B). Currently, the internal molecular mechanism of water transfer from the stigma papillae cells to the pollen is unknown although it is thought to occur in a highly polarized fashion. Lipids on both the pollen and papillae surfaces are also hypothesized to play a role in initiating water transfer (Preuss et al. 1993; Lolle and Cheung 1993). As for a possible water source, recent work by Iwano et al. (2007) suggests the vacuolar network may be involved. In compatible pollinations the vacuole of the papillae cell seems to reach up to the point of
pollen contact, and this organelle orientation is not seen in an incompatible self-pollination event (Iwano et al. 2007).

The cuticular layer of the “dry” stigma serves as a barrier to regulate water transfer during selective pollen hydration. This barrier virtually eliminates the occurrence of indiscriminate pollination allowing for species-specificity in this process (Sarker et al. 1988; Hulskamp et al. 1995; Zinkl et al. 1999). The lipid-rich pollen coat and the cuticular layer of the stigma at the point of pollen contact are believed to form a unique hydraulic conduit that allows for water flow from the stigma to the pollen grain during a compatible pollination (Elleman et al. 1992; Dickinson 1995). The mechanism that allows hydrophobic lipids to form such a liquid conduit is not known, but numerous studies show that lipids from the pollen coat and stigma surface are both necessary and sufficient for proper pollen hydration (Zinkl and Preuss 2000). On the female side, the Arabidopsis fiddlehead mutant has altered cuticle properties from a β-ketoacyl CoA synthase mutation, which is thought to adversely modify long-chain lipid biosynthesis, and results in aberrant organ fusion (Lolle et al. 1997; Yephremov et al. 1999; Pruitt et al. 2000). In addition, this mutation in fiddlehead resulted in inappropriate pollen hydration on non-stigmatic surfaces indicating that proper lipid content is critical for the control of pollen hydration (Lolle et al. 1993). On the male side, Arabidopsis mutants with defects in the long-chain lipid synthesis, such as the eceriferum (cer) mutants, have been reported to have altered pollen coat lipid contents and correspondingly this aberrantly coated pollen cannot hydrate properly (Preuss et al. 1993; Hulskamp et al. 1995, Zinkl et al. 1999).
1.3.4 **Pollen Tube Germination and Emergence**

Once pollen hydration has occurred, the pollen tube emerges through gaps in the exine walls known as apertures and penetrates into the stigmatic surface at the hydrophobic interface corresponding to the ‘foot’ (Elleman et al. 1992; Dickinson 1995; Edlund et al. 2004) (Figure 5B). It is thought that the ability of the pollen grain to sense a water gradient through the lipid conduit determines the initial germination orientation and polarity (Elleman et al. 1992; Dickinson 1995; Edlund et al. 2004). Water, lipids and ions have all been implicated as the signals that ultimately direct emergence of pollen tubes, although the mechanism is currently unknown.

1.3.5 **Penetration into the Stigmatic Papillae**

Ovules are located deep within the pistillar tissue and in order for pollen tubes to fertilize these reproductive organs, they must penetrate into the pistil through the papillae down to the ovary. To facilitate penetration and entry into the papillae, secreted enzymes from both the pollen tube and the pistil are thought to selectively breakdown the protective papillar surface. There are quite a few enzymes/factors involved in this process and this is not unexpected considering that penetrating the papillar surface would likely involve the breakdown (or at the very least extensive modification) of the waxy cuticle, its surrounding proteinaceous pellicle, and the underlying complex epidermal cell wall. The treatment of *Brassica* stigmas with a serine esterase inhibitor and resultant unsuccessful pollen tube invasion has allowed the identification of both pollen and stigma serine esterases as key factors in this breakdown (Hiscock et al. 2002). Various other enzymes, including cutinases, polygalacturonase, and pectin esterases have also been identified in *Brassica* pollen and/or stigma as having a role (Hiscock et al. 1994; Kim et al. 1996; Dearnaley and Daggard 2001). Cell wall modifying enzymes such as
pectin methylesterases, glucanases, expansins and peroxidases (Micheli 2001; Chivasa et al. 2002) loosen the papillar cell wall in preparation for pollen tube penetration and were recently identified in microarray experiments as being expressed specifically in the stigma (Tung et al. 2005). Thus, the emerging picture of modification or breach of the papillae surface involves a joint effort by enzymes both from the pistil and from the pollen. Once the pollen tube has penetrated the surface of the papilla (Figure 5B), it navigates its way down to the ovaries through a cascade of guidance signals (Lord and Russell 2002) to complete the fertilization at the ovaries. Being the fastest growing plant cell it can grow with rates up to 1cm per hour (Lord and Russell 2002).

1.4 Self-Incompatibility and the Importance of Outcrossing

Plants are sessile organisms and outcross pollinations ensure maintenance of the species by increasing genetic diversity, introduce new genotypes into the population, and therefore ensure the ability for the variants to adapt to new environments. In fact, the development of self-incompatibility responses is considered to be one of the chief causes for the spread and success of the angiosperms on the Earth. Self-incompatibility is the recognition and subsequent rejection of self-pollen and is a widely adopted reproductive strategy by plants to prevent detrimental in-breeding (Stone and Goring 2001). Interestingly, during an incompatible pollination, rejection appears to be controlled mainly by female components through an organized cascade of events.

Plants have developed rejection systems within their reproductive processes and different species have developed different strategies for inhibition of self-derived pollen. There are two main types of self-incompatibility (SI): gametophytic and sporophytic. In
gametophytic self-incompatibility, inhibition occurs at the pollen tube stage and is based on cytotoxic activity to inhibit tube growth so the pollen tubes do not reach the ovules to complete fertilization. This type of self-incompatibility is seen in Solanaceae and Rosaceae species (McClure et al. 1989; Sassa et al. 1993). In sporophytic self-incompatibility, inhibition occurs at the earlier stage of pollen germination on the papillae surface as well as pollen tube penetration into the stigmatic tissues. This sporophytic form of self-incompatibility was identified in the Brassicaceae family and will be discussed in detail below.

1.4.1 Self-Incompatibility Response in Brassica – Pollen Rejection Steps

Much of the knowledge that we have acquired about compatible pollen-pistil interactions is an offshoot of data collected from the study of the self-incompatible response in certain species such as Brassica whose molecular mechanism of SI is much better understood than the mechanism of compatibility.

The Brassica genus contains both self-compatible and self-incompatible species (reviewed in Takayama and Isogai 2007). Brassica oleracea, B. napus and B. rapa are those that are self-incompatible (Kao et al. 1996). Within Brassica napus, two useful cultivars for study in the self-incompatibility pathway have been used: W1 and Westar. The self-incompatible cultivar is W1 and the self-compatible cultivar is Westar.

With the “dry” type stigma, this self-incompatible response starts early by disrupting pollen adhesion and hydration, and affects stigma penetration by the pollen tube if necessary (Dickinson 1995). Pollen capture and pollen adhesion is initiated following “self” pollination, but is diminished relative to compatible pollinations (Stead et al. 1980; Luu et al. 1997). It has been speculated that both the “species”-compatible and self-
incompatible pollen recognition events have been initiated upon contact, but a small “delay” in relaying the self-incompatibility signal to the stigmatic papillae allows pollen capture and adhesion to occur to a certain degree in some cases (Samuel et al. 2009). The malformation of a suitable lipid adhesion interface between the pollen and the stigmatic papillae may account for the fact that pollen adhesion is reduced in these “self” pollinations, and as a result water transfer from the stigma is impaired and pollen germination is arrested. In a self-incompatible event, slight hydration of the pollen can occur, however, axis expansion through hydration was significantly decreased in *Brassica* self-incompatible pollen compared to compatible cross-pollen (Roberts et al. 1980; Zuberi and Dickinson 1985). Hydration of self-incompatible pollen could also be due to a humid environment, although it should be noted that some pollen may achieve sufficient adhesion to allow for some water transfer from stigma (Zuberi and Dickinson 1985; Luu et al. 1999). Furthermore, self-incompatible pollen that manage to hydrate, either do not produce a pollen tube or the germinated pollen tube is incapable of penetration of the papilla cuticular surface and remains coiled on the surface of the papilla (Ockendon 1972; Zuberi and Dickinson 1985). In fact, disruption of each successive step from adhesion to tube penetration is extremely successful at keeping undesired pollen from the fertilization process. Thus, there are many checkpoints in place ensuring that self-incompatible pollen is quickly arrested at certain stages in the pollen-pistil interaction.

### 1.4.2 Self Incompatibility Response in *Brassica* – Molecular Mechanism

From a molecular standpoint, the *S* locus region is thought to mediate the self-incompatibility response (Figure 6A) and is comprised of three tightly associated multi-allelic genes (see below) (Takayama and Isogai 2005). In order to reference the
different S alleles encoded by these tightly associated genes one would discuss a plant’s S haplotype (Dwyer et al. 1991; Stein et al. 1991; Goring and Rothstein 1992). Sporophytic self-incompatibility is controlled by the S haplotype (initially referred to as the S locus) and signaling during a self-incompatible response in Brassica is regulated by a receptor kinase-ligand interaction. Specifically, the self-incompatibility system is regulated by the S receptor kinase (SRK), S locus glycoprotein (SLG) and S cysteine-rich protein/S protein 11 (SCR/SP11) protein. SRK is expressed solely in the stigma and is the female determinant of this system (Takasaki et al. 2000; Silva et al. 2001) while SCR/SP11 is pollen-specific and the male determinant of this system (Schopfer et al. 1999; Suzuki et al. 1999; Takayama et al. 2000). Therefore, pollination is considered “self” if the S haplotype of the pollen parent (since this is a sporophytic system) matches that of the interacting stigma and conversely is considered “non-self” if the S haplotypes are unmatched (Boyes and Nasrallah 1993; Takayama and Isogai 2005). With the latter “non-self” designation, compatible pollination events can proceed leading to fertilization.

1.4.3 Identification of Male (Pollen) Molecular Components

The locus encoding the male determinant for self-incompatibility was identified independently by two different groups and is referred to as the SCR/SP11 gene (Schopfer et al. 1999; Takayama et al. 2000). During early anther development, SCR/SP11 is secreted from the anther tapetal cells and integrated into the pollen coat deposited on the pollen surface (Iwano et al. 2003). Following contact with the stigmatic surface of the papillae, SCR/SP11 is proposed to cross the cell wall and interact with the female component, SRK, thereby setting in motion a number of downstream responses leading to a self-incompatible pollen rejection response.
Figure 6. Potential models for self-incompatible and compatible pollen-pistil interactions

(A) Self-incompatible pollen-pistil interactions

The haplotype-specific SCR/SP11 pollen ligand binds to SRK in the pistil and activates the SRK/MLPK complex. A phosphorylation cascade activates the ARC1 E3 ubiquitin ligase leading to the ubiquitination (Ub) and inactivation of Exo70A1. Both water transfer and loosening of the papillar surface are blocked causing pollen rejection.

(B) Compatible pollen-pistil Interactions

Pollen coat proteins (PCPs) bind to SLG and SLR1 to promote pollen adhesion. Exo70A1 may have a role in targeting the vacuolar network and/or secretory vesicles to the plasma membrane to promote water transfer for pollen hydration and enzyme secretion to loosen the papillar surface for pollen tube growth.

Source: Full credit (Self-Incomptability in Flowering Plants, 2008, pgs. 173-191, Chapter 8: “Self” Pollen Rejection Through the Intersection of Two Cellular Pathways in the Brassicaceae: Self-Incomptability and the Compatible Pollen Response, M.A. Samuel, D. Yee, K.E. Haasen & D.R.Goring, Figure 8.2, © Springer-Verlag Berlin Heidelberg 2008) is given to the publication in which the material was originally published. With kind permission of Springer Science+Business Media.
1.4.4 Identification of Female (Pistil) Molecular Components

When the search for stigmatic SI components began, the first gene to be isolated from the S locus region was the S locus glycoprotein (SLG) (Nasrallah et al. 1985). SLG was found to be highly expressed in stigmatic papillae making it an ideal candidate for involvement in the SI response and turned out to encode a glycoprotein that localizes to the cell wall (Kandasamy et al. 1989). Although more recent studies have shown that it is not involved in haplotype specificity (Takasaki et al. 2000; Silva et al. 2001), SLG has been implicated in facilitating the accumulation of SRK therefore enhancing the self-incompatibility response (Dixit et al. 2000; Takasaki et al. 2001).

The multiallelic S locus receptor kinase (SRK) is expressed specifically in the pistil and is the female determinant of Brassica self-incompatibility (Takasaki et al. 2000; Silva et al. 2001). Being a plant receptor kinase, SRK is an integral plasma membrane protein with three domains including an extracellular domain, a transmembrane domain, and a functional cytoplasmic serine/threonine kinase (Goring and Rothstein 1992; Stein and Nasrallah 1993; Giranton et al. 2000). SRK exists as dimer in unpollinated pistils and provides a high-affinity ligand binding site for SCR/SP11 (Giranton et al. 2000; Shimosato et al. 2007). There is an S haplotype-specific interaction between SCR/SP11 and SRK following a self-pollination (Kachroo et al. 2001; Takayama et al. 2001) and an intracellular signaling pathway is then activated in the stigmatic papillae which results in “self” pollen rejection.

1.4.5 Downstream Interactors of SRK

With the female determinant identified, efforts turned to identifying effectors downstream of SRK with the intention of further defining the molecular mechanisms leading to a self-
pollination rejection. The kinase domain of membrane-bound receptor kinases can often accommodate interactions with a plethora of proteins to activate and regulate intracellular signaling pathways (Pawson 2002).

An $M$ locus protein kinase (MLPK) was shown to be phosphorylated by SRK (Kakita et al. 2007). Studies suggest that MLPK acts together with the SRK to promote self-incompatibility. MLPK is a membrane-bound serine/threonine kinase, and a recessive mutation in MLPK lead to a breakdown of the stigmatic self-incompatibility response (Murase et al. 2004). Conversely, transient expression of a functional MLPK in the stigmatic papillae of an $mlpk$ homozygous mutant was sufficient to restore the self-incompatibility response in $B. rapa$ (Murase et al. 2004). The membrane localization of MLPK is required for self-incompatibility, and this targeted localization likely facilitates interactions with, and phosphorylation by, SRK (Kakita et al. 2007). MLPK is thought to function in a complex with SRK to promote downstream signaling events following SRK activation by the pollen SCR/SP11 ligand upon incompatible pollen docking.

With MLPK being identified as a positive regulator of self-incompatibility via its interactions with SRK, a $Brassica napus$ pistil library and the kinase domain of SRK were utilized to perform a yeast two-hybrid screen to search for other potential interactors. Two interesting hits were revealed: Arm Repeat Containing 1 (ARC1) (Gu et al. 1998) and two ubiquitously expressed thioredoxin $h$ proteins (THL1 and THL2) (Bower et al. 1996).

Thioredoxin $h$ proteins, THL1 and THL2, were able to bind the inactive form of the SRK kinase domain and following site-directed mutagenesis experiments, it was determined that a conserved cysteine is required at the end of the transmembrane domain for this
interaction to occur (Bower et al. 1996; Mazzurco et al. 2001). To elucidate the inhibitory role of thioredoxin \(h\) on SRK, researchers utilized the observation that SRK was maintained in an inactive state \textit{in vivo} in unpollinated pistils, yet became constitutively active when immuno-purified, which was likely due to the loss of a soluble stigmatic inhibitor during the extraction and purification process (Cabrillac et al. 2001). The inhibitor was eventually identified as a thioredoxin-type protein, and to strengthen these findings, the researchers showed that by adding back into the extract a recombinant thioredoxin \(h\) (THL1), this was able to restore SRK to an inactive state (Cabrillac et al. 2001). In addition, it was shown that transgenic \(B. \textit{napus}\) Westar lines transformed with an antisense THL1 construct displayed low levels of pollen rejection, thought to be due to increased activity of an endogenous SRK (Haffini et al. 2004). Thus, thioredoxin \(h\) is a negative regulator of the self-incompatibility response (Cabrillac et al. 2001).

1.4.6 Discovery of ARC1 and its role in Self-Incompatibility Response

It was through a yeast two-hybrid screen in \textit{Brassica} that Armadillo repeat containing protein (ARC1) was identified as an interactor of SRK (Gu et al. 1998) through MLPK and has been shown to act downstream of SRK and as a positive regulator of self-incompatibility (Stone et al. 1999). ARC1 is a modular protein with a novel N-terminal domain (UND), U-box, and an ARM repeat domain (Samuel et al. 2006). UND is a conserved domain present in a number of predicted U-box/ARM proteins, though its function remains undetermined (Samuel et al. 2006). The U-box is a conserved motif present in the U-box family of E3 ubiquitin ligases. In the ubiquitination pathway, E3s are responsible for binding to the E2 ubiquitin conjugating enzyme as part of the ubiquitination reaction (Hatakeyama and Nakayama 2003). The ARM repeat domain mediates binding of ARC1 to the phosphorylated SRK kinase domain (Gu et al. 1998).
ARM repeat domains are composed of tandemly-repeated 42 amino acid ARM repeats which provide binding sites for interacting partners (Samuel et al. 2006).

In planta, ARC1 is expressed solely in the stigma, a pattern reminiscent of the expression of SRK, and therefore indicates a tissue-specific function (Gu et al. 1998). ARC1 plays a positive role during the self-incompatibility response as studies have shown that antisense suppression of ARC1 in self-incompatible Brassica W1 plants resulted in a partial breakdown of self-incompatibility (Stone et al. 1999). Because these ARC1-antisense W1 plants had functional SCR/SP11 and SRK genes present, it was determined that ARC1 was functioning downstream of SRK. It has been speculated that the partial suppression is likely attributed to an incomplete suppression of the ARC1 mRNA, or perhaps other potential intracellular signaling proteins were compensating for the loss of ARC1 (Stone et al. 1999). Increased levels of ubiquitinated proteins were observed 30 minutes following an incompatible self pollination demonstrating that ARC1 is a functional E3 ligase (Stone et al. 2003). To further confirm ARC1’s role as an E3 ubiquitin ligase during the self-incompatibility response, Brassica W1 pistils were pre-treated with a proteasomal inhibitor and this resulted in a reduction of the self-incompatibility response leading to an increase in pollen adhesion and pollen tube growth down the pistil on the otherwise self-incompatible species (Stone et al. 2003). Taken together, these results indicate a cascade wherein activated SRK directs ARC1 to re-localize to the proteasomes, where ARC1 presumably targets various substrates for degradation.

Consistent with MLPK functioning in a complex with SRK to activate downstream events, MLPK has been found to have a similar effect on ARC1 localization in tobacco
BY-2 cells (Stone et al. 2003). In addition, MLPK can efficiently phosphorylate ARC1 \textit{in vitro} at a much higher level than that seen with SRK (Samuel et al. 2008). This suggests that MLPK functions with SRK to activate ARC1 and furthermore, ARC1 “activation” by phosphorylation could include directing ARC1 to a targeted sub-cellular location (such as the proteasome) or perhaps cause ARC1 to have an increased binding affinity for its substrate (Figure 6A).

Considering that self-incompatibility rejection responses likely function by blocking or inhibiting compatible pollen-stigma interactions (Dickinson 1995), ARC1 substrates are predicted to be compatibility factors in the stigma whose role would be to promote or facilitate the events required for pollination under normal compatible conditions. In its active state, ARC1’s role as an E3 ubiquitin ligase would be to inhibit these factors, perhaps by proteasomal degradation, resulting pollen arrest on the stigma surface (as previously discussed) (Stone et al. 2003).

1.4.7 ARC1 Downstream Interactor Revealed

Stigma-derived compatibility factors, such as secreted enzymes that aid in the breakdown of the stigmatic papillar surface to allow pollen tube penetration, could be targeted by the self-incompatibility response to prevent pollen tube entry and therefore hinder fertilization (Hiscock et al. 2002). Recently, studies have focused on identifying the cellular events in the \textit{Brassica} stigmatic papillae which disrupt compatible pollen-stigma interactions during a self-incompatibility response with the hopes of gaining further understanding on the molecular events proceeding after compatible pollen docking. Again, following through on this theory, since ARC1 has been identified as an E3 ligase active in the self-incompatibility response, ARC1’s targets may have a role in
facilitating a compatible reaction.

In an effort to uncover potential substrates for ARC1, a yeast two-hybrid screen was performed using the N-terminus of ARC1 to screen through a *Brassica napus* pistil cDNA library and Exo70A1 was pulled out as a potential interactor (Samuel et al. 2009). *BnExo70A1* displays sequence similarity to the conserved eukaryotic protein, Exo70, a subunit of the exocyst complex which has been previously discussed in detail. This new-found partnership was also confirmed by *in vitro* pull down assays and *in vitro* ubiquitination assays of *BnExo70A1* by ARC1 (Samuel et al. 2009). In addition, using a tobacco BY-2 cell system, transient expression of an active SRK kinase domain, ARC1, and *BnExo70A1* resulted in targeting of ARC1 and *BnExo70A1* to ER-associated proteasomes in the perinuclear region. Further supporting these findings, RNAi-mediated inhibition of *BnExo70A1* in the stigmatic papillae of *Brassica* resulted in a constitutive rejection of compatible pollen with defects in pollen hydration, germination, and penetration of the pollen tube through the papillar surface (Samuel et al. 2009). Taken together, this data confirms the possibility that *BnExo70A1* could be targeted for inactivation by ARC1 during the self-incompatibility response and therefore is a potential compatibility factor necessary for fertilization.

1.5 Endomembrane Changes in the Stigmatic Papillae Following Compatible and Incompatible Pollen-Pistil Interactions in *Brassicaceae*

The *Brassica* papilla are single cell projections on the top of the stigma and the papillar cytoplasm is a very thin layer surrounding a large central vacuole and intertwined with
the network of smaller tubular or round vacuoles (Dearnaley et al. 1997; Iwano et al. 2007). Immediately following contact with a compatible pollen grain, structural changes in the cell wall occur with the outer wall expanding beneath the pollen grain. This alteration has not been documented following self-incompatible pollinations (Elleman and Dickinson 1990). Interestingly, the application of isolated pollen coat extract to the stigma resulted in multiple secretory vesicles being targeted to the stigmatic cell wall from the cytoplasm (Elleman and Dickinson 1996) and it has been proposed that a localized secretion event occurs at the point of pollen-stigma contact wherein pre-loaded vesicles carrying hydration factors and/or cell wall loosening enzymes, unload at the plasma membrane resulting in a targeted and precisely localized loosening of the cell wall matrix to allow pollen tube penetration (Elleman and Dickinson 1996). It is possible that this vesicle targeting process could be regulated by the exocyst by marking the pollen attachment site for polarized exocytosis of the vesicle cargo. In contrast, during an incompatible interaction, an over-abundance of vesicles were observed to be accumulating by the unexpanded cell wall (Elleman and Dickinson 1996). The self-incompatibility response results in a rapid inactivation and possible degradation of Exo70A1 by ARC1 in *Brassica* (Samuel et al. 2009), and in addition, Exo70A1 has been implicated as the subunit of the exocyst that is vital for land-marking at the plasma membrane for complex assembly in other systems (Boyd et al. 2004). Together, this may explain the accumulation of secretory vesicles which have migrated to the site of exocytosis but are likely unable to dock at the stigmatic plasma membrane (due to the loss of the putative spatial landmark) at the pollen-papillae point of contact, thereby arresting any further pollination steps from taking place.

Besides differences in vesicle accumulation, as previously mentioned, changes in the
vacuolar network in *Brassica* stigmatic papillae following pollination have also been recently observed (Iwano et al. 2007). During a compatible pollination, the vacuolar network appears to be organized in an orientation towards the site of pollen contact while in contrast, during an incompatible pollination, the vacuolar network appeared to be disrupted in the stigmatic papillae (Iwano et al. 2007). It has been hypothesized that these changes were related to promoting water and ion transport to compatible pollen grains while restricting transport to incompatible pollen grains therefore denying hydration factors to the incompatible pollen.

In addition to vesicular and vacuolar changes in the papillae, changes and differences in actin network dynamics have been observed in the compatible versus the incompatible pollen-pistil interactions (Iwano et al. 2007). Compatible pollinations led to an accumulation of actin bundles in the apical region of the stigmatic papilla adjacent to the pollen grain at approximately the start of pollen hydration. Conversely, self-incompatible pollinations displayed a decrease in actin filament concentration in the apical region suggesting actin depolymerization could be occurring (Iwano et al. 2007). And finally, it has been reported that while yeast Sec3p and Exo70p exocyst subunits arrive at the plasma membrane prior to exocyst assembly, the other six subunits are transported to this site with the secretory vesicles in an actin-dependent manner suggesting that without the proper actin network in place, the exocyst subunits cannot reach the plasma membrane for assembly (Boyd et al. 2004).

1.5.1 **Genetic Similarity of BnExo70A1 and AtExo70A1**

*Arabidopsis thaliana* carries non-functional SCR/SP11 and SRK genes and is naturally self-fertilizing (Kusaba et al. 2001). Interestingly, some *A. thaliana* ecotypes can confer
self-incompatibility with the addition of the self-incompatible *Arabidopsis lyrata* SCR/SP11 and SRK genes (Nasrallah et al. 2004, Boggs et al. 2009). This suggests that while *A. thaliana* has lost its self-incompatibility due to inactivation of the S locus genes, the downstream signaling components required for conferring self-incompatibility has remained intact in some instances.

*At*Exo70A1 is the *Arabidopsis* ortholog of *Bn*Exo70A1, with over 91% sequence identity at the nucleotide level (Figure 7A-B). Null *exo70A1-1* mutants have a number of growth defects, including the overall plant having a dwarfed appearance (Synek et al. 2006) and were documented to display defects in pollen hydration and germination (Samuel et al. 2009). It has been proposed that in response to a compatible pollination, Exo70A1 has a role in promoting pollen acceptance by facilitating targeted secretion of stigmatic factors at the attachment site of the compatible pollen (Samuel et al. 2009).

1.5.2 *Refining the potential roles of Exo70A1*

Consistent with previous studies of the exocyst complex in other systems, a potential role of regulating polarized secretion has emerged for *Bn*Exo70A1 when considering the cellular changes seen in the stigmatic papillae following compatible pollen-pistil interactions (Figure 6B). RNAi suppression of *Bn*Exo70A1 in the stigma of otherwise compatible *Brassica napus* Westar plants resulted in a significant reduction in seed production (Samuel et al. 2009). This result is thought to be a consequence of decreased pollen adhesion and hydration of what normally should be compatible pollen (and would normally produce a full seed set). Furthermore, the few pollen tubes that formed were incapable of penetrating the papillar surface, essentially mimicking the self-incompatibility response seen in the self-incompatible cultivar *Brassica* W1. Thus,
following a compatible pollination, *B.n*Exo70A1 is predicted to play a role in the stigmatic papillae promoting events such as pollen adhesion, pollen hydration, or penetration of the pollen tube through the stigmatic surface. A role for Exo70A1 in promoting compatible pollen-stigma interactions was also found to be conserved in *Arabidopsis* where *exo70A1-1* mutants were also found to exhibit defects in pollen hydration and germination (Samuel et al. 2009). As previously discussed, the Iwano et al. (2007) study raises the fascinating possibility that the plant exocyst could play a role in regulating the vacuolar network during compatible pollinations for the purposes of providing hydration to the docking pollen grain. Correlating the vacuole with hydration of the docking pollen grain and Exo70A1 function would not only be a novel role for Exo70A1, but it could also in part account for the huge expansion of the Exo70 gene family only found in plants (Elias et al. 2003; Cole and Fowler 2006). Transgenic *Arabidopsis* lines carrying an *At*Exo70A1 Promoter::GUS construct displayed GUS expression in several vegetative tissues and interestingly, have strong GUS activity in the stigma of mature flowers (Samuel et al. 2009). This is a typical expression profile for genes that are active in the stigma during pollen-stigma interactions.
Figure 7. Pair-wise amino acid alignment scores of the *Arabidopsis* Exo70 family members with *BnExo70A1* and amino acid sequence alignment of *BnExo70A1* to *AtExo70A1*

(A) BLAST searches of *Arabidopsis* genome databases identified *AtExo70A1* as the closest ortholog to *BnExo70A1* in the 23-member Exo70 family (Synek et al. 2006).

(B) Alignment of the predicted *BnExo70A1* and *AtExo70A1* (At5g03540) amino acid sequences.
Figure 7

A

<table>
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<tr>
<th>AtExo70</th>
<th>A1</th>
<th>D2</th>
<th>F1</th>
<th>H7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (aa)</td>
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<td>622</td>
<td>683</td>
<td>634</td>
</tr>
<tr>
<td>% identity to BnExo70A1</td>
<td>94</td>
<td>32</td>
<td>33</td>
<td>23</td>
</tr>
</tbody>
</table>

B

BnExo70A1 MVYDSRMDLL SERAELNRPS LQKSQLTITDN VVSILGFDS RLSALESAMR FTQIRTHAIR KAHENIDKTL
AtExo70A1 ----------- -------- A- ------------- -------T-- --------------- R--

BnExo70A1 YLEAIAGLQK VIRYFSSNKG FKNSDGVLNR ANSLLAQAQS KLEREFQCLL ASYSKAVEED RLFDGLPMLSL
AtExo70A1 --D------ I---M--S --S-------- ------------- --------------

BnExo70A1 LIPSRULPLI HDLAQQMVAQ GHQQLLQIQY REKRTFVLEE SLRKLVEKL SKEDVQRMQW EVLEAKIONW
AtExo70A1 ----------- ------------------ --Q----- --D--S----- --K--------

BnExo70A1 DQCFAETVS SVSMLLFSGD AIARSKRSPE KLFVLLDME IMLWEHSEIE TIFONGKACLE IRRSAGQTLK
AtExo70A1 ----------- ------------------ --------------- T--- ------------- --D-----

BnExo70A1 VHPLTSYVIN YVKELDQYQA TLKQLFSEPQ NGDSDNSQGA SYMIRMDQAL QNMLEGKSSQ YKDQAATLHLF
AtExo70A1 ----------- ------------------ T--- ------------- ------------- ---D-- ---P---

BnExo70A1 RVVQHAMLKY KRTAWTKILQ TSSAQGLISS GGGSVEGGS SGVSRLIGKE RYKOMNMQDP ELHHQRSQWT
AtExo70A1 -I-----Q- --V----- --L--- ------------- ---L----- -------------

BnExo70A1 PLVESGKNSS RYKYTAELD RLLLGEFPEG KSMNEFRR
AtExo70A1 ----------- P- K------------- ---
1.6 Thesis Objectives

Clearly there remain many questions about the precise cellular role Exo70A1 has during compatible pollinations that need to be addressed. The overall objective of my project is to take advantage of the many tools available with the plant model system, *Arabidopsis thaliana* to investigate how Exo70A1 functions in a compatible pollen response.

My hypothesis is that Exo70A1 is a crucial factor in compatible pollen-pistil interactions in *Arabidopsis* and that its conserved function can be demonstrated by complementing the *exo70A1-1* mutant with the *Brassica* ortholog of AtExo70A1.

The specific objectives of my Masters are as follows:

1. Investigation of the *exo70A1-1* female sterile defect in *Arabidopsis*
2. Rescue of *exo70A1-1* female sterile defect in *Arabidopsis* using the *Brassica* ortholog *BnExo70A1*
3. Localization of Exo70A1 before and after compatible pollinations in the papillae cell
4. Investigate any vacuolar connection Exo70A1 has in compatible pollinations
5. RNAi suppression of other Exo70s family members in *Arabidopsis* stigmas
Chapter 2: MATERIALS AND METHODS

2 Materials and Methods

2.1 Plant Growth Conditions

*Arabidopsis* T-DNA insertion lines in a Columbia-0 (Col-0) background with inserts in the *AtExo70A1* gene (SALK lines) were obtained from the *Arabidopsis* Biological Resource Centre (ABRC) and confirmed as null mutants by Yolanda Chong (Goring Lab). Seeds were sterilized in sterilization buffer (95% EtOH and 30% H2O2 in a 1:1 ratio) for 10 minutes and rinsed five times with double distilled water (ddH2O). Following sterilization, seeds were plated on (½ Murashige Skoog Salts, 0.4% phytoagar, pH5.8) media plates and cold treated at 4°C for a minimum of three days. Following cold treatments, plates were placed under long-day 16 hour light (170 micro einsteins) at 22°C for 14 days (or when the seedlings first true leaves emerge) and then transferred to soil (Première ProMix) with Plant Pro Fertilizer 20-20-20 (2ml per 1L water) under the same light/temperature conditions.

2.2 Plant Genomic DNA Extraction

Genomic DNA (gDNA) isolation was performed using fresh *Arabidopsis* leaf tissue (diameter 3-4mm) homogenized in 250μl of CTAB buffer (0.55M hexadecyltrimethyl-ammonium bromide (CTAB)), 1M NaCl, 0.02M EDTA, 0.1M Tris-HCl pH8 and added fresh 0.4% beta-mercaptoethanol) and incubated at 65°C for 10 minutes. An equal
volume of chloroform was added to each sample, inverted three times to mix and centrifuged for 5 minutes at 13,000 rpm at room temperature. The aqueous phase was transferred to a sterile tube and 150µl of isopropanol was added. Samples were inverted 5 times to mix and left to sit at room temperature for 15 minutes. After incubation, samples were centrifuged at room temperature, 13,000 rpm for 20 minutes. Supernatant was discarded and the pellet washed with 70% EtOH and air dried at room temperature for 45 minutes. Pellets were resuspended with 30µl ddH2O. Samples were stored at -20°C.

2.3 PCR Screening of Plants (DNA Genotyping)

To genotype plants, 1µg of gDNA was amplified with two primer sets (Appendix III) to determine if the plant was homozygous for the T-DNA insertion. One set of primers consisted of gene-specific forward and reverse primers spanning the insertion site and the second set utilized either the forward or reverse gene-specific primer paired with the left-border of the T-DNA insert. The following reagents were used in each reaction: 1x Tsg buffer, 0.2mM dNTPs, 1.6mM MgSO4, 0.5mM of each primer and 1 unit Tsg Polymerase (BioBasics). The PCR cycle was as follows: 95°C for 5 min for 1 cycle followed by 35 cycles of 95°C for 30 seconds (separation), 55°C for 30 seconds (annealing), 72°C for 90 seconds (extension), then one final extension cycle of 72°C for 10 minutes and held at 4°C. For viewing, 10µl of each reaction was run out on a 0.8% agarose gel with 2µg/ml EtBr and the gel picture taken using a MultiDoc-It Digital Imaging System (UVP).
2.4 Plant RNA Extraction and cDNA Synthesis

RNA extractions were carried out using a Trizol reagent (Invitrogen) as specified by the manufacturer. Briefly, equipment was washed and treated with RNase Zap (Ambion) to decrease the possibility of RNA degradation due to RNase contamination. Approximately 200mg of flower and bud tissue samples were ground to fine powder using liquid nitrogen chilled mortar and pestle. Once ground, 1mL of Trizol was added to each sample and incubated at room temperature for 5-10 minutes and transferred to a clean sterile tube. The samples were centrifuged for 10 minutes at 9,000rpm at 4°C. The clear phase was transferred to a clean sterile tube and 500µl of chloroform was added, the tubes were vigorously shaken for 20 seconds, incubated at room temperature for 3 minutes and then centrifuged at 9,000rpm for 15 minutes at 4°C. Following this, the aqueous phase was transferred to a clean sterile tube containing 500µl isopropanol and inverted five times to mix. The samples were incubated at room temperature for 10 minutes and then centrifuged at 9,000rpm for 15 minutes at 4°C. The supernatant was poured off and the pellet washed with 1ml of 75% EtOH (made with DEPC (diethyl pyrocarbonate) treated water) then centrifuged at 4,000rpm for 10 minutes at 4°C. The pellet was air-dried for 20 minutes and re-suspended with 30µl of DEPC water. RNA placed at -80°C for long term storage.

DNase treatment followed RNA extraction. Briefly, 1µg of RNA was added to 1 unit of DNasel (Amplification Grade, Invitrogen) in 1x DNase buffer and mixed gently. After 15 minutes incubation at room temperature, the DNasel was inactivated by adding 2.5mM EDTA and the tubes were placed in a 65°C water bath for 10 minutes.
cDNA synthesis was carried out using 200 units SuperScriptII RT (Invitrogen) and for each sample, 0.5µg of DNase treated RNA was added to 500ng of oligo(dT)$_{12-18}$, 1.2mM dNTPs and then incubated at 65°C for 5 minutes and chilled on ice for 2 minutes. First Strand Buffer (1x), 10mM DTT, and 40 units of RNase Out (Invitrogen) was added to each chilled sample and mixed using a pipette tip. Each sample was then divided into two fresh sterile tubes. To one tube, 2 units of SuperScriptII were added and to the other, an equal volume of DEPC treated water was added. This later served as a control for genomic contamination. Samples were then incubated in a 42°C water bath for 50 minutes and the enzyme inactivated with a 15 minute treatment at 70°C. cDNA was stored at -20°C.

2.5 Plasmid Purification and Transformations

The plasmids used in this study were purified using Qiagen mini-prep kits (Qiagen Ltd, UK), following the manufacturers instructions. Plasmid transformations of chemically competent DH5a cells were prepared for plasmid transformation (Sambrook and Russell 2001). *Escheria coli* DH5a cells were incubated with the plasmid for 5 minutes on ice and heated shocked in a 42°C water bath for 90 seconds and recovered in 500µl LB broth, shaking at 37°C for one hour. Transformed cells were then plated on LB+antibiotic plates and incubated overnight at 37°C. Colony PCR followed to ensure transformation.
2.6 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Approximately 0.3µg-0.6µg of cDNA was amplified with one of two primer sets; gene specific primers to determine if the transcript was present (detect \textit{RFP:BnExo70A1 mRNA}) and actin3 primers to serve as a positive control. The following reagents were used in each reaction: 1x Tsg buffer, 1.6mM MgSO$_4$, 0.2mM of each primer and 1 unit Tsg Polymerase (BioBasics). The PCR cycle was as follows: 95°C for 5 min for 1 cycle, followed by 28 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 90 seconds, then one extension cycle of 72°C for 1 minute. For viewing, 5µl of each reaction was run out on a 1% agarose gel with 2µg/ml EtBr and the gel picture taken using a MultiDoc-It Digital Imaging System (UVP).

2.7 Creation of \textit{Arabidopsis} RFP: \textit{BnExo70A1} transgenic lines

The Exo70A1 open reading frame (ORF) from \textit{Brassica napus} fused to RFP was subcloned behind the p1665 binary vector containing the \textit{SLR1} promoter (Franklin et al. 1996) which drives tissue-specific expression solely in the stigma. This construct was made by Dr. Marcus Samuel (Goring Lab) used to transform heterozygous \textit{Arabidopsis Exo70A1/exo70A1-1} plants by \textit{Agrobacterium}-mediated transformation and the floral dip method (Clough and Bent 1998). The segregating T$_0$ seeds were selected on ½ MS media plates containing 10mg/mL kanamycin and genotyped to isolate wild-type and \textit{exo70A1-1} homozygous mutant plants expressing \textit{RFP:BnExo70A1}. Following RT-PCR analysis (described above), two of the strongest expressing lines, Line 4 and Line 14 were used for further analysis.
2.8 Analysis of Seed Set
Hand pollinations with Col-0 pollen (3-4 flowers with dehisced anthers were used to pollinate each stigma) were carried out on pistils from Col-0, exo70A1-1 and RFP:BnExo70A1/exo70A1-1 plants to assess fertilization success using seed set as a marker. Pollinations involved Stage 12 flowers being emasculated (removal of the sepal, petals, and anthers leaving only the immature pistil) and covered in plastic wrap (to maintain humidity and allow for papillae elongation) before plants were placed in the growth chamber. After 24 hours, the mature emasculated pistils were hand pollinated with Col-0 pollen and covered again in plastic wrap for another 24 hours. The plastic wrap was then removed and the siliques allowed to mature (10-12 days). A minimum of seven (n=7) mature siliques per genotype were dissected and the seeds/fertilized ovules were counted.

2.9 Aniline Blue Staining
Stage 12 flowers from Col-0, exo70A1-1, RFP:BnExo70A1/exo70A1-1 were emasculated and hand pollinated with Col-0 pollen as above. Twenty-four hours post pollination, wrap was removed and the whole pistil placed into a well of a 24 well plate with 300µl 3:1 Ethanol: Glacial acetic acid and incubated at room temperature for 30 minutes to fix the sample. The fixative solution was removed and pistils were rinsed three times with sterile distilled water. Once washed, 500µl of 1M NaOH was added and the pistils incubated at 60°C for 1 hour followed by three rinses with sterile distilled water. Five hundred microlitres of 0.1% aniline blue (BDH Limited) (50mM KPO₄, pH7.5) was added to each well. The covered 24 well plate incubated at room temperature for
30 minutes or overnight at 4°C. Pistils were mounted on slides in Vectashield (Vector Laboratories, H-1000) to prevent photobleaching and viewed using a Zeiss Axioskop2 plus fluorescence microscope, and captured as grey scale images. A minimum of five (n=5) pistils per genotype were stained, mounted and counted.

2.10 Confocal Microscopy of the RFP: BnExo70A1 Localization in the Stigma

Wild-type and exo70A1-1 plants expressing RFP: BnExo7A1 Stage 12 flowers were emasculated as previously described. The mature, elongated pistils were mounted in Vectasheild and observed using LSM510 (Carl Zeiss) 40X oil-immersion objective. Laser levels were adjusted to an arbitrary “zero” using an untransformed Col-0 pistil to account for autofluorescence. For post-pollination experiments, stigmas were hand pollinated with Col-0 pollen and left to sit for 5 minutes prior to mounting and viewing.

2.11 Creation of Double Fluorescent Marker Lines

A flower with dehisced anthers from one marker line was used to hand pollinate the emasculated flower of the second marker line. The pollinated pistils were then covered with plastic wrap and left for 24 hours, the wrap removed and the silique allowed to develop. The seeds of the cross were harvested, sterilized and grown on media plates (½ Murashige Skoog Salts, 0.4% phytoagar, pH5.8) for 14 days (or when the seedlings first true leaves emerge) before being transferred onto soil. Co-localization was assessed by confocal imaging using an LSM510 (Carl Zeiss) Zeiss 40X oil-immersion
objective. The plasma membrane GFP marker line (GFP-PIP2A) (Cutler et al. 2000) was obtained courtesy of the Cutler Lab (University of California-Riverside) and marks a PIP2A plasma membrane channel protein (aquaporin). The vacuole membrane GFP marker line (GFP:δ-TIP) (Cutler et al. 2000) was also obtained from the Cutler Lab and marks a GFP:δ-TIP vacuolar membrane (tonoplast) protein. The Golgi apparatus GFP marker line (ST-GFP) (Saint-Jore et al. 2002) was obtained from the Hawes Lab (Oxford) and marks a sialyl transferase signal anchor protein that targets Golgi stacks. Each of the marker lines were crossed with RFP:BrExo70A1/Col-0 plants.

2.12 Arabidopsis Exo70 Family Member Expression Analysis

Expression values for Arabidopsis Exo70 family members were obtained using the tools hosted at the Bio-Array Resource (BAR) (http://www.bar.utoronto.ca/) (Toufighi et al. 2005).

2.13 Exo70 RNAi Plasmid Construction

Plasmids were purified using Qiagen mini-prep kits (Qiagen Ltd. UK) as per the manufacturer’s instructions. Exo70A1, Exo70H7, Exo70D2 and Exo70F1 sequences were obtained from The Arabidopsis Information Resource (TAIR) (www.arabidopsis.org) and unique 700 base pair sections were selected (Appendix V) for each member to be included in the hairpin constructs. Primers were designed in a forward and reverse orientation to amplify the unique 700bp region (Appendix III). Chosen 700bp regions were amplified via PCR from cDNA isolated from Col-0 tissue.
Prior to sub-cloning into plant transformation vector p1665 (driven by the stigma-specific SLR1 promoter), all amplified PCR products were first cloned into pCR2.1 Topo vector (Invitrogen) as per the manufacturer’s instructions. The sense and anti-sense fragments were assembled in pBlueScript-KS vector (Fermentas) and fully assembled hairpin constructs were ligated into the multiple cloning site of the p1665 plant transformation vector using the Xmal site. *Escheria coli* (*E. coli*) strains DH5a was used in this study. *E. coli* DH5a was grown in Luria Broth (LB) (EMD Science) at 37°C.

### 2.14 Sequencing

Constructs were purified from a gel slice with a GFX kit (Amersham) according to the manufacturer’s protocol. The samples were sequenced using primers designed to sequence along the entire insert piece. Samples were sent to NANUQ, the McGill University and Genomic Quebec Innovation Centre.

Chapter 3: RESULTS

3 CONTRIBUTION BY OTHERS

Dr. Marcus Samuel made the RFP: BnExo70A1 transformation vector that I transformed into the segregating heterozygous Exo70A1/exo70A1-1 plants. Dr. Samuel and I also worked together on the RFP: BnExo70A1 localization images in Figure 12A-E.

I would also like to thank my undergraduate student Helen (He) Sun (Summer 2008) for all her help with screening of plants and some microscope work done with the aniline blue assays.
3.1 **Exo70A1 is essential for a successful compatible pollen-pistil interaction**

Given previous research in the Goring lab on the role Exo70A1 plays during pollen-pistil interactions in *Brassica napus*, further examination of Exo70A1’s function was performed to better understand the mechanism of action of this protein in *Arabidopsis thaliana*, a self-fertile species. Null mutants for *AtExo70A1* are observed to be slow-growing, dwarf plants with an extended life span and defects in organ morphogenesis and fertility (Synek et al. 2006). In addition, work by Yolanda Chong in the Goring lab examined the defects in pollen-stigma interactions in the *exo70A1-1* mutants. When individual wild-type *Arabidopsis* pollen grains were placed on Col-0 (wild-type) stigmatic papillae, 91% of the pollen grains were fully hydrated (Samuel et al. 2009). Conversely, the *exo70A1-1* mutant stigmatic papillae from two independently isolated T-DNA insertion lines failed to support wild-type pollen hydration. Only 45% of the wild-type pollen placed on the *exo70A1-1* stigmatic papillae showed any hydration, with these pollen grains showing partial hydration (Samuel et al. 2009). To build on these findings and to confirm that the loss of pollen hydration was correlated with the loss of Exo70A1, heterozygous *Exo70A1/exo70A1-1* *Arabidopsis* plants were transformed with the *SLR1* promoter::RFP:*BnExo70A1* construct (Figure 8A-B), and two independent lines (Line 4 and Line 14) of *exo70A1-1* mutants expressing RFP:*BnExo70A1* (RFP:*BnExo70A1/exo70A1*) in the stigma were recovered. These lines were identified through PCR genotyping (Figure 8C). Col-0 was identified by the sole amplification of the gene for genomic DNA (gDNA), while the *exo70A1-1* mutant was identified by the sole amplification of the T-DNA insert. Transformed independent lines were identified by amplification across the T-DNA insert as well as of the RFP construct.
Figure 8. *Arabidopsis exo70A1-1* T-DNA insertion line, SLR1::RFP:BnExo70A1 construct and *Arabidopsis* transgenic lines used in this study

(A) Gene structure of *Exo70A1* and localization of the T-DNA insertion SALK line *exo70A1-1* as well as primer orientations for PCR screening.

(B) Schematic of the *SLR1::RFP:BnExo70A1* construct used to transform *Arabidopsis Exo70A1/exo70A1-1*. The *SLR1* promoter is tissue-specific, only driving expression in the stigma. Primer orientations for PCR screening are included.

(C) DNA genotyping of *exo70A1-1* plants expressing RFP:BnExo70A1. Two independent transgenic *Arabidopsis* lines, Line 4 and Line 14 with the *SLR1::RFP:BnExo70A1* construct were generated. This construct was introduced into a heterozygous *Exo70A1/exo70A1-1* *Arabidopsis* plant and transformed heterozygous plants were recovered. The next generation was then screened for the purpose of isolating segregating wild-type Col-0 and homozygous *exo70A1* plants carrying the RFP:BnExo70A1 construct (RFP:BnExo70A1/exo70A1-1). The presence of RFP:BnExo70A1 in the *exo70A1* in Line 4 and Line 14 was detected by PCR using primer combinations specifically designed to the RFP and the BnExo70A1 gene (RFP-int-for and BnExo70-rev). The *Arabidopsis exo70A1-1* plants were identified by the presence of T-DNA primers designed to amplify across the gene-TDNA junction (LBA1) (Figure 9A). The undisrupted *Exo70A1* gene (gExo70A1) was detected using gene-specific primers (70A1-1RP and 70A1-1LP).

(D) RT-PCR showing RFP:BnExo70A1 expression in the pistils of the transgenic RFP:BnExo70A1/exo70A1-1 *Arabidopsis* lines. Actin was present as the control.
Figure 8

A

B

C

D

RFP:Exo70A1

Actin
Furthermore, reverse transcription amplification (RT) analysis was performed on RNA isolated from both Line 4 and Line 14 to confirm expression of the transgenic construct (Figure 8D). Detailed pollination studies were then performed on stigmas from Col-0, the \textit{exo70A1-1} mutant, and the RFP:\textit{BnExo70A1}-transformed \textit{exo70A1-1} Line4 and Line14 plants.

Successful compatible pollen-pistil interactions were monitored by pollen adhesion and callose deposition during pollen tube growth by aniline blue staining of hand-pollinated pistils (Figure 9). The papillae of wild-type Col-0 (acting as a self-fertile control) were covered with adhered pollen grains and numerous pollen tubes could be detected, as expected (Figure 9A-I). When wild-type Col-0 pollen were used to pollinate the \textit{exo70A1-1} mutant stigmas, the pollen were largely rejected with very few adhered pollen grains detected (Figure 9A-II DIC) and no pollen tubes were detected following aniline blue treatment (Figure 9A-II Blue). The expression of the RFP:\textit{BnExo70A1} fusion protein in the \textit{exo70A1-1} mutant stigmas fully restored the ability of compatible wild-type Col-0 pollen to adhere to the RFP:\textit{BnExo70A1/exo70A1-1} stigmas and produce pollen tubes (Figure 9A-III).

To attempt to quantify the extent of restoration RFP:\textit{BnExo70A1} expression has in recovering the female sterile defect of \textit{exo70A1-1}, the number of pollen grains adhering to the stigma was assessed (Figure 9B). While the number of adhered pollen for the \textit{exo70A1-1} mutant is significantly lower than either Col-0 or RFP:\textit{BnExo70A1/exo70A1-1} (Line 14) (p<0.05), the “rescued” RFP:\textit{BnExo70A1/exo70A1-1} (Line 14) pollen counts are not significantly different from that of Col-0 (p>0.05) indicating essentially a full
**Figure 9. RFP: BnExo70A1 is able to rescue the pollen adhesion defect in exo70A1-1 mutants in the stigma during pollen-stigma interactions**

(A) Pollen attachment and pollen tube growth in *Arabidopsis* pistils pollinated by wild-type compatible Col-0 pollen in Col-0 (A-I), exo70A1-1 homozygous mutant (A-II), and the exo70A1-1 mutant expressing RFP: BnExo70A1 (A-III). All pistils were pollinated and then stained with aniline blue staining to visualize the pollen attachment and pollen tube growth. DIC images allowed the visualization of pollen grain attachment while the blue channel allowed the visualization of the aniline blue stained callose deposited by elongating pollen tubes. Numerous examples of pollen tube growth are observed on the compatible wild-type Col-0 stigma, as would be expected. The exo70A1-1 homozygous mutant stigma did not support any pollen attachment or pollen tube growth. The expression of RFP: BnExo70A1 in the exo70A1-1 mutant stigma (Line 14) rescues this “female sterile” defect and restores pollen attachment and pollen tube growth almost back to wild-type levels. (scale bars = 50µm)

(B) Average number of attached compatible Col-0 pollen grains on stigmas from wild-type Col-0, exo70A1-1 homozygous mutant, and the exo70A1-1 homozygous mutant expressing RFP: BnExo70A1 (Line 14) (n=5). The number of adhered pollen for the exo70A1-1 mutant is significantly different from both Col-0 and Line 14/ exo70A1-1 (t-test, p < 0.05) while the results for the rescued Line 14/ exo70A1-1 stigmas are not significantly different from Col-0 (t-test, p > 0.05). Error bars are ± standard error. * indicates a significant difference from Col-0.
Figure 9

A

I

DIC

Blue

Col-0

II

exo70A1-1

III

SLR1:RFP:

BnExo70A1

/exo70A1-1

(Line 14)

B

# pollen adhering/stigma

Col-0

exo70A1-1

BnExo70A1

/exo70A1-1

*
restoration of pollen acceptance ability. Because the final stage of stigmatic
development has a critical role in the pistil having receptivity to pollen, the elongation of
stigmatic papillae that occurs during these stages could be reflective of the ability of the
stigma to adhere pollen (Smyth et al. 1990). The \textit{exo70A1-1} mutant did display
variability in papillar elongation and was admittedly difficult to stage due to the mutant
overall defect. Thus to eliminate the possibility that selection of immature stigmas (ie.
without full, proper elongation) was the cause of decreased pollination, pollination of
\textit{exo70A1-1} mutants was done at various stages of papillar elongation. Regardless of
elongated or short papillae, the attempts at pollination of the \textit{exo70A1-1} stigma yielded
the same result: no significant adhering of compatible pollen on the stigmas (Figure
10C-D) in contrast to the other lines (Figure 10A-B, E-G). Siliques from Col-0, \textit{exo70A1-1}
and \textit{RFP:BnExo70A1/exo70A1-1} plants were also observed for seed production. The
control Col-0 pollinated pistils produced full seed pods as expected (Figure 11A). No
seeds were detected in the \textit{exo70A1-1} pistils pollinated with Col-0 pollen and these
plants had siliques that were shorter and were in fact empty and barren of any seed
(Figure 11A). The number of seeds severely attenuated in the \textit{exo70A1-1} mutant was
restored, almost back to wild-type quantities upon expression of \textit{RFP:BnExo70A1}
(Figure 11A-B). Seed set values shown for Col-0, \textit{exo70A1-1} mutant, and (Line 14)
\textit{RFP:BnExo70A1/exo70A1-1} and are all significantly different from each other (p<0.05).
Figure 10. Pollen adhesion lost in exo70A1-1 mutant at various points in papillae elongation process is rescued by the expression of RFP: BnExo70A1

(A-B) DIC images of unpollinated (A) and pollinated (B) Col-0 stigmas. Pollinated Col-0 stigmas show pollen adhesion at the papillar surface.

(C-D) DIC images of pollinated exo70A1-1 stigmas. Despite utilizing stigmas with variability in papillar elongation (stage 12 to stage 13 flowers) the attempt to pollinate exo70A1-1 mutant stigmas yields the absence of adhesion of Col-0 pollen at all stages of development. A very rare example of a pollen grain adhering to an exo70A1-1 stigma is shown in the far right panel.

(E) DIC images of unpollinated rescued Line 14 BnExo70A1/exo70A1-1 stigmas.

(F) DIC images of pollinated rescued Line 14 BnExo70A1/exo70A1-1 stigmas. Pollinated RFP: BnExo70A1/exo70A1-1 stigmas have regained the ability to accept compatible pollen.

For (A-G), flowers were emasculated, covered, and left overnight. For all pollinations, wild-type Col-0 pollen was applied the next day, and pistils were again left overnight. The following day, all pistils were collected, stained with aniline blue, and visualized under an epifluorescence microscope. 2 left panels - 10X objective (scale bars = 100µm); right panel - 20X objective (scale bars = 50µm)
Figure 10

A

B

C

D

E

F

G

Col-0 Unpollinated

Col-0 Pollinated

exo70A1-1 Pollinated

exo70A1-1 Pollinated

RFP: BnExo70A1/ exo70A1-1 Unpollinated

RFP: BnExo70A1/ exo70A1-1 Pollinated

RFP: BnExo70A1/ exo70A1-1 Pollinated

10x 10x 20x
Figure 11. RFP: *Bn*Exo70A1 is able to rescue seed set in *exo70A1-1* mutants in the stigma during pollen-stigma interactions

(A) Siliques from Col-0, *exo70A1-1*, and the *exo70A1-1* homozygous mutant expressing RFP: *Bn*Exo70A1 (Line14) pistils following a pollination with Col-0 pollen. Following application of compatible Col-0 pollen, no seeds are produced from *exo70A1-1* homozygous mutant pistils. Expression of RFP: *Bn*Exo70A1 in the *exo70A1-1* mutant stigma restored seed production.

(B) Average number of seeds per silique from wild-type Col-0, *exo70A1* homozygous mutant, and the *exo70A1-1* homozygous mutant expressing RFP: *Bn*Exo70A1 (Line14) (n=7). Seed set values for Col-0, *exo70A1-1* mutant and Line 14/ *exo70A1-1* are all significantly different from each other (t-test, p < 0.05). Error bars are ± standard error. * and ** indicate a significant difference from Col-0.
Figure 11

A

B

Bar graph showing the number of seeds per silique for different genotypes: Col-0 (control), ex070A1-1, brex070A1, and brex070A1-1. The graph indicates a significant decrease in the number of seeds in ex070A1-1 compared to Col-0.
3.2 Exo70A1 is localized to the plasma membrane of mature stigmatic papillae and relocates away from the membrane following a compatible pollination

After demonstrating the ability of RFP: *Bn*Exo70A1 to rescue the stigmatic female sterile defect in the *Arabidopsis* exo70A1-1 mutants (and therefore the fusion protein is functional), the RFP fluorescent component of the rescue construct was used to view localization of the Exo70A1 protein in the papillae cells of the stigma before and after pollination to gain insight on Exo70A1 function. RFP fluorescence could be readily visualized in the papillae of the transgenic lines using confocal microscopy. Segregating progeny from heterozygous Exo70A1/exo70A1-1 *Arabidopsis* plants transformed with the RFP: *Bn*Exo70A1 construct, were genotyped to identify both wild-type (Line14/Col-0) and homozygous mutant (Line14/exo70A1) (Figure 8C). As a control, untransformed (Col-0) plants were examined using confocal microscopy to account for any autofluorescence found in the papillae cells in unpollinated stigmas (Figure 12A). After laser levels were adjusted appropriately and confocal imaging of unpollinated Line14/Col-0 stigmas (freshly opened flowers - stage 13 [Figure 5A]) revealed RFP fluorescence localized to the periphery of the papillar cells reminiscent of plasma membrane (Figure 12B-D). Notably, RFP: *Bn*Exo70A1 is only localized at the plasma membrane at maturity of the papillae cells as observation of stigmas from unopened flower buds (stage 12) (Figure 5A) showed RFP fluorescence in an internal punctate pattern reminiscent of Golgi (Figure 12E) (Boevink et al. 1998). Interestingly, when the localization of RFP: *Bn*Exo70A1 was examined in the Line14/exo70A1-1 mutant, a mixed pattern of both cell periphery (plasma membrane) and internal punctate localization was consistently observed in the mature stigmatic papillae (Figure 12F).
Figure 12. Characterization of *Arabidopsis thaliana* RFP:*BnExo70A1 lines in unpollinated Col-0 and *exo70A1-1* mutant stigmas

(A) In mature stigmas (Stage 13) from untransformed wild-type Col-0, no RFP fluorescence is seen. Confocal laser levels adjusted to an arbitrary “zero” using a Col-0 stigma to control for autofluorescence, any fluorescence above this level is considered to be from expression of the *SLR1::RFP:BnExo70A1* construct.

(B-D) In mature stigmas (Stage 13) from the Line14/Col-0 lines, RFP:*BnExo70A1* appears to be localized at the plasma membrane.

(E) In immature stigmas (Stage 12) from the Line14/Col-0 lines, RFP:*BnExo70A1* is localized in a punctate pattern which looks to be internal structures reminiscent of Golgi.

(F) In the Line14/*exo70A1-1* mature stigmas, most of the RFP:*BnExo70A1* is localized to the plasma membrane, but not all is properly sorted as some punctate pattern is still visible. The female sterility defect is rescued in these plants and the inset shows a close up of the “rescued” papillae.

Scale bars = 20µm for A-C, E- F; 50 µm for D.
Figure 12

A. RFP/DIC merge

B. Line14/Col-0 mature

C. Line14/Col-0 mature

D. Line14/Col-0 mature

E. Line14/Col-0 immature

F. Line14/ exo70A1-1 mature
This combined immature/mature patterning perhaps points to developmental defects in the growth stunted plants that are rescued solely at the stigma (Figure 13). Nevertheless, it appears that the PM-localized RFP: \textit{Bn}Exo70A1 is sufficient to restore the acceptance of wild-type compatible pollen and fertility (as documented in Figures 9, 10, and 11).

Having established the pre-pollination positioning of RFP: \textit{Bn}Exo70A1 in transformed wild-type plants, the next step was to observe whether dynamic changes occurring in the papillar cell after a compatible pollination might also affect Exo70A1 localization patterns. For this, pollinated untransformed wild-type (Col-0) plants were examined using confocal microscopy as a control to adjust against autofluorescence levels in the papillae cells in compatibly pollinated stigmas (Figure 14A). Confocal imaging of pollinated Line14/Col-0 stigmas revealed a disappearance of the RFP fluorescence which was previously located at the plasma membrane in the unpollinated papillae to be replaced with a more internal localization (Figure 14B-C). Accounting for the time needed for sample preparation, it was consistently observed that the RFP fluorescence disappeared from the plasma membrane in stigmatic papillae within minutes of pollination.

3.3 Various organelle GFP marker lines were used to verify subcellular localization of RFP: \textit{Bn}Exo70A1 during various stages of papillae maturity and pollination.

To verify localization of RFP: \textit{Bn}Exo70A1 and as a means to better understand the subcellular localization events that occur upon compatible pollinations, various organelle
Figure 13. *SLR1::RFP:BnExo70A1/exo70A1-1 Arabidopsis* plants retain their dwarf stature as the construct is expressed specifically and solely in the stigmatic tissue of the plant.

Because *SLR1* is a tissue-specific promoter (Franklin et al. 1996) and will drive this construct only in the stigmatic tissue of the rescue plants, the other characterized phenotypes of the *exo70A1-1* mutant are retained, for example, the dwarf stature of the mutant plants (Synek et al. 2006).

A wild-type Col-0 plant is compared to an *exo70A1-1* mutant as well as a Line 14 RFP:*BnExo70A1/exo70A1-1* “rescued” plant.
Figure 13

exo70A1-1  BnExo70A1/  exo70A1-1  Col-0
Figure 14. Characterization of RFP: \textit{BnExo70A1} in pollinated Col-0 stigmas

(A) In mature stigmas (Stage 13) from untransformed wild-type Col-0 and pollinated with Col-0 pollen, no RFP fluorescence is seen in the papillae cells. Again, confocal laser levels adjusted to an arbitrary “zero” using a Col-0 stigma to control for autofluorescence, and any fluorescence above this level is from expression of the \textit{SLR1::RFP:BnExo70A1} construct.

(B) In mature stigmas (Stage 13) from the Line 14/Col-0 lines that have been pollinated with wild-type Col-0 pollen, RFP: \textit{BnExo70A1} displays a change in localization disappearing entirely from the cell periphery (plasma membrane) (Figure 13 C-D, DIC/RFP merge) and appears more internal within the cell.

Scale bars= 50μm for A; 20μm for B,C.
Figure 14

A

RFP/DIC merge

Col-0 mature pollinated

B

Line14/Col-0 unpollinated

Pollinate w/ Col-0 pollen

Line14/Col-0 pollinated

C

Line14/Col-0 pollinated

Line14/Col-0 pollinated
GFP marker lines were sought with the intent of merging both the GFP and RFP signals to establish any co-localization of BnExo70A1 with any compartment. Each organelle GFP marker line was crossed into a RFP:BnExo70A1 expressing Col-0 plant (Line14/Col-0) creating double marker lines. Prior to assessing co-localization patterns, the validity of working with each donated marker line in stigmatic tissue first needed to be established. The organelles of most immediate interest were plasma membrane, Golgi, and vacuole, and the marker lines were GFP:PIP2A, ST:GFP, and GFP:δ-TIP respectively.

3.3.1 **GFP:PIP2A Plasma Membrane Marker Lines**

To confirm the peripheral localization of Exo70A1 in RFP:BnExo70A1 expressing wild-type stage 13 stigmas was indeed plasma-membrane localized, the GFP:PIP2A marker was obtained. When it came to examining the GFP:PIP2A plasma membrane line in the pistil, it is expressed well in the underlying basal stigmatic cells, but is poorly and inconsistently expressed in the stigmatic papillae (Figure 15A-B). In an attempt to see if pollination might bring about an up-regulation or even a re-distribution of the signal, GFP:PIP2A stigmas were pollinated with Col-0 pollen, however, neither scenario materialized and there was no change in the stigmatic papillae (Figure 15C-D). When it comes to the double crosses, GFP:PIP2A could not be clearly visualized in the RFP:BnExo70A1 transgenic plants (data not shown) and therefore did not prove useful to assess any co-localization patterns between the GFP:PIP2A plasma membrane marker and RFP:BnExo70A1.
Figure 15. Expression of the GFP:PIP2A, a plasma membrane marker, in the *Arabidopsis* stigma

(A-B) The GFP:PIP2A is expressed clearly in the underlying stigmatic cells, but very poorly in the stigmatic papillae and could not be reliably visualized in the RFP:*BnExo70A1* transgenic *Arabidopsis* plants. This GFP line is from Cutler et al. (2000) and was also a generous gift from the Cutler Lab (UC-Riverside). (scale bars= 50µm)

(C-D) Pollination of papillae cells on these marker stigmas did not yield a re-distribution or up-regulation of the GFP:PIP2A signal. (scale bars= 50µm)
GFP:PIP2A mature unpollinated

GFP:PIP2A mature unpollinated

GFP:PIP2A mature pollinated

GFP:PIP2A mature pollinated
3.3.2 GFP:δ-TIP Vacuole x RFP:BnExo70A1 Double Marker Lines

Because the vacuole is what keeps the cell turgid, the vacuolar membrane can often be in close proximity to the plasma membrane in stigmatic papillae (Figure 16). As a result, a presumed plasma membrane-localization pattern needs to be distinguished from the vacuolar. Thus, the GFP:δ-TIP vacuolar marker serves as a good alternative way to verify if RFP:BnExo70A1 is localized to the plasma membrane. The GFP:δ-TIP single marker line displayed sufficient expression in the papillae cells but interestingly very low expression in the basal cells of the stigmatic tissue (Figure 17A-B). A close up of the papillae cell tips show the vacuolar tubular network (Iwano et al. 2007) clearly marked as well (Figure 17C-D). Thus, this marker line was crossed to the RFP:BnExo70A1/Col-0 line. Looking at the double RFP:BnExo70A1/GFP:δ-TIP marker line, the RFP fluorescence of RFP:BnExo70A1 is distinct from the GFP of the vacuolar membrane (including the vacuolar tubular network) at the outer edge of the papillar cell (Figure 18). Thus, the RFP:BnExo70A1/GFP:δ-TIP double marker line excludes Exo70A1 from the vacuolar membrane confirming the likelihood that the protein is plasma membrane localized at the papillar cell periphery. Previously, in Brassica papillae, Iwano et al. (2007) had documented movement of the large central vacuole toward the site of pollen contact, perhaps as a source of water for the pollen hydration process. During the course of my work with the confocal microscope, distinct images corresponding to the large centralized vacuole extending toward the pollen attachment site could be observed in the papillae following pollination (Figure 16B).
Figure 16. Confocal DIC images of the vacuolar network in the *Arabidopsis* stigmatic papillae

(A) The vacuole in the un-pollinated stigmatic papillae sits generally centered within the cell.

(B) Following a compatible pollination, the central vacuole in the stigmatic papillae appears to become directed towards the pollen grain, possibly for water transfer.

Source: Full credit for Schematic (Self-Incompatibility in Flowering Plants, 2008, pgs. 173-191, Chapter 8: “Self” Pollen Rejection Through the Intersection of Two Cellular Pathways in the Brassicaceae: Self-Incompatibility and the Compatible Pollen Response, M.A. Samuel, D. Yee, K.E. Haasen & D.R. Goring, Figure 8.2, © Springer-Verlag Berlin Heidelberg 2008) is given to the publication in which the material was originally published. With kind permission of Springer Science+Business Media.
Figure 16

A

plasma membrane
golgi

cell wall

large central vacuole

B

compatible pollen

large central vacuole

DIC

Pre-pollination

Post-pollination
Figure 17. Expression of the GFP:δ-TIP, a vacuolar marker, in the *Arabidopsis* stigma

The GFP:δ-TIP is visible in the stigmatic papillae and was used to mark the vacuolar membrane and also the tubular vacuole network in the RFP:*BnExo70A1* transgenic *Arabidopsis* plants. This line is from Cutler et al. (2000) and was a generous gift from the Cutler Lab (UC-Riverside).

(A) Global view of papillae expressing GFP:δ-TIP (scale bar= 50µm)

(B) Papillae expressing GFP:δ-TIP (scale bar= 50µm)

(C-D) View of papillae cell showing tubular vacuolar network (scale bar= 10µm)
Figure 18. Expression of the GFP:δ-TIP, a vacuolar marker, in the stigma of a RFP:BnExo70A1/Col-0 plant

A stage 13 stigmatic papillae expressing both RFP:BnExo70A1 and a GFP:δ-TIP vacuolar marker. The GFP:δ-TIP marked vacuole membrane is separate and distinct from the RFP:BnExo70A1 signal indicating likely plasma membrane localization of RFP:BnExo70A1 in the mature papillae cell. (scale bars= 10µm)
Figure 18

GFP-δTIP  RFP:βnExo70A1  Merge
In over twenty five individual pollen-papillae observations, where the central vacuole could be clearly discerned, I observed this pattern. Thus, in future studies, the RFP: \( Bn \text{Exo70A1} / \text{GFP}\):δ-TIP double marker line may be useful to look at changes to the vacuole and \( Bn \text{Exo70A1} \) following pollination events. However, this type of experiment would require a confocal microscopy system that allows live pollinations under the microscope.

3.3.3 ST:GFP Golgi x RFP: \( Bn \text{Exo70A1} \) Double Marker Lines

To confirm that the punctate localization pattern of RFP: \( Bn \text{Exo70A1} \) in immature papillae (Figure 12E), was actually localization to the Golgi as we suspected, (Line14) RFP: \( Bn \text{Exo70A1} / \text{Col-0} \) line was crossed to a ST:GFP Golgi marker line. Looking at the ST:GFP Golgi marker line alone, a similar punctate pattern can be seen in the immature papillae (Figure 19A) as well as the mature papillae (Figure 19B-C). Upon pollination with Col-0 pollen on the single Golgi marker lines, there was no change in the punctate pattern of Golgi in the stigmatic papillae (Figure 19D). The RFP: \( Bn \text{Exo70A1} / \text{ST:GFP} \) (Golgi) double marker line showed co-localization in the immature papillae (stage 12) displaying the same punctate pattern (Figure 20A-B) verifying \( Bn \text{Exo70A1} \) localization at the Golgi prior to stigma maturation. In mature (stage 13) papillae, the RFP: \( Bn \text{Exo70A1} \) shows the plasma membrane re-localization (already shown in Figure 12B-D), and the ST:GFP Golgi marker keeps its typical localization pattern within the cell (Figure 19B-C).
Figure 19. Expression of ST:GFP, a Golgi marker, in the *Arabidopsis* stigma

The ST:GFP is clearly visible in the stigmatic papillae and was used to mark the Golgi in the Col-0 *Arabidopsis* plants.

(A) ST:GFP in immature (Stage 12) stigmatic papillae (unpollinated)

(B-C) ST:GFP in mature (Stage 13) stigmatic papillae (unpollinated)

(D) ST:GFP in mature (Stage 13) stigmatic papillae (pollinated)

All stages show a punctate pattern of localization for the Golgi with no changes upon pollination. This GFP line is from Saint-Jore et al. (2002) and was a generous gift from the Hawes Lab (Oxford). Scale bars= 50µm
Figure 19

A
GFP/DIC merge

ST:GFP Immature unpollinated

B

ST:GFP mature unpollinated

C

ST:GFP mature unpollinated

D

ST:GFP mature pollinated
Figure 20. Expression of the ST:GFP, a Golgi marker, in the stigma of a RFP: BnExo70A1/Col-0 plant

As per Figure 19, the ST:GFP marker line marks the Golgi in stigmatic papillae that show no change in localization regardless of stigma maturity and pollen reception.

(A-B) ST:GFP and RFP: BnExo70A1 co-localization in immature (Stage 12) stigmatic papillae (unpollinated) (scale bar = 10µm)

White arrows show overlapping areas.

(C) ST:GFP and RFP: BnExo70A1 localization in mature (Stage 13) stigmatic papillae (unpollinated). RFP: BnExo70A1 is seen at the plasma membrane away from the punctate Golgi (ST:GFP) (scale bars = 20µm)

(D) ST:GFP and RFP: BnExo70A1 overlapping localization in mature (Stage 13) stigmatic papillae (pollinated) five minutes post-pollination. RFP: BnExo70A1 is no longer seen at the plasma membrane but rather at internal structures within the cell and co-localizing at some but not all Golgi. (scale bars = 10µm)

White arrows show overlapping areas.

(E) ST:GFP and RFP: BnExo70A1 co-localization in basal stigmatic cells in an unpollinated stigma (scale bars = 10µm). There is no change in localization of the markers in the basal stigmatic cells upon pollination of the papillae (data not shown)
Figure 20

A

B

C

D

E

RFP: BnExo70A1/ST:GFP
Immature unpollinated

RFP: BnExo70A1/ST:GFP
Immature unpollinated

RFP: BnExo70A1/ST:GFP
Mature unpollinated

RFP: BnExo70A1/ST:GFP
Mature pollinated

RFP: BnExo70A1/ST:GFP
Stigmatic basal cells unpollinated
It is further interesting to note that upon pollination of the papillae, the RFP: \textit{BnExo70A1} localization disappears from the plasma membrane, (already shown in Figure 14), and in addition to an internal localization, some signal seems to reappear back at the Golgi 10 minutes post-pollination (Figure 20D, white arrows). Although, this post-pollination co-localization between the ST:GFP Golgi marker and RFP: \textit{BnExo70A1} is not as complete as the co-localization seen in the unpollinated RFP: \textit{BnExo70A1}/ST:GFP immature papillae (Figure 20A-B). Looking in the basal cells of the stigma, it also appears that RFP: \textit{BnExo70A1} is localizing to the Golgi (Figure 20E) and despite the fact they underlie mature, pollinated papillae cells, there is no change in the co-localization patterns in these cells.

3.4 Role of other Exo70 family members expressed in the stigma using an RNAi approach

With the observation that Exo70A1 could potentially have such an essential role in compatible pollinations in the stigma, combined with the knowledge that the plant Exo70 family has expanded to 23 members while this expansion is not seen in mammals or yeast, I was interested in investigating the roles other \textit{AtExo70} family members might have in the stigma. Using the expression data of the \textit{AtExo70} family (Chong et al. 2009; AGI numbers Appendix IV) assembled using the University of Toronto’s Bio-Array Resource (BAR) (Toufighi et al. 2005), the four family members with the highest expression in the stigma were used to clone hairpin RNAi constructs to knock-out or knock-down gene expression (specifically in the stigma) to try and elucidate any pollination-related function of these members. The four genes were \textit{AtExo70A1} (At5g03540), \textit{AtExo70D2} (At1g54090), \textit{AtExo70F1} (At5g50380), and \textit{AtExo70H7} (At5g59730). The RNAi construct cloning strategy (Figure 21) involved choosing unique
700 base pair segments (Appendix V) for each gene and cloning both a sense+intron and antisense segment separately into TOPO vectors, assembling the sense+intron:antisense in pBluescript (KS) vector and finally ligating the sense+intron:antisense piece into a customized SLR1 promoter (stigma-specific) p1665 plant transformation vector. The small intron sequence, used to fuse the sense and antisense fragments in tandem, can be found in Appendix VI. During the cloning process many problems were encountered including the difficulty of obtaining a high-yield purification of the p1665 plasmid. This could be partially attributed to the modest growth of transformed bacteria carrying this vector as well as its extremely large size (14kb) and low-copy number. More significantly, it was very difficult to assemble the sense+intron and antisense fragments in pBluescript and many transformations would result in the unexplained loss of one of these fragments in the plasmid. At completion of my labwork, the four RNAi constructs were at different stages in the cloning process (Appendix VII). Currently, AtExo70A1\textsubscript{1851-1550} sense+intron is in the pBluescript vector and AtExo70A1\textsubscript{1851-1550} antisense is in the TOPO vector. AtExo70H7\textsubscript{301-1000} sense+intron:antisense is assembled in pBluescript and ready to be dropped into the p1665 plant transformation vector. AtExo70D2\textsubscript{1,700} sense+intron and AtExo70D2\textsubscript{1,700} antisense are in separate TOPO vectors ready for assembly in pBluescript. Finally, AtExo70F1\textsubscript{201-900} sense+intron:antisense is fully assembled in p1665 plant transformation vector and is ready for transformation into Agrobacterium and finally into Arabidopsis (using a floral dip transformation method; Clough and Bent 1998). This ongoing endeavour will be carried out by another member of the lab who will be subsequently continuing on this project.
Figure 21. Exo70 Family Members RNAi Cloning Strategy

This schematic shows the approach taken to clone RNAi hairpin constructs for Exo70 family members Exo70A1, Exo70D2, Exo70F1 and Exo70H7. Each construct was made from 700bp sections unique to that particular gene. Restriction sites were designed into the amplification primers to allow for ligation into the various plasmids with the final plasmid being plant transformation vector p1665. Primers used can be found in Appendix III while the unique 700bp segments used for the RNAi hairpin constructs are listed in Appendix V.
RNAi Cloning Strategy

1. Build SENSE and ANTISENSE in TOPO (Kan)

2. Assemble in pBLUESCRIPT KS (Amp)

3. Drop into p1665 Plant Transformation Vector with SLR1 promoter
Chapter 4 : DISCUSSION

4 DISCUSSION

In flowering plants, there is a cascade of ordered events upon pollen landing on the papillae cells of the stigma, leading to a successful pollination. However, the molecular mechanism behind compatibility signaling in the stigma has remained somewhat of a mystery as large-scale genetic screens for these factors have been overall unsuccessful. Recently, the Goring lab identified \textit{BnExo70A1} as an interactor and substrate for the E3 ligase, ARC1, which plays a role as a positive regulator in the \textit{Brassica} self-incompatible pollen response (Stone et al. 2003; Samuel et al. 2009). Thus, because Exo70A1 appears to be a target for ubiquitination and degradation by ARC1 during a self-incompatible pollen response, Exo70A1 has been implicated as a putative “compatibility factor” in compatible pollen-pistil interactions and the model organism \textit{Arabidopsis} was used to investigate the role of Exo70 in plants, specifically in the female reproductive tissue, the stigma.

Through this study, many previously unknown aspects of Exo70A1’s role in compatible pollen-pistil interactions have been uncovered. My research has demonstrated that Exo70A1 is indeed essential for compatible pollinations in \textit{Arabidopsis}, and in fact, likely to be essential for compatible pollen-pistil interactions in the \textit{Brassicaceae} family as \textit{BnExo70A1} and \textit{AtExo70A1} share a conserved function in the stigma. Through fluorescent marker studies, I have shown that Exo70 is likely plasma membrane
localized and, as will be discussed below, this is consistent with what is seen in other systems. Furthermore, my work has revealed that post-pollination, there is a distinct change in Exo70A1 localization – the purpose of which has yet to be determined. Thus, this work has shed insight on the role of Exo70A1, a putative compatibility factor in the pollen-pistil interactions in *Arabidopsis* and the relevance and implications of my findings are discussed below.

4.1 **Exo70A1 has been shown to be a key factor required in the stigmatic papillae for compatible pollinations**

Through analysis of T-DNA insertion lines, knocking out *Exo70A1* expression in the *Arabidopsis* stigma resulted in a “female sterile” phenotype which includes a loss of compatible pollen recognition. This is essentially mimicking the self-incompatibility rejection response seen in self-incompatible *Brassica* W1 plants. Interestingly, in the compatible *Brassica* Westar line carrying an *Exo70A1* RNAi construct, reduced pollen hydration and pollen tube growth was observed. Likewise, an increased level of *BnExo70A1* in the stigmas of self-incompatible W1 cultivar plants was able to partially overcome self-incompatibility indicating that this pathway negatively regulates *Exo70A1* (Samuel et al. 2009). Expressing *BnExo70A1* in the stigmatic tissue of these *Arabidopsis exo70A1-1* mutants was also able to rescue the “female sterile” phenotype in that pollen acceptance ability was restored. Furthermore, the *Arabidopsis exo70A1-1* mutant plants did not set any seed, however, seed set in the rescued plants (RFP:*BnExo70A1/exo70A1-1*) was also restored back to levels similar to that found in wild-type Col-0. This demonstrates that expression of RFP:*BnExo70A1* provides
essentially a complete rescue of the *exo70A1-1* female sterile phenotype in the stigma of *Arabidopsis*. Thus, these results implicate Exo70A1’s role in compatible pollen-pistil interactions in both *Brassica* (Samuel et al. 2009) and *Arabidopsis*. These two species evolutionarily diverged over 10-20 million years ago (Muller 1981; Ziolkowski et al. 2006), and yet *AtExo70A1* and *BnExo70A1* show over 90% similarity (Samuel et al. 2009). This clearly demonstrates an evolutionarily conserved function for *Exo70A1* in the dry stigma of the *Brassicaceae*. This is an exciting finding as it is the first time any component has been shown to act at the impasse between the self-incompatible pathway in certain cultivars of *Brassica* and the compatibility pathway that occurs upon compatible pollen docking in the *Brassicaceae* in general.

4.2 Exo70A1 localizes to the plasma membrane in mature stigmatic papillae and to Golgi in immature stigmatic papillae

One interesting observation out of this study is that Exo70A1 only localizes to the plasma membrane of stigmatic papillae in fully developed (stage 13) flowers, and its timing is consistent with a stigmatic papillar function of being receptive to pollen with flower opening (Smyth et al. 1990). Given the previous establishment of Exo70 as the plasma-membrane localized spatial landmark for the yeast exocyst complex recruitment during exocytosis (Boyd et al. 2004), the localization of RFP:*BnExo70A1* to the plasma membrane in *Arabidopsis* mature stigmatic cells is not unexpected. As previously discussed, Exo70 can function as a spatial landmark to essentially recruit the other subunits of the exocyst complex for assembly the plasma membrane for the purpose of tethering secretory vesicles for exocytosis (Boyd et al. 2004). Therefore, the plasma
membrane localization of RFP: *Bn*Exo70A1 in *Arabidopsis* mature stigmatic papillae would be consistent with Exo70 perhaps functioning as a spatial landmark for exocyst assembly in plants. Admittedly, the localization of Exo70A1 along the entire papillar plasma membrane (versus just the tip of the cell most likely to come into contact with the pollen grain) indicates that another, currently unknown, regulatory event is likely required to occur at the pollen contact site in addition to the PM localization of Exo70A1 to promote localized exocyst assembly and proposed vesicle docking.

Prior to stage 13 “mature” papillae plasma membrane localization of Exo70A1, in stage 12 “immature” papillae (when the flower buds are still closed), Exo70A1 appears to localize in a punctate pattern reminiscent to that of the Golgi. The Golgi apparatus is part of the eukaryotic secretory pathway and again, is not an unexpected organelle for Exo70A1 to be associated with given the exocyst complex’s traditional role in exocytotic processes. In fact, in mammalian cells, prior to polarization, the exocyst has been reported to be cytosolic or in perinuclear structures (Matern et al. 2001; Vega and Hsu, 2001). And in cells that do not undergo polarization, Exo70, Sec6 and Sec8 were enriched in the trans-Golgi network (TGN)/endosomal network (Yeaman et al. 2001). Within plant cells, the Golgi apparatus, vesicles and even vacuole networks are the fundamental elements of the dynamic endomembrane system in plant cells (as reviewed in Harris and Watson 1991). Again, it has been proposed that the *Arabidopsis* exocyst complex has a role in vesicular trafficking with the recycling endosomal compartment which is thought to reside at the TGN/endosome (Zarsky et al. 2009) and this prediction is consistent with results obtained using transiently transformed tobacco BY-2 cells (Chong et al. 2009) and also this Exo70A1 localization study.
Interestingly, when the localization of RFP: BnExo70A1 was examined in the Line14/exo70A1-1 mutant, a mixed pattern of both plasma membrane (cell periphery) and internal punctate localization was consistently observed in the stage 13 stigmatic papillae (Figure 12F). Because the stigma-specific SLR1 promoter was used to drive the expression of RFP: BnExo70A1 in the exo70A1-1 homozygous mutants, only the stigmatic fertility defect was restored in these plants and as previously mentioned, the overall growth defect remains in these plants (Figure 13) (Synek et al. 2006; Samuel et al. 2009) Therefore, it is also possible that this overall plant growth defect may also cause incomplete maturation of the pistil resulting in the partial plasma membrane localization of RFP: BnExo70A1. Previously, Synek et al. (2006) had reported that the exo70A1-1 mutant stigmas were immature in appearance at flower bud opening (stage 13). However, throughout this study, stigma maturity and papillae length was found to be variable and elongated stigmatic papillae could be observed at flower opening in the exo70A1-1 mutants (Figure 11C-D). This variable elongation found at flower opening indicates that the inability of exo70A1-1 plants to accept compatible pollen is not solely due to a delay in development but is more likely caused by the absence of an essential recognition component (Exo70A1) missing from the system.

4.3 Following pollen contact, Exo70A1 is no longer localized at the plasma membrane

Upon pollination of the RFP: BnExo70A1/Col-0 stigma, RFP: BnExo70A1 “disappears” from its former plasma membrane localization and is found in an alternative localization pattern within the papillae cell. This post-pollination absence of RFP fluorescence at the
plasma membrane appears to begin to dissipate in the papillae after contact with the pollen grain. BnExo70A1 is localized to the plasma membrane prior to contact of a pollen grain, it could be speculated that once contact is made between the papillae cell and pollen grain, Exo70A1 having performed its function at the plasma membrane and can then re-localize to an unspecified location within the cell. It is reasonable to hypothesize that perhaps after Exo70’s role has been fulfilled, it is internalized into the cell and degraded. This has not yet been explored experimentally but given the proper microscope system set-up which would allow for live pollinations to occur within the field of view, a time course movie would be ideal in determining the path and ultimate destination of Exo70A1 post-pollination. As well, the use of proteasomal inhibitors may shed insight by allowing the monitoring of levels of Exo70A1 upon treatment to see whether there is a build up of Exo70A1 post-pollination, indicating that the degradation mechanism that is no longer functional.

Because the traditional role of the exocyst is docking and tethering of vesicles to the plasma membrane, it is not unreasonable to expect a re-localization after the vesicles have presumably unloaded their cargo. What is intriguing is the re-appearance of Golgi and Exo70A1 post-pollination co-localization which raises the question: is Exo70A1 being recycled back to the Golgi or is this co-localization a result of newly synthesized protein?

Both scenarios are feasible as this post-pollination localization may implicate a role for Exo70A1 in the regulation of “recycling domains” within the cell (reviewed in Zarsky et al. 2009). Recycling domains are seen in plants mostly with the regulation of the PIN proteins when it comes to auxin transport (Geldner et al. 2003). It has been suggested
that the Exo70 subunits, along with plant Rab GTPases, are involved in the establishment and maintenance of recycling domains within a polarized plant cell. In fact, this has been seen in yeast where Exo70 controls the secretion of specific exocytotic vesicles such as Bgl2p, in that varying exocytotic pathways are regulated both at the target membrane and the trans-Golgi network (He et al. 2007; reviewed in Zarsky et al. 2009). The implication being that the different Exo70 family members in plants are diverse in function and may therefore localize to different exocytotically active domains within the same cell and upon assembly of the complex and tethering of the vesicle, the subunit returns back to its domain to facilitate further processes.

Alternatively, the post-pollination co-localization of Exo70A1 at the Golgi could be a result of newly synthesized protein. This is a possibility as 10 minutes post-mounting on a slide, the stigma is still active and as such, is likely capable of synthesizing new protein. Perhaps there is a balance of the amount of Exo70 that must be kept at the membrane and once pollination has occurred, there is a delay in the signaling cues responsible for relaying this information to the Golgi. Thus, Exo70A1 protein is synthesized for a time period post-pollination and this is the signal seen in the Golgi post-pollination. Therefore, should an experiment track time past 10 minutes post-pollination they may see a depletion of this signal as the signaling cues are finally relayed to the Golgi to cease production of Exo70A1.

4.4 Change in Exo70A1 localization in polarized tip-growing papillae cells

Recent research has revealed that several Arabidopsis exocyst subunit genes, Sec5,
Sec6, Sec8, Sec15a, and Exo70A1, have been implicated to function in polarized pollen tube growth (Cole et al. 2005; Hala et al. 2008), root hair elongation (Wen et al. 2005), and polar growth and development (Synek et al. 2006). Exocyst subunit multiple mutant combinations had a compounding effect on phenotypes, for example, accentuating the short hypocotyl phenotype seen in the exo70A1 single mutants (Synek et al. 2006; Hala et al. 2008). Recently, experiments in growing pollen tubes have shown increased localization of Sec6, Sec8, and Exo70A1 at the tip of the polarized cell indicating the likely assembly of the exocyst subunits as a complex (Hala et al. 2008). What is interesting to note in these results is that while Exo70A1 localizes to the plasma membrane in the polarized mature papillae cells, the stigmatic basal cells do not show any obvious plasma membrane localization pre-pollination or even a re-localization post-pollination. In fact, Exo70A1 resides in the Golgi seemingly at all times in the stigmatic basal cells (Figure 20E). Perhaps, in the stigmatic basal cells, movement of Exo70A1 (likely to the plasma membrane for example) occurs in a dynamic and transient fashion while a “stock” of Exo70A1 is present in the Golgi at all times. In contrast to the highly polarized stigmatic papillae cells wherein Exo70A1’s localization is more static in nature as it waits at the plasma membrane cells for pollen docking, allowing visualization of plasma membrane localization as seen in this study (Figure 12B-D; Figure 20C).
4.5 The exocyst is potentially involved in delivering valuable stigmatic resources to the site of pollen contact at the plasma membrane

Presuming that during a compatible pollen-pistil interaction, the exocyst is fulfilling its traditional role of tethering cargo-bearing exocytotic vesicles to the plasma membrane (and there is strong evidence, as discussed, that this is the case) then the question remains: what is being delivered by these tethered vesicles? The obvious answer is crucial stigmatic resources provided to the pollen upon docking, such as components required for successful pollen hydration and pollen tube penetration through the stigmatic cell wall on its journey to the ovaries. To investigate the contents of secretory vesicles, fractionation experiments could be performed to separate the vesicles from other cellular components (Walworth and Novick 1987) followed by a detergent assay to disrupt the vesicle membrane and allow access to the contents. Through mass-spectrometry work, the compounds contained could now be identified offering further insight into compatible pollen-pistil interaction requirements.

Because the *Brassicaceae* have a “dry” stigma, a reasonable hypothesis for the role of Exo70A1 in the stigma would be the delivery of vesicles containing aquaporins (plasma membrane intrinsic proteins – PIPs) for increased water permeability (to allow for pollen hydration) or perhaps cell-wall modifying enzymes (to facilitate pollen tube penetration through the stigmatic surface) as a rich exude is not present as it would be in species with a “wet” stigma. Through a literature search I have determined that the best class of aquaporins to investigate in the papillae cells is in the PIP2 family as PIP2 proteins have been shown to have high water conductivity (Luu and Maurel, 2005) whereas the PIP1 family is known to transport solutes and have low water channel activity (Luu and
Maurel, 2005). PIP2B (formally PIP2-2) and PIP2C (formally PIP2-3) are good candidates for investigating their possible role in pollen hydration upon contact with a receptive stigmatic papillae. These candidates are also highly expressed in the stigmatic tissue (BAR database: Toufighi et al. 2005) suggesting a likely function in this tissue and cDNA subtraction experiments revealed PIP2B being isolated three times more often that PIP2A in the stigma indication it may be the more prevalent PIP family member in the female reproductive tissue (Swanson et al. 2005). Although the PIP2A (formally PIP2-1) aquaporin used in the plasma membrane marker line was not well expressed in the papillae, this may just mean that PIP2A is not the predominant aquaporin in the papillae cells. PIPs are known to be mobile in the cell and have been implicated in being transported through the vesicle trafficking process (Riethmuller et al. 2008). In the animal system the exocyst complex could be tethering and docking intracellular vesicles carrying aquaporins in renal collecting duct cells which are thought to fuse with the plasma membrane to increase water permeability within the organ (Barile et al. 2005). During a compatible pollen-pistil interaction, it is possible that aquaporins are the cargo carried by secretory vesicles that are being tethered and docked at the PM by the exocyst complex. Defining the possible role of aquaporins being a putative vesicle cargo is an important next step in defining the molecular cascade occurring upon a compatible pollen-pistil interaction.
4.6 The possibility of Exo70 and the exocyst complex participating directly in the movement of the vacuolar network to the site of pollen contact remains to be seen

Recently, an intriguing finding by another research group regarding the vacuolar movement in the stigmatic papillae in response to compatible pollen has raised some interesting questions (Iwano et al. 2007). The vacuole and tubular vacuolar network were observed to orient towards the compatible pollen attachment site (Iwano et al. 2007). In contrast, self-incompatible pollinations were associated with disruption of the vacuolar network in the stigmatic papillae (Iwano et al. 2007). The source of hydration for the pollen still remains unknown, although because the source must be internal in the stigma, the vacuole is the most obvious candidate (Iwano et al. 2007). Whether Exo70 and the exocyst complex are directly participating in the movement of the vacuolar network does remain to be conclusively investigated although this would be an interesting avenue to pursue as this would present a new, novel role for the exocyst complex that is perhaps plant-specific and would begin to explain the expansion of the Exo70 gene family found only in plants.

4.7 Future Prospects

While this work reveals many previously unknown details about the involvement of Exo70A1 in compatible pollen-pistil interactions in Arabidopsis, there remains much to be answered. First, further localization studies could be performed, provided the correct confocal microscope set-up were available, regarding what happens to Exo70A1 post-pollination. These experiments would likely require a micromanipulator to place a single pollen grain on a single papillae cell to track changes. My work has shown there is a change in localization and a possible internalization of the signal, but what remains to
be seen is whether or not Exo70A1 is being degraded or perhaps recycled back into the endomembrane system. As well, this work did not address the role of actin and microtubule cytoskeletal networks and their role in compatible pollen-pistil interactions. It has been previously reported that there is a disruption of the actin network in a self-incompatible pollination in Brassica papillae (Iwano et al. 2007) and thus vesicle trafficking is halted and no “compatibility resources” cargo are made available to the pollen. Another question to be addressed is how does Exo70A1 know when its job is complete and is then dissipated from the plasma membrane and back to the internal cell, in a compatible interaction. In other words, what is Exo70A1s course post-pollination? In addition, it would be interesting to attempt some Transmission Electron Microscope (TEM) work to look at high resolution images vesicle localization as well as Exo70A1 localization pre- and post-pollination. Essentially, using the RFP:BnExo70A1 transformed plants, one could use an antibody specific to the RFP for visualization. Finally, following up from work I have already started, it will be interesting to see the effect on stigma fertility of the RNAi constructs of Exo70A1, Exo70D2, Exo70F1, and Exo70H7 and whether this sheds some light on possible specialized functions of the Exo70 family members.

4.8 Conclusion:

Exo70A1 acts at the impasse between self-incompatible pollen rejection and compatible pollen acceptance

Exo70A1 being a putative compatibility factor was found using a “creative logic” as genetic screens for compatibility factors required for pollinations were largely unsuccessful. Looking back to the self-incompatibility response in Brassica, if BnExo70A1 were inhibited by ARC1, putative vesicles containing stigmatic
“compatibility” factors would thereby be prevented from fusing with the plasma membrane and result in the rapid and effective rejection of “self” pollen by withholding necessary resources. Using this logic, Exo70A1’s role in compatible pollen-pistil interactions has been investigated.

As previously discussed, upon commencement of stage 13, floral maturation has occurred, the stigmatic papillae have elongated and Exo70A1 re-localizes to the plasma membrane (cell periphery) of the papilla cell. Based on research in yeast and animal systems, Exo70A1 is located in a prime position to perhaps function as the spatial landmark. Thus, Exo70A1 may act to indicate where the remaining subunits of the exocyst complex are to assemble, which in turn directs the tethering and docking of pollination-sustaining vesicles carrying cargo necessary for a compatible interaction. Given the necessity of Exo70A1 during successful pollination events, as addressed in this study, it is reasonable to theorize that upon compatible pollen acceptance, the PM-docked vesicles (docked by Exo70A1) provide essential stigmatic “compatibility” factors required by pollen to continue a successful interaction. Once pollen adhesion, hydration and penetration have taken place, “compatibility” factors would not longer be required. Correspondingly, Exo70A1 leaves the PM to an internal localization in the cell where it could be recycled back to the Golgi or perhaps tagged for degradation (Figure 22).

Through my research, we now have more insight into how Exo70A1 plays a key role at the interface of the pollen compatibility and self-incompatibility response pathways in the Brassicaceae stigma.
Figure 22. Potential model for Exo70A1’s role/localization during compatible pollen-pistil interactions in *Arabidopsis*

Exo70A1 resides in the Golgi in the “immature” stigmatic papillae during stage 12 of flower development.

Upon commencement of stage 13, floral maturation has occurred, the stigmatic papillae have elongated and Exo70A1 re-localizes to the plasma membrane (cell periphery) of the papilla cell. Based on research in yeast and animal systems, Exo70A1 is located in a prime position to perhaps function as the spatial landmark. Thus, Exo70A1 may act to indicate where the remaining subunits of the exocyst complex are to assemble, which in turn directs the tethering and docking of pollination-sustaining vesicles carrying cargo necessary for a compatible interaction.

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Correspondingly, Exo70A1 leaves the PM to an internal localization in the cell where it could be recycled back to the Golgi or perhaps tagged for degradation.
REFERENCES


Robinson NG, Guo L, Imai J, Toh EA, Matsui Y, Tamanoi F. 1999. Rh3 of Saccharomyces cerevisiae, which regulates the actin cytoskeleton and exocytosis, is a GTPase which interacts with Myo2 and Exo70. Mol Cell Biol 19, 3580-3587.


APPENDICES

APPENDIX I

Additional Published Material: Plant Cell Paper Reference

APPENDIX II

Additional Published Material: Springer Book Chapter Reference

### APPENDIX III

**Primer Chart – Primers utilized in this study.**

**PRIMER LIST 2007-2009**

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<th>Primer</th>
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APPENDIX IV

AGI Numbers of *Arabidopsis* Exocyst Subunits

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APPENDIX V

RNAi Strategy: 700bp Fragment Sequences

700bp fragments of AtExo70A1, H7, F1, and D2 used for RNAi

AtExo70A1

851 GAGAAAGACA AGTATGTGAC CAGATATTCC GAGGCTTCGA TTCTCTAAGT
901 GATCAGTGTT TTGCAGAAGT TACAGTGAGC AGTGTCTCAA TGCTACTTAG
951 CTGGGGGAT GCTATGCCCA GGAGCAAGAG ATCTCCAGAA AAGTTGTTTG
1001 TACTCTTAGA CATGTAAGAG ATAAATGGGG AGGTTCATAC AGAGATTGAG
1051 ACAATTTTCA AAGGAAAGGC ATGCCGCAAA ATTAGAGATT CTGCAGCAGG
1101 CTTGACAAAG CGTTTGCCCG AAATGTGCCA GGAACATATT GGTGATTGG
1151 AAGAAGCTGT TGAGAAAGAT GCTACAAAGA CTGCTGTTCT AGATGGAGAT
1201 ACTCAAACTGC CAGCTAGCA TCCGTAACGA GACAGCCTGC TCTGGACTTT
1251 CTACCAAACA ATCATGAGAC AGGTGAGGAC AAGTTGTTTG GAGGGAAGAG
1301 ACTCAAAATGC CAGCTAGCA TCCGTAACGA GACAGCCTGC TCTGGACTTT
1351 CTACCAAAATGC CAGCTAGCA TCCGTAACGA GACAGCCTGC TCTGGACTTT
1401 CTACCAAAATGC CAGCTAGCA TCCGTAACGA GACAGCCTGC TCTGGACTTT
1451 CTACCAAAATGC CAGCTAGCA TCCGTAACGA GACAGCCTGC TCTGGACTTT
1501 CTACCAAAATGC CAGCTAGCA TCCGTAACGA GACAGCCTGC TCTGGACTTT

AtExo70D2

1 ATGGCAACAC CGGAGACTCG TGGCGTTGAT TCCAGTTTAC AGACAGCGGA
51 GAGAGTAAATA CTCCGATTTG AGTTGCCTAT ACATGTGACG GCTAAAGAGA
101 ATCTGATTATT CCAAGGACGG GGTGATCATG AGAAGTTGCA AACAGCTTTG
151 AAAGCTGAGA GTGAGATCGC CTTGATGCTT CTGTTGTATT CCTGAGGACT
201 TGAAGTGCGA GCACAGGAGC CCGCCTGGCT GCTAGACTG GAGAAGCGGC
251 TAAGAGATTTT ATTCTGCTCT AAGACCTGCT CTTGCTGACT TCTGACTGTC
301 CTCTCTGCTT TCTGCTGACT TCTGCTGACT TCTGACTGTC
351 GGAGCTTTGAG TAGGACTATC GAGCTTCTGA AACAGGAGCC TCGGTGATCC
401 AACAGGACTG TCTGGTTTAT CCAGATGCTT CCGGTCATGC TCTGGTTTAT
451 GAGAGACTG TCTGGTTTAT CCAGATGCTT CCGGTCATGC TCTGGTTTAT
501 ACAAGGAGC TCTGGTTTAT CCAGATGCTT CCGGTCATGC TCTGGTTTAT
551 TAGGCTGGG CAGGATGCTG CAGGATGCTG CAGGATGCTG CAGGATGCTG
601 TACGAGATGT CGGTGTTTAT ACCTGCTGAT CAGGATTGCA GCAGCTGATG
651 GATTTGGACA CAGGAGCTG CAGGAGCTG CAGGAGCTG CAGGAGCTG
AtExo70F1

201  CCAGGAAGAG AATCAAAACG ACGCTCTTGT GGCRCGGTTTA GAGGCTGCAG
251  AATCGGTTAT TCATCGGTGG GATGGTGGTA ATGATTTCCT TCGTCACTCG
301  TCTCTCAGTCT CTGTTAACTA TCGTTTCCCT TCGTTTTCTC TTTGGTTTTGA
351  TGAATCTCCG GAGGAAGCTA CCGAGTTTCT TTTGAGCTGT GATGAGATTA
401  TCTCTTTTGCT TGGAGATTTCT TCGTCTGAGA ATCAGCTCTG AAGATGAAAT
451  TAGACGGATT TCAGTTCTCT TCTCCTTCTC TTTAGTGGGA GACGACTCTC
501  ATGAGCTCGAT CCGCGGTTCT TCTTGTGCTT TGATGTGGGT TGATGGTGGT
601  GAGGATTTTG AGAAATTTTG TTTGGTCTCT GATGAGAGTG GTAGTGAGGAG
651  TAGAGGAGGG TTGTGTTCTAT AGAGAGAGCT TCTCTTTGCT CAGAGAAGCC
701  GAGGAGTGAG AATGAAATTT GAGGATTTGT TGAGAGTGAG GAGATAGAGGA
751  TCCGGTTTCT CTTGGTTTTA TTGTTTCTCT GCTGAGCTTG AGATGAGAGT
801  GAGAAGCTGAG TTATAGAAGA GTTCAGAAGA TTGATTGGAA GTCTATGGAT
851  AGAGCTCGAG TATAGAAGGT AGAGCTGAGA TATAGAAGGT AGAGCTGAGA

AtExo70H7

301  CTTCTCGAAT CAGAGTTTCA TCGTGTTTTA AAAGCGAACC GTGAGATTTT
351  AGATCTCGGAA TCGGATCTCT TACGTCTCTT ATGATCTCTA CAGAGTGGAA
401  TCTCTGACTAC CAGATGCTGCA TCGAGATTCC AAGACGAAAG CAGTTACGAA
451  GAAAACGCAG ACGACAGAAG TAGATCTCTC TACTGGATTA TCAGTGGAGA
501  GAGATGATCTC AAGATGATTG CTGATTGTAT GATCTCTCTC GAGGCTCGAT
551  AGAGCTCGAT AATGAAATTT GAGGATTTGT TGAGAGTGAG GAGATAGAGGA
601  TGGCGGTTTC TTTAGGCTTC TTTAGGCTTC TTTAGGCTTC TTTAGGCTTC
651  AATGAGATGG GAGATGCTTG AATCAAGAG CAAACATGG CTAAAAGCTG
701  TGGATATTTGA TCGGATCTCTG TCTTCTCTTG TCTTCTCTTG TCTTCTCTTG
751  CATGTTTCTCT TCTCTGCTTG TTTAATGCTT GATAGCTTCTG ACTCTGAGA
801  TACTGATCTC ATCGACTAAA GGAGATTTTG TCTTGCTGTT TCTTGCTGTT
851  AGAGCTCGAT GAGGATTTTG TTTAGGCTTC TTTAGGCTTC TTTAGGCTTC
901  AGCTTGTGAG GAGTGAAGCT TTTAGGCTTC TTTAGGCTTC TTTAGGCTTC
951  ATCAGCTCGT GCCTGCTGTAG TCTAGGTGAT AACTCGTTA GCTAGACTCG
APPENDIX VI

RNAi Strategy: Intron Sequence

Intron sequence used in the construction of RNAi hairpin constructs

5' GAGGTAAGTAGTTTTTGAGCTCATAG 3'
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**Figures 5, 6, and 16**

Full credit (Self-Incompatibility in Flowering Plants, 2008, pgs. 173-191, Chapter 8: “Self” Pollen Rejection Through the Intersection of Two Cellular Pathways in the Brassicaceae: Self-Incompatibility and the Compatible Pollen Response, M.A. Samuel, D. Yee, K.E.Haasen & D.R.Goring, Figure 8.1 and 8.2, © Springer-Verlag Berlin Heidelberg 2008) is given to the publication in which the material was originally published. With kind permission of Springer Science+Business Media.

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