2018-01-11

A Stigma-Specific Phospholipase D1 Is Required for Successful Pollination and Is Targeted by the Self-Incompatibility Response in Brassica napus

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A Stigma-Specific Phospholipase D1 Is Required for Successful Pollination and Is Targeted by the Self-Incompatibility Response in *Brassica napus*

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

GRADUATE PROGRAM IN BIOLOGICAL SCIENCES

CALGARY, ALBERTA

JANUARY, 2018

Abstract

Self-incompatibility (SI) is a genetic mechanism in hermaphroditic flowers that prevents inbreeding by rejection of self-pollen, while allowing cross or genetically diverse pollen to germinate on the stigma to successfully fertilize the ovules. In the Brassicaceae, this process is triggered by activation of a receptor kinase in the papillary cells of the stigma following binding of a haplotype-specific ligand produced by the pollen. Once activated, this phospho-relay converges on intracellular compatibility factors, which are immediately targeted for degradation by the E3 ligase, ARC1, resulting in the pollen rejection response. Through a proteomics approach using self-pollinated stigmatic proteins from canola (*Brassica napus*) phospholipase D1 (PLD1) was identified as one of the candidate proteins that is targeted for degradation following SI. In this study, I provide strong evidence for role of PLD1 as a stigmatic compatibility factor and a target of SI. Loss of PLD1 led to a reduction in pollen attachment and pollen tube penetration following compatible pollination, while overexpression of PLD1 in self-incompatible stigmas led to breakdown of SI response. PLD1 overexpression was also associated with enhanced membrane activity following SI pollination, and mimicked a compatible pollination. PLD1 can be ubiquitinated by ARC1 (ARM repeat-containing protein 1) and accumulated in ARC1-suppressed lines confirming PLD1 as direct target of ARC1 during SI response. I further showed that addition of phosphatidic acid (PA) to the PLD1 deficient stigmas could rescue compatibility and also improve self-pollen attachment on SI stigmas, establishing the essential nature of PA for a compatible interaction. I propose that PA produced by PLD1 activity during compatible pollination promotes vesicle fusion at the membrane to facilitate exocytosis necessary for pollen germination to occur, while SI response could abrogate this

process by targeting PLD1 for degradation. Identification of PLD1 as a membrane modeling factor that is directly regulated by both compatible and incompatible pollination has significantly expanded our understanding of the complex mechanisms that operate during pollen-pistil interactions.

Keywords: Canola, Phospholipase D1, Plant reproduction, Pollination, Proteasome, Self-Incompatibility, Phosphatidic acid

Acknowledgements

I would like to express my profound gratitude to my supervisor, Dr. Marcus Samuel for providing me with this opportunity to complete my Ph.D. and for always challenging me in my reasoning and approach to the field of science. I particularly appreciated his mentorship throughout my studies and all the wonderful food that we shared.

I would also like to acknowledge Dr. Dae-Kyun Ro and Dr. Douglas Muench, for being members of my supervisory committee and for their support in guiding my project and research. I would like to thank my PhD external examiners Dr. Sophia Stone and Dr. Belinda Heyne, for accepting to read my thesis and evaluate my work.

Thanks to Dr. Edward C. Yeung for sharing his precious and fascinating knowledge in TEM. Thank you for always being available and for providing me with some special kind of diamonds. I have really appreciated working with you in your lab.

Special thanks to all the current and former Samuel lab members. Jamshed, I wish you great happiness with your new family member and in your career. My dear Siyu, thank you. I would have never survived this trip without you. You inspired me with your tireless and rigorous work. Thank you for all the meals, for your company and your moral support. Subbu, thank you for introducing me to the canola world and showing me all the basics in the lab. All the best to you in your career. Thanks to Sri for helping me in launching some important steps of my project. Thanks to Matija, Logan, Neil, Mendel and Lan. You are admirable gentlemen. I appreciate my time spent with you in the lab.

To Abhi, you deserve a very special thank you. You are an incredible person and have been a perfect lab-mate. Thank you for your patience in answering my countless questions.

Thank you for your kindness and friendship. I wish you all the best in your future endeavors and in what is sure to be a fulfilling career. Warm thanks to Cate McRae for her friendship and support. Thanks to the U of C greenhouse manager, Diane White, for her friendship and for always doing her best taking care of my plants.

I am grateful for my French compatriots, especially Dr. Louise Gilbert and Dr. Amélie Bernard, who shared all their passion for plants and biology with me. You were my first mentors, and I will always be grateful for your encouragement. Thanks to Dr. Frédéric Delmas who made my dream to do my Ph.D. in Canada come true.

To my parents, I take this opportunity to express my deepest gratitude for all your efforts and sacrifices. You never compromised when caring and providing for my brother and me and for wanting the best for our education and wellbeing. Thank you for never giving up on me. I would like to extend a similar thank you to my grandparents who supported my parents and were the perfect complement to this, sometimes, very complicated task. Thanks to my brother Bruno who continues to be there for me, and who always finds the right words to comfort me in difficult times. I cannot imagine a better brother. A special thought to Laure and my two nephews Alice and Martin. Thanks to my aunt Tatie Nina for always sending me beautiful cards and letters with the latest family news and neighborhood gossips.

Thanks to everyone outside of my lab who helped me discover the beauty of Canada, Calgary, and their community (Andrea M and Andrea W, Lindsey, MJ, Blaire, Ashley, Amanda, and Tamika). Finally, thanks to my partner Donna, for her infinite kindness, patience, love and for introducing me to the performing arts. I cannot wait for our next adventures.

Dedication

In memory of my mother Danielle Scandola and my grandmother Maria Ricard.

You are deeply missed.

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List of Symbols, Abbreviations, and Nomenclature

^{o}C	degree celsius
%	percentage
2D-DIGE	two dimensional – differential gel electrophoresis
ABA	abscisic acid
ARC1	Arm repeat containing 1 protein
ARM	Armadillo
At	*Arabidopsis thaliana*
ATP	adenosine triphosphate
Bn	*Brassica napus*
bp	base pair
cDNA	complementary deoxyribonucleic acid
CP	compatible pollination
CTAB	cetyltrimethylammonium bromide
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphate
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylenediaminetetra acetic acid
EGTA	ethylene glycol tetra acetic acid
Exo70A1	exocyst subunit exo70 family protein A1
EXO70	exocyst complex component 70
g	gravitational force
GLO1	glyoxalase 1
gm	gram(s)
GSH	glutathione
GSI	gametophytic self-incompatibility
GST	glutathione S-transferase
h	hour(s)
H_2O	water
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IB	immunoblot
IP	immunoprecipitation
K_3PO4	tripotassium phosphate
kb	kilobase pairs
kD	kilo Dalton
L	litre
LB	Luria-Bertani
M	molar
mg	milligrams

MG	methylglyoxal
$MgSO_4$	magnesium sulfate
min	minute (s)
ml	milliliters
mM	millimolar
mRNA	messenger ribonucleic acid
MS mixture	Murashige and Skoog salt
MVB	multivesicular body
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram
nm	nanometer
OD	optical density
PA	phosphatidic acid
PAGE	polyacrylamide gel electrophoresis
PC	phosphatidylcholine
PCD	programmed cell death
PCP	pollen coat protein
PCP-A1	pollen coat protein class A1
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
pH	decimal logarithm of the reciprocal of the hydrogen ion activity
PIP2	phosphatidylinositol biphosphate
PLD1	phospholipase D1
PMSF	phenylmethylsulfonyl fluoride
RbohD/F	respiratory burst oxidase homolog D/F
RFP	red fluorescent protein
RFU	relative fluorescence unit
RNA	ribonucleic acid
RNAi	RNA interference
RNAse	ribonuclease
ROS	reactive oxygen species
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse-transcription polymerase chase reaction
SDS	sodium dodecyl sulfate
SI	self-incompatibility
SLB	s-locus F-box
SLF	s-locus F-box
SLG	s-locus glycoprotein
SLR1	s-locus-related gene 1
SLR1-BP	s-locus-related 1-binding pollen coat protein

SP11/SCR	s-locus protein 11 or S-locus Cys-rich
SRK	s-locus-receptor kinase
S-RNase	S-locus ribonuclease
SSI	sporophytic self-incompatibility
TEM	transmission electron microscopy
Tris	tris (hydroxymethyl)aminomethane
T-DNA	transfer DNA
UP	unpollinated
v	volume
V	volts
v/v	volume/volume
w/v	weight/volume
µg	micrograms
µl	microliters
µm	micrometers
µM	micromolar

Chapter 1: General Introduction

Brassicaceae is one of the largest families of land plants, species of which play an economically important role in global food production and food security. There are 4600 species in Brassicaceae which include crop species such as *Brassica oleracea* (broccoli, cabbage, cauliflower), *Brassica rapa* (turnip, Chinese cabbage), *Brassica napus* (rapeseed or canola) and also the model organism *Arabidopsis thaliana* (thale cress). Of these species, canola is the most important cash crop in Canada contributing ~ 27 billion dollars annually to the Canadian economy. Canola is mainly cultivated for its seed oil, rich in omega three and six fatty acids, widely used for cooking as well as for biodiesel production. Given the agronomic importance of canola and the constant demand to increase production on available arable land, understanding the mechanism behind plant reproduction, particularly in Brassicaceae, has become a major research focus. Identifying the major players in the reproduction pathway which is initiated and dependent on the successful interaction between the pollen and the stigmatic papillary cell, would allow us to strategize to improve canola yield.

1.1. Reproduction in Angiosperms

Angiosperms represent 90% of all land plants, and evolution of the flowers allowed its radiance and dominance over all land terrains. Another major reproductive adaptation in angiosperms is improved efficiency of pollination and pollen tube growth. During pollination, when a pollen (male gametophyte) produced in the anther, lands on the stigma (part of the maternal reproductive tissue covered with unicellular papillae) (Figure 1), if

the pollen is compatible, the pollen will germinate and produce a pollen tube, which allows the transport of the sperm cells to the ovaries where the fertilization takes place. Emergence of the pollen tube is called siphonogamy, an evolutionary progress that allowed land plants to reproduce in the absence of external water (Hiscock and Allen, 2008). During pollination, a series of crucial events and constant cross-talk occurs between the pollen and the female reproductive tissues (Dresselhaus and Franklin-Tong, 2013). First, the pollen is captured and allowed to adhere to the stigmatic papillae. In species of Brassicaceae with dry stigmas, this is where exchange of molecular information takes place between these two cell types. If this interaction is favorable, the pollen germinates, and the pollen tube enters the style through penetrating the papillary cells. The pollen tube will then proceed through the stylar transmitting tract, towards the ovule. Once the pollen tube penetrates the ovule, the sperm cells are released to fertilize the egg cell to accomplish the fertilization event (Heslop-Harrison, 1975; Dresselhaus, 2006; Chapman and Goring, 2010). Because

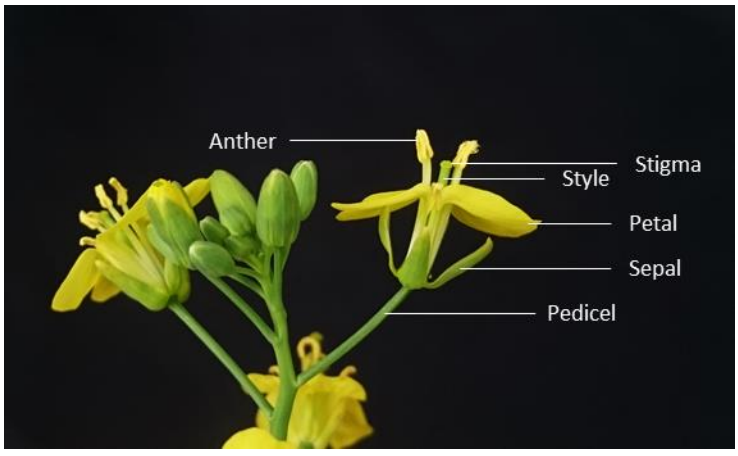


Figure 1: Canola inflorescence showing the different floral structures.

most of the angiosperm flowers are hermaphrodites and homomorphic, they usually can self-pollinate, which could lead to inbreeding depression (Ferrer and Good, 2012). A mechanism that likely played a significant role in their success to avoid inbreeding is self-incompatibility (Hiscock and Allen, 2008). Self-incompatibility or SI is a genetic mechanism that allows the plants to reject their own or genetically similar pollen in order to avoid inbreeding and promote outcrossing. In dry stigmas of Brassicaceae, the self-pollen or incompatible pollen is detected as self and blocked even before hydration and germination, while in wet stigmas, they are detected as self in the stylar transmitting tract (Hiscock and Allen, 2008).

1.2. Self-Incompatibility: A Genetic Mechanism for Plants to Avoid Inbreeding

The stigmatic papillary cells of the flowers function to capture the pollen and they have no control over the type of pollen that land on them. These stigmas are classified as either wet stigmas with exudates on them or dry stigma as in Brassicaceae without any exudates (Heslop-Harrison and Shivanna, 1977). When the pollen is released from the anther, it is in a quiescent, dehydrated state, which allows them to be transported more efficiently and allows to preserve their cellular integrity longer. This means that the dry pollen needs to be hydrated and usually, hydration is provided on the stigma following pollination (Hiscock and Allen, 2008). The wet stigmas are not selective and cannot discriminate self or non-self at this stage and therefore allow the hydration of all pollen. Wet stigmas are characterized by abundant secretions composed of lipids, carbohydrates and a wide range of proteins; the stigma also provides a gradient of water that directs the growth of the pollen

tube and in the absence of a cuticle, wet stigmas are very unselective (Hiscock and Allen, 2008).

In contrast with wet stigma, pollen hydration on dry stigma is highly regulated. First, the pollen is captured following wind or animal transportation. The adhesion step is very species-specific in Brassicaceae. For example, in *Brassica oleracea* the adhesion has been found to be strong with pollen within the family and decreases with species from other brassica family and it becomes insignificant with pollen from other plant families (Luu *et al.*, 1998). The exine, the outermost pollen component is required in the early stages of capture and adhesion (Gaude and Dumas, 1984; Zinkl *et al.*, 1999). Although the precise mechanism implying exine in adhesion is not clear, exine is indispensable, as mutants defective in its components, sporopollenin and pollen coat, are mostly male sterile (Quilichini *et al.*, 2014). The exine could play a role in the modeling of the sporopollenin in order to form the foot, an "appressorium-like" structure that hooks the pollen to the stigmatic papilla (Elleman and Dickinson, 1990b). One of the roles of the exine could be to mix with the waxy cuticle and the proteinaceous pellicle, covering the papillary cell of the stigma, to form the pollen foot (Elleman and Dickinson, 1990b; Kandasamy *et al.*, 1994). This coat conversion is accompanied by a change in the chemical and physical properties which can be observed by transmission electron microscopy (TEM) to observe the pollen coat becoming opaque and resistant to the removal by cyclohexane and remains attached to the stigma (Elleman and Dickinson, 1990a). The development of the foot would create a hydrophilic channel where molecules and factors required for recognition, hydration and germination could be shared. Among these elements, S-locus glycoprotein

(SLG) and s locus related 1 (SLR1) are proteins secreted by the stigma in *Brassica napus* (Nasrallah *et al.*, 1985; Isogai *et al.*, 1988). SLG and SLR1 are known to bind to Pollen Coat Proteins (PCPs); SLG binds to Pollen Coat Protein class A1 (PCP-A1) (Doughty *et al.*, 1998) and SLR1 binds to S Locus Related 1-Binding Pollen coat protein (SLR1-BP) (Takayama *et al.*, 2000). These proteins interactions could be the first step that initiates the foot formation and conferring the adhesion property (Doughty *et al.*, 1998; Luu *et al.*, 1999; Takayama *et al.*, 2000).

In Brassica, SI is sporophytic (SSI), which means SI is determined by the diploid genome of its parents (sporophyte) (Figure 2). In families of Solanaceae or Papaveraceae, SI is determined by the genetic makeup of the gametophyte and is called gametophytic self-incompatibility (GSI) (de Nettancourt, 2013). In each of these systems, there are male and female determinants, expressed exclusively in the pollen or the pistil to mediate SI response. During Solanaceae GSI, on the wet stigmas of Solanaceae species, a self-pollen is blocked in the style after pollen tube germination and penetration. Solanaceae GSI is governed by a non-self S-RNAse recognition based system with the female determinant S-RNAse functioning as a secreted ribonuclease in the extracellular matrix (ECM) of the style (Huang *et al.*, 1994). The S-RNAse is responsible for the degradation of the pollen RNA, thereby slowing down pollen tube growth and arrest causing SI (McClure *et al.*, 1990). The male determinant, S-locus F-box (SLF/SLB), is an F-box protein, a member of the E3-ubiquitin complex, responsible for the degradation of non-self S-RNAse (Bedinger *et al.*, 2017). S-RNase acts as a ligand for the recognition by SLF/SLB and as a cytotoxin

which is not recognized by SLF following self-pollination allowing it to degrade pollen RNA and stopping its growth (Bedinger *et al.*, 2017).

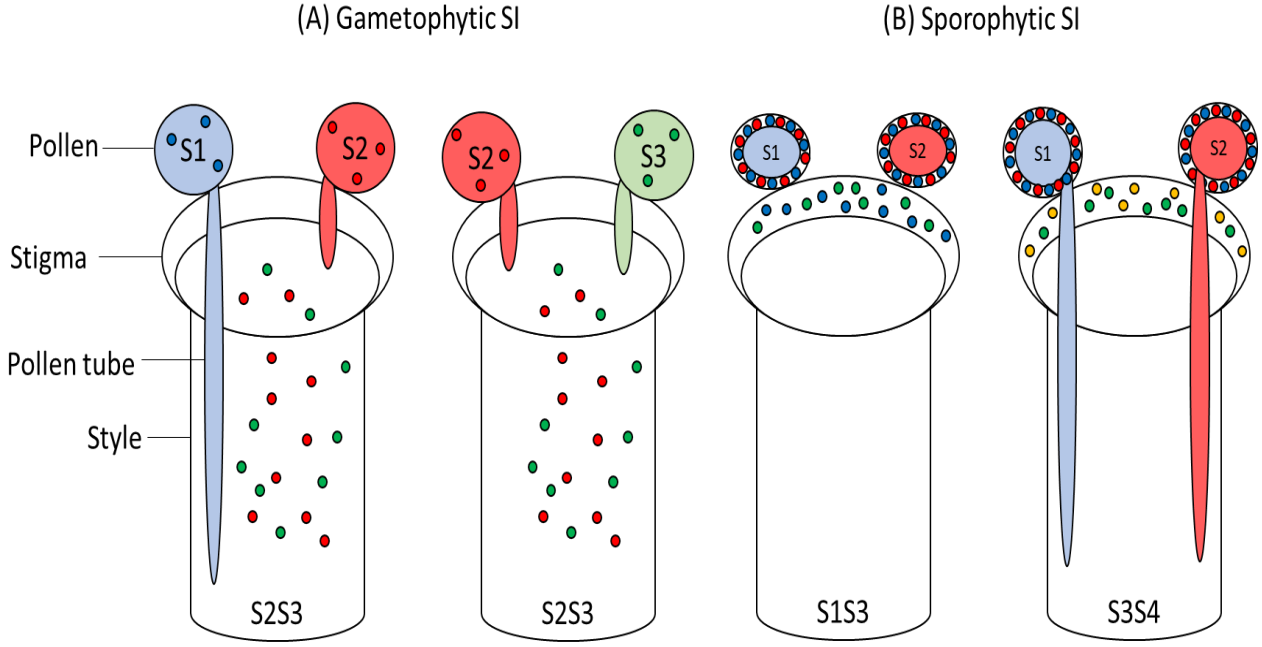


Figure 2: Schematic representation of the GSI and SSI.

(A) Gametophytic SI is characterized by the allele-specific non-recognition of stylar S-RNAse by its own SLF allele. If one of the pollen determinants corresponds to one of the female determinants, it is not recognized as a non-self RNAse and therefore is not processed for degradation. The RNAse can then degrade pollen mRNA and stop pollen tube elongation.

(B) In Sporophytic SI, allelic determinants have co-evolved to recognize each other. The male determinants are expressed in the diploid tapetum and deposited on the pollen coat of the haploid "microspore" and is therefore, from a diploid or sporophytic origin. When these ligands are recognized by the same stigmatic allelic determinant, SI is activated.

Another example of GSI is in *Papaver rhoeas*, but its mechanisms differ from the one found in Solanaceae. In *P. rhoeas*, a receptor-ligand recognition system confers the SI, where attachment of self-pollen on the stigma leads to activation of SI mechanism that increases intracellular calcium inside the pollen and eliciting a programmed cell death (PCD) of the pollen (Foote *et al.*, 1994; Wheeler *et al.*, 2009). The molecular components involved include *Papaver rhoeas* pollen S (PrpS), a pollen membrane receptor, which binds to the *Papaver rhoeas* stigma S (PrsS) secreted by the stigma (Foote *et al.*, 1994; Wheeler *et al.*, 2009) and this interaction activates channels which allows calcium and potassium to be delivered to the pollen (Wu *et al.*, 2011). Following calcium uptake by the pollen, multiple signaling cascades are activated, which includes depolymerization of the actin cytoskeleton, reduction of the pyrophosphatase activity of p26 (de Graaf *et al.*, 2006), activation of a putative mitogen-activated protein kinase (MAPK) (Rudd *et al.*, 2003) and the activation of the caspase-like proteases (Bosch and Franklin-Tong, 2007). All these events together would activate the programmed cell death (PCD), leading to the self-destruction of the pollen (Wheeler *et al.*, 2010).

1.3. Self-Incompatibility in Brassicaceae

Dry stigmas of Brassicaceae are the first barrier that the pollen needs to overcome in order to penetrate the papillary cells. The stigma must be triggered either by positive interactions with the pollen in order to release water and nutrients required for pollen hydration or interact with self-pollen in order to shut down the basal exocytosis machinery required for delivery of cargo for pollen germination (Doucet *et al.*, 2016).

During pollen hydration, for the pollen to germinate, fluid transfer for hydration can take different morphological patterns (Hiroi *et al.,* 2013) and it is important to note that relative humidity can also participate in pollen hydration (Safavian *et al.,* 2014). In *A. thaliana,* pollen hydration can occur on any surface under high humidity conditions (65%-100%), but the pollen will only germinate on the stigmatic surface (Ma *et al.*, 2012). This indicates that hydration can be controlled passively and independently of other factors required during pollen-pistil interactions and while it can hydrate on any surface, it requires the specific cell-cell signaling with the papillary cell, to promote its germination following hydration (Ma *et al.*, 2012).

Hydration of the pollen is indispensable for pollen tube germination and requires transfer of water and other elements from the stigma (Dickinson, 1995). The main mode of SI mechanism in Brassicaceae is through blocking the pollen in the earliest of its hydration phase, thus preventing hydration. It is proposed that self-pollen recognition leads to rapid blocking of the machinery required for delivery of germination promoting cargo to the pollen attachment site (Doucet *et al.,* 2016). The *S*-locus or the *S*-haplotype in Brassicaceae codes for the male and female determinant and possesses several alleles (Bateman, 1955). In general, the *S*-haplotype contains 3 genes (Suzuki *et al.,* 1999), SLG (Nasrallah *et al.,* 1985), *S*-locus receptor kinase (SRK) (Stein *et al.,* 1991) and *S*-locus protein 11 or *S*-locus Cys-rich (SP11/SCR) (Schopfer *et al.,* 1999; Shiba *et al.,* 2001). SLG is thought to confer adhesion property to the pollen and is involved in the formation of the pollen foot through interaction with PCPs (Doughty *et al.,* 1993). The SCR/SP11 protein is rich in cysteine and

is produced in the tapetum of the anther that is also responsible for the production of several pollen coat components during microgametogenesis (Doughty *et al.*, 1998).

SCR/SP11 has been proven to be the male determinant of SI and has been shown to bind the female localized, SRK (Ma *et al.*, 2016). The binding works as a 2:2 receptor-ligand where two molecules of SCR/SRK bind to 2 receptors and forms a heterotetramer, but the timing of binding and SRK homodimerization is not precisely known (Ma *et al.*, 2016). SRK structural analysis provides evidence that homodimerization is necessary for SRK activation (Ma *et al.*, 2016). Activation of SRK results in autophosphorylation of the kinase domain of SRK, which facilitates the association of another protein kinase, MLPK (*M* Locus protein kinase), anchored to the membrane (Murase *et al.*, 2004). MLPK is a serine-threonine kinase with an autophosphorylation activity and lack of MLPK can result in breakdown of SI (Murase et al., 2004). Yeast two-hybrid analysis led to the discovery of Thioredoxin H-Like proteins (THL-1 and THL-2), which function as negative regulators of SRK (Bower *et al.*, 1996). During compatible interactions, association of THL-1 and THL-2 with SRK can inhibit SRK (Cabrillac *et al.*, 2001). The inactivation mechanism of SRK is not well known, but SRK internalization in endosomes has been observed, where they co-localize with THL and this likely functions to inactivate SRKs (Ivanov and Gaude, 2009).

One of the most important interactors of SRK isolated to date is ARC1, an Arm-Repeat containing protein (Gu *et al.*, 1998). ARC1 functions as a positive regulator of SI, as suppression of *ARC1* resulted in breakdown of SI response (Stone, Arnoldo, & Goring, et al., 1999). ARC1 possesses arm repeat domains at the C-terminus that interact with the

kinase domain of SRK and is especially abundant in the stigma (Gu *et al.*, 1998). ARC1 is a U-box E3 ligase and through colocalization experiments in BY-2 cells was shown to be targeted to the proteasome by the kinase domain of SRK (Stone *et al.*, 2003). Through *in vitro* assays, ARC1 was also shown to be an E3 ubiquitin ligase that was able to ubiquitinate compatible pollination factors (Exo70A1 and GLO1) (Stone *et al.*, 2003; Samuel *et al.*, 2009; Sankaranarayanan *et al.*, 2015). The current hypothesis is that, SI-induced activation of ARC1 leads to proteasome-mediated degradation of compatibility factors, resulting in SI response.

One particular compatibility factor ubiquitinated by ARC1, that is relevant to this thesis, is exocyst subunit exo70 family protein A1 (Exo70A1), which is targeted for degradation by ARC1 following SI (Samuel *et al.*, 2009). Exo70A1 was shown to function as a compatibility factor as its down-regulation resulted in decreased pollen adhesion, hydration, and germination, while reciprocally, over-expression of Exo70A1 resulted in breakdown of SI in canola (Samuel *et al.*, 2009). Exo70A1 is a member of the exocyst complex which includes seven other subunits SECRETORY3 (SEC3), SEC5, SEC6, SEC8, SEC10, SEC15, and EXO84, which are involved in vesicle exocytosis (Heider and Munson, 2012). The exocyst complex, during pollen-pistil interactions, likely functions to tether secretory vesicles to the plasma membrane at the point of contact of the pollen with the stigma to release water and stigmatic factors required for the pollen hydration (Samuel *et al.*, 2009). In *A. thaliana*, it was shown that accumulation of exocytotic vesicles at the membrane occurred following compatible pollination (Safavian and Goring, 2013). In *B. napus*, instead of vesicles, formation of multivesicular bodies (MVB) were observed

following compatible pollination (Safavian and Goring, 2013). MVBs are large membrane-bound structures containing nanovesicles, and can be related to exosomes (30-100nm) with an endomembrane origin (Raposo and Stoorvogel, 2013). MVBs are able to fuse with the plasma membrane and release their contents in the extracellular matrix (Raposo and Stoorvogel, 2013). Following self-incompatible pollination in *B.napus*, compatible pollination in *A. thaliana exo70a1-1* lines and SI pollination in *A. lyrata* (self-incompatible *Arabidopsis* line), there was no fusion of MVBs or vesicles with the plasma membrane observed, suggesting that SI could block this response (Safavian and Goring, 2013). Over-expression of *RFP-Exo70A1* rescues this response in self-incompatible W1 canola lines, and exocytosis was likely abrogated in *Exo70A1* RNAi canola lines making them incompatible (Samuel *et al.*, 2009; Safavian *et al.*, 2015a). Furthermore, RNAi study of each of the seven members of the exocyst complex also demonstrated that they are all required for a successful pollination (Safavian *et al.*, 2015b). Each single mutant showed a significant defect in pollen hydration and germination (Safavian and Goring, 2013). These studies provide a direct link between the function of the exocyst complex and all the subunits, with the event of exocytosis being indispensable for pollen hydration. The larger size of the *B. napus* stigmas could explain the need for larger structures like MVBs to store more vesicles and increase the hydration potential as MVBs have not been observed in species with smaller stigmas such as *A. thaliana* or *A. lyrata*. During SI, MVBs and vesicles are mainly observed in the cytosol (*A. thaliana*) and the vacuole in *B. napus* and *A. lyrata* (Safavian and Goring, 2013). The absence of secretory vesicles fusing with the membrane observed after SI pollination, further confirms the model that active exocytosis is required

for successful pollen germination to occur. Interestingly, SI has also been associated with accumulation of autophagic bodies indicating that vesicles could be processed and degraded through this process and that autophagy is likely part of the SI response (Safavian *et al.*, 2015b).

Fusion of exocytotic vesicles at the papillary cell membrane at the pollen attachment site, is likely involved in providing, water, nutrients to the growing pollen and probably enzymes like pectinase required for the penetration of the pollen tube by loosening the papillary cell wall. Another component required for the exocyst complex to function would be its binding to phosphoinositols (Chapman and Goring, 2011). The studies on *Arabidopsis thaliana ROOT HAIR DEFECTIVE 4-11 (rhd4-11)* and *phosphatidylinositol 4- kinases β1/β2* (*pi4k β1/β2)* mutant lines show altered accumulation of phosphatidylinositol-4-phosphate (PI4P) and demonstrated that these two genes regulated the levels of PI4P at the membrane surface (Chapman and Goring, 2011). It has been proposed that PI4P is required for Exo70A1 activation at the membrane which will lead to tethering of the exocytotic vesicles facilitating hydration of the pollen (Chapman and Goring, 2011). This would be similar to animal systems where the exocyst subunits Exo70 and Sec3 are known to interact with phosphoinositol 4,5 phosphate at the plasma membrane (He and Guo, 2009). But *rhd4-1* and *pi4kβ1/β2* mutants display only a slight defect in pollen hydration suggesting that Exo70A1 may still function in the absence of these proteins (Chapman and Goring, 2011). It is possible that these activities are compensated by other phosphatases or kinases activities.

Another downstream compatibility factor that is directly regulated by SI is glyoxalase I (GLO1) (Sankaranarayanan *et al.,* 2015). GLO1, was shown to be down-regulated by ARC1-mediated ubiquitination during SI (Sankaranarayanan *et al.*, 2015). GLO1 is an enzyme responsible for the detoxification of methylglyoxal (MG) in cells. MG is a glycolysis by-product that is highly reactive with proteins, nucleotides, and phospholipids *in-vivo*, producing advanced glycation end-product which are dysfunctional (Thornalley, 1990; Thornalley, 2008). GLO1 down-regulation during SI corresponds to an increase in MG-modified products in the papillary cells and these MG modified compatibility factors are more efficiently targeted by ARC1 for degradation (Sankaranarayanan *et al.*, 2015).

Screening for possible components in the SI pathway through transcriptome and proteome analysis has helped to identify some key players of pollination in angiosperms. However, numerous proteins identified or differentially expressed during pollination events have not been analyzed in depth. Characterization of each of these proteins could help to decipher the mechanism involved during pollination and pollen rejection and enrich our knowledge about this essential biological process (Figure 3). Previously, 2D-DIGE study followed by MS/MS analysis identified 23 unique proteins (Samuel *et al.,* 2011). All these down-regulated proteins are possible candidates for compatibility factors and also as targets for degradation by ARC1 or following autophagy. This thesis focuses on elucidating the role of the Phospholipase D1 alpha protein, which was one of the proteins identified to be down-regulated following SI (Figure 4). Since PLD1 isoforms have been implicated in calcium signaling, exocytosis and cytoskeleton modeling, it could be an interesting candidate to pursue to understand its function during pollen-pistil interactions.

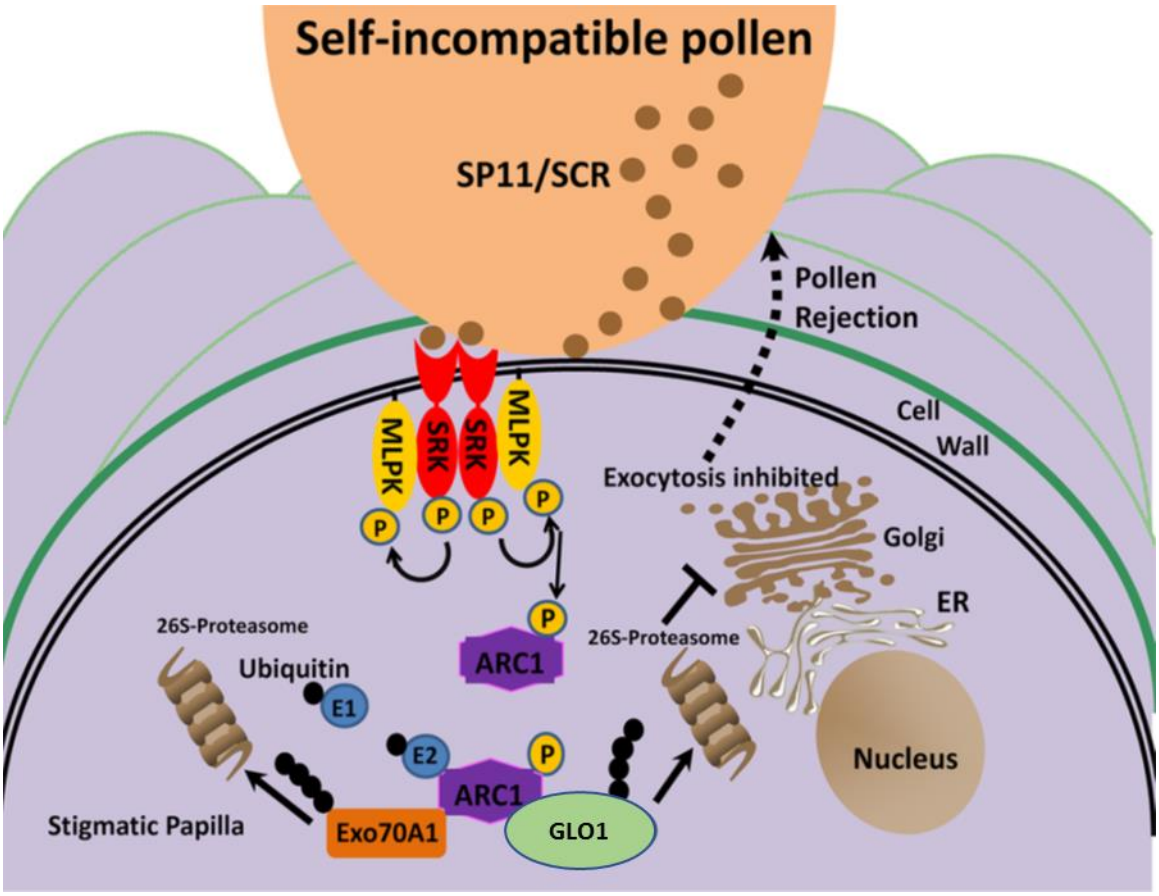


Figure 11: Self-Incompatibility interactions in Brassicaceae (adapted from Sankaranarayanan *et al.*, 2013a).

The S-locus receptor kinase (SRK), the female determinant in SSI, binds to the male determinant, the S-locus cysteine-rich protein (SCR/SP11) in a haplotype-specific manner. During a self-incompatible pollination, SCR/SP11 interaction with SRK leads to autophosphorylation of SRK and downstream activation of MLPK. Both MLPK and SRK can activate ARC1, which then targets compatibility factors such as Exo70A1, GLO1 and possibly others compatible factors for proteasomal degradation. Loss of these compatibility factors will result in blocking of the secretory processes required for pollen hydration and germination to occur resulting in rejection of self-pollen.

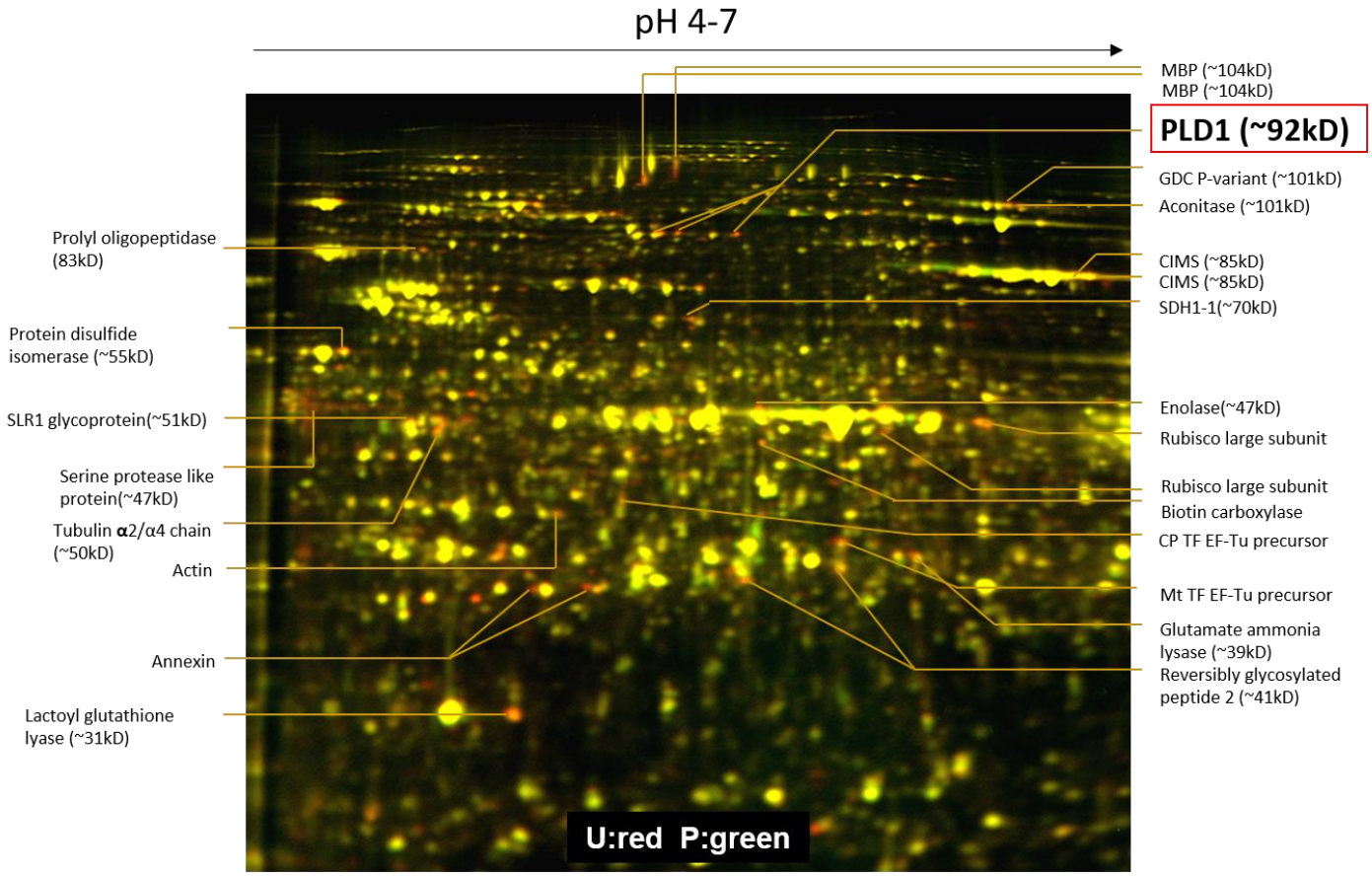


Figure 4: 2D-DIGE proteome analysis resulted in identification of 23 unique proteins down-regulated during SI after 60 min of self-incompatible pollination in *Brassica napus* stigma (adapted from Samuel *et al.*, 2011).

PLD1 (highlighted in red) is a 92 kD protein identified..

1.4. Phospholipase D 1

Phospholipases D (EC 3.1.4.4) hydrolyze the phosphodiester bond of the polar head group of membrane phospholipids, principally phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Figure 5) that form 68-80 % of membrane lipids (Furt *et al.*, 2011). PLDs can be found widely in numerous organisms like animals, plants (Bargmann and Munnik, 2006), bacteria, yeast and viruses (Rudge and Wakelam, 2009; Peng and Frohman, 2012). The product of the catalytic activity of PLDs is phosphatidic acid (PA) and the corresponding soluble polar head group (choline, ethanolamine, etc.). PA has a small head group and is negatively charged which confers it spatial properties that can curve membranes negatively and lead to their fusion and fission. Besides this fascinating feature, PA is also involved in cellular signaling, phospholipids metabolism, and vesicle trafficking (Hong *et al.*, 2016).

In plants, PLD activity was detected in carrot and cabbage for the first time in 1947 and from rat brain preparation in 1975 and this activity was called lecithinase or phosphatidohydrolase activities (Hanahan and Chaikoff, 1948; Saito and Kanfer, 1975). These studies described the release of different amounts of choline and phosphatidylethanolamine from lipid extracts of raw and steam processed carrots (Hanahan and Chaikoff, 1948; Saito and Kanfer, 1975). PLD was then purified independently from cabbage (Abousalham *et al.*, 1993) and castor bean, then the respective gene was cloned from castor bean (Wang *et al.*, 1993; Wang *et al.*, 1994). Since then, numerous PLDs have been characterized in various plant species such as Oryza, Brassicaceae or Solanaceae (Eliás *et al.*, 2002).

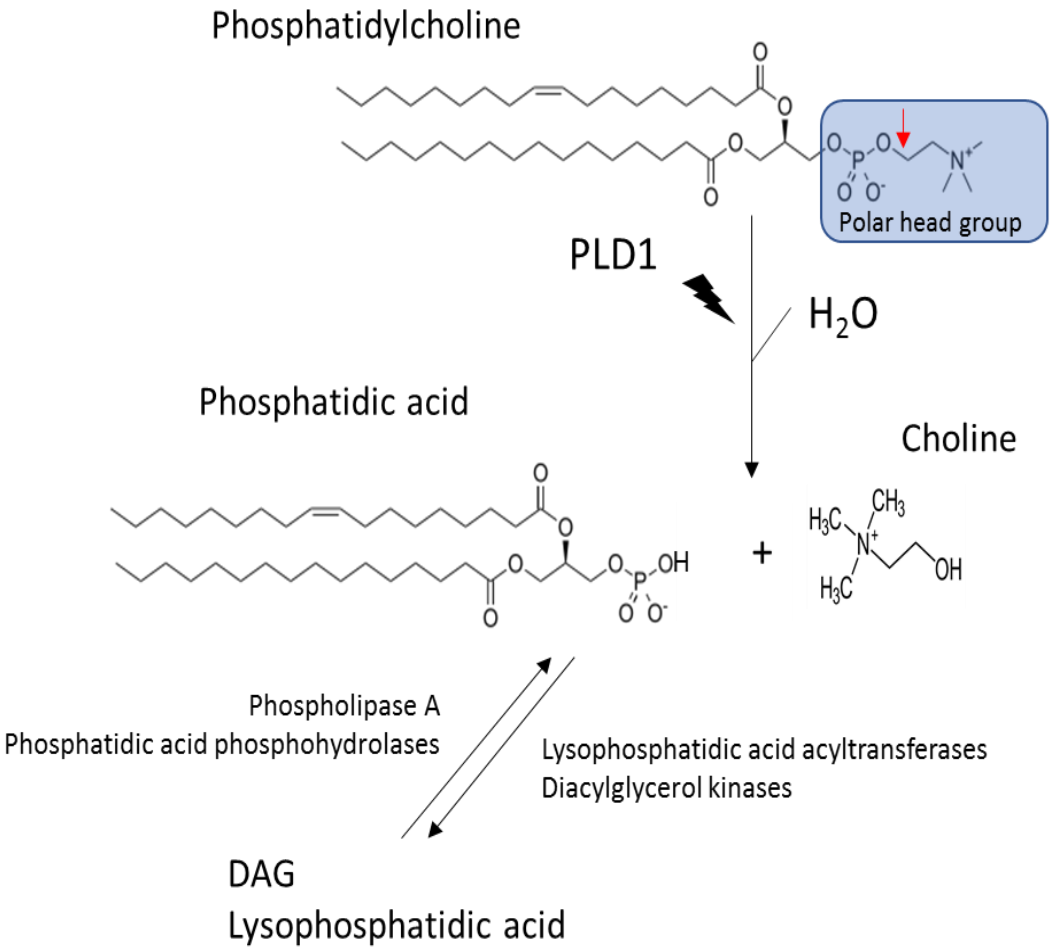


Figure 5: Reaction scheme for the hydrolysis of phosphatidylcholine by phospholipase D.

PLD1 hydrolyzes the phosphodiester bond (red arrows position) of phosphatidylcholine (1-palmitoyl-2-oleoyl-phosphocholine) or phosphatidylethanolamine to release phosphatidic acid (PA) and choline (or ethanolamine). PA can then be metabolized to either DAG or lysophosphatidic acid by diacylglycerol kinases and lysophosphatidic acid acyltransferases respectively. Conversely, DAG and Lysophosphatidic acid can be converted to PA by Phospholipase A or Phosphatidic acid phosphohydrolases.

1.4.1. PLD superfamily and structure in plants

PLDs are characterized by multiple isoforms classified into two sub-families the C2-PLDs and PXPH-PLDs. All PLDs contain the conserved HxKxxxD motif (Figure 6). In *A. thaliana,* there are 12 isoforms, and most of them contain the C2 motif (Qin and Wang, 2002a). The PX PH-PLDs are largely represented in mammals and fungi. Yeast contains only one PLD gene that codes for a PC specific PLD. In mammals, there are two PLDs, PLD1 and PLD2 (Hammond *et al.*, 1995; Colley *et al.*, 1997). In contrast to animal models, plants have multiple isoforms of PLDs. Rice (*Oryza sativa*) has 16 PLDs identified so far (Li *et al.*, 2007); in *A. thaliana*, PLDs are designated as PLDα (1-3), β(1, 2), γ(1-3), δ, ε, and ζ(1, 2) and are classified into two sub-families, the C2 and PXPH PLDs (Figure 6). The C2 domain of PLD is a Ca^{2+} binding domain of about 130 amino acids, highly conserved between the various PLDs from the C2 family (Wang *et al.*, 2006). Differences in the amino acids of the C2 domain may explain difference in the amount of calcium (µM to mM range) required to activate the PLDs (Zheng *et al.*, 2000).

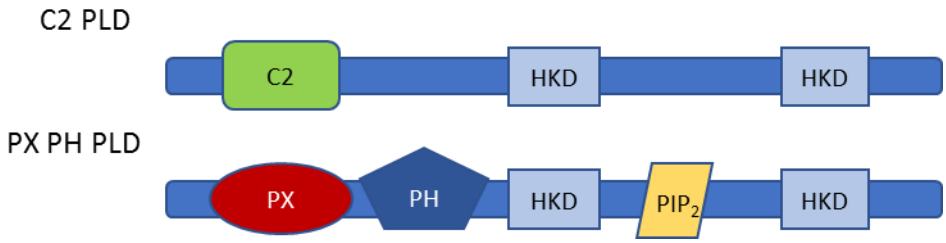


Figure 6: Principal structures of PLDs.

PLDs can be classified in two groups, the C2 PLD and PX PH PLDs. The C2 PLD has a C2 binding domain for lipids and calcium and the 2 HKD catalytic domain. The PX PH PLDs characterized by the PH plekstrin homology domain and PX Phox homology domain.

PLDζ (1&2) belong to PX PH PLDs subfamily (Qin and Wang, 2002b; Taniguchi *et al.*, 2010), where the PX and PH domains are used to bind phosphoinositides; PX can also bind to proline-rich proteins similar to Src homology 3 domain (Ito *et al.*, 2001). Similar to how C2 domains have varied affinity to calcium, PX and PH domains have differential binding affinity to phosphatidylinositol biphosphate (PIP_2) (Qin and Wang, 2002a). PX, but not PH, is critical for PLD activity and the exact function of both domains remains to be elucidated in plants (Hong *et al.*, 2016). These plant PLDs are more closely related to mammalian PLDs that possess in general the PHPX domain, which do not require calcium, but instead, need phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) to hydrolyze PC selectively (Qin and Wang, 2002b). Studies have shown that these PLDζs are involved in environmental stress regulation. In particular, PLDζ2 expression is drastically increased under phosphate deprivation (Li *et al.*, 2006) and is triggered by exogenous auxin and phosphate (Li *et al.*, 2006; Li and Xue, 2007). At a cellular level, plant PXPH PLDs have also been shown to mediate vesicular trafficking, phosphate recycling, and root gravitropism (Galvan-Ampudia *et al.*; Li *et al.*, 2006)

The HKD domain of PLDs is highly conserved and confers catalytic function to PLD and is duplicated in the sequence to form two HKD domains. These two domains come together to form one active site in a monomer and act in a ping-pong fashion (Appendix A, Figure A3, A4) (Leiros *et al.*, 2000). The catalytic action is initiated by the N-terminal histidine residue of the HKD domain, which attacks the phosphate group of the phosphatidyl glycerophospholipid, and forms a covalent phospho-histidine intermediate (Selvy *et al.*, 2011). Immediately, the polar head is reduced and released, which is rapidly followed by

a second nucleophilic attack by the histidine of the second HKD domain on water or primary alcohol which releases PA or the phosphatidyl alcohol product (Selvy *et al.*, 2011). The use of primary alcohol instead of water is an essential characteristic of PLD activity, and this reaction is called transphosphatidylation (Yang *et al.*, 1967). In addition to HKD domains, PLDα1 also have the DRY motif which bind to the G α subunit of heterotrimeric G proteins (Zhao and Wang, 2004). In addition to these above-mentioned domains, actin and oleate binding motifs can be found on C2 PLDs (Hong *et al.*, 2016). The presence of these multiple domains demonstrates the richness of the possible networks that can converge on regulation of the PLD enzymes and also the numerous downstream pathways it could potentiate.

1.4.2. Regulation of PLD activity

PLD activity is influenced by various factors such as pH levels, calcium, oleate, PIP_2 and also by the presence of SDS in the reaction mixture that strongly activates PLD activity; the key modulators are calcium and $PI(4,5)P_2$ (Pappan and Wang, 2013). The C2-PLDs require presence of Ca^{2+} presence (Li *et al.*, 2009) and the differences observed in the C2 sequence between the PLDs could explain whether or not the PLD requires mM or µM level of Ca^{2+} (Pappan and Wang, 2013). Calcium binding triggers the phospholipid binding to the C2 domain and make them accessible to the catalytic site (Hong *et al.*, 2016). Very similar to calcium, $PI(4,5)P_2$, is required for the activity of PLDγ, and ζ activity (Pappan *et al.*, 1998a; Qin and Wang, 2002b). PLDs are characterized by their substrate preferences with PLDα family members preferably hydrolyzing PC, PE and phosphatidylglycerol (PG)

(Dippe and Ulbrich-Hofmann, 2009). In addition to these phospholipids, PLDβ and γ can use N-acylphosphatidylethanolamine (NAPE) and release PA and N-acylethanolamine respectively (Pappan *et al.,* 1998b). The PLDγ and PLDδ members have a preference toward PE (Pappan *et al.,* 1998b; Qin *et al.,* 2002). PLD activity will also be influenced by the acyl chain length, with them usually preferring short acyl chains and recent techniques like multiple reaction monitoring (MRM) using mass spectrometry, allow us to precisely analyze the nature of the acyl chains of the PA produced under different stresses (Rainteau *et al.,* 2012)

These substrate preferences explain the range of PLD activity and the various sources for origin of PA further reinforce the idea that one of the main functions of PLD is the regulation of the phospholipids on the membranes.

1.4.3. Plant PLD role in cellular signaling

PLDs in plants are mainly involved in development, environmental stress and in defense responses. In this section, the mechanistic and functional roles of AtPLDα1 mechanisms are discussed, since BnPLD1 sequence identified from the proteomics approach is very similar to AtPLDα1 (Appendix A, Figure A4).

In *Arabidopsis thaliana*, PLDα1 has been shown to be involved in seed aging where it functions to negatively regulate the aging process (Devaiah *et al.,* 2007). *pldα1* knock-out and knock-down mutants resulted in better germination and growth rates after aging treatment (seeds kept at 43 ^{o}C, with 100% of humidity for different amount of time) which was very similar to natural aging (seeds stored at RT for 3 and 6 years) (Devaiah *et al.,*

2007). Lipid analysis of the mutant lines showed less reduction in fatty acids than the control and less secondary end-product of lipids peroxidation (Devaiah *et al.*, 2007). It was therefore proposed that PA produced by PLDα1 is either the target of lipids peroxidation or they enhance lipids peroxidation causing rapid seed aging and deterioration (Devaiah *et al.*, 2007). Several studies have shown that PLDα1 is an important factor in *Arabidopsis thaliana* abscisic acid (ABA) signaling. PLDα1 can mediate ABA-induced stomatal closure by RbohD and F (NADPH oxidase) activation, where RbohD and F bind and are activated by PA to produce reactive oxygen species (ROS) required for stomatal closure (Zhang *et al.*, 2009). Moreover, it appears that there was a shift in PA species during the ABA signaling with an increase in 34:1 (16:0,18:1), 34:2 (16:0,18:2), 36:3(18:1,18:2), which have increased binding affinity to Rbohs as confirmed by PA binding assay (Zhang *et al.*, 2009).

The same study showed that Abscisic Acid Insensitive 1 (ABI1), the protein phosphatase 2C and a negative regulator of ABA signaling, also has a PA binding domain and this can lead to stomatal closure, since PA binding inactivates ABI1, in turn activating ABA signaling (Zhang *et al.*, 2009). *ABI1* allele that is deficient in PA binding had normal levels of ROS and nitric oxide (NO) compared to the *abi1* mutant and only the non-PA binding mutant showed a stomatal aperture defect when ABA, H_2O_2 and NO were applied (Zhang *et al.*, 2009). This demonstrated that under drought stress ABI1 needs to be inhibited (by PA binding) to shut the stomatal aperture (Zhang *et al.*, 2009). But this pathway is independent from the RbohD and F mediated ROS production and could act downstream of ROS production (Zhang *et al.*, 2009). Salt stress responses are also regulated by PA

production (Yu *et al.*, 2010). In the presence of NaCl, PA is able to bind the Mitogen-Activated Protein Kinase 6 (MAPK6), which controls a plasma membrane Na^+/H^+ antiporter (SOS1) that can exclude Na^+ (Yu *et al.*, 2010). PLDα1 activity was increased in presence of NaCl and the *pldα1* mutant displayed a decrease of PA species that were shown to bind MAPK6, demonstrating again the influence of PA species in signaling (Yu *et al.*, 2010). Under salt-stress, PA produced from PLDα1 activity promotes microtubule stabilization through binding microtubule-associated protein 65-1 (MAP65-1) which increase salt-stress tolerance (Zhang *et al.*, 2012).

One of the interesting functions of PLDs is the one associated with membrane modeling and vesicular trafficking (Ammar *et al.*, 2013a). In animal models, it has been proposed for a long time that PA produced from PLDs could modify the membrane curvature to allow fusion of secretory vesicles to the plasma membrane (Figure 7). It appears that plant PLDs could have the same function. A strong line of evidence for this comes from the observation that treatment of pollen tubes with 0.5% n-butanol, which inhibits PA production by PLD, resulted in a decrease of more than 50% of vesicles targeted to the membrane (Pleskot *et al.*, 2012). Another evidence comes from the observation that AtVPS2 (Vacuolar Protein Sorting 2) a component of the ESCRT-III (Endosomal sorting complex required for transport) can co-immunoprecipitate PLDα1 as a potential interactor from protein extracts from roots (Ibl *et al.*, 2012). Also, subcellular studies have indicated that PLD could localize in various cellular compartments such as plasma membrane, nucleus,

mitochondria, tonoplast or the cytoplasm in different tissue types, where they are found in either soluble fractions or membrane fractions (Hong *et al.*, 2016).

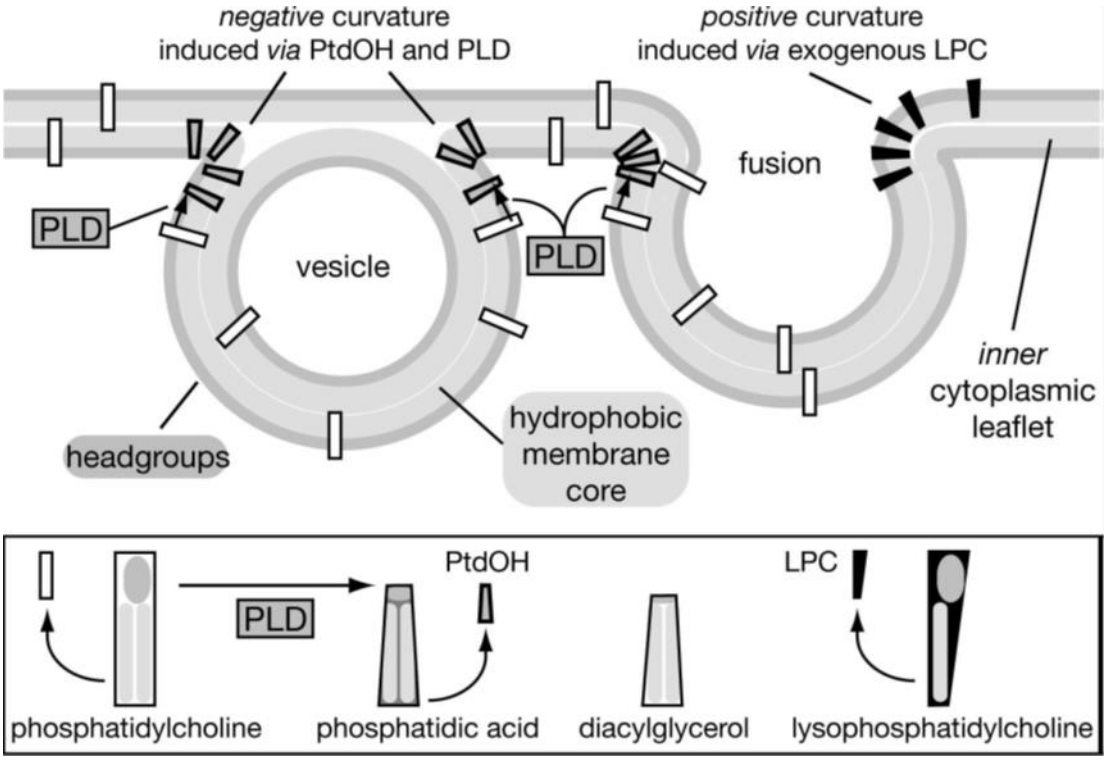


Figure 7: Model showing vesicle fusion at the plasma membrane (Taken from (Cazzolli *et al.*, 2006)).

The fusion is facilitated by the shape of the phosphatidic acid or the lysophosphatidic acid which take different cone shape that curve the membranes lowering energy required for the process.

1.4.4. Phospholipase D in *Brassica napus*

The first *Brassica napus* PLD was purified from seeds in 1997 (Novotná *et al.*, 1997), and since then only few PLDs have been characterized. *In-vitro* studies have identified PLD activity in different subcellular protein fractions from hypocotyls and seeds (Novotná *et al.*, 2003). This activity was divided into a PIP_2-dependent PLD activity and a PIP_2-independent activity which is associated to the PLDα class and does not require PIP_2 but instead milli molar levels of calcium (Pappan and Wang, 1999). PLD activity in *B. napus*, is also associated with copper stress signaling and increases 3 to 4-fold in the seedling roots after copper treatment (Russo *et al.*, 2008).

In *B. napus,* eight genes from PLD β, γ, and δ families have been cloned, sequenced and annotated (Janda *et al.*, 2015). These are PIP_2-dependent and their expression is strongly induced by benzothiadiazole (BTH) and methyl jasmonate (MeJa) (Janda *et al.*, 2015). Overexpression of PLDε in canola can stimulate nitrate metabolism and the overexpressors yielded more seeds under nitrogen-depleted conditions (Lu *et al.*, 2016).

1.5. Rationale, Hypothesis and Thesis Objectives

In spite of intense scrutiny of the signaling mechanisms during the complex self-incompatibility (SI) response in Brassicaceae, not much progress has been achieved beyond the receptor-ligand interaction that triggers a proteasomal degradation pathway to target compatibility factors. Other than a few compatibility factors such as Exo70A1 and GLO1, most of these compatibility factors necessary for pollination still remain unknown. SI is one of the most studied mechanism in plant reproduction (Watanabe *et al.*, 2008) and

identification of factors that influence SI can directly contribute to potential crop improvement and hybrid seed production (Gowers, 2000). Based on the degradome data from the 2D-DIGE proteomics study, a reduction in the amount of BnPLD1 occurs following SI pollination (Figure 4). Cloning of this gene and the subsequent sequence analysis of this BnPLD1 revealed that it was very similar to C2 type PLDs (Appendix A, Figure A4) and shows 94% identity to AtPLDα1. Based on these observations, I hypothesize that SI pollination induced reduction in the level of BnPLD1α would result in a concomitant reduction of its PA product inside the papillary cell at the contact sites with the self-pollen. Lack of localized PA near pollen attachment site could thus prevent the membrane curvature required for exocytosis of vesicles containing factors necessary for the pollen germination.

I propose that BnPLD1 is a compatibility factor during pollen-pistil interactions and that SI mechanisms can target degradation of PLD1, leading to rejection of self-pollen. The objectives of my Ph.D. thesis are to a) examine whether PLD1 is a compatibility factor during pollination in Brassicaceae b) Explore whether PLD1 is down-regulated by ARC1 following SI reaction c) Investigate if production of PA is involved in the exocytosis of vesicles containing the factors necessary for pollen germination.

Specific objectives:

1) Investigation of the involvement of phospholipase D 1 in pollen-pistil interaction

- To test the role of PLD1 in pollination through a pharmacological approach.
- Functional analysis of role of PLD1 during pollen-pistil interactions through transgenics approach.

- To investigate whether SI response and pollination defects induced by lack of PLD1 could be rescued by external PA.
- To examine if overexpression or suppression of PLD1 would alter vesicle movement following pollination through TEM.

2) PLD1 regulation during pollination

- Characterization of PLD1 expression and regulation following pollination
- Analyzing sub-cellular localization and regulation of PLD1 in PLD1-RFP overexpressors through confocal microscopy
- Examining the interaction of ARC1 with PLD1 through genetic and biochemical assays

3) Investigation of functional conservation of PLDα family in *Arabidopsis thaliana*

Chapter 2: Materials and Methods

1.6. Plant Material and Growth Conditions

Experiments performed with canola (*Brassica napus)* plants were done using W1 (self-incompatible), Westar (compatible) cultivars and *ARC1* antisense transgenic plants (*arc1*) in the self-incompatible W1 background as previously described (Stone *et al.*, 1999). The seeds were sterilized for 2 min in 70% ethanol, followed by a 7 min treatment with 30% bleach and carefully rinsed three times with water. The sterilized seeds were then germinated on ½ Murashige and Skoog (MS) plates with 1% sucrose, and 7 g/L of plant agar (Phytotech), pH 5.8. After 3 to 4 days of stratification at 4 ºC in the dark, plates containing seeds were transferred to long-day conditions (16 h light at 22 ºC and 8 hours dark at 18 ºC) for germination. After 2 weeks on germination medium, the seedlings were transferred to soil (PRO-MIX®BX MYCORRHIZAE™) and grown under long-day conditions.

For experiments using *Arabidopsis thaliana*, T-DNA insertional lines in Columbia-0 (Col-0) background with inserts in the *AtPLDα1* (SALK line SALK_053785.56.00.x), *AtPLDα2* (GABI-KAT line GABI_212E06) and *AtPLDα3* genes (SALK line SALK_130690) were obtained from the Arabidopsis Biological Resource Centre. The seeds were sterilized and stratified as indicated previously for canola seeds. After stratification, the seed containing plates were placed under long-day conditions (16 h light at 22 ºC for 7-14 days (or until emergence of the first true leaves) and then transferred to soil and maintained under long-

day conditions (16 h light and 8 h dark at 22 ºC). They were genotyped to verify the presence of the T-DNA insertion (see list of primers Table 1).

Table 1: List of primer for PLDα T-DNA mutants screening.

Primer	Sequence 5' to 3'
PLDa1SALK_053785.56.0 LP	ATTAAGTGCAGGGCATTGATG
PLDa1SALK_053785.56.0 RP	CAAGGCTGCAAAGTTTCTCTG
plda3 SALK_130690 RP	ATGCACAAGTTTGTTTGGAGG
plda3 SALK_130690 LP	GAGCTAACGACACTTGGTTCG
LBa1 of pBIN-pROK2 for SALK lines	TGGTTCACGTAGTGGGCCATCG
LBb1.3	ATTTTGCCGATTTCGGAAC
PLD2GABI_212E06RP	AGCCGAATGAAAAACCAAAAC
PLD2GABI_212E06LP	GATTTCCTGACTCCCCTGAAG
PLD2GABI_212E06LBo84	ATATTGACCATCATACTCATTGC

1.7. PLD1 Inhibition Assay

Stage 12 flowers from self-incompatible W1 line, were immersed overnight in either 5 mM or 10 mM of N-methylethanolamine or with either 11 µM or 44 µM of 1-butanol. The flowers were then pollinated with Westar pollen to initiate a compatible reaction. Six hours after pollination, flowers were collected and subjected to aniline blue assay for observation of pollen tubes using a Leica DMR epifluorescence microscope.

1.8. Aniline Blue Treatment

Aniline blue assay was performed as previously described (Samuel *et al.*, 2011). After pollination, pistils were fixed for 30 min in 3:1 ethanol and glacial acetic acid. The pistils were washed three times with distilled water and incubated in 1N NaOH at 60 ºC for 1 h. The pistils were thoroughly washed three times with water. Then they were stained with basic aniline blue (0.1% aniline blue in 0.1 M K_3PO_4) for 30 min followed by mounting in 70 % glycerol and observation through the blue channel using a Leica DMR epifluorescence microscope.

1.9. Molecular Cloning of *Brassica napus* PLD1

Complementary DNA (cDNA) was prepared using total mRNA isolated from *Brassica napus*, W1 stigmas using Superscript II Reverse Transcriptase (Invitrogen). Using the cDNA as the template, PCR was performed using *PLD1* specific primers PLDa1InfFw and PLDa1InfRev (Appendix B, Table B1) to obtain an amplicon of *PLD1*, that was then sequenced (Appendix A, Figure A2) and inserted in the p1666 plasmid.

1.10. *Brassica napus* Transformation

PLD1 RNAi Westar lines were generated by insertion of the double-stranded RNAi sequence (Table 2) into the binary p1665 vector, under the control of the stigma-specific *SLR1* promoter (Samuel *et al.*, 2009). The insertion of the construct into the vector was made using Infusion cloning (Clontech) (see list of primers Table 3). *Agrobacterium tumefaciens* (GV3101) – mediated transformation was used to transform Westar plants with

this construct (UC Davis, plant transformation facility). Kanamycin resistant transformants were analyzed by PCR (see list of primers Table 4) to confirm the presence of the construct and protein quantities in unpollinated stigma were checked by Western blot with anti-PLD1 antibodies (Appendix A, Figure A5, A6). For *PLD1* overexpressing W1 and Westar lines, *PLD1* fragment was introduced in the p1666 vector under the *SLR1* promoter and fused with RFP. The construct was introduced into W1 plants by *Agrobacterium tumefaciens* (GV3101) – mediated transformation (UC Davis, plant transformation facility for OX1 line). The transformation of OX3 and OX6 and WestPLOX (Westar lines overexpressing PLD1) lines was performed in the laboratory following the method described previously (Bhalla and Singh, 2008) (Borjian and Arak, 2013). For transformation, W1 and Westar seeds were sterilized as described earlier. They were sown in Magenta GA-7 plant culture box (Cat no V8505) containing ½ MS, 1% sucrose, and 7.5 g/L plant agar. The seeds were stratified for 3 to 4 days at 4 ^{o}C after which the jars were kept for 3-4 days in the dark at 22 ^{o}C for inducing hypocotyl elongation. The hypocotyls were cut into 1 cm pieces and placed with 1 cm spacing, on a plate containing a co-cultivation media (1/2 MS, 1 % sucrose, 6 g/L Agar and 1 mg/L of 2, 4-Diclorophenoxyacetic acid, pH 5.7) for three days under long daylight conditions. *Agrobacterium tumefaciens* (GV3101) containing the binary vector p1666 containing PLD1-RFP, were cultured overnight at 28 ^{o}C in 50 ml of LB broth containing kanamycin and rifampicin at 50 mg/L from a preculture, spun at 4000 rpm for 10 min, resuspended in 1/2 MS and 3 % sucrose until OD reached 0.3 to 0.6 at 600 nm. Acetosyringone (100 mM) was added to the resuspended culture and the hypocotyls were incubated 30 min in this solution and transferred to new co-cultivation media plates.

After 2 days in long day condition, the hypocotyls were transferred to callus induction media (1X MS media with 3 % sucrose 5 g/L of agar, 0.5 mg/L of NAA, 0.5 mg/L of BAP, 0.5 mg/L of 2, 4-D, 0.5 mg/L of $AgNO_3$, 300 mg/L of timentin, 5 mg/L of kanamycin, pH 5.8). After appearance of callus, they were transferred to the shoot induction media (1X MS, 2 % sucrose, pH 5.8, agar 5.5 g/L, 0.2 mg/L of NAA, 3 mg/L of BAP, 5 mg/L of $AgNO_3$, 0.01 mg/L of GA3, 300 mg/L of timentin, 5 mg/L of kanamycin for the first 2 weeks and then 25 mg/L of kanamycin). After appearance of shoots, they were separated from the callus and placed in the shoot growth media (½ MS, 2% sucrose, pH 5.8, 6 g/L agar, 1.5 mg/L of IBA, 160 mg/L of timentin, 25 mg/L of kanamycin) for 2 weeks. Plantlets that grew out on the shoot induction media were trimmed and transferred to root induction media (½ MS, 2% sucrose, pH 5.7, 6 g/L of agar, 1.5 mg/L of IBA, 160 mg/L of timentin, 25 mg/L of kanamycin). Once the roots were established, kanamycin resistant, green plants were genotyped for the presence of the construct (see list of primers in Table 4). The true positives were then transplanted on to soil and maintained in growth chambers and the best lines with high PLD1 expression in the stigmas were identified through Western blotting using anti-PLD1 antibodies.

Table 2: N-terminal 625 nucleotides sequence of *Brassica napus* Phospholipase D1 coding sequence selected for RNAi vector (highlighted in grey).

>KJ755984.1 *Brassica napus* cultivar Westar phospholipase D1 mRNA, partial cds
ATGGCGCAGCATCTGTTGCATGGTACTTTGCACGCTACGATCTATGAAGTTG
ATGACCTCCACACTGGTGGACTCAGGTCCGGCTTCTTCGGCAAGATTCTGGC
TAATGTAGAAGAGACCATTGGTGTTGGCAAAGGAGAAACACAGCTATACGC
AACGATCGATCTCCAAAGAGCCAGAGTTGGTCGAACAAGAAAGATCAAGG
ACGAAGCCAAAAACCCAAAATGGTACGAGTCCTTTCACATCTACTGCGCCC
ACTTGGCTTCCGACATCATCTTCACCGTCAAGGACGACAACCCCATCGGCGC
CACCCTCATCGGAAGAGCCTACGTCCCCGTCGACCAAGTCATCCACGGCGA
GGAAGTCGACCAGTGGGTTGAGATATTAGACAACGACAGAAACCCCATCCA
CGGAGGGTCCAAGATCCACGTGAAGCTACAGTACTTCGGCGTCGAGGCGGA
TCGTAACTGGAACCAAGGTATCAAGAGCGCTAAGTTCCCTGGAGTCCCTTA
CACGTTCTTCTCCCAGAGGCAGGGATGCAAAGTCTCTCTCTACCAAGACGCT
CACATCCCTGATAACTTCGTCCCGAGGATCCCTCTCGCTGGAGGGAAGAACT
ACGAGCCTCAGAGGTGCTGGGAGGATATTTTCGACGCGATAAGCAACGCGC
AGCATATGATCTACATCACTGGATGGTCTGTGTATACTGAGATTGCCTTGGT
TAGAGACTCCAGGAGGCCGAAGCCTGGAGGAGACGTGACCGTTGGCGAGCT
GCTTAAAAAGAAAGCTAGCGAAGGTGTTAGGGTTCTTCTCCTTGTGTGGGA
CGATAGAACATCCGTCGATGTGTTGAAGAAAGACGGTCTCATGGCTACTCA
TGATGAAGAAACGGAGAATTTCTTCAGAGGAAGCGACGTTCATTGTATTCT
CTGTCCTCGTAACCCTGATGACGGTGGTAGCATAGTCCAGAACTTGCAGGTC
TCAGCCATGTTCACGCACCATCAGAAGATCGTTGTTGTGGACAGCGAGATG
CCGAGCCGAGGAGGTTCACAGATGAGGAGGATCGTGAGTTTTGTTGGTGGG
ATCGATCTCTGTGATGGACGTTACGACACTCCTTTCCACTC

Table 3: List of primers used for transgenic constructs.

Construct	Primer	Sequence 5' to 3'
PLD1 RNAi	P1 SLRinfFor-dSma-PLD1For	CTCTAGAGGATCcccATGGTACGAGTCCTTTCA
	P2 EcoR1-Int-PLD1-Rev	GCgaattcCTATGAGCTGCAAAAACTACTTACCTCCTTCAACACATCGACGGA
	P2' SLRinfR	TCGAGCTCGGTAcccggggCgaattcCTATGAGCTG
	P4 SLRinfR-PLD1 For	TCGAGCTCGGTAcccgggATGGTACGAGTCCTTTCA
	P5 InfINt2PLD1 Rev	ATAGgaattcGCcccgggCTTCAACACATCGACGGA
PLD1 Overexpression	PLDa1InfFw	ACTCTAGAGGATcccgggGATGGCGCAGCATCTGTTG
	RFPinfR-PLD1SmaRev	CGGAGGAGGCCATcccgggGGTTGT

1.11. Genomic DNA Extraction and PCR of Putative Transformants

Leaf disc punched from a young leaf (Putative transformants of W1, Westar, and *A. thaliana*) was ground in 250 µL of CTAB buffer (2% CTAB, 20 mM EDTA, 100 mM Tris-Cl pH 8.0, 1.4 M NaCl and 0.2% (v/v) β-Mercaptoethanol) and incubated at 65 °C for 40 min in a water bath. Following this, 250 µL of chloroform was added to the microcentrifuge tube and gently vortexed for 1 min and centrifuged at 12000 rpm for 5 min at RT. Approximately 200 µL of the upper aqueous layer was then transferred to new tubes and mixed with 150 µL of isopropyl alcohol. The tube was left for 5-10 min at RT and then centrifuged at 12000 rpm for 10 min (RT). The supernatant was removed and the pellet was washed with 70% ethanol by centrifugation (12000 rpm, 5 min). The ethanol was

drained off and the samples were left to dry for 30 min. The pellet containing the DNA was suspended in sterile distilled water. The DNA concentration was quantified by absorbance at 260 nm. Putative *PLD1* RNAi transgenics were genotyped using PCR using forward and reverse primers to amplify sense and antisense strands of *PLD1* respectively (Table 4). For screening *PLD1-RFP* overexpression lines, genotyping was performed using *SLR1*-For and *RFP-Int-Rev* primers (Table 4). The presence of *SRK* was also checked by PCR in these over-expression lines using *SRK910*-For and *SRK910*-Rev primers (Table 4). The PCR reaction (25 µL) contained at least 100 ng of genomic DNA and 50 ng of binary vector DNA carrying the appropriate construct as positive control for PCR. Around 20 pmol each of forward and reverse primers were used (0.75 µL each per reaction) along with 12.5 µL of 2X Taq Plus Master Mix Red (Lamda biotech). The PCR reactions were performed in a BIO-RAD S1000TM Thermal Cycler, using following thermal cycling regime: 1 cycle 95 °C for 4 min; 95 °C for 30 sec; 53 °C for 45 sec, 72 °C for 2 min (Go to step 2, repeat 30 cycles), 72 °C for 10 min and 4 °C forever. The PCR amplification product was analyzed by gel electrophoresis on 1 % agarose.

Table 4: Primers used for screening putative transgenic lines.

Construct	Primer	Sequence 5' to 3'
PLRI Sense	SLR1 For	TAATGAGTGGCTGGAAAGTCA
	EcoRIIntPLD1 Rev	GCgaattcCTATGAGCTGCAAAAACTACTT ACCTCCTTCAACACATCGACGGA
PLRI Anti Sense	InfINT2 PLD1 Rev	ATAGgaattcGCcccgggCTTCAACACATCGA CGGA
	SLR1-Rev	CGCAAGACCGGCAACAGGATT
PLOX	PLD1infsmaI For	ACTCTAGAGGATCCCCGGGATGGCG
	RFP-*Int*-Rev	GATCTCGAACTCGTGGCCGTT
SRK	SRK910-For	CCTACGATAGTTCTTACACT
	SRK910-Rev	CCATGATGTCGGAGTGAACGTT

1.12. Protein Preparation and Western Blot Analysis

For protein isolation following pollination, *Brassica napus* flowers were collected in the morning before anthesis. After different pollination time (10 min, 30 min and 60 min), the stigmas were excised from the pistil and pulverized in an extraction buffer containing 50 mM HEPES, 5 mM EDTA, 1 mM DTT, 1M PMSF, 10 % glycerol (v/v) and 1X protease inhibitor cocktail tablets (Roche). The samples were then centrifuged at 13000 g for 20 min at 4 ºC to remove the debris.

Protein concentration was determined using the Bio-Rad protein reagent assay (Cat no 5000006). Approximately 5 to 7 µg of the total protein was loaded on a 10% Acrylamide/Bis-acrylamide gel (40% Acrylamide /Bis-acrylamide, 29:1, Cat no 1610146) at 180 V for 2 h. The total protein was transferred (semi-dry) to a PVDF membrane (0.45 µm, EMD Millipore, Cat no 1620177) using BioRad Tris/Glycine buffer (Cat no 1610771) for 1 h at 25 V followed by blocking the membrane for 1h at RT in 7% Skim Milk (EMD Millipore, Cat no 115363) made in 1X TBST. The membrane was probed with an anti-PLD1 antibody (Genscript) at 1:5000 in 7% milk in 1X TBST for 2h at RT or overnight at 4ºC. The membrane was thoroughly washed in 1X TBST between the steps and secondary anti-mouse antibody (1:15000 in 5% milk in TBST) coupled with HRP-conjugated sheep anti-mouse (Amersham NXA931) was used for 1h at RT. The chemiluminescence from the HRP substrate, Luminata™ Forte (Western HRP Substrate, Millipore) was detected using an X-Ray film (Amersham Hyperfilm ECL) or using the Amersham Imager 600. The RFP signal from RFP-PLD1 was detected after stripping the antibodies from the membrane in 2 M of $MgCl_2$ and 100 µL of acetic acid for 20 min. Then the membrane was washed

thoroughly and blocked 1h at RT in 7 % Skim Milk (EMD Cat no 115363) in 1X TBST. Rabbit Anti-RFP polyclonal (Rockland) was used at a dilution of 1: 3000 in 7 % skim milk in 1X TBST for 2h at RT or overnight at 4 ºC. Goat anti-rabbit antibodies (Invitrogen) in 1:6000 dilution in 5 % (w/v) skimmed milk was used as the secondary antibody to detect anti-RFP antibody.

1.13. Production of Anti-PLD1 Polyclonal Antibodies

Affinity-purified anti-PLD1 polyclonal antibodies were raised and affinity purified by GenScript (Piscataway, NJ, USA). A 14–amino acid PLD1 specific peptide (corresponding to amino acids 636 to 649 of *Brassica napus* PLD1) with an additional N-terminal Cysteine residue, EPAERPDADSSYMKC, was synthesized, conjugated with keyhole limpet hemocyanin, and used to raise antibodies against PLD1 in mouse.

1.14. Phosphatidic Acid (PA) treatment of stigmas

Flowers were incubated by immersing of the pedicel, overnight with either water or 44 µM 1-butanol or 44 µM of 1-butanol and 150 mM of 1-acyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-sn-glycero-3-phosphate (NBD-PA) (ammonium salt) (Avanti ® Polar lipids) or 150 mM of NBD-PA alone. They were then pollinated for 24 h, with saturating amount of pollen after which aniline blue assay was performed and the pollen was counted and observed with an epifluorescence microscope.

1.15. TEM Imaging of stigmas

TEM imaging was performed as previously described (Huang and Yeung, 2015; Sumner, 2015; Safavian *et al.*, 2016). Stigmas were clipped off the pistils and cut in two halves to allow better penetration of the fixatives. The stigmas were fixed in 4 % glutaraldehyde in PIPES buffer 0.05 M, pH 6.9. The samples were degassed by keeping them in vacuum for 10 min and fixed for 4 h at room temperature. The stigmas were then rinsed three times for 10 min each with 0.05 M PIPES buffer. Post-fixation was performed in 2% OsO4 in PIPES buffer for 3 h on ice. Dehydration of the samples was then carried out in a series of increasing concentration of acetone (50%, 70%, 80%, 90%, 100%) and the stigmas were then embedded in Spurr's resin (EMS Cat no 14300). Using an ultramicrotome, thick sections of 60 μm thickness from the area of interest were cut, stained with toluidine blue for 1 min and rinsed with a drop of water (Appendix A, Figure A8). Once the area of interest was isolated, longitudinal sections (80 nm thick) were cut using a Leica Ultramicrotome EM UC7 fitted with a diamond knife. The sections were collected on a copper grid and were stained with uranyl acetate and lead citrate. The grids were observed, and images were acquired using a transmission electron microscope Hitachi H-7650 at 80 kV.

1.16. Confocal Microscopy

The flowers were placed with their pedicel in water and were pollinated with either W1 pollen or with compatible Westar pollen for different time points. Following indicated times, the stigmas were clipped off the pistils and mounted on a glass slide with 50% glycerol. The RFP signals from the papillary cells of the stigmas were then observed using

a Leica SP5 laser confocal microscope using the 40X oil-immersion objective through the Leica HyD detector using the HeNe 543 laser (excitation 543; emission 585-649).

1.17. Proteasome Inhibitor Treatment

Proteasome inhibitor treatments were performed as described previously (Stone *et al.*, 2003). W1 flowers were collected one day before anthesis and incubated in 50 μM of MG132, a proteasomal inhibitor (10 mM stock in DMSO), overnight. W1 flowers incubated in DMSO alone were used as control. The following day, open flowers were pollinated with fresh W1 pollen for different experimental time points followed by total protein extraction, quantification and Western blotting with anti-PLD1 antibodies as described previously.

1.18. Microsomal Extraction

Microsome extraction was performed as described previously (Northey *et al.*, 2016). Plant tissues (stigmas before anthesis) were ground in liquid nitrogen and resuspended in extraction buffer (100 mM Tris/HCl pH 8.0, 25% sucrose, 5% glycerol, 10 mM EDTA, 10 mM EGTA, 5 mM KCl, 1 mM DTT and 1X protease inhibitor cocktail (cOmplete™, Mini, EDTA-free (Roche)). The homogenate was then transferred to pre-equilibrated 5% PVPP pellet (PVPP equilibrium buffer: 200mM Tris/HCl pH 8.0, 40% sucrose, 20 mM EDTA, 20 mM EGTA, 10 mM KCl) for 5 minutes followed by centrifugation at 1000 g for 3 min. The supernatant was then transferred to ultracentrifuge tubes (Beckman Coulter) and centrifuged at 21000 g for 10 min. The supernatant from this 20K was then centrifuged

again at 100000 g for 1 h and the resultant supernatant was used as the cytosolic fraction. All centrifugations were performed at 4 ºC. The various pellets from each centrifugation step were re-suspended in membrane solubilization buffer (50 mM Tris/HCl pH 8.0, 50 mM NaCl, 0.1% SDS, 5 mM EDTA, 3 mM DTT and 1X protease inhibitor cocktail (cOmplete™, Mini, EDTA-free (Roche)). The three solubilized pellet fractions along with the cytosolic fraction were used for Western blotting with anti-PLD1 antibodies (1:5,000 dilution).

1.19. PLD1 Activity Assay

Acyl 06:0 NBD-PC 1-acyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl-sn-glycero-3-phosphocholine (ammonium salt) (Avanti ® Polar lipids) (in Chloroform) was evaporated under N_2 flux and suspended by sonication in dH_2O. Proteins from stigmas were extracted following the method described previously in a buffer composed of 50 mM Tris-HCl pH7.5, 10 mM KCl 1 mM EDTA 2 mM DTT and 0.5 mM PMSF. The reaction mixture contained 20 to 40 µg of stigma proteins in 100 mM of MES pH 6.5 and 100 µM of NBD-PC, 0.5 mM of SDS and 0.5% of 1-butanol, in a final volume of 100 µL. The reaction was started by the addition of the NBD-PC and incubated for 30 min at 30 °C in an incubator with shaking at 200 rpm. The assay was terminated by addition of 250 µL of chloroform/methanol (2:1, v/v) followed by the addition of 100 µL of 2 M KCl. The mixture was vortexed for 1 min and then centrifuged at 12000 g for 5 min at RT to separate the lipids. The lower phase was taken and dried under N_2 flow, resuspended in 5 µL of chloroform: methanol (1:2) and using a 10 µL gastight syringe (Hamilton) applied on a

TLC pre-run in a special tank containing the solvent to be used for migration (isooctane:acetic acid:H_2O:ethyl acetate (2:3:10:12)). The migration was allowed to the top of the plate to remove any contaminant from the silica gel plates (Silica gel on TLC alu foil Sigma). The TLC plates were then read using a Typhoon FLA 7000 Imager (GE Healthcare) (excitation, 450 nm; emission, 520 nm).

1.20. Recombinant Protein Purification, *In Vitro* Ubiquitination Assay

All fusion proteins were expressed and purified from Escherichia coli strain BL21 (DE3) or Rosetta™ (DE3) pLysS cells as previously described (Samuel et al., 2009). PLD1 and ARC1 were cloned in pGEX-4T-1 (as described previously (Sankaranarayanan *et al.*, 2015). *PLD1* ORF was amplified by PCR Phusion using the primers (Inf-PGEX-Eco-PLD1For and Inf-PGEX-EcoPLD1Rev) (Table 5) and inserted into pGEX-4T-1 by ligation. In vitro ubiquitination assays were performed using purified GST: PLD1 protein using GST:ARC1 as the E3 ligase as previously described (Stone et al., 2003). For the ubiquitination assay, the reaction mixtures (25 μL) contained 0.5 μg of yeast E1 (BostonBiochem, Cambridge, MA), 0.5 μg of *Arabidopsis thaliana* UBC7 or UbcH5a human E2 (BostonBiochem, Cambridge, MA), 1 μg of E3, 25 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 25 mM NaCl, 2 mM ATP, 0.3 mM DTT, 1 μg of ubiquitin, 1 mM creatine phosphate, and 1 unit of phosphocreatine kinase (Sigma-Aldrich). Reactions were incubated at 30 °C for 2 h and terminated by adding 2X SDS sample buffer and heating at 95 °C for 5 min. The samples were resolved on an 8% SDS-PAGE gel followed by protein gel blot analysis using either anti-PLD1 (GenScript) as previously described.

Ubiquitination detection with anti-Ubiquitin antibody was performed after the membrane was stripped (as described previously), autoclaved (121 ºC for 20 min) and blocked for 2h in 5% skim milk. Following blocking and three 5 min washes with 1X TBST buffer, the membrane was incubated overnight with anti-Ubiquitin antibody (1:100) (Sigma Aldrich) in 3% skim milk. After four 5 min washes, the membrane was incubated with HRP conjugated secondary antibody (1:5000 in 3% skim milk; Goat anti-rabbit, Invitrogen). The membrane was then washed six times with 1X TBST at 5 min interval, and the bound antibodies were visualized with LuminataTM Forte (Western HRP Substrate, Millipore).

Table 5: List of primers used for pGEX-PLD1 construct.

Construct	Primer	Sequence
pGEX-PLD1	Inf-PGEX-Eco-PLD1For	5'-TGGATCCCCGGAATTCATGGCGCAG-3'
	Inf-PGEX-EcoPLD1Rev	5'-GTCGACCCGGGAATTCCTAGGTTGTA-3'
	pGEX-FOR	5'-GGGCTGGCAAGCCACGTTTGGTG-3'
	pGEX-REV	5'-CGGGAGCTGCATGTGTCAGAGG-3'

1.21. Immunoprecipitation Assay

Stigmatic protein extracts were prepared by grinding the unpollinated and pollinated stigmas in extraction buffer (100 mM HEPES, pH 7.5, 5 mM EDTA, 5 mM EGTA, 0.2% Triton X-100, 1 mM PMSF supplemented with 1X protease inhibitor cocktail (cOmplete™, Mini, EDTA-free (Roche)). Protein (30 µg) were added to 750 µL of IP

buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 0.5% Triton X-100, 1 mM PMSF supplemented with 1X protease inhibitor cocktail (cOmplete™, Mini, EDTA-free (Roche)). To prevent non-specific binding to the beads, pre-clarification was carried out by adding 5 µL of Sigma EZ view Protein A beads to the IP buffer with proteins, and incubated for 90 min at 4 °C. After centrifugation at 8200 g for 30 seconds, the supernatant was transferred to a new tube and 3 µg of anti-PLD1 antibody was added and incubated at 4 °C on a rotatory shaker for 2 h. EZ -view beads (10 µL) were added to the mix and incubated at 4 °C for 2 h. After centrifugation at 4000 g at 4 ^{o}C for 1 min, the samples were mixed with 1 mL of IP buffer to wash three times. After the final wash, 25 µL of IP buffer was added to the beads and boiled for 5 min with 2X SDS protein loading buffer. The immunoprecipitated proteins were separated on a 10% SDS-PAGE gel followed by protein blot analysis with anti-PLD1 antibodies as previously described.

Chapter 3: Functional analysis of the role of PLD1 during pollination in *Brassica napus*

3.1. Introduction

Reproduction in plants is an important developmental event that ensures species survival by genetic mixing. In flowering plants, the earliest of events during reproduction is controlled at the stigmatic surface where after landing of the pollen on the stigma, it establishes a molecular dialogue with the stigma components to initiate the reproductive process. The dry stigma of Brassicaceae is a highly evolved structure that can selectively choose a suitable pollen and allow its hydration and tube germination for the transport of the sperm cells through the style to the egg cells (Dresselhaus and Franklin-Tong, 2013). In the sporophytic self-incompatible Brassica species that possess a dry stigma, this interaction is possible only if the pollen has a different haplotype than the female (Goring and Indriolo, 2010). Following recognition of the self-pollen, the stigma actively blocks delivery of hydration promoting factors and also prevents water and nutrient release which are carried out by exocytosis (Goring and Indriolo, 2010). Several members involved in this exocytotic pathway have been characterized with the most important being Exo70A1 which is critical for exocytosis to occur (Samuel *et al.*, 2009; Sankaranarayanan *et al.*, 2015). RNAi-mediated suppression of Exo70A1 resulted in compatible pollination defects such as poor pollen adhesion or pollen germination, as well as a decrease in pod size and reduced number of seeds (Samuel *et al.*, 2009; Sankaranarayanan *et al.*, 2015).

In this study, to evaluate the role of PLD1 during pollination, I have used pharmacological and transgenic approaches to investigate the importance of PLD1 for compatible pollination and the possibility that it could be targeted during SI response. Through inhibition of PLD1 by a pharmacological approach using primary alcohol such as 1-butanol and methylethanolamine (MEA) that are excellent PLD1 inhibitors, I show that PLD1 is necessary for compatible pollination. Furthermore, through generating canola transgenics that are suppressed in *PLD1* expression, the role of PLD1 as a compatibility factor was further confirmed. I was also able to show that PLD1 overexpression could break-down self-incompatibility in W1 self-incompatible lines, thus raising the possibility that PLD1 could be targeted by SI response.

3.2. Results

3.2.1. Inhibition of PLD1 in *Brassica napus* stigmas results in reduced pollen attachment following compatible pollination

PLD1 produces PA by hydrolyzing the polar head of membrane phospholipids, principally phosphatidylcholine (PC) and phosphatidylethanolamine (PE). One of the most important characteristics of PLD activity is the ability of the enzyme to use a primary alcohol to perform a reaction called transphosphatidylation (Yang *et al.*, 1967). This reaction leads to the formation of a phosphatidyl alcohol instead of a phosphatidic acid (PA). Unlike PA, which can be quickly transformed into DAG (diacylglycerol) or lysoPA, the phosphatidyl alcohols are very stable in cells (Wang, 2005). First, this property is convenient for the elucidation of PLD1 activity in cells. Secondly, since PA production by PLD1 is blocked

by the primary alcohols, any PA-dependent programming is blocked downstream. To verify how PLD1 activity can affect pollination or if PLD1 is directly involved in pollen attachment or pollen tube growth, W1 flowers were incubated overnight with different primary alcohols (1-butanol or methylethanolamine (MEA)), followed by compatible pollination for 6 h. Treatment with these primary alcohols resulted in a significant decrease in pollen attachment on the stigmatic surface (Figure 8). Moreover, observation of the pollen tubes through aniline blue staining revealed that the pollen tubes have difficulty in penetrating the papillary cells unlike the controls (Figure 8A). Since alkali and heat treatment during aniline blue assay wash off the weakly attached pollen, the ones that were able to attach strongly were visualized for pollen tube penetration. Treatment with MEA and 1-butanol thus resulted in a decrease in both pollen attachment and pollen tube germination (Figures 8B, C), suggesting that PA produced by phospholipases D is required for compatible pollination and pollen tube elongation through the papillary cells.

To directly confirm the role of PLD1 during compatible pollination, transgenic Westar (compatible) lines were created in which *PLD1* expression was selectively suppressed in the stigmas through RNAi approach, using the stigma-specific *SLR1* promoter, which has been found to have a strong localized expression in the stigma (Hackett *et al.*, 1996). The RNAi lines (R4, R5, R6), produced homomorphic flowers with no defect. Flowers from these lines were first evaluated for the level of suppression of PLD1 expression through a Western blot using anti-PLD1 antibodies (Figure 9A).

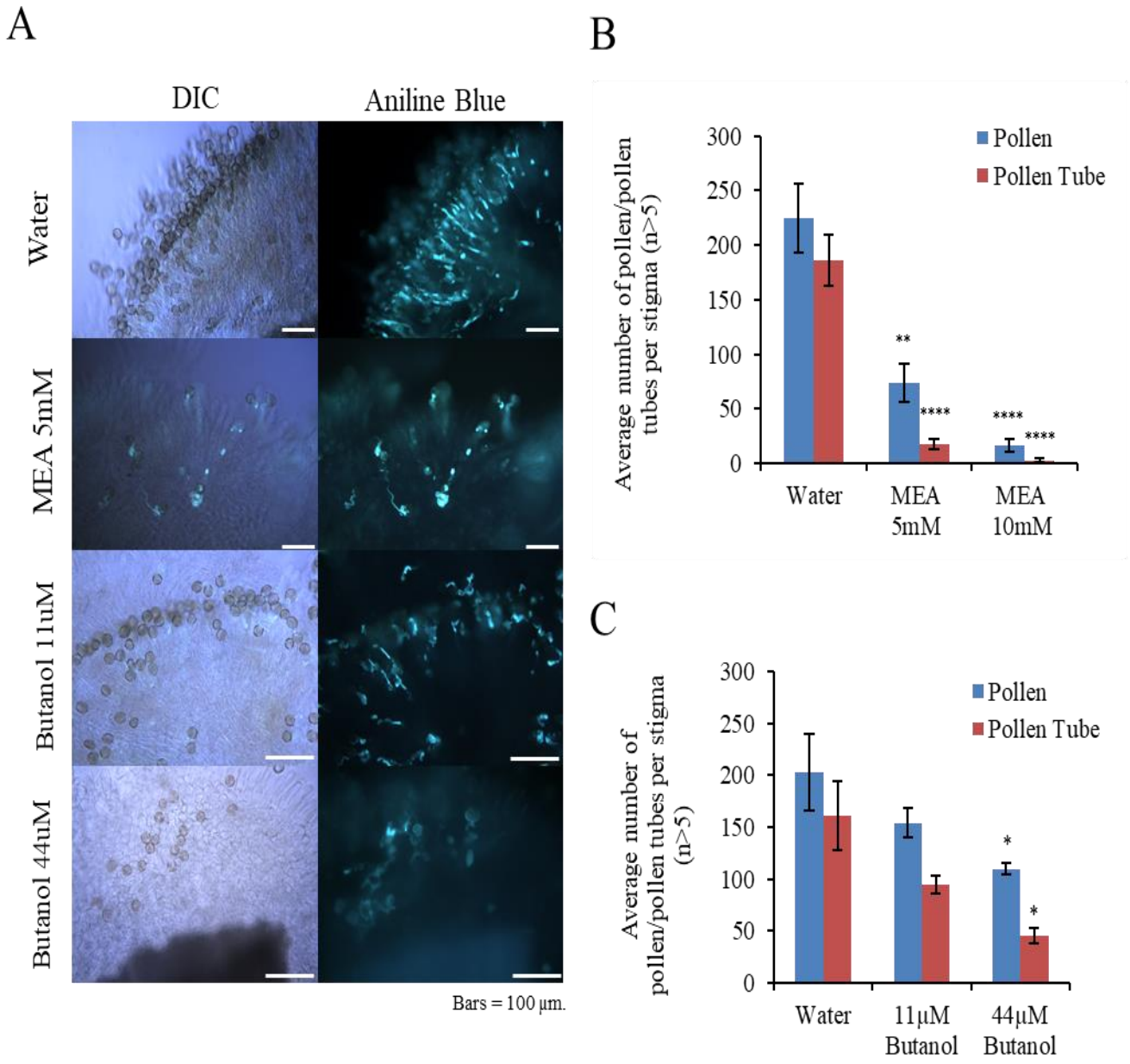


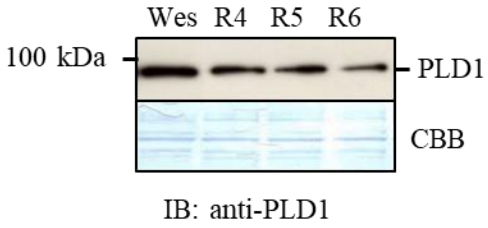
Figure 8: Disruption of PLD1 activity decreases pollination in *Brassica napus*.

(D) Aniline blue assay of W1 flowers incubated overnight with PLD1 inhibitors methylethanolamine (MEA) or 1-butanol and pollinated 6 h with compatible pollen (Westar).

(E) Pollen and pollen tube count after overnight incubation with MEA and 6 h pollination with Westar pollen (t-Test, equal variance, **$p<0.005$, ****$p<0.00005$; $n>5$). Error bars indicate ± SE.

(F) Pollen and pollen tube count after overnight incubation with 1-Butanol and 6 h pollination with Westar pollen (t-Test, equal variance, *$p<0.05$; $n>5$). Error bars indicate ± SE.

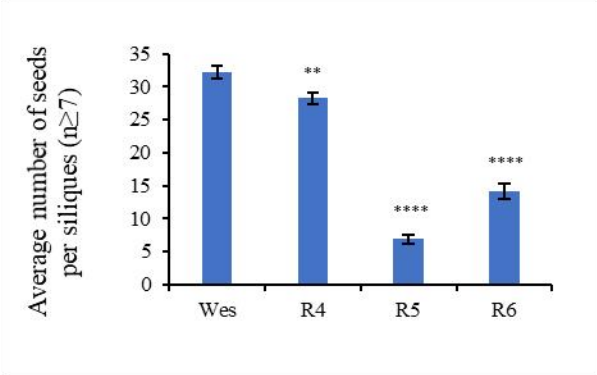
A



B

Stigma: Wes R4 R5 R6
Pollen: ----------Wes----------

C



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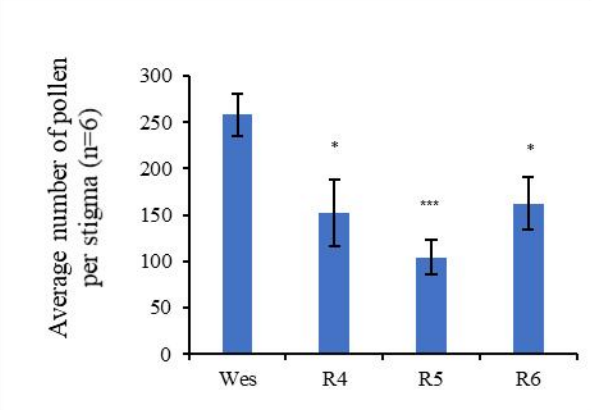

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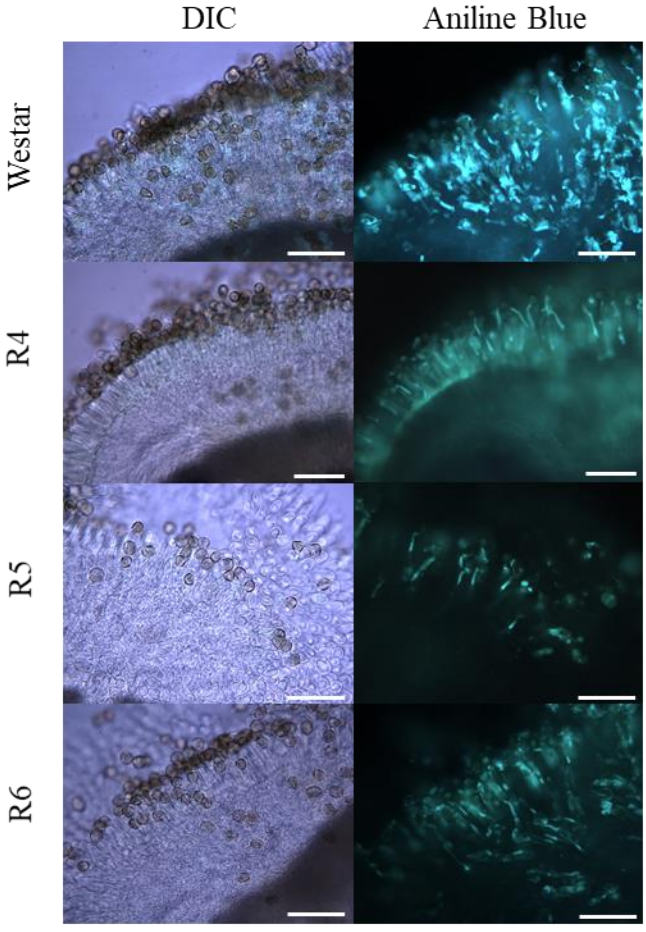


Bars = 100 μm.

Figure 9: RNAi-mediated suppression of *PLD1* in compatible Westar background leads to decreased pollen attachment and a reduction in seed set.

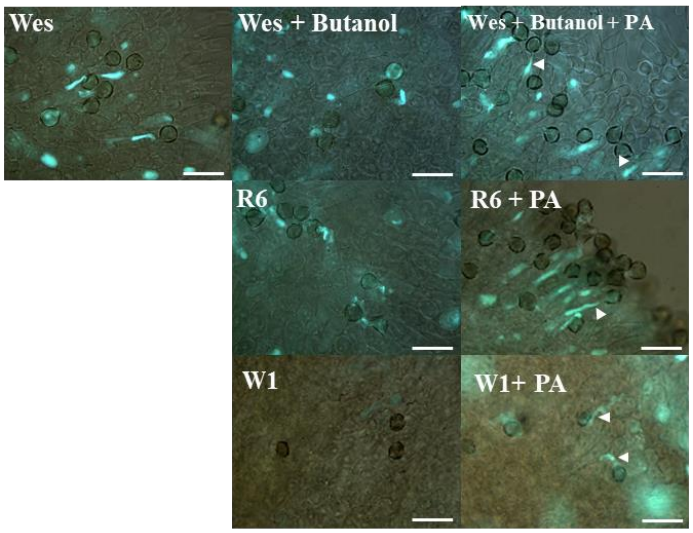
(A) Western blot showing PLD level in RNAi-mediated suppression lines.
(B) Seed pod phenotype indicating a decrease in pod size of the RNAi lines compared to the control Westar.
(C) Average seed per silique between Westar and RNAi lines (t-Test, equal variance, **$p<0.005$, ****$p<0.00005$; $n\geq7$). Error bars indicate ± SE.
(D) Pollen count per stigma (t-Test, equal variance, *$p<0.05$, ***$p<0.0005$; $n>5$; $n=6$). Error bars indicate ± SE.
(E) Aniline-blue assay after 24 h pollination with compatible pollen Westar on Westar line (control) and RNAi lines (R4, R5, R6) showing pollen on top of the papillary cells (DIC) and pollen tubes penetrating the stigma (left).

The suppression was also verified through PLD1 activity assays using one of the dominantly suppressed lines (R6) (Appendix A, Figure A7). When these lines were pollinated with Westar pollen and allowed to develop pods, a decrease in the size of the pods (Figure 9B) associated with a concomitant reduction in the number of seeds per pod (Figure 9C) was observed. When examined for pollen attachment, the number of pollen attached after aniline blue treatment was also significantly reduced in the RNAi lines (Figure 9D, E).

3.2.2. Phosphatidic acid from PLD1 activity is an essential component required for pollination.

Since a major product of PLD1 activity is generation of PA and lack of either PLD1 activity or *PLD1* expression in the RNAi lines resulted in compromised pollination, I hypothesized that lack of PLD1-mediated PA production could be the cause for the observed pollination defects. To assess the importance of PLD1 produced PA for pollination, flowers from compatible Westar lines were incubated with either 1-butanol or 1-butanol in the presence of PA, followed by compatible pollination. In all these treatments, transpirational uptake was used so as to not disturb the dry stigmatic surface. Like observed previously, 1-butanol inhibited compatible pollination and the presence of PA led to the rescue the pollen adhesion and penetration defects induced by 1-butanol treatment (Figure 10A, B). This suggests that PA derived from PLD1 activity is required for early pollination events. In accordance with this, PA was also able to rescue the pollination defects observed in the *PLD1*-RNAi line (R5, R6) (Figure 10A, B). The average number of pollen and pollen tubes were significantly higher than the control in the presence of PA (Figure 10B). PA treatment led an increase of 38 % more pollen on Westar flowers incubated with 1-butanol and PA compared to the Westar flowers with 1-butanol alone (Figure 10B). For the RNAi lines, there was an increase of 50.9 % and 46 % and pollen attachment in line 5 and 6 respectively, in the presence of PA. These observations establish that PA is required for early pollination events to occur, and this PA could be produced by the activation of PLD1 following compatible pollination.

A



Bars = 100 µm.

B

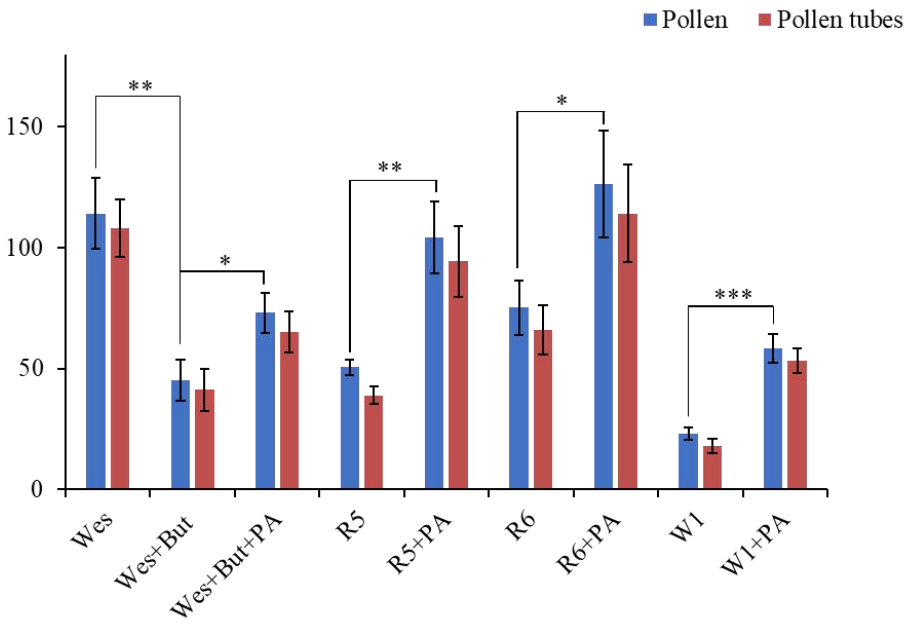


Figure 10: Phosphatidic Acid is able to rescue PLD1 RNAi lines and W1 pollen attachment defect.

(A) Aniline blue assay showing the pollen tubes elongation in flowers incubated in water or with 44 µM of 1-Butanol and complemented with 150µM PA or 150µM of PA alone and pollinated with compatible pollen (Westar and line R6) and incompatible pollen (W1) for 24h.

(B) Pollen and pollen tube count of flower either incubated in water, 44 µM of 1-butanol or butanol and PA and pollinated 24h.

Given that PLD1 was identified as a protein downregulated after SI response, I next asked if through targeting PLD1, SI was blocking PA production necessary for compatible pollination.

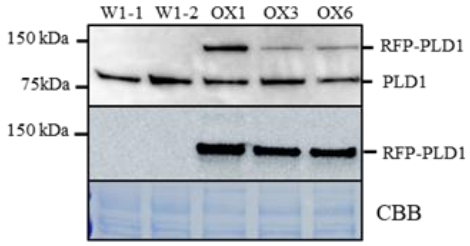
To test this, W1 self-incompatible flowers were treated with PA followed by SI pollination. As expected, a strong and significant increase in pollen attachment was observed following incompatible pollination and pollen tubes could be observed penetrating the papillary cells (Figure 10A). In W1 stigmas, PA treatment led an increase of 63.7% pollen attachment relative to self-pollinated W1 stigmas (Figure 10B). Treatment of PA leading to breaking down of SI response, provides strong evidence that one of the major targets of SI pathway could be PLD1 suppression in order to block PA production.

3.2.2. Overexpressing PLD1 in W1 self-incompatible line partially breaks down the SI response

The ability of PA to breakdown the SI response and downregulation of PLD1 after SI pollination suggested that PLD1 could be one of the targets of SI. If this were true, stabilizing PLD1 levels in self-incompatible stigmas following SI pollination could lead to blocking of SI response thereby promoting germination of self-incompatible pollen.

To investigate this possibility, PLD1-RFP overexpressing self-incompatible W1 lines were generated using the stigma-specific SLR1 promoter. The *PLD1-RFP* lines (OX1, OX3 and OX6) displayed increased expression of total PLD1 (PLD1 + PLD1-RFP) compared to control lines (Figure 11A). When *PLD1-RFP* overexpressing W1 lines were examined for their ability to reject self-incompatible pollen, SI pollen was able to break down the SI

A



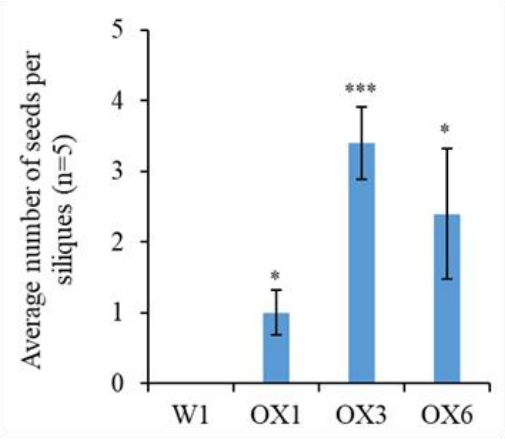
IB: anti-PLD1/anti-RFP

B

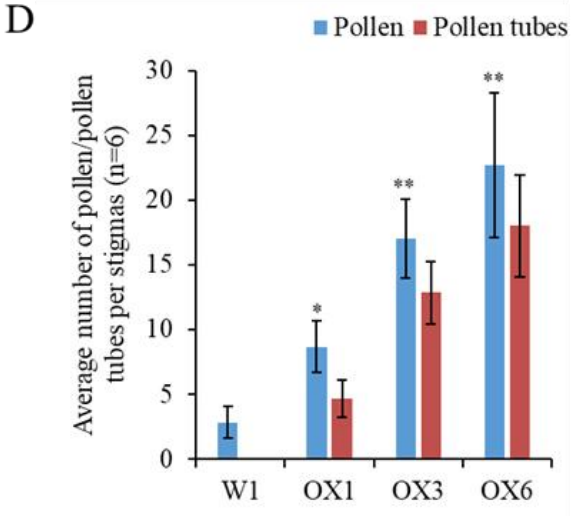
Stigma: Wes W1 OX1 OX3 OX6

Pollen: ----------W1----------

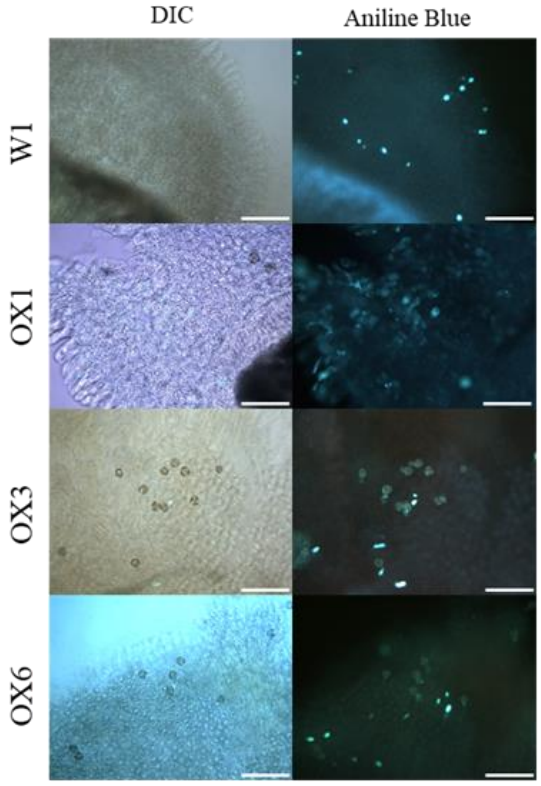
C



D



E



Bars = 100 μm.

Figure 11: Overexpressing PLD partially breaks SI reaction in SI W1 lines.

(A) Analysis by Western-blot of PLD1 and RFP-PLD1 in W1 line and overexpressor lines (OX1, OX3, and OX6).
(B) Seed pods showing extension in overexpressor lines compared to the W1 lines after self-pollination.
(C) Average seed count per silique after pollination with W1 pollen between W1 and OE lines (t-Test, equal variance, *$p<0.05$, ***$p<0.0005$; n=5).
(D) Pollen and pollen tubes count per stigma 24h after pollination with W1 (t-Test, equal variance, *$p<0.05$, **$p<0.005$; n=6).
(E) Aniline blues assay after self-pollination during 24h revealing pollen attached and pollen tubes growing after self-incompatible pollination.

response and led to development of increased pod size with significant number of seeds in each of these pods compared to the control W1 lines (Figure 11B, C). When SI pollinated stigmas were subjected to aniline blue assays, a significant increase in pollen attachment and pollen tube growth could be observed on the *PLD1-RFP* overexpressors compared to W1 stigmas (Figure 11D, E).

3.2.3. PLD1 is involved in membrane trafficking

Based on the results in this chapter, I showed that PLD1 is a key player during compatible pollination and PA produced from PLD1 is essential for pollen hydration and germination to occur. Since PLDs play a major role in membrane modeling in plants (Ammar *et al.*, 2013b), I hypothesized that PLD1-mediated PA production could play an essential role in pollen hydration by facilitating vesicle exocytosis. To decipher the role of PLD1 in delivery of vesicles or multivesicular bodies (MVB) at the pollen attachment site after pollination, stigmas from the various transgenic lines were subjected to imaging using transmission

electron microscopy (TEM). MVBs have been previously reported during pollination in *Brassica napus*, during exocytosis following compatible pollination and their sequestration in the cytosol or the vacuole following SI (Safavian and Goring, 2013). When stigmas from PLD1 overexpressing OX1 line was subjected to TEM prior to SI pollination, no difference could be observed between OX1 line and the control W1 line stigma (Figure 12A, B). Therefore, PLD1 overexpression did not result in any constitutive activation of exocytosis and not much membrane activity could be observed (Figure 12A, B). However, following

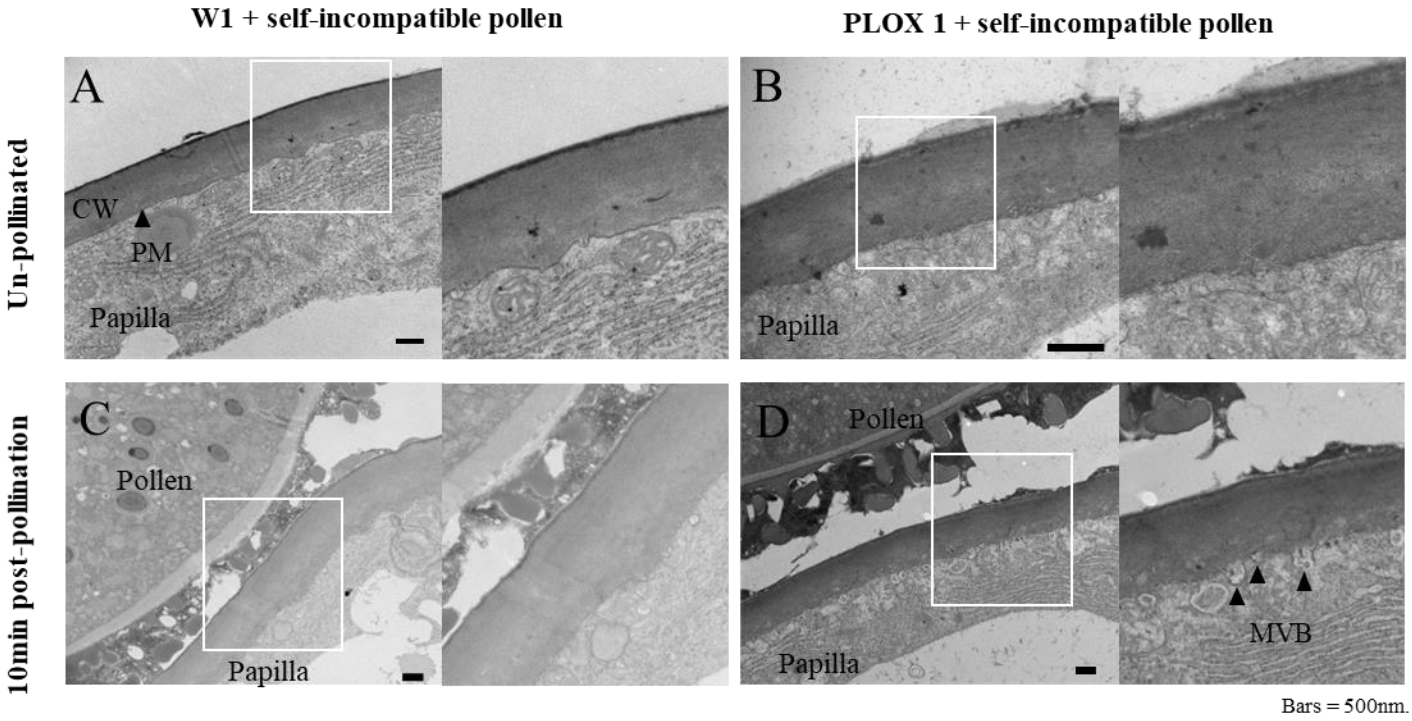


Figure 12: PLD1 is involved in membrane trafficking.

TEM imaging of W1 and PLD1 over-expressing line 1 (PLOX 1) papillary cells pollinated (C, D) or not (A, B) with self-incompatible pollen. The images focus on the plasma membrane and the presence of MVBs (black arrows) at the contact point with pollen grains. Left images are a magnification of the white box from the right picture.

10 min of SI pollination, while no membrane activity was observed in the W1 stigmas, in OX1 papillary cells, vesicles were observed fusing with the plasma membrane at the contact point with the pollen (Figure 12C, D). It was also evident that these large vesicles resembled MVBs with a number of smaller vesicles inside them as previously reported following a compatible interaction in canola (Safavian and Goring, 2013). These results demonstrate that there is a link between PLD1 function and the modeling of the membrane during canola pollination. More importantly, MVBs fusing with the plasma membrane is indicative of a compatible response further confirming the observation that SI pollen could trigger a compatible response when PLD1 was overexpressed in W1 background.

3.3. Discussion

In dry stigmas of Brassicaceae, where the SI response is regulated at the stigmatic surface, once the pollen is recognized as a compatible mate, the papillary cells release water and resources necessary for pollen germination to occur. Although we know the receptor-ligand interaction that occurs during SI response, the factors that impart a compatible response are still poorly understood. Other than some pollen coat proteins that can interact with membrane or secreted proteins such as SLR1 and SLG (Doughty *et al.,* 1998; Takayama *et al.,* 2000), *bona fide* triggers for compatible pollination in either the pollen or the stigma remain as yet unidentified. One of the key proteins that was identified to be essential for pollination is Exo70A1, which is responsible for exocytosis of vesicles required for pollination to occur and it is also targeted by the SI response that blocks this exocytosis from occurring (Samuel et al., 2009). The RNAi-*Exo70A1* canola lines were shown to

accumulate MVBs in the cytosol and were impaired in the machinery that could deliver them for fusion at the pollen attachment site (Safavian and Goring, 2013). In spite of this, other factors that could influence membrane re-modeling that could allow exocytotic vesicle or MVBs have remained unknown. The identification of PLD1 as a compatibility factor, in addition to Exo70A1, further enhances our understanding of the molecular mechanisms behind compatible pollination. Both pharmacological inhibition of PLD1 activity and direct genetic evidence through RNAi-mediated suppression of *PLD1* in stigmas provided evidence that PLD1 is required for compatible pollination (Figure 8, 9).

The experiments with PA supplementation suggests that PA generated from PLD1 activity is likely responsible for promoting compatible pollination possibly through facilitating delivery of factors necessary for pollen hydration and germination (Figure 10). One mechanism through which PA could accomplish this could be due to its cone shape, which could bend the membrane negatively, allowing fusion of MVBs at the contact point of pollen with the stigma, allowing the delivery of water and nutrients (Safavian and Goring, 2013). Its localization at the inner leaflet of the plasma membrane could initiate membrane curvature required for membrane fusion during exocytosis, and this is a recurring theme in phospholipid studies (Roth, 2008). Alternatively, PA could serve as an anchorage for proteins involved in exocytosis (Roth, 2008).

One of the interesting observations from the TEM was that there was no constitutive activation of the exocytotic machinery in the PLD1-OX lines without pollen attachment and only after pollen attachment, membrane activity could be observed. This observation suggests that either PLD1 has to be induced after pollination or the exocyst complex should

be activated only after pollination to deliver vesicles at the membrane where PA generated from PLD1 activity could aid in membrane fusion of the vesicles. One second messenger that could play a crucial role in this activation process could be calcium, since PLD1 possesses the calcium-binding C2 domain and PLD1 can be catalytically activated by binding of calcium (Zheng *et al.*, 2000). In agreement with this, calcium spikes are consistently observed following compatible pollinations (Iwano et al., 2004).

Overexpression of PLD1 was able to compromise the SI response suggesting it could be a direct target of SI pathway (Figure 11). Previous studies have suggested that Exo70A1 is also targeted for degradation by SI response (Samuel et al., 2009) and therefore, in the PLD1 overexpressing lines, the assumption would be that Exo70A1 will still be targeted by ARC1 for degradation. Thus, the observed breakdown of SI in the OX line suggests that PLD1 should act downstream of Exo70A1 during SI. In the PLD1-OX lines, although Exo70A1 is degraded, overexpressed PLD1 could resist degradation by ARC1 and thus could promote fusion of MVBs close to the membrane (Figure 12), resulting in partial breakdown of SI. Since SI reaction is complex and has multiple targets to shut down the exocytotic machinery, overexpression of PLD1 could counter only certain aspects of the SI pathway, resulting in the observed partial breakdown.

Taken together, the observations from this study have provided clear evidence that PLD1 is a compatibility factor where PA produced by PLD1 activity is likely required for membrane remodeling to occur, allowing fusion of exocytotic MVBs or vesicles, to deliver the cargo required for compatible pollination.

Chapter 4. Phospholipase D1 is targeted for degradation by the Self-Incompatibility Response

4.1. Introduction

Recognition of the pollen encoded ligand SCR/SP11 by the female determinant SRK, leads to phosphorylation-mediated activation of ARC1, a member of the plant U-box (PUB) family (Stone et al., 2003). Blocking of this degradation machinery through either inhibitor treatment or by suppression of *ARC1* resulted in breakdown of SI response (Stone et al., 2003). This ARC1-mediated degradation of compatibility factors is known to be the primary process in the stigma during SI that shuts off the transfer of factors necessary for pollen growth (Doucet and Goring, 2017). ARC1 was shown to be responsible for targeting the compatibility factors Exo70A1 and GLO1 for degradation to the proteasome (Samuel *et al.*, 2009; Sankaranarayanan *et al.*, 2015). Recombinant ARC1 was shown to ubiquitinate both Exo70A1 and GLO1 and in a heterologous system and ARC1 can target both these factors to the proteasome in the presence of activated SRK kinase domain (Samuel *et al.*, 2009; Sankaranarayanan *et al.*, 2015). More specifically, GLO1 was found to be accumulating either when treated with MG132 or in the *ARC1* suppression lines (Stone *et al.*, 2003; Samuel *et al.*, 2011).

Analysis of the *B. napus* degradome following SI pollination through 2D-DIGE revealed that phospholipase D1 was down-regulated (Sankaranarayanan *et al.*, 2013a). This led me to hypothesize that PLD1 could be a compatibility factor that is likely targeted by the SI pathway. It is also important to note that only one PLD isoform was identified from the

2D-DIGE to be differentially expressed, indicating that PLD1 could play a significant role during pollination. In Chapter 3, through pharmacological and genetic approaches I was able to show that PLD1 is required for compatible pollination and overexpression of PLD1 could compromise SI. This observation along with the 2D-DIGE data showing that PLD1 could be downregulated strongly suggest that PLD1 is regulated by SI response.

To conclusively demonstrate that PLD1 expression could be regulated through the SI response, I have characterized PLD1 expression and also assessed its localization in canola stigmas after compatible and incompatible pollinations. My results indicate that PLD1 expression is very strong in the stigmas and absent in the leaves and SI response could target PLD1 for ARC1-mediated degradation.

4.2.Results

4.2.1. PLD1 is specifically expressed in floral tissue and its expression is regulated following SI and CP pollination.

In order to characterize the role of PLD1 during pollination, the specificity of its expression in various tissues was first assessed through Western blot using anti-PLD1 antibodies. PLD1 was found to be specific to floral tissue with strong expression in the stigma and petals, while weak expression was observed in the sepals and PLD1 was absent in anther, pollen and leaf tissues (Figure 13A). Stigmas showed the strongest PLD1 expression with stage 10 and 11, having stronger expression than stage 12, which coincides with anthesis (Figure 13A). Increased expression in stage 10 and 11 could indicate the need for high PLD1 and PA produced from it, for polarized elongation of the papillary cells and the levels

stabilize in stage 12 in order to be sufficient for promoting pollination (Figure 13A). Having established this, to confirm the observations from the 2D-DIGE that PLD1 was downregulated by SI (Appendix A, Figure A1), PLD1 level was assessed at various times after SI pollination. PLD1 level was found to decrease as early as 10 min post-pollination, and continued to stay low at 30 and 60 min post-SI (Figure 13B), Interestingly, across many replications, PLD1 level was found to increase after 60 min, and the level was more than 10 and 30 min after SI, but was lower than the unpollinated state (Figure 13B). In contrast to SI pollination, following compatible pollination, mostly PLD1 level was slightly enhanced after 10 min of compatible pollination, although not consistently and the levels dropped below unpollinated state after 60 min of compatible pollination (Figure 13B). When PLD1 activity assays were performed using NBD-PC as a substrate in the presence of n-butanol, and observing the levels of NBD-phosphatidyl butanol produced by PLD1 activity, compatible pollination led to increase in PLD1 activity after 10 and 30 min after pollination, while no significant changes could be observed after SI pollination (Figure 13C). The lack of correlation between reduction in PLD1 levels after SI and an absence of reduction in PLD1 activity could be due to interference from other PLD1 isoforms that mask the reduction in SI.

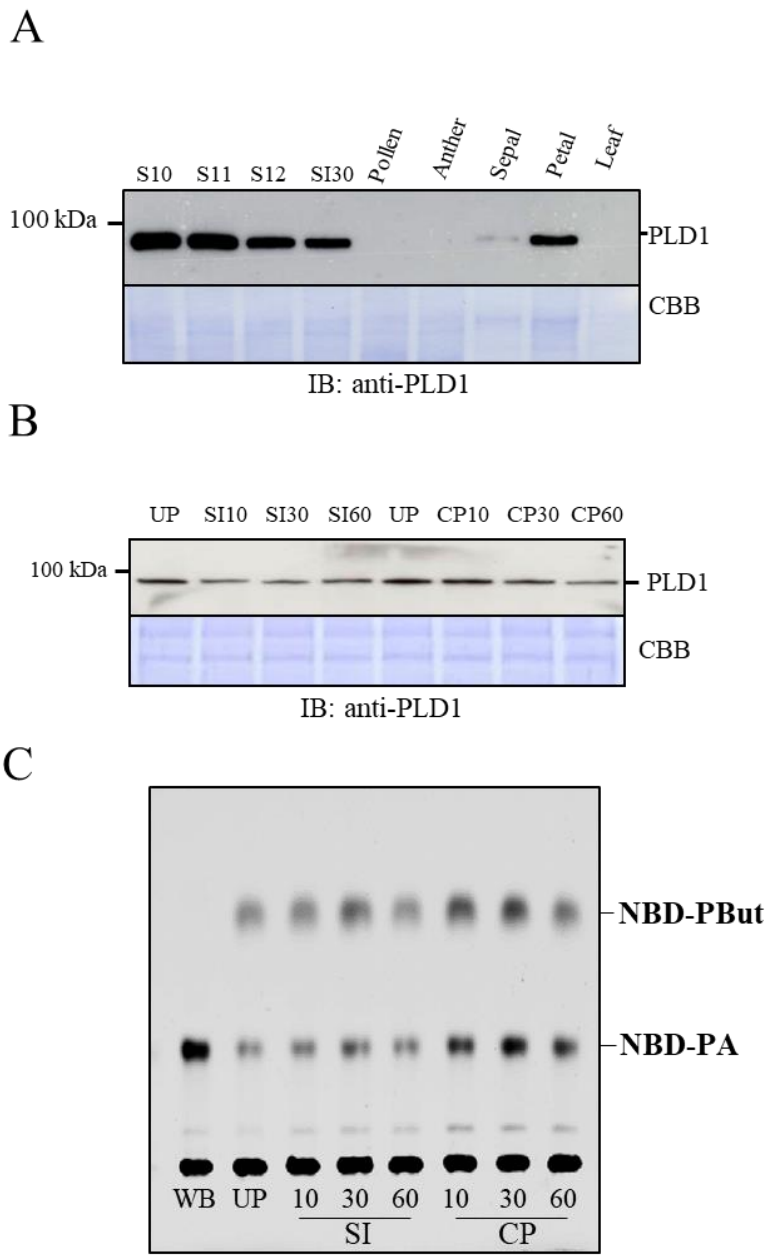
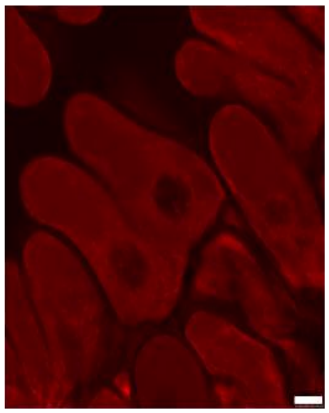


Figure 13: PLD1 is regulated following SI and CP.

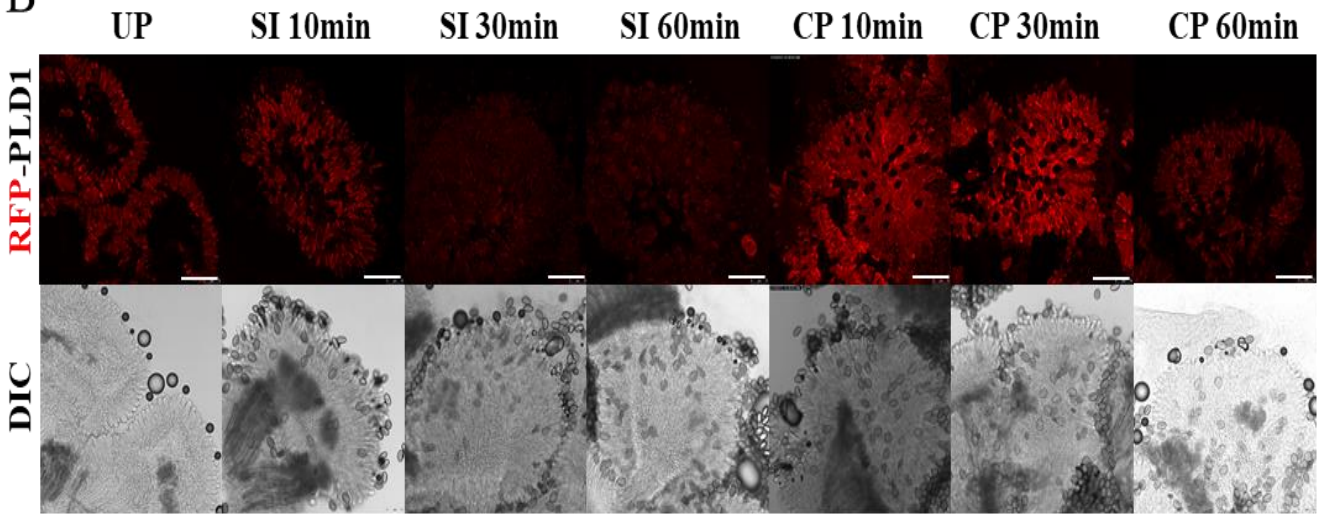
(A) Western blot analysis of BnPLD1 expression in various tissues (Stage 10, 11, 12 stigmas, Stage 12 stigmas pollinated with SI for 30 min, pollen, anther, sepal, petal, leaf).
(B) Western blot BnPLD1 in stigmas either left unpollinated (UP) or pollinated for 10, 30 and 60 min with SI or compatible pollen. CBB stained membrane is shown below for loading.
(C) TLC plate showing stigmatic PLD activity at various times after pollination, assessed by the formation of NBD-Phosphatidylbutanol (NBD-Pbut) from NBD-Phosphatidylcholine (NBD-PC) in presence of 0.5% 1-butanol. WB: Without butanol in the reaction mixture. NBD-PA: NBD-phosphatidic acid.

A



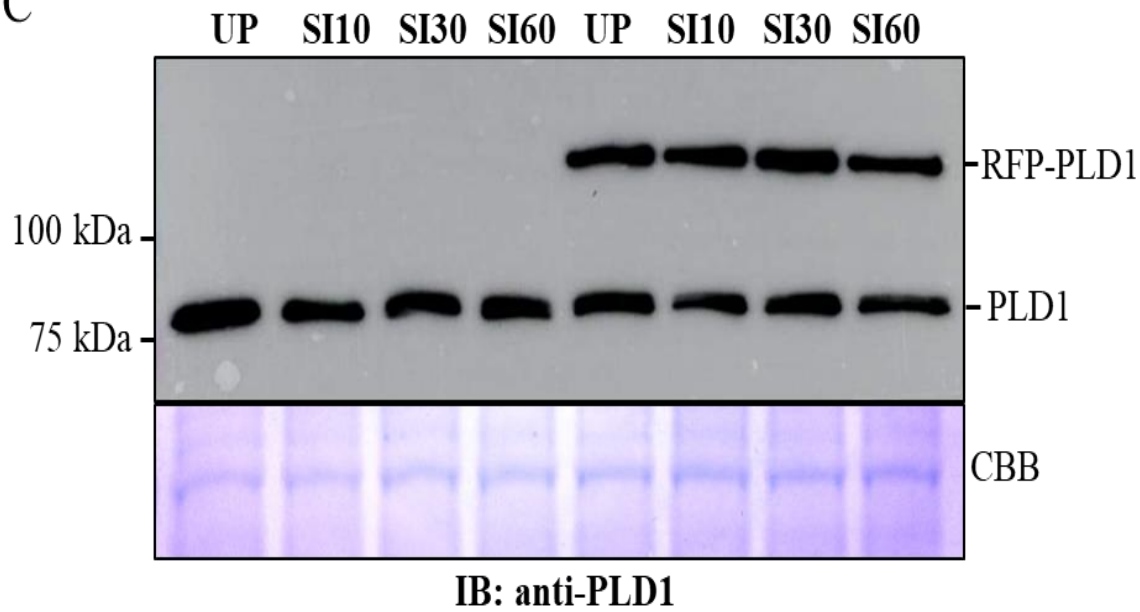
Bars=10 μm.

B



Bars=150 μm.

C



IB: anti-PLD1

Figure 14: PLD1 expressing RFP-PLD1 under SLR1 promoter show regulation after SI and CP reaction.

(A) Subcellular localization of PLD1-RFP in the papillary cells of PLD1-OX line. Scale: 10 µM
(B) Confocal microscopy imaging of overexpressor PLOX1 line stigma showing PLD1-RFP expression after either 10, 30 and 60 min after W1 pollination or compatible pollination with Westar pollen.
(C) Western-Blot showing temporal regulation of PLD1 in wild-type W1 stigma and PLD1-RFP overexpressor line pollinated with W1 pollen for various times (10, 30 and 60 min).

Temporal regulation of PLD1 expression was also confirmed through confocal microscopy using PLD1-RFP overexpressing lines. In the PLD1-OX lines, at the subcellular level, PLD1-RFP was expressed throughout the cell including cell membrane and the cytosol (Figure 14A), and this was corroborated with the observation that PLD1 was found both in cytosolic and microsomal fractions (Appendix A, Figure A9). When PLD1-OX stigmas were imaged after various times after SI and compatible pollination, a reduction in signal intensity was observed following SI pollination and 60 min after compatible pollination consistent with the Western blotting (Figure 14B). When protein extracts from PLD1-OX lines were examined for regulation by SI response, they showed a similar trend to W1 extracts, except there was significantly increased levels of PLD1 in the overexpressing line (Figure 14C).

4.2.2. Both ARC1 and proteasome are required for PLD1 downregulation following SI

The ability of SI to cause the downregulation of PLD1 expression raised the possibility that ARC1, the E3-ubiquitin ligase, which has a central role in targeting for degradation of

compatibility factors during SI (Stone *et al.*, 2003; Samuel *et al.*, 2009; Sankaranarayanan *et al.*, 2015), could also be involved in regulation of PLD1 expression. To examine the influence of the proteasome in downregulation of PLD1, flowers from incompatible W1 plants were treated with the proteasome inhibitor MG132, followed by analysis of the stigma extracts for PLD1 expression. MG132 treatment led to stabilization of PLD1 and prevented degradation of PLD1 after SI pollination (Figure 15A). This accumulation shows that the proteasome system is required for degrading PLD1 and indicates that it is likely that the ubiquitin pathway is associated with this proteasome-mediated degradation during the SI response. Having established this, I next examined if ARC1 was required for this proteasome-mediated downregulation of PLD1 after SI. For this, PLD1 expression was assessed in flowers from *ARC1*-suppressed plants after SI pollination (Figure 15B). Expression of PLD1 was very similar to the MG132 treated flowers with stabilization of PLD1 in unpollinated flowers and lack of downregulation of PLD1 after SI treatment (Figure 15B). Rapid ubiquitination of PLD1 after SI was also confirmed through immunoprecipitating with anti-PLD1 antibodies followed by detection using PLD1 antibodies or anti-ubiquitin antibodies (Appendix A, Figure A11). To further confirm that ARC1 is directly responsible for ubiquitinating PLD1, an *in-vitro* ubiquitination assay was performed with recombinant GST-PLD1 protein using GST-ARC1 as the E3 ubiquitin ligase (Figure 16). Higher molecular weight forms, consistent with poly-ubiquitinated GST-PLD1 were observed with the addition of E1, UBC7 (E2) and ARC1 (E3) and this smear was not present in the lane without these enzymes (Figure 16).

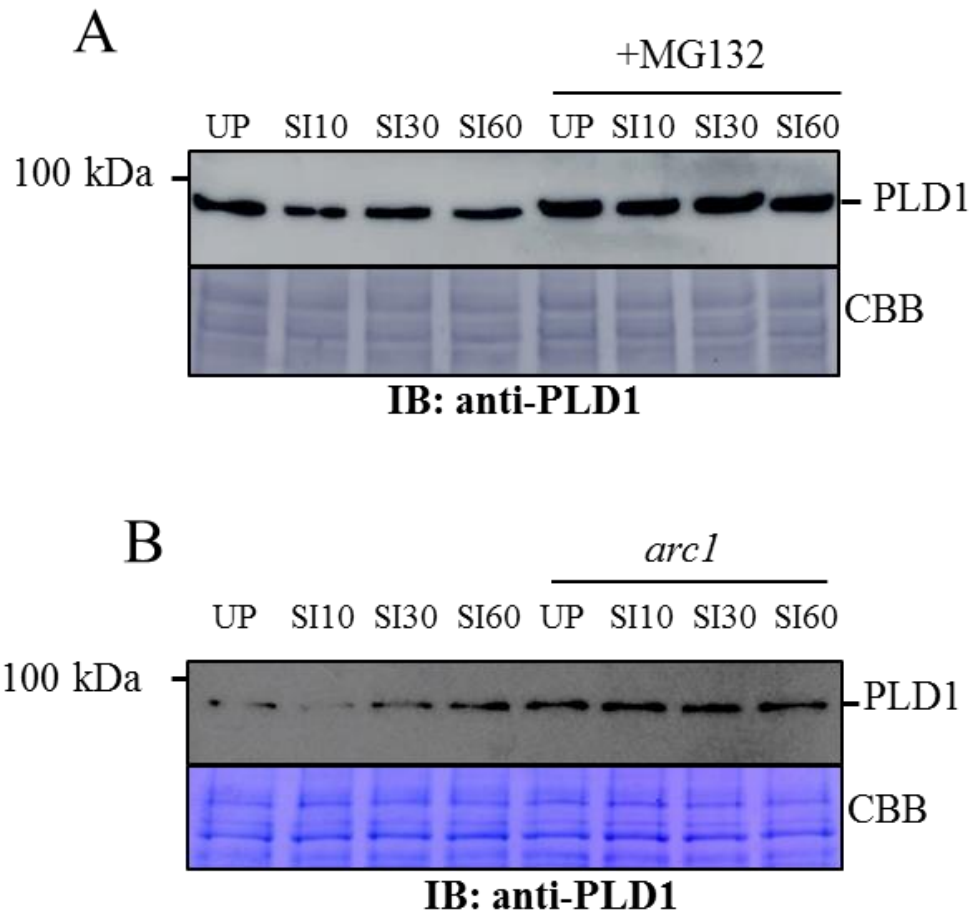


Figure 15: PLD1 downregulation after SI is dependent on ARC1 and the proteasome.

(A) PLD1 expression was detected through Western blot analysis of stigma extracts collected from W1 flowers either left untreated or treated with MG132 for 3h, followed by self pollination for 10,30,60 min.

(B) PLD1 expression was detected in self-pollinated stigmas from W1 line or ARC1 suppressed W1 line.

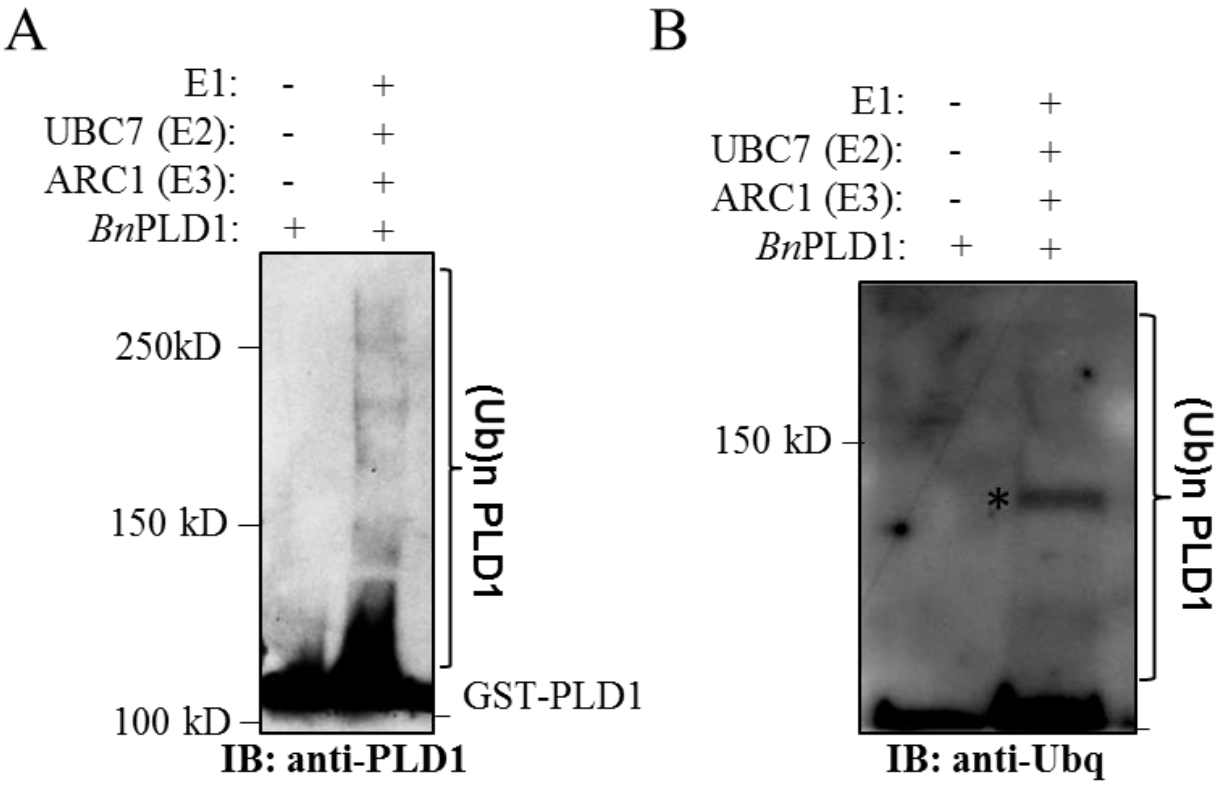


Figure 16: PLD1 *in-vitro* ubiquitination assay.

(A) Western blot using anti-PLD1 antibodies to detect ubiquitination of GST-PLD1 following in vitro ubiquitination assay using ARC1 as the E3 ligase.
(B) The same blot was stripped and probed with anti-Ubiquitin antibodies to detect ubiquitination of GST-PLD1 (* indicates the E1 enzyme used in the assay).

4.2.3. Resetting of the pollination signaling machinery in W1 stigmas

Investigating PLD1 level fluctuations in the stigma at different time points after pollination revealed that PLD1 expression decreases at 10 and 30 min after SI, but at 60 min after SI, its expression was elevated, although it was still lower than the unpollinated state (Figure 13A). This observation allowed me to hypothesize that, SI pollinated stigmas could be resetting themselves after a wave of SI response and initial rejection of the self-pollen. To further investigate this, self-incompatible W1 stigmas were pollinated with self-pollen to induce the SI reaction for 60 min (SI triggered stigmas), and this was followed by either SI pollination or compatible pollination. This would allow to assess if following saturated SI pollination, whether an incompatible stigma could accept compatible pollen. Westar pollination of SI triggered stigmas resulted in significant increase in pod size, seed numbers which were associated with increased pollen attachment and pollen tube penetration consistent with a compatible pollination (Figure 17A-D). Therefore, application of SI pollen did not prevent compatible Westar pollen from being accepted in spite of the SI response being active for over 60 min. When PLD1 levels were examined at various times after SI pollination in SI triggered stigmas, self-pollination after 60 min of SI reaction, seemed to trigger another cycle of down-regulation (Figure 17D). The pattern was very similar to a new SI reaction with PLD1 downregulated after 10 and 30 min of SI and increased again after 60 min (Figure 17D).

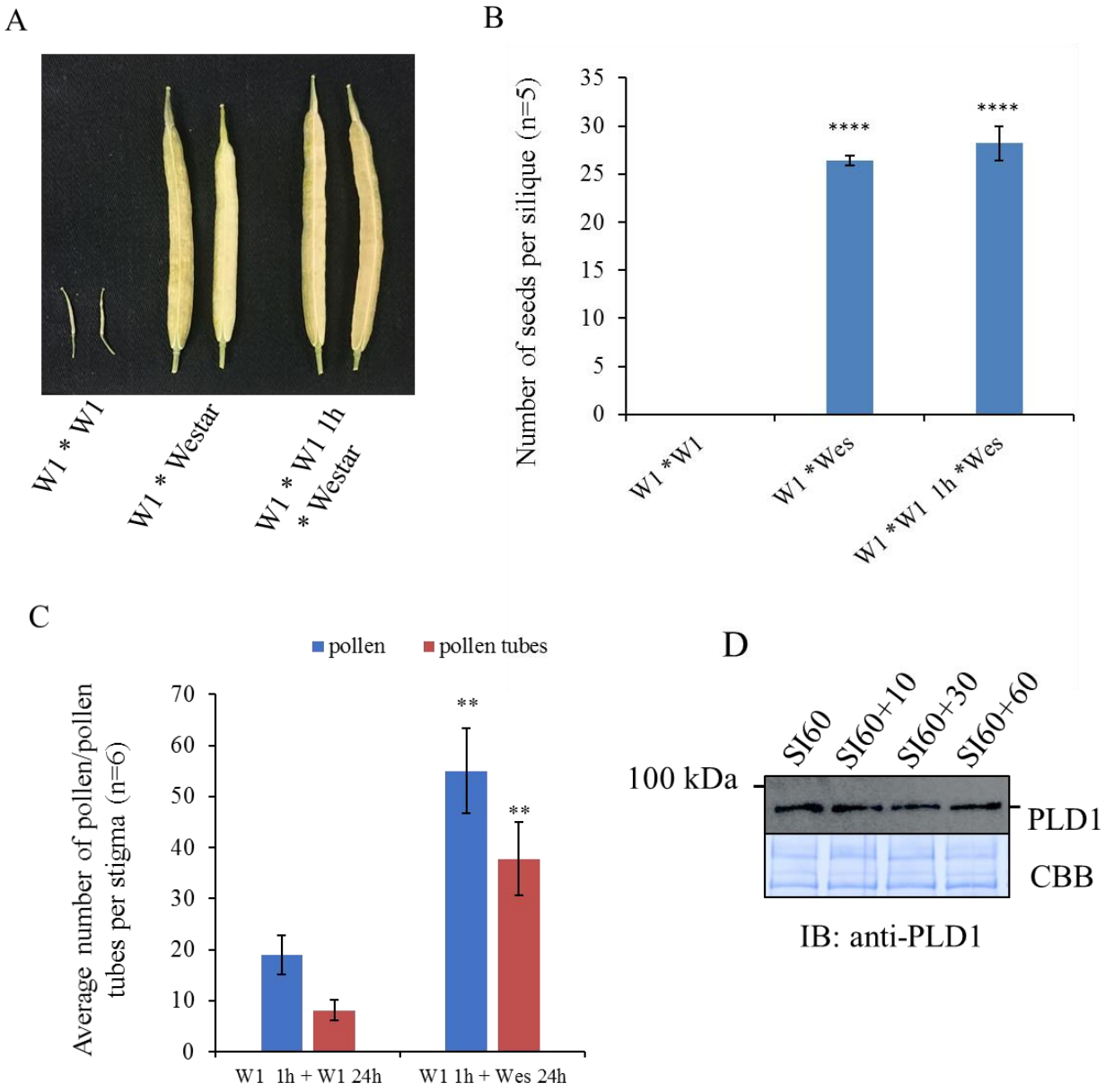
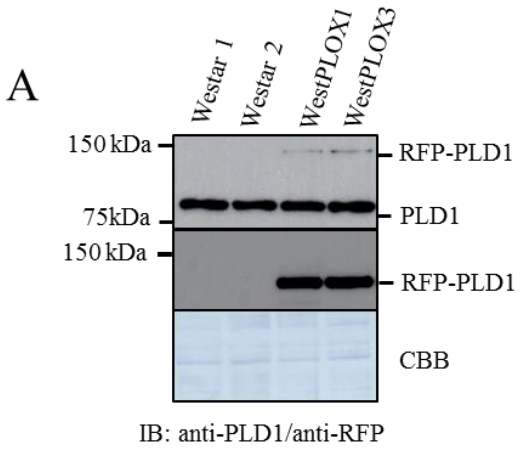


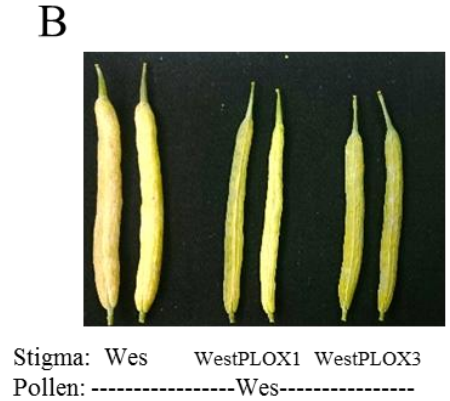
Figure 17: Resetting of the pollination signaling machinery in W1 stigmas.

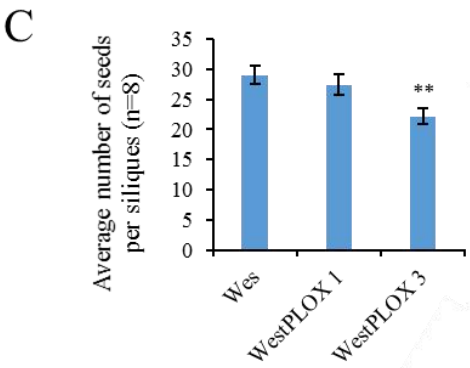
(A) Pods from flowers that were heavily pollinated W1 stigmas for 1h with self-pollen and then applying Westar pollen.
(B) Average number of seeds per siliques from these pods are shown.
(C) Pollen/pollen tube counted through aniline blue imaging of W1 stigmas self-pollinated for one hour followed by 24h pollination with either W1 of Westar pollen.
(D) Western blot analysis to detect PLD1 expression following pollination of SI-induced W1 stigmas (pollinated with W1 for one hour) with Westar pollen for 10, 30, or 60 min.

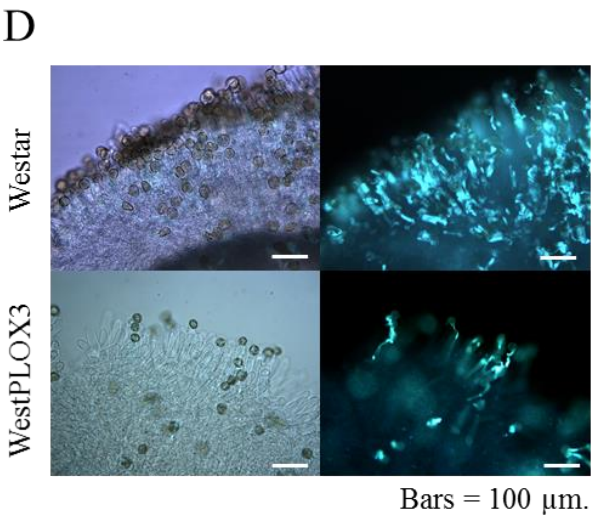
4.2.4. Over-expression of PLD1 in Westar self-compatible line leads to a slight decrease in pollen attachment.

In contrasting fashion, while SI reaction led to a slight increase in PLD1 levels after 60 min of SI pollination, compatible reaction led to slight decrease in PLD1 levels after 60 min of pollination. Based on this, I hypothesized that once function of PLD1 is accomplished, it should be reduced or eliminated in order for the stigmas to allow pollen tube growth. In addition to this, I also observed that it was difficult to obtain seeds from PLD1 overexpressing W1 lines. Therefore, to test if overexpression of PLD1 could actually reduce the ability of stigma to accept compatible pollen, Westar lines overexpressing PLD1-RFP were created (Figure 18A). When these transgenic overexpression lines were assessed for ability to accept compatible pollen, a slight decrease in pod size and a decrease in their number of seeds compared to the Westar line was observed (Figure 18 B, C). Aniline blue assay also revealed less abundant pollen attachment and pollen germination compared to the Westar control line (Figure 18D, E).


A
Westar 1
Westar 2
WestPLOX1
WestPLOX3
150 kDa
RFP-PLD1
75kDa
PLD1
150 kDa
RFP-PLD1
CBB
IB: anti-PLD1/anti-RFP


B
Stigma: Wes WestPLOX1 WestPLOX3
Pollen: ----------Wes----------


C
Average number of seeds per siliques (n=8)
35
30
25
20
15
10
5
0
**
Wes
WestPLOX 1
WestPLOX 3


D
Westar
WestPLOX3
Bars = 100 µm.

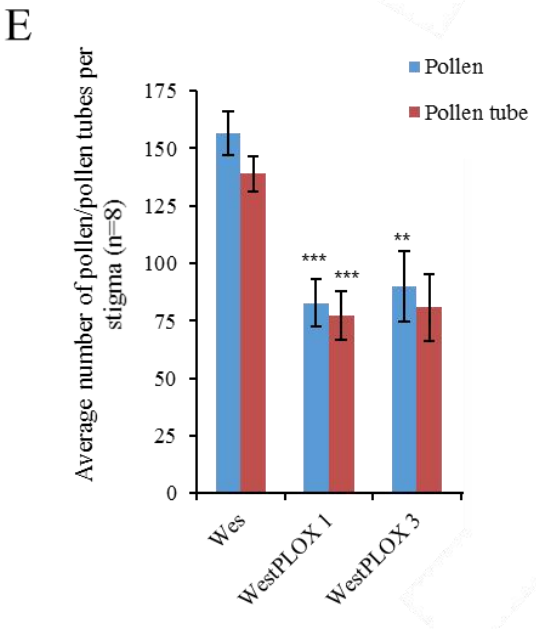

E
Pollen
Pollen tube
Average number of pollen/pollen tubes per stigma (n=8)
175
150
125
100
75
50
25
0
***
***
**
Wes
WestPLOX 1
WestPLOX 3

Figure 18: PLD1 overexpression in self-compatible Westar stigmas results in reduced pollen attachment and seed set.

(A) Stigmatic proteins from unpollinated Westar line and two independent transgenic PLD1-overexpressing lines in compatible Westar background (WestPLOX1 and WestPLOX 3) were analyzed for PLD1-RFP expression by Western blot using PLD1 and RFP antibodies.
(B) Comparison of mature pods following pollination with compatible Westar pollen.
(C) Average number of seeds per pod in control Westar line and PLD1-OX lines after pollination with Westar pollen. All the pods were allowed to develop for an equal length of time (t-Test, equal variance, **p<0.005, ****p<0.00005; n=8). Error bars indicate ± SE.
(D) Aniline blue assay showing pollen attachment and pollen tube germination on stigma of PLD1 overexpressing Wester lines compared to pollen on Westar line following 24 h pollination *in-planta.*
(E) Average number of pollen/pollen tubes in control Westar line and PLD1-OX lines after pollination with Wester pollen for 24h (t-Test, equal variance, *p<0.05, **p<0.005, ****p<0.00005; n=8). Error bars indicate ± SE.

4.3.Discussion

Protein regulation by ubiquitin-mediated degradation has been shown to be an integral part of pollen rejection system during SI. In the gametophytic system, ubiquitination is relayed by the SCF/SLF E3 complex that targets toxic S-RNases from non-self pistils for degradation by the proteasome (Liu *et al.*, 2014). Degradation by the 26S proteasome/COP9 signalosome (CSN) is a relatively fast way to control protein expression as the proteasome is a large protein complex that recognizes protein bound to at least four ubiquitin molecules (at Lys-48), denature the protein and breaks it down to peptides (2-30 amino acids each) (Glickman and Ciechanover, 2002). The strongest evidence of ubiquitin-26S proteasome system involved during SI in Brassicaceae is the break-down of SI in *arc1* lines (Stone *et al.*, 1999). Also, *in vitro* ubiquitination of Exo70A1 and GLO1 by ARC1

and breakdown of SI by MG132 have confirmed the role of the ubiquitin-26S proteasome system in SI (Samuel *et al.*, 2009; Sankaranarayanan *et al.*, 2015). Recently it was proposed that this SI system uses autophagy, which is usually used when proteins start to aggregate in the stigmas (Bence *et al.*, 2001) (Goring, 2017). Multivesicular bodies (MVB) are often found in structures related to autophagic bodies in *B. napus*, and autophagic bodies are also found in *A. lyrata* (Safavian and Goring, 2013; Goring, 2017).

In this chapter, PLD1 was shown to be regulated by both self and cross-pollination. Both Western blot analysis and PLD1-RFP observation by confocal microscopy confirmed the 2D-DIGE analysis. Accumulation of PLD1 in *ARC1* suppressed line and stigma treated with MG132 indicate that PLD1 is regulated by ARC1-mediated proteasomal degradation of proteins. In addition, *in-vitro* ubiquitination assay further confirmed that PLD1 is a direct substrate of ARC1. This result adds PLD1 to the list of already established compatibility factors GLO1 and Exo70A1, which are down-regulated during SI and further reinforces the position of ARC1 as a positive effector of self-incompatibility response (Samuel *et al.*, 2009; Sankaranarayanan *et al.*, 2015).

The accumulation of PLD1 in unpollinated stigmas following either MG132 treatment or in *ARC1* suppressed lines (Figure 15) suggests that PLD1 could be targeted for proteasomal degradation at a basal level by the SI system. This observation is similar to the regulation of GLO1 levels by the proteasome and ARC1, the absence of which resulted in accumulation of GLO1 in the unpollinated stigmas (Sankaranarayanan *et al.*, 2015). Based on these results, the default state for the SI stigma is to have basal activity of ARC1 which will continue to target compatibility factors and this pathway can be hyperactivated by

binding of a self-pollen. The observed ubiquitination of immunoprecipitated PLD1 from unpollinated stigma extract is also indicative of basal activation of SI response (Appendix A, Figure A10). The fact that SRK exists as a dimer prior to pollination suggests that it could have some basal activity in the absence of the ligand that could trigger basal SI (Nasrallah and Nasrallah, 2014). If SI is the default state, then a compatible pollen has to be able to shut down the basal SI in order to promote exocytosis-mediated delivery of factors for pollination. Immediate accumulation of methylglyoxal following compatible pollination, which can block the proteasome could be one of the mechanisms through which SI could be abrogated temporarily (Sankaranarayanan *et al.*, 2015).

Overexpression of PLD1 in W1 background led to breakdown of SI (Chapter 3) and this indicated that stabilization of PLD1 above a threshold that is required for its function as a compatibility factor could be responsible for the observed breakdown. Analysis of temporal regulation of overexpressed PLD1 revealed that PLD1 (endogenous and overexpressed PLD1-RFP) was regulated very similarly to endogenous PLD1 in W1 background (Figure 14). The only difference being, there was a delay (after 60 min) in PLD1 downregulation after SI pollination and there was considerably more PLD1 in the overexpressed line. This fits with the hypothesis that inability of ARC1 to target the total pool of PLD1 likely resulted in continued activity of PLD1 following SI pollination, that resulted in enhance pollen attachment and pollen tube germination.

The increase in PLD1 observed after 60 min of SI pollination suggested that the pollination machinery in the stigma could reset itself after the initial wave of SI reaction to inhibit self-pollen. Previous studies have shown that at the papillary cell level pre-pollination with a

self-pollen grain can affect cross-pollen germination after 5 min, suggesting that SI reaction diffuses across the papillary cell (Iwano *et al.*, 2015) which countered the hypothesis that SI response is very localized. In my study, after one hour of SI pollination, the stigma was able to accept compatible pollen and produce seeds. This approach indicates that the SI response does not shut down the ability of the stigma to accept pollen and it is able to either go for another round of SI response wave or proceed to CP reaction (Figure 17). Application of SI pollen therefore did not prevent Westar pollen from being accepted even if SI had elicited degradation of compatible factors. In canola, it has been shown previously that pollen-pistil interaction is extremely localized, and one stigma can accept both types of pollen (Goring, 2000). While senescence-associated genes were induced by compatible pollination, these genes were not altered by SI pollination suggesting that, following SI reaction, the stigma is able to keep itself viable to accept compatible pollen (Sankaranarayanan *et al.*, 2013b). In the same study, it was shown that cell survival after SI and CP pollination show an increase in dead papillary cells following CP compared to SI (Sankaranarayanan *et al.*, 2013b). The contradictory results between my study and Iwano *et al.* study, could be due to the difference in the induction of SI (5min vs. 1h) or a simple difference such as the size of papillary cells in canola vs *Arabidopsis thaliana*. Iwano *et al*. used SI reconstituted *Arabidopsis thaliana* stigma, which are relatively very small compared to canola stigmas that were used in my study. In the smaller stigmas of *Arabidopsis thaliana*, a localized response may not be possible while the larger papillary cells of canola stigma can allow compartmentalization or localization of SI response.

I also observed that PLD1 expression itself was also able to go through another wave of SI pollination following an initial SI reaction, suggesting that the stigma can reset after an initial rejection response. These observations raise questions about level of compatibility factor availability in SI stigmas during the pollination phase and if the same resetting of SI can be observed with other compatibility factors down-regulated during SI (Exo70A1 or GLO1). While PLD1 regulation in early pollination has been related to ARC1s ability to control SI by ubiquitination and degradation of PLD1 by the proteasome, the decrease of PLD1 during CP and the increase of PLD1 during SI60 has raised more questions about stigma biology post-pollination and should be investigated in the future.

Chapter 5. Investigation of Functional Conservation of Phospholipase D alpha Family in *Arabidopsis thaliana*

5.1. Introduction

Compared to *Brassica napus* genome which is predicted to contain around 45 phospholipases D genes (NCBI database), the *Arabidopsis thaliana* Phospholipase D family comprises of 12 members (Qin and Wang, 2002a). Despite this, PLD1 has been the only PLD, to be identified as being differentially expressed following SI pollination in canola, and the absence of which resulted in compatibility defects (Chapter 3). Since *Arabidopsis thaliana* is an established model organism, I wanted to investigate if PLD had a conserved function during pollen-pistil interaction in Brassicaceae. Our RNAi-mediated suppression system in canola was incomplete as PLD expression and activity could still be observed in the best RNAi lines (Chapter 3). The T-DNA insertional mutant library available in *Arabidopsis thaliana* and the ability to have knockouts as opposed to knockdowns would allow me to assess the requirement of PLD1 in pollination and also the possibility of functional redundancy in *Arabidopsis thaliana*. Identification of functional conservation of phospholipase D function in *Arabidopsis thaliana* model could lead to parallels with other Brassicaceae species, further increasing our knowledge on PLD function in pollination. There are three PLDα in *Arabidopsis thaliana* (PLDα1, PLDα2 and PLDα3) sharing 94%, 86.7% and 60% of amino acid identity with BnPLD1, respectively (Appendix A, Figure A4). According to microarray data, all three PLDs are expressed in the stigma (Appendix A, Figure A12), but PLDα1 seems to be predominantly expressed in stage 10 and 12 flowers.

To study potential functional conservation between PLD1 function in *Brassica napus* and *Arabidopsis thaliana*, three T-DNA knock-out lines for the 3 PLDα genes were generated (Figure 19). These PLD mutants either alone or in combination were evaluated for pollination defects.

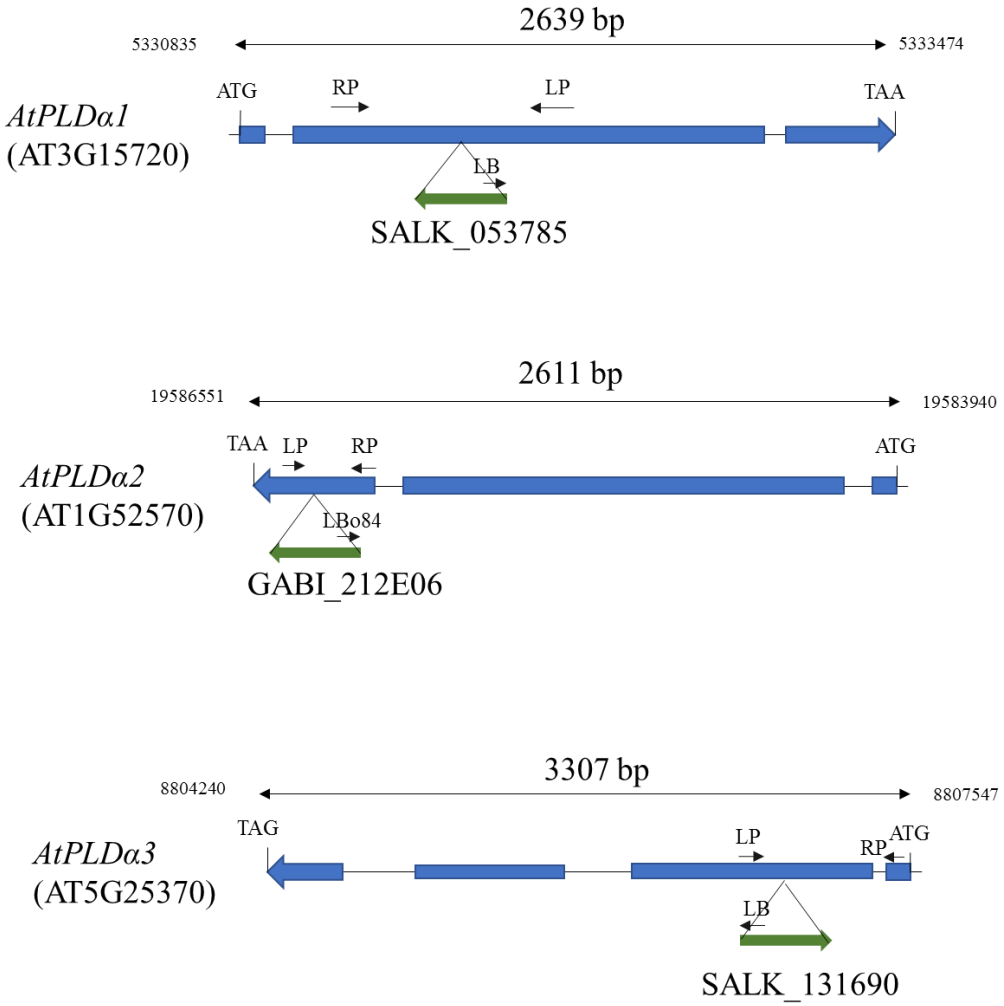


Figure 19: Schematic representation of the T-DNA insertion site in *AtPLDα1, AtPLDα2* and *AtPLDα3.*

Exons are represented by blue boxes. The green boxes represent the T-DNA inserts. LP is left primer, RP, right primer and LB, left border.

5.2. Results

5.2.1. Single *pldα1*, *pldα2*, and *pldα*3 mutants have reduced acceptance of wild-type compatible pollen.

Homozygous, exonic, T-DNA insertional mutants of PLDα1, PLDα2 and PLDα3 (At3g15730, At1g52570 and At5g25370), were obtained from the Arabidopsis Biological Resource Centre and were grown to confirm homozygosity (Appendix A, Figure A13). Homozygous plants were tested for defects in pollination, through aniline blue assays after 3h pollination with wild-type Col-0 pollen (Figure 20A). In all three individual mutants, a significant reduction in pollen attachment was seen although this reduction in pollen attachment did not result in any reduction in seed set (Figure 20B). The single mutants showed only 47% to 50% pollen attachment when compared to the pollen adherence on wild-type stigmas (Figure 20B).

5.2.2. Double knockout mutant *pldα1/2*, *pldα1/3*, *pldα2/3* also display reduced wild-type compatible pollen without any additive effect.

PLD*α* single mutants were crossed together to generate double mutants, *pldα1/2*, *pldα1/3,* and *pldα2/3*. The F3 generation from this cross was screened to isolate homozygous double mutants (Appendix A, Figure A14). These three double mutant lines were examined for pollination defects by pollinating the mutant lines with wild-type Col-0 pollen. There was

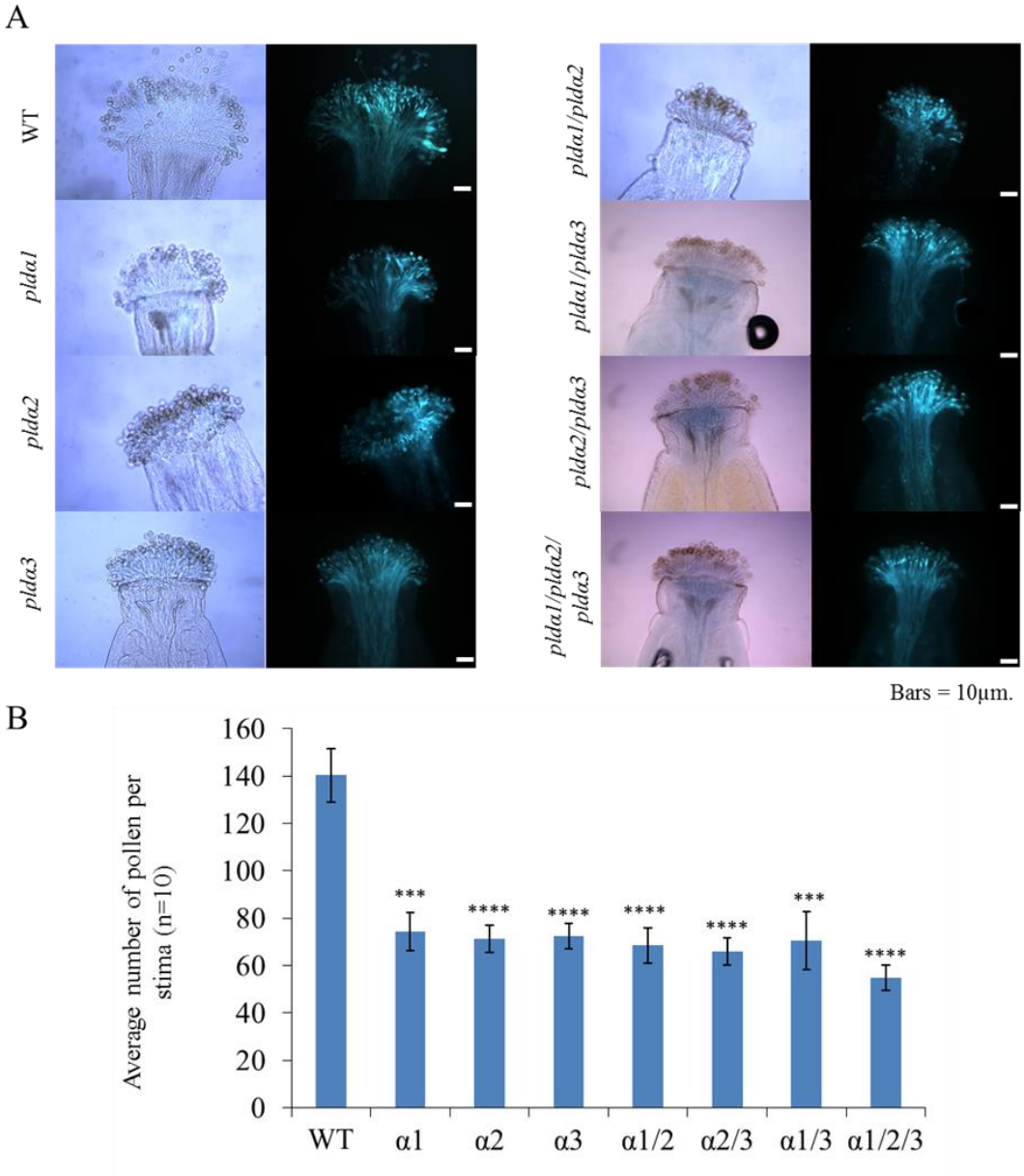


Figure 2023: Pollen grain attachment and pollen tube growth following pollination of pistils from single, double and triple PLD1 alpha family T-DNA mutants with wild-type Col-0 pollen.

(A) Aniline blue treatment of PLD alpha mutant with wild-type Col-0 pollen at 3h post-pollination. All the mutants showed reduced compatible pollen grain adhesion (DIC images) and pollen tube growth (aniline blue images).

(B) Graph showing average number of adhered pollen grains per stigma at 3h post-pollination following aniline blue stain. Each stigma was pollinated with wild-type Col-0 pollen (t-Test, equal variance, ***$p<0.005$, ****$p<0.00005$; n=10). Error bars indicate ± SE.

a significant reduction in the number of pollen attached to the stigma at 3h post-pollination compared to the control, but no differences compared to the single mutants (Figure 20B). These mutants displayed 50% to 53% reduction in the number of pollen compared to the wild-type (Figure 19B). There were no differences between the double mutants observed.

5.2.3. Pollination of *pldα1/pldα2/pldα3* triple knockout mutant does not show differences with the double knockout *pldα* mutants.

The triple mutant *pldα1/pldα2/pldα3* was generated (Appendix A, Figure A15) using the double mutant combinations to examine if there would an additive effect that can reveal functional redundancy. A drastic reduction in the number of pollen on stigma after 3h of pollination was expected in these lines compared to the double mutants, but there was no difference, as the reduction in pollen numbers were similar to the single and double mutants (Figure 20B). The only reduction observed between the triple mutant compared to the wild-type was a 61% reduction in the number of pollen attached and there was a slight 8.5% decrease in the number of pollen compared to the single mutant but this was not significant.

5.3 Discussion

In this study, I have found that all the mutants generated (single, double and triple) showed 50% or more reduction in the number of wild-type pollen attached after 3h of pollination. It was expected an addition of the effects in the double and triple mutants. The lack of additive effects in the double and triple mutants are intriguing and genetically suggest a linear pathway having similar phenotypes across the various classes of mutants. The

observed enhanced reduction of pollen attachment in the triple mutants indicates that all three isoforms are important for this response although they are not absolutely essential for successful pollination. When further investigating the *Arabidopsis thaliana* phospholipase D isoforms, PLDα1, α2, and α3 are the most related *A. thaliana* phospholipase D isoforms to PLD1 from *B. napus*. All the PLDαs share the same regulation domains except the putative DRY motif interacting with the heterotrimeric G protein subunit Gα is only present in PLD1 and PLDα1 (Appendix A, Figure A4). Other differences between them include substrate preference, amount of Ca^{2+} required and PIP_2 requirement (Hong *et al.*, 2016). However, it is also important to take into account the possibility that other members of PLD family might be involved in PA production required for pollination. In this scenario, it is likely that PLD mutations allow for an availability of more PC or PE for other PLDs, which could lead to maintenance of PA pool required to maintain compatibility.

One interesting phenotype observed was that the *PLD* mutant stigmas were smaller than wild-type suggesting that PA produced by these PLDs could be essential for stigma development. This may actually help to explain the observed lack of additive phenotypes across the combination mutants. It is plausible that these PLDs are involved in stigma development and the observed pollination defect was conferred because of the developmental defect in these stigmas. This hypothesis could also be challenged as there was also a lack of additive phenotype in the stigma size in the double or triple mutants (Figure 20A). Further characterization of whether PA is required for pollination in *Arabidopsis thaliana* through inhibitors, followed by the assessing the source of these PAs

in the smaller stigmas of *Arabidopsis thaliana* might provide clues as to the requirement of PLD or PA for pollination.

One major difference between canola and *Arabidopsis thaliana* during pollination is how exocytotic process occurs. In *A. thaliana*, the exocyst complex mediates the exocytosis of vesicles while in *B. napus*, multivesicular bodies are exocytosed instead of vesicles (Safavian and Goring, 2013; Goring, 2017). It is possible that PA derived from PLD1 is specific and required for fusion of the larger multivesicular bodies, but may not be involved or required for vesicle trafficking in *Arabidopsis thaliana*. A previous study examining the role of PA produced from PLD1 in mediating MTI (MAMP-triggered immunity) or ETI (Effector-triggered immunity) was very similar to our observations with PLD1 alpha family (Johansson *et al.*, 2014). A high level of redundancy was found across all 12 PLDs mediating MTI and ETI in *Arabidopsis thaliana* when assessed for growth virulent and avirulent pathogens (Johansson *et al.*, 2014). Although, treatment with n-butanol resulted in reduction of HR induction in leaves infected by AvRpm1, none of the single, double or triple *PLD* mutants displayed any defect in HR or resistance toward virulent and avirulent *Pst*DC3000 (Johansson *et al.*, 2014). This suggests that extreme functional redundancy could exist across all members of phospholipase D family in *Arabidopsis thaliana* in producing PA required for MTI and ETI. During ABA signaling, PLDα1 and PLDδ work in cooperation and only the double mutant displayed more ABA insensitivity than their respective single mutants (Uraji *et al.*, 2012). In this regard, it is also likely that different classes of PLDs could interact with PLD alpha (1-3) to mediate pollination or stigma development.

Chapter 6. Summary and Perspectives

6.1 General Discussion

Pollination in Brassicaceae is a complex and selective process, which has the ultimate goal to ensure successful fertilization of the ovules that ultimately leads to the development of viable seeds. During this process Brassicaceae species are quite adept at preventing genetically similar mates from developing on their stigmas, while specifically allowing cross pollen to germinate, develop and fertilize the ovules. This genetic self-incompatibility mechanism has been under intense scrutiny for many decades and many players have been identified and studied, which has led to our understanding of the signaling mechanisms that leads to self-pollen rejection and compatible cross-pollination (Doucet *et al.,* 2016). It is now broadly accepted that in *Brassica napus*, self-pollen recognition sets off a succession of events in the stigma that leads to degradation of compatible factors that prevent self-pollination. Self-pollen is recognized by allele-specific interaction of the pollen secreted S-locus Cys-rich/S-locus protein11 (SCR/SP11) with its cognate receptor, the stigma-encoded S-receptor kinase (SRK) (Stein *et al.*, 1991; Schopfer *et al.*, 1999). This recognition brings the receptor to dimerize and recruits MLPK receptor, and through a phosphorylation cascade (Murase *et al.*, 2004) leads to activation of ARC1 E3 ligase which targets compatibility factors such as GLO1 and Exo70A1 for degradation by the proteasome (Samuel *et al.*, 2009; Sankaranarayanan *et al.*, 2015). Besides this, as a direct or indirect effect of the receptor activation MVBs accumulate in the cytosol or auto-phagocyted in the vacuole (Safavian and Goring, 2013). From a metabolic point of view,

both calcium and methylglyoxal are known to increase in the papillary cells following receptor activation (Iwano *et al.*, 2015; Sankaranarayanan *et al.*, 2015).

In contrast, we do not have sufficient understanding of the early pollen-papillary cell signals that induces the compatible signaling process. Intracellularly, we know that the favorable pollination signals converge on a) the exocyst complex which tethers vesicles to the membrane, b) allows secretion of MVBs c) increase in GLO1 levels that detoxifies MG, a by-product of glycolysis (Samuel *et al.*, 2009; Safavian and Goring, 2013; Sankaranarayanan *et al.*, 2015). There a number of other compatibility proteins identified, but these are few proteins/processes that are directly regulated by the SI system. In *Arabidopsis thaliana*, calcium spikes during pollination have been observed and autoinhibited $Ca^{(2+)}$-ATPase13 (ACA13) is proposed to be a pump for calcium export to the pollen (Iwano *et al.*, 2004). PCP-B, a pollen coat protein has been proposed to the pollen component that triggers stigma response (Wang *et al.*, 2017), but no stigmatic receptor for this protein has been discovered.

6.2. PLD1 is Required for Successful Pollination and is Targeted by the Self-Incompatibility Response in *Brassica napus*

Based on my studies with PLD1 function as a compatibility factor and a target of SI response, we can add PLD1 to the list of compatibility factors that are required for pollination and are direct targets of SI in *Brassica napus*. My research has unveiled the role of PLD1 as crucial protein required for early pollination processes such as pollen adhesion and pollen tube germination. Suppression of PLD1 in the stigmas correlated with a loss of

pollen adhesion and pollen tube growth which are comparable to the pollen rejection response observed during self-incompatible response. The most direct evidence for PA produced by PLD1 was responsible for these phenotypes came from the fact that external PA addition was able to complement and rescue the pollination defects observed in the *PLD1* RNAi lines and the W1 self-incompatible line, as well as in the Westar compatible lines treated with PLD1 inhibitor (Chapter 3). At the cellular level, PLD1 overexpression improved membrane trafficking following SI pollination likely enhancing MVBs delivery at the contact point of the pollen and the stigma. Interestingly, constitutive activation of this response was absent as increased trafficking was observed only after pollination, suggesting that PLD1 activity could be induced by other upstream signals. Calcium has been proposed as one of the upstream second messengers which is known to increase following pollination and also known to be a direct activator of PLD1 (Qin *et al.*, 1997). In this regard, combining previous studies, we can also hypothesize that PLD1 activation could also lead to breaking down of microtubules (Samuel et al., 2011) which are required for pollination response to occur as there is precedence of PLD1 generated PA causing breakdown of polymerized MTs (Zhang *et al.*, 2012).

PLD1 protein levels were found to fluctuate during different time points after pollination, including downregulation after SI pollination. ARC1 could be still the principal E3 ligase that targets PLD1 for proteasomal degradation as PLD1 was found to accumulate in *ARC1* suppressed lines or when the proteasome was inhibited by MG132 (Chapter 4). Interestingly, regulation of PLD1 other than SI have been highlighted in my thesis. First,

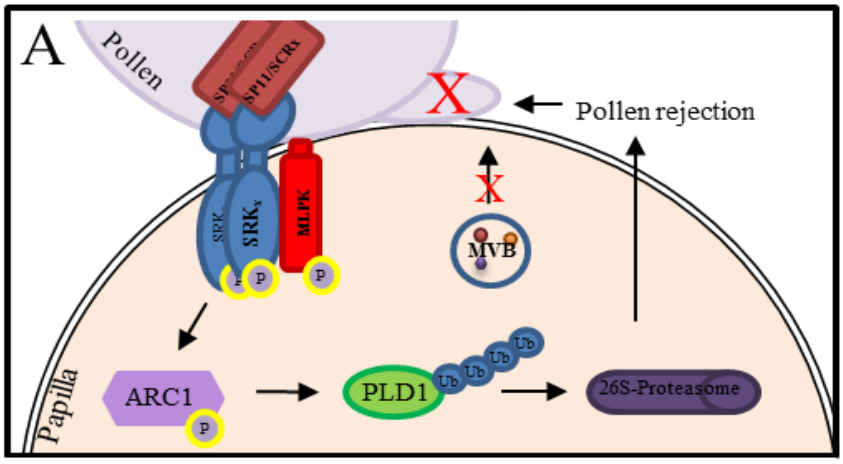
PLD1 level was found to increase after 1h of SI pollination, a phenomenon that could be coupled with a general mechanism of resetting of the stigma after an initial wave of SI response has progressed through which, the pool of proteins down-regulated during SI pollination could be replenished to allow the stigma to stay receptive for the right mate. Secondly, in a contrasting fashion, PLD1 levels decreased 1h after successful pollination, suggesting that after promoting successful compatible pollination events, it could be downregulated concomitant with the initiation of the stigma senescence response that has been previously observed with upregulation of senescence-associated genes as early as 1h after successful pollination (Sankaranarayanan *et al.*, 2013b).

In *Arabidopsis thaliana*, characterization of the various single and combinatorial phospholipase D α mutants during pollination have not allowed to discern the requirement of PLDα activity for successful pollination. The extreme functional redundancy reported with the various PLD classes could be responsible for this lack of a strong phenotype (Johannson et al., 2014). The knockout screens revealed that the three PLD1-alphas could contribute to pollination but are not essential for this response to occur (Chapter 5).

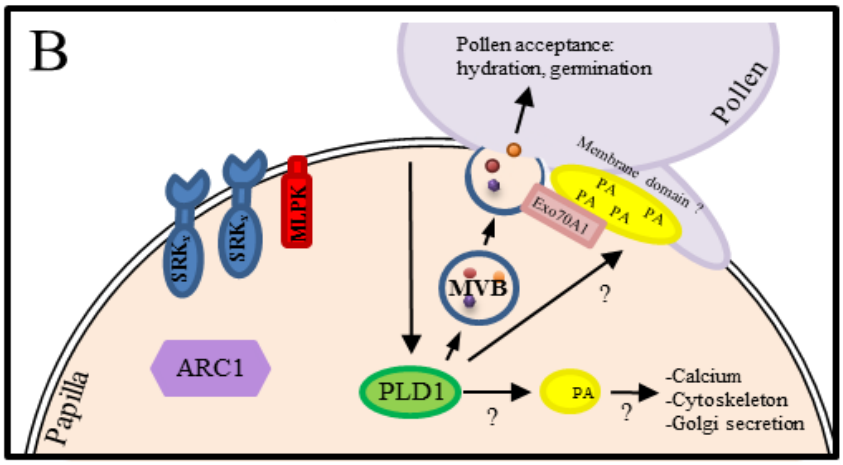
6.3. Proposed Model for PLD1 Contribution during Pollen-Pistil Interaction in *Brassica napus*

The various observations described in this thesis have allowed me to come up with a model showing the role of PLD1 during pollination in *Brassica napus* (Figure 21). Accumulation of PLD1 during SI in *arc1* and MG132 treated stigmas demonstrate that PLD1 is regulated by ARC1-mediated degradation. The breakdown of SI response in PLD1 overexpressing

lines also provided direct evidence that PLD1 is a target of SI. Following SRK activation, ARC1 can ubiquitinate PLD1 and target it to the proteasome for degradation (Figure 21A). This would arrest MVB exocytosis because reduction of PA from PLD1 activity in either n-butanol inhibited stigmas or *PLD1* RNAi lines result in a loss of pollen adhesion and pollen tube germination (Chapter 3). During a compatible response, PLD1 activity could be induced causing to stimulate MVBs secretions (Figure 21B). Evidence for this came from the observation that increased MVBs were found fusing with the membrane in the PLD1 overexpressing lines in W1 background. The exact nature of the signals that can converge on PLD1 are not known at this point. More importantly, elucidating the nature of the cargo delivered by these MVBs would provide us clues as to what factors (proteins or metabolites) are essential for pollen hydration and germination to occur.



Self-Incompatible Response



Compatible Response

Figure 21: Proposed model for PLD1 role in stigmatic papillae following pollination.

(A) Landing of self-pollen on the stigma leads to SP11/SCR-SRK interaction at the papillary cell plasma membrane. Activation of SRK and MLPK results in downstream interaction and activation of ARC1 by phosphorylation. Activated ARC1 can ubiquitinate PLD1 for degradation by the proteasome causing an inhibition of exocytosis of MVBs thereby rejecting the self-pollen

(B) During compatible pollination, upstream signals activate PLD1, which then promotes exocytosis of MVBs likely through facilitating their fusion at the membrane. PLD1 can convert phospholipids (PC, PE…) to phosphatidic acid (PA) that could facilitate Exo70A1-mediates MVBs tethering to the membrane at the pollen-stigma contact point.

6.4. Conclusions and Future Perspectives

Given the pleiotropic nature of PA, it would not be surprising to find PA interacting with several identified pollination factors. In *Arabidopsis thaliana*, proteins can bind directly to PA produced by PLDα1 at the plasma membrane and get activated by it, like the NADPH oxidases (RbohD and F) during ABA-mediated stomatal closure, binding to specific PA species (Zhang *et al.*, 2009). In this regard, it is possible that PA produced by PLD1 are used as anchorage to Exo70A1 or members of the exocyst complex to facilitate the tethering process. In *A. thaliana*, there is evidence showing that PLDα1 is recruited by the ESCRT-III (Endosomal Sorting Complex Required for Transport) by binding to one of its component, AtVPS2 (Vacuolar Sorting Protein) (Ibl *et al.*, 2012). However, whether PLD1 is directly responsible for the activation of MVBs secretion remains to be elucidated. *Exo70A1* RNAi *Brassica* lines accumulated MVBs inside the papillary cell following compatible pollination suggesting a relationship between the exocyst system and MVBs secretion (Safavian and Goring, 2013) and Exo70A1 is known to be a major target for ARC1 (Samuel *et al.*, 2009).

Alternatively, PLD1 could play a critical role in Exo70A1 docking at the membrane or could produce PA required for fusion of the vesicles. Exo70A1 is a member of the exocyst complex, responsible for the docking of exocytic vesicles on the plasma membrane with fusion sites during polarized or regulated secretion (Zarsky *et al.*, 2013). Furthermore, PA is a phospholipid with a conical shape able to induce negative curve of the cytosolic leaflet of the plasma membrane making it a perfect candidate involved in membrane modeling (Roth, 2008). In mammals, PLD1 and PLD2 have been extensively studied during

exocytotic events (Ammar *et al.*, 2013a) revealing accumulation of PA at the membrane when exocytosis is stimulated and binding with soluble N-Ethylmaleimide-sensitive Factor (NSF) Attachment Protein Receptors (SNAREs) proteins in chromaffin cells (Zeniou-Meyer *et al.*, 2007). PAs are also known to bind with several well-known secretion regulators such as GTPases and syntaxins (Jang *et al.*, 2012). Others possible interactors of PLD1 or PA during pollen-pistil interaction could be cytoskeleton elements since MAP65-1 (microtubules associated protein) can bind to PA (Pleskot *et al.*, 2013) and microtubules reorganization is a critical event during canola pollination (Samuel *et al.*, 2011). Since annexin 1 is a potential compatible factor (Sankaranarayanan *et al.*, 2013a), which can be activated by calcium and can bind to PA, one more of action of PLD1 could be through annexins (Blackwood and Ernst, 1990; Lizarbe *et al.*, 2013). Annexin can bind to PA, attracted by negative charges and can also function as a calcium channel that could correlate with the calcium exchange that occurs between the stigma and the pollen during the compatible pollination (Lizarbe *et al.*, 2013).

Composition of the acyl chains of PA could greatly influence the various targets that it can bind to and it is now possible to identify the composition of the acyl chains of PA. With the differences in chains often linked to a different target (Rainteau *et al.*, 2012), determination of the lipid profile following pollination could allow us to have a thorough understanding of the lipids metabolome and provide us clues as to which lipid interactions could be occurring during pollination. This could lead to revealing a new mechanism involved in pollen-pistil interactions.

Co-localization, binding assays, are some of the future experiments that would be necessary to identify the precise the place of PLD1-PA in the pollen-interaction puzzle. Further efforts should be dedicated to identifying PLD1 and PA interactors involved during pollination and how PLD1 enzymatic activity is induced and identify whether PLD1 translocation or accumulation at the papillary cell plasma membrane occurs at the contact point with the pollen. On a larger theme, future studies should be conducted to, identify ARC1 relationship with autophagosome formation following SI, characterize other compatibility factors including the stigmatic receptor of compatibility and identify the cargo in the MVBs. These would tremendously improve our understanding of the self-incompatibility and compatibility response during pollen-pistil interactions in plants.

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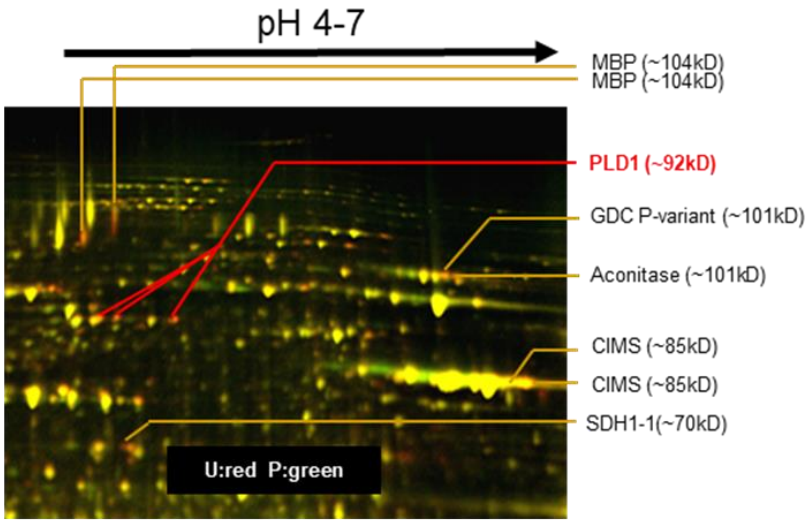
Appendix A: Supporting Figures



Protein ID	Ratio Fold change relative to unpollinated (P/U)	T-test
PLD1	-1.94	0.027
PLD1	-1.61	0.0005
PLD1	-1.57	0.026

Figure A1: PLD1 identification as a down-regulated protein after 60 min of SI pollination in *Brassica napus* stigmas.

2D-DIGE analysis of stigma protein after 60min SI (U: Un-pollinated, P: 60min after self-incompatible pollination).

Brassica napus cultivar Westar phospholipase D1 mRNA, partial cds

GenBank: KJ755984.1

FASTA Graphics Item in clipboard

Go to:

LOCUS KJ755984 2430 bp mRNA linear PLN 15-JUL-2014
DEFINITION Brassica napus cultivar Westar phospholipase D1 mRNA, partial cds.
ACCESSION KJ755984
VERSION KJ755984.1
KEYWORDS .
SOURCE Brassica napus (rape)
ORGANISM Brassica napus
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae;
Pentapetalae; rosids; malvids; Brassicales; Brassicaceae;
Brassiceae; Brassica.
REFERENCE 1 (bases 1 to 2430)
AUTHORS Scandola,S. and Samuel,M.
TITLE Direct Submission
JOURNAL Submitted (28-APR-2014) Biological Sciences, University of Calgary,
2500 University Drive N.W., Calgary, Alberta T2N1N4, Canada
COMMENT ##Assembly-Data-START##
Sequencing Technology: Sanger dideoxy sequencing
##Assembly-Data-END##

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>KJ755984.1 *Brassica napus* cultivar Westar phospholipase D1 mRNA, partial cds

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Figure A2: GenBank submission and identification of *Brassica napus* cultivar Westar phospholipase D1 mRNA, partial cds (PLD1).

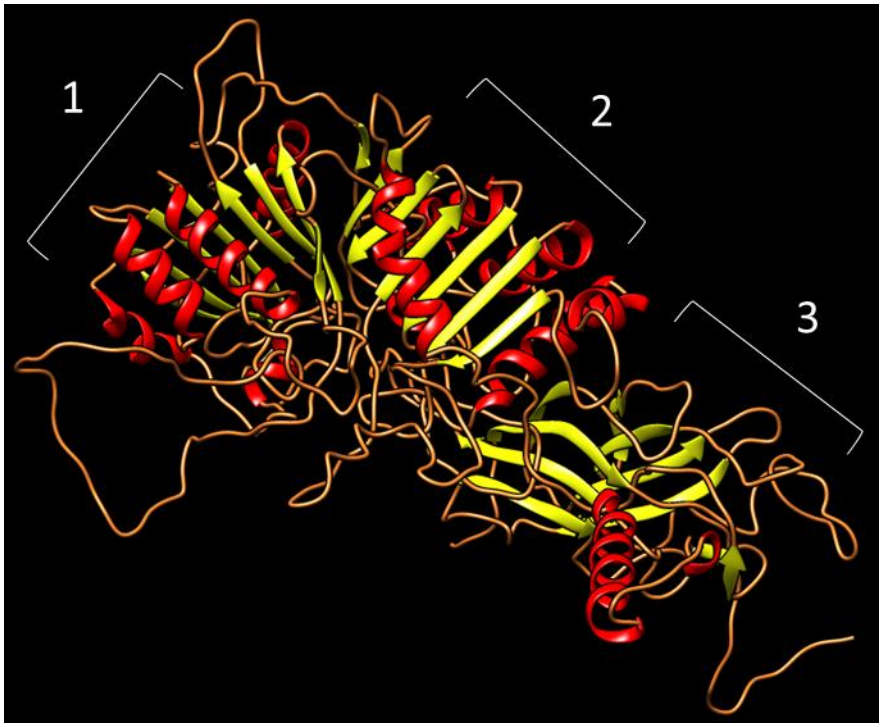

Figure A3: PLD1 prediction structure by RaptorX (Visualisation Chimera).

The PLD1 polypeptide chain is folded into 3 domains. The active site is formed at the interface between the first two domains, that come together to reunite the HKD domains. Molecular graphics and analyses were performed with the UCSF Chimera package. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311) (Pettersen et al., 2004).

Figure A4: PLD1 alignment.

BnPLD1 (accession number: KJ755984.1) is aligned with AtPLDα1 (accession number: BAB02304.1), AtPLDα2 (accession number: AAD55607), AtPLDα3 (NP_197919) and OsPLDα1 (accession number: Q43007). Different conserved domains are shown highlighted. The C2 domain is shown in purple and the two HKD domain are shown in green. Putative PIP_2 domain is highlighted in blue and the putative DRY motif in yellow.

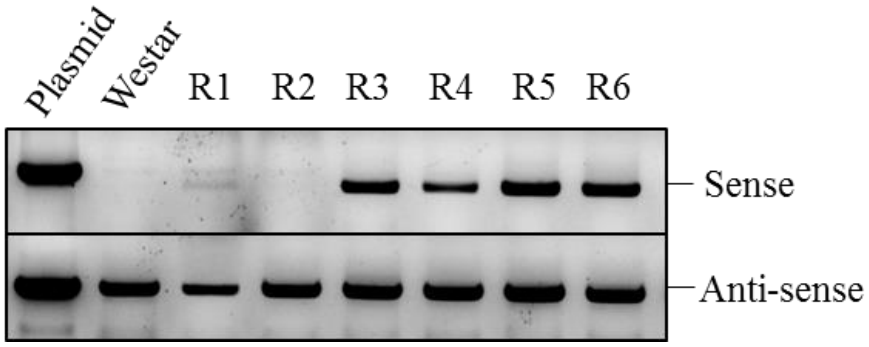

Figure A5: Genotyping of transformed *Brassica napus* Westar lines for the presence of RNAi transgene by PCR.

Lines R1 and R3 to R6 shows amplification for the sense and anti-sense version of *PLD1* DNA sequence.

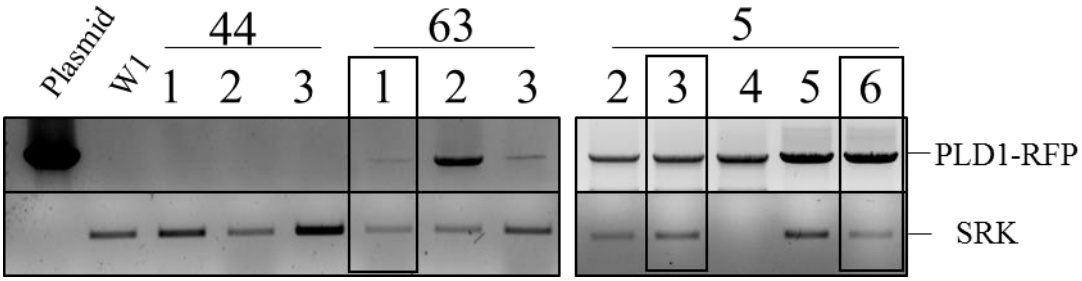

Figure A6: Genotyping of *PLD1* overexpressor lines in *Brassica napus* W1 background by PCR.

PLD1-RFP construct was detected along with the presence of SRK.

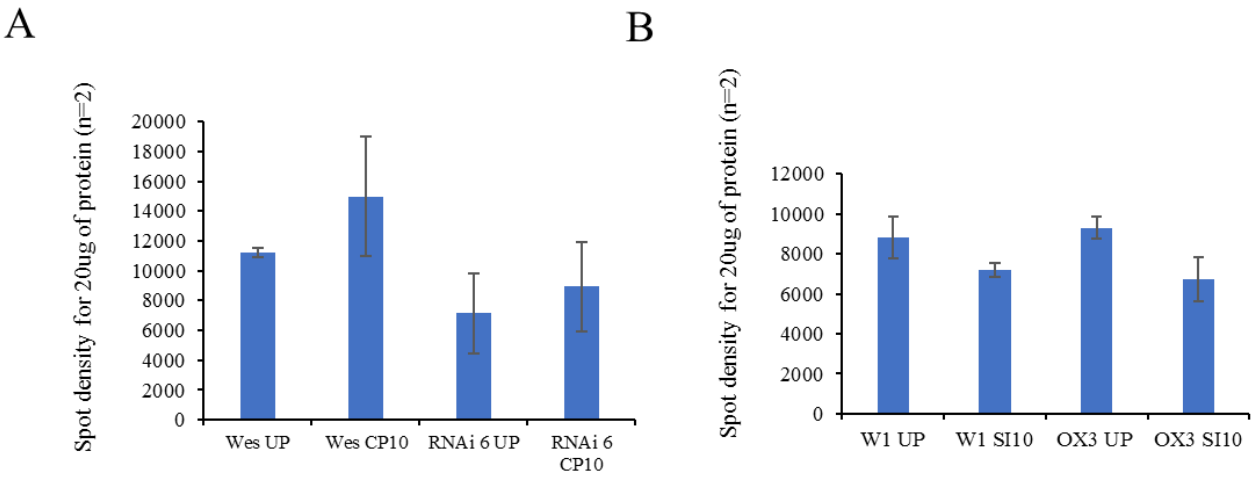

Figure A7: PLD activity assay.

(A) PLD activity in Westar and RNAi 6 line at unpollinated (UP) stage and 10 min pollination (CP10) with Westar pollen.

(B) PLD activity in W1 line and PLOX 3 line (OX3) at UP stage and 10 min after self-pollination (SI10).

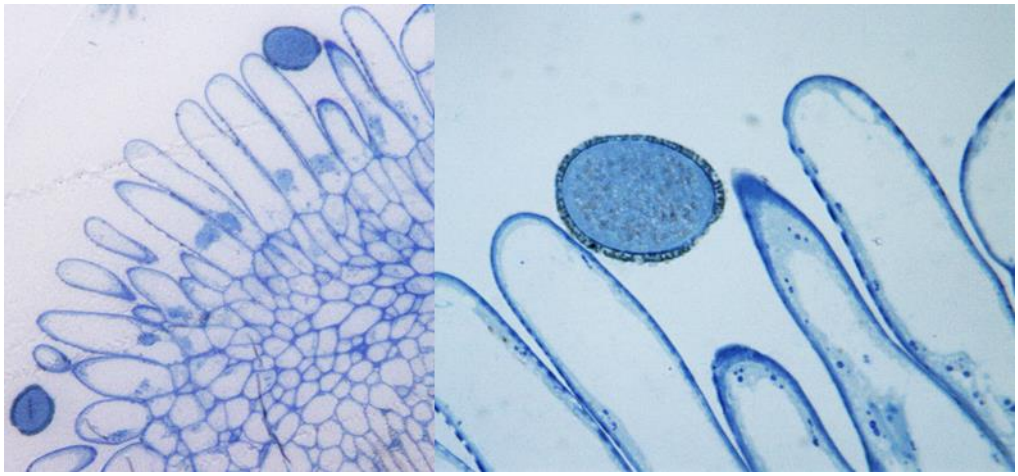

Figure A8: Preview of Westar *Brassica napus* stigmatic papilla in contact with a compatible pollen grain visualized by staining with toluidine blue.

Sections of 60 µm thickness from the embedded pistil were observed to check the area of interest before ultrathin sections.

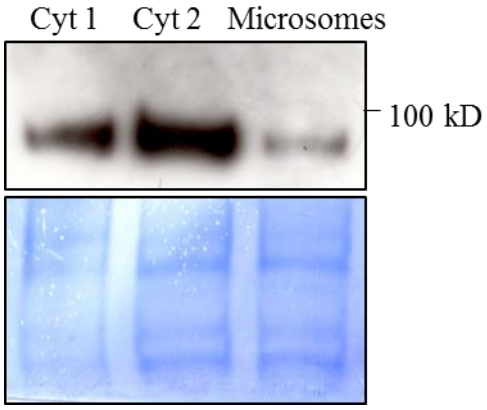

Figure A9: PLD1 detection in microsomal and cytosolic fractions.

PLD1 localization in cytosol (Cyt 1 and Cyt 2) and microsomal fraction were assessed by Western blot with anti-PLD1 antibodies.

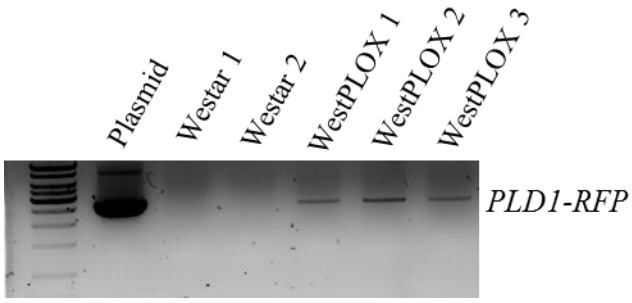

Figure A10: Genotyping of transformed *Brassica napus* Westar lines by PCR for the presence of over-expressor construct *PLD1-RFP* under *SLR1* promoter.

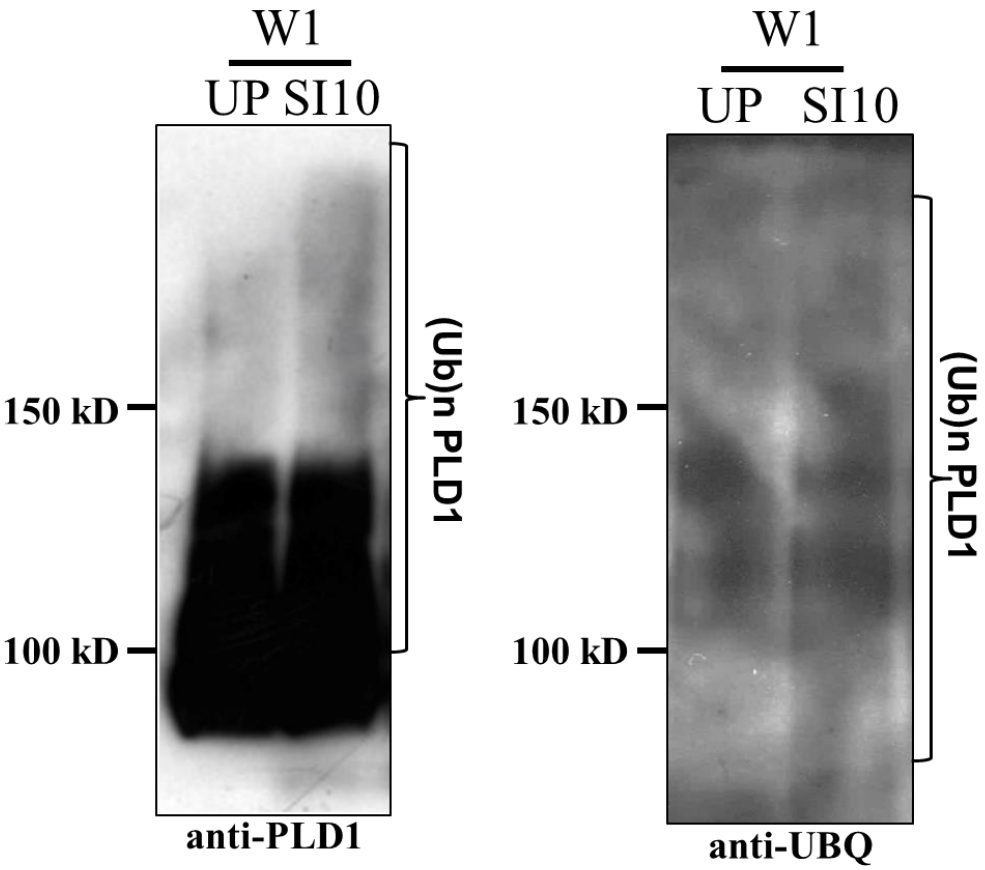

Figure A11: Endogenous PLD1was immunoprecipitated from either unpollinated stigmas (UP) or SI 10 min pollinated stigmas followed by Western blotting with either anti-PLD1 or anti-Ubiquitin antibodies.

	Stage 10	Stage 12
PLDalpha1	718	941.75
PLDalpha2	148.5	177
PLDalpha3	110.5	150

Figure A12: Microarrays analysis showing *PLDα1, 2* and *3* mRNA expression levels (raw intensities) in *Arabidopsis thaliana* stigma during different stages (Jamshed and Samuel unpublished).

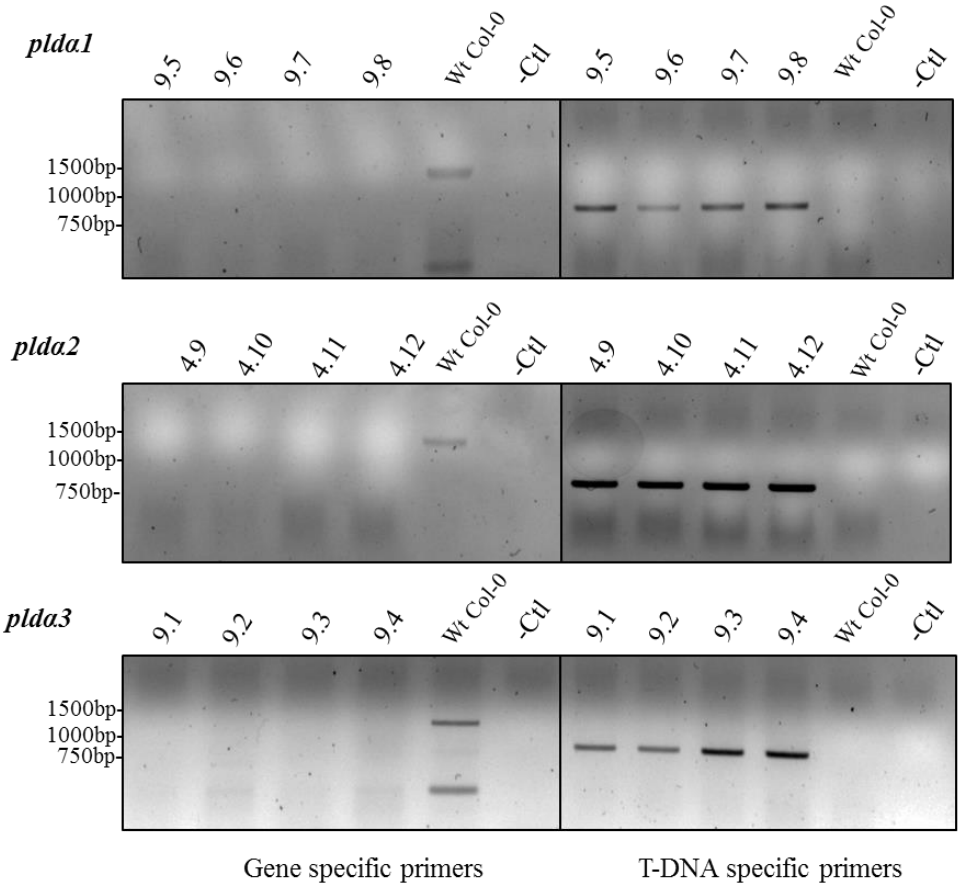

Figure A13: Genotyping of single *pldα* mutant lines by PCR.

Each homozygote is characterized by the absence of an amplicon when using gene specific primers and by the presence of an amplicon when using T-DNA specific primers. In the negative, control DNA is replaced by water.

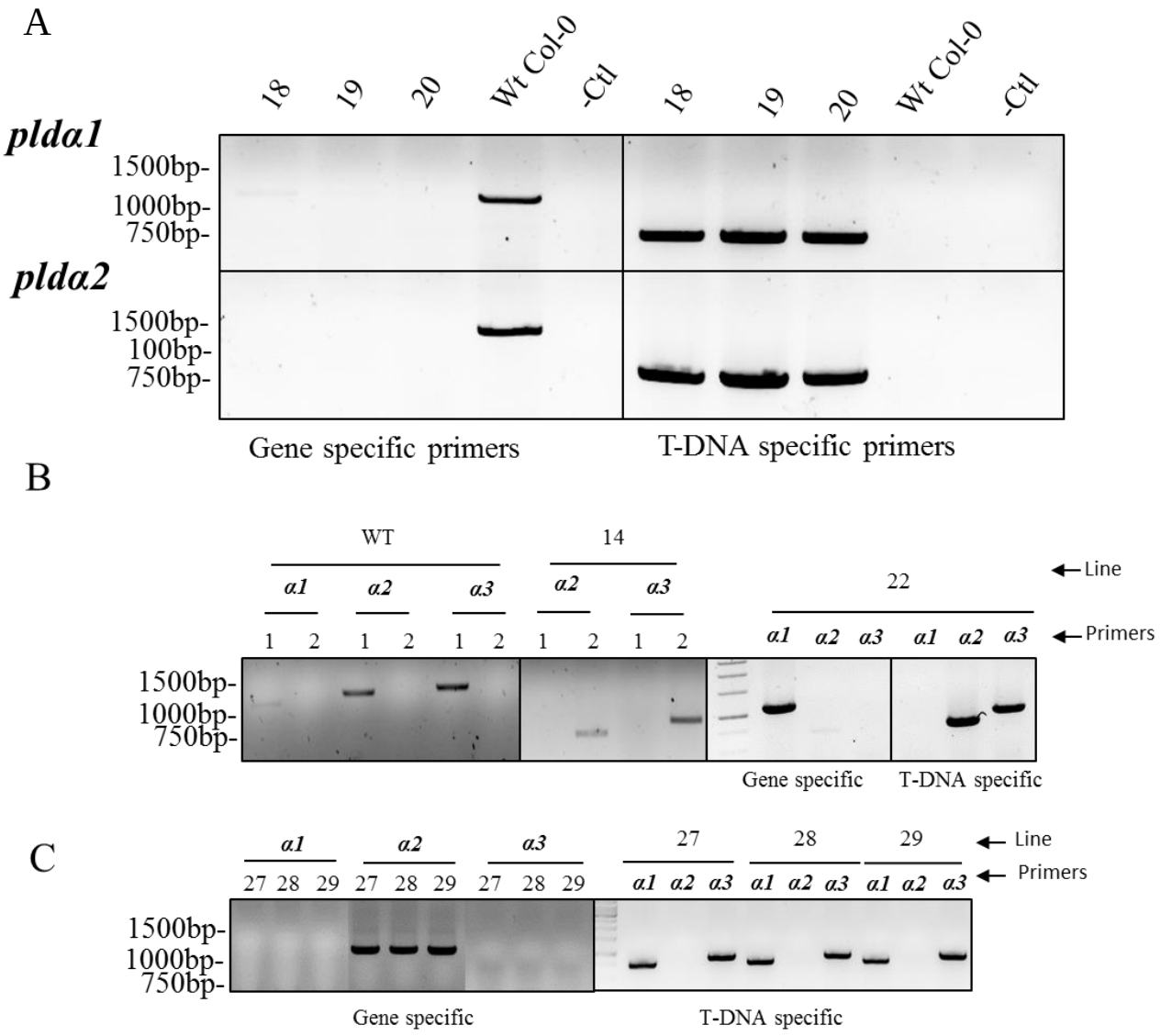

Figure A14: Genotyping of double *pldα* mutant lines by PCR.

(A) *pldα1/2* mutants genotyping (line 18, 19 and 20);
(B) *pldα2/3* mutants genotyping (line 14 and 22);
(C) *pldα1/3* mutants genotyping (line 27,28 and 29);
(1) Gene specific primers. (2) T-DNA Specific primers.

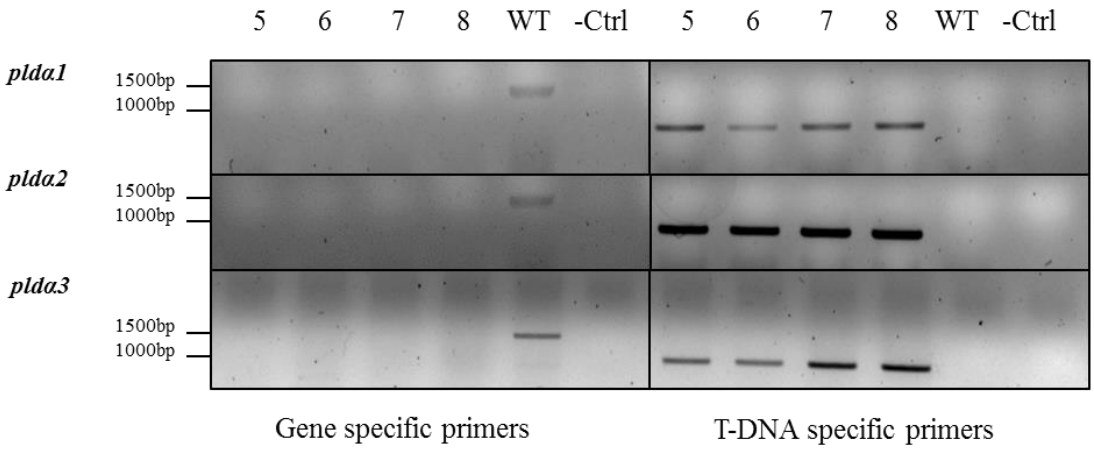

Figure A15: Genotyping of triple *pldα1/2/3* mutants genotyping (line 5, 6, 7 and 8).

Each homozygote is characterized by the absence of amplicon in presence of gene-specific primers and by the presence of an amplicon with T-DNA specific primers.

Appendix B: Additional Tables

Table B1: Complete list of primers used during this thesis.

PLDa1SALK_053785.56.0 LP	ATTAAGTGCAGGGCATTGATG
PLDa1SALK_053785.56.0 RP	CAAGGCTGCAAAGTTTCTCTG
plda3 SALK_130690 RP	ATGCACAAGTTTGTTTGGAGG
plda3 SALK_130690 LP	GAGCTAACGACACTTGGTTCG
LBa1 of pBIN-pROK2 for SALK lines	TGGTTCACGTAGTGGGCCATCG
LBb1.3	ATTTTGCCGATTTCGGAAC
PLD2GABI_212E06RP	AGCCGAATGAAAAACCAAAAC
PLD2GABI_212E06LP	GATTTCCTGACTCCCCTGAAG
PLD2GABI_212E06LBo84	ATATTGACCATCATACTCATTGC
SLR FOR	TAATGAGTGGCTGGAAAGTCA
SLR REV	CGCAAGACCGGCAACAGGATT
pGEX-FOR	GGGCTGGCAAGCCACGTTTGGTG
pGEX-REV	CCGGGAGCTGCATGTGTCAGAGG
SRK910-FOR	CCTACGATAGTTCTTACACT
SRK910-REV	CCATGATGTCGGAGTGAACGTT
RFP-int-FOR	CCACCTACATGGCCAAGAAGC
RFP-int-REV	GATCTCGAACTCGTGGCCGTT
P1 SLRinfFor-dSma-PLD1For	CTCTAGAGGATCcccATGGTACGAGTCCTTTCA
P2 EcoR1-Int-PLD1-Rev	GCgaattcCTATGAGCTGCAAAAACTACTTACCTCCTTCAACACATCGACGGA
P2’ SLRinfR	TCGAGCTCGGTAcccggggCgaattcCTATGAGCTG
P4 SLRinfR-PLD1 For	TCGAGCTCGGTAcccgggATGGTACGAGTCCTTTCA
P5 InfINt2PLD1 Rev	ATAGgaattcGCcccgggCTTCAACACATCGACGGA
PLDa1InfFw	ACTCTAGAGGATcccgggGATGGCGCAGCATCTGTTG
PLDa1InfRev	CGGAGGAGGCCATCCCGGTTGTAAG

RFPinfR-PLD1SmaRev	CGGAGGAGGCCATcccgggGGTTGT
SLR For	TAATGAGTGGCTGGAAAGTCA
EcoRIIntPLD1 Rev	GCgaattcCTATGAGCTGCAAAAACTACTTACCTCCTTCAACACATCGACGGA
InfINT2 PLD1 Rev	ATAGgaattcGCcccgggCTTCAACACATCGACGGA
SLR Rev	CGCAAGACCGGCAACAGGATT
PLD1infsmaI For	ACTCTAGAGGATCCCCGGGATGGCG
RFPInt Rev	GATCTCGAACTCGTGGCCGTT
SRK910 For	CCTACGATAGTTCTTACACT
SRK910 Rev	CCATGATGTCGGAGTGAACGTT

Appendix C: List of Publications

Peer Reviewed

Sankaranarayanan, S., Jamshed, M., Kumar, A., Skori, L., **Scandola, S**., Wang, T., Spiegel, D., Samuel, M.A. (2017). Glyoxalase Goes Green: The Expanding Roles of Glyoxalase in Plants. ***International Journal of Molecular Sciences***, *18*(4), 898. doi:10.3390/ijms18040898.

Conference Communication

1. **Scandola, S.**, and Samuel, M.A. 2017. Understanding role of Phospholipase D1 during pollen-pistil interactions in canola. **Plants from Sea to Sky: The Canadian Society of Plant Biologists and the Canadian Society for Horticultural Science joint meeting. Vancouver (British Columbia). Canada. Minisymposium talk.**

2. **Scandola, S.**, and Samuel, M.A. 2017. Imaging pollen-pistil interactions to understand the Phospholipase D1 role during compatible and self-incompatible pollination in *Brassica napus*. **Visualizing Science – An Integrative Cell Biology Symposium. Calgary (Alberta). Canada. Talk.**

3. **Scandola, S.**, and Samuel, M.A. 2014. Investigating the Role of Phospholipase D During Compatible and Self-Incompatible Pollination in *Brassica napus.* **Annual Meeting of The American Society of Plant Biologists and the Canadian Society of Plant Biologists. Portland (Oregon). USA. Poster.**

Appendix D: Permissions and Copyrights

D1. American Chemical Society – Journal of Proteome Research (Figure 3) (Sankaranarayanan et al., 2013). **Permission attached.**

D2. John Wiley and Sons - International Union of Biochemistry and Molecular Biology Life (Figure 7) (Cazolli et al., 2006). **Permission attached.**

Title: Proteomics Approaches Advance Our Understanding of Plant Self-Incompatibility Response

Author: Subramanian Sankaranarayanan, Muhammad Jamshed, Marcus A. Samuel

Publication: Journal of Proteome Research

Publisher: American Chemical Society

Date: Nov 1, 2013

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