Report

Current Biology

A Flower-Specific Phospholipase D Is a Stigmatic Compatibility Factor Targeted by the Self-Incompatibility Response in *Brassica napus*

Highlights

- PLDα1 is a flower specific phospholipase D enzyme involved in pollination
- Suppression of PLDα1 results in reduced pollen germination and pollen tube growth
- PLDα1 is ubiquitinated by ARC1 and targeted for degradation during SI response
- PLDα1 overexpression in incompatible stigmas can break down SI response

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In Brief

To avoid inbreeding, most plants use self-incompatibility to reject self-pollen and promote cross pollination to generate genetic diversity and hybrid vigor. Scandola and Samuel identify phospholipase D α1 as a compatibility factor that is required for early pollination events and is targeted by the ARC1-mediated self-incompatibility response.

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A Flower-Specific Phospholipase D Is a Stigmatic Compatibility Factor Targeted by the Self-Incompatibility Response in *Brassica napus*

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SUMMARY

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 *Brassica*, SI is initiated by the allele-specific recognition of pollen-encoded, secreted ligand (SCR/SP11) by the stigmatic receptor kinase S-locus receptor kinase (SRK), resulting in activation of SRK through phosphorylation [1–3]. 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 [4, 5]. Through proteomics approach using proteins from SI activated stigmas of *Brassica napus*, we identified phospholipase D α1 (PLDα1) as one of the candidates that is most likely targeted for degradation after SI [6]. PLDα1 is enriched in the stigmas and functions as a stigmatic compatibility factor as loss of PLDα1 compromised compatible pollination, while overexpression of PLDα1 in self-incompatible stigmas led to breakdown of SI response. PLDα1 can be ubiquitinated by ARC1 and accumulate in *ARC1*-suppressed lines, confirming PLDα1 as a target of ARC1 during SI response. Addition of phosphatidic acid (PA) to PLDα1-deficient stigmas was sufficient to rescue compatibility, suggesting an essential role for PA generated by PLDα1 for compatible interactions. We propose that PA produced by PLDα1 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 PLDα1 for degradation.

RESULTS

Most hermaphroditic flowering plants have evolved various mechanisms to avoid self-pollination in order to prevent inbreeding depression from occurring. One of these is the self-incompatibility (SI) response, a genetic mechanism that rejects self- or genetically similar (incompatible) pollen, while accepting non-self- or cross- (compatible) pollen to promote genetic diversity and to improve fitness. During SI response, function of ARC1 is central to mediating rejection, as loss of ARC1 can compromise SI [5]. Phosphorylated ARC1 can interact with and target compatibility factors such as EXO70A1 and GLO1 for proteasomal degradation, thus causing rejection of self-pollen [5, 7, 8]. EXO70A1 is part of the octameric exocyst complex that mediates exocytosis of vesicles targeted for delivery at the membrane. Absence of EXO70A1 in stigmas compromises compatible pollinations, and it has been proposed to facilitate delivery of vesicles carrying cargo necessary for pollen hydration and germination. In spite of this, the nature of signals at the plasma membrane that aid fusion of the exocytotic vesicles in stigmas has remained unknown.

When analyzing the SI-induced degradome (proteins downregulated after SI), we identified phospholipase D α1 (PLDα1; Genbank: KJ755984.1) as one of the potential candidate targets to be downregulated by the SI response [6]. PLDα1 can hydrolyze the polar head from phospholipids, principally phosphatidylcholine, to release phosphatidic acid (PA), a major signaling messenger involved in membrane modeling and vesicle fusion [9, 10]. In plants, PLDα1 and its homologs contain a C2 domain (Ca^{2+}-dependent phospholipid binding), which enhances PLDα1 binding to phospholipids [11]. There are two HKD domains that form the catalytic site, a phosphoInositide (PI) binding site and a DRY motif that allows the binding of G proteins [11]. In plants, PLDs are involved in a wide range of processes, including drought stress, where PA from PLD activity has been shown to bind to Rboh, an NADPH (reduced nicotinamide adenine dinucleotide phosphate) oxidase to control stomatal aperture via the production of reactive oxygen species [12].

PLDα1 Is Essential for Compatible Pollination

Proteomic analyses indicated that PLDα1 is downregulated after SI response and thus could function as a compatibility factor and a target of SI during SI pollination. When spatial regulation of PLDα1 expression was examined, PLDα1 was found to be specific to floral tissues, with strong expression in the stigma and petals, while weak expression was observed in the sepals and was absent in anther, pollen, and leaf tissues (Figure 1A). Stigmas from stage-10 to stage-12 flowers, which are stages prior to anthesis and are known to progressively show higher expression of factors required for both compatible and

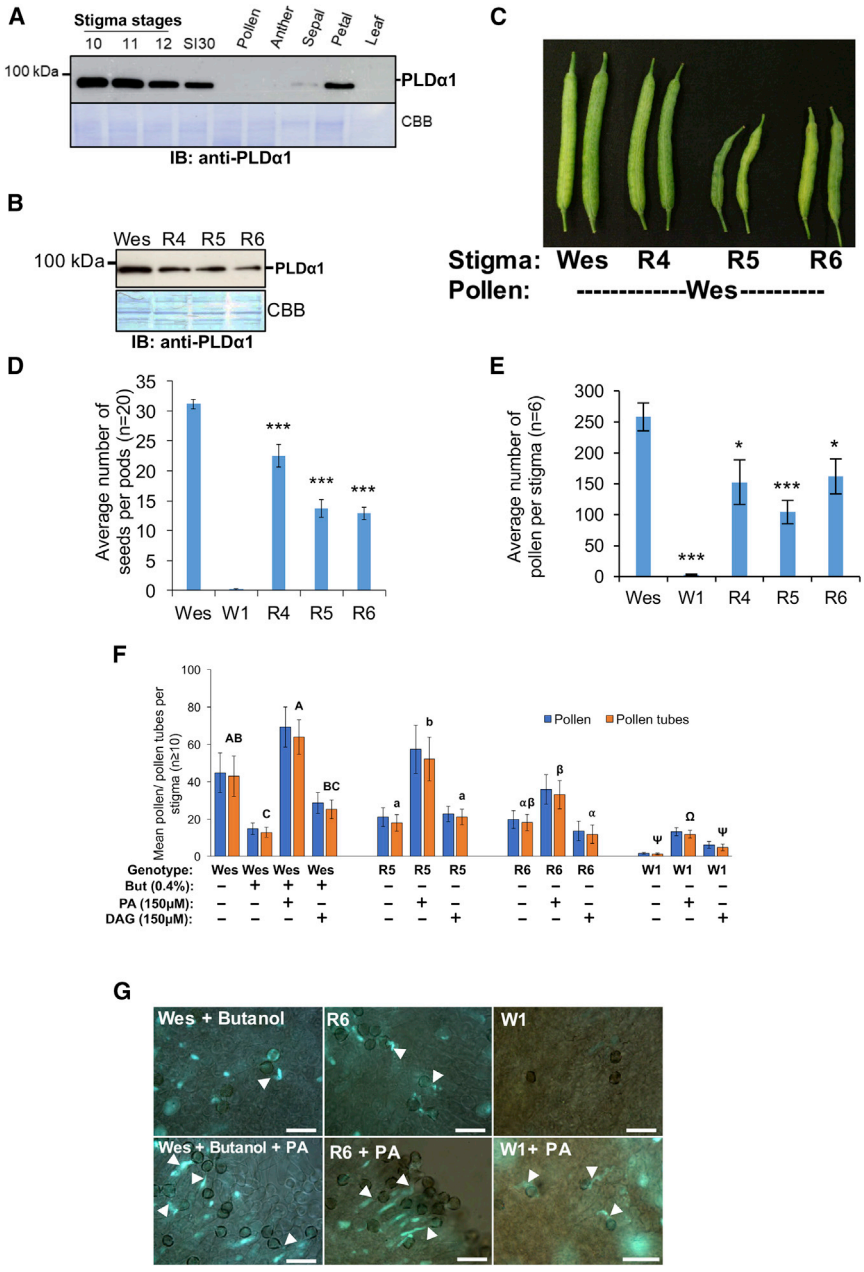

Figure 1. PLDα1 Is Required for Compatible Pollination

(A) Western blot analysis showing expression of PLDα1 in various canola floral structures and leaves. PLDα1 is enriched in stigmas during different stages of flower development before anthesis (stages 10, 11, and 12; SI30, expression on PLDα1 in stage-12 stigmas after 30 min of SI pollination).

(B) Western blot showing suppression of PLDα1 in *PLDα1-RNAi* lines.

(C) Pod phenotype in *PLDα1-RNAi* lines indicating a decrease in pod size in *PLDα1*-suppressed lines.

(D) Graph showing average seeds per pod following compatible pollination in Westar and RNAi lines along with self-pollinated W1 (t test, ***$p < 0.0005$; $n = 20$). Error bars indicate ±SEM.

(E) Graph showing average number of pollen attached per stigma after compatible pollination in Westar and RNAi lines along with self-pollinated W1 (t test, *$p < 0.05$, ***$p < 0.0005$; $n = 6$). Error bars indicate ±SEM.

(F) Phosphatidic acid (PA) is able to rescue pollination defect in RNAi lines and can also break down SI response. The graph shows the average number of pollen and pollen tubes per stigma either left untreated or treated with 1-butanol and/or PA or DAG followed by either compatible pollination in Westar and *PLDα1-RNAi* (R5 and R6) lines or with self-incompatible pollination on self-incompatible W1 stigmas. Error bars indicate ±SEM. Different letters over the bars indicate significant differences based on Tukey's multiple comparison analyses of the number of pollen tubes in each treatment within the same genetic background ($p < 0.05$; $n \geq 10$).

(G) Images showing pollen attachment and pollen tube growth on stigmas (indicated with white arrows) described in (F). Scale bars, 100 μm.

See also Figures S1 and S2.

incompatible response, displayed very strong expression of PLDα1 (Figure 1A).

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 to generate phosphatidyl alcohol instead of a PA [13]. Unlike PA, which can be quickly transformed into diacylglycerol (DAG) or lysoPA, the phosphatidyl alcohols are very stable in cells [14]. This property is convenient for both the elucidation of PLDα1 activity in cells and to assess the essential nature of PA produced by PLDα1 as any PA-dependent programming is blocked downstream. When W1 flowers were incubated overnight with different primary alcohols (methylethanolamine [MEA] or 1-butanol), followed by compatible pollination for 6 h, a significant decrease in pollen attachment and pollen tube growth was observed (Figure S1A–S1C). Treatment with a tertiary alcohol such as tert-butanol was unable to affect the pollination response (Figure S1D). During aniline blue assay, since alkali and heat treatment result in washing off the weakly attached pollen, only pollen that is able to strongly attach to stigmatic surface is visualized. The reduction in pollen development on the stigma after inhibition of PLDα1 suggests that PA produced by phospholipases D is required for compatible pollination.

To directly confirm the role of PLDα1 during compatible pollination, we created transgenic Westar (compatible) lines in which *PLDα1* expression was selectively suppressed in the stigmas through an RNAi approach, using the stigma-specific *SLR1* promoter [15]. The RNAi lines (R4, R5, and R6) displayed strong suppression of *PLDα1* transcripts, while the closely related Bn*PLDα2* was largely unaffected (Figures S2A and S2B). Western blot analysis using anti-PLDα1 antibodies further confirmed the reduction in PLDα1 expression in these RNAi lines (Figure 1B; Figure S2C). When these lines were pollinated with Westar pollen and allowed to develop pods, a decrease in the size of the pods

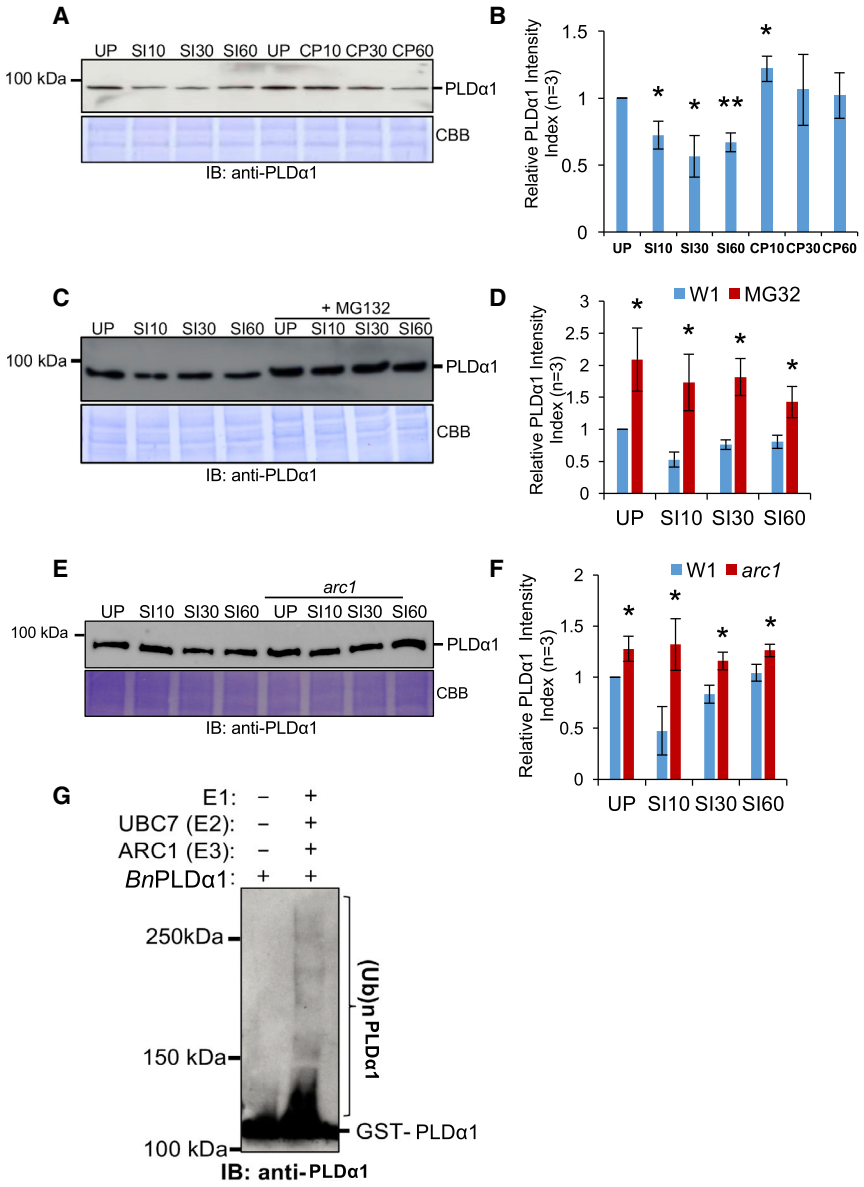

Figure 2. PLDα1 Downregulation following SI Is Dependent on ARC1 and the Proteasome

(A) Western blot analysis to detect PLDα1 expression in stigmas either left unpollinated or pollinated for 10, 30, or 60 min with SI or compatible pollen. Coomassie-brilliant-blue-stained membrane is shown below for loading.

(B) Average relative intensity of PLDα1 signals from three independent biological replicates of the treatments represented in (A) were quantified from the western blot assays using the ImageJ program (https://rsb.info.nih.gov/ij/) (t test, *p < 0.05, **p < 0.005; n = 3). Error bars indicate ±SEM.

(C) PLDα1 expression was detected through western blot analysis of proteins extracted from W1 stigmas either left untreated or treated with MG132 for 3 h, followed by pollination with self-incompatible W1 pollen for 10, 30, or 60 min.

(D) Average relative intensities from three biological replicates of the blot represented in (C) (t test, *p < 0.05, **p < 0.005; n = 3). Error bars indicate ±SEM.

(E) PLDα1 expression was detected in proteins from stigmas from W1 or ARC1-suppressed (W1 background) lines either left unpollinated or pollinated with self-incompatible W1 pollen for 10, 30, or 60 min.

(F) Average relative intensities from three biological replicates of the blot represented in (E) (t test, *p < 0.05, **p < 0.005; n = 3). Error bars indicate ±SEM.

(G) *In vitro* ubiquitination assays followed by western blotting using anti-PLDα1 antibodies to detect ubiquitination of GST-PLDα1 using ARC1 as the E3 ligase. The higher-molecular-weight smear indicates ubiquitinated GST-PLDα1.

(Figure 1C) associated with a concomitant reduction in the number of seeds per pod (Figure 1D) was observed. When examined for pollen attachment, the number of pollen attached after aniline blue treatment was also significantly reduced in these RNAi lines (Figure 1E). These observations both from a pharmacological approach and the genetic approach provide strong evidence for the role of PLDα1 as an essential compatibility factor that is required for successful pollination to occur.

PA from PLDα1 Activity Is an Essential Component Required for Pollination

We next tested whether PA treatment could rescue these pollination defects after either suppression of *PLDα1* or inhibition of PLDα1 activity. For this, Westar flowers were incubated with 1-butanol, 1-butanol in the presence of PA, or 1-butanol in the presence of DAG, followed by compatible pollination. In all of these uptake experiments, transpirational uptake through pedicel of the flowers was used so as to not disturb the dry stigmatic surface. As observed previously, 1-butanol inhibited compatible pollination, while addition of PA in the presence of 1-butanol rescued the pollen adhesion, and penetration defects induced by 1-butanol treatment and DAG treatment was unable to substitute for PA (Figures 1F and 1G). This suggests that PA derived from PLDα1 activity is required for early pollination events. In accordance with this, PA was also able to rescue the pollination defects observed in the *PLDα1*-RNAi line (R5 and R6) (Figures 1F and 1G), further confirming the importance of PA generated by PLDα1 for compatible pollination. The fact that PLDα1 was identified as a protein that is downregulated after SI response raised the possibility of whether, through targeting of PLDα1, SI was blocking the PA production necessary for compatible pollination. To test this, we treated W1 self-incompatible flowers with PA followed by SI pollination. A strong and significant increase in pollen attachment was observed after incompatible pollination, and pollen tubes could be observed penetrating the papillary cells (Figure 1G). The ability of PA to break down SI response suggests that one of the major targets of SI pathway could be to suppress PLDα1, thereby blocking PA production.

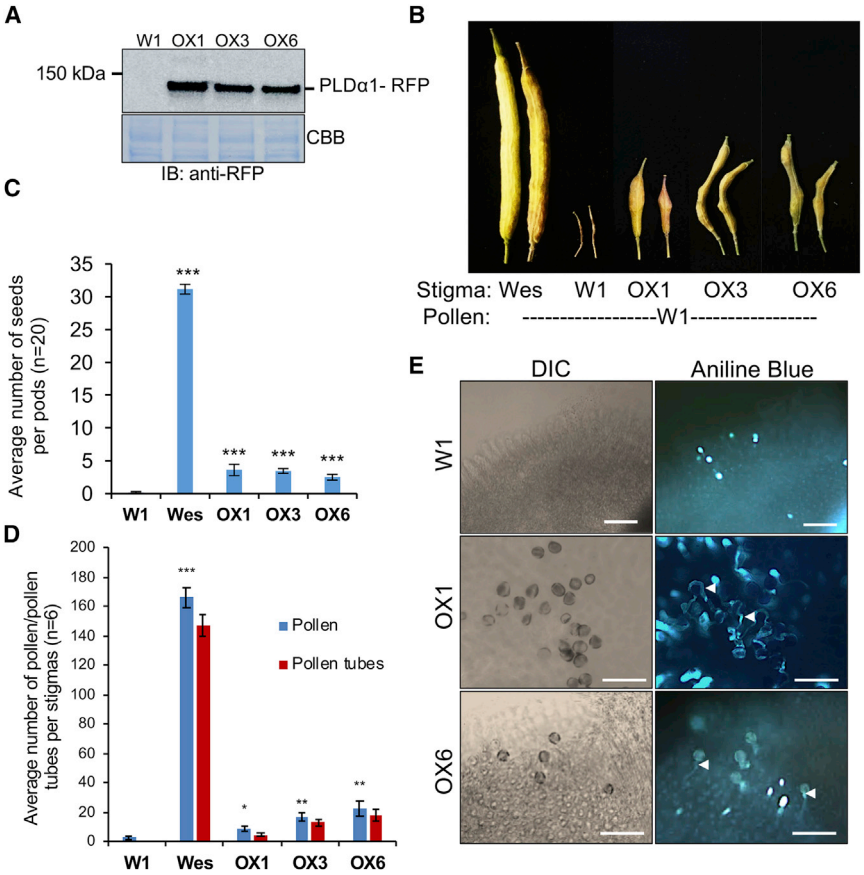

Figure 3. PLDα1 Overexpression Partially Breaks Down Self-Incompatibility Response

(A) Western blot analysis showing PLDα1-RFP expression in W1 lines overexpressing PLDα1-RFP (OX1, OX3, and OX6).

(B) Seed pods showing pod growth in the overexpressor lines compared to the W1 lines after self-pollination.

(C) Average seeds per pod in W1 and OX lines (t test, ***$p < 0.005$; $n = 20$). Error bars indicate ±SEM.

(D) Graph showing average number of pollen attached per stigma following 24 h of pollination with W1 pollen (t test, *$p < 0.05$, **$p < 0.005$, ***$p > 0.005$; $n = 6$). Error bars indicate ±SEM.

(E) Aniline blues assay performed after 24 h of pollination of W1 and OX lines using self-incompatible W1 pollen. Scale bars, 100 μm.

See also Figure S3.

PLDα1 Is a Target of ARC1 during SI Response

To investigate whether PLDα1 expression was regulated by SI, we assessed PLDα1 expression at various times after SI pollination. PLDα1 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 2A; Figure S2D). In contrast to SI pollination, after compatible pollination, PLDα1 level was mostly unaltered (Figure 2A; Figure S2D). The ability of SI to cause the downregulation of PLDα1 expression raised the possibility that ARC1, the E3-ubiquitin ligase, which targets compatibility factors for degradation during SI [5, 7, 8], could be involved in regulation of PLDα1 expression. Our proteasome inhibition assays using MG132 resulted in stabilization of PLDα1 and prevented degradation of PLDα1 after SI pollination (Figure 2B; Figure S2E), further supporting the notion that ARC1 could be involved. To directly test the role of ARC1, when stigmas from *ARC1*-suppressed plants were analyzed for PLDα1 expression after SI pollination, PLDα1 expression was very similar to the MG132-treated flowers with stabilization of PLDα1 in unpollinated flowers and lack of downregulation of PLDα1 after SI treatment in *ARC1*-suppressed background (Figure 2C; Figure S2F). To further confirm that ARC1 is directly capable of ubiquitinating PLDα1, we performed an *in vitro* ubiquitination assay with recombinant GST-PLDα1 protein using GST-ARC1 as the E3 ubiquitin ligase (Figure 2D). Higher-molecular-weight forms, consistent with poly-ubiquitinated GST-PLDα1, 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 2D). Based on these results, we propose that during SI response, activated ARC1 targets PLDα1 for degradation, thereby blocking production of PA that is most likely required for successful pollination to occur.

Overexpressing PLDα1 in W1 Self-Incompatible Line Partially Breaks Down the SI Response

The ability of PA to break down SI response and the observed ARC1-mediated downregulation of PLDα1 after SI pollination suggested that blocking PLDα1 could be a mode of action of SI. Therefore, overexpression of *PLDα1* in the stigmas could stabilize the PA pools after incompatible pollination and lead to breakdown of SI. To investigate this possibility, we generated PLDα1-RFP overexpressing self-incompatible W1 lines using the stigma-specific *SLR1* promoter. The *PLDα1-RFP* lines (OX1, OX3, and OX6) were confirmed for their expression of the transgene using anti-RFP antibodies (Figure 3A), followed by evaluation of their SI status. When these lines were pollinated with self-incompatible pollen, SI pollen were able to establish on these stigmas and led to development of pods with significant number of seeds in each of these pods compared to control untransformed W1 lines (Figures 3B and 3C). When SI-pollinated stigmas from these plants were subjected to aniline blue assays, a significant increase in pollen attachment and pollen tube growth could be observed on the *PLDα1-RFP* overexpressors compared to W1 stigmas (Figures 3D and 3E), demonstrating a breakdown of SI response after overexpression of PLDα1.

When stigmas of *PLDα1-RFP (OX1)* line were subjected to confocal microscopy after SI pollination, a reduction in signal intensity was observed (Figures S3A and S3B). When the responding papillary cells were imaged, a progressive reduction in PLDα1-RFP signal intensity could be observed in the papilla that was interacting with the SI pollen at 10, 30, and 60 min after pollination (Figure S3C). When protein extracts from *PLDα1-RFP (OX1)* line were examined for regulation by SI response, they

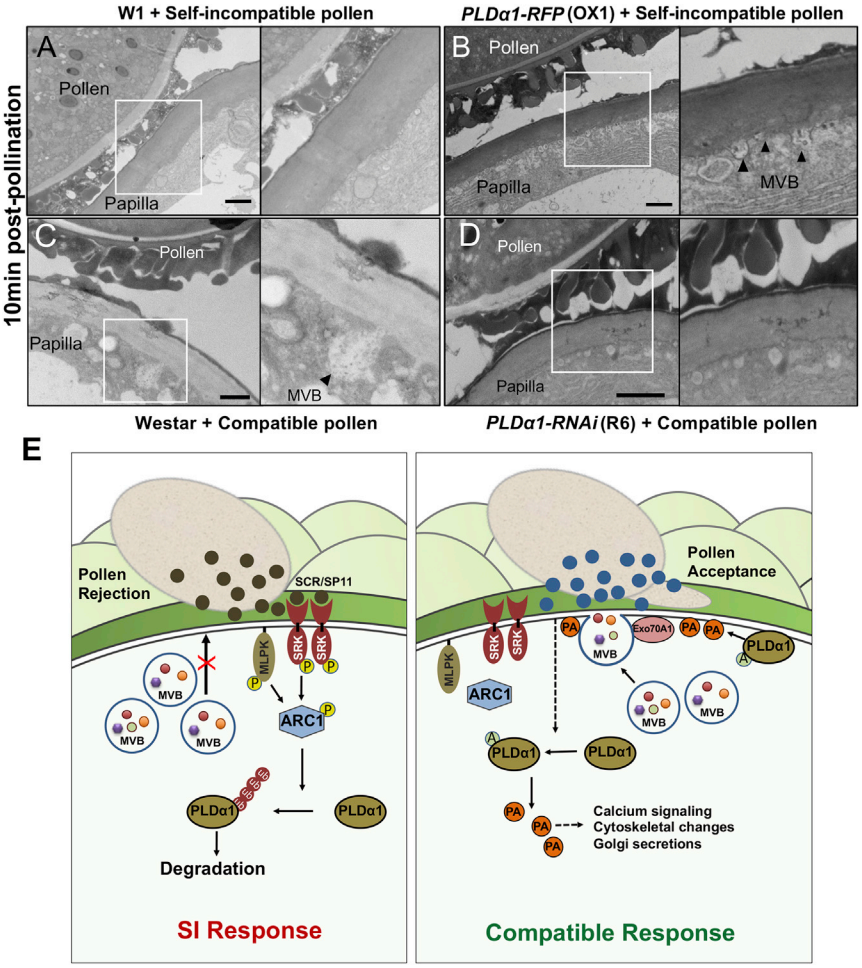

Figure 4. PLDα1 Is Involved in Increased Membrane Trafficking during Pollination

(A and B) TEM imaging of papillary cells of W1 (A) and OX1 (*PLDα1-RFP* overexpressor in W1 background; B) lines 10 min after self-incompatible pollination with W1 pollen. Scale bar,1 μm.

(C and D) TEM imaging of papillary cells of Wes (C) and R6 (*PLDα1-RNAi*; D) lines 10 min after compatible pollination with Westar pollen. Imaging was performed at the contact point between pollen and papillary cell.

(A–D) The images to the right of each panel show magnifications of the white boxed regions from the respective panel. Multivesicular bodies (MVBs) are indicated with black arrows. Scale bar,1 μm.

(E) Proposed model for PLDα1 role in stigmatic papillae after pollination. During SI response, landing of self-pollen on the stigma leads to SP11/SCR-SRK interaction and along with MLPK results in downstream phosphorylation-mediated activation of ARC1. Activated ARC1 can ubiquitinate PLDα1 and direct PLDα1 for degradation by the proteasome, causing inhibition of exocytosis of MVBs and thereby rejecting the self-pollen. During compatible pollination, upstream signals activate PLDα1 (PLDα1-A), which then catalyzes the production of phosphatidic acid (PA), promoting exocytosis of MVBs, most likely through facilitation of their fusion at the membrane, leading to successful pollination.

See also Figure S4.

showed a similar trend to W1 extracts, except that there was significantly increased levels of PLDα1 in the overexpressing line (Figures S3D and S3E). Therefore, we propose that under normal circumstances, SI response would target PLDα1 for degradation in order to hinder the production of PA required for compatible pollination. In the *PLDα1-RFP (OX1)* line, we predict that although PLDα1 is targeted by the SI response, it was maintained at a level that could still support PA production, thereby facilitating pollen germination, leading to a breakdown of SI response.

PLDα1 Is Involved in Membrane Trafficking

Multivesicular bodies (MVBs) have been previously reported during pollination in *Brassica napus* during exocytosis after compatible pollination and their sequestration in the cytosol or the vacuole after SI [16]. When stigmas from the PLDα1-overexpressing *PLDα1-RFP (OX1)* line were subjected to transmission electron microscopy (TEM) prior to SI pollination, no difference could be observed between the *PLDα1-RFP (OX)* line and the control W1 papillary cells (Figures S4A and S4B). Therefore, PLDα1 overexpression did not result in any constitutive activation of exocytosis and membrane activity. However, after 10 min of SI pollination, while no membrane activity was observed in the W1 papilla (Figure 4A), in OX1 papillary cells, MVBs were observed fusing with the plasma membrane at the contact point with the pollen (Figure 4B). The MVB fusion in the OX1 line is indicative of a compatible response, further confirming the observation that SI pollen could trigger a compatible response when PLDα1 is overexpressed in a W1 background.

In comparison, Westar papillary cells displayed large MVBs fusing with the plasma membrane (Figure 4C), and suppression of PLDα1 levels in the *PLDα1-RNAi 6* stigmas resulted in the absence of any MVB fusing at the papillary membrane close to the contact site with the pollen, after compatible pollination (Figure 4D; Figures S4C and S4D). We propose that during compatible pollination, activation of PLDα1 would lead to re-modeling of the plasma membrane through PA derived from PLDα1 activity to promote exocytosis required for pollen germination. In the RNAi line, due to suppression of *PLDα1*, this exocytotic process may either be impaired or slowed down after compatible pollination, while this process is abrogated after SI response due to targeting of PLDα1 for degradation.

DISCUSSION

In dry stigmas of Brassicaceae where 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. Other than a few pollen coat proteins that can interact with the membrane or secreted proteins such as SLR1 and SLG [17, 18], *bona fide* triggers for

compatible pollination in either the pollen or the stigma remain as yet unidentified. One of the key stigmatic proteins that was identified to be essential for pollination is Exo70A1, which is responsible for the exocytosis of vesicles that is required for pollination to occur and it is also targeted by the SI response that blocks this exocytosis from occurring [7]. 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 [16]. In spite of this, other factors that influence membrane re-modeling that could promote exocytosis of vesicles or MVBs have remained unknown. Identification of PLDα1 as a compatibility factor and a potential contributor to membrane remodeling further enhances our understanding of the molecular mechanisms behind compatible pollination.

The experiments with PA supplementation suggest that PA generated from PLDα1 activity is most likely responsible for promoting compatible pollination, possibly through facilitating delivery of factors necessary for pollen hydration and germination (Figure 4E). One mechanism through which PA can accomplish this could be its cone shape, which is capable of bending the membrane negatively, allowing fusion of MVBs at the contact point of pollen with the stigma and thereby the delivery of water and nutrients [16]. Its localization at the inner leaflet of the plasma membrane could initiate membrane curvature required for membrane fusion during exocytosis, which is a recurring theme in phospholipid studies [19]. Alternatively, PA could serve as an anchorage for proteins involved in exocytosis [19].

One of the interesting observations from the TEM was that membrane activity could be observed in the PLDα1-OX lines only after pollen attachment as there was lack of constitutive activation of the exocytotic machinery without any pollen attachment. This observation suggests that either PLDα1 has to be induced after pollination or the exocyst complex should be activated post-pollination to deliver vesicles at the membrane where PA generated from PLDα1 activity could aid in membrane fusion of the MVBs and vesicles. One second messenger that could play a crucial role in this activation process is calcium, and PLDα1 possesses the calcium-binding C2 domain and can be catalytically activated by binding of calcium [20]. In agreement with this, calcium spikes are consistently observed after compatible pollinations [21]. Taken together, our results have provided clear evidence that PLDα1 is a compatibility factor and a target of SI, where PA produced by PLDα1 activity is most likely required for membrane remodeling to occur, allowing fusion of exocytotic MVBs or vesicles, to deliver the cargo required for compatible pollination.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- METHOD DETAILS
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  - Protein extraction and western blot analysis
  - Proteasome inhibitor treatment
  - Recombinant Protein Purification, *In Vitro* Ubiquitination Assay
  - Aniline Blue assay
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SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and one table and can be found with this article online at https://doi.org/10.1016/j.cub.2018.12.037.

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AUTHOR CONTRIBUTIONS

S.S. conducted the experiments. S.S. and M.A.S. designed the experiments and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-PLD1	Genscript	153652_1
Anti-mouse IgG	Amersham	NXA931; RRID:AB_772209
Anti-RFP (Rabbit) Min X Hu Ms and Rt Serum Proteins	Rockland	600-401-379; RRID:AB_2209751
Goat anti-rabbit	Invitrogen	N/A
anti-Ubiquitin	Sigma Aldrich	N/A
Bacterial and Virus Strains		
Agrobacterium tumefaciens GV3101		N/A
BL21 (DE3) pLysS		N/A
Rosetta (DE3) pLysS	Novagen	70956
Chemicals, Peptides, and Recombinant Proteins		
MS medium with vitamins - 100L	PhytoTechnology laboratories	M519
Bx Mycorrhizae	Pro-Mix	1000635262
cOmplete, EDTA-free Protease Inhibitor Cocktail	Roche	11836170001
Protein reagent assay	Bio-Rad	5000006
40% Acrylamide /Bis-acrylamide, 29:1	Bio-Rad	1610146
PVDF membrane 0.45 μm	Bio-Rad	1620177
Tris/Glycine buffer	Bio-Rad	1610771
Skim Milk	EMD Millipore	1620177
LuminataTM Forte	Milipore	N/A
Amersham Hyperfilm ECL	GE Healthcare	28906836
MG132	APExBIO	A2585
Acyl 06:0 NBD PA	Avanti Polar lipids	810138C
Spurr's resin	EMS	14300
Human UbcH5a/UBE2D1	BostonBiochem	E2-616
Ubiquitin Activating Enzyme (UBE1)	BostonBiochem	E-301
pGEX PLD1	This study	N/A
pGEX ARC1	This study	N/A
PhosphoCreatine Kinase		N/A
Ubiquitin	Sigma	U6253
Critical Commercial Assays		
In-Fusion HD Cloning Plus	Clontech	638909
GSH agarose beads	Sigma-Aldrich	G4510
Deposited Data		
Brassica napus cultivar Westar phospholipase D1 mRNA	This study	Genbank: KJ755984.1
Experimental Models: Organisms/Strains		
Westar		N/A
W1		N/A
ARC1		N/A
Oligonucleotides		
P1 SLRinfFor-dSma-PLD1For: CTCTAGAGGATCcccATGGTACGAGTCCTTTCA	This study	N/A
P2 EcoR1-Int-PLD1-Rev: GCgaattcCTATGAGCTGCAAAAACTACTTACCTCCTT CAACACATCGACGGA	This study	N/A
P2′ SLRinfR: TCGAGCTCGGTAcccggggCgaattcCTATGAGCTG	This study	N/A
P4 SLRinfR-PLD1 For TCGAGCTCGGTAcccgggATGGTACGAGTCCTTTCA	This study	N/A

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
P5 InfINt2PLD1 Rev: ATAGgaattcGCcccgggCTTCAACACATCGACGGA	This study	N/A
PLDa1InfFw: ACTCTAGAGGATcccgggGATGGCGCAGCATCTGTTG	This study	N/A
RFPinfR-PLD1SmaRev: CGGAGGAGGCCATcccgggGGTTGT	This study	N/A
SLR1 For: TAATGAGTGGCTGGAAAGTCA	This study	N/A
EcoRIIntPLD1 Rev: GCgaattcCTATGAGCTGCAAAAACTACTTACCTCCTTCAAC ACATCGACGGA	This study	N/A
InfINT2 PLD1 Rev: ATAGgaattcGCcccgggCTTCAACACATCGACGGA	This study	N/A
SLR1-Rev: CGCAAGACCGGCAACAGGATT	This study	N/A
PLD1infsmaI For: ACTCTAGAGGATCCCCGGGATGGCG	This study	N/A
RFP-*Int*-Rev: GATCTCGAACTCGTGGCCGTT	This study	N/A
SRK910-For: CCTACGATAGTTCTTACACT	This study	N/A
SRK910-Rev: CCATGATGTCGGAGTGAACGTT	This study	N/A
Inf-PGEX-Eco-PLD1For: TGGATCCCCGGAATTCATGGCGCAG	This study	N/A
Inf-PGEX-EcoPLD1Rev: GTCGACCCGGGAATTCCTAGGTTGTA	This study	N/A
pGEX-FOR: GGGCTGGCAAGCCACGTTTGGTG	This study	N/A
pGEX-REV: CGGGAGCTGCATGTGTCAGAGG	This study	N/A
PLD1 qPCR Fw CATCCGTCGATGTGTTGAAG	This study	N/A
PLD1 qPCR Rev TCTTCTGATGGTGCGTGAAC	This study	N/A
PLD2 qPCR FW ACAACGACAGGAACCCAATC	This study	N/A
PLD2 qPCR Rev GCTTTTGACTCCCATGTTCC	This study	N/A
EF1a qRP CAACACTCTTGATAACACCG	This study	N/A
EF1a qFP CTTCTCTGAGTACCCACCAC	This study	N/A
Other		
Amersham Imager 600		N/A

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Marcus Samuel (msamuel@ucalgary.ca).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

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 [4]. 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°C in the dark, plates containing seeds were transferred to long-day conditions (16 h light at 22°C and 8 h dark at 18°C) for germination. After 2 weeks on germination medium, the seedlings were transferred to soil (PRO-MIX BX MYCORRHIZAE) and grown under long-day conditions.

METHOD DETAILS

Brassica napus Transformation

PLDα1 RNAi Westar lines were generated by *Agrobacterium tumefaciens* (GV3101) – mediated transformation (UC Davis, plant transformation facility). The double-stranded RNAi sequence was mobilized into the binary p1665 vector, under the control of the stigma-specific *SLR1* promoter [4] through Infusion cloning (Clontech) (see list of primers in key sources table, See also Table S1). Agrobacterium harboring this binary vector was used to transform Westar plants and kanamycin resistant transformants were isolated and analyzed by PCR (see list of primers in key sources table) to confirm the presence of the construct. PLDα1 levels in unpollinated stigma were examined by western blotting with anti-PLDα1 antibodies. For *PLDα1* overexpressing W1 and Westar lines, *PLDα1* fragment fused with RFP at the C terminus was introduced into the p1666 vector under the *SLR1* promoter. This construct was introduced into W1 plants through *Agrobacterium tumefaciens* (GV3101) – mediated transformation (UC Davis, plant transformation

facility for PLDα1-OX1 line). The transformation of OX3 and OX6 and WestPLOX (Westar lines overexpressing PLDα1) lines was performed in the laboratory following the method described previously [7, 22]. The true kanamycin resistant positives were then transplanted on to soil and maintained in growth chambers and the best lines with high PLDα1 expression in the stigmas were identified through western blotting using anti-PLDα1 antibodies.

Production of Anti-PLDα1 Polyclonal Antibodies

Affinity-purified anti-PLDα1 polyclonal antibodies were raised and affinity purified by GenScript (Piscataway, NJ, USA). A 14–amino acid PLDα1 specific peptide (corresponding to amino acids 636 to 649 of *Brassica napus* α) with an additional N-terminal Cysteine residue, EPAERPDADSSYMKC, was synthesized, conjugated with keyhole limpet hemocyanin, and used to raise antibodies against PLDα1 in mouse.

Protein extraction and western blot analysis

For protein isolation following pollination, *Brassica napus* flowers were collected in the morning before anthesis and pollinated for different times with either compatible or self-incompatible pollen. After different pollination times (10 min, 30 min and 60 min), the stigmas were excised from the pistil and pulverized in 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°C to remove the debris. Protein concentration was determined using the Bio-Rad protein reagent assay. Approximately 5 to 7 μg of the total protein was loaded on a 10% Acrylamide/Bis-acrylamide gel (40% Acrylamide /Bis-acrylamide, 29:1) 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 for 1 h at 25 V followed by blocking the membrane for 1 h at RT in 7% Skim Milk (EMD Millipore) made in 1X TBST. The membrane was probed with an anti-PLDα1 antibody (Genscript) at 1:5000 in 7% milk in 1X TBST for 2 h at RT or overnight at 4°C. 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 1 h at RT. The chemiluminescence from the HRP substrate, Luminata Forte (Western HRP Substrate, Millipore) was detected either using an X-Ray film (Amersham Hyperfilm ECL) or using the Amersham Imager 600. The RFP signal from RFP-PLDα1 was detected after stripping the antibodies from the membrane in 2 M $MgCl_2$ and 100 μL of acetic acid for 20 min. The stripped membrane was washed thoroughly and blocked 1 h at RT in 7% Skim Milk (EMD) in 1X TBST. Rabbit anti-RFP polyclonal (Rockland) was used at a dilution of 1: 3000 in 7% skim milk in 1X TBST for 2 h at RT or overnight at 4°C. 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.

Proteasome inhibitor treatment

Proteasome inhibitor treatments were performed as described previously [5]. W1 flowers were collected one day before anthesis and incubated overnight in 50 μM of MG132, a proteasomal inhibitor (10 mM stock in DMSO). 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-PLDα1 antibodies as described previously.

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 [7]. PLDα1 and ARC1 were cloned in pGEX-4T-1 (as described previously [8]. *PLDα1* ORF was amplified by PCR Phusion using the primers (Inf-PGEX-Eco-PLDα1For and Inf-PGEX-EcoPLDα1Rev) (Key sources table) and inserted into pGEX-4T-1 by ligation. *In vitro* ubiquitination assays were performed using purified GST: PLDα1 protein as the substrate using GST:ARC1 as the E3 ligase as previously described [8]. For the ubiquitination assay, the reaction mixtures (25 μL) contained 0.5 μg of yeast E1 (Boston-Biochem, Cambridge, MA), 0.5 μg of *Arabidopsis* 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-PLDα1 (GenScript) as previously described. Ubiquitination detection with anti-Ubiquitin antibody was performed after the membrane was stripped (as described previously), autoclaved (121°C for 20 min) and blocked for 2 h 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 Luminata Forte (Western HRP Substrate, Millipore).

Aniline Blue assay

Aniline blue assay was performed as previously described [6]. After pollination, pistils were fixed for 30 min in 3:1 ethanol-glacial acetic acid. The pistils were washed three times with distilled water and incubated in 1 N NaOH at 60°C for 1 h. The stigmas were thoroughly rewashed three times with water. Then they were stained with basic aniline blue (0.1% aniline blue in 0.1 M K_3PO_4). The pistils were mounted in 70% glycerol and observed with the blue channel of a Leica DMR epifluorescence microscope.

PLD inhibition assay

Pedicels of self-incompatible W1 canola, stage 12 flowers were immersed overnight in either 5mM or 10mM N-methylethanolamine (MEA), with either 0.1% or 0.4% Butanol or with 0.4% Tert-Butanol. Flowers were then pollinated with Westar pollen to initiate a compatible reaction. Six hours after pollination, flowers were collected and treated subjected to aniline blue assay for observation of pollen and pollen tube growth through epifluorescence microscopy.

PA rescue

Flowers were incubated overnight by immersion of their pedicel in either water, 0.4% 1-butanol and 150 μM of 14:0 PA 1,2-dimyristoyl-sn-glycero-3-phosphate (sodium salt) (Avanti Polar lipids) or 150μM of PA alone, 0.4% 1-butanol and 150 μM of 16:0 DG 1,2-dipalmitoyl-sn-glycerol (sodium salt) (Avanti Polar lipids). 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.

Confocal microscopy

Isolated flowers were pollinated for various times with either W1 or Westar pollen. The stigmas were clipped off the pistils and mounted using 50% glycerol on a glass slide. The stigmas were observed using a Leica SP5 laser confocal microscope with a 40X oil-immersion objective and a Leica HyD detector. The stigmas were scanned after the indicated treatments using a HeNe 543 (excitation 543; emission 585-649).

TEM imaging

TEM imaging was performed as previously described [23–25]. 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 followed by rinsing 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. 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.

RNA isolation and cDNA synthesis

Total RNA was isolated using a modified single step TRIzol method [26]. RNase free labware (tips, microcentrifuge tubes) were used. Briefly, 6 to 8 frozen stigmas were homogenized in iced for 10 s with 250 μL of TRIzol. Subsequently, 50 μL of chloroform was added to the mixture and shaken vigorously for 15 s and the samples were kept for 3 min at room temperature. The samples were centrifuged at 12000 x g for 15 min at 4°C and the aqueous phase (supernatant) thus obtained was transferred to another microfuge tube by gentle pipetting, avoiding the interphase slurry. RNA was precipitated by adding 125 μL of 100% isopropanol and incubating the tubes 10 min at RT and the samples were centrifuged at 12000 x g for 10 min at 4°C. After decanting isopropanol, the pellet was washed with 250 μL of 75% ethanol in diethyl pyrocarbonate (DEPC) treated water and briefly centrifuged at 7500 x g for 5 min at 4°C. Total RNA was suspended in DEPC water and stored at −80°C for further use.

RNA quantification was performed using a NanoDrop ND 1000 spectrophotometer.

The RNA was treated with DNase I, RNase-free (Thermo Scientific) following the instructions provided by the manufacturer. The purified RNA thus obtained was reverse transcribed using a high capacity cDNA reverse transcript kit (Applied Biosystems, ON, Canada), according to manufacturer's instructions.

Expression analysis by RT-qPCR

A hundred-microliter reaction mixture was set up (cDNA, gene specific primers (Key Resources Table), 10 μL of 2X SYBR green reagent and 5 μL DEPC water) in a StepOne Real-Time PCR System (Applied Biosystems, CA). The *Brassica napus* Elongation factor 1-α (*EF1-α*) (NM_001315606.1) gene was used to normalize transcript abundance. The following genes were used in this study; *BnPLDα1* (KJ755984.1) and *BnPLD2* (XM_013827355.2). The PCR conditions used were as specified in the manufacturer's instructions manual, including heat activation- 95°C, 10 min, denaturation- 95°C for 15 s, annealing and final extension at 60°C for 1 min followed by 40 cycles. Relative transcript levels were analyzed using the $2^{-\Delta\Delta}$Ct method [27] which calculates the difference in transcript levels compared to control.

QUANTIFICATION AND STATISTICAL ANALYSIS

t-Test, equal variance was performed with Microsoft Excel. Details of statistical evaluations are provided in the figure legends, and the numbers of samples indicated in the figures. Western blotting was replicated at least 3 times with consistent results.