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Chapter 4

Isolation of the Pistil-Stimulated Pollen Tube Secretome

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Abstract

Detection of secreted proteins and peptides during pollen tube guidance has been impeded due to lack of techniques to capture the pollen tube secretome without contamination from the female secreted proteins. Here we present a protocol to detect tobacco pollen tube secreted proteins, semi-in vivo pollen tube secretome assay (SIV-PS), following pollen tube crosstalk with the female reproductive tissues. This method combines the advantages of in vivo pollen tube–pistil interaction and filter-aided sample preparation (FASP) techniques to obtain an in-depth proteome coverage. The SIV-PS method is rapid, efficient, inexpensive, does not require specialized equipment or expertise, and provides a snapshot of the ongoing molecular interplay. We show that the secretome obtained is of greater purity (<1.4% ADH activities) and that pollen tubes are physiologically and cytologically unaffected. A compendium of quality controls is described and a rough guide on downstream bioinformatics analysis is outlined. The SIV-PS method is applicable to all studies of protein secretion using pollen tube as a model and can be easily adapted to other flowering species with modification. The overall duration for this protocol is approximately 8 hours spanning 4 days (an average of 2 h/day per two workers) excluding microscopy and LC-MS/MS analysis.

Key words Sexual plant reproduction, Cell–cell communication, Pollen tube guidance, Protein secretion, LC-MS/MS, Glycosylation

1 Introduction

At the interface between two adjacent cells, the extracellular matrix provides a hub for cell–cell communication. Small peptides secreted to the extracellular space are a unique signature of the cellular response to the external signals perceived. Therefore, studying the secretome or more accurately the "peptidome" of the extracellular matrix, provides an excellent system to obtain a detailed account of the short and long-distance signalling events and more importantly the molecular interplay of the cells involved. The extracellular matrix constitutes of cleaved mature peptides (peptidome), hormones, modified polysaccharides as well as secreted nanovesicles that serve as long-distance signalling vesicles in almost all forms of life [1].

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In flowering plants, precise guidance of the pollen tube (the male gametophyte) toward the embryo sac of the ovule (the female gametophyte) involves persistence response by the pollen tube to pre-laid secreted peptide signals from the female gametophyte in the transmitting tract tissues [2]. Several of the female secreted peptides have been identified by cell-specific transcriptomic studies [3–6] and modified carbohydrate oligomers by genetic approaches using female mutants defective in pollen tube attraction [7]. This was feasible owing to the accessibility of the female gametophyte in several plant species in particular *Torenia fournieri* where the embryo sac is markedly protruded [5, 8]. Therefore, the crosstalk between the two gametophytes, involving a single polar cell (the pollen tube) and a multiplex of female reproductive tissues (the transmitting tract and female gametophyte) provides an excellent system to study short- and long-distance signalling as well as single cell versus complex tissues mechanisms of communications. For this a network of crosstalk events involving secreted ligands and perceiving receptors needs to be established. Unfortunately, development of methods for isolating the pollen tube secretome responsive to the female guidance signals has proven difficult due to the inaccessibility of the pollen tubes within the female reproductive tissues. Given this limitation, we developed a straightforward and easily adaptable method for studying the pollen tube secretome following penetration through the pistil tissues.

1.1 Purification of Pollen Tube Secretome Using SIV-PS Method

Here we demonstrate an efficient capture of the pollen tube secretome (peptidome) in a seminative environment after the pollen tube penetration through the stigma and style (Figs. 1, 2, 3, and 4). We named this improvised method from the original technique [9], semi-in vivo pollen tube secretome assay (SIV-PS). The procedure involves preparation of in-planta pollinated pistils and control unpollinated pistils in an improvised pollen tube germination chamber, incubation for 24 h, concentration of the secretome with 3/10 kDa protein filters followed by FASP processing and protein identification by liquid chromatography mass spectrometry. The apparatus used in a step-by-step set up and the anticipated results are shown in Figs. 1, 2, 3, and 4. SIV-PS has several advantages over in vitro pollen tube secretome; (1) it first allows interaction of the pollen tubes with the female reproductive tissues, thus rendering the pollen tubes competent to perceive female secreted signals that considerably influence the pollen tube secretome as well as transcriptome [10–13] (2) the pollen tube secretome captured is most likely directly influenced by the perceived female emitted cues, and therefore offers the possibility to identify candidate molecules of the male–female interaction (3) the setup of the protocol does not require specialized equipment and hence remains inexpensive (4) when coupled with FASP processing [14] and gel-free tandem

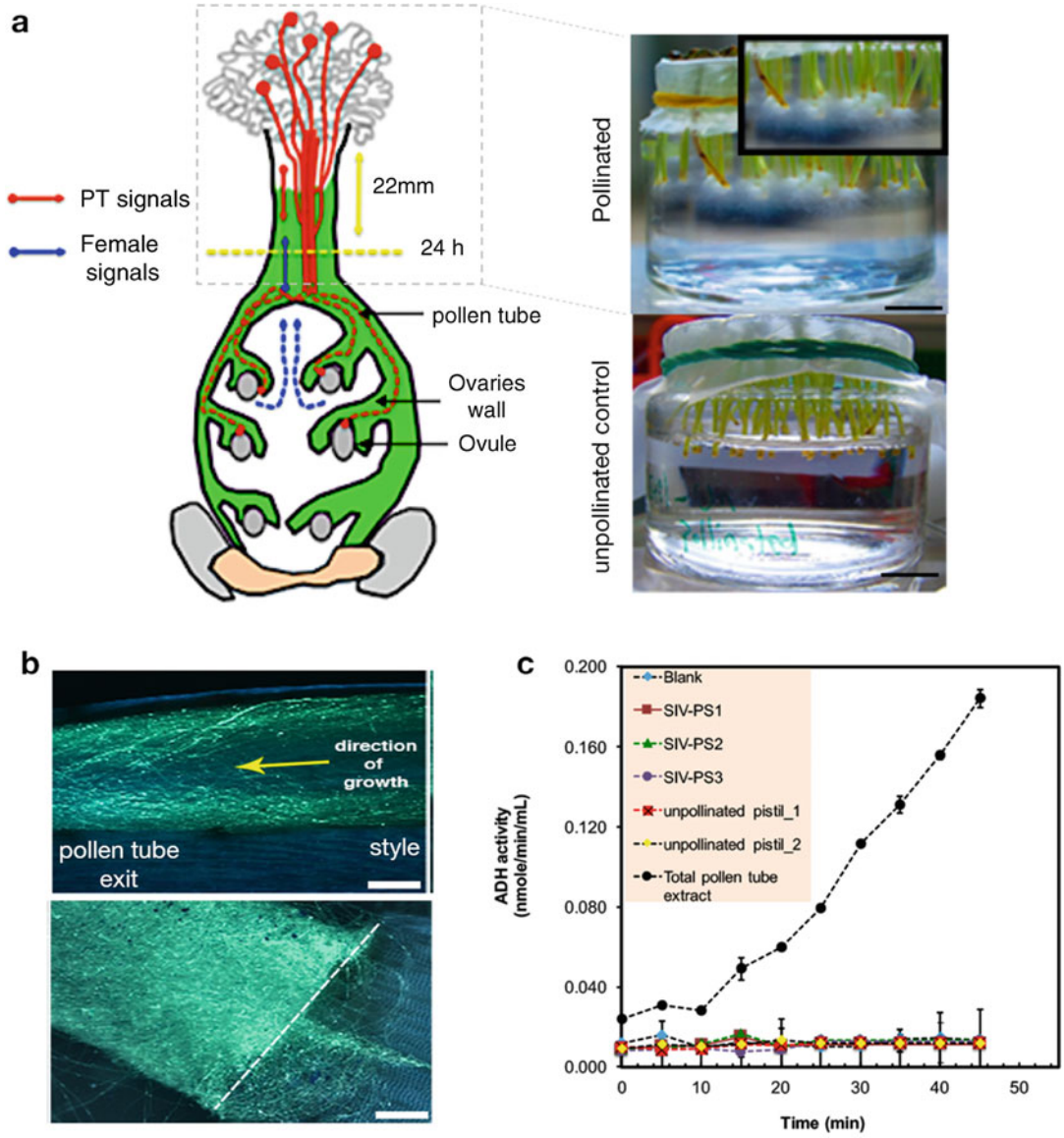

Fig. 1 The SIV-PS system for capturing semi-in vivo pollen tube secretome. (**a**) Schematic of Nicotiana tabacum pollen tube growth through the transmitting tissue of the style and entry into an ovule of the female gametophyte. After 24 h of in-planta germination, an excision is made on the pistil 22 mm (yellow dotted line) from the stigma shoulders and the pistil is transferred to the secretome chamber for in vitro pollen tube germination and secretome collection. Right panel shows an actual secretome chamber of the pollinated pistil (upper panel) and the unpollinated pistil control (lower panel) with successful pollen tube growth through the style tissues into the secretome chamber highlighted in the inset. (**b**) Aniline blue stain of the pollen tube bundle direction and exit from the cut pistil. Scale bars, ~5 μm. (**c**) Exemplified expected results of the secretome purity test from the SIV-PS derived secretome samples conducted using the ADH assay. Purity of the secretome is assessed by comparing maximum ADH activities of the secretome samples after 45 min incubation at 37 °C to the ADH activity of the total pollen tube extract

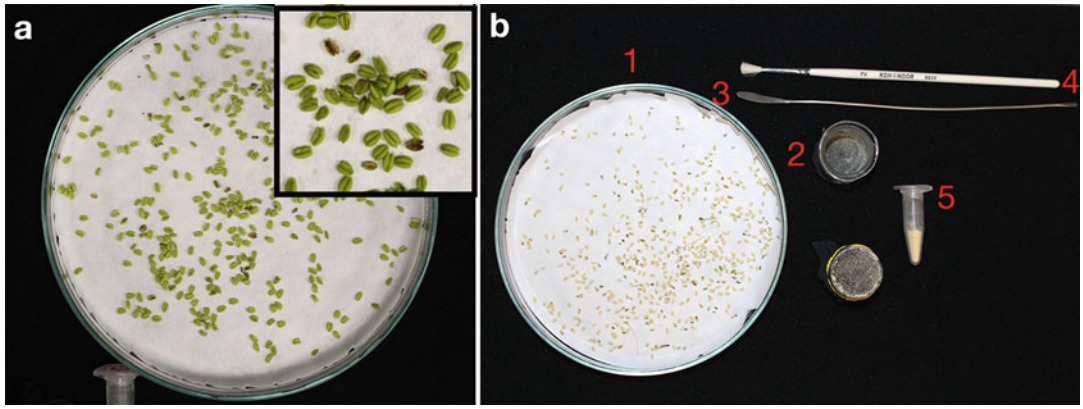

Fig. 2 Collection of dry pollen. (**a**) Anthers at pre-anthesis are collected into a petri dish covered with filter paper and incubated at room temperature overnight (**b**) 1; fully dry anthers 24 h after incubation, 2; chamber for sieving pollen grains into a freshly arranged filter paper, 3; spatula for scooping filtered pollen into an Eppendorf tube, 4; exemplified brush size used for pollination of stigma (*see* **step 18**), 5; collected dry pollen grains for immediate use or storage at −20 °C

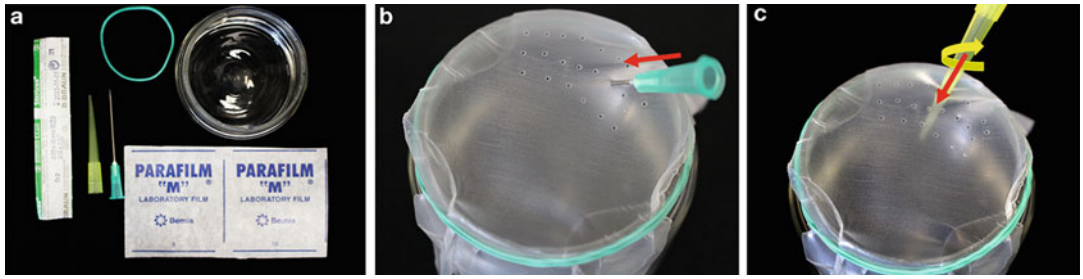

Fig. 3 Preparation of the SIV-PS secretome chamber. (**a**) A 50 mL capacity glass beaker is sterilized by autoclaving. Sterile 21G1 needle, parafilm (ethanol sterilized), yellow tip, and a rubber band are prepared. (**b**) In the laminar flow cabinet, decant tobacco pollen tube germination media into the chamber and seal with parafilm by slight stretching and secure with a rubber band. Perforate holes with the 21G1 needle ~2 mm apart in a sequential order. (**c**) Enlarge the holes for easy pistil penetration using a yellow tip by applying a vertical pressure (red arrow) and a twist (yellow arrow). Secretome chamber ready to use. Prepare just before pistil excision

LC-MS/MS, peptides of low molecular weight (<10 kDa) and of low abundance (as low as 0.45 parts per million) can be detected [10] (5) and unlike in vitro assay, the SIV-PS method has extremely high pollen tube growth reproducibility and allows set up of negative controls to quantitatively assess true secreted proteins (Fig. 1 [10]). The samples obtained by the SIV-PS method are also useful to study posttranslational modifications of secreted proteins as well as pollen tube metabolome to identify secreted modified carbohydrates and other potential signalling macromolecules. It is important to test the purity of the secretome samples. To estimate contamination by cytosol, one of the most abundant cytosolic enzymes, alcohol dehydrogenase (ADH), is used as an indicator

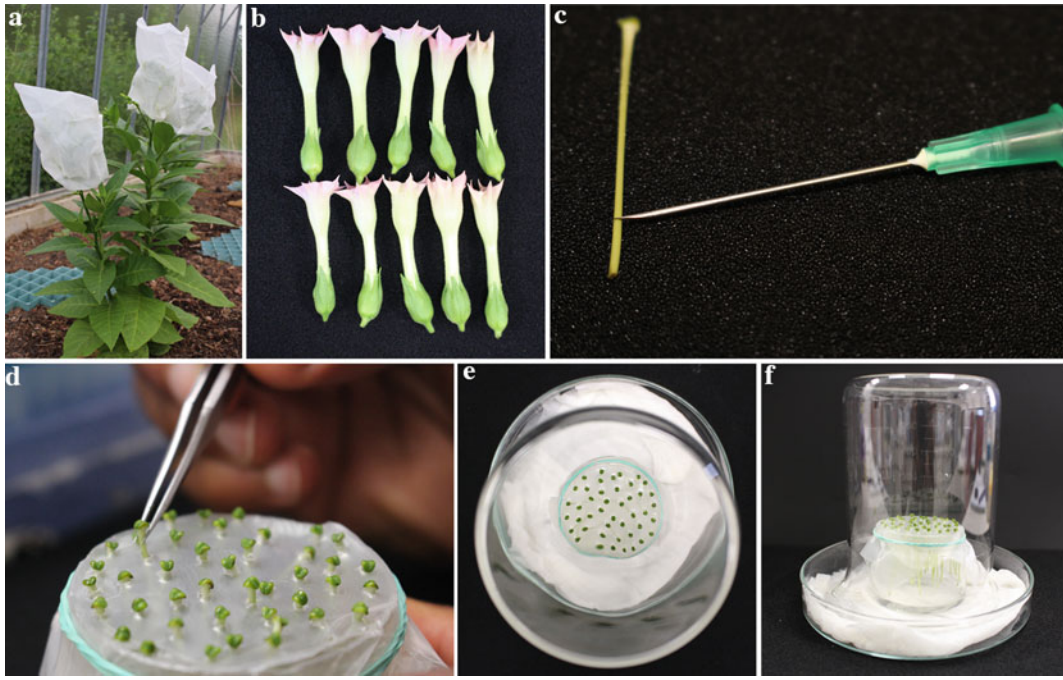

Fig. 4 Purification of the pollen tube secretome with the SIV-PS system. (**a**) The capture of the secretome start with the flower emasculation and in-planta pollination. (**b**) 24 h post-pollination, pistils are collected for processing. (**c**) Pollinated and unpollinated pistils are excised ~22 mm from the stigma shoulders. (**d**) All pistils are then gently inserted into prearranged secretome chamber using a sterilized forceps. (**e–f**) A humid chamber is constructed and the complete SIV-PS system with prearranged pistils is incubated at 28 °C for 24 h. On the next day, the secretome is ready for collection

[10]. Because of the noninvasive nature of the protocol, the purity of the sample is high, nearly 15-fold below detection of the sensitive ADH activities. The benefits of the SIV-PS method are also applicable to several other research areas. In particular, studies of endomembrane dynamics and secretion mechanisms including endocytosis, exocytosis, and unconventional protein secretion in a single cell system, influence of signal transductions on transcription and translation in pollen tubes, cytoskeleton dynamics, architecture of plant cell wall composition as well as in pathogen–plant interaction; e.g., by comparative genome-omics and secretome dynamics when pollen tube penetrates the pistil tissues as to a fungal hyphae penetrating leaf tissues. Lately, we have applied the SIV-PS method and coupled it with phospholipase C cleavage to enrich potential pollen tube secreted glycosylphosphatidylinositol-anchored proteins (GPI-anchor) as first in-line transient receptor proteins that could perceive female guidance signals (Hafidh et al., unpublished). The SIV-PS method can be easily adapted to other flowering species with modification.

1.2 Maximizing Protein Detection by FASP and Label-Free Quantitative LC-MS/MS

Since secreted peptides are predominantly small in size and of low abundance, their detection can be challenging. Efficient ionization of small tryptic peptides for MS/MS detection is highly influenced by downstream processing of the protein samples. Two major strategies are used in preparation of peptides suitable for mass spectrometry. The first method involves solubilization of proteins with detergents, separation of proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and in-gel digestion [15]. The in-gel processing is free from interfering impurities but suffers from lower peptide recovery and hence protein detection. The second method is detergent-free, comprising protein extraction with strong chaotropic reagents such as urea and thiourea, protein precipitation, and in-solution digestion of proteins under denaturing conditions [16]. The disadvantage of the gel-free approach is that the proteome may be incompletely solubilized and the digestion may be impeded by interfering substances. Therefore, we opt to follow a recently described method, filter-aided sample preparation (FASP), which combines the advantages of both methods above [16]. The essential steps of the FASP method include depletion of detrimental components in urea-containing buffer, carboamidomethylation of thiols, on-filter digestion of proteins, and elution of peptides [16]. With the FASP method, the number of unique peptides identified can double the number of peptides identified through in-gel processing [14]. By combining FASP method with label-free LC-MS/MS, we have identified over 800 candidate protein accessions from tobacco SIV-PS using <2 μg of the total secretome sample with an average of 62% of peptides that are less than 20 kDa and identified by >3 total peptides reproducible in at least 3/4 replicates [10]. The majority of the identified proteins were detected by 2–10 total peptides suggesting great sensitivity even of low abundant proteins [10].

1.3 Comparisons with Other Secretome Methods

Prior to our studies [10, 11], there have not been any other studies of pollen tube secretome. We presume the reason was simply due to lack of a technique that can allow capture of pure pollen tube secreted proteins after a crosstalk with the female reproductive tissues. Nevertheless, prevailing methods exist for studying the secretome of the apoplast (extracellular matrix) in plants. They use either vacuum-assisted infiltration collection method (VIC) [17] or a gentle gravity-assisted extraction method (GEM) [18]. Both of these methods have been commonly used in the studies of plant–pathogen interaction with an effort to identify plant/pathogen secreted proteins to the apoplast during plant defense against the infection [19]. Although these methods suffer from cytosolic contaminations due to cell breakage and insensitive detection of the apoplastic proteins because of the collection procedures and amount of materials recovered, they have spearheaded discovery of elicitor secreted peptides, pathogen-associated

molecular patterns (PAMPs), as well as PAMP-triggered immunity response proteins of the host cells [20]. Application of these methods is feasible because of the type of tissues used, rosette leaves which constitute of cells with rigid cell wall structures. The only gametophytic secretome study conducted was that of isolated ovule secretome which used a modified GEM method, tissue free (tfGEM) [21]. We therefore set out to develop a rapid, efficient, and an informative method for capturing pollen tube secreted proteins following pollen tube penetration through the female reproductive tissues. To our knowledge, this is the only method that has been tested and published so far [10].

1.4 Limitations of the SIV-PS Method

Our improvised SIV-PS protocol enables efficient and representative identification of male factors that participate in the male–female communication during fertilization without a need for higher level of expertise [10]. As any other technique, the SIV-PS method has its limits. Compared to the in vitro pollen tube secretome method (duration of 1–2 days), the SIV-PS method is time consuming. It requires up to 4 days (with an average of 2 h/day/2 coworkers) to obtain the secretome samples with completed purity and microscopic viability test. This step is independent of the number of workers and therefore its duration cannot be optimized. However, the limited hours spent per day means the SIV-PS method permits running of parallel experiments. Another disadvantage of our SIV-PS method is that it requires more starting materials: approximately 50 emasculated pistils per sample to obtain ~300 μL of 1–2 μg/μL of the pollen tube secretome per sample. However, with two or more coworkers, this might not be a limiting factor, instead more materials can be obtained by more coworkers, though this can increase the chance of sample variability. The main constraint of the SIV-PS method is that it cannot be paused at any time until the secretome samples have been collected.

2 Materials

2.1 Starting Material

1. It is strongly recommended to prepare a minimum of four biological replicates to obtain meaningful reproducibility. Approximately 20–30 flowering tobacco plants are required to obtain four replicates of ~1–2 μg/μL (total of 200–300 μL) protein concentration per sample per single run.
2. Multiple runs can be performed during the summer period if the plants are grown in outdoor green houses. At the first bolting, the primary inflorescence should be cut to encourage formation of secondary inflorescences. Approximately fifty emasculated flowers are required per sample per run.

2.2 Preparation of the Plant Material

1. *Critical*: Even though pistils are not considered fully sterile, all beakers used as germination chambers as well as flasks and falcon tubes must be sterile throughout the procedure to reduce potential fungal contamination. It is important that sample visualization be done immediately and purity tests should be conducted ideally before freezing the secretome samples at −80 °C.

2.3 General Reagents

1. Prepare all solutions using deionized (dH_2O) ultrapure water. Use of RNase free water is not essential unless for a specific application. Prepare and store all reagents at room temperature (unless indicated otherwise). For the N-glycosylation test, the detection buffer (4-chloro-1-naftol, methylalcohol in 10 mM Tris–HCl pH 6.8) should preferably be prepared fresh. Carefully follow all waste disposal regulations when disposing waste materials. The pollen tube germination media can be used for months if stored in cold (<8 °C) and only opened in flow box.
2. EDTA–disodium salt ($C_{10}H_{14}N_2O_8Na_2{\cdot}2H_2O$, Mw 372.24).
3. DAPI for nucleic acid staining (C16H15N5·2HCl, Mw 350.25). *Caution*: DAPI is a suspected mutagen; wear gloves and work in a flow hood while preparing the stock solution.
4. *N*,*N*-dimethylformamide (DMF, $HCON(CH_3)2$, Mw 73.09). *Caution*: DMF is potentially toxic; wear protective gloves under a chemical hood while handling the solution.
5. Propidium iodide (PI, $C_27H_{34}I_2N_4$, Mw 668.39). *Caution*: PI is suspected as a mutagen; wear gloves while preparing the stock solution.
6. Liquid nitrogen (N_2, Mw 28.01). *Caution*: Wear safety glasses or a face shield when handling liquid nitrogen; wear gloves when you are touching any object cooled by liquid nitrogen.
7. Ultrapure ethanol (C_2H_6O, Mw 46.07).
8. Acetic acid, glacial ($C_2H_4O_2$, Mw 60.05). *Caution*: Always pour acid slowly into the solution.
9. Glycerol ($C_3H_8O_3$, Mw 92.09).
10. Triton X-100 ($(C_2H_4O)nC_{14}H_{22}O$).
11. Tris–HCl.
12. Boric acid (H_3BO_3, Mw 61.83). *Caution*: Boric acid can cause respiratory sensitilization and a range of health hazards, wear gloves and mask while preparing the stock solution.
13. Calcium nitrate hydrous ($Ca(NO_3)_2.4H_2O$), Mw 236.19). *Caution*: Calcium nitrate is corrosive and toxic, wear safety glasses or a face shield, gloves and mask while preparing the stock.

14. Magnesium sulfate heptahydrous ($MgSO_4 \cdot 7H_2O$, Mw 246.47).
15. Potassium nitrate (KNO_3), Mw 101.1.
16. 2-(N-Morpholino)ethanesulfonic acid sodium salt (MES), Mw 209.26.
17. Sucrose.
18. Murashige-Skoog (MS) medium.
19. SAVO bleach.
20. Agar.
21. Acrylamide/Bis solution.
22. Sodium dodecylsulfate (SDS), Mw 288.37.
23. Ammonium persulfate (APS), Mw 228.2.
24. Glycine.
25. Dithiothreitol (DTT).
26. Coomassie Bromophenol Blue (CBB).
27. Isopropanol (C_2H_6O, Mw 46.07).
28. Methanol.
29. NaCl.
30. $MgCl_2 \cdot 6H_2O$, Mw 203.3.
31. Milk powder.
32. Bovine serum albumin.
33. Nitro blue tetrazolium chloride (NBT).
34. 5-Bromo-4-chloro-3-indolyl phosphate (BCIP).
35. 1-StepTM NBT/BCIP ready-made mix.
36. Concavalin A.
37. Horseradish peroxidase.
38. 4-Chloro-1-naftol.
39. Hydrogen peroxide.
40. Primary antibodies.
41. Secondary antibodies.
42. Prestained protein marker.
43. Alcohol dehydrogenase activity assay kit.
44. ProteoSilver silver stain kit.

2.4 General Equipment

1. Nitrocellulose membranes.
2. Blotting papers Whatman, thickness 5 (alternatively four pieces of Whatman papers, thickness 3), size cca 7 × 9.5 cm per one blot.

3. Mini PROTEAN® 3 System, glass plates, filter pads, gel holder cassettes, buffer tank, Plastic spatula, and lid.
4. Ice blocks (MiniPROTEAN) for cooling.
5. Plastic trays for processing the gels and assembly of blot sandwiches.
6. Polypropylene boxes 10 × 10 cm square.
7. Disposable cuvettes.
8. Spectrophotometer capable of measuring absorbance at 480 nm.
9. Soil composition—Jiffy-7 tablets (seed pellets, peat pellets; Jiffy Products International; http://www.Jiffygroup.com), diameter 41 mm.
10. Square plastic plant pot (3.2 × 3.2 cm).
11. Tweezers.
12. Scissors.
13. 2× 1 L plastic beakers.
14. 50 mL polypropylene conical centrifuge tubes.
15. Eppendorf 5810R centrifuge with swing-bucket rotor or similar alternative, max. RCF 4500 × *g*.
16. Pipettes.
17. 0.80 × 120 mm needle for protein loading.
18. 2-ml syringe to aspirate the gel holes before loading.
19. 1.5-ml transparent cap-locked microtube.
20. Microscope slides.
21. Coverslips of 0.13–0.16 mm thickness; 18 × 18 mm.
22. Laminar flow box.
23. Magnetic stirrer.
24. Glass mortar and pestle (inner diameter 8 cm). *Critical*: Store these in a refrigerator or in a cold room (4 °C) 1 day before homogenization.
25. Outdoor green house or a growth room.
26. Epifluorescence or, ideally, a confocal laser scanning microscope equipped with a spinning disk module.
27. Knife (blade).
28. 28 °C incubator.
29. Millipore 3 and 10 kDa filters.
30. −20 and −80 °C freezers.
31. Garden tying strings.
32. Optional; eBlot TM semidry transfer system.

2.5 Pollination, Dissection Tools, Germination Chambers, and Pollen Tube Germination Media

1. Bright brush bristle (#2/4) (KOH-I-NOOR Hardtmuth a.s; http://eshop.koh-i-noor.eu/shop/bright-brush-9936-bristle-4, cat. no. 9936006011BL).
2. 21G1–1.5 needles.
3. A sterile 3.5 cm × 4.5 cm round beaker (40 mL capacity).
4. A 500 mL glass beaker to create humidity chamber.
5. Sterile PARAFILM M.
6. Elastic rubber bands.
7. Tobacco pollen tube germination media (SMM-MES): dissolve 60 g of sucrose in 500 mL deionized water, 0.1 g H_3BO_3, 0.708 g $Ca(NO_3)_2{\cdot}4H_2O$, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.1 g KNO_3, and 4.88 g 2-(N-Morpholino)ethanesulfonic acid sodium salt (MES). Make up the volume to 900 mL and adjust the pH with KOH to 5.9. Adjust the final volume to 1 L. Sterilize the media in water bath >100 °C for 40 min. Repeat the sterilization after 24 h. Store the media in a cold room (<8 °C).

2.6 In-Gel N-Glycosylation Test

1. Concavalin A.
2. Horseradish peroxidase.
3. Hydrogen peroxide.
4. Detection buffer: 45 mg 4-chloro-1-naftol, 15 ml methylalcohol, and 60 ml 10 mM Tris–HCl pH 6.8.

2.7 Alcohol Dehydrogenase Assay (ADH)

1. ADH kit.
2. Disposable cuvettes.
3. Spectrophotometer capable of measuring absorbance at 480 nm.

2.8 SDS Polyacrylamide Gel

1. Resolving gel buffer: 1 M Tris–HCl, pH 8.8. Weigh 36.33 g Tris–HCl and transfer to a beaker containing 900 mL of water. Mix and adjust the pH with HCl (*see* **Note 1**). Make up the volume to 1 L with water. Store at 4 °C.
2. Stacking gel buffer: 0.5 M Tris–HCl, pH 6.8. Weigh 18.24 g Tris–HCl, mix with 900 mL of water, and adjust the pH as above. Adjust the volume to 1 L solution and store at 4 °C.
3. 30% acrylamide/Bis solution (29.2:0.8) acrylamide:Bis: Weigh 29.2 g of acrylamide monomer and 0.8 g Bis (cross-linker) and transfer to a 100 mL graduated cylinder containing about 40 mL of water. Add a spatula of AG 501-X8 (D) mixed-resin beads and mix for about 30 min. Make up to 100 mL with water and filter through a 0.45 μm Corning filter (*see* **Note 2**). Store at 4 °C, in a bottle wrapped with aluminum foil (*see* **Note 3**).

4. 10% (w/v) Sodium dodecylsulfate (SDS): Dissolve 30 g SDS in 200 mL water. Adjust the volume to 300 mL and store at room temperature.
5. 40% (w/v) Ammonium persulfate (APS): Dissolve 20 mg in 50 μL of water (*see* **Note 4**).
6. Electrode buffer (10× stock solution without SDS): Weigh 30.3 g Tris (250 mM) and 144 g of glycine (1.92 M) and mix with 800 mL of water. Make up the volume to 1 L.
7. SDS-PAGE running buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS. Dilute 100 mL of 10× electrode buffer to 990 mL with water and add 10 mL of 10% SDS. Care should be taken not to mix the solution vigorously since SDS generates bubbles.
8. 3× 1D sample buffer; Mix 15 mL of 0.5 M Tris–Cl pH 6.8 (final 0.15 M), 20 mL of 75% (v/v) glycerol (final 30%), 3 g SDS (final 6% w/v), 1.157 g DTT (final 0.15 M), 1.5 mL of 1% bromophenol blue in 50% isopropanol (final 0.03%) (*see* **Note 2**). Adjust the volume to 50 mL, aliquot in to 2 mL tubes, and store at −20 °C. To prepare a working 1D sample buffer, simply dilute the 3× 1D sample buffer by one-third with water or protein sample.

2.9 Immunoblotting

For wet transfer system (Bio-Rad MiniProtean Blotter, Catalogue 170-3930).

1. Nitrocellulose membranes.
2. Blotting buffer: 0.025 M Tris–HCl, 0.192 M glycine, 20% methanol (*see* **Note 3**).
3. Tris-buffered saline (TBS; 10×): 1.5 M NaCl, 0.1 M Tris–HCl, pH 8.0.
4. TBS containing 0.05% Tween-20 (TBST 1×): Mix 100 mL 10× TBS with 0.5 mL Tween 20. Adjust to 1 L final volume.
5. Blocking solution: 5% milk in TBS (*see* **Note 5**). Store at 4 °C.
6. 1% Bovine serum albumin (BSA): Dissolve 3 g of bovine serum albumin V (Sigma, cat. A4503) IN 300 mL of 1× TBST
7. Primary antibodies: Dilute 500–5000× with 1× TBST with 0.5% BSA in a 25 mL solution per blot (*see* **Note 6**).
8. Secondary antibodies: Dilute 5000× with 1× TBST with 0.5% BSA in a 25 mL solution per blot (*see* **Note 6**).
9. Mini PROTEAN® 3 System glass plates, filter pads, gel holder cassettes, buffer tank, and lid.
10. Plastic trays for processing the gels and assembly of blot sandwiches.

11. Blotting papers Whatman, thickness 5 (alternatively four pieces of papers Whatman, thickness 3), size cca 7 × 9.5 cm per one blot.
12. Ice blocks (MiniPROTEAN) for cooling.
13. 1 M Tris–Cl (pH 9.5): Dissolve 12.12 g to 80 mL of water. Adjust the pH to 9.5 with HCl. Make up the solution to 100 mL.
14. 2 M NaCl: Dissolve 11.7 g to 100 mL of water.
15. 1 M $MgCl_2 \cdot 6H_2O$: Dissolve 20.3 g of $MgCl_2 \cdot 6H_2O$ to 100 mL water. Autoclave at 120 °C for 30 min and store at 4 °C.
16. Alkaline phosphate buffer (AP buffer): Mix 10 mL of 1 M Tris–Cl (pH 9.5), 5 mL of 2 M NaCl and 0.5 mL of 1 M $MgCl_2 \cdot 6H_2O$. Adjust to a total volume of 100 mL.
17. 70% Dimethylformamide (DMF). Mix 1.4 mL dimethylformamide with 0.6 mL water.
18. Nitro blue tetrazolium chloride (NBT). Dissolve 50 mg in 1 mL of 70% dimethylformamide. Store at 4 °C.
19. 5-Bromo-4-chloro-3-indolyl phosphate (BCIP): Dissolve 25 mg BCIP in 1 mL of 70% DMF. Protect from light, store at −20 °C.
20. Ready to use AP buffer: Add 132 μL of BCIP and 132 μL of NBT to 20 mL of alkaline phosphatase buffer just before adding to membrane. Alternatively, use 1-StepTM NBT/BCIP ready-made mix.

3 Methods

3.1 Overview of the Method

1. The first step of the protocol involves sowing of tobacco seeds (*Nicotiana tabacum*, cv. Samsun) and growing tobacco plants.
2. Day 1: Flowers at 1 day before anthesis are emasculated.
3. Day 2: The pistils are pollinated and pollen tube germination chambers are then prepared for the incubation of pollinated as well as unpollinated control pistils.
4. Day 3: Pollinated and unpollinated flowers from day 2 are harvested, excised 22 mm below the stigma shoulder, arranged in germination chambers, and incubated for 24 h at 28 °C under full humidity.
5. Day 4: The pollen tube germination media containing secreted proteins are collected and concentrated using protein size exclusion filters and samples are split for purity check, SDS-PAGE separation, LC-MS/MS peptide identification, and for long-term storage. Pollen tubes emerged from the cut

pistil can also be excised and stored if desired for total protein extraction or for RNA sequencing.

6. The duration allocated to some steps can be reduced if more coworkers are involved, however, high care must be taken as this might result in bigger variabilities between samples as a result of individual handling.

3.2 Seed Germination and Plant Growth

1. For plants that will be grown to the outdoor green house, seeds should be sown 3 months before the start of the experiment.
2. Compost mixture preparation: Rehydrate Jiffy tablets with water in an appropriate container (e.g., a beaker). After rehydration, transfer the soaked tablets to pots and/or trays for subsequent sowing and/or planting (*see* **Note 5**).
3. Seed germination: Use the compost mixture to fill each pot (8 cm in diameter) and resurface the top soil. Sow approximately 100 seeds per pot and slightly cover the seeds with soil using a sieve. Label the pots and place them on a large tray to facilitate easy watering from the bottom of the tray.
4. Place the pots in a cold room (7 °C) for 2 days to stratify the seeds. Next, transfer the pots to the growth room for seed germination (*see* Subheading 2. Materials for plant material).
5. Alternative seed germination for plant growth in growth chamber: sterilize the seeds in 1% (w/v) sodium hypochlorite (90% bleach SAVO, Biochemie group a.s.) for 10 min, wash with sterilized water four times, and plate on half strength MS agar. After stratification at 4 °C in the dark for 3 days, move the plates to the growth chamber set at 25 °C under a 16-h-light/8-h-dark photoperiod for plant growth. After 8–10 days, transplant the seedlings to soil.
6. *Pause point*: Seedlings should be grown for 2 weeks.
7. Seedling planting: Fill 20–40 18-cm-diameter pots with a compost mixture. Make a small indentation into the soil with an index finger. Transfer the seedlings into the holes and let them grow under standard greenhouse conditions, 22–25 °C under short day for 6 weeks.
8. Growing plants in an outdoor green house under natural day–night photoperiods in spring and summer: Cut the stems of adult plants with fully developed roots to leave four bottom leaves. Let the new inflorescence branches to emerge (~2 weeks) and transfer into soil spacing the plants approximately one meter apart.
9. *Pause point*: Plantlets should be grown for 4–6 weeks.

3.3 Collection of Mature Pollen Grains for Pollination (Timing 90 min)

1. Approximately one week prior to the start of SIV-PS experiment, mature pollen grains to be used for pollination need to be collected on a daily basis and stored at −20 °C . Most effective method is to collect the immature flowers from 20–30 plants at stage 6 of development (52–55 mm in length) containing immature pollen grains 1 day before anthesis (Fig. 2) [22]. These anthers are dried overnight at room temperature and pollen grains are easily filtered out using prearranged glass cylinder and sieve.
2. Prepare one or two 1 L plastic beakers layered with wet paper towels. First remove and dispose all open flowers containing dehisced mature pollen grains. Collect all stage 6 flowers that are 1 day before anthesis (52–55 mm) into the beakers (*see* **Note 6**).
3. Bring the flowers to the laboratory, prepare two-third 16 cm glass Petri dishes layered with 0.1 cm Whatmann paper or equivalent. Manually open the flower petals longitudinally to expose the anthers and pull-off the anthers into the Petri dish (Fig. 2). Continue until the bottom of the Petri dish is covered with anthers with enough space between anthers, approximately 5/cm^2 density. If more flowers are left, start a new Petri dish (*see* **Note 7**).
4. Leave the anthers on a laboratory bench to dry at room temperature overnight.
5. On the next day, install a legging net (or equivalent) slightly stretched on an open ends glass cylinder 3 cm × 3 cm and secure it with a rubber band (Fig. 2). Gather the now dehisced anthers into the prearranged glass cylinder and filter through the pollen grains by gently milling the anthers with a spatula.
6. Transfer the obtained pollen grains powder into a sterile 1.5 ml microcentrifuge tube.
7. Weight the pollen grains collected and store at −20 °C (*see* **Note 8**).

3.4 Flower Emasculation [Day 1] (Timing 120 min)

1. Counted as SIV-PS Day 1, the next step is the emasculation of N. tabacum flowers (Fig. 4).
2. Prepare 30–40 netting bags (approximately 10 cm × 10 cm) and equivalent number of garden tying strings.
3. Remove all open flowers containing dehisced mature pollen and discard them.
4. Manually open stage 6 flowers (52–55 mm closed buds containing undehisced anthers [22]) and remove all the anthers. Continue to do so until all flowers available from a single plant have been emasculated. (*see* **Notes 9** and **10**).

5. Using the netting bags, cover the inflorescences holding the emasculated flowers and secure them with tying strings (Fig. 4).
6. Leave the flowers overnight for stigma to fully mature.

3.5 Pollination of Emasculated Flowers [Day 2] (Timing 60 min)

1. On day 2, using a bright brush bristle, load the brush with pollen and pollinate each stigma sufficiently without over saturating the stigma. Pollinated flowers are covered with the net and left in planta for another 24 h.
2. Remove, from −20 °C, an aliquot of frozen tobacco mature pollen grain powder and let it acclimatize to room temperature for 5 min (*see* **Note 11**).
3. Remove the netting bags with care not to damage the flowers or the inflorescences.
4. Using a bright brush bristle, pollinate each stigma (*see* **Note 12**).
5. After completing the pollination of all emasculated flowers, cover back all the inflorescences with the net bag and leave for pollen tubes to germinate overnight in planta.

3.6 Harvesting of Pollinated Flowers [Day 3] (Timing 60 min)

1. After the 24 h of pollen tube growth in vivo, pollinated pistils are collected for the final step of in vitro germination. Flowers are collected into a plastic beaker layered with wet paper towels to avoid pistil dehydration especially during hot summers. The collection should not last more than 30 min and ideally should be done in the early morning. Collection of bigger number of flowers at once is not recommended. If many flowers have been pollinated to obtain several replicates, the collection can be done in batches of four replicates per round of collection by two efficient coworkers.
2. If more than one worker is involved, prepare an equivalent number of 1 L plastic beakers layered with 2–3 wet paper towels.
3. Remove all the netting bags and manually detach each emasculated flower (pollinated and unpollinated flowers) and place them in to the 1 L beaker.
4. Cover the collected flowers with one wet paper towel for transportation to the lab.
5. Leave the plants for 1–2 days to recover before the second experiment can be repeated.

3.7 Preparation of Germination Chamber (Timing 20 min)

1. Unpack the germination chambers from aluminum foil in the flow box.
2. Titrate 40 mL of tobacco pollen tube germination media into each germination chamber (Fig. 3).
3. Seal the top of the germination chambers with sterile parafilm and secure with a rubber band (Fig. 3, *see* **Note 13**).
4. Puncture holes in a clockwise manner with a 21 G × 1–1.5″ needle leaving 2–3 mm gaps between holes (Fig. 3). Continue until the center of the parafilm has been perforated. This normally equates to approximately ×50 holes. Enlarge the hole using a sterile yellow tip for easy penetration of the pistils (Fig. 3).
5. Label the side of each germination chamber and transfer them to the bench.

3.8 Arrangement of Pistils on Pollen Tube Germination Chamber (Timing 120 min)

1. Prepare new 21 G × 1–1.5″ needle, ruler, and clean forceps.
2. Take a flower and manually detach the pistil at the attachment with the ovary.
3. Align the pistil with a ruler on a paper towel. Using the 21 G × 1–1.5″ needle, excise the pistil at 22 mm below the stigma shoulders (Fig. 4, *see* **Note 14**, *see Troubleshooting*—Table 1).
4. Using forceps, place the excised pistil into one of the holes of the germination chamber containing fresh pollen tube germination media (SMM-MES). Arrange the pistils spirally (Fig. 4). The pistil cut end should be fully submerged in to the germination media (Fig. 1, *see* **Note 15**).
5. Follow similar steps for pollinated and unpollinated pistils.
6. Continue until all flowers have been processed.

3.9 Semi-In Vivo Pollen Tube Germination (Timing 20 min)

1. Prepare an equivalent number of Petri dish bottoms to pollen germination chambers layered with wet paper towels.
2. Transfer the pistil-loaded germination chambers on to a Petri dish bottoms,
3. Cover the germination chambers with 500 mL beakers to create a humid chamber (*see* **Note 16**).
4. Incubate the complete germination chambers for 24 h at 28 °C without shaking.

3.10 Secretome Collection [Day 4] (Timing 30 min)

1. The PT secretome is collected after 24 h of SIV pollen tube growth; suspended pistils with protruded pollen tubes are removed and pollen tubes can be excised and collected or discarded if not required. The media containing pollen tube secreted proteins is transferred into falcon tubes and kept on

Table 1
Troubleshooting

Step	Problem	Possible reason	Solution
3.8	No pollen tube growth from pistil explant	Old pollen or bad media	Check the stored pollen and the media by in vitro pollen tube germination
3.10	No pollen tube emergence from the pistil	Mutilated excision of the pistil	When excising the pistil, use a sharp 21G1 needle to minimize tissue damage
3.12	Very low secretome protein concentration	Poor pollen tube germination Not enough pistils arranged The secretome sample was not concentrated enough High amount of proteases in the media	Make sure you have pollen germination >90% of the pollinated pistils Arrange enough pistils (~50) per secretome chamber Concentrate the samples further if necessary to <500 μl or use less starting pollen germination media depending on the type of chamber used Addition of proteases inhibitors is not recommended as it might influence pollen tube secretome
3.13	Excessive cytosolic contamination	Bad pollen tube germination media or not well buffered	Make fresh media and check the pH regularly. Check pollen tubes integrity after incubation
3.18	High rate of nonviable pollen tubes	pH of the media is off Pollen tubes were left too long Staining solution induced pollen tube burst	Check the pH Assess pollen tube viability immediately after collection Check and quantify the pollen tubes by light microscopy prior to any staining

ice. Pistil materials are used for cytological tests including pollen tube viability and pharmacological treatments to assess pollen tube endomembrane secretion activities. For pollen tube excision (Subheading 3.11) from the cut pistils, pistils are placed on a sterile pre-wet glass slide under a dissection microscope and pollen tubes are excised along the cross-section with a sterile 21 G × 1–1.5″ needle. The pollen tube pellet is immediately transferred into a cool 1.5 mL microcentrifuge tube on ice containing buffer with RNase inhibitor. Once the collection of pollen tube is completed, the samples are flash frozen in liquid nitrogen and stored at −80 °C. The media containing secreted proteins is then processed (Subheading 3.12).

2. Check if the pollen tube bundles have emerged from the pistil explant before proceeding (Fig. 1, *see Troubleshooting*—Table 1).

3. If pollen tube bundles need to be collected, first perform pollen tube excision prior to collecting the secretome (*see* Subheading 3.11).
4. Remove gently the rubber band and the parafilm from the germination chamber.
5. Decant all the germination media containing secreted proteins into sterile 50 mL Falcon tubes. Leave the tubes on ice.
6. Proceed with secretome concentration (Subheading 3.12).
7. *Pause point*: Collected secretome can be stored at −20 °C for shorter time or at −80 °C for longer time storage prior to protein concentration.

3.11 Excision of Pollen Tube Bundles (Timing 30 min)

1. Sterilize a microscope glass slide by wiping with 100% pure ethanol.
2. Place the slide under dissection microscope. Wet the slide with approximately 20 μL of the pollen tube germination media.
3. Using forceps, lift off a pistil explant with pollen tube bundle and lay it on a slide so that the pollen tubes are extended horizontally along the slide without too much tangling.
4. Use a sterile 21 G × 1–1.5″ 1 needle and excise the pollen tube bundle longitudinally.
5. Transfer the pollen tube bundles using the needle into a prearranged cooled 1.5 mL microcentrifuge tube.
6. Once all pistil explants have been processed, resuspend the pollen tube bundles with RNA extraction buffer containing RNases inhibitors or a protein extraction buffer containing protease inhibitors for later RNA or protein extraction. Freeze the samples immediately in liquid nitrogen and store at −80 °C (*see* **Note 17**).

3.12 Concentration of the Secretome (Timing 60–90 min)

1. Obtained secretome samples are generally in low ng/μL levels in 50 mL pollen tube germination medium. For more reliable and accurate downstream applications, secretome samples should be concentrated using 3 kDa or a 10 kDa ultracentrifugation filters. This also allows partial dialysis of the samples from metabolized amino acids, salts, and sugars present in pollen tube germination medium that could interfere with LC-MS/MS. The duration for sample concentration is approximately 30–60 min to reduce the volume to ~300 μL per sample. This might vary depending on the protein concentration of the secretome. To avoid freeze-thaw cycles, concentrated samples are aliquoted for multiple applications and stored.
2. Arrange a required number of Amicon Ultra 2 mL 10K Centrifugal filters (Merk Millipore) (*see* **Note 18**).

3. Load each filter with 2 mL of the secretome supernatant.
4. Gently arrange the filters in a fixed angle rotor and centrifuge at 7500 × *g* for 10 min at 15 °C (*see* **Note 19**).
5. Discard the flow through and reverse spin to collect the retained secretome.
6. Continue until the whole secretome has been concentrated from 50 mL down to 0.5–2 mL (*see* **Note 19**).
7. Load the filter with the remaining 2 mL of the secretome and centrifuge at 1000 × *g* for 2 min at 15 °C. Continue until 300–500 μL is achieved (*see* **Note 20**).
8. Aliquot approximately 150–200 μL to be used for protein concentration measurements (e.g., 2-D Quanti kit, GE-healthcare), purity test by enzymatic assay and SDS-PAGE analysis (*see Troubleshooting*—Table 1).
9. Store the remaining samples at −80 °C for gel-free LC-MS/MS protein identification.
10. *Pause point*: Store the SIV-PS secretome at −80 °C until further use.

3.13 Purity Test Using ADH Calorimetric Assay (Timing 90 min)

1. All secretome samples are subjected to contamination by proteins from the cytoplasm and to a laser extent from the cell wall depending on the method of secretome collection used. Therefore, purity test must be conducted to estimate the levels of contamination and evaluate whether the obtained samples are of reliable quality for downstream analysis. Enzymatic assays provide sensitive, reliable, and quantitative estimation of secretome purity due to their abundance. Commonly used enzymes as biomarkers of cytosolic contamination include alcohol dehydrogenase (ADH), glucose-6-phosphate dehydrogenase (G6PDH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and malate dehydrogenase (MDH). Secretome purity is conducted by measuring ADH activities in secretome samples and compared with ADH activities in a total cell extract. Ideally, the same materials used to obtain the secretome should be used to obtain the total cell extract. Instead of ADH activities, western blot can be performed to compare ADH levels between the samples (Subheadings 3.14–3.16). The advantage of the ADH assay is that it provides a direct quantitative measure with reliable estimation of contamination, whereas western blot does not unless it is done very carefully with specialized imaging equipment and appropriate controls [23]. ADH activity is measured over time and secretome samples purity is established (Fig. 1).
2. Extract total proteome from SIV pollen tubes and quantify amount of proteins for each sample.

3. Switch on a spectrophotometer capable of measuring absorbance at 450 nm (*see* **Note 21**).
4. Acclimatize the ADH assay buffer to room temperature.
5. Reconstitute the NADH standard and the developer solution with ultrapure water.
6. Prepare NADH standard by diluting 10 μL of the 10 mM NADH stock solution with 90 μl of the ADH assay to generate a 1 mM standard solution. Pipette 0, 2, 4, 6, 8, and 10 μL of the1 mM standard solution into a 96 well plate in duplicates. Add ADH Assay Buffer to each well to bring the total volume to 50 μL per well (*see* **Note 22**).
7. Aliquot 3, 6, and 9 μg (or at least two different concentrations) of the secretome samples and their controls in duplicate into the same 96 well plate. Bring the final volume to 50 μL per well with the ADH buffer.
8. Prepare 100 μL of reaction mix per sample by mixing 82 μL ADH assay buffer, 8 μL developer solution, and 10 μL of 2 M UV pure ethanol (*see* **Note 23**).
9. Pipette 100 μL of the appropriate reaction mix to each well. Mix well using a horizontal shaker.
10. Incubate the plate at 37 °C in a dark without shaking.
11. After 2 min of incubation (T_{initial}), record the initial absorbance at 450 nm ($A450_{\text{initial}}$).
12. Return the plate to the incubator and continue recording the absorbance at 5 min intervals.
13. When the absorbance value of the most active sample is greater than the value of the highest standard (10 nmol/well), stop with the measurements to avoid values exceeding the end of the linear range of the standard curve.
14. For calculating ADH activities, use the final measurement ($A450_{\text{final}}$) and time (T_{final}) of the most active sample prior to exceeding the end of the linear range of the standard curve.
15. Subtract the final measurement $A450_{\text{final}}$ of the 0 (blank) NADH standard from the $A450_{\text{final}}$ of the standards and the samples.
16. Plot the NADH standard curve of the absorbance $A450_{\text{final}}$ (nm) against time (min).
17. Obtain the change in absorbance ($\Delta A450$) between T_{initial} and T_{final} by subtracting $A450_{\text{final}} - A450_{\text{initial}}$, i.e., $\Delta A450 = A450_{\text{final}} - A450_{\text{initial}}$.
18. Use this absorbance value to obtain the amount of NADH generated between T_{initial} and T_{final} (B) from the standard

curve using a Y-intersection formula (Microsoft Excel or similar).

19. Compute the ADH activities (nmol/min/mL, mU/mL) for each sample as follows:

$$\text{ADH activity (nmol/ min /mL)} = \frac{B \times \text{dilution factor of the sample}}{T_{\text{final}} - T_{\text{initial}} \times V(\text{sample volume added})}.$$

B = amount (nmol) of NADH between T_{initial} and T_{final}.

20. Plot a scatter graph of ADH activities against time for all secretome samples and their respective positive controls for comparison and estimation of contamination levels (*see* **Note 24**, *see Troubleshooting*—Table 1).

3.14 SDS-PAGE Analysis for Western Blotting (Timing 60 min)

1. SDS-PAGE of the secretome samples is not essential in the SIV-PS method since protein detection is done by gel-free LC-MS/MS. However, SDS-PAGE needs to be performed when western blot is a desired method for the secretome purity test or when performing a glycosylation test to establish the fraction of glycosylated secreted proteins. Secretome samples are mixed with SDS-PAGE running buffer in a 1:1 (v/v) ratio, sonicated for 5 min, and standard SDS-PAGE procedures are followed using *12.5% Mini PROTEAN® 3*. To obtain higher sensitivity of protein bands on the gel, silver stain should be used instead of Coomassie Brilliant Blue G250 stain. Generally, ~40 μg per well is run on an SDS-PAGE to obtain reliable sensitivity and visualization of differential protein profiles.
2. Protein transfer to nitrocellulose membrane for downstream application such as immunoblotting or N-glycosylation test can be done using wet transfer or a semidry transfer system. The wet transfer system is lengthier (at least 20 min of preparation and approx. 45 min running) but provides an overall efficient transfer of all protein size range and abundance. Conversely, the semidry transfer system is much more convenient in handling (10 min of preparation) and has shorter running time (10 min) but it is less efficient in overall protein transfer. For routine usage and in longer runs, the semidry system is also more expensive. We recommend usage of the semidry system only during immunoblotting of known secreted protein or for a trial test of any downstream application. Wet transfer should be performed for any unknown secretome sample at least for the first attempt as it improves transfer of low MW proteins.
3. Mix 2.5 mL of resolving buffer, 3.33 mL of acrylamide mixture, and 4 mL water in a 50 mL conical flask. Degas with helium for 10 min. Add 100 μL of SDS, 80 μL of ammonium persulfate, and 10 μL of TEMED and cast gel within a 7.25 cm × 10 cm × 1.5 mm gel cassette. Allow space for stacking gel and gently overlay with isobutanol or water.

4. Prepare the stacking gel by mixing 1.25 mL of resolving buffer, 0.67 mL of acrylamide mixture, and 3 mL water in a 50 mL conical flask. Degas with helium for 10 min. Add 100 μL of SDS, 40 μL of ammonium persulfate, and 5 μL of TEMED. Insert a 10-well gel comb immediately without introducing air bubbles.
5. Heat aliquots of bovine Ro 60, RBC membranes, and human spectrin antigens at 95 °C for 5 min. Do not add lysis buffer to the prestained protein standard or subject it to heat. Centrifuge the heated samples at 3000 × *g* for 30 s to bring down the condensate. Load increasing amounts of Ro antigen (1–4 μg) on one gel and the same amounts of spectrin (3 μg/lane) or RBC membrane antigens on two other gels along with protein standards (10 μL/well–2 μg/marker/lane). Add protein standards in every other lane (alternating with spectrin) in the gel with spectrin. Electrophorese at 15 mA until the sample has entered the gel and then continue at 25 mA till the dye front (from the BPB dye in the samples) reaches the bottom of the gel.
6. Following electrophoresis, pry the gel plates open with the use of a spatula. The gel remains on one of the glass plates. Rinse the gel with water and transfer carefully to a container with western blot transfer buffer.
7. Cut a nitrocellulose membrane to the size of the gel and immerse in methanol. Rinse twice in distilled water and once with transfer buffer.
8. Protein transfer to nitrocellulose membrane can be performed in a wet system (Subheading 3.15) using Mini PROTEAN® 3 protein transfer system (Bio-Rad, Subheading 3.15) or by a semidry method (Subheading 3.16) using eBlot protein transfer system.

3.15 Western Blot Using Mini PROTEAN® 3 Protein Transfer System (Timing 60 min)

1. After gel electrophoresis (Subheading 3.14), carefully wrench the gel plates with the use of a plastic spatula. The gel remains attached on one of the glass plates. Using a glass spatula, cut off the stacking gel and carefully transfer the resolving gel into a plastic container containing blotting buffer.
2. Submerge the gel into blotting buffer and incubate for 10 min.
3. Change to a fresh blotting buffer and incubate for another 10 min.
4. Repeat **step 3** and incubate for additional 10 min (*see* **Note 25**).
5. Cut a nitrocellulose membrane to the size of the gel and immerse in blotting buffer. Incubate for 30 min.

6. Assemble the blotting sandwich in a plastic tray containing blotting buffer according to MiniProtean manufacture instructions (*see* **Note 26**).
7. Remove any air bubbles by rolling a glass rod on the blotting sandwich.
8. Load the blotting sandwich into the electrode module with the black side of the sandwich facing the black side of the electrode module. Place the complete module on top of magnetic table stirrer.
9. Slot the ice container to the electrode module to allow cooling during blotting.
10. Place and start the magnetic stirrer.
11. Fill the electrode tank with blotting buffer and plug the electrodes to the powerpack.
12. Blot the gel for 1 h at 350 mA.
13. Remove the blots out of the sandwich and proceed with immunoblot protein detection.

3.16 Western Blot Using GenScript eBlot Semidry Transfer System (Timing 20 min)

1. After electrophoresis (Subheading 3.14) rinse the gel with deionized water for 10 min on a horizontal shaker.
2. Decant the water and repeat **step 1** two more times.
3. Set up the eBlot apparatus according to manufacture instructions.
4. Equilibrate a precut nitrocellulose membrane for 1 min in equilibration buffer.
5. Place the equilibrated membrane on the eBlot Anode pad and aspirate any air bubbles using a plastic shovel.
6. Carefully place the pre-run SDS-PAGE gel on top of the membrane and remove any air bubbles.
7. Place an appropriate size gel window (88 × 78 mm) ensuring the gel window fully covers the margins of the membrane.
8. Place the eBlot Cathode pad and close the lid.
9. Set running time to 10 min and start the eBlot run.
10. Remove the blots and proceed with immunoblot protein detection and glycosylation test (*see* **Note 27**).

3.17 Pollen Tube Viability, Intactness, and Secretory Activities

1. Viability and intactness is assessed by visualizing cytoplasmic streaming of the pollen tubes using differential interference contrast microscopy (DIC) (Subheading 3.18) as well as using Alexander staining (Subheading 3.19) and propidium iodide (PI) (Subheading 3.20) to estimate proportions of pollen tube burst. Pollen tubes from the SIV-PS assay are

reproducibly intact with active cytoplasmic streaming. The secretion activities of the SIV-PS pollen tubes can be tested using a combination of pharmacological with endocytic markers.

2. Secreted proteins that follow the canonical secretory pathway are glycosylated while translocated into the lumen of endoplasmic reticulum. To detect the N-glycoproteome of the SIV-PS secretome, secreted proteins are separated on SDS-PAGE, and thereafter transferred onto nitrocellulose filter by western blot. Obtained blots are incubated with Concavalin A (ConA) as primary antibody followed by the incubation with horseradish peroxidase (HR) as a secondary antibody. Glycosylated fraction of the secretome is then visualized by monitoring bound peroxidase activities in the presence of 4-chloro 1-naftol and 30% hydrogen peroxide as a substrate. The chemiluminescent reaction results in the development of purple colored bands as sites of HR activity. The reaction is stopped by the addition of water. Stained protein bands can be excised and identified by LC-MS/MS.

3.18 Cell Viability: Pollen Tubes Cytoplasmic Streaming (Timing 20 min)

1. Place a single pollen tubes bundle onto a microscope glass slide containing 20 μL of fresh pollen tube germination media. Cover the slide with a cover slip.
2. Using a confocal microscope or any DIC equipped microscope, set the microscope to DIC mode, and observe streaming of the pollen tube cytoplasm. A viable pollen tube should show a fast inwards fountain like flow of the cytoplasm originating from the pollen tube base toward the tip. Simultaneously, quantify integrity of the pollen tubes (*see* **Note 28**, *see Troubleshooting*—Table 1).

3.19 Cell Viability: Alexander Staining (Timing 30 min)

1. Transfer a single pollen tube bundle onto a microscope glass slide containing 10 μL of Alexander staining solution. Cover the slide with a cover slip.
2. Incubate the pollen tube samples in the staining solution at room temperature for few minutes.
3. Visualize by bright-field microscopy. Viable pollen tubes are stained pink-to-purple, nonviable cells are stained green.

3.20 Cell Viability: PI Staining (Timing 30 min)

1. Transfer a single pollen bundle onto a microscope glass slide containing 10 μL of 20 μg/mL PI solution. Cover the slide with a cover slip and incubate in the dark for 10 min.
2. Visualize the staining with appropriate filter setting, e.g., TRITC HYQ filter set (590–650 nm, Nikon) in the Nikon TE2000 fluorescence microscope or the RFP channel configuration using the 560 nm laser on the confocal microscope.

Viable cells should be stained only in the pollen tube cell wall and the dye should not have penetrated the pollen tube. Viability can be estimated by scoring the ratio of viable:nonviable pollen tubes.

3.21 N-Glycosylation Test

1. Secreted proteins that follow the ER-Golgi pathway are post-translationally modified by addition of glucan moieties termed N-glycosylation. Detection of protein glycosylation provides independent verification of the pathway of protein secretion.
2. Resolve the secretome, ~40 μg per sample, on SDS-PAGE (Subheadings 3.14–3.16) and perform western blot.
3. Stain the nitrocellulose membrane with Ponceau S staining to verify western blot protein transfer.
4. Incubate the membrane in TTBS buffer and shake at 25 rpm for 30 min.
5. Exchange with fresh TTBS buffer, incubate for another 30 min.
6. Incubate the membrane with Concavalin A (2.5 mg/100 mL) in TTBS buffer for 1 h.
7. Wash the membrane with TTBS buffer for 30 min.
8. Incubate the membrane with horseradish peroxidase (5 mg/100 mL) in TTBS buffer for 45 min.
9. Transfer the membrane to a container containing detection buffer, 45 mg 4-chloro 1-naftol in 15 mL methyl alcohol and 60 ml 10 mM Tris–HCl, pH 6.8.
10. To visualize the staining, slowly add 25 μL of 30% hydrogen peroxide at one time to the detection while constantly mixing.
11. Continue to add 25 μL of 30% hydrogen peroxide (maximum 75 μL) until desired intensity of the bands has been achieved.
12. Stop the reaction by replacing the detection buffer with deionized water (*see* **Note 29**).

3.22 Tentative Bioinformatics Workflow

Following label-free quantitative LC-MS/MS, evaluate secreted proteins as follows;

1. Consider protein groups as secreted based on Top3 protein algorithms stipulating that (a) the ratio of the calculated median of a protein group in a sample to the average median of the control unpollinated samples is >3 and (b) the number of total peptides is, at a minimum 3, and that (c) the protein group must be upregulated in at least half of the replicates. For sample comparison, individual protein accessions from replicates of all sample types are combined into supergroups (SGs).

2. Filter out protein groups according to the algorithms above. Alternative (less stringent) approaches can be adopted after thorough evaluations based on custom LC-MS/MS output.
3. *Using the obtained putative "secreted list" perform a batch analysis as follows*:
   (a) Assess presence of N-terminal signal peptide using SignalP v5.0 (http://www.cbs.dtu.dk/services/SignalP/) and iPSORT (http://ipsort.hgc.jp) algorithms.
   (b) For the remaining non-signal peptide containing proteins, assess their secretion probabilities as unconventionally secreted proteins using SecretomeP v2.0 server (http://www.cbs.dtu.dk/services/SecretomeP/) with animal sequence trained algorithms.
   (c) In parallel, use TargetP v2.0 (http://www.cbs.dtu.dk/services/TargetP-2.0/index.php) and WoLF PSORT (https://wolfpsort.hgc.jp) servers to predict subcellular localization of all accessions from the identified putative secreted list.
   (d) For posttranslational modification, use NetNGlyc v1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/) to predict protein glycosylation and complement the conventional or unconventional secretion pathway predicted from the above analysis.
   (e) To assess membrane affinity, use PredGPI (http://gpcr.biocomp.unibo.it/predgpi/), FragAnchor and big-PI (http://www.embl-heidelberg.de/beisenha/gpi_p_prediction.html) and CSS-Palm v4.0 (http://csspalm.biocuckoo.org) to predict if a protein contains a glycosylphosphatidylinositol (GPI)-anchor regions for plasma membrane anchoring or sites for posttranslational palmitoylation.
   (f) Use TMHMM v2.0 (http://www.cbs.dtu.dk/services/TMHMM/), DAS-TMFilter (http://www.enzim.hu/DAS/DAS.html), HMMTOP v2.0 (http://www.enzim.hu/hmmtop/) and PROSITE (https://prosite.expasy.org) servers to eliminate plasma membrane destined proteins that could have contaminated the extracellular secretome.
   (g) Above workflow is a guideline only; consider using additional and latest servers for more confident prediction.
   (h) From the verified in silico list, select representative candidate accessions to verify their secretion by immunolocalization and fluorophore tagging by live cell imaging.

3.23 Anticipated Results

Capturing and purification of pollen tube secreted proteins using the SIV-PS workflow yields typically 4 μg/μL of protein, but varies between 1 and 6 μg/μL depending on the efficiency of pollen tube germination from the pistil explants. This amount of protein is sufficient for performing LC-MS/MS mass spectrometry for sequencing of secreted peptides, ADH purity test, glycosylation test, SDS-PAGE protein profiling, and immunodetection. For detection of externally anchored proteins, such as GPI-anchored proteins, an enrichment step is necessary to increase detection based on our empirical results. A treatment of the pollen tubes after germination prior to secretome collection with phospholipase C should allow enrichment of the GPI-anchored proteins in the SIV-PS secretome. The estimated cytosolic contamination of the SIV-PS procedure is expected to be <1.5% ADH activities, whereas pollen tube burst consistently remained under 13%. The number of detected proteins (peptides) is variable between samples (likely reflecting secretome dynamics). In our hands we observed an average of 800 protein groups that are reliably detected. The number of groups obtained combined with quantitative LC-MS/MS allows critical evaluation of secretome dynamic between replicates and in different treatments (such as with/without ovule stimulation/BSA/Wortmanin pharmacological treatment) as well as authenticity of the pollen tube secreted proteins. Application of stringent algorithms prior to bioinformatics analysis is necessary to construct a pollen tube specific pistil induced secretome.

4 Notes

1. Preferably should be <5 months old to achieve better protein band resolution.
2. To prepare 1% (w/v) bromophenol blue, mix 0.5 g bromophenol blue with 50 mL of 50% (v/v) isopropanol.
3. To prepare blotting buffer, mix 200 mL of methanol with 100 mL of 10× electrode buffer. Adjust the volume to 1 L with water.
4. Preferably prepare the working stock fresh. However, a pre-prepared 40% stock can be stored at −20 °C for a week.
5. When the tablets are rehydrated, the volume increases fivefold. This fact should be taken into consideration when planning the container size. The diameter of a fully swollen tablet is ca 3.5 cm.
6. Stage 6 flowers can be easily identified by the pink coloration of the petals tip ends in the closed state. Care must be taken not to collect younger flowers. If younger flowers are collected, the anthers will not mature and dehisce to release mature pollen

grains. It is better to collect flowers at early mornings, at 7–8 a.m., as greenhouses get warmer in later times during the summer period.

7. If too many anthers are collected in one Petri dish, drying of the anthers will not be efficient and fewer pollen grains will be recovered.
8. Pollen grains should be collected for at least two weeks consecutively (or can be randomized over the summer to achieve an equivalent of two weeks collection) to ensure that enough materials are available for pollination. Collected tobacco pollen grains are viable for at least 2 years if stored properly at −20 °C. Pollen grain viability can be tested by in vitro germination at an interval of three months following storage. One summer collection provides enough pollen material for 2–3 years worth of SIV-PS experiment or for a general pollen grain usage.
9. It is highly recommended to set up a negative control for the secretome study. Therefore, when emasculating flowers, it should be taken into consideration that half of those flowers will be left unpollinated to establish the background set of proteins present in the media that would originate from the unpollinated pistil itself.
10. If desired, gloves can be used to avoid tanning stain on fingers. Although rarely happens, processed flowers should be netted overnight to avoid potential cross pollination and allow pistil maturity (Fig. 4). Emasculation of higher proportion of immature flowers, <48 mm, increases the chance of lack of pollen tube germination through the pistil, and thus substantially reducing the amount of protein recovered in the secretome.
11. We estimate that approximately 300 μL (~200 mg) equivalent of pollen grains are sufficient for a limited pollination of 50 emasculated flowers. This amount is adjustable depending on the individual worker.
12. Do not over pollinate the stigmas as excess pollen grains will germinate not only through the pistil but also sideways and likely to encourage fungal growth on top of the germination chamber. Fungal secreted proteins might then contaminate the secretome of the pollen tube.
13. Parafilm can be sterilized simply with 100% ethanol for 1 min and let to dry in the flow box before use.
14. For 50 pistils (equivalent of one replicate), a single 21 G × 1–1.5″ needle is sufficient for the excision of the style ends before the needle loses its sharpness.
15. If the germination medium is not sufficient to cover the pistil bottoms, additional medium can be added by pipetting through one of the pre-made holes.

16. Ensure that the interface between the beaker and the petri dish bottom is fully sealed with a wet paper towel to maintain humidity. More coworkers can be recruited to shorten the time of flower collection.
17. It is important to proceed promptly with the dissection of the pollen tube bundles to avoid potential loss of pollen tube viability and RNA degradation.
18. For faster secretome concentration, more than one filter can be used per secretome sample. At later stages of concentration, these can be pooled again to represent a single sample.
19. See Merck Millipore ultracentrifugation guide lines for your specific centrifuge.
20. Do not centrifuge at higher RCF as this will permeabilize the membrane leading to protein loss, particularly low MW proteins.
21. Where available, a plate reader provides a much more convenient way as well as accuracy of recording absorbance at the time intervals required and should be used instead of a standard single sample spectrophotometer.
22. If performing the ADH assay for the first time, include a positive control supplied. The standard curve generated does not need to be repeated if all measurements will be performed within 1 week. Where possible, use the black bottom 96 well plate to minimize light interference during incubation.
23. Free NADPH and NADH in the samples will generate background signal, therefore for each sample prepare a reaction mix without ethanol. The ethanol can be substituted with ADH assay buffer. The obtained background values will be subtracted from each sample reading prior to calculating ADH activities of the samples.
24. Always use ultrapure water to reconstitute the reagents. When reconstituting the developer solution, mix well by pipetting but do not vortex the mixture. Aliquot the developer solution in 100 μL into nontransparent 1.5 ml microcentrifuge tubes or wrap the tubes with aluminum foil to protect from direct light. Store at −20 °C and use within 2 months. After reconstituting the NADH standard, also use within 2 months.
25. The blotting buffer must be prepared fresh and should not be reused after gel equilibration.
26. The order of the sandwich layers must be kept to avoid protein transfer to the wrong layer. The orientation of the gel will be reversed (mirrored) after blotting, this should be taken into consideration with the orientation of the protein marker.

27. After the eBlot run, do not leave the membrane in the assembly unit for too long as this will result in protein diffusion and loss of protein band resolution. To ensure efficient transfer, make sure the titanium plates of the eBlot system are clean and free from salts and the nitrocellulose membrane is sufficiently equilibrated.
28. The cytoplasmic streaming events can be recorded in a time series as a proof of viability.
29. The 30% hydrogen peroxide has to be added gradually to avoid oversaturation of the blot. Once oversaturated, the staining is not reversible.

Troubleshooting advice can be found in Table 1.

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