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Application Article

Application of proteomics to the study of pollination drops[1]

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- *Premise of the study:* Pollination drops are a formative component in gymnosperm pollen-ovule interactions. Proteomics offers a direct method for the discovery of proteins associated with this early stage of sexual reproduction.
- *Methods:* Pollination drops were sampled from eight gymnosperm species: *Chamaecyparis lawsoniana* (Port Orford cedar), *Ephedra monosperma*, *Ginkgo biloba*, *Juniperus oxycedrus* (prickly juniper), *Larix ×marschlinsii*, *Pseudotsuga menziesii* (Douglas-fir), *Taxus ×media*, and *Welwitschia mirabilis*. Drops were collected by micropipette using techniques focused on preventing sample contamination. Drop proteins were separated using both gel and gel-free methods. Tandem mass spectrometric methods were used including a triple quadrupole and an Orbitrap.
- *Results:* Proteins are present in all pollination drops. Consistency in the protein complement over time was shown in *L. ×marschlinsii*. Representative mass spectra from *W. mirabilis* chitinase peptide and *E. monosperma* serine carboxypeptidase peptide demonstrated high quality results. We provide a summary of gymnosperm pollination drop proteins that have been discovered to date via proteomics.
- *Discussion:* Using proteomic methods, a dozen classes of proteins have been identified to date. Proteomics presents a way forward in deepening our understanding of the biological function of pollination drops.

Key words: conifers; gnetophytes; gymnosperm; mass spectrometry; pollination drop; proteomics.

Pollination drops are unique to gymnosperms. Receptive ovules secrete a liquid that mediates pollen capture and triggers germination. Understanding the composition of pollination drops is key to elucidating their role in pollen-ovule interactions. Drops are produced by nucellar tissue and secreted into the micropyle. Visible to the naked eye, these drops range in volume from 10–1000 nL. Depending on the species, drops are released either to coincide with pollen release or with egg receptivity. The differences in the timing of drop release varies among gymnosperms, because prefertilization ovule and pollen development differs among the major extant clades, i.e., cycads, conifers, *Ginkgo* L., and gnetophytes. Pollen-ovule interactions are especially diverse among particular groups, such as Pinaceae and Podocarpaceae. In this application paper, we demonstrate that proteomics provides powerful tools for revealing pollination drop biochemistry, which is currently very poorly understood.

Although a great deal has been written about the diversity and evolution of pollination drops within a morphological context (Doyle, 1945; Owens et al., 1998; Gelbart and von Aderkas, 2002), far less is known about their biochemistry (Nepi et al., 2009) and physiology (Tomlinson et al., 1997; Runions et al., 1999; Mugnaini et al., 2007). In particular, the active biological role of proteins in pollination drops has only recently come to light (von Aderkas et al., 2012). Drops are able to modify extracellular carbohydrate composition with secreted invertases, which favors conspecific pollen germination over heterospecific pollen germination (von Aderkas et al., 2012). Drops also have functional chitinases that play a role in ovule defense (Coulter et al., 2012). This extracellular defense is an adaptation in gymnosperm ovules, which, unlike angiosperm ovules, are open to the outside.

Understanding the biological role(s) of these liquids was made possible by advances in mass spectrometry–based proteomics. Mass spectrometry–based proteomics depends on the

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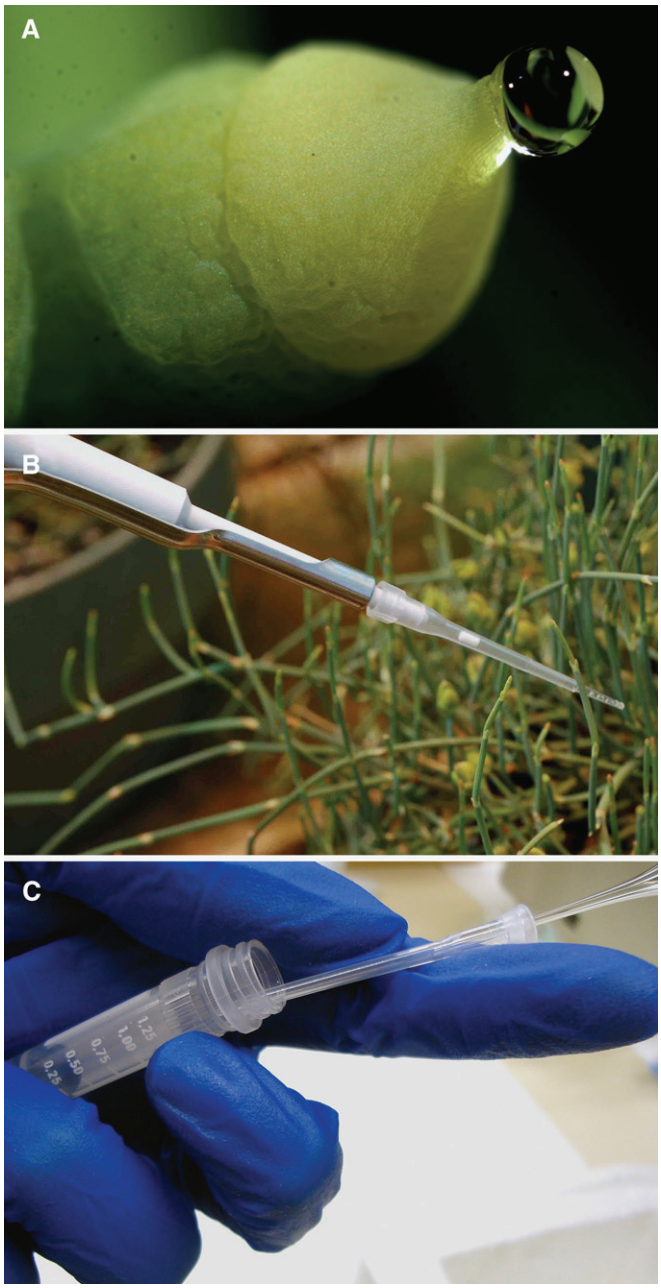

Fig. 1. Pollination drop collection. (A) Pollination drop of *Ginkgo biloba*, 20×. (B) RNA-ase free micropipette tip with filter. (C) Drops are aggregated into a 2-mL microtube by blowing out the pipette tip.

ability to apply a charge to proteins or their component peptides, allowing separation of highly complex mixtures as a function of their mass-to-charge ratios. This technology enables the identification of these peptides, which can then be related back to their parent proteins (Steen and Mann, 2004). Pollination drops are not only a good starting point for studying pollen-ovule interactions, but the protein identifications provide a valuable link to the study of gene expression in the secretory nucellar tissue that can be developed in future.

The proteome of the pollination drop is the set of expressed proteins that are found in the drop. There are generally two objectives in proteomics research: (1) discovery and identification of proteins, and (2) quantitation of known proteins. To date, all studies on pollination drops have been directed toward protein discovery. Quantification of individual proteins has not yet been attempted using targeted proteomics methods such as multiple reaction monitoring (Picotti and Aebersold, 2012; Maiolica et al., 2012). Proteomics investigations have been followed up by biochemical assays to verify in situ and in vitro functionality of pollination drop enzymes (Coulter et al., 2012; von Aderkas et al., 2012). Discovery of proteins is accomplished by generating peptides whose sequences can be used to query protein databases providing unequivocal identification when sequence information is sufficient (Steen and Mann, 2004). Ideally, this approach can result in identification of all of the proteins present in a pollination drop. However, in practice the number of proteins that are identifiable is significantly less, because no gymnosperm genome has been published and gymnosperm protein databases are less comprehensive compared to those of angiosperms. Nevertheless, there are many currently unexplored avenues of investigation still available including studies of protein expression levels, protein complexes, networks that interact with cell surface proteins, and posttranslational modifications.

The types of biochemical interactions, e.g., protein-mediated interactions, between male gametophytes and female reproductive tissues have been studied in angiosperms (Chae and Lord, 2011), but we are unaware of any equivalent research in gymnosperms. Gymnosperm pollination differs from that of angiosperms in that a pollen grain contacts the ovule directly. However, pollen's immediate contact with a drop does not usually result in rapid fertilization. Although the distance that gymnosperm pollen must grow to reach the eggs is typically much shorter than that which angiosperm pollen must grow to reach its eggs, gymnosperm pollen takes more time to attain fertilization (Williams, 2012). There are two reasons for this: gymnosperm pollen generally grows more slowly, and pollen growth is regulated to coincide with egg receptivity, which may occur as much as a year after pollination (Willson and Burley, 1983). The proteomics of these interactions must begin with analysis of the point of pollen's first contact, the pollination drop.

Currently, analysis of pollination drops is done by systems-scale analysis, which poses a number of challenges including sample complexity, dynamic range, and purity (Mallick and Kuster, 2010). Systems scale refers to the large amount of data that is generated by instruments and which must be handled with algorithms. Complexity refers to both the endogenous complexity of a sample, as well as the complexity that is introduced by processing. Although apoplastic solutions, i.e., extracellular liquids, are orders of magnitude less complex than cellular extracts, they still contain high numbers of proteins and other molecules such as carbohydrates, calcium, and phosphorus (Nepi et al., 2009). Separation methods such as chromatographic methods and electrophoresis can remove many of the nonprotein compounds; however, when pollination drops are directly introduced into a mass spectrometer without any preparation or separation, complexity can become a significant problem in species that have compound-rich drops, for example, the sucrose-rich drops of *Welwitschia* Hook. f.

The second challenge, dynamic range, refers to differences in concentrations between different species of proteins or peptides. These differences may span many orders of magnitude, which presents problems for instruments as well as software. For proteins present at low concentrations, low signal-to-noise

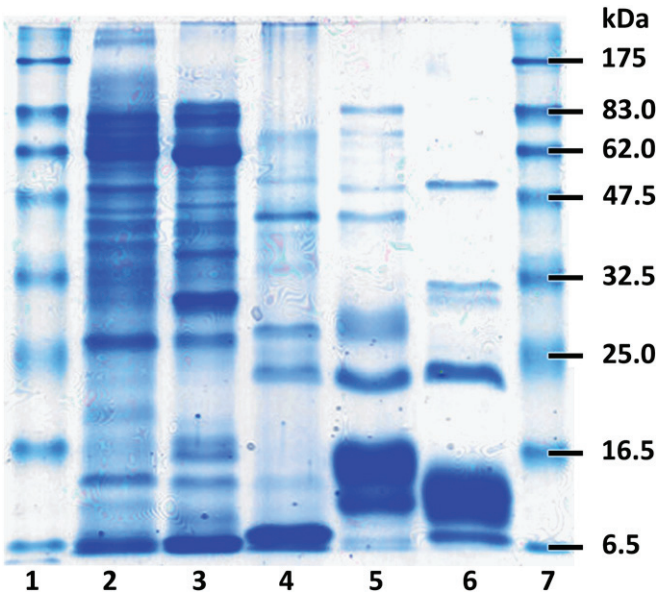

Fig. 2. One-dimensional SDS-PAGE of various conifer pollination drop proteins. The gel was stained using GelCode Blue. Fifty microliters of sample was loaded onto a precast 4–12% Invitrogen gel and run for 1 h at 4°C. The gel was run at 118 mA through the stacking gel and 70 mA through the separating gel. Lanes: (1, 7) molecular weight ladder, (2) *Pseudotsuga menziesii* (Douglas-fir), (3) *Larix ×marschlinsii* (hybrid larch), (4) *Taxus ×media* (hybrid yew), (5) *Chamaecyparis lawsoniana* (Port Orford cedar), (6) *Juniperus oxycedrus* (prickly juniper).

ratios decrease the analytical sensitivity. Both complexity and dynamic range can be influenced by ion suppression. The most abundant peptides in a sample will absorb most of the available charge, with the result that less-abundant peptides remain uncharged and undetected (Mallick and Kuster, 2010).

The third challenge, sample purity, can be compromised by contamination from other proteomes. Debris can enter open ovules and cause significant analytical problems. Because sample purity restricts all other aspects of proteomics, we have put a particular emphasis in this paper on describing collection methods that have worked well with our gymnosperm samples.

In the following applications paper, we outline best practices and strategies for collection, preparation, and processing of pollination drops for proteomics. We also describe various proteomics methods that we have applied to pollination drops on a variety of gymnosperm species. All of these methods are effective in protein identification, but some are adapted to species-specific peculiarities of pollination drop chemistry, e.g., samples with high sugar content. Because there are many types of mass spectrometers, we also outline methods appropriate for the mass spectrometers that we used. These collection and proteomics methods have proven robust and reliable and can be extensively applied to any species of gymnosperm.

METHODS

***Sampling*—**Pollination drops were collected with either a 10-µL micropipette tip (Fig. 1B) or a 10-µL glass capillary tube that had been drawn out over a flame to a fine point. The micropipette tip was suitable for collecting drops that are larger in volume, e.g., *Taxus* L. (~200 nL) or *Ephedra* L. (~10 µL), while the capillary tube was suitable for collecting pollination drops that were smaller in volume, e.g., *Chamaecyparis lawsoniana* (A. Murray) Parl. (<20 nL). A suitable alternative to a micropipette tip that we have also used is an RNA-free micropipette tip with filter. Care had to be taken that the tip did not contact human skin, as this could lead to keratin contamination of the sample. To avoid this, nitrile gloves were worn. Latex gloves were avoided, because proteins found in rubber, e.g., hevein, can also contaminate the samples. Liquid accumulated within the micropipette tip/capillary tube was transferred into a 2-mL microtube placed on ice using a Pasteur pipette (Fig. 1C).

The location of the ovule determined the choice of drop-collecting method. In most gymnosperms, pollination drops are easily collected from ovules with well-exposed micropyles, e.g., *Gingko biloba* L., *Welwitschia mirabilis* Hook. f., *Juniperus oxycedrus* L., and *Taxus ×media* Rehder. Such drops could be collected directly from the plant (Fig. 1A). When this was not practical, because a tree was either too tall or because the drops were too small to be visualized without aid of a dissecting microscope, cone-bearing branches were clipped from the tree and brought into the laboratory. To avoid knocking the pollination drops from ovules, these branches were gently placed in a plastic container lined with wetted paper towels or filter paper. The humid environment prevented pollination drop evaporation. In contrast to such easily accessed ovules, many gymnosperms bear atropous ovules in cones, i.e., micropyles face the cone axis. Unless the ovuliferous scales or scale/bract complexes (henceforth referred to collectively as scales) were well separated at pollen receptivity, it was very difficult to access the ovules to collect pollination drops. In these cases, drops were collected from scales placed in Petri dishes lined with moist filter paper from dissected ovulate cones, e.g., *Pseudotsuga menziesii* (Mirb.) Franco and *Larix ×marschlinsii* Coax.

The minimum drop volume requirement depended on the concentration of proteins in the drop as well as instrument sensitivity. Very small volumes (5–10 µL) could be analyzed if they were protein-rich. However, without preliminary runs and a Bradford assay to gauge protein concentration, this assessment of volume was difficult to judge a priori. To allow for repeated analyses, we normally collected a minimum of 100 µL for any species of interest. The 2-mL microtubes containing the pollination drops were stored at −20°C or −80°C until analysis could be performed.

Chamaecyparis lawsoniana (Port Orford cedar) was collected from the Dorena Lake Research Station of the USDA Forestry Service, Dorena Lake, Oregon, USA. Collections of *J. oxycedrus* were from trees in the Botanical Garden of the University of Siena, Siena, Italy. Drops of *Ephedra monosperma* J. G. Gmel. ex C. A. Mey. were collected from the Plant Sciences Department's research greenhouse on the campus of the University of California, Davis, Davis, California. *Welwitschia mirabilis* was collected from the University of Washington Botany Department's greenhouse, Seattle, Washington. Harvests of drops were also made from *T. ×media* and *L. ×marschlinsii* specimens on the campus of the University of Victoria, Victoria, British Columbia. The *G. biloba* (Fig. 1A) drop was photographed on a tree growing in Finnerty Garden of the University of Victoria, Victoria, British Columbia.

***Proteomics*—**Four examples of different methods used with various gymnosperm drops are provided: (1) separation by gel electrophoresis, (2) separation by reversed-phase high-performance liquid chromatography (RP-HPLC) and gel electrophoresis, (3) protein identification with a quadrupole/time-of-flight mass spectrometer, and (4) protein identification with a Thermo Scientific LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany).

1. Separation by gel electrophoresis—We used a standard procedure that we developed for pollination drops, complete details of which were published in a paper by Wagner et al. (2007). In brief, aliquots of pollination drops were centrifuged and heated to 100°C in a water bath to unfold the proteins, which were then separated by gel electrophoresis.

This method was used with most drops, with the exception of viscous, sugar-rich, and debris-laden pollination drops, such as those from *W. mirabilis*. An additional preparatory step was required to eliminate these other compounds from samples: proteins were separated from sugars by centrifuging the pollination drop through a Microcon filtration unit (EMD Millipore Corporation, Billerica, Massachusetts, USA) that had a 10 kDa nominal molecular weight limit as follows. An aliquot of approximately 25 µL was first diluted to 400 µL with dH_20. The sample was centrifuged (16,000 × g) for 5 min, then filtered by centrifugation (14,000 × g) in the Microcon unit for 30 min. The sample was recovered from the filter by centrifugation (1000 × g) into a fresh tube for 3 min. After this point, proteins were separated by electrophoresis, individual bands were excised, and the protein was reduced, alkylated, and digested with porcine trypsin. The last step was extraction of the peptides from the gel fragment.

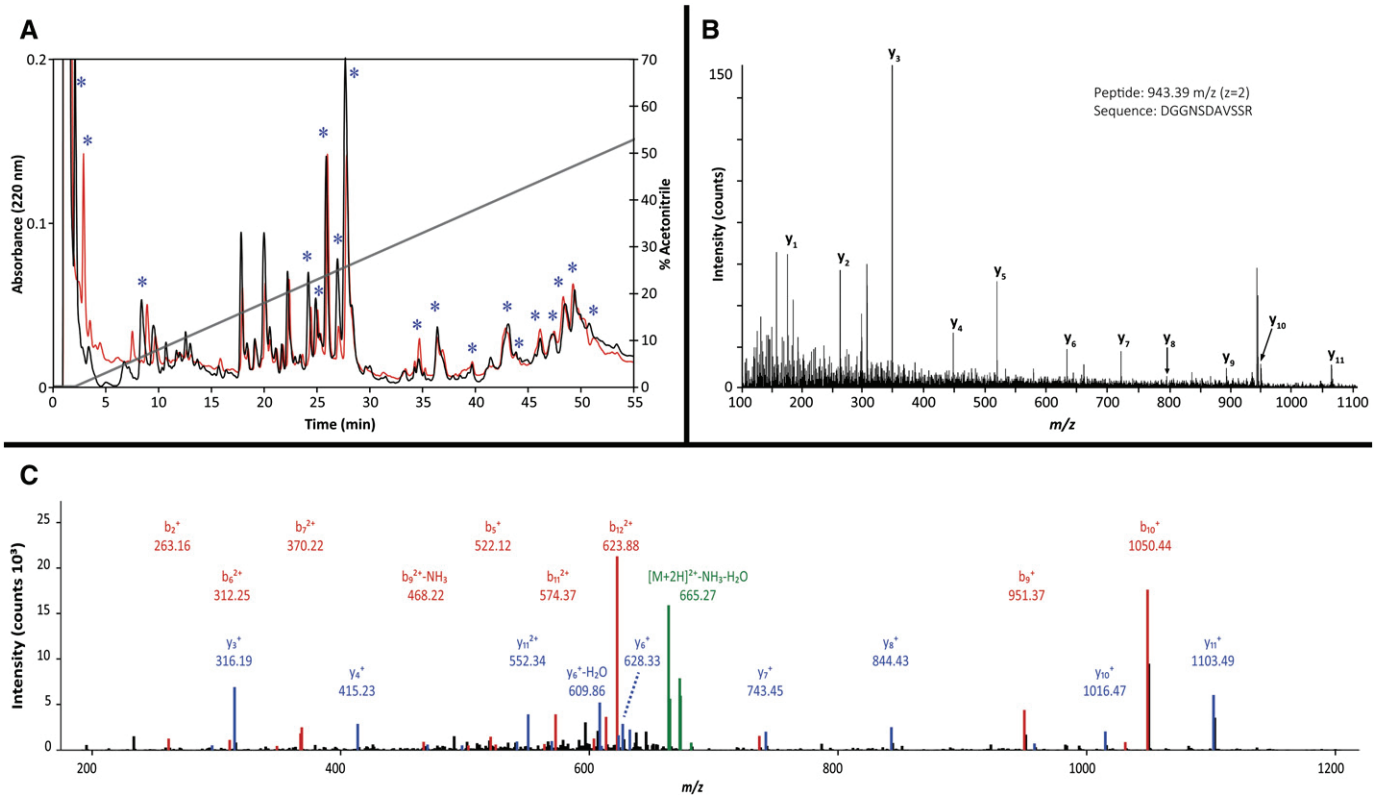

Fig. 3. Reversed-phase profile and spectra of three gymnosperm taxa. (**A**) RP-HPLC profiles of two *Larix* ×*marschlinsii* ovular secretion samples. One sample was collected at the beginning of the secretion period (red trace) and the other collected seven days later (black trace). In each experiment, 20 µL of whole sample was loaded onto a C8 column and separation occurred in a linear gradient of increasing acetonitrile concentration. UV absorbance of eluent was monitored at 220 nm. Asterisks denote fractions shown by SDS-PAGE to contain protein. (B) MS/MS fragmentation data. Tryptic peptide from chitinase protein found in *Welwitschia mirabilis* pollination drops introduced by nanospray electrospray ionization into a QSTAR Pulsar I Hybrid Quadrupole-TOF MS/MS mass spectrometer (Applied Biosystems/MDS Sciex). Data were managed with PEAKS (Bioinformatics Solutions) and Bioanalyst software (Applied Biosystems/MDS Sciex). (C) MS/MS fragmentation data. Peptide (VYSGDTDGRVP) from serine carboxypeptidase II-3 protein found in *Ephedra monosperma* pollination drops introduced by nanospray electrospray ionization into the LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). Data were managed with Proteome Discoverer (Thermo Fisher Scientific) and Mascot version 2.2.1 (Matrix Science) software.

2. Separation by RP-HPLC and gel electrophoresis—Proteins were also separated by a combination of RP-HPLC and gel electrophoresis. A 20-µL aliquot of a *L.* ×*marschlinsii* sample was loaded onto a Brownlee narrow-bore C8 column (PerkinElmer, Waltham, Massachusetts, USA). The drop was in 0.1% trifluoroacetic acid (TFA) in HPLC-grade water. The flow rate for loading was 0.25 mL/min for 2 min. Fractions were eluted in a linear gradient of 0.1% TFA in water to 90% acetonitrile containing 0.075% TFA over 90 min at a flow rate of 0.25 mL/min. Dried fractions were later suspended in 10 µL of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer for electrophoretic separation. Electrophoresis was used to verify protein presence in a particular HPLC fraction. This was done for two different samples: one was from the start of the pollination drop secretory period; the second was collected seven days later.

3. Protein identification with a quadrupole/time-of-flight mass spectrometer—Samples of *W. mirabilis* and *J. oxycedrus* that had been separated by gel electrophoresis, then reduced, alkylated, and extracted, were subsequently introduced into a quadrupole/time-of-flight (TOF) system (i.e., QSTAR Pulsar I Hybrid Quadrupole-TOF MS/MS mass spectrometer; Applied Biosystems/MDS Sciex, Framingham, Massachusetts, USA), via on-line reversed-phase capillary liquid chromatography that separated the peptides by hydrophobicity. The eluted peptides were sequentially introduced into the mass spectrometer where they were ionized and then separated on the basis of their mass-to-charge ratio (m/z), selected, and fragmented using collision-induced dissociation (CID), before introduction into a time-of-flight (TOF) mass spectrometer for measurement of the fragment ion masses.

Details of the following method are from Wagner et al. (2007). Digested samples were acidified with 3 µL of formic acid and then desalted with a custom-made POROS R2 50 µm Reversed-Phase column (4 mm) (Applied Biosystems, Carlsbad, California, USA). The bound sample was washed with a 0.1% (v/v) formic acid solution in dH_2O. The sample was then eluted into an Au/Pd coated capillary (Proxeon Biosystems, Odense, Denmark) with 4 µL 60% (v/v) methanol and 3% (v/v) formic acid solution in dH_2O. Nanospray electrospray ionization was used to introduce ions into the QSTAR Pulsar I Hybrid Quadrupole-TOF MS/MS mass spectrometer (Applied Biosystems/MDS Sciex).

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis was performed using an integrated Famos autosampler, Switchos switching pump, and UltiMate Micro Pump system (LC Packings, Oakville, Ontario, Canada) interfaced to a QTRAP Hybrid Triple Quadrupole/Linear Ion Trap MS/MS Mass Spectrometer equipped with a nano-electrospray ionization source (Applied Biosystems/MDS Sciex) and fitted with a 10-µm fused-silica emitter tip (New Objective, Woburn, Massachusetts, USA). Solvent A consisted of 0.05% formic acid (v/v) and 2% acetonitrile in dH_20, while solvent B consisted of 2% dH_20 (v/v) and 0.05% (v/v) formic acid in acetonitrile. Sample was injected in 95% solvent A and washed on the trapping column for 5 min. The trapping column was switched inline, and the sample was eluted onto a 75 µm × 15 cm column (New Objective) packed with 5 µm 100 Å Magic C18AQ packing material (Michrom Bioresources, Auburn, California, USA). Separations were performed using a linear gradient of 95%:5% to 40%:60% A:B over 35 min. The composition was then changed to 20%:80% A:B over the course of 3 min before re-equilibrating for 15 min at 95%:5% A:B.

Mass spectrometry data were acquired automatically using Analyst 1.4.1 software (Applied Biosystems/MDS Sciex). An information-dependent acquisition method was run and included an enhanced mass spectrometry (EMS), an enhanced resolution (ER) precursor ion scan of mass range 400–1200 amu, and two

Table 1. Summary of methods and taxa.

Proteomics technique	Species
Fractionation of proteins	
One-dimensional gel electrophoresis[1]	*Pseudotsuga menziesii* (Douglas-fir) *Larix ×marschlinsii* (hybrid larch) *Taxus ×media* (hybrid yew) *Chamaecyparis lawsoniana* (Port Orford cedar) *Juniperus oxycedrus* (prickly juniper)
RP-HPLC	*Larix ×marschlinsii* (hybrid larch)
Mass spectrometry and protein identification	*Juniperus oxycedrus* (prickly juniper)[1] *Welwitschia mirabilis*[1] *Ephedra monosperma*

[1] Wagner et al., 2007.

enhanced product ion (EPI) scans of mass range 100–1500 amu. The resultant MS/MS spectra were converted into Mascot Generic Format (MGF) files using Analyst software, and individual MGF files from one protein sample were merged into a single MGF file. Merged MGF files for each protein sample were submitted to PEAKS 3.0 software (Bioinformatics Solutions, Waterloo, Ontario, Canada) for auto de novo sequencing. Peptide amino acid sequences generated by PEAKS were submitted to a protein BLAST search (short, nearly exact matches) of the National Center for Biotechnology Information (NCBI) nonredundant protein database. Peptide sequences from *W. mirabilis* were submitted to a BLAST search of *W. mirabilis* expressed sequence tag (EST) data sets at the Plant Genome Network (http://pgn.cornell.edu/blast/blast_search.pl).

All peptide sequences were manually verified using Analyst software with Bioanalyst (Applied Biosystems/MDS Sciex). Combined peptide sequences were submitted for protein identification to a nonredundant protein sequence database at Bork Group's MS Blast search (http://dove.embl-heidelberg.de/Blast2/msblast.html) under default settings. Hits were considered significant if their protein score exceeded the threshold score calculated by MSBlast software (Habermann et al., 2004).

4. Protein separation and identification with a Thermo Scientific LTQ Orbitrap Velos mass spectrometer—Deionized water (500 µL) was added to the *Ephedra* sample (120 µL). To this, 1 mL of dichloromethane (DCM) and 100 µL of formic acid were added. The solution was thoroughly mixed, then centrifuged (3000 rpm) at 4°C for 6 min. The organic phase was removed and another 1 mL of DCM added. The sample was centrifuged in the same conditions for 4 min. The organic phase was again removed, leaving the aqueous phase with the sample, which was then desiccated on a vacuum centrifuge.

The complete protocol for a sample run on the LTQ Orbitrap Velos (Thermo Fisher Scientific) is as follows. *Ephedra* samples were separated by on-line reversed-phase chromatography using a Thermo Scientific EASY-nLC II system with a reversed-phase peptide trap ReproSil–Pur C18-AQC18 A1 EASY column (100 µm in diameter, 2 cm length, 5 µm, 120 Å; Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) and in-house prepared reversed-phase analytical column Michrom Magic C-18AQ (75 µm I.D., 15 cm length, 5 µm, 100 Å), at a flow rate of 300 nL/min. Solvent A consisted of 0.05% formic acid (v/v) and 2% acetonitrile in dH_20, while solvent B consisted of 2% dH_20 (v/v) and 0.05% (v/v) formic acid in acetonitrile. The sample was injected in 95% solvent A and washed on the trapping column for 5 min. After the trapping column was switched inline, separations were performed using a linear gradient of 0–10% solvent B for 45 min to 10–40% B for 45 min, then 40–100% B for 10 min followed by 100% B for 2 min, then 0% B for 5 min, before re-equilibrating for 15 min at 95% : 5% A : B. The chromatography system was coupled on-line with an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) equipped with a Nanospray I source. Data-dependent MS/MS acquisition was used with CID fragmentation. The full mass spectrometry scans were acquired over a mass range of 400–2000 m/z with detection in the LTQ Orbitrap Velos mass analyzer at a resolution setting of 60,000. MS/MS spectra were acquired in the ion trap, and the method was set to analyze the top eight most intense ions when fragmentation was performed using CID with activation time for resonance set to 10 ms.

The raw data files were searched using Thermo Scientific Proteome Discoverer software version 1.2 (Thermo Fisher Scientific) with the Mascot version 2.2.1 search engine (Matrix Science, Boston, Massachusetts, USA) against the UniProt-SwissProt 20110104 (523,151 sequences; 184,678,199 residues) Viridiplantae database.

RESULTS

The protein profiles of pollination drops show species-specific differences including the kinds and concentrations of proteins (Fig. 2). Closely related conifers show greater similarities in their protein profiles than do distantly related conifers. For example, profiles of closely related pinaceous conifers *P. menziesii* (Douglas-fir) and *L. ×marschlinsii* (hybrid larch) are more similar to one another than they are to the profiles of nonpinaceaeous conifers such as *C. lawsoniana* (Port Orford cedar), *J. oxycedrus* (prickly juniper), and *T. ×media* (hybrid yew) (Fig. 2). Drops of different species also differ in the number of proteins: *P. menziesii* and *L. ×marschlinsii* are protein rich, having two to three dozen bands each, whereas protein-poor drops of *C. lawsoniana*, *J. oxycedrus*, and *T. ×media* have less than a dozen bands (Fig. 2). Closely related conifers share a number of bands in common, e.g., *C. lawsoniana* and *J. oxycedrus* have major bands in common at 23 kDa and 50 kDa. These protein separations also indicate large differences in concentration of proteins within a species. *Pseudotsuga menziesii* has high concentrations of proteins in bands of the following approximate molecular weights: 14 kDa, 27 kDa, 48 kDa, and 50–80 kDa. In comparison, the high concentrations of proteins in closely related *L. ×marschlinsii* are at 17 kDa, 18 kDa, 27 kDa, 30 kDa, 36 kDa, and 50–80 kDa. Only very few of these high-concentration protein bands overlap with high-concentration bands in the other species.

Within a species, the protein complexity in drops does not appear to vary over the period of drop secretion. Drops of *L. ×marschlinsii* collected at different times in the secretory period have identical HPLC profiles (Fig. 3A, Table 1). This confirmation of compositional stability of pollination drops allowed us to search for proteins from drops collected at any time during the secretory period. We could be certain that the relative abundance of particular proteins was unchanged. Protein separation (Fig. 4) was followed by processing of proteins into peptides that were subsequently introduced into the mass spectrometer for analysis. Two examples of spectra are provided. The first is an MS/MS spectrum showing the y-ion series from a tryptic peptide from a chitinase protein found in pollination drops from *W. mirabilis* (Fig. 3B, Table 1) that had been generated using a QSTAR Pulsar I Hybrid Quadrupole-TOF MS/MS mass spectrometer (Applied Biosystems/MDS Sciex). The second example is an MS/MS spectrum showing a y-ion series from a peptide (VYSGDTDGRVP) from a serine carboxypeptidase II-3 found in *E. monosperma* pollination drops (Fig. 3C) that had been generated by tandem mass spectrometry using an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific).

Gymnosperm protein identifications were generated for three of the protein bands of *J. oxycedrus* (Fig. 2, lane 6; Table 2). We found three putative defense proteins: chitinase, glucanase-like protein, and a thaumatin-like protein. The remaining bands were unidentifiable because of limitations in gymnosperm databases, i.e., no sequenced genomes or limited number of expression studies.

DISCUSSION

In the past decade, proteomics has been a highly versatile tool in the identification of proteins in pollination drops. This

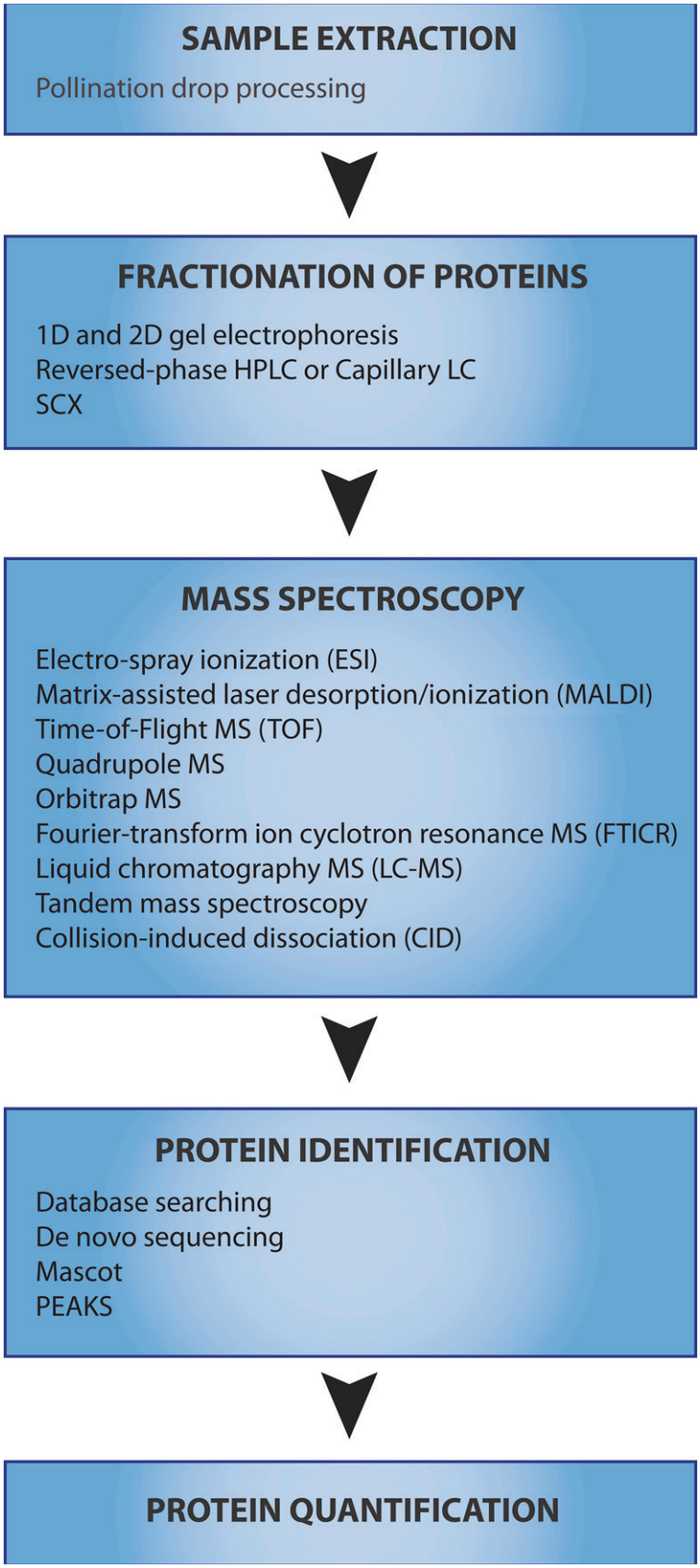

Fig. 4. Flow chart of proteomics protocol for pollination drops.

Table 2. Protein sequences of *Juniperus oxycedrus*.[1]

Molecular weight (kDa)	Peptide sequence	Protein ID
~32.5	FGLFETNK	Glucanase-like protein
	STPHAATVLSK	*Thuja occidentalis*
	GWPSAGTSVATVDNAR	(Q5RZ68)
~30	DLVAQQADVAFK	Chitinase
	FYTYDGFLSAAK	*Cryptomeria japonica*
	QQLNVDPGSNLR	(Q5NTA4)
	QLTWNYNYGAAGK	
~25	GCSFDNSR	Thaumatin-like protein
	WAAASPGGGR	*Cryptomeria japonica*
	TCLSDLNSK	(Q8H995)
	CPQAYSYAK	
	WAAASPGGGR	
	TLQVAAGTTQGR	
	STFTCPSGTNYK	

[1] Modified from Wagner et al., 2007.

new knowledge has changed how we view the first contact of pollen and ovule in gymnosperms. Previously, pollination drops have been treated as mere receiving agents of pollen (Singh, 1978), but we now know, thanks to proteomics, that different species have unique pollination drop protein composition, which influences germination of conspecific vs. heterospecific pollen (von Aderkas et al., 2012). Pollen germinates in a relatively microbe-free environment because drops in some species have a number of antimicrobial protein classes present (Poulis et al., 2005; O'Leary et al., 2007; Wagner et al., 2007). Because peptide sequence identity is determined by homology to sequences in databases, it is still necessary to verify the role of the protein. For example, identification of a chitinase is not confirmation that the chitinase is functional in situ. To prove function, we have recently shown that an antifungal chitinase identified from *Pseudotsuga* drops exhibits enzyme activity both in situ and in vitro (Coulter et al., 2012). In addition, we have shown that drop-identified invertases are able to alter the carbohydrate composition of drops in situ, creating different osmotic environments in which pollen germinates (von Aderkas et al., 2012). Protein identifications were also used as a starting point to devise experiments to prove that these proteins originate from nucellus (Poulis et al., 2005). Much more work can be done along these lines. Another line of research arising from such protein surveys is comparative biology. Proteomes of pollination drops show similarities (Nepi et al., 2009; Coulter et al., 2012; von Aderkas et al., 2012) with other extracellular exudates, such as root secretions (Hawes et al., 2011) and nectar (Thornburg et al., 2003; Heil, 2011).

All pollination drops sampled to date contain proteins (Table 3). As the gel figure in this paper shows, gymnosperms range in the diversity, concentrations, and sizes of these proteins (Fig. 2). Drops of a particular species, e.g., *L.* ×*marschlinsii*, are relatively stable in composition, which implies that these drops represent a constant environment over their period of secretion. Pollen is captured and germinates in this proteome (Poulis et al., 2005; O'Leary et al., 2007; Wagner et al., 2007). The pollination drop represents a relatively species-specific germination medium (von Aderkas et al., 2012). This medium also selects against microbes, and as the example from *J. oxycedrus* shows, defense proteins are abundantly well-represented. It is reasonable to conclude that proteins serve a variety of prezygotic roles, including osmotic regulation, defense, and alteration of extracellular carbohydrates.

This raises the question of the origin of the drop. For some species, this proteome can be considered to be a secretome, but in others, the proteins found in the drop are due to cell breakdown of the nucellus during formation of the pollination chamber. This structure appears at, or around, the time of pollination drop secretion (Singh, 1978). The protein profile that includes many breakdown products due to cell death and proteinase and peptidase activity could be considered a degradome. This creates interpretive challenges. For example, species of gymnosperms such as those of *Pinus* (Owens et al., 1981) and *Ginkgo* (Douglas et al., 2007), in which cellular degradation during formation of pollination chambers occurs simultaneously with pollination drop formation, will require more careful proteomic analysis if we are to separate the origins and functions of the different protein components.

Drop collection has its particular challenges. Further advances in the field of pollination drop biology require understanding species-specific reproductive phenology to collect drops at the correct time. In addition, the details of morphology of reproductive structures are needed if collection methods are to be further refined. A significant problem in collection is finding enough plants that produce a sufficient volume of pollination drop. If it is possible to gain access to well-maintained, healthy collections of gymnosperms, a great deal of money can be saved, as the most expensive part of collection is the price of labor required during collection. We estimate that costs for drop collection from Port Orford cedar are approximately USD$1500–2000/mL, which is a third of the costs of Douglas-fir drops (USD$6000/mL). Cycad pollination drops are three or more times more expensive than Douglas-fir, because of a combination of low volume of each pollination drop and excessive labor required to dissect ovulate cones. An important final consideration is that material identified in breeding programs can be assigned a genotype. Choosing such material should be a priority, as it improves repeatability. Drop volume ranges from as little as 10 nL in *C. lawsoniana* to more than 1 μL in *Welwitschia*. To perform repeated analyses, a minimum of 100 μL should be collected. One-shot preliminary analysis can be done with as little as 5–8 μL of sample that is processed either by direct trypsin digest or liquid-liquid extraction before mass spectrometric analysis.

Separation techniques coupled with mass spectrometry have allowed us to make many identifications (Nepi et al., 2009). The choice of whether to use electrophoretic gel separation or gel-free separation methods depends on whether the goal is to select individual proteins or all proteins. There are many more gel-free methods, e.g., liquid-liquid extraction, two-dimensional liquid chromatography, or solid-phase exchange. Gel-free methods permit very small volumes (5–10 μL) to be used, even when they have low concentrations of proteins, but our experience is that they work best with drops that have relatively simple protein profiles.

The major limitation in protein identification from gymnosperm pollination drops is not mass spectrometry, but in finding homologous sequences with known identities in databases. Half the proteins in *Juniperus* were unidentifiable because they did not score hits in the database that we used. This is typical of gymnosperms, no matter the database. Although peptide sequences may be of high quality, in the absence of gymnosperm genomic information, no identification is possible. As the quality of publicly available proteomics and genomics databases

TABLE 3. Proteins identified by mass spectrometry from pollination drops of gymnosperm species.

Pollination drop protein	Species
Aspartyl protease	*Pseudotsuga menziesii*[2]
Chitinase	*Juniperus communis*[4], *J. oxycedrus*[4], *P. menziesii*[1], *Welwitschia mirabilis*[4]
Galactosidase	*P. menziesii*[2]
Glucan 1,3-β-glucosidase	*Chamaecyparis lawsoniana*[4], *J. communis*[4]
Glucanase-like protein	*J. oxycedrus*[4]
Glycosyl hydrolase	*J. communis*[4]
Invertase	*P. menziesii*[2]
Peroxidase	*P. menziesii*[2]
Serine carboxypeptidase-like protein	*Ephedra monosperma*[5], *P. menziesii*[2]
Subtilisin-like proteinase	*C. lawsoniana*[4], *J. communis*[4]
Thaumatin-like protein	*C. lawsoniana*[4], *J. communis*[4], *J. oxycedrus*[4], *Taxus ×media*[3]
Xylosidase	*P. menziesii*[2]
β-D-glucan exohydrolase	*C. lawsoniana*[4]

Note: 1. Poulis, 2004; 2. Poulis et al., 2005; 3. O'Leary et al., 2007; 4. Wagner et al., 2007; 5. This paper.

continues to improve for gymnosperms, protein identifications will become easier.

Proteomics is a rich and diverse field, and there are other methods that we have not listed. For an excellent overview, we recommend a recent review by Mallick and Kuster (2010). Two examples of proteomic techniques that can be applied in the future and that would allow different kinds of investigations include protein analysis by MALDI imaging (matrix-assisted laser desorption/ionization time-of-flight) and protein quantification and dynamics. The first, MALDI imaging, has been used to image proteins in tissue sections, thus combining mass spectrometry with histology (Grassl et al., 2011; Chaurand, 2012). Locating the areas of protein secretion in the nucellus would add significant knowledge to the field of pollination drop biology. The second, elements of protein dynamics, includes the study of protein turnover and changes in concentrations. For all of these, absolute and relative quantitative methods could be used (Elliot et al., 2009; Schulze and Usadel, 2010). More sophisticated proteomics, in which modifications to proteins, such as phosphorylation (Kirkpatrick et al., 2005) and dimerization, are considered, have not yet been attempted with proteins in pollination drops. As we begin to understand more about the dynamics of these solutions and how the protein component interacts with other apoplastic compounds, as well as surface membranes of pollen and nucellus, it will become clearer which proteins warrant closer study. Much depends on getting sequenced genomes in the next few years. Such data would improve databases used in proteomics. Furthermore, RNA sequencing of transcriptomes from the secretory tissue, i.e., nucellus, will provide gene expression information that can be linked directly to protein expression. Those studies, in turn, will begin to generate models of ovule activity during pollination. The ability to study and compare gene families critical to pollen-ovule interactions will provide a new perspective on the evolution of seed plant reproduction.

Plant researchers who wish to study pollination drops using proteomics will find that their expectations are easily rewarded at the discovery phase. Now that we know that proteins are both present and can be abundant in pollination drops, significant biological advances can be made in applying proteomics to more comprehensive molecular and cell biological experimental approaches that will elucidate pollen-ovule interactions in gymnosperms.

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