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 receptivity. The differences in the timing of drop release varies among gymnosperms, because prefertilization ovule and pollen receptivity.

Results: Proteins are present in all pollination drops. Consistency in the protein complement over time was shown in L. ×marschlii. 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.

1 Manuscript received 19 January 2013; revision accepted 16 March 2013.
2 The authors thank Genome BC, Genome Canada, and the Natural Sciences and Engineering Research Council of Canada; University of California, Davis; Arnold Arboretum of Harvard University; and the University of Victoria for material and financial support. Editorial help from Dr. Carol Parker was appreciated.
3 Current address: National Research Council Canada, 1411 Oxford Street, Halifax, Nova Scotia B3H 3Z1, Canada
4 Author for correspondence: naprior@uvic.ca

doi:10.3732/apps.1300008

Applications in Plant Sciences 2013 1(4): 1300008; http://www.bioone.org/loi/apps © 2013 Prior et al. Published by the Botanical Society of America. This work is licensed under a Creative Commons Attribution License (CC-BY-NC-SA).
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 ratio
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., *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., *Juniperus oxycedrus* L., and *Taxus xmedia* Rehd. 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 ovaliform 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 xomarschilinsii* 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, 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. xomarschilinsii* 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 ddH₂O. 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.
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. snarlschinski 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 the mass spectrometer by nanospray electrospray ionization. The bound sample was washed with a 0.1% (v/v) formic acid solution in dH2O. 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 dH2O. 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 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 dH2O, while solvent B consisted of 2% dH2O (v/v) and 0.05% (v/v) formic acid in acetonitrile. Sample injection was 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 enhanced resolution (ER) precursor ion scans of mass range 400–1200 amu, and two
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 automatic sequencing. Peptide amino acid sequences generated by PEAKS were subsequently introduced into the mass spectrometer (Thermo Fisher Scientific). The second example is an MS/MS spectrum showing 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/MD Sciex). The second example is a MS/MS spectrum showing a y-ion series from a peptide (YSGDTDGRVP) 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

**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 such as *C. lawsoniana* (Port Orford cedar), *J. oxycedrus* (prickly juniper), and *T. media* (hybrid yew) are more similar to one another than they are to the profiles of nonpinaceous 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. × smaragdina* 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. 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. × smaragdina* 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/MD Sciex). The second example is a MS/MS spectrum showing a y-ion series from a peptide (YSGDTDGRVP) 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.

**TABLE 1. Summary of methods and taxa.**

| Fractionation of proteins | Species |
|---------------------------|---------|
| One-dimensional gel electrophoresis¹ | *Pseudotsuga menziesii* (Douglas-fir) |
| Larix ×smaragdina* (hybrid larch) | |
| Taxus × media* (hybrid yew) | |
| Chamaecyparis lawsoniana* (Port Orford cedar) | |
| Juniperus oxycedrus* (prickly juniper) | |
| RP-HPLC | *Larix ×smaragdina* (hybrid larch) |
| Mass spectrometry and protein identification | *Juniperus oxycedrus* (prickly juniper)¹ |
| | *Wollethesia mirabilis*³ |
| | *Ephedra monosperma* |

¹Wagner et al., 2007.
Fig. 4. Flow chart of proteomics protocol for pollination drops.
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

| Molecular weight (kDa) | Peptide sequence | Protein ID |
|------------------------|------------------|------------|
| ~30                    | FGLFETNK         | Glucanase-like protein |
|                        | STPHAAVL5K       | Thuja occidentalis (Q5R268) |
|                        | GWPSAGTVATVDNAR  |                        |
|                        | DVAQOAWFVK       | Chitinase |
|                        | FYTIDFLSAAK      | Cryptomeria japonica (Q5NTA4) |
|                        | QQLNVDPSGSLR     |                        |
|                        | QLTWNYNGASA8GK   |                        |
| ~25                    | GCSDFNSR         | Thaumatin-like protein |
|                        | WAAASPGGGR       | Cryptomeria japonica (Q8H995) |
|                        | TCLSDLNSK        |                        |
|                        | CPQAYSYK         |                        |
|                        | WAAASPGGGR       |                        |
|                        | TLQVAGTQQR       |                        |
|                        | STFTCPSGTNYK     |                        |

1 Modified from Wagner et al., 2007.
TABLE 3. Proteins identified by mass spectrometry from pollination drops of gymnosperm species.

| Pollination drop protein                      | Species                                                                 |
|-----------------------------------------------|-------------------------------------------------------------------------|
| Aspartyl protease                             | *Pseudotsuga menziesii*                                                 |
| Chitinase                                      | *Juniperus communis*, *J. oxycedrus*, *P. menziesii*, *P. lawsoniana*    |
| Galactosidase                                  | *P. menziesii*                                                          |
| Glucan 1,3-β-glucosidase                      | *Chamaecyparis lawsoniana*, *J. communis*                               |
| Glucanase-like protein                        | *J. oxycedrus*                                                          |
| Glycosyl hydrolase                            | *J. communis*                                                           |
| Invertase                                      | *P. menziesii*                                                         |
| Peroxidase                                    | *P. menziesii*                                                          |
| Serine carboxypeptidase-like protein           | *Ephedra monosperma*, *P. menziesii*                                   |
| Subtilisin-like protein                        | *C. lawsoniana*, *J. communis*                                         |
| Thaumatin-like protein                        | *C. lawsoniana*, *J. communis*, *J. oxycedrus*, *Taxus媒体*              |
| Xylosidase                                    | *P. menziesii*                                                         |
| β-glucan exohydrolase                         | *C. lawsoniana*                                                        |

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

more comprehensive molecular and cell biological experimental approaches that will elucidate pollen-ovule interactions in gymnosperms.

LITERATURE CITED

CHAE, K., and E. M. LORD. 2011. Pollen tube growth and guidance: Roles of small, secreted proteins. *Annals of Botany* 108: 627–636.

CHAURAND, P. 2012. Imaging mass spectrometry of thin tissue sections: A decade of collective efforts. *Journal of Proteomics* 75: 4883–4892.

COULTER, A., B. A. D. POUlis, and P. von ADErkAS. 2012. Pollination drops as dynamic apoplastic secretions. *Flora* 207: 482–490.

DOUGLAS, A. W., D. W. Stevenson, and D. P. LittLe. 2007. Ovule development in *Ginkgo biloba* L., with emphasis on the collar and nucellus. *International Journal of Plant Sciences* 168: 1207–1236.

DOYLE, J. 1945. Developmental lines in pollination mechanisms in the Coniferales. *Scientific Proceedings of the Royal Dublin Society, Series A* 24: 43–62.

ELLIOT, M. H., D. S. SMITH, C. E. PARKER, and C. BORCHERS. 2009. Current trends in quantitative proteomics. *Journal of Mass Spectrometry* 44: 1637–1660.

GELBART, G., and P. von ADErkAS. 2002. Ovular secretions as part of pollination mechanisms in conifers. *Annals of Forest Science* 59: 345–357.

GRASSI, J., N. L. TAYLOR, and A. H. MILLAR. 2011. Matrix-assisted laser desorption/ionization mass spectrometry imaging and its development for plant protein imaging. *Plant Methods* 7: 21.

HABERMAN, B., J. OREGMA, S. SUNYAEN, and A. SHEVCHENKO. 2004. The power and the limitations of cross-species protein identification by mass spectrometry-driven sequence similarity searches. *Molecular & Cellular Proteomics* 3: 238–249.

HAWES, M. C., G. CURLANG-RIVERA, Z. G. XIONG, and J. O. KESSLER. 2011. Roles of border cells in plant defense and regulation of rhizosphere microbial populations by extracellular DNA ‘trapping’. *Plant and Soil* 355: 1–16.

HEIL, M. 2011. Nectar: Generation, regulation and ecological functions. *Trends in Plant Science* 16: 191–199.

KIRKPATRICK, D. S., S. A. GERBER, and S. P. GYGI. 2005. The absolute quantification strategy: A general procedure for the quantification of proteins and post-translational modifications. *Methods (San Diego, Calif.)* 35: 265–273.

MAJOLICA, A., M. A. JUNGER, I. EZKURDIA, and R. AEBSROLD. 2012. Targeted proteome investigation via selected reaction monitoring. *Journal of Proteomics* 75: 3495–3513.

MALICK, P., and B. KUSTER. 2010. Proteomics: A pragmatic perspective. *Nature Biotechnology* 28: 695–709.

MUGNAINI, S., M. NEPI, M. GUARNIERI, B. PIOTTO, and E. PACINI. 2007. Pollination drop in *Juniperus communis*: Response to deposited material. *Annals of Botany* 100: 1475–1481.

NEPI, M., P. von ADErkAS, R. WAGNER, S. MUGNAINI, A. COULTER, and E. PACINI. 2009. Nectar and pollination drops: How different are they? *Annals of Botany* 104: 205–219.

O’LEARY, S. J. B., B. A. D. POUlis, and P. von ADErkAS. 2007. The identification of two thaumatin-like proteins (TLPs) in the pollination drop of hybrid yew that may play a role in pathogen defence during pollen collection. *Tree Physiology* 27: 1649–1659.

OWENS, J. N., S. J. SIMPSON, and M. MOLDER. 1981. Sexual reproduction of *Pinus contorta*. I. Pollen development, the pollination mechanism, and early ovule development. *Canadian Journal of Forest Research* 11: 36–50.

OWENS, J. N., T. TAKASO, and C. J. RUNIONS. 1998. Pollination in conifers. *Trends in Plant Science* 3: 479–485.

PICA, P., and R. AEBSROLD. 2012. Selected reaction monitoring-based proteomics: Workflows, potential, pitfalls and future directions. *Nature Methods* 9: 555–566.

POULIS, B. A. D. 2004. Safe sex in conifers. Ph.D. Thesis, University of Victoria, Victoria British Columbia, Canada.

POULIS, B. A. D., S. J. B. O’LEARY, J. D. HADDOW, and P. von ADErkAS. 2005. Identification of proteins present in the Douglas-fir ovular
secretion: An insight into conifer pollen selection and development. *International Journal of Plant Sciences* 166: 733–739.

RUNIONS, C. J., K. H. RENSING, T. TAKASO, AND J. N. OWENS. 1999. Pollination of *Picea orientalis* (Pinaceae): Saccus morphology governs pollen buoyancy. *American Journal of Botany* 86: 190–197.

SCHULZE, W. X., AND B. USADEL. 2010. Quantitation in mass-spectrometry-based proteomics. *Annual Review of Plant Biology* 61: 491–516.

SINGH, H. 1978. Embryology of gymnosperms. Gebrüder Borntraeger, Stuttgart, Germany.

STEEN, H., AND M. MANN. 2004. The ABC’s (and XYZ’s) of peptide sequencing. *Nature Reviews. Molecular Cell Biology* 5: 699–711.

THORNBURG, R. W., C. CARTER, A. POWELL, R. MITTLER, L. RIZHISKY, AND H. T. HORNER. 2003. A major function of the tobacco floral nectary is defense against microbial attacks. *Plant Systematics and Evolution* 238: 211–218.

TOMLINSON, P. B., J. E. BRAGGINS, AND J. A. RATTENBURY. 1997. Contrasted pollen capture mechanism in Phyllocladaceae and certain Podocarpaceae (Coniferales). *American Journal of Botany* 84: 214–223.

VON ADERKAS, P., M. NEPI, M. RISE, F. BUFFI, M. GUARNIERI, A. COULTER, K. GILL, ET AL. 2012. Post-pollination prefertilization drops affect germination rates of heterospecific pollen in larch and Douglas-fir. *Sexual Plant Reproduction* 25: 215–225.

WAGNER, R. E., S. MUGNAINI, R. SNIEZKO, D. HARDIE, B. POULIS, M. NEPI, E. PACINI, AND P. VON ADERKAS. 2007. Proteomic evaluation of gymnosperm pollination drop proteins indicates highly conserved and complex biological functions. *Sexual Plant Reproduction* 20: 181–189.

WILLIAMS, J. 2012. Pollen tube growth rates and the diversification of flowering plant reproductive cycles. *International Journal of Plant Sciences* 173: 649–661.

WILLSON, M. F., AND N. BURLEY. 1983. Mate choice in plants: Tactics, mechanisms and consequences. Princeton University Press, Princeton, New Jersey, USA.

http://www.bioone.org/loi/apps