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Degradome and Secretome of Pollination Drops of *Ephedra*

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Abstract Although secreted proteins (a secretome) are known to occur in gymnosperm pollination drops, this study shows evidence for the presence of a protein degradome for the first time. A protein degradome is composed of protein and peptide fragments, a product of protein breakdown, whereas a secretome is composed of whole, secreted, and often biologically active extracellular proteins. Harvested *Ephedra* pollination drops from seven species were pooled either by collection date or, in the case of less abundant sample volumes, by species. Samples were processed by one of two methods: 1. gel electophoresis or by 2. liquid-liquid extraction, followed by chromatographic separation. Processed samples were trypsin-digested and analyzed with a Thermo Scientific LTQ Orbitrap Velos. On average, two-thirds of the detected and characterized proteins found in *Ephedra* spp. pollination drops were intracellular proteins, such as ubiquitin. The remaining third represent proteins known to be secreted, often involved in apoplastic processes such as defense and carbohydrate-modification, typical of known conifer pollination drop proteins. Characterized proteins detected in our comparative study of *Ephedra* spp drops ranged from 6 in *E. monosperma* to 20 in *E. foeminea*. We propose that the intracellular proteins detected are present as the result of nucellar tissue degeneration during pollination drop formation; previous proteomic investigations of pollination drops were in taxa that lack nucellar degeneration during drop formation Discovery of a degradome in pollination drops is novel and significant in that its presence has biological implications for pollination biology. We predict that degradomes in pollination drops are not restricted to *Ephedra*, but should also occur in species with nucellar tissue breakdown that coincides with pollination drop formation, such as in cycads and *Ginkgo* and some Pinaceae. Analysis of several collection dates of *E. monosperma* shows a large number of proteins that change over the course of the pollination drop secretion period, which suggests that variation in pollination drop contents over time may be important in the pollination biology of *Ephdera*.

Keywords: degradome • *Ephedra* • gymnosperm reproduction • pollination drop • proteomics • secretome

Introduction

Gymnosperm pollination drops are involved at some point in the capture and delivery of pollen into ovules, followed by pollen germination and fertilization (Gelbart and von Aderkas, 2002). The role of the pollination drop varies according to the pollination mechanism in which it occurs (Tomlinson et al., 1997). In *Ephedra,* pollination drops (Fig. 1) perform both the pollen capture and delivery function (Endress, 1996). Pollen can be delivered by wind or by insects, but in the latter case, pollination drops also function as a nectar/reward for the pollinator (Moussel et al., 1980; Meeuse et al., 1990). *Ephedra* species are not obligately insect-pollinated, as wind pollination may also occur at the same time (Karl Niklas, this volume). In this respect, *Ephedra* is similar to other gnetophytes (*Welwitschia* and *Gnetum*) (Endress, 1996).

Ephedra pollination drops contain abundant sucrose, but are also abundant in phosphate compounds, amino acids, and polypeptides (Ziegler, 1959). Until this study, no proteins have yet been documented, although Ziegler (1959) found acid phosphatase activity in the nucellus, the sporogenous tissue that produces the pollination drop. He wrote that such nucellar proteins likely are responsible for processing cellular compounds that are secreted into the drop. We hypothesize that *Ephedra* pollination drops contain proteins, given that rich and diverse pollination drop proteomes have been recently described from a wide range of gymnosperms (Wagner et al., 2007). To this end, we embarked on the first proteomic study of *Ephedra* pollination drops. The aim was to test for the presence of proteins, and if present, to understand the variation in protein composition in the pollination drops of *Ephedra*.

Ziegler (1959) also first reported the presence of mineral and organic compounds released into pollination drops by the nucellus. The developmental stage of the nucellus at the time of pollination drop release can vary widely among different gymnosperm taxa. In *Ephedra*, the nucellus is post-meiotic (Rydin et al., 2010), whereas nucellus of *Taxus* is premeiotic (Dupler, 1920). The nucellus of *Ephedra* differs from many other gymnosperms in that a central apical portion degenerates to form a pollen chamber (Rydin et al., 2010). Pollen chambers are known from the earliest fossils of Gnetales (Rothwell and Stockey, 2013). Nucellar degradation to form a pollen chamber also occurs in *Ginkgo* (Douglas et al., 2007) and cycads (Norstog and Nicholls, 1997). By comparison, *Taxus* and most other conifers have whole, undegraded nucellus throughout pollination and into early embryo development (Singh, 1978). Thus we not only hypothesize the presence of proteins in *Ephedra* pollinations drops, but we also expect that such degenerative processes in *Ephedra* at the time of pollination drop formation would influence the type of proteins present, such as protein breakdown products that accompany tissue death.

Literature Review

Ephedra reproductive biology, of which the pollination drop is just a part, deserves detailed investigation. Gnetales (*Ephedra*, *Gentum*, *Welwitschia*) is a distinct lineage among the six major groups of extant gymnosperms which has occupied various and contested positions in hypothesized seed plant phylogenies (Graham and Iles, 2009; Mathews, 2009; Rydin and Korall, 2009). Regardless of the various possible sister-group relationships that Gnetales may have, detailed understanding of this group is important for any interpretations of evolutionary history among seed plants. Among other gymnosperms, such as the Pinaceae and Cupressaceae, some taxa have pollination drop proteins in common (Wagner et al., 2007). In this review, a brief history of the study of *Ephedra* pollination drops will be followed by a summary of what is known of its pollination drop physiology and biochemistry. To highlight some unique and poorly understood aspects of the pollination mechanism of *Ephedra,* we will compare it with other, better-studied gymnosperm species. We will also provide a rationale for using proteomics in the study of pollination drops. In spite of the fluid phylogeny of extant spermatophytes, it is clear that the pollination mechanism of *Ephedra* is of ancient origin. Ever since Doyle's seminal paper in 1945 in which information on extant conifer species was combined with transformational series of key ancestral fossils, pollination drops have been considered a basic component of even the earliest pollination mechanisms of gymnosperms. Some groups have wide variation in pollination drop capture, *i.e.* Podocarpaceae, including capture of pollen by mechanisms that do not involve pollination drops, *e.g. Saxegothea* (Doyle, 1945). Mechanisms that lack pollination drops are common in only two groups, Araucariaceae and some Pinaceae. Pollination drops are a prevalent feature of gymnosperm pollination and have been documented in one fossil (Rothwell, 1977) and are suspected to be present in most fossil groups (Doyle, 1945; Doyle 2008). Tomlinson (2012) incorporated morphological and physiological aspects of ovule behaviour in his analysis of the evolution of pollination mechanisms. In his scheme, pollination drops are ancestral in conifers. Little and co-authors (2014) used phytochrome gene duplication rooting of seed plants (Mathews, 2009) in combination with sister-group relations of major plant extant seed plant lineages as a backbone for constraining a morphological matrix that includes extinct seed plants (Doyle, 2008). Little et al. (2014) concluded that the pollination mechanism of *Ephedra* traces its origins to an ancient conserved suite of traits among seed plants.

Pollination drops of *Ephedra* have attracted attention for over 140 years. Observations of their role in pollen capture were included along with those of 14 other gymnosperm genera in the first detailed study of pollination drop biology (Strasburger, 1871). Since then, *Ephedra*'s pollination drop has been the subject of periodic investigation. Questions regarding insect-pollination (Bino et al., 1984, Porsch, 1910) and wind-pollination (Buchmann et al., 1989, Niklas & Buchmann, 1987; Niklas & Kerchner, 1986; Niklas et al., 1986) have received the most attention. More recently, a comparison of ovule morphology and anatomy among *Ephedra* species (Rydin et al., 2010) has provided detailed information on ovule organization, including variation in pollination drop secretory tissue, i.e. nucellus. Ziegler (1959) compared *Ephedra* with *Taxus* in a physiological study on some components of pollination drops. To put the published effort

on *Ephedra* in perspective, *Taxus*, the other taxon used in Ziegler's study is, historically, the best-studied of all gymnosperm genera. *Taxus* drops were not only among the very first to be described (Vaucher, 1841), but Strasburger (1871) provided detailed, reliable observations on their secretion and retraction. More importantly, they have an abundance of ovules with easily accessible pollination drops that, compared with most other gymnosperm taxa, have relatively large volumes (~ 250 nL). *Ephedra* produces an even larger drop (~ 1 µl). Thus, given enough ovulate plants, collection is relatively easy. Early chemical analysis of pollination drops of various conifers revealed components such as calcium and various carbohydrates (Fujii, 1903; Schumann, 1903), which were later found in *Ephedra* also (Ziegler, 1959). Proteins of conifer pollination drops were identified by immunohistochemistry (arabinogalactans; O'Leary et al., 2004) and mass spectrometry (thaumatin-like proteins; O'Leary et al., 2007), but to date similar investigations have not been carried out on *Ephedra*.

Ephedra has a pollination mechanism that is among the most common in gymnosperms. Pollination mechanisms can be divided into those that have pollination drops, and a small number of species that do not (Little et al., 2014). Those with drops are classified into one of six pollen capture mechanisms, based on how pollination drops are involved in either pollen capture or post-capture (Little et al., in press). *Ephedra* is characterized by pollination drops that both capture and deliver non-saccate pollen into the ovule (Little et al., 2014; Tomlinson, 2012). A mechanism that lacks a drop is known as an "extra-ovular capture and germination" type. In this mechanism, pollen lands near or on the ovule where it germinates; at no point is a pollination drop involved. The pollen tube enters the opening of the ovule, the micropyle, and reaches the interior of the ovule to undergo sperm release and fertilization (Endress, 1996). Gymnosperms with extra-ovular capture and germination are known only from a small number of conifers, such as Araucariaceae*, Saxegothea* (Podocarpaceae), and some Pinaceae, e.g. *Abies* and a few species of *Tsuga* (Doyle, 1945). There are six pollen capture mechanisms that have a drop, and perhaps three extra-ovular capture and germination mechanisms that do not have drops. In the evolution of gymnosperm pollination mechanisms, extra-ovular and germination mechanisms are derived from pollination mechanisms that have drops (Little et al., 2014; Tomlinson, 2012). Although *Ephedra*'s pollination mechanism is familiar, we know less about a number of its features, in particular, pollination drop composition, component stability, and how pollen interacts with pollination drops.

Pollination drops are produced by the nucellus (Fujii, 1903). However, the components need not arise locally, e.g. carbohydrates found in the drop may be the result of long distance transport as well as local production. In contrast to what is known about sucrose production in flowering plant nectar (Heil, 2011), we do not know how much pollination drop sucrose originates from extracellular or apoplastic transport versus intracellular or symplastic processes. Proteins active within the *Ephedra* nucellus have an influence on pollination drop composition (Ziegler, 1959). The first protein to be mentioned in the pollination drop literature was acid phosphatase, but this protein was not found in pollination drops; it was located by immunohistology in the nucellus of *Ephedra helvetica* (= *E. distachya*) (Ziegler, 1959). Cellular acid phosphatase was thus considered to be involved in processing compounds destined for secretion into the pollination drop. However, acid phosphatase may not be restricted to the nucellus as it was found by immunohistochemistry in the pollination drop of the related gnetophyte, *Welwitschia*

mirabilis (Carafa et al., 1992). Later, the enzyme chitinase was identified by mass spectrometry in the drops of *W. mirabilis* (Wagner et al., 2007). In contrast to the only two proteins known from gnetophytes, there are numerous proteins known in Pinaceae and Cupressaceae (Nepi et al., 2009).

Ephedra pollination drop secretion is not currently understood from a mechanistic standpoint. Our lack of understanding of the process of secretion and retraction of the pollination drop across gymnosperms in general has fueled contradictory interpretations of the evolution of pollination mechanisms (for discussion see Mugnaini et al., 2007). Some pollination drop secretion models have been proposed that are passive. Other models have been proposed that depend on the degree of active secretion that is occurring from the nucellus (Tomlinson et al., 1997).

The passive mechanisms include both pollination drops and substitutes for pollination drops. At one extreme is Ziegler's (1959) suggestion that pollination drop secretion and retraction is a passive, purely physico-chemical phenomenon that lacks active cellular secretion. He based this idea on the fact that his application of metabolic poisons to kill nucelli of *Taxus* and *Ephedra* did not halt pollination drop secretion. Thus, he surmised that extracellular substances, such as sucrose, were sufficient to draw water from nucellar tissue by osmosis to form drops. Under this scheme, withdrawal would also be a passive process, one driven by evaporation. However, at the other extreme, some studies suggest that pollination drops are not essential for pollen capture and delivery, but can be replaced by simple rainwater capture mechanisms that wholly or partially substitute for biologically produced pollination drops. Various mechanisms involving rainwater substitution of some kind have been proposed for *Abies* (Chandler and Owens, 2004), *Cedrus* (Takaso and Owens, 1995), *Picea* (Runions et al., 1996), and *Pinus* (Brown and Bridgwater, 1986; Greenwood, 1986), although in the latter case rainwater capture has been dismissed in a recent study by Leslie (2010). A rainwater-based capture mechanism has never been suggested for *Ephedra*. Drops that are exposed to the air, such as those of *Ephedra*, which are without surrounding or enclosing structures, are destroyed by rain. In addition, it is known that rain disturbs pollen uptake in species such as *Taxus* (Tison, 1911). Such overly exposed ovules cannot receive pollen until later, after a new drop is secreted.

In contrast to these passive models of pollen uptake, more active roles for the ovule have been proposed. The ovule appears, at least in some cases, to be active and possibly interacting with pollen. In a wide variety of species, observations have been published in which drop secretion and retraction occurred quickly (Jin et al., 2012; Mugnaini et al., 2007; Tomlinson et al., 1997), with retraction speed too high to be accounted for by evaporation alone. Furthermore, in some members of the Podocarpaceae, secretion and retraction occurs repeatedly. Liquid spreads across the ovule's neighbouring surfaces, collecting buoyant saccate pollen (Tomlinson et al., 1997). Retraction and drop emergence repeats several times to continue pollen scavenging. Mugnaini et al. (2005) proposed a two-step drop secretion mechanism for some cupressaceous species that was based on both active and passive components. For example, some genera of Podocarpaceae (*Podocarpus* - Tomlinson et al., 1997) and some Cupressaceae, (*Chamaecyparis* – Owens et al. 1980) have the ability to repeatedly secrete pollination drops, whereas ovules of other Podocarpaceae (*Phyllocladus* - Tomlinson et al., 1997) are able to produce a drop only once, which recedes after pollen capture, never

to be replaced. There are suggestions based on fossil evidence of pollinator-ovule interactions that imply gain and loss of insect pollination because of evolutionary turnover of pollinators, possible compositional shifts of sucrose concentrations, and changes in ovule morphological features (Labandeira et al., 2007). Among extant gymnosperms, a molecular or cell biological mechanism needs to be developed that can account for the active processes involved in drop secretion and retraction. The current bottleneck to such work is the paucity of studies of molecular components of pollination drops, including the lack of published genomes, nucellus transcriptomes and comparative physiological studies. In *Ephedra*, the pollination mechanism is relatively simple: pollen is captured by a secreted pollination drop that subsequently recedes. If a drop is removed, the nucellus is capable of producing another one (Moussel, 1980). What is different, though not unique, about the drop in *Ephedra* compared to that of most conifers studied is that drop production co-occurs with nucellus tissue breakdown. This cell degradation forms the pollen chamber where captured pollen sinks prior to germination (Moussel, 1980).

There are several reasons why the process of secretion is not clearly understood, particularly in gnetophytes. Although a functioning enzyme, i.e. acid phosphatase, was detected in the pollination drop of *Welwitschia*, it is not known whether it was secreted into the drop by nucellar tissue, or it arrived in the drop after degenerative formation of the pollen chamber. Pollen chambers are found in *Ephedra* (Rydin et al., 2010) and some other gymnosperms, such as cycads (Norstog and Nicholls, 1997), *Gingko* (Douglas et al., 2007), *Pinus* and *Picea* (Singh, 1978). In comparison, many gymnosperms do not have pollen chambers. *Taxus* has an intact nucellus, i.e. a solid dome of parenchymatous tissue that shows no sign of degeneration before or during pollination drop formation (O'Leary et al., 2004). Since *Taxus* pollination drops have proteins secreted from intact cells, it follows that ovules with cell degradation-derived pollen chambers, such as those of *Ephedra, Ginkgo* and *Pinus*, may have drops that contain proteins of two origins: 1. secreted from intact cells 2. released by cell lysis.

Protein degradomics is a systems approach to mass spectrometry that investigates proteases and their substrates, as well as proteolytic events (López-Ortiz and Overall, 2002). The portion of proteins that originate from the degraded tissues are appropriately called the degradome. However, a degradome can arise from a number of processes occurring concurrently or independently. One source of degradome already considered above is cellular debris due to senescence during pollen chamber formation (Roberts et al., 2012). A second source may be from the activity of extracellular proteases and peptidases, if present in pollination drops, that would generate breakdown products from extracellular proteins. If this occurs then both these peptidases and proteases would be detected along with polypeptide fragments of other proteins. Degradomes may form biochemically complex networks, but these remain relatively unstudied in plants (Huesgen and Overall, 2012). Some of the breakdown products may function in providing signals that regulate defense responses of living cells. Proteomics provides identification with high confidence, but proof of functionality of constituents of the degradome within the pollination drop requires further study of substrate processing. Furthermore, it must be shown that these compounds are functional *in situ*.

Secretome proteins characteristically have signal cleavage peptides that permit their active export across the plasmalemma. In gymnosperms, such cleavage signal

peptides have been described for thaumatin-like proteins found in pollination drops (O'Leary et al., 2007). Since cleavage peptides are removed during export of the proteins from cells, confirmation requires querying peptide sequences against gene databases, and then isolating the gene from the plant material to verify the presence of a cleavage peptide coding sequence. The identification of enzymes has altered our view of how pollination drops function and provided new insights into the biochemical role played by pollination drops during reproduction (Prior et al., 2013). A wide variety of proteins have been identified in *P. menziesii* (Poulis et al., 2005), *Larix* x *marschlinsii* (O'Leary et al., 2007), *Juniperus communis, J. oxycedrus, Welwitschia mirabilis*, and *Chamaecyparis lawsoniana* (Wagner et al., 2007). Protein identifications from these taxa suggest roles in antimicrobial defense, carbohydrate modification, alteration or maintenance of osmotic levels, and pollen selection (Nepi et al. 2009). Some of these roles have been confirmed with enzyme assays. Douglas-fir pollination drop proteins identified as invertases have, after closer biochemical study, been proven to cleave sucrose in situ. Invertases in this system act as regulators of the pollination drop's carbohydrate composition. In turn, this change in solute concentration of the major pool of molecules in the drop has a direct influence on the selection of conspecific over heterospecific pollen in Douglas-fir and larch (von Aderkas et al., 2012). In comparison with conspecific pollen that prefer these osmotic conditions and readily germinate, heterospecific pollen much less frequently, and show poor germination rates. Another example of enzyme assay confirmation of identified proteins is that of chitinases. These were proven to process chitin substrates in situ (Coulter et al., 2012), suggesting that these proteins have a defensive role during reproduction, defending the ovule and pollen against airborne pathogenic fungi.

The chemical composition of the pollination drop of *Ephedra* species must be considered in a biological and ecological context. Certain components may qualitatively enhance the ecological services already provided by the plant, e.g. the quality of the nectar reward for insects (Fig. 2). In *Ephedra*, the high amounts of sucrose attract insects, as we have ourselves seen on many occasions, confirming published studies (Bino et al. 1984; Meeuse et al., 1990; Moussel, 1980). *Ephedra* also is relatively rich in amino acids, especially glutamine and glutamic acid (Ziegler, 1959). In angiosperm nectar, free amino acids are the next most abundant group of compounds after carbohydrates. Free amino acids influence sensory preferences in insects (Linander et al., 2012). A variety of insects have been recorded from *Ephedra* spp., including dipterans, as well as hymenopterans such as vespids, braconids and chalcids, but not bees (reviewed in Bino et al., 1984). It is likely that pollination drop composition, like plant nectar composition, may be highly influenced by plant phylogeny versus pollinator preferences (Nicolson, 2011). Drops in *Ephedra* having evolved in an arid environment, it is also possible that high solute concentrations, i.e. sucrose, are necessary to prevent drops from evaporating too quickly, which may have been a possible pre-adaptation to insect pollination. If advances are to be expected in the study of chemecological aspects of insect pollination in *Ephedra*, more thorough chemical analysis as well as insect behavioural studies will be required.

Many components of pollination drops influence pollen growth and development. Sucrose has a universal role in *Ephedra* of also providing a nutrient source for pollen germination and pollen tube growth, regardless of whether the species is insect- or wind-pollinated. *Ephedra* pollen germinates rapidly and the pollen tube grows quickly, reaching the egg in 14 hours, which is much faster than other gymnosperms (El-Ghazaly

et al., 1997; Williams, 2012). The pollen can even germinate while in the pollination drop outside the micropyle (Bino et al., 1984). It would appear that the tubes do not have to be in close proximity of the nucellus to be able to grow long distances. The pollination drop with its carbohydrate and other substances is able to support long distance growth of these tubes (Bino et al., 1984). Sucrose is also the major contributor to the osmotic potential of the drop. *In vitro* assays of other gymnosperms have also shown that carbohydrate concentrations can play a critical role in germination success (Dumont-BéBoux et al., 1999). Ziegler (1959) showed that calcium is present in *Ephedra*. Because calcium is critically important in pollen germination for most seed plants, it is a major component of pollen germination media (Brewbaker and Kwack, 1963). *Ephedra* also contains a variety of amino acids (Ziegler, 1959), which may contribute to pollen germination as suggested in studies of *Juniperus* pollen growth in vitro on media supplemented with the major amino acids found in pollination drops (Duhoux and Pham Thi, 1980; Seridi-Benkaddour and Chesnoy, 1988). The other compounds that Ziegler (1959) found in *Ephedra* include polypeptides and phosphate-rich compounds. These compounds were only identified as to general class, and remain uncharacterized. As should be apparent with *Ephedra*, there are many unrealized opportunities for researchers who would like to enter this field. We would like to reiterate that *Ephedra* has a pollination drop of enormous volume compared to some gymnosperms (*Ephedra* ~1000 nL versus *Chamaecyparis lawsoniana* ~10 nL). A consequence is that many thousand fewer drops need to be collected for a chemical analysis. This advantage is multiplied by the fact that several species of *Ephedra* are small easy-to-grow plants, some becoming sexually productive in a less than a year if vegetatively propagated.

In spite of a history of study of various aspects *Ephedra* pollination drop biology, including secretion and retraction (Strasburger, 1871), ecological features (Bino et al., 1984, Buchmann et al., 1989), nucellus morphology (Rydin et al., 2010) and physiology, and composition (Ziegler, 1959), we still need to address fundamental questions concerning drop composition and the influence, if any, of pollen chamber formation in this composition. A more detailed and thorough analysis of components, especially protein composition, needs to be undertaken before the ecological services that *Ephedra* pollination drops provide can be considered.

Materials and Methods

A - Sample collection.. *Ephedra* pollination drop samples were collected by touching the drops with a micropipette tip. Drops were expelled into an Eppendorf tube and stored at -20°C until analysed. *Ephedra likiangensis* and *E. minuta* drops were collected from plants in the botanical greenhouse at Stockholm University from January 17 through February 16, 2012 and December 21 through January 10, 2012 respectively. *E. foeminea* drops were collected in Asprovalta, Greece in July 2011. *E. distachya* drops were collected in Nea Vrasna, Greece May 30 and June 2, 2011. *E. trifurca* drops were collected at the Aqua Fria River Bottom, Maricopa County, Arizona, U.S.A. on March 17, 2012. *E. monosperma* drops were collected from March to April, 2011 from greenhouse-grown plants at the Orchard Park Facility, University of California at Davis. *E. compacta* drops were collected in Laguna de Alchichica, Puebla, Mexico from April 10 to 23, 2012. In addition, samples of *Ginkgo biloba* and *Larix* x *marschlinsii* were collected from trees growing outdoors on the campuses of University of California at Davis and University of Victoria, respectively. A separate comparative study was carried out on pollination drops of *E. monosperma* collected on three sample dates, March 9, 24 and April 10, 2011.
B - 1D SDS PAGE.. 20 µL of pollination drop sample was mixed with 5 µL NuPage MES SDS Buffer and 1 µL of 1M DDT. Samples were boiled at 99 °C for 10 min, and then loaded on to a NuPage Novex 4 - 12 % Bis-Tris precast gel. 5 µL of BLUeye Prestained Protein Ladder was run alongside the samples. The gel was fixed with a 40 % ethanol / 10 % acetic acid solution for 10 min, and then stained with 0.1 % G250 Coomassie Brilliant Blue overnight. The gel was then destained with 10 % acetic acid solution.

C - LC-MS/MS analysis.. Samples were reduced with dithiothreitol (30 min at 37 °C), and cysteine sulfhydryls were alkylated with iodoacetamide (30 min at 37 °C in darkness). Trypsin (2 µg; Promega) were added to each sample, which was digested at 37 °C for 16 hr. The samples were de-salted on a Waters HLB Oasis column, speed vac-concentrated and then stored at -80 °C prior to LC-MS analysis.

Peptide mixtures were rehydrated to 100 µL with 2 % acetonitrile/water/2 % formic acid and separated by on-line reversed phase chromatography using a Thermo Scientific EASY-nLC II system with a reversed-phase pre-column Magic C-18AQ (100 µm internal diameter, 2 cm length, 5 µm, 100 Å, Michrom BioResources Inc, Auburn, CA) pre-column and a reversed phase nano-analytical column Magic C-18AQ (75 µm internal diameter, 15 cm length, 5 µm, 100 Å, Michrom BioResources Inc, Auburn, CA) both in-house prepared, at a flow rate of 300 nl/min. The chromatography system was coupled to an LTQ Orbitrap Velos mass spectrometer equipped with a Nanospray II source (Thermo Fisher Scientific). Solvents were A: 2 % acetonitrile, 0.1 % formic acid; B: 90 % acetonitrile, 0.1 % formic acid. After a 249 bar (~ 5 µL) pre-column equilibration and 249 bar (~ 8 µL) nanocolumn equilibration, samples were separated by a 90 min gradient (0 min: 5 % B; 80 min: 45 % B; 2 min: 90 % B; 8 min: 90 % B).
D - Data analysis parameters.. Raw LCMS files were converted to Mascot Generic Format and processed with PEAKS Client 6 (Bioinformatics Sofware Inc, Waterloo, ON, Canada) with Peaks DB and Spider searches enabled against the Uniprot/Trembl and

Uniprot/Swiss-Prot Allspecies taxonomy databases. Only plant species were selected. Settings were as follows: instrument type set as FT-ICR/Orbitrap; high energy CID as fragmentation mode; parent ion error tolerance 8 ppm; fragment ion error tolerance 0.03 Da; trypsin as enzyme; up to one missed cleavage allowed; carbamidomethylation as a fixed modification; deamidation and oxidation as variable modifications. Peptide spectrum match false discovery rate (FDR), peptide FDR and protein FDR all set to ≤ 1 %. The quality of the spectra were verified for proteins that were identified by only a single peptide sequence.

E - Scanning electron microscopy.. Ephedra monosperma ovules were collected from the Bev Glover Greenhouse, University of Victoria. Ovules were removed from branches and mounted on a Deben MK3 cold stage in a Hitachi S-3500N variable pressure scanning electron microscope (VP SEM). The microscope was operated at 20 kV, 50 Pa variable pressure in backscattered electron mode using a Robinson BSE detector.

Results

A - Comparative Study of Seven Ephedra Species.. All *Ephedra* pollination drops contained proteins (Fig. 3). The relatively light bands of *Ephedra* proteins run at native concentrations indicate lower amounts of protein, compared to that of larch and *Ginkgo* (Fig. 4). Proteins identified from liquid extractions of pollination drops can be separated into degradome and secretome proteins (Tables 1, 2). We did not include proteins that had good spectra that matched uncharacterized proteins, e.g. inferred protein from *Picea sitchensis* cDNA, although these could be as many as a third of the high quality identities for any one species, e.g. *E. foeminea* pollination drops contained 29 proteins, of which only 20 were characterized.

The number of characterized proteins in pollination drops of *Ephedra* species ranged from 6 to 20, averaging 13.4 $\pm$ 5.3 identified proteins/species (Table 3). *Ephedra foeminea* and *E. trifurca* contained more proteins (20), compared to *E. distachya* (15), *E. compacta* (13), *E. minuta* (11), *E. likiangensis* (9), and *E. monosperma* (6). These proteins could be divided into intracellular (64 %) and extracellular proteins (36 %). The percentage of intracellular proteins ranged from 44 – 100 %: *E. likiangensis* (44 %), *E. minuta* (45 %), *E. trifurca* (50 %), *E. compacta* (54 %), *E. monosperma* (67 %), *Ephedra foeminea* (80 %) and *E. distachya* (100%).

In all pollination drops a variety of intracellular proteins were detected (Tables 1, 3). The most frequently detected intracellular proteins - ubiquitin and polyubiquitin - were in five species (Table 1). Dessication-related proteins were detected in four species.Cyclophilin-α, histones, and elongation factor 1-α were detected in three different species. Four of the most common proteins, i.e. detected in more than three or more species, were detected in drops of *E. foeminea*. However, this might be expected given that the *E. foeminea* had the most proteins of any species in this comparative analysis. *E. compacta* had three of the commonly shared proteins. The remaining proteins on Table 1 were detected one or two times only.

Extracellular proteins were less abundant than intracellular proteins (Tables 2, 3). The most commonly shared extracellular proteins were xylosidases (Table 2), which were detected in drops of four *Ephedra* species. Aspartic protease, β-galactosidase, peroxidase and serine carboxypeptidase were detected in three *Ephedra* species. The remaining seven proteins on Table 2 were detected only once or twice.

On a species level, proteins detected in drops represented a wide variety of enzymes. The proteins are either water-soluble proteins secreted into the pollination drop, or are from the water-soluble portion of plant cells: no membrane-anchored proteins were detected in any samples. *Ephedra foeminea* drops had a probable defense protein (chitinase), two carbohydrate-modifying enzymes (β-xylosidase, glycosyl-hydrolase-like protein), and proteases (aspartic protease, serine carboxypeptidase). The largest number of proteins were associated with the cytoplasm, including histone proteins, citrate synthase, elongation-factor-1-α, cyclophilin, calreticulin, luminal-binding protein 4, a probable glycerophosphoryl diester phosphodiesterase, polyubiquitin, peptidyl-prolyl cis-trans isomerase, BIP isoform A, and granule bound starch synthase. *Ephedra trifurca* had a similar number of characterized proteins as *E. foeminea,* divided evenly between secretome and degradome. *Ephedra trifurca* had some of the same proteins as *E.*

foeminea (histone, elongation-factor-1-α, ubiquitin, chitinase, β-xylosidase, aspartic proteinase, serine carboxypeptidase). The proteins found in drops of *E. trifurca* were divided evenly between degradome and secretome. *Ephedra trifurca* had defense proteins, including a chitinase and an alpha amylase inhibitor, peroxidase and endoglucanases, as well as a carbohydrate-modifying enzymes, e.g. β-D-xylosidase and β-galactosidase, and a serine carboxypeptidase. Some other apoplastic enzymes, such as malate dehydrogenase, were detected.

In drops of *E. likiangensis*, intracellular and extracellular proteins were equally present; among the symplastic proteins, ubiquitin and proteases were predominant. *Ephedra minuta* drops had abundant symplastic ubiquitins (Table 3), as well as apoplastic carbohydrate-modifying enzymes (β-xylosidase, β-glucosidase) and defense proteins (thaumatin-like proteins). Cellular proteins not normally found in the apoplast included Elongation factor 1-α, ubiquitin, acyl-CoA-binding domain-containing protein, actin. *E. compacta* had a number of ubiquitin and polyubiquitin proteins, as well as acyl-CoA-binding domain-containing protein, calmodulin, a peptidase, and α-amylase; all of these were degradome proteins. Among the secretome proteins were β-xylosidase, β-galactosidase, SOD, aspartic protease and peroxidase. *Ephedra monosperma* had mostly degradome proteins (profilins, desiccation-related protein, the GTP-binding protein RAN-1, and ceramidase) and had only two secretome proteins that we could detect in this initial comparative study – serine carboxypeptidase and glucan endo-1,3-β-glucosidase. *Ephedra distachya* was unique among the species sampled, because all of its 15 proteins were degradome proteins (Table 3).

B - Comparative Study of Ephedra monosperma Drops from Three Dates.. We were able to get samples of *Ephedra monosperma* pollination drops from three different dates (Table 4). Thirty-two proteins were identified from these samples, more than four times the number found in *E. monosperma* sample used in the comparative study of different *Ephedra* species (Table 3). The number of proteins declined with time, with the largest number of proteins (22) found in the first sample (Mar. 9), which was not long after pollination drops began to be produced in the greenhouse. On the next two dates, progressively fewer proteins were found until only 14 proteins could be detected on the final date (Apr. 10). Four proteins, a homolog of serine carboxypeptidase-like 32 protein found in *Arabidopsis thaliana,* a histone 4 in *Pisum sativum,* α-galactosidase and a predicted protein homologous to one in *Populus trichocarpa*, were found at all three time points. Fourteen proteins were detected at two time points and 14 were only found at one time. Most proteins (20/32) were degradome proteins. The exceptions were extracellular proteins, such as serine carboxypeptidase, thaumatin-like protein, acid α- and β-galactosidase, peroxidase, as well as α –xylosidase.

Discussion

Pollination drops of *Ephedra* contain proteins. Although this has not been reported previously in *Ephedra*, it was expected, as all other pollination drops analyzed to date contain proteins. However, the protein profiles of in this study exhibit some notable differences from those of other gymnosperms we have measured, most of which were conifers (Wagner et al., 2007). *Ephedra* spp. not only have lower concentrations of protein, judging from the lightness of the bands in the gels, but also contain fewer total proteins. In addition, the protein profiles of *Ephedra* show substantial amounts of intracellular proteins not found in conifer pollination drops. In short, *Ephedra* has a degradome, consisting of proteins, and presumably shorter peptide fragments. The most likely source of the protein degradome is from nucellar degeneration which forms the flask-shaped pollen chamber during pollination drop production, causing intracellular proteins to be added to the other pollination drop compounds. This assumption is logical, since pollen chamber formation occurs prior to and during pollination drop secretion (Rydin et al., 2010). A protein that is characteristic of this degradome is ubiquitin, which plays a major role in recycling proteins inside a cell. It is not known to function outside of the cytoplasm, *i.e.* in the apoplastic fluids of plants. Protein profiles of both degradome and secretome are composed of a few dozen proteins at most. Compared to other gymnosperms, the average number of proteins, which is about a dozen per *Ephedra* species, is slightly greater than in pollination drops of the Cupressaceae sampled to date, which range from half-a-dozen to a dozen (Wagner et al., 2007), but much less than those of pinaceous species, such as *Pseudotsuga menziesii* (Poulis et al., 2005) and *Larix* x *marschlinsii* (O'Leary et al., 2007), which have many dozens each.

In *Ephedra* pollination drops there are also proteins that are not part of the degradome. These proteins are likely formed inside cells and discharged into the apoplastic fluid by active cellular processes, and together these constitute a secretome of substances exported into pollination drops, similar to what has been found in most gymnosperms investigated using proteomics. Chitinase is an example of a protein that belongs to the secretome. In the results reported here, chitinases were present in both *E. foeminea* and *E. trifurca*. Chitinase is also found in pollination drops of another gnetophyte, *Welwitschia mirabilis*, as well as a number of conifers (Wagner et al., 2007). In Douglas-fir drops, chitinases are able to process chitin substrates in situ, which suggests that they are active in anti-fungal defense during sexual reproduction (Coulter et al., 2012). Should the chitinases in the pollination drop of *Ephedra* prove functional, they may also protect ovules, which like those of other gymnosperms are exposed to the elements and are, therefore, more vulnerable to wind-borne pathogens than those of angiosperms which are enclosed within a protective ovary.

The percentage of characterized cellular versus secretory proteins in the drops ranged from 44 % to 100 %, depending on species. Other gymnosperms, such as *Juniperus*, typically have no intracellular proteins in their pollination drops (Wagner et al., 2007). The most common intracellular protein found in *Ephedra* pollination drops is ubiquitin, which is found in five of the seven species. Of the 24 intracellular proteins detected, only 10 are found in more than one species. This implies that although a degradome is universal in *Ephedra* pollination drops, its composition may widely differ among the species. To provide a better idea of variation of protein profiles, studies need

to be undertaken that focus on variation among individual plants as well as over the period of pollination drop secretion.

A measure of the variation in degradome is given by our samples of *E. monosperma* plants from the same greenhouse population over three time points from the early to late in the pollination drop period. There were more proteins at the beginning of the period than at the end, which implies that proteins initially present in drops are broken down over time. Most of the proteins were clearly intracellular proteins, e.g. GTP-binding nuclear protein RAN 1, confirming that a degradome is constantly present in the drops. Only a few proteins are found across all time points, e.g. histone 4, the majority varying widely. This was equally true for secretome and degradome profiles. *Ephedra monosperma* has as much variation over time as there is among all species of *Ephedra* (Table 3). Investigations into variation within a species are important, as they will better allow us to isolate proteins that may have biological function.

The question of function must be considered carefully. Caution must be exercised for many reasons. These drops not only capture pollen, but fungi, bacteria, viruses and dust. We have been able to show in previous studies that enzymes in the drop, in particular, chitinases and invertases are able to function in situ, but this work is difficult because of the small amount of liquid with which one has to work. As a consequence, it is one thing to find proteins with identities and therefore, functions, but it is quite another to prove that the proteins function as expected from their sequence-based identities.

We assume that the degradome proteins, for example, ubiquitin, and histones are not functional in the drop, because they are outside the cell where they are normally located. Cytoplasmic proteins such as ubiquitin are involved in recycling proteins and peptides targeted for breakdown *inside the cell*. Ubiquitin has not been previously found in pollination drops of Pinaceae in which pollen chambers are not formed and the nucelli do not undergo a degradation at the time of drop release, e.g. *Pseudotsuga* and *Larix*. Other proteins that are strictly cytoplasmic include cyclophilin A (a plant immunophilin), which is restricted to cell organelles: its presence in the drop is likely due to cell death and subsequent leakage of cellular contents.

Focusing on two species in the comparative study, *E. foeminea* and *E. distachya*, *E. foeminea* had the most detected proteins, half of which are degradome proteins, where *E. distachya* had only degradome proteins. Having about 50 percent degradome proteins is close to the average for the seven species that we measured. In addition to ubiquitin, just discussed, notable degradome proteins in *E. foeminea* are histones which are normally restricted to the nucleus and involved in chromosome organization, Granule-bound starch synthase which synthesizes amylose in the chloroplast, BIP isoform A which is a molecular chaperone located on the endoplasmic reticulum, and immunophilins such as peptidyl-prolyl cis-trans isomerase which are found in a number of locations within the cell. The predominance of these cytoplasmic proteins among the degradomic fraction is probably due to either their abundance in degrading cells, and/or in their slower rate of degradation compared to that of other proteins (i.e. already reduced to small peptides or amino acids). The profile of proteins detected in pollination drops of *E. distachya* consists entirely of intracellular proteins, none of which are normally found in apoplastic secretions including: proteins involved in signal transduction, e.g. small Ran-related GTP-binding protein; calmodulin 4 which is a regulatory protein controlled by calcium; nucleoside diphosphate kinase that regulates metabolic pools of nucleoside

diphosphates; histones that control chromosome organization; heat-shock proteins that regulate a plant cell's response to stress. Recently, there have been papers that suggest a few of these proteins may function in the apoplast. For example, root border cells of angiosperms and gymnosperms (Wen et al., 2008b) upregulate gene expression that results in secretion of intracellular proteins such as DNA-bound histones that act a trap for pathogens (Hawes et al., 2012).

In other gymnosperm pollination drops, most proteins do not appear to be related to a degradome, but are secreted by cells directly into the apoplast. In these cases the collective secreted protein component is known as a secretome. The secretome proteins that we have been able to identify from our analyses of various species of gymnosperms were from many classes of enzymes. We detected a variety of defense proteins, including among others, thaumatin-like protein, peroxidase, glucan-endo-β-1,3-glucanase, and superoxide dismutase. However, the proteins of the secretome are probably not all involved in defense. In addition there are carbohydrate-modifying enzymes such as α- and β-galactosidase proteins. In roots of peas, galactosidases operate on cell wall fragments to produce galactose, which is inhibitory to root growth (Wen et al., 2008a). All of the proteins that we have designated as part of the secretome, e.g. peroxidase, malate dehydrogenase, superoxide dismutase and thaumatin-like proteins, have been found apoplastically in other plants. Some protein classes have many members that have diverse functions, e.g. serine carboxypeptidases. These include serine carboxypeptidases that have regulatory functions both in the cytoplasm, as well as in the extracellular spaces. Until these proteins are shown to function in situ in the pollination drop, they are, like all other enzymes included in our lists, assigned to the secretome because they or members of their class of protein have been detected in the apoplast of other plants. In our survey of *Ephedra* presented here, no proteins are common to the secretomes of all species. The number of proteins ranges among the *Ephedra* species between 2 and 10 per pollination drop/species.

We expected to find acid phosphatase in the drop, since two different laboratories have reported its presence *via* activity assays in Gnetales. Ziegler (1959) detected it in the nucellus of *E. helvetica (=E. distachya* subsp. *helvetica*) as well as in the non-gnetalean *Taxus baccata* (Taxaceae) and Carafa et al. (1992) reported its presence in pollination drops of *W. mirabilis*. However, we did not detect this enzyme in any pollination drops of the seven *Ephedra* species that we analyzed using proteomics methods. We have never found it in any conifers, but a proteomic analysis of the nucellus has yet to be completed.

There are more proteins in these species than we have been able to describe. In all *Ephedra* species, there was a relatively high percentage of uncharacterized proteins. Although the mass spectra of proteins to which no identity can be assigned are of high quality, the databases against which we search this information often have insufficient depth, particularly with regards to gymnosperms. This situation should improve if, in future, databases improve. For example, genomes of *Picea abies* (Nystedt et al., 2013) and *P. glauca* (Birol et al., 2013) will be useful once they are annotated. As more gymnosperms are covered, the improved depth of the databases will assist in protein identification. Molecular biologists will be able to use these databases to make better protein identifications and to improve the prediction of functions for these proteins. However studies of the distantly related Gnetales may not benefit to as a large degree compared to those of conifers.

Ephedra pollination drops may be acting as nectar. However, in spite of high sucrose concentrations among all *Ephedra* pollination drops measured to date, not all species are insect-pollinated, e.g. *E. campylopoda* (Porsch, 1910): some are insect- and wind-pollinated, e.g. *E. aphylla* (Meeuse et al., 1990), and others are only wind-pollinated, e.g. *E. trifurca* (Buchmann et al., 1989). Insects that are not pollinators, such as ants, are also attracted to *Ephedra* drops (Porsch, 1910). Ziegler (1959) mentioned the high concentrations of amino acids in drops, which would influence the palatability of these drops to some types of insects. Insect pollination is certainly widespread among gnetophytes (Endress 1996), although it may not be obligate in any *Ephedra* species.

Until this study, any proteins in pollination drops were considered to probably be a functional portion of the drop (Nepi et al., 2009). The possibility that proteins may also be byproducts of pollen chamber formation that have been washed into the drop has never been explored. This is due to the fact that the species investigated to date did not have pollen chambers formed from nucellar breakdown. Thus the pollination drops of *Ephedra* are probably a mixture of functional and formerly functional, as well as biologically inactive proteins and/or peptides. As such, *Ephedra* differs from conifers analyzed to date, such as Pinaceae and Cupressaceae. It will be interesting to expand pollination drop analysis into *Pinus*, *Ginkgo* and cycads, all of which have pollen chambers. The low amount of protein in *Ephedra* drops suggests a less important role, if any, for these proteins during reproduction. The higher sucrose concentrations in these drops result in higher osmotic pressure in these drops, which may prevent foreign pollen from germinating (von Aderkas et al., 2012) and pathogens from establishing and growing.

Ephedra pollination drops have proteins that can be divided into those that belong to the degradome, itself a result of pollen chamber formation, and those that are exported by the cytoplasm into the drop and form an active part of the secretome that is, based on similarity to other gymnosperms, involved in carbohydrate modification, defense and other apoplastic activities.

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Captions

Figure 1. Scanning electron micrographs of *Ephedra monosperma* ovules. a. An open micropyle. b. A pollination drop partially exuded from the micropyle. c. A pollination drop fully exuded from the micropyle. Bar = 500 μm

Figure 2. *Ephedra* ovules. a. Ovule of *E. compacta* with pollination drop. b. *E. monosperma* with an insect feeding on the pollination drop.

Figure 3: 1D SDS-PAGE of proteins at native concentrations in *Ephedra* pollination drops. Lanes from left to right: molecular weight ladder (kDa), 1. *E. distachya*, 2. *E. distachya*, 3. *E. foeminea*, 4. *E. minuta*, 5. *E. likiangensis*, 6. *E. monosperma*.

Figure 4: 1D SDS-PAGE of native concentrations of proteins in pollination drops of three gymnosperms: Lane 1. *Larix* x *marschlinsii*, Lane 2. *E. monosperma*, Lane 3. *Gingko biloba.* Figure is only to show number of bands and relative band intensity.

Table 1: Degradome proteins found in pollination drops of *Ephedra* spp.

Protein	Species
Ubiquitins	*E. compacta* *E. foeminea* *E. likiangensis* *E. minuta* *E. trifurca*
Dessication-related protein	*E. compacta* *E. likiangensis* *E. minuta* *E. monosperma*
Cyclophilin A	*E. distachya* *E. foeminea* *E. minuta*
Elongation factor 1- α	*E. distachya* *E. foeminea* *E. trifurca*
Histones	*E. distachya* *E. foeminea* *E. trifurca*
Acyl-CoA-binding domain-containing protein 6	*E. compacta* *E. trifurca*
α -Amylase	*E. compacta* *E. likiangensis*
Calmodulin	*E. compacta*

	E. distachya
Glycosyl hydrolase	*E. foeminea* *E. trifurca*
GTP-binding nuclear protein	*E. distachya* *E. monosperma*
α -Amylase inhibitor	*E. trifurca*
Auxin response factor	*E. distachya*
Calreticulin	*E. foeminea*
Ceramidase	*E. monosperma*
Citrate synthase	*E. foeminea*
Cysteine proteinase	*E. likiangensis*
α -Gliadin	*E. trifurca*
Glycerophosphoryl diester phosphodiesterase	*E. foeminea*
Granule-bound starch synthase	*E. foeminea*
Heat shock proteins	*E. distachya*
Lactoylglutathione lyase	*E. trifurca*
Luminal-binding protein	*E. foeminea*
Profilin	*E. monosperma*
Thiol protease aleurain	*E. likiangensis*

Table 2: Secretome proteins found in pollination drops of *Ephedra* spp. Proteins that could also be considered degradome are marked with an asterisk.

Protein	Species
Xylosidases	*E. compacta* *E. foeminea* *E. minuta* *E. trifurca*
Aspartic proteinase*	*E. compacta* *E. likiangensis* *E. trifurca*
Galactosidases	*E. compacta* *E. minuta* *E. trifurca*
Peroxidase	*E. compacta* *E. likiangensis* *E. trifurca*
Serine carboxypeptidases*	*E. foeminea* *E. monosperma* *E. trifurca*
Chitinase	*E. foeminea* *E. trifurca*
Glucan endo-1,3- β -glucosidase	*E. monosperma* *E. trifurca*
Malate dehydrogenase	*E. trifurca*
Peptidase*	*E. likiangensis*
Superoxide dismutase*	*E. compacta*
Thaumatin-like protein	*E. minuta*

Table 3: Peptide sequences and identities of pollination drop proteins found in *Ephedra* spp. Proteins in red are degradome proteins, those in black are secretome.

Species	Peptide amino acid sequence obtained	Protein identification
E. compacta	K.SSEEAME(sub N)DYITK.V M.GLKEEFEEY(sub H)AEK.V R.AKWDAWK.A	Acyl-CoA-binding domain-containing protein 6 OS=*Arabidopsis thaliana*
	K.EGIPPVQQR.L R.TLADYNIQK.E E.VESSDTIDNVK.A	Ubiquitin-NEDD8-like protein RUB2 OS=*Oryza sativa* subsp. *japonica*
	R.TLADYNIQK.E K.EGIPPVQQR.L	Polyubiquitin 2 OS=*Zea mays*
	R.TLADYNIQK.E E.VESSN(+.98)TIDNVK.A	Putative polyubiquitin (Fragment) OS=*Arabidopsis thaliana*
	R.NIQVVDGSNNLKAPK.G	Putative carboxyl-terminal peptidase OS=*Arabidopsis thaliana*
	R.VFDKDQNGFISAAELR.H	Calmodulin (Fragment) OS=*Pyrus communis*
	K.AVADIVINHR.C	Alpha amylase (Fragment) OS=*Cuscuta reflexa*
	L.GVESGQDAVIR.G R.TPEEILR.I	Dessication-related protein_ putative; 70055-71849 OS=*Arabidopsis thaliana*
	K.VTEQDLE(sub A)DTYNPPFK.S	Putative beta-xylosidase (Fragment) OS=*Triticum aestivum*
	R.STPEMWPDIIQK.A	Beta-galactosidase OS=*Picea sitchensis*
	R.AVVVHADPDDLGK.G	Superoxide dismutase [Cu-Zn] OS=*Pinus sylvestris*
	K.GEHTYVPVTK.K	Aspartic proteinase (Fragment) OS=*Cucumis sativus*
	R.FDNNYYK.D	Peroxidase (Fragment) OS=*Lupinus polyphyllus*
E. distachya	K.ATAGDTHLGGEDFDNR.M R.IINEPTAAAIAYGLDKK.A R.VEIIPNDQGNR.T K.NKITITNDKGR.L	Heat shock 70 kDa protein OS=*Glycine max*
	K.ATAGDTHLGGEDFDNR.M R.IINEPTAAAIAYGLDKK.A R.VEIIANDQGNR.T K.NKITITNDKGR.L	Heat shock cognate 70 kDa protein 1 OS=*Solanum lycopersicum*
	R.ELISNSSDALDKIR.F K.ADLVNNLGTIAR.S D.AIDEYAIGQLK.E R.FESLTDK.S	Heat shock protein 81-2 OS=*Arabidopsis thaliana*
	K.IGGIGTVPVGR.V	Elongation factor 1-alpha

	N.IVVIGHVDSGK.S	OS=*Zea mays*
	R.VETGVIKPG.M	
	F.DKDQNGFISA.A	Calmodulin 4 (Fragment) OS=*Daucus carota*
	MADQLTDDQISEFK.E	
	FDKDGDGC(+57.02)ITTK.E	Calmodulin protein (Fragment) OS=*Pinus taeda*
	R.DNIQGITKPAIR.R	Histone H4 OS=*Solanum melongena*
	L.FEDTNLC(+57.02)AIHAK.R	Histone H3-like 1 OS=*Arabidopsis thaliana*
	R.NVIHGSDAVESAQ(sub R)K.E	Nucleoside diphosphate kinase OS=*Arabidopsis lyrata* subsp. *lyrata*
	K.AGFAGDDAPR.A	Actin-3 OS=*Glycine max*
	R.GNGTGGESIYGEK.F	Peptidyl-prolyl cis-trans isomerase OS=*Zea mays*
	R.VLQISGER.N	18.1 kDa class I heat shock protein (Fragment) OS=*Medicago sativa*
	R.VLQISGER.S	Small heat shock protein hsp10.4 (Fragment) OS=*Quercus suber*
	K.LVIVGDGGTGK.T	GTP-binding nuclear protein Ran2 OS=*Solanum lycopersicum*
	K.LVIVGDGGTGK.T	Small Ran-related GTP-binding protein OS=*Triticum aestivum*
	R.TFVKVYK.S	Auxin response factor 12 OS=*Oryza sativa* subsp. *indica*
E. foeminea	K.EALQAEVGLPVDR.N K.VVGTPAYEEM(+15.99)VR.N R.FAFSDYPELNLPER.F K.SSFDFIDGYEKPVEGR.K K.MGDGYETVR.F R.VLTVSPYYAEELISGIAR.G	Granule-bound starch synthase 1_ chloroplastic/amyloplastic OS=*Zea mays*
	R.FAFSDYPELNLPER.F K.VVGTPAYEEM(+15.99)VR.N K.EALQAEVGLPVDR.N K.MGDGYETVR.F K.SSFDFIDGYEKPVEGR.K R.VLTVSPYYAEELISGIAR.G	Granule-bound starch synthase OS=*Zea mays* subsp. *mays*
	R.EAEEFAEEDKK.V K.FELSGIPPAPR.G R.VEIESLFDGVDFSEPLTR.A K.DYFDGKEPNK.G R.LSQEEIER.M	BiP isoform A OS=*Glycine max*
	K.EAEEFAEEDKK.V R.VEIESLFDGVDFSEPLTR.A K.DYFDGKEPNK.G R.LSQEEIER.M	Luminal-binding protein 4 OS=*Nicotiana tabacum*
	K.TFASGILVPK.S	Probable

		glycerophosphoryl diester phosphodiesterase 3 OS=*Arabidopsis thaliana*
	DNIQGITKPAIR.R	Histone H4 (Fragment)
	R.ISGLIYEETR.G	OS=*Daucus carota*
	R.DNIQGITKPAIR.R	Histone H4 OS=*Silene latifolia*
	R.ISGLIYEETR.G	
	K.KPEGYDDIPK.E	Calreticulin OS=*Zea mays*
	K.LDC(+57.02)GGGYVK.L	
	K.KPEGYDDIPK.E	Calreticulin OS=*Prunus armeniaca*
	R.FEDGWDKR.W	
	R.EIAQDFK.T	Histone H3-like 1 OS=*Arabidopsis thaliana*
	R.TLADYNIQK.E	Polyubiquitin 9 OS=*Arabidopsis thaliana*
	R.ALGLPLERPK.S	Citrate synthase OS=*Picea sitchensis*
	R.ALGLPLERPK.S	Citrate synthase 5_ mitochondrial OS=*Arabidopsis thaliana*
	R.GNGTGGESIYGEK.F	Peptidyl-prolyl cis-trans isomerase OS=*Zea mays*
	R.IGGIGTVPVGR	Elongation factor 1-alpha (Fragments) OS=*Pseudotsuga menziesii*
	K.AGLQFPVGR.I	Histone H2A OS=*Euphorbia esula*
	V.VTQ(+.98)QDLDDTYQPPFK.S	Beta-xylosidase/alpha-L-arabinofuranosidase 2 OS=*Medicago varia*
	R.VWVYSGDTDGR.V	Serine carboxypeptidase-like 32 OS=*Arabidopsis thaliana*
	R.AINSM(+15.99)ECNGGNPSAVQ(sub D)DR.V	Class IV chitinase OS=*Nepenthes alata*
	R.C(+57.02)YESYSEDPS(sub K)IVK.A	Glycosyl hydrolase-like protein (Fragment) OS=*Picea sitchensis*
E. likiangensis	K.IQDKEGIPPDQQR.L	Ubiquitin OS=*Triticum aestivum*
	E.VESSDTIDNVK.A	
	R.TLADYNIQK.E	
	K.YNGGIDTEEA(sub S)YPYK.G	Thiol protease aleurain OS=*Hordeum vulgare*
	R.EDGIVSPVK.N	
	L.GVESGQDAVIR.G	Dessication-related protein_ putative; 70055-71849 OS=*Arabidopsis thaliana*
	R.TPEEILR.I	
	K.AVADIVINHR.C	Alpha amylase (Fragment) OS=*Cuscuta reflexa*
	R.FDNNYYK.D	Peroxidase (Fragment) OS=*Lupinus polyphyllus*
	R.NIQVVDGSNNLKAPK.G	Putative carboxyl-terminal peptidase OS=*Arabidopsis thaliana*
	A.Q(+.98)GSGEYFTR.I	Aspartic proteinase

		nepenthesin-1_ putative OS=*Ricinus communis*
	K.GEHTYVPVTK.K	Aspartic proteinase (Fragment) OS=*Cucumis sativus*
	R.EDGIVSPVK.D	Cysteine proteinase OS=*Elaeis guineensis* var. *tenera*
E. minuta	R.LIFAGKQLEDGR.T K.EGIPPVQQR.L R.TLADYNIQK.E E.VESSDTIDNVKAK.I	Ubiquitin-NEDD8-like protein RUB2 OS=*Oryza sativa* subsp. *japonica*
	K.VESSDTIDNVKAK.I R.LIFAGKQLEDGR.T R.TLADYNIQK.E	Ubiquitin OS=*Musa acuminata*
	R.LIFAGKQLEDGR.T R.TLADYNIQK.E K.EGIPPVQQR.L	Polyubiquitin 2 OS=*Zea mays*
	L.GVESGQDAVIR.G R.TPEEILR.I	Dessication-related protein_ putative; 70055-71849 OS=*Arabidopsis thaliana*
	R.GNGTGGESIYGEK.F	Cyclophilin A (Fragment) OS=*Triticum aestivum*
	K.FFKGQC(+57.02)PQAYSYAK.D K.DDATSV(sub T)FTC(+57.02)PSP(sub G)TNYK.V K.GQC(+57.02)PQAYSYAK.D	Thaumatin-like protein OS=*Cryptomeria japonica* Thaumatin-like protein OS=*Pinus taeda*
	R.STPEMWPDIIQK.A K.NVVFNTAK.I K.WGHLKEL.H	Beta-galactosidase OS=*Picea sitchensis*
	R.YAVNYVR.G	Beta-glucosidase_ putative OS=*Ricinus communis*
	A.VNQDSLGVQGK.K K.ALADYVHAK.G	Alpha-galactosidase OS=*Oryza sativa* subsp. *japonica*
	R.WEVPYNLLPR.E	Alpha-xylosidase OS=*Arabidopsis thaliana*
E. monosperma	K.YM(+15.99)VIQGEPGVVIR.G	Profilin-1 (Fragment) OS=*Triticum aestivum*
	K.YM(+15.99)VIQGEPGVVIR.G	Profilin OS=*Zea mays*
	L.LGVESGQDAVIR.G	Dessication-related protein_ putative; 70055-71849 OS=*Arabidopsis thaliana*
	K.LVIVGDGGTGKT.T	GTP-binding nuclear protein Ran-A1 OS=*Nicotiana tabacum*
	R.SPSAYLNNPP(sub A)EER.N	Ceramidase_ putative OS=*Ricinus communis*
	R.VWVYSGDTDGRVP.V	Serine carboxypeptidase 1 OS=*Zea mays*
	L.FNENLKPGPTG(sub S)ER.N	Glucan endo-1_3-beta-glucosidase 11 OS=*Arabidopsis thaliana*

E. trifurca	K.SSEEAME(sub N)DYITK.V M.GLKEEFEEY(sub H)AEK.V R.AKWDAWK.A	Acyl-CoA-binding domain-containing protein 6 OS=*Arabidopsis thaliana*
	K.EGIPPVQQR.L R.TLADYNIQK.E K.IQDKEGIPPDQQR.L E.VESSDTIDNVK.A	Ubiquitin-NEDD8-like protein RUB2 OS=*Oryza sativa* subsp. *japonica*
	K.EGIPPVQQR.L R.TLADYNIQK.E K.IQDKEGIPPDQQR.L L.EVESSDTIDNVK.A	Ubiquitin_ putative OS=*Ricinus communis*
	K.ITSFLDPDGWK.T	Lactoylglutathione lyase OS=*Gossypium hirsutum*
	K.V(sub T)VLVDNEDFLK.E Q.QLPQFEEIR.N	Alpha-gliadin OS=*Triticum aestivum*
	K.VTE(sub L)QDLEDTYNPPFK.S	Os11g0291000 protein OS=*Oryza sativa* subsp. *japonica*
	R.IGGIGTVPVGR	Elongation factor 1-alpha (Fragments) OS=*Pseudotsuga menziesii*
	K.EHGAQEGQAGTGAFPR.C	Alpha-amylase inhibitor 0.19 OS=*Triticum aestivum*
	K.EHGAQEGQAGTGAFPR.C	Dimeric alpha-amylase inhibitor OS=*Aegilops umbellulata*
	K.AGLQFPVGR.I	Probable histone H2A.1 OS=*Oryza sativa* subsp. *japonica*
	K.VTQ(+.98)QDLEDTYNP(sub V)PFK.S E.TMIGNYAGK.A E.WWSEALHGISDVGPGT(sub A)K.F	Beta-D-xylosidase 1 OS=*Arabidopsis thaliana*
	H.T(sub S)AITSGQGFGGTIK.A R.ELAAFFANVMHETS(sub G)GL.C	Class IV chitinase Chia4-Pa2 variant (Fragment) OS=*Picea abies*
	S.WNYNYGAAGK.S R.STPEMWPDLIQK.A A.FRTDNEPFKA.A	Beta-galactosidase OS=*Pyrus communis*
	R.STPEMWPDLIR.K	Beta-galactosidase (Fragment) OS=*Mangifera indica*
	K.MELIDAAFPLLK.G	Malate dehydrogenase OS=*Picea sitchensis*
	R.VWVYSGDTDGRVPVT.S	Serine carboxypeptidase II-3 OS=*Hordeum vulgare*
	I.GGYYDAGDNVK.F	Endoglucanase 20 OS=*Arabidopsis thaliana*
	GGYYDAGDNVK.F	Putative endo-1_4_-beta-glucanase (Fragment) OS=*Solanum lycopersicum*
	R.FDNNYYK.D	Peroxidase (Fragment) OS=*Lupinus polyphyllus*
	K.GEHTYVPVTK.K	Aspartic proteinase (Fragment) OS=*Cucumis sativus*

Table 4: *Ephedra monosperma* pollination drop proteins from three collection dates. Degradome proteins are at the top of the list, marked by a red "x".

Protein	Mar 9	Mar 24	Apr 10
Histone H4 OS=Pisum sativum PE=1 SV=2	x	x	x
Predicted protein OS=*Populus trichocarpa* GN=POPTRDRAFT_642406 PE=4 SV=1	x	x	x
Putative uncharacterized protein OS=*Selaginella moellendorffii* GN=SELMODRAFT_143620 PE=4 SV=1	x	x	
Putative uncharacterized protein OS=*Glycine max* PE=2 SV=1	x	x	
Acyl-CoA-binding protein (Fragment) OS=*Jatropha curcas* PE=2 SV=1	x	x	
Glycosyl hydrolase family-like protein OS=*Salvia miltiorrhiza* PE=2 SV=1	x	x	
GTP-binding nuclear protein Ran-A1 OS=Nicotiana tabacum GN=RAN-A1 PE=2 SV=1		x	x
Eukaryotic initiation factor 4A OS=Triticum aestivum PE=2 SV=1		x	x
RAS-like protein (Fragment) OS=*Arabidopsis thaliana* PE=2 SV=1		x	x
Translation initiation factor OS=*Zea mays* GN=eIF-4A PE=2 SV=1		x	x
Acid beta-fructofuranosidase OS=Solanum lycopersicum GN=TIV1 PE=2 SV=1	x		
Alpha-glucosidase OS=Hordeum vulgare PE=2 SV=1	x		
Multicystatin OS=*Helianthus annuus* GN=smc PE=2 SV=1	x		
Polyubiquitin 11 OS=Arabidopsis thaliana GN=UBQ11 PE=1 SV=1	x		
Predicted protein OS=*Populus trichocarpa* GN=POPTRDRAFT_1090916 PE=4 SV=1	x		
Endoglucanase 23 OS=Oryza sativa subsp. japonica GN=GLU12 PE=2 SV=1	x		
NtPRp27-like protein OS=*Solanum tuberosum* PE=2 SV=1		x	
Ubiquitin-like protein (Fragment) OS=*Solanum lycopersicum* GN=ubiquitin-like PE=2 SV=1		x	
Cyclophilin A (Fragment) OS=*Triticum aestivum* GN=CYP18-3 PE=3 SV=1			x
Photosystem II Q(B) protein (Fragment) OS=*Kochia scoparia* GN=psbA PE=4 SV=1			x
Alpha-galactosidase OS=Coffea arabica PE=1 SV=1	x	x	x
Serine carboxypeptidase-like 32 OS=Arabidopsis thaliana GN=SCPL32 PE=2 SV=1	x	x	x
Alpha-galactosidase OS=Oryza sativa subsp. japonica GN=Os10g0493600 PE=1 SV=1	x		x
Acid alpha galactosidase 1 OS=*Cucumis sativus* PE=2 SV=1	x		x
Alpha-xylosidase OS=*Arabidopsis lyrata* GN=ARALYDRAFT_894626 PE=4 SV=1	x	x	
Peroxidase (Fragment) OS=Lupinus polyphyllus PE=2 SV=2	x	x	
Alpha-xylosidase OS=Arabidopsis thaliana GN=XYL1 PE=1 SV=1	x	x	
Beta-galactosidase 1 OS=Oryza sativa subsp. japonica GN=Os01g0533400 PE=2 SV=1		x	x
Beta-galactosidase 8 OS=Arabidopsis thaliana GN=BGAL8 PE=2 SV=2	x		
Thaumatin-like protein OS=*Cryptomeria japonica* GN=Cry j 3.1 PE=2 SV=1	x		
Alpha-galactosidase OS=*Coffea canephora* GN=gal1 PE=2 SV=1		x	
Beta-galactosidase 9 OS=Oryza sativa subsp. japonica GN=Os06g0573600 PE=2 SV=1			x

Figure 1

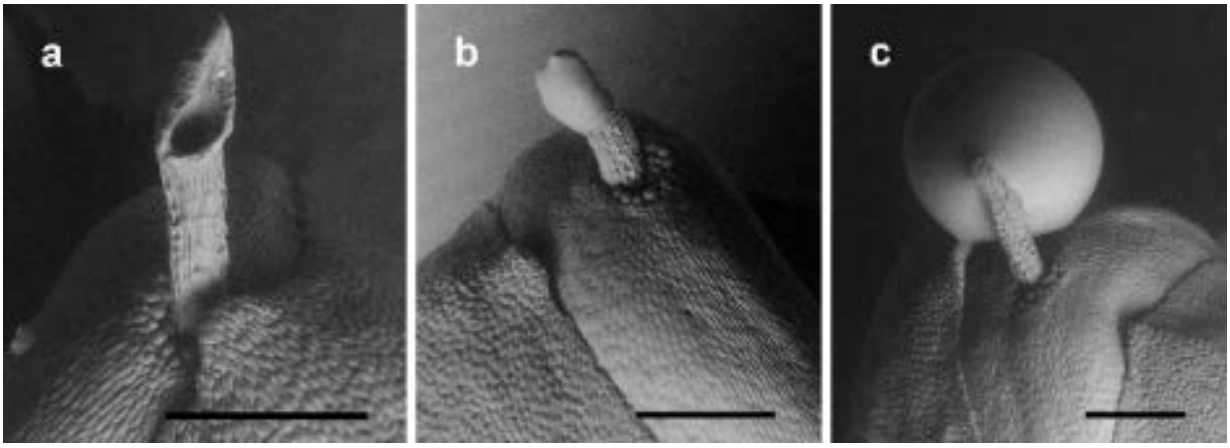

Figure 2

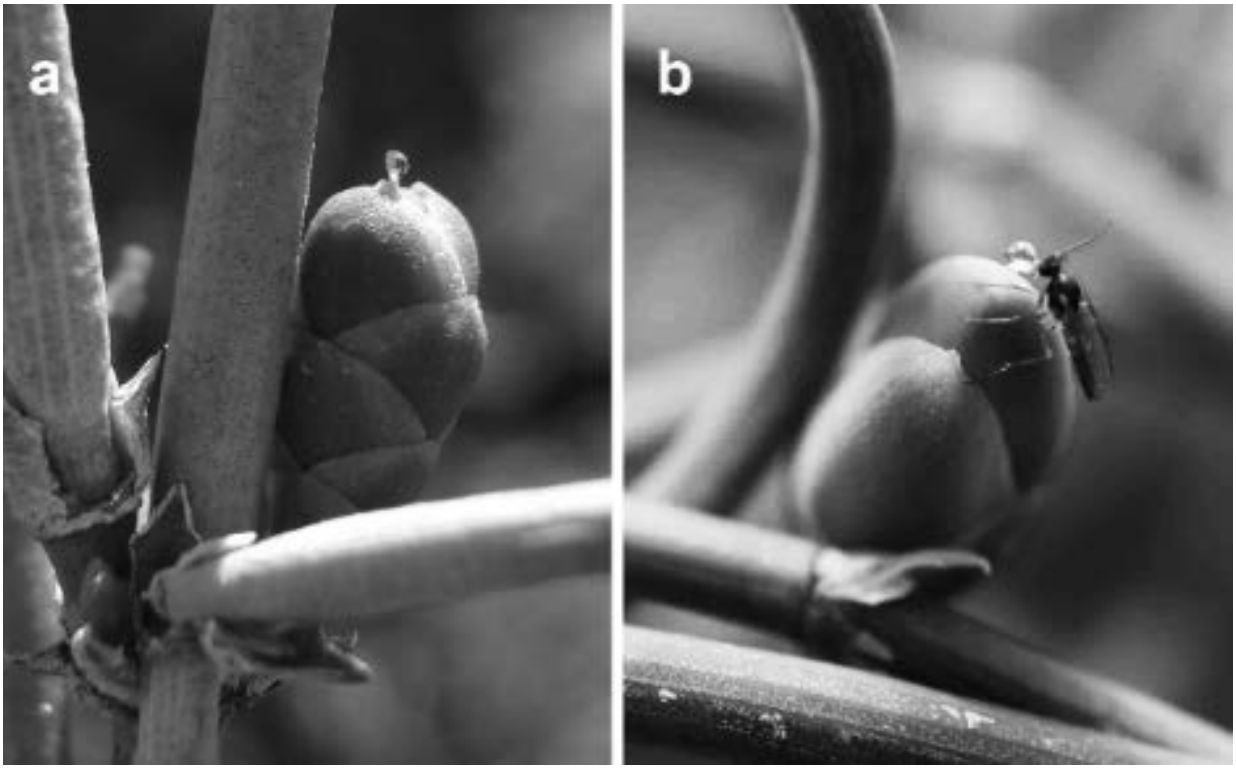

Figure 3

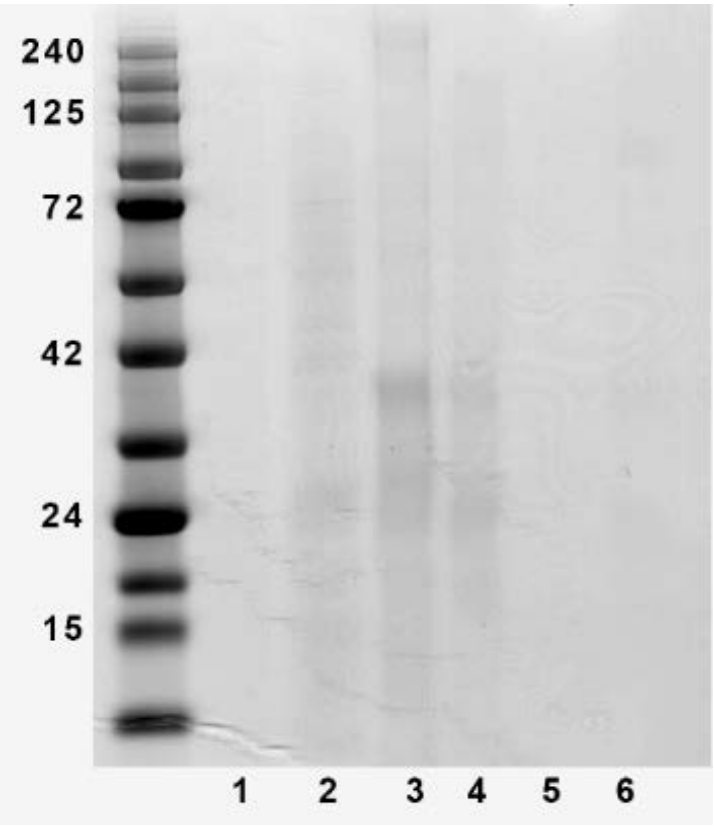

Figure 4