Annals of Botany **117**: 973–984, 2016
doi:10.1093/aob/mcw026, available online at www.aob.oxfordjournals.org

ANNALS OF BOTANY
Founded 1887

Insights from the pollination drop proteome and the ovule transcriptome of *Cephalotaxus* at the time of pollination drop production

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Received: 20 July 2015 Returned for revision: 12 October 2015 Accepted: 8 January 2016 Published electronically: 4 April 2016

• **Background and Aims** Many gymnosperms produce an ovular secretion, the pollination drop, during reproduction. The drops serve as a landing site for pollen, but also contain a suite of ions and organic compounds, including proteins, that suggests diverse roles for the drop during pollination. Proteins in the drops of species of *Chamaecyparis*, *Juniperus*, *Taxus*, *Pseudotsuga*, *Ephedra* and *Welwitschia* are thought to function in the conversion of sugars, defence against pathogens, and pollen growth and development. To better understand gymnosperm pollination biology, the pollination drop proteomes of pollination drops from two species of *Cephalotaxus* have been characterized and an ovular transcriptome for *C. sinensis* has been assembled.

• **Methods** Mass spectrometry was used to identify proteins in the pollination drops of *Cephalotaxus sinensis and C. koreana*. RNA-sequencing (RNA-Seq) was employed to assemble a transcriptome and identify transcripts present in the ovules of *C. sinensis* at the time of pollination drop production.

• **Key Results** About 30 proteins were detected in the pollination drops of both species. Many of these have been detected in the drops of other gymnosperms and probably function in defence, polysaccharide metabolism and pollen tube growth. Other proteins appear to be unique to *Cephalotaxus*, and their putative functions include starch and callose degradation, among others. Together, the proteins appear either to have been secreted into the drop or to occur there due to breakdown of ovular cells during drop production. Ovular transcripts represent a wide range of gene ontology categories, and some may be involved in drop formation, ovule development and pollen–ovule interactions.

• **Conclusions** The proteome of *Cephalotaxus* pollination drops shares a number of components with those of other conifers and gnetophytes, including proteins for defence such as chitinases and for carbohydrate modification such as β-galactosidase. Proteins likely to be of intracellular origin, however, form a larger component of drops from *Cephalotaxus* than expected from studies of other conifers. This is consistent with the observation of nucellar breakdown during drop formation in *Cephalotaxus*. The transcriptome data provide a framework for understanding multiple metabolic processes that occur within the ovule and the pollination drop just before fertilization. They reveal the deep conservation of *WUSCHEL* expression in ovules and raise questions about whether any of the *S*-locus transcripts in *Cephalotaxus* ovules might be involved in pollen–ovule recognition.

Key words: Cephalotaxus, proteome, transcriptome, development, gymnosperm, pollen selection.

INTRODUCTION

In most extant gymnosperms, pollination relies on the wind-mediated transfer of pollen to ovulate cones. Many gymnosperm species produce an ovular secretion, the pollination drop, which extends beyond the micropyle and forms a liquid surface on which the pollen lands. Subsequently, the drop withdraws and transports pollen grains into the ovule where they germinate, form pollen tubes and ultimately release sperm that fertilize eggs (Doyle and O'Leary, 1935; Singh, 1978). The sole function of the pollination drop was once thought to be transport of pollen into the ovule. These ovular secretions, however, contain a suite of organic and inorganic compounds including sugars, amino acids, organic acids, proteins and calcium (Ziegler, 1959; Seridi-Benkaddour and Chesnoy, 1988; Carafa *et al*., 1992). The nature of the proteins in the drops suggests that in addition to nourishing the developing pollen, they play diverse roles in pollination (Gelbart and von Aderkas, 2002; Nepi *et al*., 2009; Coulter *et al*., 2012).

Studies of pollination drops using immunological methods detected arabinogalactans in *Taxus* × *media* (O'Leary *et al*., 2004), while chemical methods detected acid phosphatase in *Welwitschia mirabilis* (Carafa *et al*., 1992). Studies using proteomic methods have focused on the conifers *Pseudotsuga menziesii* (Poulis *et al*., 2005), *Taxus* × *media* (O'Leary *et al*., 2007), *Juniperus communis*, *Juniperus oxycedrus* and *Chamaecyparis lawsoniana*; and on the gnetophytes, *Welwitschia mirabilis* (Carafa *et al*., 1992; Wagner *et al*., 2007) and several species of *Ephedra* (von Aderkas *et al*., 2015).

TABLE 1. *Proteins previously identified in the pollination drops of gymnosperms*

	Chae. laws.	Jun. com.	Jun. oxy.	Pseu. men.	Tax. × med.	Wel. mir.	Eph. comm.	Eph. foe.	Eph. min.	Eph. tri.	Eph. lik	Eph. mon.	Eph. comp.
β-D-Glucan exohydrolase	x												
Glucan 1,3-β-glucosidase (or precursor)	x	x								x		x	
Subtilisin-like proteinase	x												
Glycosyl hydrolase		x											
Glucanase-like protein			x										
Chitinase			x			x		x		x			
Thaumatin-like protein	x	x	x						x				
Xylosidase				x			x	x	x	x			x
Galactosidase				x			x		x	x			x
Peroxidase				x			x			x	x		x
Invertase				x									
Aspartyl protease				x			x			x	x		x
Serine carboxypeptidase (-like) protein				x				x		x		x	
Arabinogalactan protein					x								
Malate dehydrogenase										x			
Peptidase											x		
Superoxide dismutase							x						

Plant names are abbreviated as follows: Chae. laws., *Chamaecyparis lawsonia*; Jun. com., *Juniperus communis*; Jun. oxy., *Juniperus oxycedrus*; Pseud. menz., *Pseudotsuga menziesii*; Tax. × med., *Taxus × media*; Wel. mir., *Welwitschia mirabilis*; Eph. comm., *Ephedra communis*; Eph. foe., *Ephedra foeminea*; Eph. min., *Ephedra minuta*; Eph. tri., *Ephedra trifurca*; Eph. lik., *Ephedra likiangensis*; Eph. mon., *Ephedra monosperma*; Eph. comp., *Ephedra compacta*.

Adapted from Coulter *et al*. (2012) and von Aderkas *et al*. (2015).

These studies detected thaumatin-like proteins, chitinases, invertase, galactosidase, peroxidase and subtilisin-like protease, among others (Table 1). Putative functions of the proteins include the conversion of sugars, cleavage of polysaccharides, defence against pathogens, and the expansion and growth of pollen tubes (Table 1) (Gelbart and von Aderkas, 2002; Poulis *et al*., 2005; Wagner *et al*., 2007). Chitinases in *Pseudotsuga menziesii* have antifungal activity (Coulter *et al*., 2012). Some of the proteins in Table 1 are conserved among taxa, such as chitinase and glucosidase, while others, such as galactosidase, have so far been observed in fewer species.

Developmental studies suggest that pollen selection conceivably could occur within pollination drops. In the ovules of several species of Pinaceae, differences in pollen germination and pollen tube growth and development were observed, depending on whether the pollen was conspecific, heterospecific (McWilliam, 1959; Fernando *et al*., 2005) or heterogeneric (von Aderkas *et al*., 2012) with respect to the ovule. The underlying mechanisms controlling these differential responses of pollen and pollen tubes are unknown, however. It is possible that nutritional requirements of male gametophytes are species specific, leading some to thrive in certain pollination drops while others languish or die (Gelbart and von Aderkas, 2002). Alternatively, protein–protein interactions may occur between the pollen and the ovule, beginning with pollen recognition and culminating in the destruction or inhibition of the growth of certain pollen types. Protein–protein interactions between pollen and/or pollen tubes and ovules have been well documented in angiosperms, including those of the incompatibility reactions of S-locus proteins. Self-incompatibility (SI) systems are found in at least 100 angiosperm families (Igic *et al*., 2008), and comprise diverse molecular mechanisms, the most well characterized of which are the sporophytic self-incompatibilty (SSI) system found in members of the Brassicaceae, and the gametophytic self-incompatibilty (GSI) systems found in the Papaveraceae, Solanaceae, Plantaginaceae and Rosaceae (for reviews, see Takayama and Isogai, 2005; Franklin-Tong, 2008; Iwano and Takayama, 2012; Gibbs, 2014).

Proteomic studies are needed in additional gymnosperms to better understand pollination drop composition and function in conifers, cycads, *Ginkgo* and gnetophytes. Here, we documented the proteins present in the pollination drops of *Cephalotaxus*, for which no data were previously available. *Cephalotaxus*, or 'plum yew' (Fig. 1), is native to southern and eastern Asia, and includes 8–11 species (Bassett *et al*., 2005). It is the sole genus in the Cephalotaxaceae, although some taxonomic treatments do not recognize Cephalotaxaceae, but consider *Cephalotaxus* to be a divergent genus within the Taxaceae (Rai *et al*., 2008). Regardless, the divergence of *Cephalotaxus* from Taxaceae *sensu stricto* is ancient, having occurred about 150 Ma (Leslie *et al*., 2012). We focused on *Cephalotaxus sinensis* and *Cephalotaxus koreana*. We also generated RNA-sequencing (RNA-Seq) data to document the transcripts present in ovules of *C. sinensis* at the time of pollination drop production and to provide additional insight on ovule metabolic processes and pollination drop functions. This is the first transcriptome assembled from a *Cephalotaxus* ovule at the time of pollination drop production, and the first transcriptome of any gymnosperm ovule.

MATERIALS AND METHODS

Pollination drop and ovule collection

Pollination drops and ovules were collected from species of *Cephalotaxus* at the Arnold Arboretum of Harvard University (Boston, MA, USA). Pollination drops were collected in April 2011 from each of *C. sinensis* Rehder and Wilson and *C. koreana* Nakai using a flame-drawn capillary tube. Drops were pooled to obtain a minimum of 100 µL per species and were stored at –20 °C until analysis.

FIG. 1. *Cephalotaxus koreana*. (A) Branch with male cones before pollen shedding. (B) Female cones with pollination drops.

In April 2012, cones were collected from *C. sinensis* at the time of pollination drop production and were frozen immediately in liquid nitrogen for RNA-Seq analyses.

Protein preparation, electrophoresis, mass spectrometry and data analysis

Sample preparation, electrophoresis and protein sequencing followed previous methods (Prior *et al.*, 2013). In brief, 1-D SDS–PAGE was performed, and gels were stained with Coomassie blue. Bands were excised from gels, digested with trypsin, and analysed at the University of Victoria Genome BC Proteomics Center via high-performance liquid chrtomatography–tandem mass spectrometry (HPLC-MS/MS). HPLC was performed on a RP nano-analytical column Magic C-18AQ (Michrom BioResources Inc., Auburn, CA, USA). The chromatography system was coupled online with an LTQ Orbitrap Velos mass spectrometer equipped with a Nanospray II source (Thermo Fisher Scientific).

The raw data files were searched using Thermo Scientific Proteome Discoverer software version 1.2 (Thermo Fisher Scientific) with Mascot version 2.2.1 (Matrix Science, Boston, MA, USA) and PEAKS Studio v. 6 (Bioinformatics Solutions Inc., Ontario, Canada) against the UniProt-SwissProt and Uniprot-Trembl databases. Several post-translational modifications were tested for during searches. These included the fixed carbamidomethylation of cysteines when iodoacetamide was used in sample processing, oxidation of methionine and deamidation (N, Q). Due to the paucity of gymnosperm data available in these databases, *de novo* discovery of peptides was also performed using PEAKS Studio. SPIDER homology searches implemented in PEAKS Studio were also performed to compensate for sequencing errors and mutations which may otherwise inhibit the correct identification of peptides (Han *et al.*, 2005). PEAKS 'In Chorus' was used to summarize the various search results. In both MASCOT and PEAKS, peptides were accepted as correctly identified if their scores had values of at least $P < 0.01$. The false discovery rate, as determined by a decoy database search, was kept below 1 %. Proteins were considered as correctly identified if they contained at least one unique peptide that fulfilled the above criteria. We did not include 'uncharacterized protein' hits in this report.

RNA-Seq

Ovules, including the nucellus and integuments, were dissected from cones on ice and pooled into a single sample. RNA was extracted using a CTAB–PVP–Tris–HCl extraction buffer as described previously (Chang *et al.*, 1993). However, we excluded spermidine from our extraction buffer, used only 100 mM Tris–HCl as opposed to 1 M, and adjusted our centrifugation speed from 14 000 rpm to 10 000 rpm. Library preparation and sequencing were performed by the FAS Center for Systems Biology (Harvard University). In brief, RNA was amplified using the PrepX SPIA RNA-Seq Library Kit (Nugen), sheared, and made into 200 bp insert libraries using the PrepX ILM DNA Library Preparation kit (IntengenX, Pleasanton, CA, USA). Samples were sequenced on an Illumina HiSeq 2000.

Quality of sequences was assessed using FastQC (Andrews). Adaptors were removed using CutAdapt (Martin, 2011), and low quality sequences were trimmed using Sickle (Joshi and Fass, 2011) and Trimmomatic (Lohse *et al.*, 2012). Trimmed sequences were assembled using Trinity (Grabherr *et al.*, 2011) with default parameters. Similarity searches were performed using BLASTx against the NCBI non-redundant (nr) database and Uniprot [SwissProt (SP) and TReMBL (TR)] databases. Transcripts were annotated by using blast2go (Conesa *et al.*, 2005) against the Gene Ontology (GO) database.

RESULTS

Proteome

Proteins ranged in molecular weight from approx. 7 to 50 kDa. Six SDS–PAGE gel bands from *C. koreana* extracts and eight

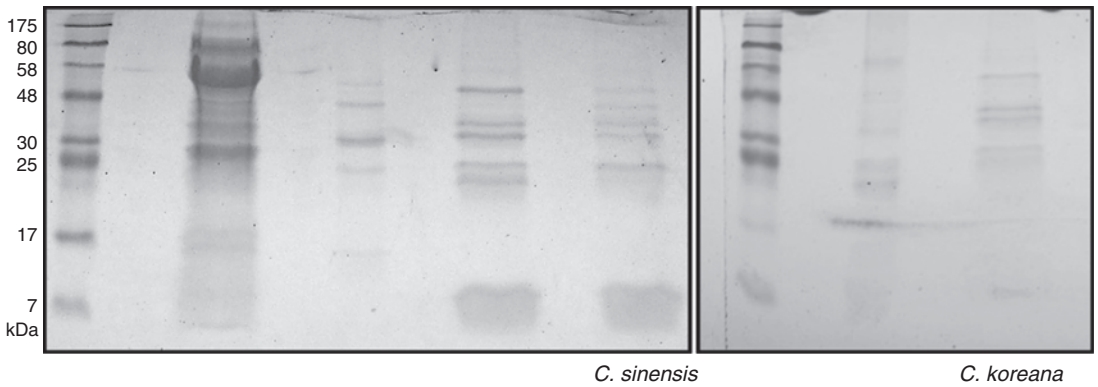

FIG. 2. SDS–PAGE of pollination drops from *C. koreana* and *C. sinensis* and others. Lanes 1 and 6, protein marker; lanes 2–4 and 7, samples not discussed in this paper; lane 5, *C. sinensis*; lane 8, *C. koreana*.

bands from *C. sinensis* extracts were sequenced (Fig. 2). A total of 30 proteins from *C. sinensis* and 32 from *C. koreana* were identified (Table 2). Peptides can be found in Supplementary Data Table S1. Twelve proteins were detected in both samples: chitinase IV, chitinase I, peroxidase, thaumatin-like protein (TLP), pollen allergen CJP-38, α-galactosidase, β-galactosidase, α-amylase, cup a 3 protein, pathogenesis-related protein, PR5 allergen jun r/cup s, and luminal binding protein (BiP) (Table 2).

Transcriptome

Paired-end sequencing yielded 314 781 368 paired-end reads from a 200 bp insert library, encompassing 21.8 Gb of data. After stringent quality filtering, only reads of QV > 20 were accepted (mean QV = 37). Sequences have been deposited in the NCBI SRA database under the accession number SRP058054.

Using the Trinity *de novo* assembly program, 402 215 transcripts were assembled with N50 = 390, with 56 370 transcripts above 500 bp and 17 201 transcripts above 1000 bp (Fig. 3). Searches against the NCBI nr database and the combined SP and TR Uniprot databases, using BLASTx and an e-value cut-off of 10^{-5}, yielded 49 277 transcripts with significant matches against the nr database, 27 837 against SP and 47,558 against TR (Supplementary Data Table S2). In total, 49 769 transcripts were matched to putative homologues using this approach.

BLAST searches will not find matches for all sequences because short reads are rarely matched to known genes. For example, only 7·8 % of all transcripts <500 bp in length had blast hits against the nr database, but 36·0 % of transcripts sized ≥500 bp had hits. The paucity of gymnosperm data available in the databases further limits the total number of BLAST hits.

Transcripts with BLAST matches against the nr database were annotated using Gene Ontology (GO) terms to predict their possible functions. A total of 9644 transcripts were annotated (Supplemetnary Data Table S3). GO annotations classify the function of transcripts into three categories: biological processes, cellular components and molecular functions. Within these categories, the greatest numbers of transcripts were assigned to the sub-categories 'binding' and 'catalytic' (molecular processes), 'cellular process' and 'metabolic process' (biological processes), and 'cell' and 'cell part' (cellular component) (Fig. 4).

DISCUSSION

Proteome of the pollination drops of C. koreana *and* C. sinensis

The pollination drops of *C. koreana* and *C. sinensis* harbored at least 30 proteins (Table 2). Their potential roles include defence of nutrient-rich pollination drops from microbial pathogens, promotion and support of pollen tube growth, metabolism during drop production, and response to stress. It is likely that some of these proteins are actively secreted into the pollination drop (O'Leary *et al*., 2007), while others may enter the drop as a result of nucellar breakdown (von Aderkas *et al*., 2015). As pollination drops form in *Cephalotaxus* (C. Pirone-Davies, unpubl. res.) and in some other gymnosperms, the most micropylar cells of the nucellus break down (O'Leary *et al*., 2004).

Several of the proteins we detected in *Cephalotaxus* are found in the pollination drops of other gymnosperms, while others are reported here for the first time (Table 2). Defensive, or pathogenesis-related proteins (PR proteins), are conserved across all species examined to date. PR proteins are classified into diverse families, including chitinases (PR-3), TLPs (PR-5) and peroxidases (PR-9) (van Loon *et al*., 2006). Chitinases have been detected previously in two species of *Juniperus*, *Ephedra foeminea*, *E. trifurca* and *Welwitschia mirabilis* (Wagner *et al*., 2007), TLPs have been detected in species of *Juniperus*, *E. minuta* and *Taxus* × *media* (O'Leary *et al*., 2007), peroxidase in *Pseudotsuga menziesii*, *E. compacta*, *E. likiangensis* and *E. trifurca*, and galactosidase in *E. minuta*, *E. compacta*, *E. trifurca* and *P. menziesii* (Poulis *et al*., 2005). Three of the six proteins shared by both species of *Cephalotaxus* are PR proteins, i.e. they are induced in the presence of a pathogen. The mechanisms by which the defence-related proteins function in defence are diverse. Chitin is the primary component of fungal cell walls, and thus chitinases, which break down various polymers including chitin (Grover, 2012), often function as antifungals, as has been shown in the pollination drops of *Pseudotsuga menziesii* (Coulter *et al*., 2012). TLPs may affect fungal growth via β-glucanase activity, or the hydrolysis of β-1,3-glucan in fungal cell walls

TABLE 2. *Proteins identified in the pollination drops of* C. koreana and, C. sinensis

C. sinensis (outdoor)	*C. koreana* (outdoor)
Alpha-**galactosidase**	Alpha-**galactosidase**
Alpha-amylase type B isozyme	Alpha-amylase type B isozyme
Beta-galactosidase	Beta-galactosidase
Class I **chitinase**	Class I **chitinase**
Class IV chitinase	Class IV **chitinase**
Cup a 3 protein	Cup a 3 protien
Luminal-binding protein (BiP)	Luminal binding protein (BiP)
Pathogenesis-related protein	**Pathogenesis-related protein**
Peroxidase	Peroxidase
Pollen allergen CJP38	Pollen allergen CJP38
PR5 allergen Jun r	PR5 allergen Jun r
Thaumatin-like protein	**Thaumatin-like protein**
Alpha-1_4-glucan-protein synthase	Acidic endochitinase
Alpha-amylase isozyme	**Acidic thaumatin-like protein**
Ascorbate peroxidase	Allergen Ara h 1
Aspartate aminotransferase	Allergen Arah3/Arah4
Calmodulin	Alpha-amylase
Cytosolic glyceraldehyde-3-phosphate dehydrogenase	Ara h 1 allergen
Elongation factor 1-alpha	Arachin
Elongation factor	Conarachin
Enolase	Endo-beta-mannanase
Eukaryotic translation elongation factor	**Glucan 1_3-beta-glucosidase**
Glutathione-S-transferase	**Glucan** endo-**1_3-beta-glucosidase**
Glyceraldehyde 3-phosphate dehydrogenase	Glutathione reductase
Heat shock protein 70	Gly1
Histone H2A variant 1	Glycinin
Isocitrate dehydrogenase [NADP]	Iso-Ara h3
Monodehydroascorbate reductase	Malate dehydrogenase
Probable histone H2A variant 1	Neutral ceramidase
Triosephosphate isomerase	Polygalacturonase
UDP-glucose:protein transglucosylase-like protein SlUPTG	Storage protein
	Zinc finger protein_ putative

Bold indicates proteins that were detected in previous studies.

The first 12 proteins are found in both samples, followed by proteins unique to each sample. Proteins in each are listed in alphabetical order.

(Grenier *et al*., 1999), and they may also exhibit α-amylase activity that hinders the digestion of plant starches and proteins in the insect gut, thus deterring insect predation (Franco *et al*., 2002). All of these proteins, however, have other known roles in plants. Chitinases are involved in various growth and developmental processes (Grover, 2012). TLPs accumulate in tissue in response to some environmental stresses and developmental cues (Liu *et al*., 2010), and some TLPs can inhibit the formation of ice crystals (Hon *et al*., 1995). Peroxidases are involved in auxin metabolism, lignin and suberin formation, and the linking of cell wall components (Passardi *et al*., 2005). Nonetheless, in *Cephalotaxus*, other conifers and gnetophytes, it is likely that the function of one or more of these proteins is to defend the nutrient-rich drop from pathogens (Gelbart and von Aderkas, 2002).

Cup s and Jun r are members of the PR-5 protein family and thus may play a role in defence. They have been identified as pollen allergens in *Cupressus sempervirens* and *Juniperus rigida*, respectively (Cortegano *et al*., 2004; Breiteneder and Mills, 2005). Other pollen allergens, such as CJP-38, share homology with β-1,3-glucanase, which degrades β-1,3-glucan, or callose. Enzymes with glucanase or glucosidase activity are also found in *Chamaecyparis lawsoniana* and *Juniperus oxycedrus* (Table 1). Functions of β-1,3-glucanase are diverse, and include the regulation of plasmodesmata (Levy *et al*., 2007), hydrolysis of β-1,3-glucan in fungal cell walls and pollen tube growth in angiosperms (Kotake *et al*., 2000; Sela-Buurlage *et al*., 1993). In the pollination drops of *Cephalotaxus*, CJP-38 may have antifungal activity, or, if it originates from nucellar cells, it may regulate plasmodesmata, and thus cell–cell communication within the ovule. It is unclear whether CJP-38 is involved in pollen tube growth in *Cephalotaxus* as is the case in angiosperms. Callose is present in the pollen tubes of some gymnosperms (Yatomi *et al*., 2002), but it is not ubiquitous, and its distribution within the tube varies depending on species and developmental stage (for a review, see Fernando *et al*., 2010). In contrast, the angiosperm tube wall is composed predominantly of callose, and callose septae, or plugs, are formed throughout the tube as it grows (Abercrombie *et al*., 2011).

α-Amylase hydrolyses starch, thus mobilizing energy for growth and development (Huang *et al*., 1992). Starch has been observed to accumulate in the nucellar cells of some gymnosperms just before drop formation (Carafa *et al*., 1992; Takaso and Owens, 1995) and, in some species, it decreases at the time of pollination drop production (Owens and Simmons, 1987).

Galactosidase enzymes are less widespread in pollination drop proteomes than defence-related proteins, but they occur in drops of *Pseudotsuga mensziesii* and some species of *Ephedra*. They are also present in various plant tissues where they metabolize a variety of polysaccharides and are involved in fruit ripening, growth and the hydrolysis of lactose. In flowering plants, galactosidases are observed in both the stigma exudate and the pollen, and are hypothesized to loosen the cell wall components of the intine and assist in pollen germination and elongation (Hruba *et al*., 2005; Rejon *et al*., 2013). It has been suggested that galactosidase and xylosidase present in the pollination drops of *Pseudotsuga menziesii* may also help loosen the cell walls of pollen via degradation of xyloglucan support chains of the pollen intine, thus promoting pollen tube growth (Poulis *et al*., 2005).

The luminal binding protein (BiP) occurs in the endoplasmic reticulum (ER) lumen, where it assists in the proper folding of proteins (Boston *et al*., 1996; Galili *et al*., 1998). Based on its typical sub-cellular location, BiP is probably present in the drop as a result of nucellar breakdown. It is conserved across both samples, suggesting that it may have an important metabolic role at the time of pollination drop production. BiP activity increases during biotic and abiotic stress responses to pathogens, nutrient deficiency, temperature changes and water stress (Alvim *et al*., 2001). It has been proposed that increased BiP production is needed to support an increase in the synthesis of PR proteins (Jelitto-Van Dooren *et al*., 1999). Numerous PR proteins are present in the pollination drop; thus, it is possible that BiP could support their synthesis.

Additional proteins were detected in either *C. koreana* or *C. sinensis*, but not in both (Table 2). Technical replicates are needed to verify that their presence is species specific (Elias

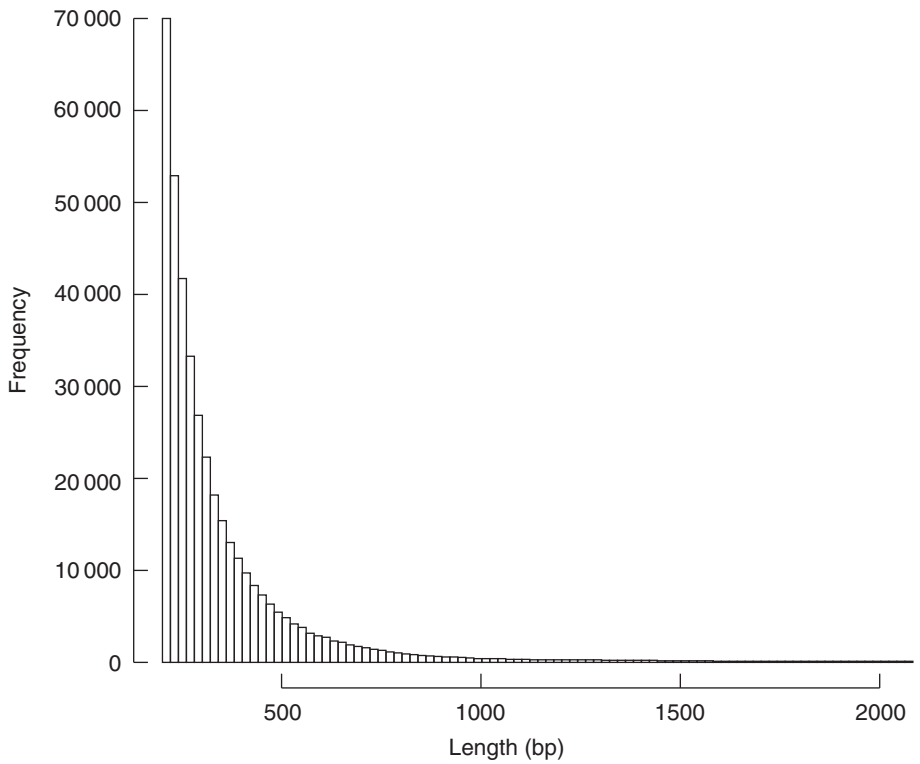

Fig. 3. Histogram of Trinity-assembled transcript sizes.

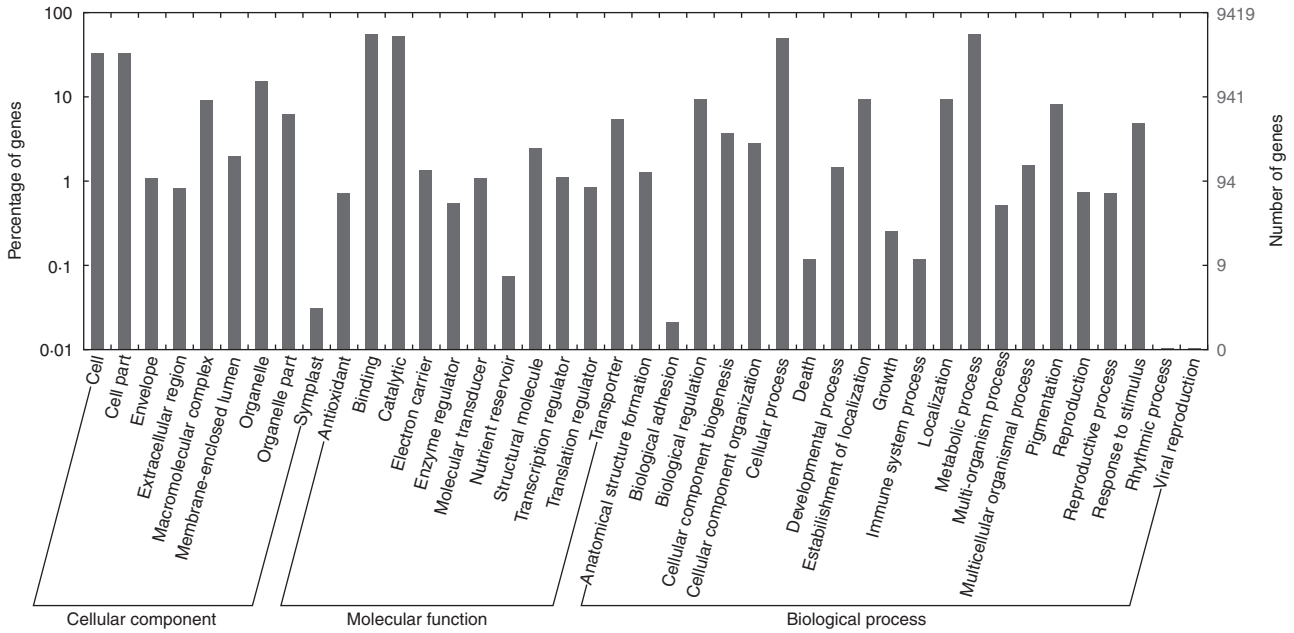

Fig. 4. Histogram of gene ontology classifications, sub-divided into biological processes, cellular components and molecular functions. The right *y*-axis indicates the number of genes in a category. The left *y*-axis indicates the percentage of genes in a category.

et al., 2005; Ham *et al*., 2008). Many of these other proteins are involved in cellular metabolic processes, and may have been released from nucellar cells, as appears to be the case for similar proteins recently detected in pollination drops of seven species of *Ephedra* (von Aderkas *et al*., 2015).

The transcriptome of C. sinensis *at the time of pollination drop production*

The sequences we identified in the pollination drop proteome are a small sub-set of the sequences in our RNA-Seq data. This

is not surprising given that one sample is from an aqueous secretion and the other from a multicellular tissue. Also, the synthesis and degradation of mRNA and protein are differently affected by various factors including numerous post-transcription regulatory processes that affect mRNA stability, the timing of protein synthesis and rates of protein turnover (for a review, see Vogel and Marcotte, 2012). The degree of overlap in the identities of transcripts and the proteins is small, with just 174 matching hits (Supplementary Data Table S4).

The transcripts detected in the ovule of *C. sinensis* represent 41 GO categories broadly distributed across biological processes, cellular components and molecular functions (Fig. 4). Among the more surprising results, we detected homologues of S-locus proteins, which could function in pollen–ovule interactions, and B-class MADS-box genes, which are expected in male cones based on previous studies (Sundstrom and Engstrom, 2002; Thiessen and Becker, 2004). We focus our discussion here on transcripts for proteins that potentially are involved in pollen recognition, pollination drop formation and reproductive development.

Pollen recognition

We detected several transcripts that may be involved in pollen recognition, based on BLAST results and GO annotations (Supplementary Data Table S4). Among the five proteins with 'recognition of pollen' in the GO annotation were one transcript that matched an S-locus lectin protein kinase and four transcripts that matched a g-type lectin S-receptor-like serine threonine-protein kinase in either the NCBI nr or the SP database. Additional transcripts had top blast hits to a g-type lectin S-receptor-like serine/threonine kinase, but did not include pollen recognition in the GO annotation (Supplementary Data Table S3). S-locus proteins determine the specificity of pollen rejection in angiosperm SI systems, but SI systems differ with respect to the proteins encoded by the *S*-loci (Takayama and Isogai, 2005). It is the SSI system of Brassicaceae that uses S-receptor kinase (SRK) as the female determinant of pollen rejection (Stein *et al*., 1991; Sherman-Broyles *et al*., 2007). It belongs to the diverse receptor-like kinase (RLK) family and is the best characterized member of the S-domain RLKs (SRLKs) (Shiu and Bleecker, 2003; Xing *et al*., 2013). SRLKs are differentiated from other RLKs by the presence of an extracellular S-domain composed of three sub-domains, B_Lectin, SLG and PAN_APPLE, one of which, SLG, is responsible for binding the male determinant during SI reactions (Kemp and Doughty, 2007). Apart from SRK in *Brassica*, the functions of SRLK members are largely unknown. They are present in non-reproductive tissues and are predicted to be involved in roles other than pollen recognition, including development and defence (Dwyer *et al*., 1994; Bassett *et al*., 2005). A recent transcriptomic profile for soybean under salt stress detected one SRLK that was upregulated when stressed (Ge *et al*., 2010). A subsequent study sought to validate the role of this SRLK but relied heavily on its overexpression in arabidopsis, leaving open the question of its function in soybean (Sun *et al*., 2013).

Self-incompatibility is widespread in angiosperms, and the fact that unrelated proteins are encoded by *S*-loci in different SI systems is consistent with independent evolution of multiple SI systems, both of the SSI and the GSI type (Gibbs, 2014). In GSI and SSI, pollen either fails to germinate on the stigma or growth of pollen tubes arrests in the style. There is genetic evidence for a third type of SI, which may be more widespread than GSI and SSI. Late-acting self-incompatibility (LSI), in the strict sense, describes self-sterility resulting from the failure of pollen tubes to penetrate the ovule or failure of egg and sperm to fuse (reviewed in Gibbs, 2014). LSI may be widespread in angiosperms, and it may be the ancestral type of SI in angiosperms (Gibbs, 2014). A similar phenomenon has been observed in *Picea glauca*, where both abortion of pollen tubes and failure of the tubes to release sperm were observed after self-pollination but not after cross-pollination (Runions and Owens, 1998), and in *Abies alba*, where archegonia broke down in self- but not in cross-pollinated ovules (Kormatuk, 1999). These pre-zygotic events are distinguished from cases in which expression of lethal genes in the zygote or embryo causes breakdown of the ovule and low seed set. Post-zygotic breakdown may in fact explain other cases in conifers in which self-pollination results in low seed set.

There are intriguing suggestions from pollination studies in conifers of signalling between pollen and ovules, which, if present, could function in LSI or other SI systems. These include the dependence of ovule development on the germination and growth of the pollen tube in species of *Pinus* and *Tsuga* (Pinaceae) (McWilliam, 1959; Dogra, 1967; Owens and Blake, 1983; Owens *et al*., 2005) and *Thuja* (Cupressaceae) (Owens *et al*., 1990), aberrant pollen tube growth and/or ovular breakdown in heterospecific crosses in *Pinus* (McWilliam, 1959) and *Abies alba* (Kormatuk, 1999), and the retraction of the pollination drop in the presence of conspecific, but not heterospecific pollen in *Juniperus communis* (Mugnaini *et al*., 2007). Signalling between pollen and ovule also could explain pollen selection in the pollination drop. In several cross-pollination studies, conspecific pollen germinated and grew better in the pollination drop than heterospecific or heterogeneric pollen (McWilliam, 1959; Fernando *et al*., 2005; von Aderkas *et al*., 2012). These studies, however, did not distinguish whether this resulted from nutritional needs being better met by conspecific pollination drops, or by pollen–ovule signalling, or by both. Outcrossing in *Cephalotaxus* is promoted by dioecy, although dioecy does not necessarily prevent crossing among individuals with nearly identical genotypes. Outcrossing would be further promoted, however, if pollen selection was also occurring in the pollination drop.

Self-incompatibility systems help promote outcrossing and are often cited as a unique feature of angiosperms that contributes to reproductive isolation and speciation (Stebbins, 1957; Jain, 1976). The presence of SI systems in gymnosperms would require a revision of this hypothesis. The observations pointing to pollen–ovule signalling are perhaps the most compelling, whereas the SRLK transcripts in the ovule might be for proteins that function in ovule development or defence. In the latter case, one might expect SRLK members in the proteome of the pollination drop. We did not detect SRLK proteins in this study. However, it is possible that SRLK was present in our samples, but in a quantity too low to be detected by the methods used here. Also still needed from studies of pollination in conifers and other gymnosperms are data from the diallel crosses that can reveal the presence of SI (Gibbs, 2014), and a sufficient

number of histological observations of self- and cross-pollination events.

Pollination drop formation

The pollination drop, like nectar, is a liquid secretion from reproductive tissue, and it is possible that the mechanisms controlling secretion in these systems are similar. Ovule transcripts in our RNA-Seq data may have a bearing on the question of whether similar mechanisms are used. The pollination drop originates in the nucellus, as shown by immunolocalization studies of pollination drop proteins (Poulis *et al*., 2005), and then passes through the micropyle to form a droplet. Ultimately, it retracts, bringing pollen into the ovule. In *Cephalotaxus*, drop production and retraction follow a diurnal cycle, with drops produced in the early morning and slowly retracting throughout the day, a phenomenon that has been observed in podocarpaceous conifers (Tomlinson *et al*., 1991). Drop retraction is also induced when pollen enters the drops of *Cephalotaxus* (C. Pirone-Davies, pers. comm.); this also has been noted in other conifers, including the Pinaceae, Podocarpaceae and Cupressaceae (Doyle and O' Leary, 1935; Tomlinson *et al*., 1997; Mugnaini *et al*., 2007). The regulation of water movement and its role in drop production is not well understood. Conifer ovules are not vascularized, and thus water associated with pollination drop formation and retraction apparently is not associated with the osmotic potential of the xylem. Pollination drop formation must therefore be controlled by water dynamics within the ovule or cone (O'Leary and von Aderkas, 2005). One explanation is that changes in the osmotic potential of the drop facilitate the movement of water (Ziegler, 1959; Coulter *et al*., 2012). Sugars are proposed to regulate water movement during nectar secretion in angiosperms (Lin *et al*., 2014), and a similar process could be occurring during drop formation in gymnosperms, with the various sugars present in the drops serving as osmotic regulators (Seridi-Benkaddour and Chesnoy, 1988; Nepi *et al*., 2009). Consistent with this idea, we detected several transcripts for proteins involved in the transport of sugars (Supplementary Data Table S5), including several members of the SWEETS protein family, a superfamily of sugar transporters (Xuan *et al*., 2013), some of which are involved in nectar secretion (Lin *et al*., 2014). Transcripts of β-glucosidase and P-loop-containing nucleoside triphosphate hydrolases, additional proteins involved in nectar production (Bender *et al*., 2012), also are present in the *Cephalotaxus* drops.

Reproductive development

Ovules are a signature feature of the seed plants, and the presence of the ovule and placental tissue connecting it to a megasporophyll determines female identity. Ovules and pollen typically are borne in separate structures in gymnosperms. This leads to the expectation that genes known to control ovule development and female identity in angiosperms will be expressed in the ovulate cones of gymnosperms, while those controlling male identity will be expressed in pollen cones. In particular, one might expect similar sets of genes to be expressed in ovules across seed plants, since ovules are conserved, unlike the structures on which they are borne (Mathews and Kramer, 2012). Transcripts we detected in the ovules of *Cephalotaxus* that are homologues of genes involved in reproductive development in angiosperms bear on these expectations. These include *ULTRAPETALA* (*ULT1*), MADS-box transcription factors, *AP2*-related, *CLAVATA* (*CLV*), *WUSCHEL* (*WUS*), *JOINTLESS* and *LEAFY* (*LFY*) (Supplementary Data Table S3). The roles of several of these loci are understood in angiosperms, but fewer data are available for gymnosperms (for a review, see Mathews and Kramer, 2012).

Ovule identity in angiosperms is determined by loci in the AGAMOUS (AG) protein lineage (Pinyopich *et al*., 2003), and we detected several *AG* and *AG-like* transcripts in the *C. sinensis* ovule. Angiosperm ovules also express *WUS*, a member of the WUSCHEL-related homeobox domain (*WOX*) family, but they do not express *CLAVATA3* (*CLV3*). In angiosperm shoot apical meristems (Laux *et al*., 1996) *WUS* and *CLV* signalling pathways interact to maintain the stem cell niche of the central zone. In arabidopsis ovules, *WUS* is critical for initiation of the integuments, consistent with the origin of the nucellus of the ovule from a shoot apical meristem (Gross-Hardt *et al*., 2002; Mathews and Kramer, 2012), and the presence of *WUS* in the ovules of *Gnetum* (Nardmann *et al*., 2009) and *C. sinensis* suggests that the role of WUS during integument formation may be conserved across the seed plants. Consistent with their determinate nature, *CLV3* is not expressed in arabidopsis ovules. It is thus interesting that we detected *CLV* transcripts in the dissected ovules of *C. sinensis*. This could point to the presence of a stem cell niche, maintained by the signalling of *WUS* and *CLV*. Alternatively, the presence of *WUS* and *CLV* in the ovule could result from meristematic activities in the integuments during early stages of their development. Perhaps less probably, they could represent a novel set of functions in ovules.

Within both shoot and floral meristems of arabidopsis, control of cell accumulation involves *ULT1* (Fletcher, 2001). *ULT1* also positively regulates floral meristem determinacy, possibly through the *AG* pathway (Prunet *et al*., 2008). *ULT1* encodes a trithorax group protein, a class of proteins involved in chromatin remodelling. In arabidopsis, *ULT1* interacts with *KANADI* to organize the gynoecium along two polarity axes (Pires *et al*., 2014) In *Cephalotaxus*, the *ULT1* homologue probably is also involved in chromatin remodelling and may be controlling some aspect of ovule development. It would be interesting to determine whether it is involved in polarity determination in the ovule.

MADS-box genes are transcription factors that contain a MADS DNA-binding domain, which is conserved across eukaryotes and metazoans (Gramzow *et al*., 2014). MADS-domain proteins are sub-divided into Type 1, or serum response factor (SRF)-like proteins, and Type 2, or myocyte enhancer factor (MEF)-like proteins. Few Type 1 genes have been functionally characterized, but Type 1 genes in arabidopsis are involved in female gametophyte, embryo sac and seed development (for a review, see Gramzow and Theissen, 2010). Type 1 genes were detected in several members of the Pinaceae as well as in *Sciadopitys verticillata*. However, detection of transcripts is infrequent and is limited to shoot, bud, male cone

and embryo tissues (Gramzow *et al*., 2014). The functions of these transcripts are unknown. Our data set contains a single Type 1 MADS-box transcript.

Type 2 MADS-box genes, in contrast, have been extensively studied in angiosperms, where they are best known for their roles in floral organ identity. They also are involved in diverse developmental processes in fruits, seeds, embryos, roots and leaves (for reviews, see Becker, 2003; Theissen, 2001). Determination of organ identity in flowers is described by the ABCDE model, with A + E class genes specifying sepals, A + B + E class petals, B + C + E class stamens, C + E class carpels, and D + E class ovules (for reviews, see Causier *et al*., 2010; O'Maoileidigh *et al*., 2014). Most genes in these classes are Type 2 MADS-box genes. In gymnosperms, B and C class genes appear to be involved in the specification of reproductive structures and the differentiation of male and female cones (Melzer *et al*., 2010). Using BLAST searches, we detected several *Cephalotaxus* transcripts that showed greatest similarity to Type 2 MADS-box genes, including B class and B-sister (Bs) transcripts, *AG* and *AG-like* (C class), *TM8* and *DEFICIENS AGAMOUS-LIKE 10* (*DAL10*). We verified via phylogenetic analyses (S. Mathews, E. Kramer, C. Pirone-Davies, unpubl. res.) that among the BLAST hits are single homologues of B class, Bs and AG transcripts.

The MADS-box genes from the A class have not previously been detected in gymnosperms (Melzer *et al*., 2010). However, the A class gene *AP2* from the AP2/ERF family of transcription factors has been detected in several conifers (e.g. Nilsson *et al*., 2007). In arabidopsis, *AP2* specifies the identity of sepals and petals, regulates C class genes, is involved in seed development and may play a role in development of non-floral organs (Jofuku *et al*., 1994). Homologues of *AP2* from *Picea* probably control diverse developmental events, but also share features of their angiosperm counterparts, as Pi*AP2* has the capacity to substitute for an A class gene in arabidopsis (Nilsson *et al*., 2007). We detected one *AP2*-related transcript in *C. sinensis*.

The MADS-box genes from the B class have been found in *Ginkgo*, *Gnetum gnemon* and some conifers (Mouradov *et al*., 1999; Sundstrom *et al*., 1999; Gramzow *et al*., 2014), where their expression is largely restricted to male cones. It appears that the role of these genes in the development of pollen-bearing structures is conserved across seed plants (Sundstrom and Engstrom, 2002; Theissen and Becker, 2004). B class transcripts were, however, found by Gramzow and colleagues (Gramzow *et al*., 2014) in transcriptomes assembled from the female cones of *Picea abies*. Our detection of B class and Bs transcripts in the ovules of *C. sinensis*, suggests that B class genes are indeed involved in a role beyond male reproductive development. Since we collected ovules from outdoor sites where pollen was likely to be present, we cannot completely rule out the possibility that we detected transcripts from the pollen. It is unclear, however, whether B class transcripts would occur in mature pollen. *In situ* data indicate their presence in various pollen cone tissues, but no transcripts were detected in the pollen mother cells, and mature pollen was not analysed (Sundstrom and Engstrom, 2002). The expression of one or more B gene homologues in ovules is not uncommon in angiosperms (for a review, see Kramer and Irish, 2000), so it may be that this pattern is also common among gymnosperms.

Homologues of the C class gene *AG* have been identified in all major gymnosperm lineages (Rutledge *et al*., 1998; Tandre *et al*., 1998; Winter *et al*., 1999; Kramer *et al*., 2003), and *AG* and *AG-like* are expressed in the female cones of four cycads and several conifers, the male cones of *Cryptomeria*, the shoots of *Gnetum gnemon*, non-reproductive tissues of *Picea abies* and in the nucellus of *Taxus globosa* (Englund *et al*., 2011; Gramzow *et al*., 2014). In flowering plants, *AG* is involved in stamen and carpel identity and in establishing determinancy of the floral meristem (Bowman *et al*., 1989). In gymnosperms, *AG* genes are generally involved in the development of both male and female cones (Melzer *et al*., 2010), and may be involved in the development of ovuliferous scales (Tandre *et al*., 1998) and in the transition from vegetative to reproductive identity (Carlsbecker *et al*., 2013).

Additional Type 2 MADS-box genes in the *Cephalotaxus* ovular transcriptome include *TM8*, *DAL10* and *JOINTLESS*. *TM8* and *DAL10* belong to a large clade that is sister to the C class clade (Melzer *et al*., 2010). *TM8* expression occurs in reproductive and non-reproductive tissues of diverse gymnosperms (Gramzow *et al*., 2014), including the ovules of *Taxus baccata* and *G. biloba* (Lovisetto *et al*., 2012). Although the function of *TM8* remains poorly understood, it may be involved in controlling A class expression in tomato (Daminato *et al*., 2014). Given the diversity of *TM8* expression patterns across seed plants, further research into its function is needed. *DAL10* has also been found in numerous gymnosperm species and tissues, but is absent from angiosperm lineages (Carlsbecker *et al*., 2003). Similar to *TM8*, little is known about the function of this gene, although it appears to be involved in the shift from vegetative to reproductive buds, and its presence in developing seed and pollen cones is similar to that of B and C class genes (Carlsbecker *et al*., 2003, 2013). *JOINTLESS* is involved in the formation of the abscission zone in tomato (Mao *et al*., 2000). It works at least in part via the regulation of transcription factors involved in meristem identity, genes involved in cell wall formation and lipid metabolism, and phytohormones (Nakano *et al*., 2012).

The presence in *Cephalotaxus* ovules of transcripts similar to the transcription factor gene *LFY* is consistent with its activity in early female and male cone development (Vasquez-Lobo *et al*., 2007). It may regulate some ABC class MADS-box proteins as it does in angiosperms (Moyroud *et al*., 2010). In angiosperms, *LFY* enables the transition from vegetative to floral meristems (Weigel *et al*., 1992), and also activates floral homeotic genes (Weigel and Meyerowitz, 1994). Members of the *LFY* lineage have been detected in non-reproductive and reproductive tissues of diverse non-flowering plants (Moyroud *et al*., 2010), including the nucellus of *Picea* (Carlsbecker *et al*., 2013).

In summary, our proteome data strongly support the hypothesis that pollination drop proteins are important in defence, polysaccharide metabolism and pollen tube growth. We detected additional, novel proteins that may be involved in defence (Cup a3, Cup s and Jun r), starch degradation (α-amylase) and callose degradation (pollen allergen CJP-38), while others are likely to be by-products of nucellar degradation (luminal binding protein, histone and others). The implications of the latter in pollination drop function, if any, are unclear. Examination of the transcriptome of *Cephalotaxus* ovules revealed several

transcripts that may be important in pollen–ovule recognition, pollination drop formation and retraction, and reproductive and developmental processes within the ovule. Functional validation of the pollination drop proteins and ovular transcripts characterized here will deepen our knowledge of pollination biology in gymnosperms. Transcriptome data, moreover, from additional stages of ovule development and from a broad phylogenetic sample across gymnosperms will greatly advance our understanding of gymnosperm reproductive development. Finally, we note the need for histological and genetic data from rigorous self- and cross-pollination experiments to explore the intriguing observations that suggest that pollen–ovule signalling occurs in at least some gymnosperm reproductive systems.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: peptides and proteins identified in the pollination drops of *C. koreana* and *C. sinensis*. Table S2: BLAST results (nr, SwissProt and trEMBL) for the ovule transcriptome of *C. sinensis*. Table S3: Blast2go annotations for the ovule transcriptome of *C. sinensis*. Table S4: list of transcripts and proteins found in both the transcriptome and proteome data sets. Table S5: transcripts identified in the ovules of *C. sinensis* with the putative functions of sugar transport, pollen–ovule interactions and development.

ACKNOWLEDGEMENTS

We thank Elena Kramer for her insight on ovule development and for sharing with us her data matrix of MADS-box genes. This work was supported by a Putnam Fellowship from the Arnold Arboretum of Harvard University to C.P.D. The Natural Sciences and Engineering Research Council of Canada's PGS and Discovery Grant Programs provided financial support to N.A.P. and P.vA., respectively.

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