Gymnosperm Pollination Drop Proteins and their Relation to Function and Phylogeny

by

Rebecca Elizabeth Wagner
B.Sc. University of Victoria, 2003

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MASTER OF SCIENCE

In the Department of Biology

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Abstract

The pollination drop is a conservative pollination mechanism observed in all major gymnosperm taxa. Despite its ubiquity and essentiality to gymnosperm reproductive success, it remains poorly understood. Recent studies identifying conifer ovular secretion proteins have indicated a more complex role for ovular secretions than pollen receipt. We used a proteomics approach to analyze the pollination drops of four gymnosperm species (*Juniperus communis* (common juniper), *Juniperus oxycedrus* (prickly juniper), *Chamaecyparis lawsoniana* (Port Orford cedar), and *Welwitschia mirabilis*). Pollination drop proteins were separated by SDS-PAGE, and the most abundant proteins were analyzed by mass spectrometry and sequenced. Based on BLAST searching of combined amino acid sequences, several proteins were identified: an 83 kDa subtilisin-like proteinase, a 62 kDa glycosyl hydrolase, a 47.5 kDa glucan 1,3-β-glucosidase precursor, a 30 kDa chitinase, and a 25 kDa thaumatin-like protein in *J. communis*; a 30 kDa chitinase, a 25 kDa thaumatin-like protein, and a 32.5 kDa glucanase-like protein in *J. oxycedrus*; an 83 kDa subtilisin-like proteinase, a 62 kDa β-D-glucan exohydrolase, a 47.5 kDa glucan 1,3-β-glucosidase, and two 25 kDa thaumatin-like proteins in *C. lawsoniana*, and a 25 kDa chitinase in *W. mirabilis*.

Gymnosperm phylogeny is a highly debated topic, particularly following the widespread adoption of molecular phylogenetic analyses which conflict with historical morphological phylogenies. The gymnosperms are a difficult group to classify because of their deep evolutionary history and lack of conservative features. Considering that the pollination drop is a highly conservative feature of gymnosperm reproduction, we propose that analysis of pollination drop protein (PDP) variation could be used as an alternative method to resolve gymnosperm phylogeny. PDP variation was analyzed at three taxonomic levels: genus, family, and gymnosperm clade. Based on variation in SDS-PAGE banding patterns, identified peptides, amino acid sequences, and protein identification, we conclude that PDP variation has a phylogenetic component. Further research is necessary to develop this method into a tool used to predict phylogenetic relationships.

Based on protein identifications, there is strong evidence that the pollination drop functions in both pathogen defense and pollen development. The observation of hydrogen peroxide and peroxidase activity in the ovular secretions of *J. communis*, *C. lawsoniana*, *Pseudotsuga menziesii* (Douglas fir), and *Larix* x *marschlinsii* (hybrid larch) provided further support for the assumed functions of ovular secretions.

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List of Abbreviations

BLAST:	Basic Local Alignment Search Tool
BSA:	Bovine serum albumin
DAB:	Diaminobenzidine
DMSO:	Dimethylsulfoxide
DTT:	Dithiothreitol
EST:	Expressed sequence tag
HRP:	Horseradish peroxidase
m/z:	Mass-to-charge ratio
NCBI:	National Center for Biotechnology Information
PDP:	Pollination drop protein
PTM:	Post-translational modification
ROI:	Reactive oxygen intermediate
(1D/2D) SDS-PAGE:	(One dimensional/Two dimensional) Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SA:	Salicylic acid
SI:	Self-incompatibility
TLP:	Thaumatin-like protein

Acknowledgements

I would first and foremost like to thank my supervisor Dr. Patrick von Aderkas. Without his support, guidance and strong coffee, the book may never have been written. I am further indebted to Dr. Brett Poulis, Dr. Stephen O'Leary and Dr. Michael Wilson for teaching me how to think like a scientist. Many thanks to Andrea Coulter, Adrienne Robb, and Jenni Robb for assistance with collection, great conversation, and the occasional ride to Oregon. Thank you to all past and present members of the PvA lab. My project would not have been possible without the help of Darryl Hardie, Phil Jakubowski, Leanne Ohlund, Derek Smith, and all staff at the University of Victoria-Genome BC Proteomics Centre: their expertise, patience and kindness helped me overcome many stumbling blocks. I would also like to thank Richard Sniezko, Leslie Elliott, Jerry Hill, Scott Kolpak, Angelia Kegley, Jerry Berdeen, and all staff at the US Department of Agriculture Dorena Lake Genetic Resource Centre for allowing me to sample from their Port Orford cedar trees. Thank you to Serena Mugnaini, Massimo Nepi, Ettore Pacini at the University of Siena in Italy for supplying juniper samples. I am grateful to Doug Ewing at the University of Washington greenhouse for allowing me to collect from his *Welwitschia mirabilis* plants. Many helpful people at the University of Victoria have been instrumental to my success: thank you to past and present members of the Constabel lab, Koop lab, and Levin lab for great ideas and technical assistance. Thank you to Eleanore Floyd for clarifying the fine print. I am very thankful to all of my wonderful friends and family for their support. My research was funded by NSERC, and the Biology Department and Faculty of Graduate Studies of the University of Victoria.

Chapter 1: Gymnosperm evolution: a review

Introduction

The term gymnosperm, literally meaning "naked seed", describes all non-flowering seed plants. Gymnosperms are a difficult group to classify, partly because they are defined in relation to angiosperms, their more diverse and prevalent cousin. In the words of Stewart and Rothwell (1993), gymnosperms are "defined by the absence of flowering-plant characters, rather than by a uniquely derived character or set of characters. When considered in this light, the gymnosperms do not appear to be a natural lineage (that is, not a monophyletic group)." Perhaps more importantly, gymnosperms are difficult to classify because they are a group that radiated rapidly long ago, and therefore have a deep evolutionary history (Donoghue and Doyle 2000).

For two centuries, plant biologists have vehemently debated gymnosperm classification based on extant morphology and fossil evidence. In the last two decades, the use of DNA sequences in classification systems has introduced more excitement and complexity to this debate. Despite modern advances, gymnosperm evolution remains somewhat of a mystery.

The Gymnosperms

At present, four groups of extant gymnosperms are recognized: the cycads, *Ginkgo biloba*, the conifers and the Gnetales. Fossil species from the four extant groups, the seed ferns, the cycadeoids and other unclassified species (e.g. some Mesozoic seed ferns) comprise the extinct gymnosperms. Whether or not they represent a natural grouping, gymnosperms can be roughly defined based on their vegetative and reproductive features.

Vegetative features

Gymnosperms are united by the presence of secondary xylem, either of a pycnoxylic (dense) or manoxylic (loosely textured) type (Gifford and Foster 1989). Aside from all exhibiting secondary growth, gymnosperms vary widely in morphology including habit (e.g. trees to shrubs), leaf type (e.g. simple to pinnate compound), leaf venation (e.g. dichotomous to parallel), branching pattern (e.g. apical to dichotomous) among other morphological characters (Gifford and Foster 1989).

Reproductive features

Gymnosperm seeds are relatively exposed to the environment: they are borne on sporophylls, scales or comparable structures and lack an enclosing structure (e.g. the angiosperm carpel). Reproductively, gymnosperms are adapted to utilize environmental forces: they are anemophilous (wind-pollinated), with the exception of some entomophilous (insect-pollinated) species. Pollen is the vehicle for the endosporic male gametophyte, which is composed of only a few cells upon release into the environment. The ovule scavenges airborne pollen by a variety of mechanisms, one of the most common being the ovular secretion (Gelbart and von Aderkas 2002). Examples of ovular secretions, including the external pollination drop and the internal post-pollination pre-fertilization drop, are found in all extant gymnosperm phyla (Gelbart and von Aderkas 2002; Gifford and Foster 1989).

Upon receipt by the ovule, pollen germinates to form a pollen tube. Pollen tube structure and function varies in different gymnosperm phyla, probably in relation to evolutionary history. For example, some gymnosperms produce highly branched pollen tubes that serve a nutritive function (i.e. they grow laterally in nucellar tissue and absorb nutrients) in addition to gamete delivery (Fernando et al. 2005). Gymnosperms employing these strategies are termed zooidogamous. In contrast, siphonogamous gymnosperms produce relatively unbranched pollen tubes that deliver non-motile gametes directly to the egg.

With some exceptions, these variations in male reproduction correspond to variations in female reproduction. Ovules of zooidogamous gymnosperms form a pollen chamber by nucellar degeneration; however, some conifer species (all conifers being siphonogamous) form pollen chambers (Singh 1978). The function of the pollen chamber is to receive pollen and provide a suitable environment for pollen germination. Most gymnosperms produce archegonia (structures housing the egg cell), enclosed in archegonial chambers. In zooidogamous species, a fluid filled archegonial chamber receives motile sperm released from ruptured pollen tubes. In siphonogamous species, sperm are directly conveyed to the egg within the archegonia. Interestingly, *Welwitschia mirabilis* Hook does not produce archegonia at all, and fusion of egg and sperm results when upward tubular growth of the female gametophyte, in the form of female gametophytic tubes, fuses with pollen tubes. All gymnosperms undergo free nuclear division during early stages both of gametophyte development and embryogenesis.

Historical vs. Modern Classification

Historical classification systems of gymnosperms, or systems predating the advent of cladistics, attempted to define gymnosperms based on direct observation. By comparing leaf type, branching pattern and stem morphology, Chamberlain (1935) devised a system of classification dividing all living and fossil gymnosperms into the "cycadophytes" and "coniferophytes". Cycadophytes were defined as gymnosperms with fern-like compound leaves, weakly branching trunks, and stems with a large pith and small amount of manoxylic secondary xylem (Chamberlain 1935). In contrast, coniferophytes were defined as gymnosperms with simple leaves (i.e. needles), profusely branching trunks, and stems with a small pith and large amount of pycnoxylic xylem (Chamberlain 1935). More recent studies have shown that these categories represent growth habits rather than evolutionary lineages; therefore, the cycadophyte and coniferophyte groupings are artificial (Stewart and Rothwell 1993).

Many historical classification systems of seed plants were attempts to solve Darwin's "abominable mystery" of the origin of angiosperms (Darwin 1859), and therefore classified gymnosperms in relation to angiosperms. In 1907, Wettstein proposed that Gnetales are ancestral to angiosperms (Wettstein 1907). Wettstein's ideas were widely accepted, because they explained unique Gnetalean features (e.g.. ovule surrounded by an additional envelope) that were otherwise restricted to angiosperms (Doyle and Donoghue 1986). The proposal that Gnetales are the sister group of the angiosperms became known as the anthophyte hypothesis. Despite its popularity, the anthophyte hypothesis was not universally accepted: Bailey (1944) rejected the anthophyte hypothesis based on his observation that Gnetalean and angiosperm vessels differed in origin. On the basis of xylem morphology, Bailey grouped Gnetales, conifers and *Ginkgo biloba* separately from angiosperms (Bailey 1944).

In 1950, the German entomologist Willi Hennig argued that classification systems should reflect only phylogenetic relationships (Futuyma 1998). Modern classification, or cladistics, attempts to unite organisms with common ancestry into monophyletic groups (clades) based on the discovery of shared uniquely derived character traits (Futuyma

1998). Cladistic analyses avoid arbitrary groupings by explicit inclusion of two criteria: 1) shared characters must be derived (apomorphic) rather than ancestral (plesiomorphic); and 2) shared characters must represent a single evolutionary change in the common ancestor (Futuyma 1998). Following Hennig's publication, cladistics became the main method used in gymnosperm classification.

Until the last two decades however, even phylogenetic analyses of gymnosperms were based entirely on morphological data. Features of vegetative and reproductive anatomy, both from extant and fossil seed plants, were scored as characters with two or more states (e.g. a leaf character with a simple or compound state). Currently, phylogenetic analyses are based predominantly on DNA sequence data.

Gymnosperm Classification Based on Morphology

"Seed plant phylogeny and the origin of angiosperms" (Doyle and Donoghue 1986) is one of the most widely cited studies of gymnosperm evolution and classification. The historical tendency to view gymnosperm evolution as a means to discover angiosperm origins is immediately obvious in the title; however, all living and fossil gymnosperms are represented in the analysis. Doyle and Donoghue fine-tuned their 1986 findings, and presented a reanalysis of their data in response to critiques and recent discoveries (Doyle and Donoghue 1992). Various vegetative and reproductive features were scored as characters with two or more states, and analyzed by statistical methods designed to find the most parsimonious and stable arrangement of groups.

In all of Doyle and Donoghue's cladograms, seed plants are a monophyletic group (Fig.1.1, Fig. 1.2). The most ancestral seed plants are the Devonian seed ferns, succeeded by the Paleozoic seed fern families Medullosaceae and Callistophytaceae (Fig. 1.1). Depending on the cladogram, the oldest living seed plants are the cycads (Fig. 1.2b, Fig. 1.2d), the conifers (Fig. 1.2e), ginkgos and the conifers (Fig. 1.2a) or ginkgos, the conifers and the cycads (Fig. 1.2c).

In all complete trees, extant conifers, the extinct conifer Cordaitales, and ginkgos are closely related (Fig. 1.1). The only consistent feature of Doyle and Donoghue's cladograms is the close relationship between angiosperms and the monophyletic Gnetales (Fig. 1.2). In cladograms of extant seed plants, Gnetales and angiosperms are sister groups, with the Gnetales diverging after the angiosperms (Fig. 1.2). In complete cladograms, angiosperms and the Gnetales are not sister groups, but part of a monophyletic anthophyte clade with the Mesozoic seed fern *Pentoxylon* and the cyacadeoid Bennettitales (Fig. 1.1).

What implications do these results have on gymnosperm evolution? Primarily, Doyle and Donoghue's (1992) results support the anthophyte hypothesis: The Gnetales are more closely related to angiosperms than to other gymnosperms, and gymnosperms are a paraphyletic group. Therefore, the group "gymnosperm" does not represent a natural lineage. Another interesting implication is that Chamberlain's coniferophytes are not an evolutionary lineage, as conifers and ginkgos often form a paraphyletic group (Fig. 1.2d, Fig. 1.2e). Therefore, the shared features of conifers and ginkgos (e.g. pycnoxylic secondary xylem) may be an example of convergent evolution.

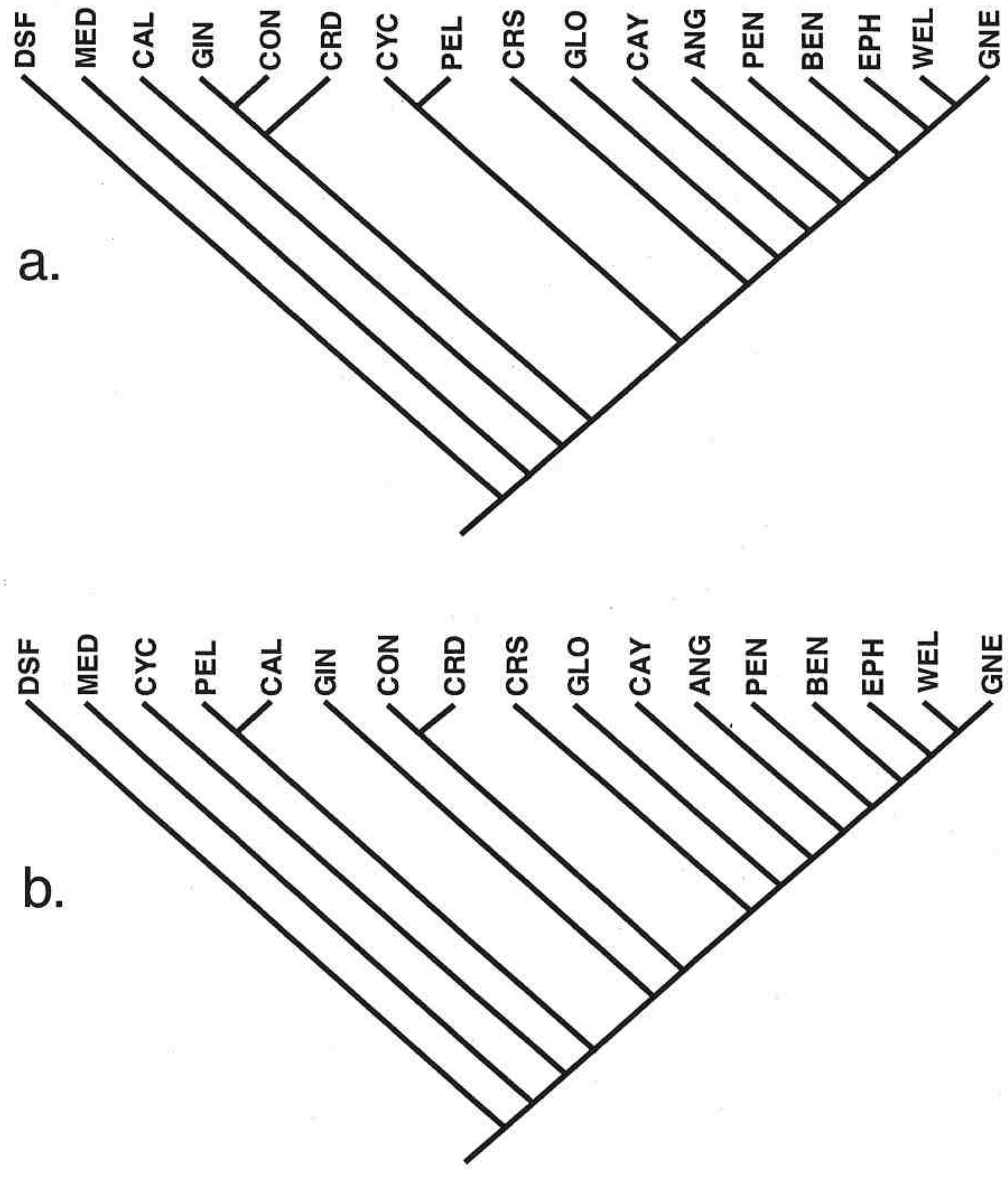


Figure 1.1: Representative most parsimonious seed plant cladograms. DSF: Devonian seed fern; MED: Medullosaceae; CAL: *Callistophyton*; GIN: Ginkgoales; CON: Coniferales; CRD: Cordaitales; CYC: Cycadales; PEL: *Peltaspermum*; CRS: Corystospermaceae; GLO: Glossopteridales; CAY: Caytoniaceae; ANG: angiosperms; PEN: *Pentoxyln*; BEN: Bennettitales; EPH: *Ephedra*; WEL: *Welwitschia*; GNE: *Gnetum*. Modified from Doyle and Donoghue (1992)

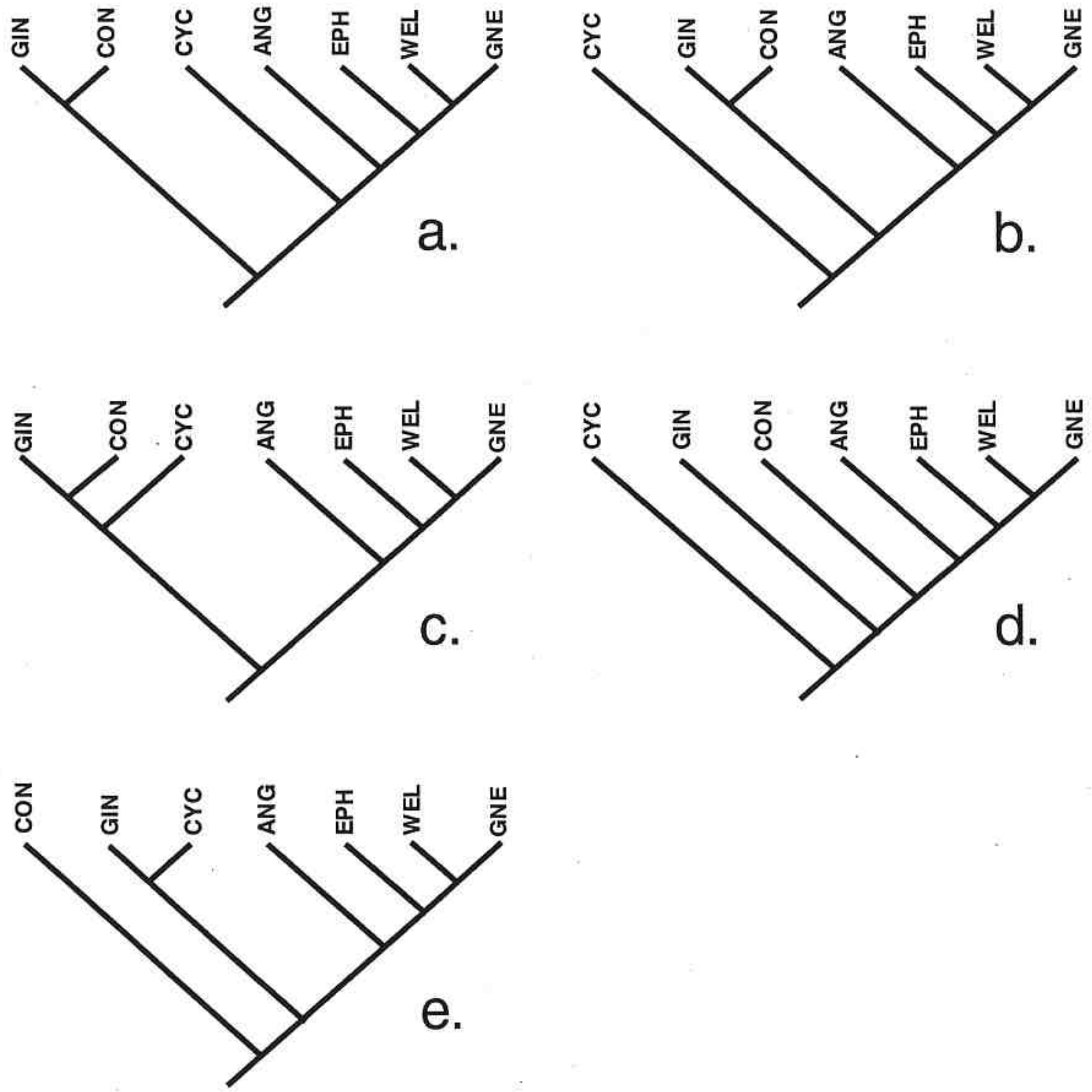
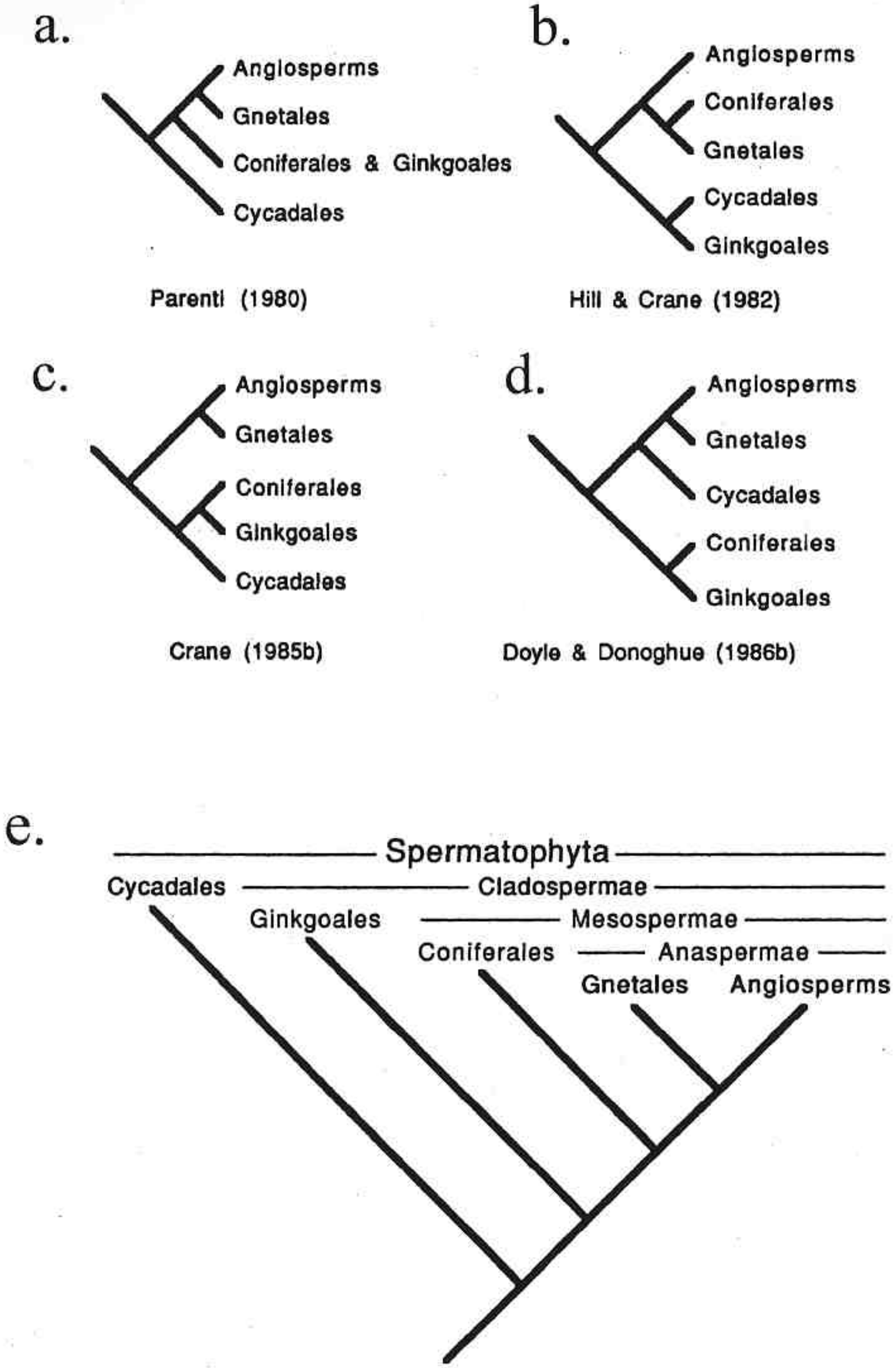


Figure 1.2: Cladograms of recent seed plants. Revised analysis of Recent and fossil groups yielded all five arrangements, A-E. GIN: Ginkgoales; CON: Coniferales; CYC: Cycadales; ANG: angiosperms; EPH: *Ephedra*; WEL: *Welwitschia*; GNE: *Gnetum*. Modified from Doyle and Donoghue (1992).

In an overall view of gymnosperm evolution, the ancestral gymnosperm was cycad-like, with manoxylic secondary xylem, pinnate compound leaves, and non-saccate pollen (Doyle and Donoghue 1992). However, the course of character evolution is equivocal, with different combinations of derived character acquisitions (i.e. pycnoxylic secondary xylem, saccate pollen, siphonogamy etc.) in each tree (Fig. 1.1).

One of the major critiques of Doyle and Donoghue's original 1986 study came from Loconte and Stevenson (1990), who objected to the *a priori* inclusion of fossil data in phylogenetic analyses. The use of fossils in cladistic analyses is a contentious issue, as some researchers view fossil data as either irrelevant or misleading (Doyle and Donoghue 1992). Loconte and Stevenson (1990) argued against the *a priori* inclusion of fossil data based on discrepancies between cladograms including and excluding fossil data. However, Doyle and Donoghue view such discrepancies as a reason to include fossil data, as fossils "strengthen key relationships" and "clarify the course of character evolution, even when they do not alter cladogram topology" (Doyle and Donoghue 1992). For example, conifers and anthophytes are closely related in one of Doyle and Donoghue's extant cladograms (Fig. 1.2d), but the complete cladograms show that synapomorphies uniting conifers and anthophytes are more likely homoplastic characters, as plesiomorphic fossil species are interpolated between the two groups (Fig. 1.1).

Many other published phylogenies agree with Doyle and Donoghue's 1992 results. In all cladograms in Figure 1.3, gymnosperms are a paraphyletic group, with the Gnetales sister group to the angiosperms.



Parenti (1980)

Hill & Crane (1982)

Crane (1985b)

Doyle & Donoghue (1986b)

Figure 1.3: Diagrams of phylogenetic relationships of seed plants. Fig. 1.3a-d from previously published studies, modified by Loconte and Stevenson (1990). Fig 1.3e modified from Loconte and Stevenson (1990).

An interesting exception is Hill and Crane's 1982 cladogram (Fig. 1.3b), which groups conifers and the Gnetales as a monophyletic group sister to the angiosperms. Loconte and Stevenson's (1990) results also support a close relationship between the three groups (Fig. 1.3e), based on shared reproductive advances such as siphonogamy. However, Doyle and Donoghue (1992) argue on the basis of fossil evidence that the shared features of conifers, the Gnetales and angiosperms are the result of convergent evolution. Like Doyle and Donoghue's (1992) results, there is no agreement as to the oldest extant gymnosperms (Fig. 1.3). Unlike Doyle and Donoghue's (1992) results, cladograms in Fig. 1.3a, c and d support a coniferopsid sister relationship between conifers and ginkgos.

In 1996, Doyle reassessed seed plant phylogeny, primarily in response to emerging molecular evidence refuting the anthophyte hypothesis. In order to bring his ideas up to date and reevaluate Gnetalean relationships within the gymnosperms, Doyle reassessed his data in response to criticism and recent discoveries. Specifically, Doyle (1996) included data from the recently described *Piroconites* fossil, a putative ancestor to the Gnetales. Furthermore, he included additional angiosperm and conifer taxa to elucidate their relationships with the Gnetales (Doyle 1996). Most importantly, Doyle (1996) interpreted his results in light of molecular evidence refuting the anthophyte hypothesis.

In his new analysis, Doyle (1996) found that the Gnetales and angiosperms were not part of an anthophyte clade, but paraphyletic groups within a larger clade he termed the glossophytes (Fig. 1.4).

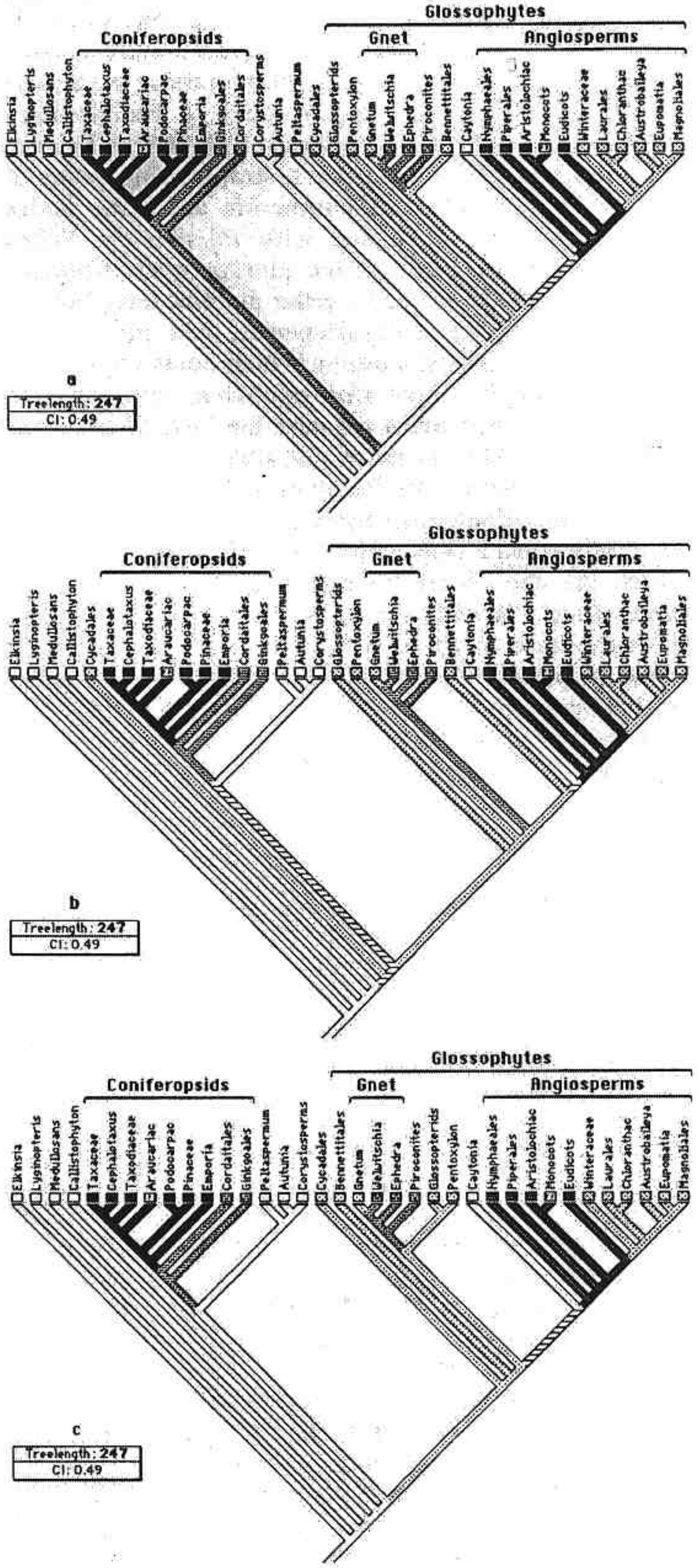


Figure 1.4: Representative most parsimonious trees. Modified from Doyle (1996).

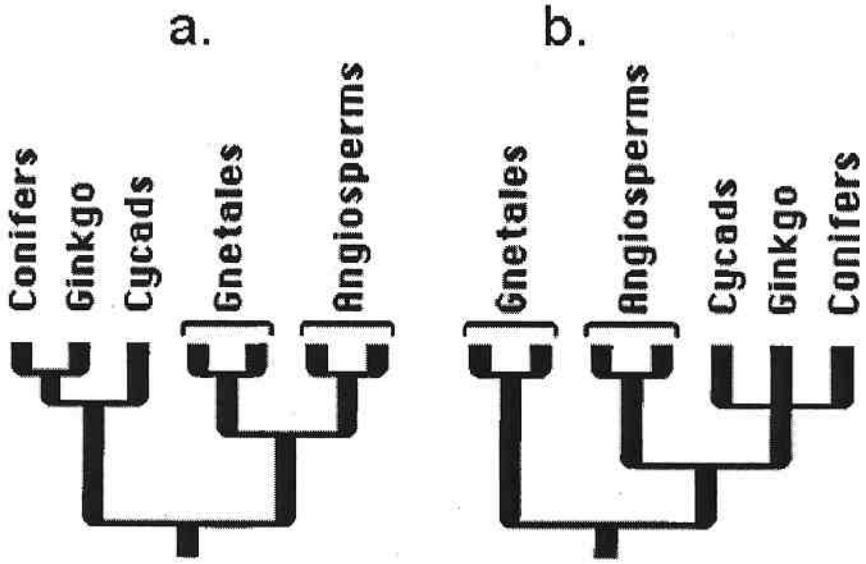
The glossophyte clade includes the Mesozoic seed fern glossopterids in a basal position, the early Jurassic fossil *Piroconites* as sister group to the Gnetales, the Mesozoic seed fern *Caytonia* basal to the angiosperms, and previously described anthophyte taxa (e.g. Bennettitales) in various arrangements (Fig. 1.4). These results are interesting because "flowers" are not a shared feature of the glossophytes; therefore, these features may have evolved independently in the Gnetales and the angiosperms (Doyle 1996). Another change from previous studies is the monophyletic grouping of extant conifers, Cordaitales and ginkgos, which supports a coniferopsid evolutionary lineage (Fig. 1.4). Extant conifers form a monophyletic clade within the coniferopsid clade (Fig. 1.4). The oldest extant gymnosperm again varies, in this case between cycads (Fig. 1.4b) and the conifer family Taxaceae (Fig. 1.4a, Fig. 1.4c). The topology of the monophyletic seed plants clade is seed ferns, coniferopsids, Mesozoic seed ferns, and glossophytes. Cycads are alternately a sister group to the glossophytes (Fig. 1.4a, Fig. 1.4c) or basal to the coniferopsids (Fig. 1.4b).

It seemed that Doyle (1996) had integrated the new molecular evidence with existing views of gymnosperm evolution: angiosperms and the Gnetales were closely related, but not as closely related as previous morphological analyses had suggested. Doyle's model suggested that "angiosperm like" characters (i.e. highly aggregated male and female reproductive organs) evolved independently in the Gnetales and angiosperms, but the two groups shared a relatively recent common ancestor, the Glossopterids (Doyle 1996). Therefore, the anthophyte theory was not completely incorrect, and the gymnosperms remained a paraphyletic group.

Gymnosperm Classification Based on Molecular Evidence

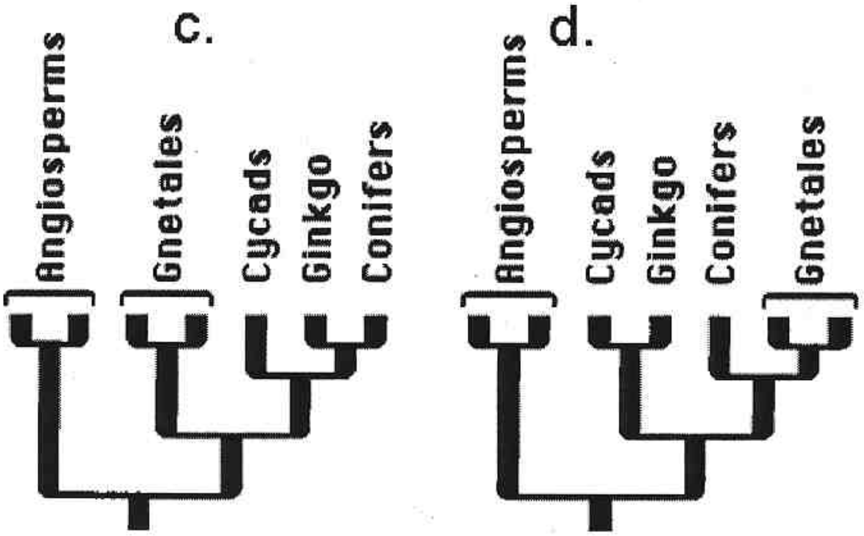
Beginning in the early 1990s, researchers attempted to reconstruct the evolutionary history of gymnosperms using DNA sequence data. Molecular systematics became very popular because it was a relatively objective approach, and "no one will argue whether a base at a particular site is an A or a G, as they might argue whether layers around the ovule are the same" (Doyle 1998). Early molecular studies challenged long held assumptions about gymnosperm evolution. With the introduction of this approach, a major controversy arose in the field of gymnosperm evolution.

On many points, molecular and morphological systematic analyses reached the same conclusions. All early molecular studies agreed on the monophyly of cycads, the Gnetales, angiosperms, and seed plants as a whole (Doyle 1998), and many molecular phylogenetic trees posited conifers and ginkgos as sister groups (Fig. 1.5). The most interesting and most unexpected implication of the early molecular studies was that angiosperms and the Gnetales are not closely related (Doyle 1998). Depending on the study, results suggested that either angiosperms or the Gnetales are the oldest extant seed plants, and a sister group to all other seed plants (Fig. 1.5b); (Albert et al. 1994; Hamby and Zimmer 1992). In some studies, Gnetales are more closely related to conifers than to angiosperms (Fig. 1.5d); (Chaw et al. 1997). A surprising result by Chase et al. (1993) was that extant conifers are paraphyletic, with the Pinaceae and all other conifers forming two separate clades. However, this result is based on chloroplast gene sequence data and cannot be replicated in nuclear DNA studies (Doyle 1998).



rRNA, Hamby & Zimmer 1992

rRNA, Hamby & Zimmer 1992
rbcL, Albert et al. 1994



rbcL, Hasebe et al. 1992
28S, Stefanovic 1996

cpITS, Goremykin et al. 1996
18S, Chaw et al. 1997
coxI, Bowe & DePamphilis 1997

Figure 1.5: Molecular phylogenetic trees. The gene analyzed is indicated to the left of each citation. Modified from Doyle (1998).

Despite general trends, results were inconclusive. Primarily, results varied widely with choice of genome (i.e. nuclear vs. plastid) (Doyle 2000). Other than the distant relation of the Gnetales and angiosperms, different genomes and genes seemed to disagree on gymnosperm relationships. This variation was so extensive that one rRNA study even supported a sister group relationship between the Gnetales and angiosperms (Hamby and Zimmer 1992). In addition, low levels of statistical support and inconsistencies with the fossil record further discredited these early molecular studies (Donoghue and Doyle 2000). Because the emerging molecular evidence was mixed, some reconciliation could be made between morphological and molecular phylogenies.

In his 1996 paper on seed plant phylogeny, Doyle reasserted the close relationship between the Gnetales and angiosperms in light of molecular evidence. The results showed that the Gnetales and angiosperms are related, which fits with morphological phylogenies, but not sister groups, which fits with molecular phylogenies (Doyle 1996).

This resolution was short lived. In the year 2000, two independent multi-gene analyses produced results that strongly supported gymnosperms as a monophyletic group sister to the angiosperms (Bowe et al. 2000); (Chaw et al. 2000). Furthermore, the results of Bowe et al. (2000) and Chaw et al. (2000) suggested a close relationship between Gnetales and conifers, even raising the possibility that the Gnetales were derived from conifers.

Bowe et al.'s (2000) comprehensive molecular phylogeny, in addition to Chaw et al.'s (2000) study in the same issue of PNAS, provided strong evidence against the anthophyte hypothesis. Gene sequences from three genomes (mitochondrial, plastid and nuclear) of all extant seed plant lineages were analyzed, supporting a sister group

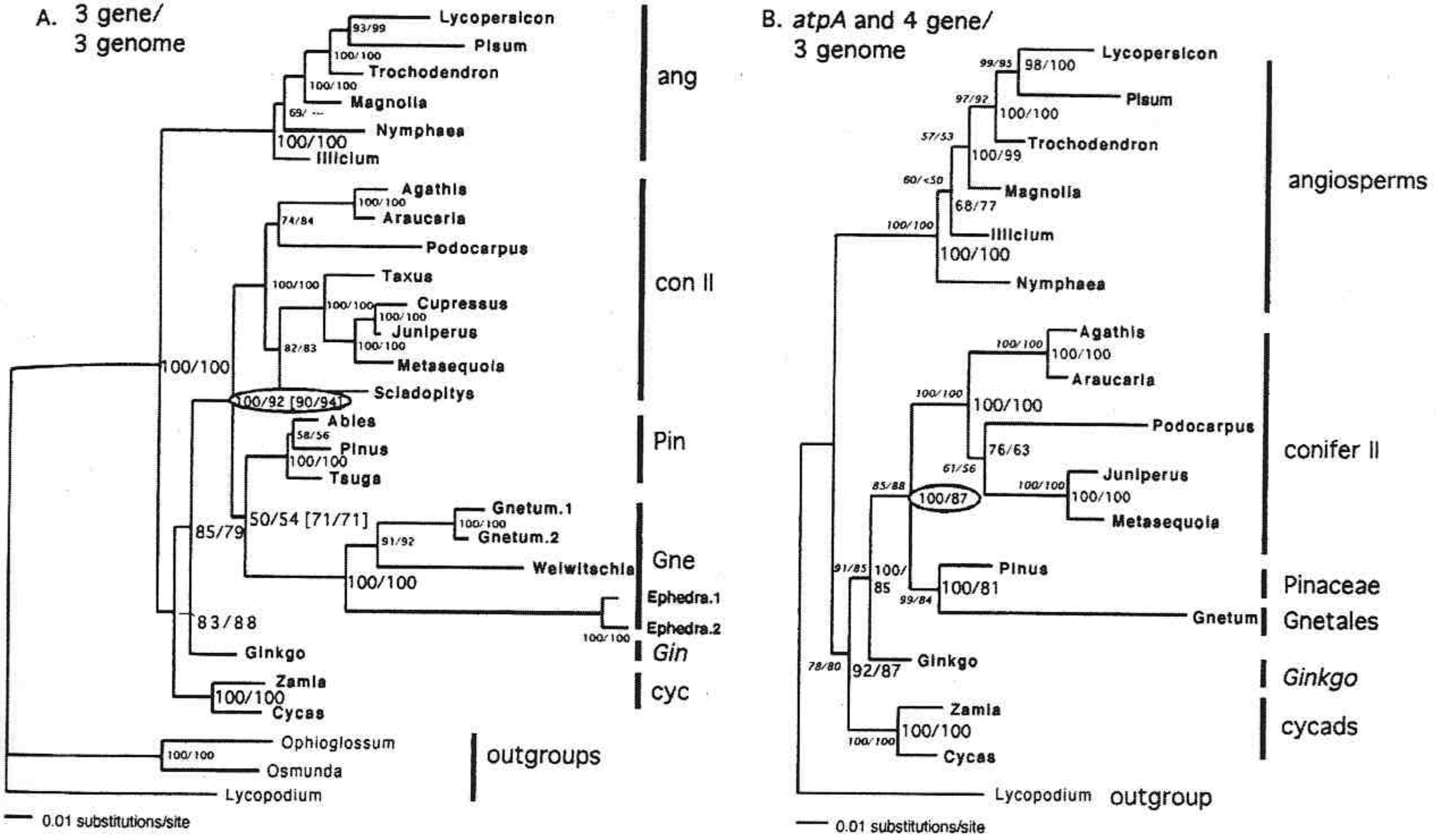


Figure 1.6: Rooted seed-plant phylogenies using three-genome data. Data were analyzed from (A) *cox1* (mitochondrial gene), *rbc*L (chloroplast gene), and 18S rDNA (nuclear gene), and (B) including *atp*A (mitochondrial gene). Modified from Bowe et al. (2000).

relationship between angiosperms and gymnosperms, and monophyly of cycads, ginkgos and Gnetales (Fig. 1.6). The inclusion of mitochondrial gene sequences is crucial, as the mitochondrial genome has "slowly evolving" sequences, which may contain enough information (phylogenetic signal) to resolve the deep phylogenetic history of the gymnosperms. Conifers form a paraphyletic group, with Gnetales and Pinaceae as sister groups (Fig. 1.6) in three of four gene analyses. Following the initial divergence of angiosperms from gymnosperms, cycads diverge first, followed by ginkgos and the conifer/Gnetales assemblage (Fig. 1.6). Bowe et al.'s (2000) results support a close relationship between conifers and Gnetales, with a Conifer II clade (conifers excluding Pinaceae) sister to a Pinaceae/Gnetales clade (Fig. 1.6). The specific association between Gnetales and Pinaceae is recognized as a preliminary result, but the evidence strongly suggests angiosperms and Gnetales are paraphyletic groups. Chaw et al. (2000) report similar findings, despite sampling different taxa and genic regions, as well as using different statistical methods.

In molecular systematics, analysis of a single gene or genome can lead to biased phylogenetic arrangements (Magallón and Sanderson 2002). Both Bowe et al.'s (2000) Chaw et al.'s (2000) phylogenetic analyses were based on multiple genomes; therefore, both studies provide strong evidence to reject the anthophyte hypothesis. However, Donoghue and Doyle (2000) express doubt over some of Bowe et al.'s (2000) and Chaw et al.'s (2000) conclusions. According to Donoghue and Doyle (2000), different rootings of Bowe et al.'s (2000) or Chaw et al.'s (2000) unrooted cladograms can result in trees with different topologies; therefore, one can just as easily produce a tree with gymnosperms as a paraphyletic group. Donoghue and Doyle (2000) recommend further statistical analyses of alternate rootings before accepting the conclusion that gymnosperms are monophyletic and sister to the angiosperms. Furthermore, Donoghue and Doyle (2000) argue that a close relationship between Gnetales and conifers would contradict evidence that all conifers—but not Gnetales—have lost one copy of the inverted repeat in the chloroplast genome (Raubeson and Jansen 1992). One result that Donoghue and Doyle (2000) cannot deny, however, is that the Gnetales and angiosperms do not form a clade.

Currently, there is agreement in the rejection of the anthophyte hypothesis (Magallón and Sanderson 2002); however, all other areas of gymnosperm phylogeny are in a flux. According to Magallón and Sanderson (2002), "available results suggest that particular types of molecular data, treatment of the primary data and the combination of two or more genes from different genomic compartments usually result in one of three distinct phylogenetic hypotheses among major lineages of seed plants." The three phylogenetic hypotheses are: 1) Gnetales are the sister taxon to a clade that includes all other seed plants; 2) Gnetales are sister to Pinaceae, conifers are paraphyletic, and angiosperms are the sister taxon to a clade that includes all other seed plants; and 3) Gnetales are sister to conifers, conifers are monophyletic, and angiosperms are the sister taxon to a clade that includes all other seed plants (Magallón and Sanderson 2002). Therefore, there continues to be disagreement over the phyletic status of the gymnosperm lineage, and the exact relationship of the Gnetales and conifers.

A major problem in gymnosperm molecular phylogenetics is disagreement among phylogenetic trees depending on genome choice (Magallón and Sanderson 2002). A recent study by Burleigh and Mathews (2004) attempted to identify biases in data (e.g.

genome choice) and data analysis that result in "preference" for particular cladograms. Burleigh and Mathews (2004) examined the gnepine (Gnetales sister to the Pinaceae) and gnetifer (Gnetales sister to the conifers) hypotheses, and attempted to identify the data and data analyses that tend to support each hypothesis. In examining rate heterogeneity, the authors elsewhere found that sites evolving at intermediate rates tend to support the gnepine hypothesis, whereas sites evolving at rapid rates tend to support the gnetifer hypothesis (Burleigh and Mathews 2004). In his paper "On the best evolutionary rate for phylogenetic analysis." (Yang 1998), Yang suggested that slowly evolving sites may have little phylogenetic information, whereas intermediate sites have the most, and the amount of information in the fastest sites may decrease slightly due to heterogeneity in the signal. Based on Yang's ideas, Burleigh and Mathews (2004) eliminated rapidly evolving sites from some of their analyses. In the complete data analysis (including rapidly evolving sites), Maximum Likelihood Analysis favoured the gnepine hypothesis, whereas Maximum Parsimony Analysis favoured the gnetifer hypothesis (Burleigh and Mathews 2004). However, when rapidly evolving sites were excluded from the Maximum Parsimony Analysis, the gnepine hypothesis was favoured (Burleigh and Mathews 2004). Burleigh and Mathews (2004) therefore suggested that the presence of rapidly evolving sites, to which parsimony is particularly sensitive (Felsenstein 1978), is one biasing factor in gymnosperm molecular phylogenetics.

Discrepancies between Morphological and Molecular Phylogenies

At present, morphological and molecular phylogenies are diametrically opposed in their main conclusions: Gymnosperms are a paraphyletic group according to morphological phylogenies, and a monophyletic group according to molecular phylogenies. This discrepancy is based on disagreements over the relationships of three groups: the Gnetales, the conifers, and the angiosperms. Though they agree on the monophyly of seed plants, morphological and molecular phylogenies disagree on the relationship between gymnosperms and angiosperms. In morphological phylogenies, gymnosperms are ancestral to angiosperms; therefore, gymnosperms are a paraphyletic group. The inherent assumption in this evolutionary scheme is that the Gnetales and angiosperms are sister groups, an idea which is well supported by morphological phylogenies. However, molecular phylogenies predict that gymnosperms and angiosperms are sister groups; therefore, gymnosperms are a monophyletic group. This prediction is based on strong molecular support of the monophyly of both angiosperms and gymnosperms, and weak molecular support of a close relationship between angiosperms and gymnosperms. Furthermore, a close relationship between the Gnetales and angiosperms is not supported by molecular phylogenetic analyses; therefore, angiosperms do not appear to be derived gymnosperms, and any Gnetalean and angiosperm similarities are likely convergent. According to molecular phylogenies, the closest relative of the Gnetales are the conifers.

At this point, one is left with more questions than answers. As far as providing an accurate reflection of evolutionary history, which set of phylogenies is better? At present, most scientists have rejected the anthophyte hypothesis based on overwhelming molecular evidence (Donoghue and Doyle 2000). In this area of gymnosperm evolution (the relationship of Gnetales and angiosperms), molecular phylogenies have been more informative. However, this does not mean that morphological data should be excluded from phylogenetic analyses, as this would exclude key fossil species. If one method cannot be proven better than the other, one should have a good understanding of the strengths and weaknesses of both morphological and molecular data in gymnosperm cladistics. In understanding the limitations of each approach, perhaps it will become clear how to integrate the two.

Understanding Gymnosperm Evolution: Caveats and Pitfalls

Regardless of the approach used, tracing phylogenetic relationships among seed plant lineages is a complex endeavor. Gymnosperms have a deep evolutionary history, in that all extant lineages radiated rapidly long ago (Donoghue and Doyle 2000). In the stratigraphic record, seed plants appear in the Late Devonian (approximately 370 million years ago), with divergence of three of the five lines within the Late Carboniferous (approximately 290 to 320 million years ago) (Donoghue and Doyle 2000). Given the amount of time each gymnosperm lineage has evolved, both uniquely derived and convergent characters have probably originated (Magallón and Sanderson 2002). Therefore, any similarities in morphology or gene sequence between extant gymnosperm lineages could be the result of either homoplasy or common ancestry (Magallón and Sanderson 2002). Another problem in gymnosperm classification is a result of extinction: extant gymnosperm diversity represents only a fraction of historical gymnosperm diversity (Magallón and Sanderson 2002). Given the incomplete nature of the fossil record and the immeasurability or absence of key features in fossils, it is difficult to determine the evolutionary history of gymnosperms.

The high divergence and low diversity exhibited by gymnosperms are both problematic in molecular phylogenetic analyses. In a molecular phylogenetic tree, a long-branch represents a lineage with a high rate of divergence, where divergence is often measured by rate of base substitution (Graur and Li 1999). The cladistics term "long-branch attraction" describes the spurious attraction of long-branches based on the tendency of parsimony to cluster long-branches together (Graur and Li 1999). With the highly divergent extant outgroups (i.e. ferns) and ingroups (i.e. *Gnetum*) used in molecular phylogenetic analyses, long-branch attraction can be a problem (Donoghue and Doyle 2000). Burleigh and Mathews (2004) suggest that the monophyly of angiosperms and gymnosperm observed in molecular phylogenetic trees may be an artifact of long-branch attraction. In their study, Burleigh and Mathews (2004) observed that the outgroup branch consistently attached to the long branch connecting angiosperms with extant gymnosperms, suggesting that both groups were monophyletic. Molecular phylogenetic analyses are further restricted by the relatively low diversity of extant gymnosperms, as lineages with few species (i.e. ginkgos, Gnetales) provide limited information.

Morphological cladistic analyses are not subject to the same limitations as molecular phylogenetic analyses, as fossil gymnosperms can fill in gaps in and between extant gymnosperm groups. However, morphological phylogenies are limited by the fact that the data is often based on subjective decisions (Doyle 1998). As a result, relevant characters in morphological cladistic analyses are often interpreted and reinterpreted many times, and morphological homologies are notoriously difficult to ascertain (Bowe et al. 2000).

Resolving Gymnosperm Phylogeny

Molecular and morphological data are both useful in constructing gymnosperm phylogenies, and should therefore be integrated. Within certain limits, molecular phylogenetic analyses provide an objective means to compare extant groups. Morphological phylogenies, particularly those including fossil species, can clarify the course of character evolution. A complete understanding of gymnosperm evolution may only be achieved with further fossil investigation.

Early gymnosperm evolution is a subject of great interest, and one that can only be investigated by morphological cladistic analysis. Because the gymnosperms radiated rapidly early in their evolution (Hilton 1998), an understanding of early gymnosperm evolution will prove crucial to understanding gymnosperm evolution as a whole. For example, if angiosperms diverged before the extant gymnosperms (as predicted by some molecular phylogenies), then only the fossil record can elucidate the origin of the angiosperms. The recent discovery of the Gnetalean fossil *Paleognetaleana* is an excellent example of the importance of fossils in understanding gymnosperm evolution. *Paleognetaleana* is dated to 270 million years ago, extending previous estimates of Gnetalean divergence from the Mesozoic to the early Permian (Wang 2004). This evidence proves that all extant gymnosperms had established separate lines early in the Permian, which satisfies a precondition for molecular estimates of gymnosperm monophyletic origins (Wang 2004). The discovery of *Paleognetaleana* provides evidence for the monophyly of gymnosperms, and thus lends support to current molecular phylogenies (Wang 2004).

Fossil evidence and morphological phylogenies are also important in clarifying character evolution. For example, the morphology of *Paleognetaleana* provides evidence for a relationship between Gnetales and conifers early in the Permian, as the cone of *Paleognetaleana* shares key reproductive features with the Gnetales (integument with three envelopes with the inner cuticle extended into the micropylar tip) and the Paleozoic conifers (axillary fertile units arranged in a helical fashion) (Wang 2004). In this case, the intermediate morphology of *Paleognetaleana* may help explain the evolution of two highly divergent groups from a common ancestor.

Resolution is still required, particularly of disputed issues in early gymnosperm evolution. One such issue is whether seed plants are monophyletic or polyphyletic. If the seed originated more than once, as Galtier and Rowe's (1989) fossil evidence may indicate, then it is possible that angiosperms and gymnosperms evolved along completely different lines. The identity of the progymnosperm ancestor to the seed plants is another unresolved issue. Because Archaeopteridalean progymnosperms are heterosporous and present in early seed plant fossil assemblages, many consider *Archaeopteris* the closest relative of the seed plants (Hilton 1998). However, the recently discovery of *Runcaria*, a middle Devonian seed plant precursor, suggests that the Aneurophytales are the closest relative to the seed plants (Gerrienne et al. 2004). Gerrienne et al. (2004) base their assumption on the overlap of *Runcaria* and the Aneurophytales in the fossil record.

Alternative Methods

Understanding gymnosperm evolution is a complex problem requiring a multi-faceted approach. In the last decade, other methods to analyze gymnosperm evolution have emerged.

MADS box genes, members of a family of DNA-binding proteins, are homeotic genes controlling floral organ identity (Coen and Meyerowitz 1991). MADS genes were first characterized in angiosperms, but homologs have been found in gymnosperms. In a study by Shindo et al. (1999), four MADS genes of *Gnetum parvifolium* were characterized in terms of DNA sequence and expression pattern. A comparison of this data with published data of angiosperm and conifer (Pinaceae) MADS genes indicated that female reproductive organs of Gnetales and conifers evolved from a common ancestor, whereas female reproductive organs of Gnetales and angiosperms evolved independently (Shindo et al. 1999).

A different approach to phylogenetic components was taken by Leitch et al. (2001), who examined variation in gymnosperm C-value (the amount of DNA contained in a haploid cell). In their study, nuclear DNA C-values were superimposed on the molecular phylogenetic trees of Bowe et al. (2000) and Chaw et al. (2000) in order to determine the relationship between C-value and gymnosperm phylogeny. Results were too vague to be useful, but the authors suggest measuring cells of fossil gymnosperms, based on the assumption that cell size is correlated with genome size, to clarify patterns in extant groups (Leitch et al. 2001).

As early as 1965, Zuckerkandl and Pauling predicted that protein sequence data contained evolutionary information (Zuckerkandl and Pauling 1965). Research beginning in the 1980s showed that proteomics could be useful in phylogeny construction. Penny et al. (1982) compared five conserved proteins in 11 mammalian species, and found that each 11 species tree for all five proteins showed a similar pattern of branching at all levels. The proteins examined are functionally conservative (e.g.

Cytochrome *c*, Haemoglobin A). Therefore, cross-species comparison was possible for the following two reasons: 1) proteins were present in all study species, 2) proteins were equivalent in all study species (Penny et al. 1982).

There have been disputes since the 1980s over how best to infer phylogenetic relationships from molecular data (Navas and Albar 2004). Unlike DNA sequence data, proteomic data is amenable to cladistics—a method of analysis based on the identification of shared derived traits (synapomorphies). Specifically, proteomic data can be displayed in a binary form (e.g. presence or absence of a certain protein) that can be analyzed by non-statistical methods such as parsimony (reduction of number of evolutionary steps) and character compatibility. A combined proteomics/cladistics approach could identify proteins with evolutionary value, and could result in phylogenies based on data from entire organisms (e.g. genomes and proteomes) rather than single genes.

Proteomics has been used to resolve phylogenetic relationships in angiosperms. Zivy et al. (1995) compared 2D SDS-PAGE profiles of *Triticum* (wheat) *Hordeum* (barley) and *Secale* (rye) from the Triticeae tribe—a group with controversial phylogeny. Cladograms were constructed based on the presence or absence of protein spots in 2D SDS-PAGE profiles, and results were in accordance with classical taxonomy. A similar study of the Brassicaceae by Marques et al. (2001) identified common and unique spots of *Brassica* species (i.e. mustards) and *Raphanus* sp. (radishes). The resulting cladogram accurately reflected genetic relationships.

The proteome approach used in the aforementioned studies is informative because it gives consensus results of hundreds of expressed genes; however, it is unfortunately restricted to closely related species (Thiellement et al. 1999). In future studies, it may be useful to construct phylogenetic trees based on spot comparison and sequence comparison of functionally conserved proteins (e.g. Cytochrome *c*) (Penny et al. 1982). Many features of gymnosperm reproduction have been highly conserved throughout evolution, particularly female reproduction. A proteomics approach to identify conserved proteins in gymnosperm female reproduction could reveal gymnosperm phylogenetic relationships.

From the Palaeozoic genus *Lebachia* to virtually all groups of extant gymnosperms, the mature gymnosperm ovule consists of an outer integument with a micropyle, an apical layer of nucellar tissue, and the haploid megagametophyte tissue containing one or more egg cells within an archegonium (Singh 1978). Although ovules are pollinated at different stages of development, almost all gymnosperm pollination mechanisms involve liquid secretions—the most common being the external pollination drop (Gelbart and von Aderkas 2002). By contrast, male gymnosperm reproduction is variable across taxa. In conifer pollen, the wall structure, chemical composition, and number of cells upon release can vary within and between families (Fernando et al. 2005).

The nucellus is an important and conservative feature of gymnosperm reproduction. Because gymnosperm ovules are relatively exposed to the environment, the nucellus acts as a barrier to both desirable and undesirable would-be colonists of the ovule. In many fossil species and the living cycads, the nucellar epidermis is thick and heavily cutinised (Singh 1978). In angiosperms, there is a chemical component to the nucellar barrier: the nucellus produces attractants for conspecific pollen (Herrero 2001).

The same may be true for gymnosperms, given that *Tsuga* and *Araucaria* species exhibit extreme siphonogamy (i.e. pollen germinates at some distance from the micropyle and somehow finds the ovary). It is also possible that conifer nucellus produces defense chemicals against pathogens (Poulis 2004).

How conservative are nucellar chemical barriers in gymnosperm reproduction? In other words, would an analysis of nucellar chemical barriers be useful in gymnosperm phylogenetics? Proteins putatively involved in pollen tube growth and pathogen defense were found in the pollination drop of *Taxus x media*, and production of one protein was localized to the nucellus (O'Leary 2004). In this thesis, we chose to analyze the pollination drop—a highly conserved feature of gymnosperm evolution. We assume that pollination drops and pollination drop proteins (PDP's) are both functionally conservative, and may therefore be useful in phylogeny construction.

Pollination Drops

Pollination drops are a common type of pollination mechanism, and are exhibited by all extant and extinct gymnosperm phyla. The presumed direct ancestors to the gymnosperms (the pteridosperms) and the earliest gymnosperms (i.e. *Lebachia*) all produced pollination drops (Doyle 1945). In 1977, Rothwell discovered fossil evidence of pollination drops exuding from ovules of the seed fern *Callospermarion pusillum*. Of the extant gymnosperms, pollination drop-producing species are found in all four proposed phyla (Cycadophyta, Ginkgophyta, Gnetophyta, and Coniferophyta).

In 1841, Vaucher published the first observation of pollination drops in the four-volume treatise "Histoire Physiologique des Plantes d'Europe". Three decades later, Strasburger (1871) conducted a more thorough investigation of pollination drops of various conifer genera (including *Juniperus*, *Taxus*, and *Thuja*). Following the early phenomenological studies of pollination drops, Fujii published the first report of the chemical composition of the pollination drop in 1903, reporting the presence of glucose, calcium and amino acids in the pollination drop of *Taxus* sp. A similar study by Tison (1911) reported the presence of glucose, calcium and malic acid in the pollination drop of *Cupressus funebris*. A study by Ziegler (1959) found many amino acids, peptides, inorganic phosphate, malic acid and citric acid in *Ephedra* and *Taxus* species.

The early studies of chemical composition indicated that pollination drops were composed of a variety of relatively simple water-soluble compounds (Gelbart and von Aderkas 2002). The first in-depth investigations of the presence and role of proteins in conifer ovular secretions is the published study by Poulis et al. (2005), as well as in the PhD thesis of O'Leary (2004). Poulis et al. (2005) described nine proteins in the ovular secretion of *Pseudotsuga menziesii* (Mirbel) Franco, and proposed that the proteins influence pollen selection and development. The idea that conifer ovular secretion proteins also function in pathogen defense is presented in unpublished work in the PhD theses of O'Leary (2004) and Poulis (2004).

The main problem in studying pollination drops is lack of sample. Small volumes of sample are collected (i.e. one to two mL/year) because individual pollination drops are small and they are produced only for a short period of time. The small amount of protein isolated from ovular secretions further increases the challenge. In the PhD thesis of Poulis (2004), it was reported that ovular secretion of *Pseudotsuga menziesii* collected in 2003 contained 0.917 mg/mL protein.

Fortunately, highly sensitive tools such as tandem mass spectrometry allow the analysis of small amounts of protein. Although it is possible to determine the primary amino acid sequences of conifer ovular secretion proteins (Poulis et al. 2005), the proteomics approach can fall short in the functional identification of sequenced proteins. In general, gymnosperms are not well represented in DNA and protein sequence databases. In the NCBI taxonomy database, gymnosperm sequence entries (both nucleotide and protein) represent 4 % of all plant entries and 8×10^{-3} % of all cellular organism entries (Wheeler et al. 2000). Therefore, functional identifications of gymnosperm proteins can be made based on sequence homology with distantly related species (e.g. *Arabidopsis thaliana*). With cross-species identification, there is a danger of trying to "pigeon-hole" protein function entirely from sequence homology. There are examples in the literature of homologous proteins with very different functions in different organisms (i.e. glutathione transferase) (Tomarev and Zinovieva 1998); therefore, one must be careful when assigning function.

Proteomics provides a powerful tool, even given these constraints. Since it has only been recently discovered that pollination drops contained proteins, the research possibilities are great. In order to investigate gymnosperm evolution from a proteomics perspective, we studied three closely related species in the Cupressaceae (*Juniperus communis* Linnaeus, *Juniperus oxycedrus* Linnaeus, and *Chamaecyparis lawsoniana* (Murray) Parlatore), and a non-conifer species (*Welwitschia mirabilis* Hook). The following three hypotheses were examined in this thesis:

H_o^1 The gymnosperm pollination drop contains proteins

H_o^2 Variation in pollination drop protein (PRP) profiles has a phylogenetic component

H_o^3 Gymnosperm pollination drops may function in pathogen defense and reproduction

Chapter 2: Pollination drop proteins in four gymnosperm species of varying evolutionary relatedness

Introduction

Virtually all gymnosperms depend on wind to deliver pollen from male to female cones. The pollen capture strategy employed by the female cone is highly evolved. Pollination mechanisms include the presence of sticky hairs, the engulfment of pollen by asymmetric ovular growth, extreme pollen tube formation, and secretions from the ovule (Gelbart and von Aderkas 2002). These secretions transport pollen inside the ovule. Pollination drops exude from the ovule, capture pollen directly from the air, and retract into the ovule, carrying the pollen with them.

Pollination drops are crucial to seed plant reproduction, yet the first in-depth studies of the nature and content of ovular secretions have only appeared in the last few years. A study by Poulis et al. (2005) identified proteins in ovular secretions of conifers and postulated functions for these proteins. In this thesis, we studied the pollination drop producing cupressad species *Juniperus communis* (common juniper), *Juniperus oxycedrus* (prickly juniper), *Chamaecyparis lawsoniana* (Port Orford cedar), and the Gnetalean species *Welwitschia mirabilis*.

The Cupressaceae is the third largest family of conifers, and includes approximately 135 species in 28 genera (Farjon 1998). Cupressad species are distributed worldwide: the species *J. communis* has the largest distribution of any woody plant, ranging south from the Arctic to approximately 30° N latitude in North America, Europe, and Asia (Farjon 1998). Based on molecular systematics, the Cupressaceae have been reorganized with into 7 subfamilies and now include members of the former Taxodiaceae

(Gadek et al. 2000). The discovery of new species has also altered the classification of the Cupressaceae: a cupressad species in Vietnam led to the reclassification of (Nootka cypress) and the description of a new genus (*Xanthocyparis* (D. Don) Farjon & Harder) to accommodate both species (Little et al. 2004).

For many species in the Cupressaceae, pollination of ovules takes place in early spring (Farrar 1995). The arrangement of ovules in the globular conelet results in pollination drops forming at the tip of the conelet. Pollen in this family is spherical and sinks into the drop (Gelbart and von Aderkas 2002). Inside the pollination drop, the pollen grain swells and the outer pollen coat (exine) is ruptured and cast off before germination (Singh 1978).

Welwitschia mirabilis has very unusual reproductive features (e.g. lack of archegonia), many of which led scientists to assume it a close relative of the angiosperms. Ovulate cones arise from branched reproductive shoots, and each cone consists of four rows of oppositely arranged bracts (Gifford and Foster 1989). In the axil of each bract are two pairs of bracteoles, with one pair fused to form an envelope around the ovule. The integument of the ovule forms a long micropylar tube, which protrudes from the subtending bract. Viscous pollination drops are produced at it the tip of the micropyle. Male cones of *W. mirabilis* produce microsporangiophores that surround a sterile ovule. Sterile ovules differ in shape from fertile ovules—the long micropylar tube of the sterile ovule is apically expanded into a flat plate—but both produce secretions (Endress 1996). Some researchers suppose that insects play a role in *W. mirabilis* pollination, given the presence of sugar-rich pollination drops on both male and female cones (Endress 1996). Carafa et al. (1992) studied *W. mirabilis* pollination drop from female cones and identified sugars, uronic acids, and a small amount of free amino acid and an enzyme, acid phosphatase.

Following Poulis et al.'s (2005) identification of proteins in the ovular secretion of *Pseudotsuga menziesii* (Douglas fir), we investigated whether pollination drop proteins (PDPs) were present in other gymnosperm species, and whether these proteins may function in reproduction and/or pathogen defense. Furthermore, we investigated gymnosperm PDP variation. In our study, we used a proteomics approach to analyze the pollination drops of three closely related cupressad species (*Juniperus communis*, *Juniperus oxycedrus*, and *Chamaecyparis lawsoniana*) and the more distantly related Gnetalean species (*Welwitschia mirabilis*).

Methods and Materials

Plant material

Trees were used from two juniper species located in the Tuscany region of Italy. Pollination drop samples from *Juniperus communis* and *Juniperus oxycedrus* were collected February 2005 in Greve in Chianti near a cement works (Cementificio Sacci), and November 2003 in Campiglia Marittima in a stone quarry (Cave di Campiglia), respectively. Ovule-bearing branches with no visible pollination drops were cut to 40 cm lengths and transported to the laboratory at the University of Siena. Branches were placed in water for 2 to 3 d until the pollination drops were visible. The *Chamaecyparis lawsoniana* trees were located in a greenhouse at the US Department of Agriculture Dorena Lake Genetic Resource Centre, and pollination drops were collected in February 2005 and 2006. Collection from *Welwitschia mirabilis* plants was performed in July

2005 in the xeric room of the University of Washington Botany Greenhouse. Pollination drops from both *C. lawsoniana* and *W. mirabilis* were collected directly from the plants. Sample from all species was collected in 10 μL glass capillary tubes pulled to a fine point over flame. Daily collections were pooled in a 1.5 mL storage tube and stored at -20 °C until analysis.

The volume of a *C. lawsoniana* pollination drop was estimated using a hand held counter and 20 μL glass capillary tubes. The number of drops required to fill the capillary was counted, averaged over three trials, and used to estimate the average volume of one pollination drop.

One dimensional sodium dodecylsulphate polyacrylamide gel electrophoresis (1D SDS-PAGE)

All buffers for SDS-PAGE were prepared according to Laemmli (1970). Prior to electrophoresis, pollination drop collection vials were thawed and centrifuged at 16,000 x g for 1 min to pellet any suspended debris. A sample aliquot ranging from 20 to 50 μL was desiccated to dryness at room temperature in a Jouan RC 10.22 centrifugal evaporator. The *W. mirabilis* sample was too viscous to analyze by SDS PAGE and was therefore diluted. The protein component of the *W. mirabilis* pollination drop sample was separated using a Microcon® centrifugal filtration unit with a 10,000 Dalton nominal molecular mass limit (Millipore). Approximately 25 μL of *W. mirabilis* pollination drop were diluted to 400 μL with dH_20, centrifuged at 16,000 x g for 5 min to pellet any suspended debris, spun at 14,000 x g for 30 min (filtration step), and spun at 1,000 x g for 3 min (recovery step). The recovered protein fraction (approximately 60 μL) was desiccated to dryness at room temperature in the centrifugal evaporator. The desiccated samples were diluted to 10 μL with 5 μL 2X glycine sample buffer and 5 μL dH_20. Samples were boiled for 3 min prior to loading. Electrophoresis was performed using the Bio-Rad Mini-Protean 3 system (BioRad) with a 4 % polyacrylamide stacking gel and a 12 % polyacrylamide resolving gel. The stacking and resolving gels were run in succession at 10 and 20 mA, respectively. Electrophoresis was carried out until the tracking dye reached the bottom of the resolving gel.

Colloidal CoomassieBlue staining

Electrophoresed gels were gently agitated overnight in a fixing solution (50 % ethanol (v/v) and 3 % phosphoric acid (v/v) in dH_20) at room temperature. Post fixation, gels were washed three times, 30 min each in dH_20. Gels were equilibrated for one hour in Neuhoff solution (16 % ammonium sulfate (w/v), 25 % methanol (v/v), and 5 % phosphoric acid (v/v) in dH_20). After equilibration, 1 g of Coomassie blue stain (Coomassie Brilliant Blue G250 pure powder, Serva) was added to the Neuhoff solution. Staining was carried out for 1 to 3 d until protein bands were easily visible. Stained gels were stored at 4 °C in post stain storage buffer (20 % ammonium sulfate (w/v) in dH_20).

In-gel protein digest

To minimize contamination, all procedures (excluding incubations and centrifugation) were carried out in a laminar flow hood. Protein bands were excised from gels using a razor blade. Individual bands were cut into 1 to 2 mm^3 gel pieces and placed in 1.5 mL Eppendorf tubes. Gel cores were de-stained overnight at room temperature in 200 μL of wash solution (50 % (v/v) methanol and 5 % (v/v) acetic acid in dH_20).

The wash solution was replaced the following morning; subsequently, the gel cores were de-stained for a further 3 h at room temperature. After discarding the wash solution, the gel cores were dehydrated for 5 min at room temperature in 100 µL acetonitrile. The acetonitrile was discarded and the dehydrated gel cores were vacuum-desiccated for 5 min at ambient temperature in the centrifugal evaporator. The proteins embedded in the gel cores were reduced in a 30 min incubation at 56 °C with 50 µL of 50 mM dithiothreitol (DTT) in 100 mM ammonium bicarbonate. The DTT solution was discarded, and the proteins were alkylated in a 30 min dark incubation at 45 °C with 50 µL of 100 mM of iodoacetamide in 100 mM ammonium bicarbonate.

The iodoacetamide solution was discarded and the gel cores dehydrated in 100 µL of acetonitrile. The acetonitrile solution was in turn discarded and the gel cores rehydrated in 100 µL of 100 mM ammonium bicarbonate. The ammonium bicarbonate was then discarded, and the dehydration and vacuum desiccation steps were repeated. Trypsin reagent was prepared by adding 1.0 mL of ice-cold 50 mM ammonium bicarbonate to 20 µg of Sequencing Grade Modified Trypsin (Promega). The desiccated gel cores were rehydrated with 30 µL trypsin solution for 10 min on ice. Excess trypsin solution was discarded, an extra 5 µL of 50 mM ammonium bicarbonate added to each eppendorf, and proteins digested overnight at 37 °C.

To extract the digested peptides, gel cores were incubated in 30 µL ammonium bicarbonate for 1 hour at 37 °C. Extracted peptides from comparable gel cores (isobaric bands from samples of the same species) were pooled.

Mass spectrometry peptide sequencing

Digested samples to be analyzed by manual nanospray were acidified with 3 µL formic acid and desalted by loading onto a 4 mm column containing POROS 50 R2 Reversed-Phase packing material (Perseptive Biosystems) packed in a NanoES Spray

Capillary (Proxeon Biosystems). The column with bound sample was held in a microfuge-mounted micropurification device (Proxeon Biosystems) and washed with a 0.1% (v/v) formic acid solution in dH_2O. The sample was then eluted into an Au/Pd coated capillary (Proxeon Biosystems) with 4 μL 60 % (v/v) methanol and 3 % (v/v) formic acid solution in dH_2O. Nanospray electrospray ionization (ESI) was used to introduce ions into the QSTAR Pulsar I Hybrid Quadrupole-TOF MS/MS Mass Spectrometer (Applied Biosystems/MDS Sciex).

LC-MS/MS analysis was performed using an integrated Famos autosampler, Switchos switching pump, and UltiMate micro pump system (LC Packings) interfaced to a Q TRAP hybrid triple quadrupole/Linear Ion Trap MS/MS Mass Spectrometer equipped with a nano-electrospray ionization source (Applied Biosystems/MDS Sciex) and fitted with a 10 μm fused-silica emitter tip (New Objective). Solvent A consisted of 0.05 % formic acid (v/v) and 2 % acetonitrile in dH_20, while solvent B consisted of 2 % dH_20 (v/v) and 0.05 % (v/v) formic acid in ACN. Sample were injected in 95 % solvent A and washed on the trapping column for 5 min. The trapping column was switched inline, and the sample was eluted onto a 75 μm x 15 cm column (New Objective) packed with 5 μm 100Å Magic C18AQ packing material (Michrom Bioresources). Separations were performed using a linear gradient of 95:5 to 40:60 % A:B over 35 min. The composition was then changed to 20:80 % A:B over the course of 3 min before re-equilibrating for 15 min at 95:5 % A:B.

MS data were acquired automatically using Analyst 1.4.1 software (Applied Biosystems/MDS). An information-dependent acquisition method was run and included an enhanced MS (EMS) and enhanced resolution (ER) precursor ion scan of mass range

400 to 1200 amu and two enhanced product ion (EPI) scans of mass range 100 to 1500 amu. The resultant MS/MS spectra were converted into Mascot generic format (MGF) files using Analyst software, and individual MGF files from one protein sample were merged into a single MGF file. Merged MGF files for each protein sample were submitted to Peaks 3.0 software (Bioinformatics Solutions Inc.) for auto de novo sequencing. Peptide amino acid sequences generated by Peaks were submitted to a protein BLAST search (short nearly exact matches) of the NCBI non-redundant database. Peptide sequences from *W. mirabilis* were submitted to a BLAST search of *W. mirabilis* EST datasets at the Plant Genome Network (http://pgn.cornell.edu/blast/blast_search.pl).

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

Results

Plant material

The estimated volume of one *C. lawsoniana* pollination drop was 10 nL.

One dimensional sodium dodecylsulphate polyacrylamide gel electrophoresis (1D SDS-PAGE)

Purification by 1D SDS PAGE indicated that there were proteins in the pollination drops of all study species. For both *Juniperus* species and *W. mirabilis*, there were approximately 10 protein bands in total (Figure 2.1). The PDP profile of *C. lawsoniana* was more complex, with approximately 20 protein bands (Figure 2.1).

The PDP profiles of *J. communis* and *J. oxycedrus* were relatively similar, with *J. communis* bands D and E approximately isobaric to the *J. oxycedrus* bands G and H.

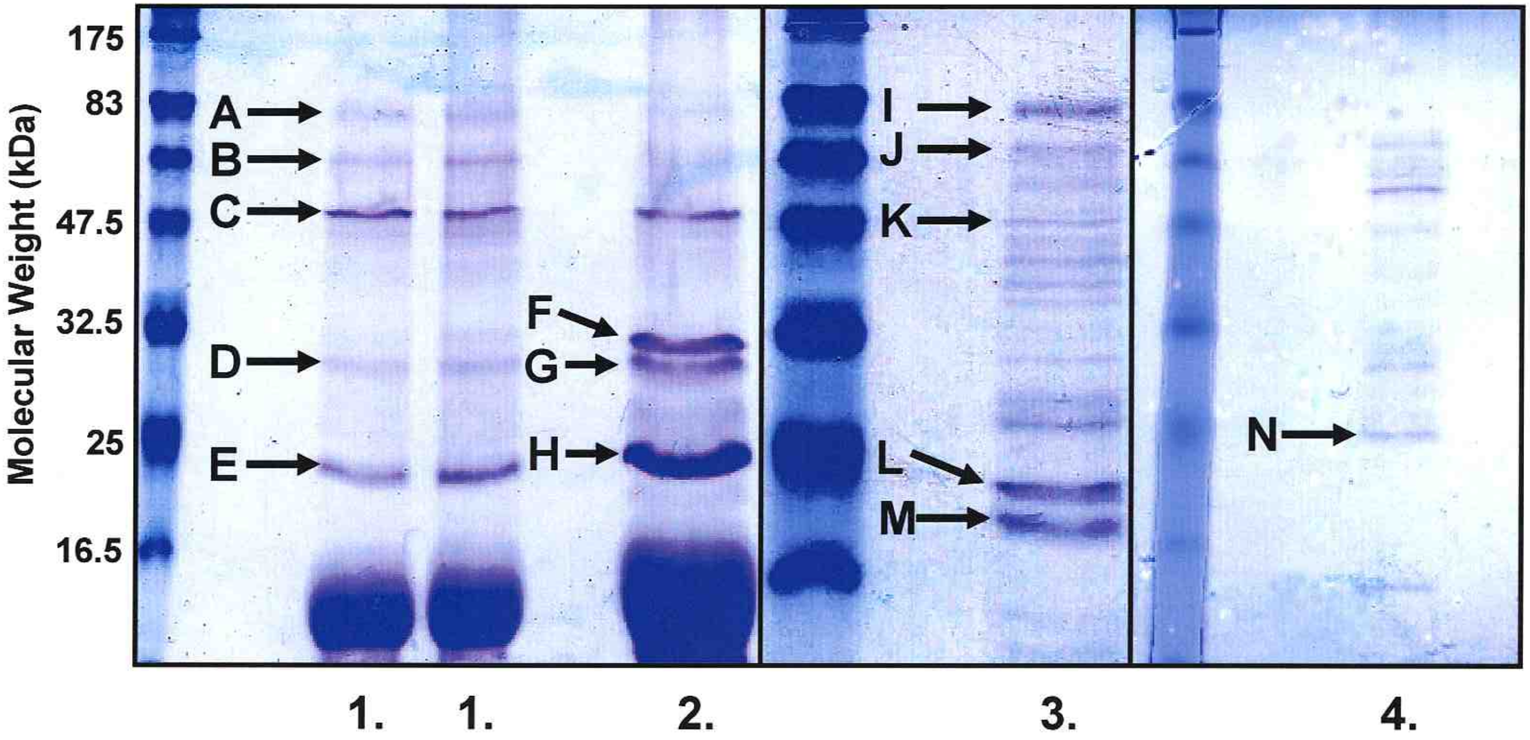


Figure 2.1: 1D SDS-PAGE gel separation of gymnosperm PDPs. The gel was incubated in colloidal Coomassie Blue stain solution until bands were visible (approximately 2 d). Protein bands (indicated A-N) were cored, and prepared by in-gel digestion for mass spectrometric analysis.
1=*Juniperus communis* (40, 50 µL)
2=*Juniperus oxycedrus* (40 µL)
3=*Chamaecyparis lawsoniana* (7.5 µL)
4=*Welwitschia mirabilis* (25 µL)

(Figure 2.1). The *J. communis* high molecular weight protein bands A, B, and C exhibited a similar banding pattern to the high molecular weight protein bands in *J. oxycedrus* pollination drop (Figure 2.1).

Both *Juniperus* species share similarities with *C. lawsoniana* in terms of PDP profiles; bands E and H were approximately isobaric to protein L, and bands A and C were approximately isobaric to bands I and K (Figure 2.1). The PDP profile of *W. mirabilis* does not resemble that of the other study species; however, it was difficult to make comparisons given the apparent low amount of protein in *W. mirabilis* pollination drop.

Protein identification

Tryptic peptides were fragmented and sequenced for each cored protein band (Figures 2.2-2.15, Tables 2.1-2.14). The number of peptides sequenced for each sample varied from 2 to 7, with two peptides being the minimum number used for protein identification. The proteins identified in the pollination drop of *J. communis* were an 83 kDa subtilisin-like proteinase, a 62 kDa glycosyl hydrolase, a 47.5 kDa glucan 1,3-β-glucosidase precursor, a 30 kDa chitinase, and a 25 kDa thaumatin-like protein (Table 2.15). A 30 kDa chitinase and a 25 kDa thaumatin-like protein were also identified in the pollination drop of *J. oxycedrus,* in addition to a 32.5 kDa glucanase-like protein (Table 16). An 83 kDa subtilisin-like proteinase, a 62 kDa β-D-glucan exohydrolase, a 47.5 kDa glucan 1,3-β-glucosidase, and two 25 kDa thaumatin-like proteins were identified in the pollination drop of *C. lawsoniana* (Table 2.17). One 25 kDa chitinase was identified in the pollination drop of *W. mirabilis* (Table 2.18).

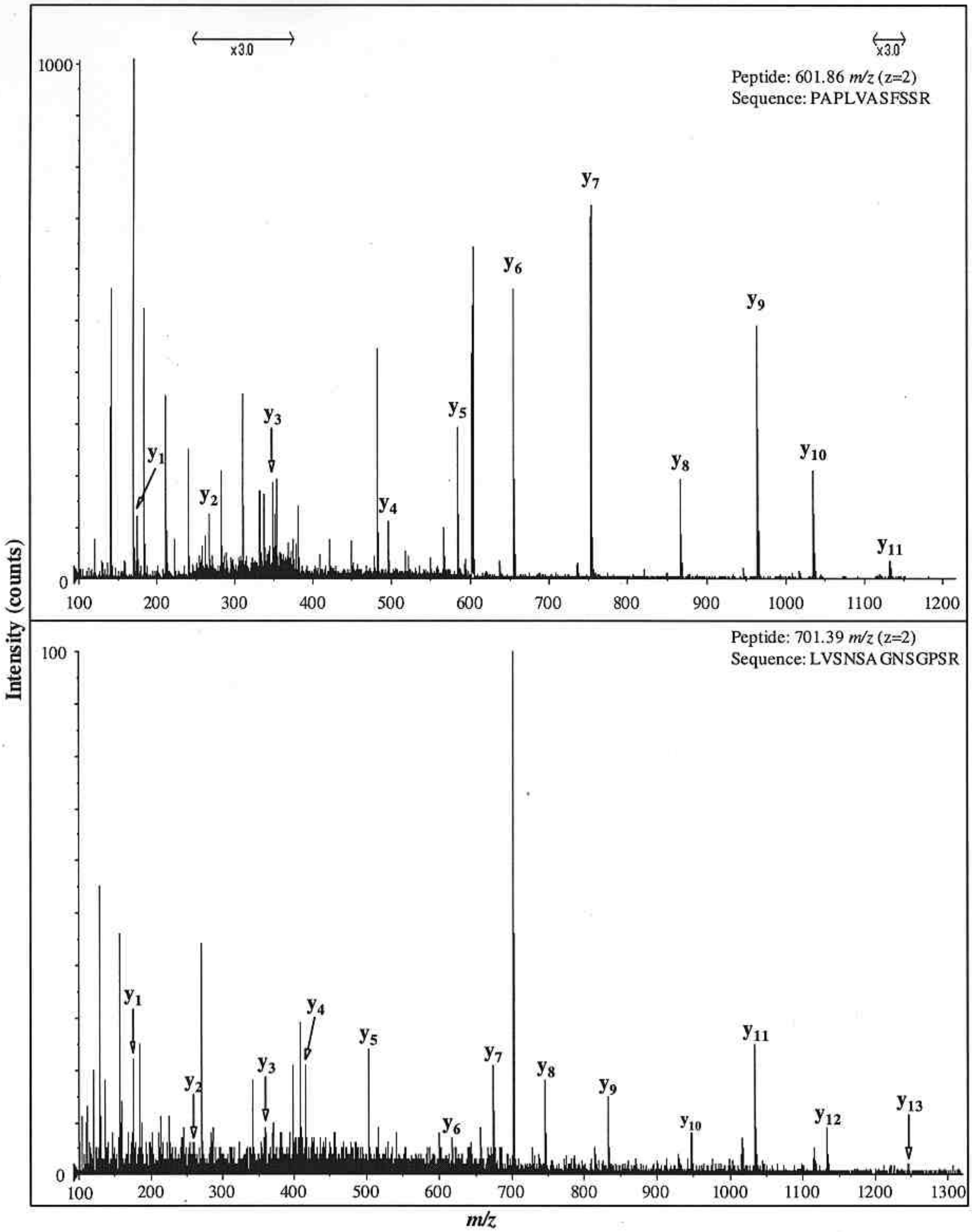


Figure 2.2: Protein A tandem MS fragmentation data.
Tryptic peptides from protein A (Figure 2.1, *J. communis*) were introduced into the Q-STAR*i* quadropole time-of-flight mass spectrometer (Applied Biosystems) by nanospray electrospray ionization (ESI). Data were managed with Peaks (BSI) and Bioanalyst Software (PE-SCIEX).

	Peptide *m/z* (z = 2)	
y ions	**601.86**	**701.39**
	R	**R**
1	175.12	175.12
	S	**S**
2	262.15	262.15
	S	**P**
3	349.18	359.2
	F	**G**
4	496.25	416.23
	S	**S**
5	583.28	503.26
	A	**N**
6	654.32	617.3
	V	**G**
7	753.39	674.32
	L	**A**
8	866.47	745.36
	P	**S**
9	963.52	832.39
	A	**N**
10	1034.56	946.43
	P	**S**
11	1131.61	1033.47
		V
12	-	1132.53
		L
13	-	1245.62

Table 2.1: Protein A peptide amino acid sequences.
The y-ion series obtained from tandem MS fragmentation of trypsin digested protein A (Figure 2.2) and the deduced amino acid sequence of each tryptic peptide. The monoisotopic mass difference between individual y-ions is the monoisotopic residue mass of an amino acid.

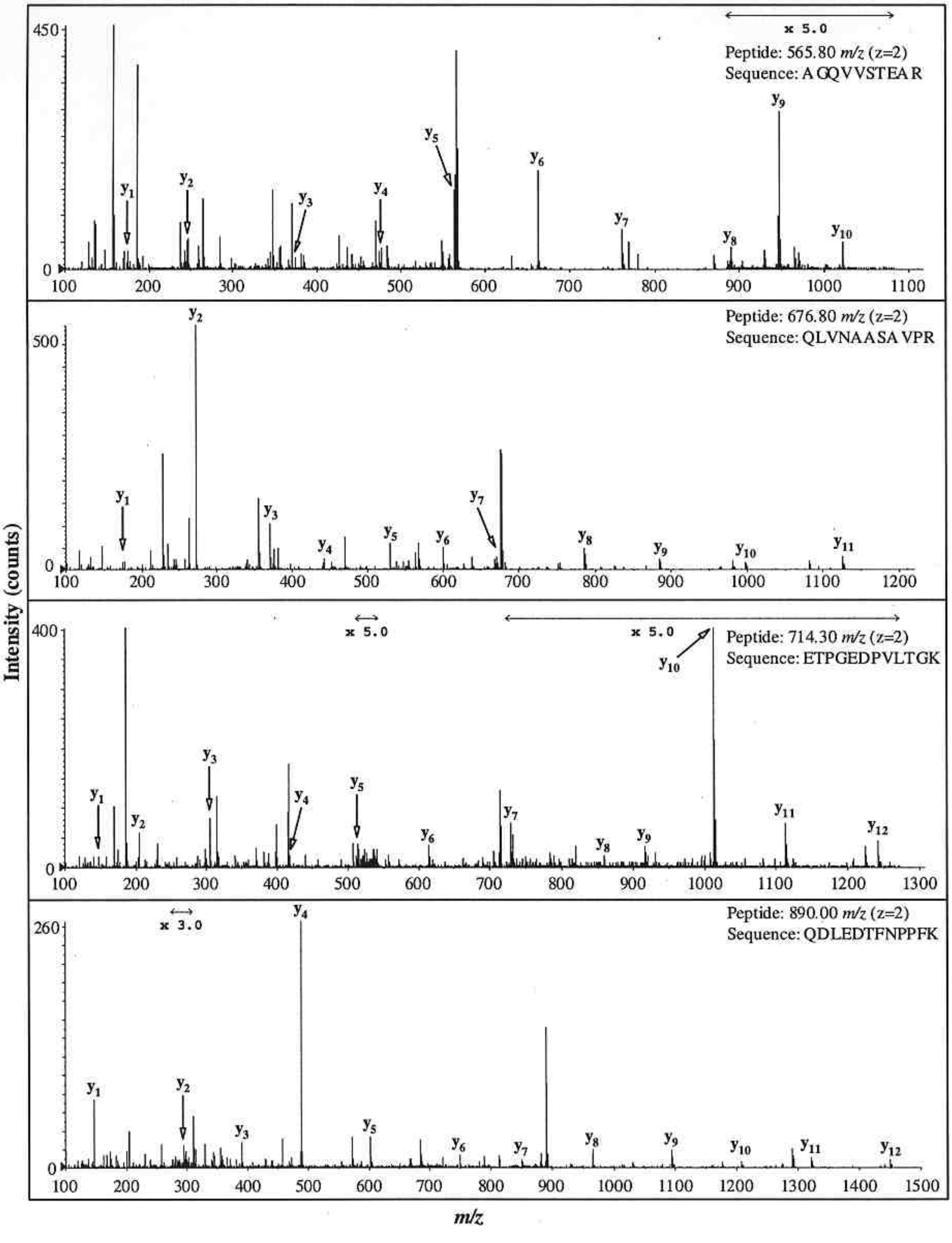


Figure 2.3: Protein B tandem MS fragmentation data.
Tryptic peptides from protein B (Figure 2.1, *J. communis*) were introduced into the Q-STAR*i* quadropole time-of-flight mass spectrometer (Applied Biosystems) by nanospray electrospray ionization (ESI). Data were managed with Peaks (BSI) and Bioanalyst Software (PE-SCIEX)

	Peptide *m/z* (z = 2)			
y ions	**565.80**	**676.80**	**714.30**	**890.00**
1	**R** 175.12	**R** 175.12	**K** 147.11	**K** 147.11
2	**A** 246.16	**P** 272.17	**G** 204.13	**F** 294.18
3	**E** 375.20	**V** 371.24	**T** 305.18	**P** 391.23
4	**T** 476.25	**A** 442.28	**L** 418.27	**P** 488.29
5	**S** 563.28	**S** 529.31	**V** 517.33	**N** 602.33
6	**V** 662.35	**A** 600.35	**P** 614.39	**F** 749.40
7	**V** 761.42	**A** 671,38	**D** 729.41	**T** 850.45
8	**Q** 889.47	**N** 785.43	**E** 858.46	**D** 965.47
9	**G** 946.50	**V** 884.49	**G** 915.48	**E** 1094.52
10	**A** 1017.53	**L** 997.58	**P** 1012.53	**L** 1207.60
11	-	**Q** 1125.64	**T** 1113.58	**D** 1322.63
12	-	-	**E** 1242.62	**Q** 1450.68

Table 2.2: Protein B peptide amino acid sequences.
The y-ion series obtained from tandem MS fragmentation of trypsin digested protein B (Figure 2.3) and the deduced amino acid sequence of each tryptic peptide. The monoisotopic mass difference between individual y-ions is the monoisotopic residue mass of an amino acid.

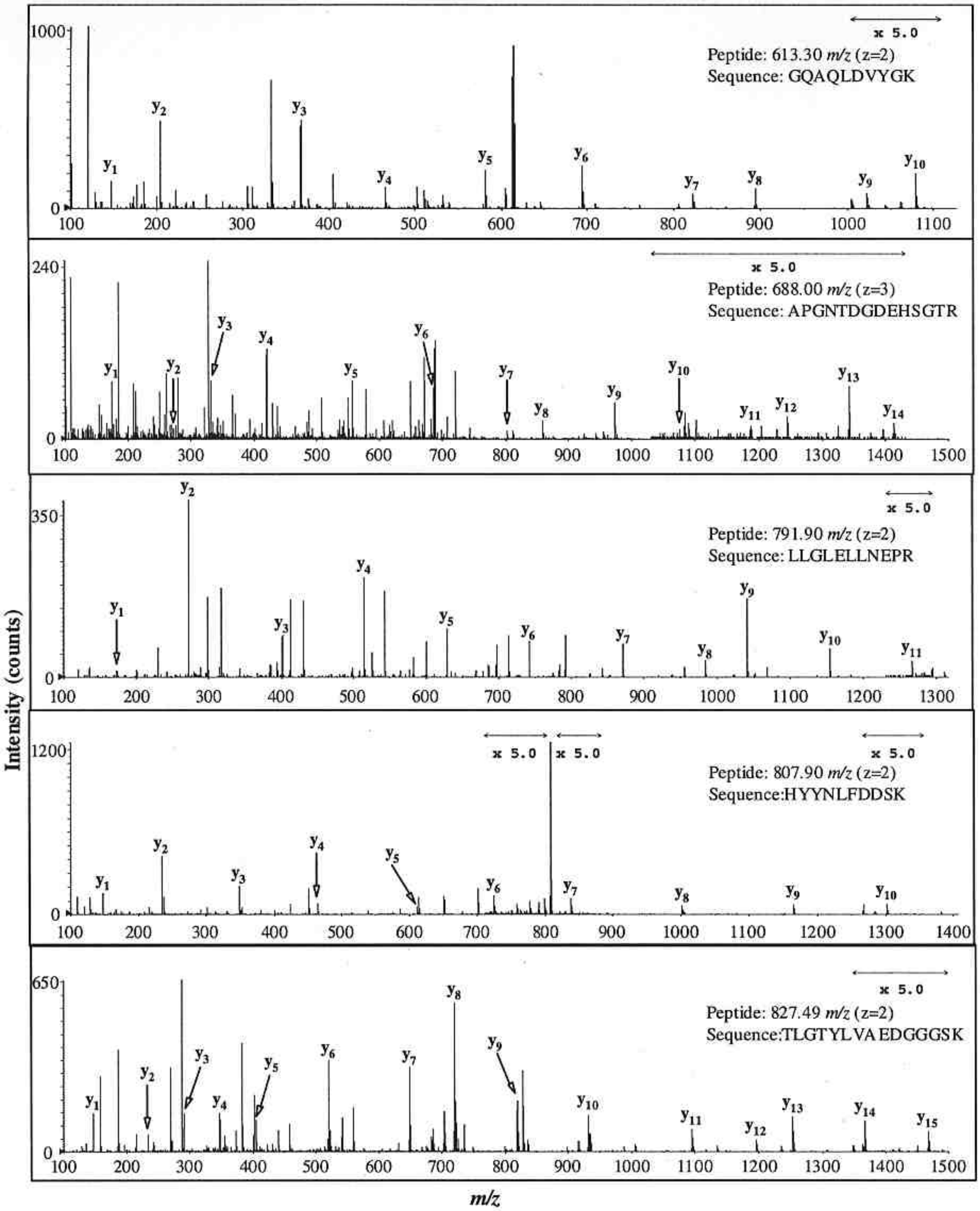


Figure 2.4: Protein C tandem MS fragmentation data.
Tryptic peptides from protein C (Figure 2.1, *J. communis*) were introduced into the Q-STAR*i* quadropole time-of-flight mass spectrometer (Applied Biosystems) by nanospray electrospray ionization (ESI). Data were managed with Peaks (BSI) and Bioanalyst Software (PE-SCIEX).

	Peptide *m/z* (z = 2 or 3)				
y ions	**613.30**	**688.00**	**791.90**	**807.90**	**827.49**
1	**K** 147.11	**R** 175.12	**R** 175.12	**K** 147.11	**K** 147.11
2	**G** 204.13	**T** 276.17	**P** 272.17	**S** 234.14	**S** 234.14
3	**Y** 367.20	**G** 333.19	**E** 401.21	**D** 349.17	**G** 291.17
4	**V** 466.27	**S** 420.22	**N** 515.26	**D** 464.20	**G** 348.19
5	**D** 581.29	**H** 557.28	**L** 628.34	**F** 611.27	**G** 405.21
6	**L** 694.38	**E** 686.32	**L** 741.43	**L** 724.35	**D** 520.24
7	**Q** 822.44	**D** 801.35	**E** 870.47	**N** 838.39	**E** 649.28
8	**A** 893.47	**G** 858.37	**L** 983.55	**Y** 1001.45	**A** 720.32
9	**Q** 1021.53	**D** 973.40	**G** 1040.57	**Y** 1164.52	**V** 819.38
10	**G** 1078.55	**T** 1074.44	**L** 1153.66	**H** 1301.58	**L** 932.47
11	-	**N** 1188.49	**L** 1266.74	-	**Y** 1095.53
12	-	**G** 1245.51	-	-	**T** 1196.58
13	-	**P** 1342.56	-	-	**G** 1253.60
14	-	**A** 1413.60	-	-	**L** 1366.68
15	-	-	-	-	**T** 1467.73

Table 2.3: Protein C peptide amino acid sequences.
The y-ion series obtained from tandem MS fragmentation of trypsin digested protein C (Figure 2.4) and the deduced amino acid sequence of each tryptic peptide. The monoisotopic mass difference between individual y-ions is the monoisotopic residue mass of an amino acid.

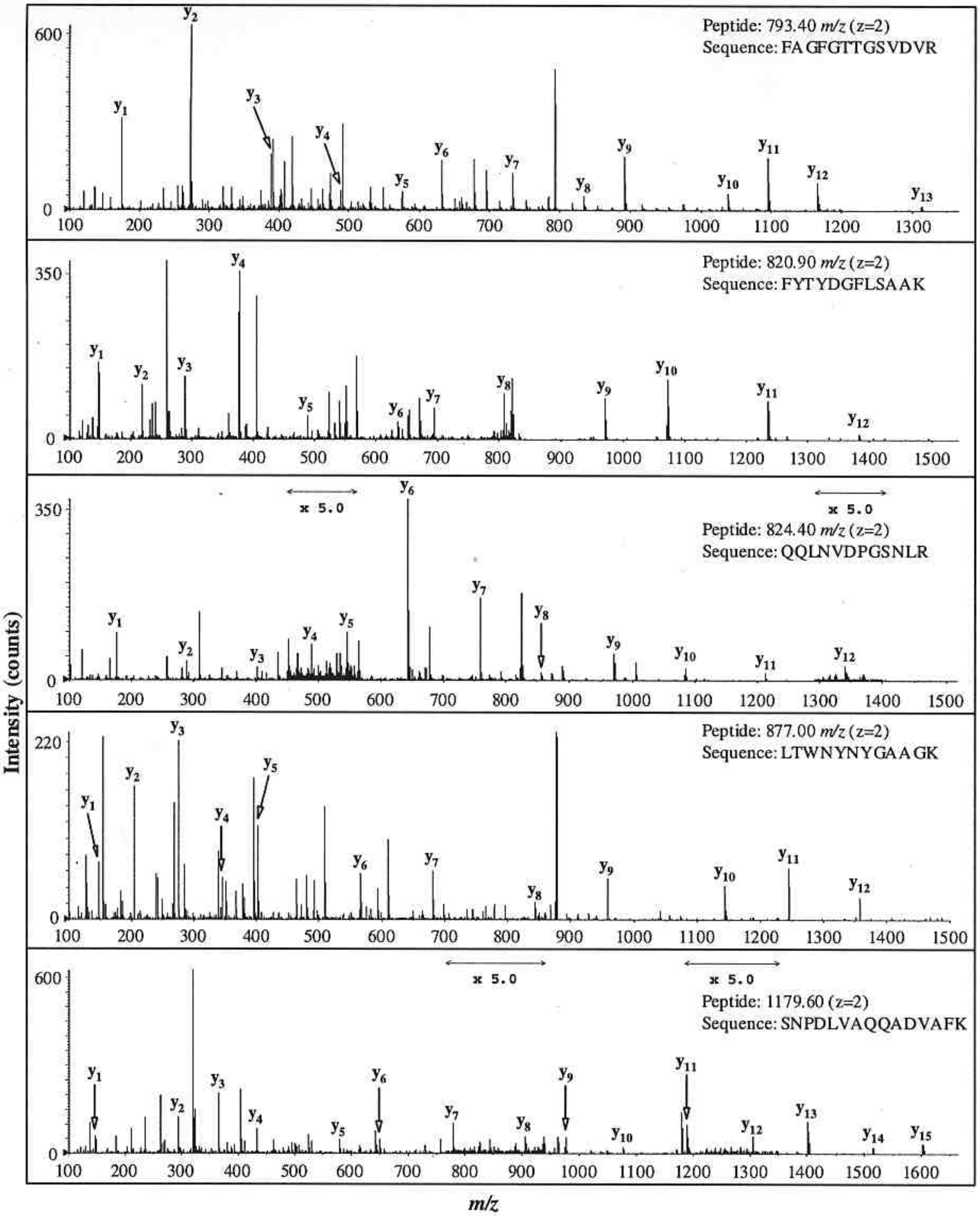


Figure 2.5: Protein D tandem MS fragmentation data.
Tryptic peptides from protein D (Figure 2.1, *J. communis*) were introduced into the Q-STAR*i* quadropole time-of-flight mass spectrometer (Applied Biosystems) by nanospray electrospray ionization (ESI). Data were managed with Peaks (BSI) and Bioanalyst Software (PE-SCIEX).

	Peptide *m/z* (z = 2)				
y ions	793.40	820.90	824.40	877.00	1179.60
	R	K	R	K	K
1	175.12	147.11	175.12	147.11	147.11
	V	A	L	G	F
2	274.19	218.15	288.20	204.13	294.18
	D	A	N	A	A
3	389.21	289.19	402.25	275.17	365.22
	V	S	S	A	V
4	488.28	376.22	489.28	346.21	464.29
	S	L	G	G	D
5	575.31	489.30	546.30	403.23	579.31
	G	F	P	Y	A
6	632.34	636.37	643.35	566.29	650.38
	T	G	D	N	Q
7	733.38	693.39	758.40	680.34	778.41
	T	D	V	Y	Q
8	834.43	808.42	857.45	843.40	906.47
	G	Y	N	N	A
9	891.45	971.48	971.49	957.44	977.51
	F	T	L	W	V
10	1038.52	1072.53	1084.57	1143.52	1076.57
	G	Y	Q	T	L
11	1095.54	1235.59	1212.63	1244.57	1189.66
	A	F	Q	L	D
12	1166.58	1382.66	1340.69	1357.65	1304.68
	F				P
13	1313.65	-	-	-	1401.73
					N
14	-	-	-	-	1515.78
					S
15	-	-	-	-	1602.81

Table 2.4: Protein D peptide amino acid sequences.
The y-ion series obtained from tandem MS fragmentation of trypsin digested protein D (Figure 2.5) and the deduced amino acid sequence of each tryptic peptide. The monoisotopic mass difference between individual y-ions is the monoisotopic residue mass of an amino acid.

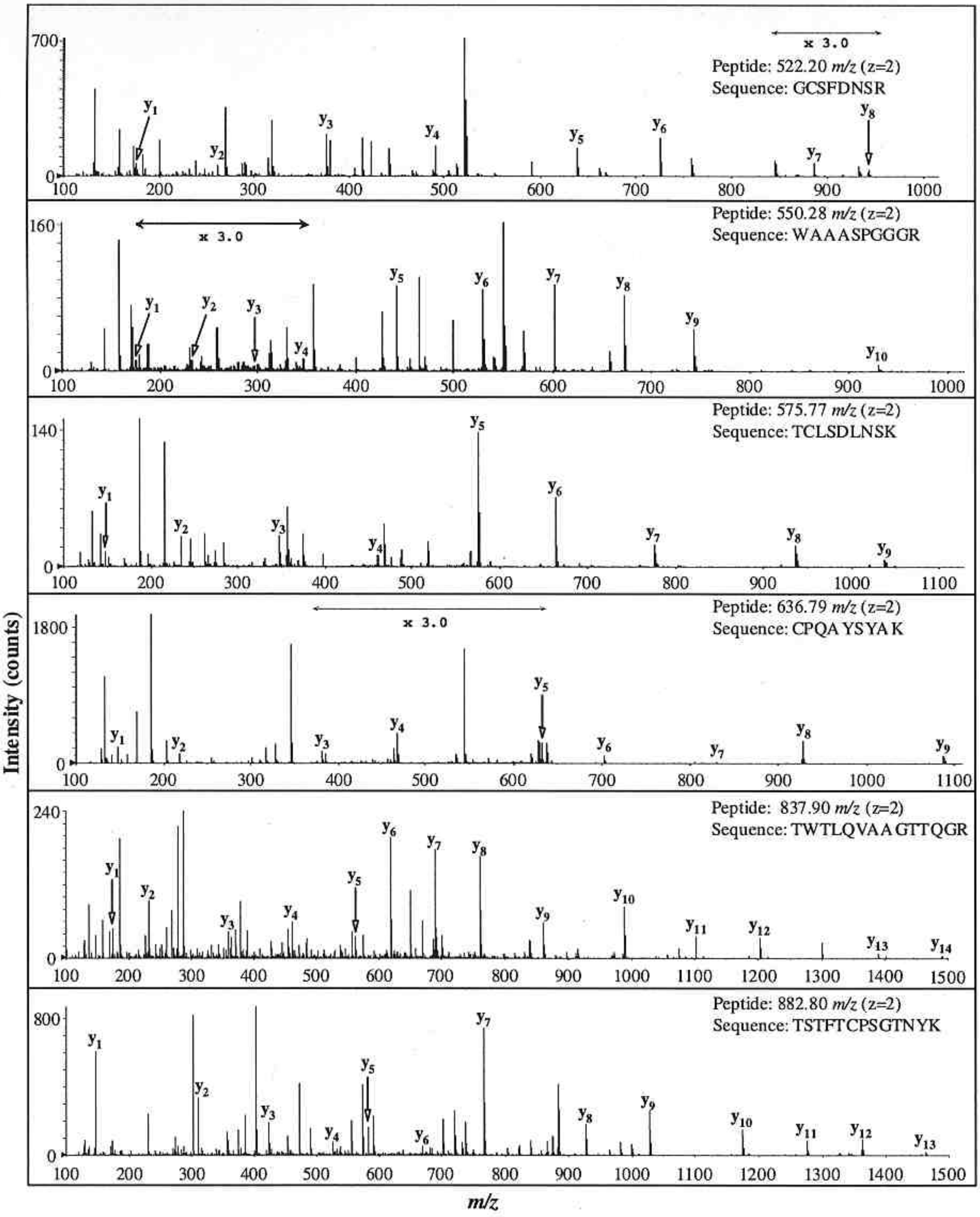


Figure 2.6: Protein E tandem MS fragmentation data.
Tryptic peptides from protein E (Figure 2.1, *J. communis*) were introduced into the Q-STAR*i* quadropole time-of-flight mass spectrometer (Applied Biosystems) by nanospray electrospray ionization (ESI). Data were managed with Peaks (BSI) and Bioanalyst Software (PE-SCIEX).

	Peptide *m/z* (z = 2)					
y ions	522.20	550.28	575.79	636.79	837.90	882.80
	R	R	K	K	R	K
1	175.12	175.12	147.11	147.11	175.12	147.11
	S	G	S	A	G	Y
2	262.15	232.14	234.14	218.15	232.14	370.18
	N	G	N	Y	Q	N
3	376.19	289.16	348.19	381.21	360.20	424.22
	D	G	L	S	T	T
4	491.22	346.18	461.27	468.24	461.25	525.27
	F	P	D	Y	T	G
5	638.29	443.24	576.30	631.31	562.29	582.29
	S	S	S	A	G	S
6	725.32	530.27	663.33	702.35	619.32	669.32
	C	A	L	Q	A	P
7	885.35	601.31	776.41	830.40	690.35	766.37
	G	A	C	P	A	C
8	942.37	672.34	936.45	927.46	761.39	926.40
		A	T	C	V	T
9	-	743.38	1037.49	1087.49	860.46	1027.45
		W			Q	F
10	-	929.46	-	-	988.52	1174.52
					L	T
11	-	-	-	-	1101.60	1275.57
					T	S
12	-	-	-	-	1202.65	1362.60
					W	T
13	-	-	-	-	1388.73	1463.65
					T	
14	-	-	-	-	1489.78	-

Table 2.5: Protein E peptide amino acid sequences.
The y-ion series obtained from tandem MS fragmentation of trypsin digested protein E (Figure 2.6) and the deduced amino acid sequence of each tryptic peptide. The monoisotopic mass difference between individual y-ions is the monoisotopic residue mass of an amino acid.

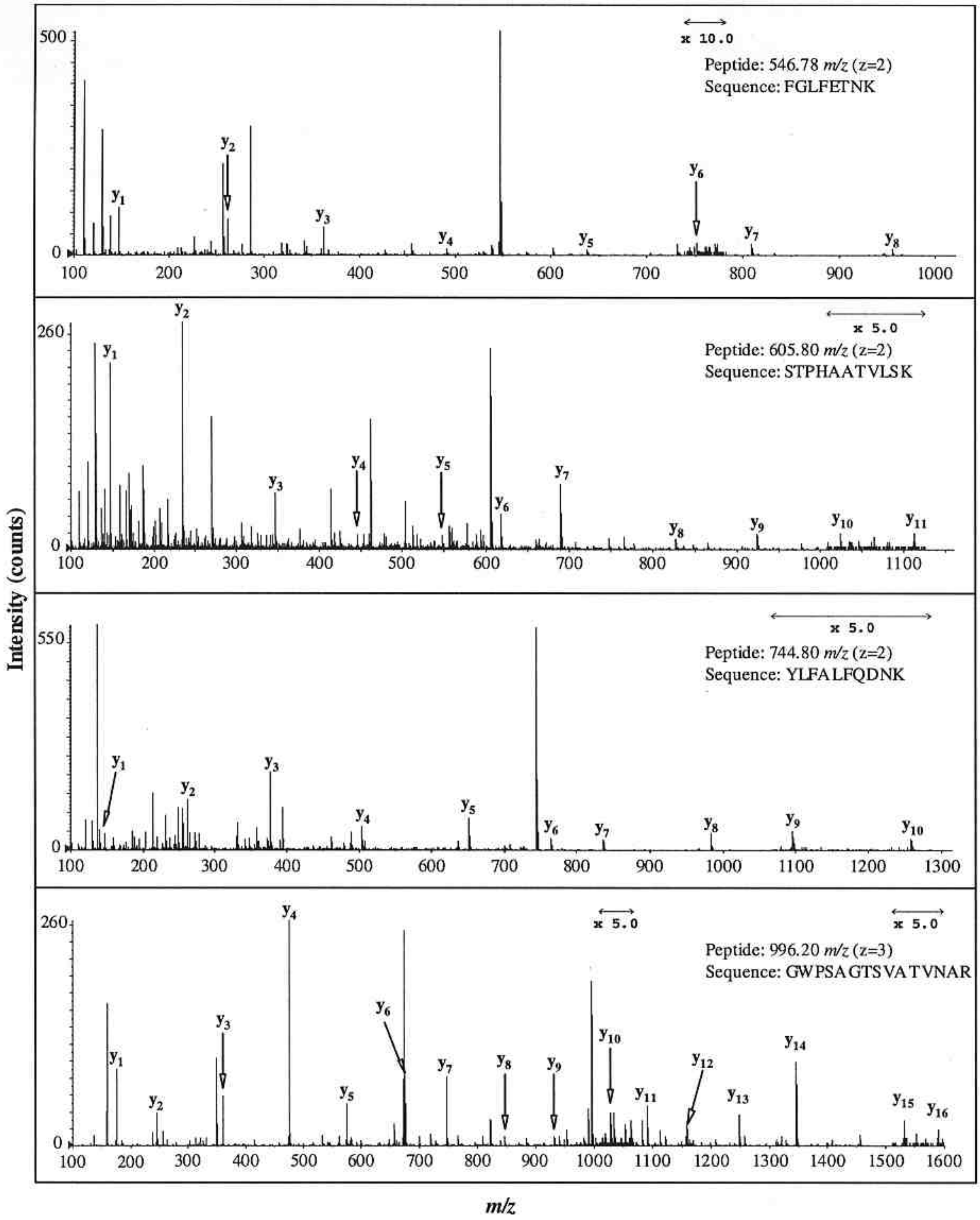


Figure 2.7: Protein F tandem MS fragmentation data.
Tryptic peptides from protein F (Figure 2.1, *J. oxycedrus*) were introduced into the Q-STAR*i* quadropole time-of-flight mass spectrometer (Applied Biosystems) by nanospray electrospray ionization (ESI). Data were managed with Peaks (BSI) and Bioanalyst Software (PE-SCIEX).

	Peptide m/z (z = 2 or 3)			
y ions	**546.78**	**605.80**	**744.80**	**996.20**
1	**K** 147.11	**K** 147.11	**K** 147.11	**R** 175.12
2	**N** 261.16	**S** 234.14	**N** 261.16	**A** 246.16
3	**T** 362.20	**L** 347.23	**D** 376.18	**N** 360.20
4	**E** 491.25	**V** 446.30	**Q** 504.24	**D** 475.23
5	**F** 638.31	**T** 547.35	**F** 651.31	**V** 574.29
6	**L** 751.40	**A** 618.38	**L** 764.39	**T** 675.34
7	**G** 808.42	**A** 689.42	**A** 835.43	**A** 746.38
8	**F** 955.49	**H** 826.48	**F** 982.50	**V** 845.45
9	-	**P** 923.53	**L** 1095.58	**S** 932.48
10	-	**T** 1024.58	**Y** 1258.65	**T** 1033.23
11	-	**S** 1111.61	-	**G** 1090.55
12	-	-	-	**A** 1161.59
13	-	-	-	**S** 1248.62
14	-	-	-	**P** 1345.67
15	-	-	-	**W** 1531.75
16	-	-	-	**G** 1588.77

Table 2.6: Protein F peptide amino acid sequences.
The y-ion series obtained from tandem MS fragmentation of trypsin digested protein F (Figure 2.7) and the deduced amino acid sequence of each tryptic peptide. The monoisotopic mass difference between individual y-ions is the monoisotopic residue mass of an amino acid.

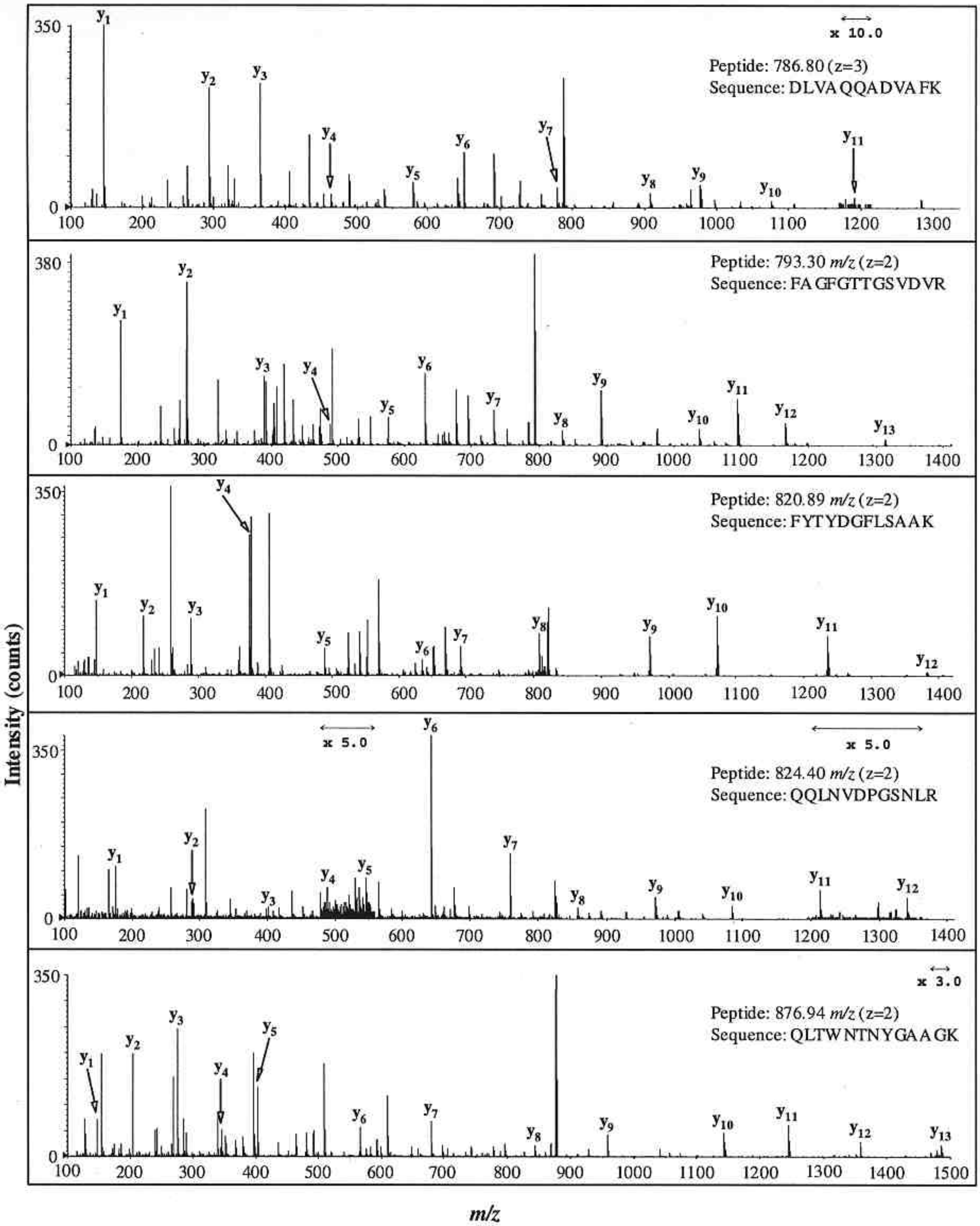


Figure 2.8: Protein G tandem MS fragmentation data.
Tryptic peptides from protein G (Figure 2.1, *J. oxycedrus*) were introduced into the Q-STAR*i* quadropole time-of-flight mass spectrometer (Applied Biosystems) by nanospray electrospray ionization (ESI). Data were managed with Peaks (BSI) and Bioanalyst Software (PE-SCIEX).

	Peptide *m/z* (z = 2 or 3)				
y ions	**786.80**	**793.30**	**820.89**	**824.40**	**876.94**
	K	**R**	**K**	**R**	**K**
1	147.11	175.12	147.11	175.12	147.11
	F	**V**	**A**	**L**	**G**
2	294.18	274.19	218.15	288.20	204.13
	A	**D**	**A**	**N**	**A**
3	365.22	389.21	289.19	402.25	275.17
	V	**V**	**S**	**S**	**A**
4	464.29	488.28	376.22	489.28	346.21
	D	**S**	**L**	**G**	**G**
5	579.31	575.31	489.30	546.30	403.23
	A	**G**	**F**	**P**	**Y**
6	650.35	632.34	636.37	643.35	566.29
	Q	**T**	**G**	**D**	**N**
7	778.41	733.38	693.39	758.38	680.34
	Q	**T**	**D**	**V**	**Y**
8	906.47	834.43	808.42	857.45	843.40
	A	**G**	**Y**	**N**	**N**
9	977.51	891.45	971.48	971.49	957.44
	V	**F**	**T**	**L**	**W**
10	1076.57	1038.52	1072.53	1084.57	1143.52
	L	**G**	**Y**	**Q**	**T**
11	1189.66	1095.54	1235.59	1212.63	1244.57
	D	**A**	**F**	**Q**	**L**
12	-	1166.58	1382.66	1340.69	1357.65
		F			**Q**
13	-	1313.65	-	-	1485.71

Table 2.7: Protein G peptide amino acid sequences.
The y-ion series obtained from tandem MS fragmentation of trypsin digested protein G (Figure 2.8) and the deduced amino acid sequence of each tryptic peptide. The monoisotopic mass difference between individual y-ions is the monoisotopic residue mass of an amino acid.

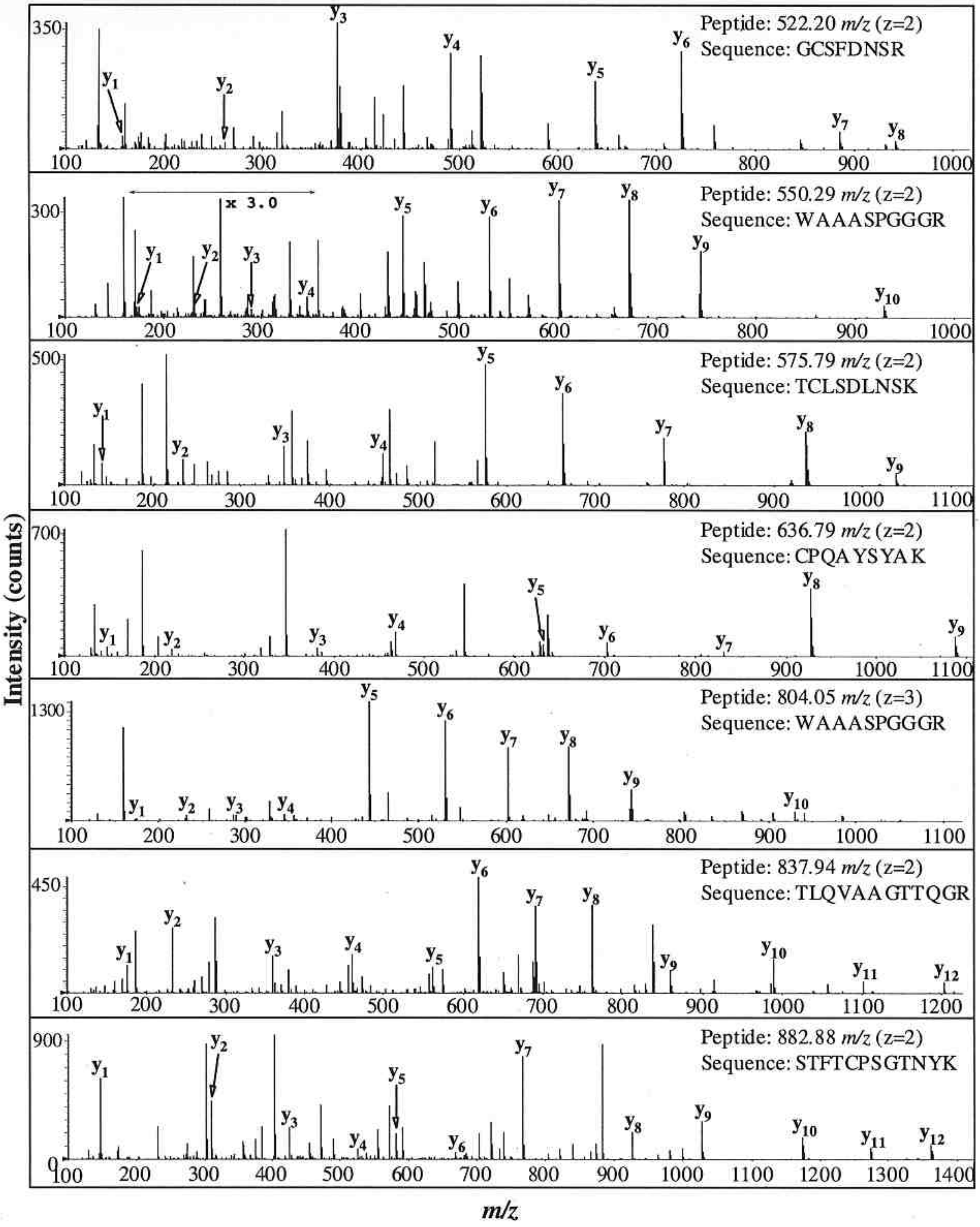


Figure 2.9: Protein H tandem MS fragmentation data.
Tryptic peptides from protein H (Figure 2.1, *J. oxycedrus*) were introduced into the Q-STAR*i* quadropole time-of-flight mass spectrometer (Applied Biosystems) by nanospray electrospray ionization (ESI). Data were managed with Peaks (BSI) and Bioanalyst Software (PE-SCIEX).

	Peptide *m/z* (z = 2,3)						
y ions	**522.20**	**550.29**	**575.79**	**636.79**	**804.05**	**837.94**	**882.88**
1	**R** 175.12	**R** 175.12	**K** 147.11	**K** 147.11	**R** 175.12	**R** 147.11	**K** 147.11
2	**S** 262.15	**G** 232.14	**S** 234.14	**A** 218.15	**G** 232.14	**G** 232.14	**Y** 310.18
3	**N** 376.19	**G** 289.16	**N** 348.19	**Y** 381.21	**G** 289.16	**Q** 360.20	**N** 424.22
4	**D** 491.22	**G** 346.18	**L** 461.27	**S** 468.25	**G** 346.18	**T** 461.25	**T** 525.27
5	**F** 638.29	**P** 443.24	**D** 576.30	**Y** 631.31	**P** 443.24	**T** 562.29	**G** 582.29
6	**S** 725.32	**S** 530.27	**S** 663.33	**A** 702.35	**S** 530.27	**G** 619.32	**S** 669.32
7	**C** 885.35	**A** 601.31	**L** 776.41	**Q** 830.40	**A** 601.31	**A** 690.35	**P** 766.37
8	**G** 942.37	**A** 672.34	**C** 936.45	**P** 927.46	**A** 672.34	**A** 761.39	**C** 926.40
9	-	**A** 743.38	**T** 1037.49	**C** 1087.49	**A** 743.38	**V** 860.46	**T** 1027.45
10	-	**W** 929.46	-	-	**W** 929.46	**Q** 988.52	**F** 1174.52
11	-	-	-	-	-	**L** 1101.60	**T** 1275.57
12	-	-	-	-	-	**T** 1202.65	**S** 1362.60

Table 2.8: Protein H peptide amino acid sequences.
The y-ion series obtained from tandem MS fragmentation of trypsin digested protein H (Figure 2.9) and the deduced amino acid sequence of each tryptic peptide. The monoisotopic mass difference between individual y-ions is the monoisotopic residue mass of an amino acid.

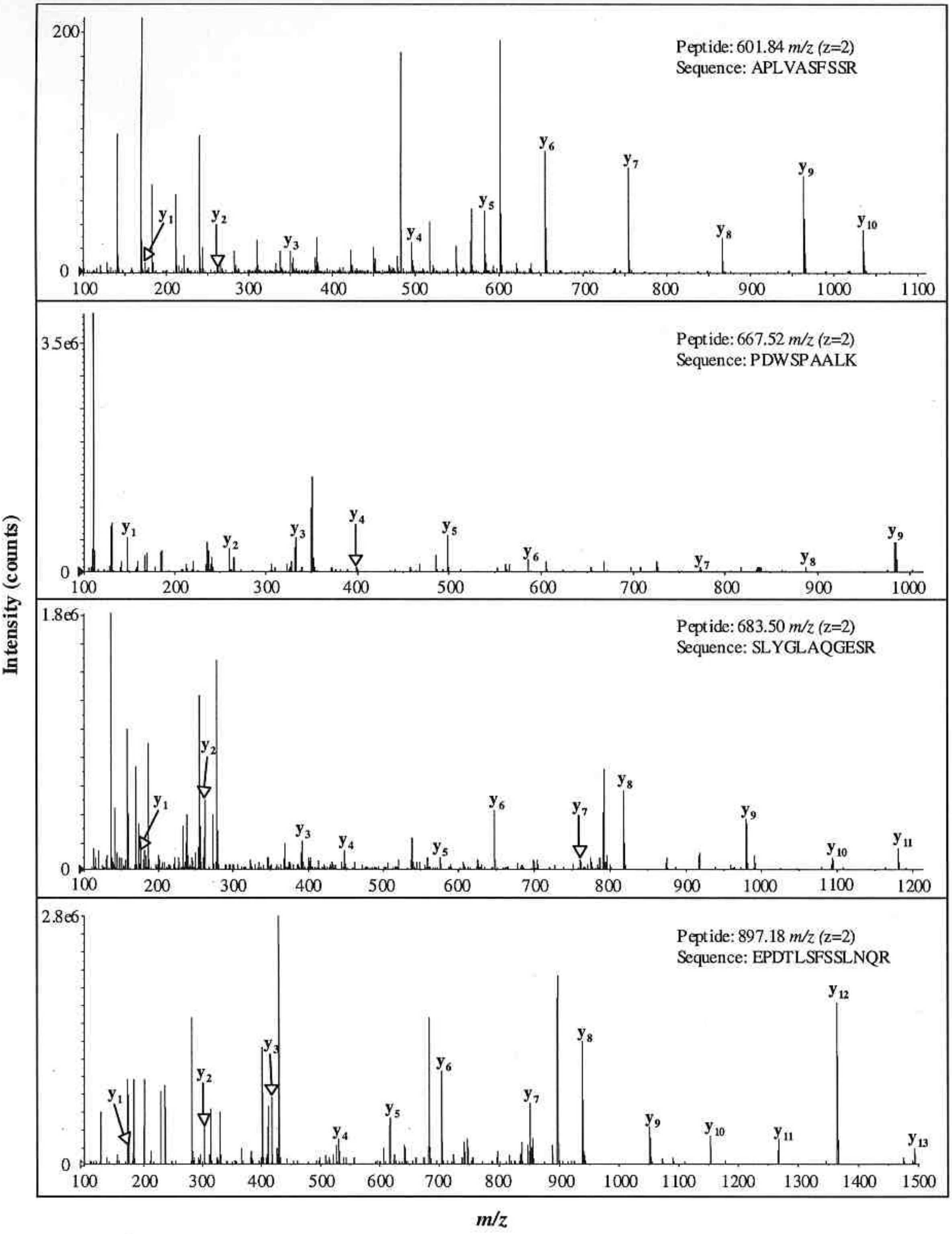


Figure 2.10: Protein I tandem MS fragmentation data. Tryptic peptides from protein I (Figure 2.1, *C. lawsoniana*) were introduced into either the Q-TRAP quadrupole ion-trap mass spectrometer or the Q-STAR*i* quadropole time-of-flight mass spectrometer (Applied Biosystems) by nanospray electrospray ionization (ESI). Data were managed with Peaks (BSI) and Bioanalyst Software (PE-SCIEX).

	Peptide *m/z* (z = 2)			
y ions	**601.84**	**667.52**	**683.50**	**897.18**
1	**R** 175.12	**K** 147.11	**R** 175.12	**R** 175.12
2	**S** 262.15	**L** 260.20	**S** 262.15	**Q** 303.18
3	**S** 349.18	**A** 331.23	**E** 391.19	**N** 417.22
4	**F** 496.25	**A** 402.27	**G** 448.22	**L** 530.30
5	**S** 583.28	**P** 499.32	**Q** 576.27	**S** 617.34
6	**A** 654.32	**S** 586.36	**A** 647.31	**S** 704.37
7	**V** 753.39	**W** 772.44	**L** 760.39	**F** 851.44
8	**L** 866.47	**D** 887.46	**G** 817.42	**S** 938.47
9	**P** 963.52	**P** 984.51	**Y** 980.48	**L** 1051.55
10	**A** 1034.56	-	**L** 1093.56	**T** 1152.60
11	-	-	**S** 1180.60	**D** 1267.63
12	-	-	-	**P** 1364.68
13	-	-	-	**E** 1493.72

Table 2.9: Protein I peptide amino acid sequences.
The y-ion series obtained from tandem MS fragmentation of trypsin digested protein I (Figure 2.10) and the deduced amino acid sequence of each tryptic peptide. The monoisotopic mass difference between individual y-ions is the monoisotopic residue mass of an amino acid.

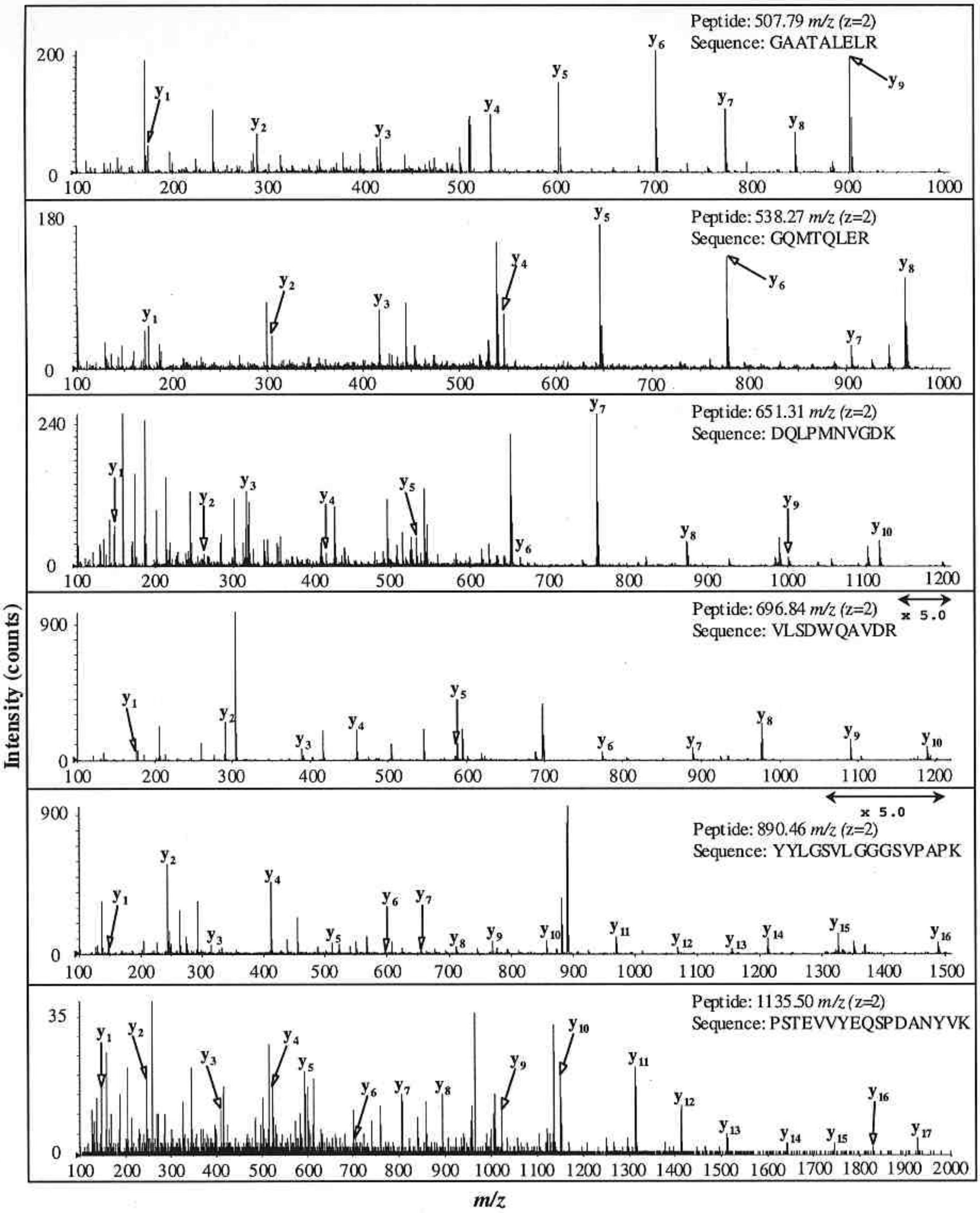
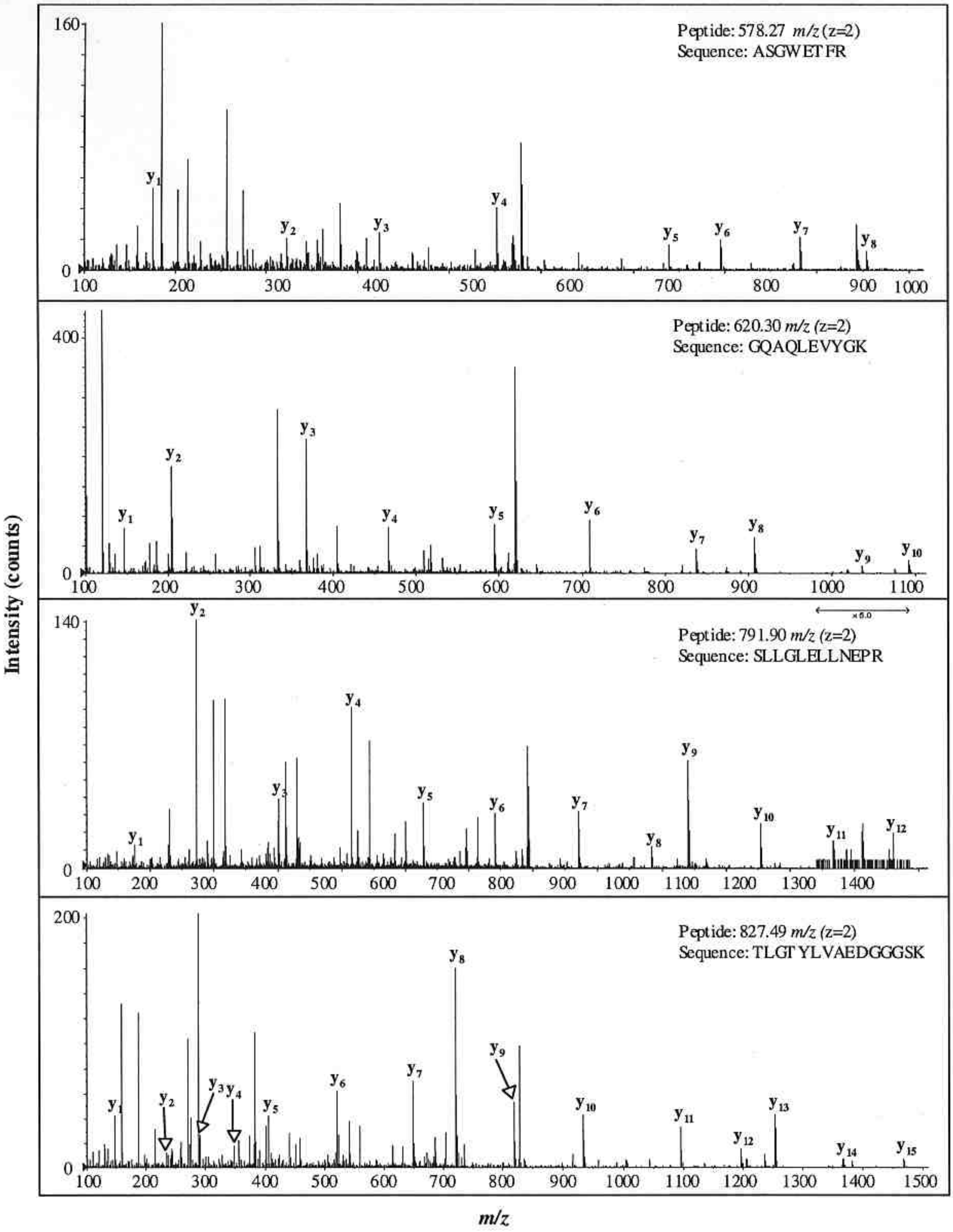


Figure 2.11: Protein J tandem MS fragmentation data.
Tryptic peptides from protein J (Figure 2.1 *C. lawsoniana*) were introduced into the Q-STAR*i* quadropole time-of-flight mass spectrometer (Applied Biosystems) by nanospray electrospray ionization (ESI). Data were managed with Peaks (BSI) and Bioanalyst Software (PE-SCIEX).

	Peptide *m/z* (z = 2)					
y ions	507.79	538.27	651.31	696.84	890.46	1135.50
1	R 175.12	R 175.12	K 147.11	R 175.12	K 147.11	K 147.11
2	L 288.20	E 304.16	D 262.14	D 290.15	P 244.17	V 246.18
3	E 417.25	L 417.25	G 319.16	V 389.21	A 315.20	Y 409.24
4	L 530.33	Q 545.30	V 418.23	A 460.25	P 412.26	N 523.29
5	A 601.37	T 646.35	N 532.27	Q 588.31	V 511.32	A 594.32
6	T 702.41	M 777.39	M 663.31	W 774.39	S 598.36	D 709.35
7	A 773.45	Q 905.45	P 760.37	D 889.42	G 655.38	P 806.40
8	A 844.49	G 962.47	L 873.45	S 976.45	G 712.40	S 893.44
9	G 901.51	-	Q 1001.51	L 1089.53	G 769.42	Q 1021.49
10	-	-	D 1116.54	V 1188.60	S 856.45	E 1150.54
11	-	-	-	-	L 969.54	Y 1313.60
12	-	-	-	-	V 1068.60	V 1412.67
13	-	-	-	-	S 1155.64	V 1511.74
14	-	-	-	-	G 1212.66	E 1640.78
15	-	-	-	-	L 1325.74	T 1741.83
16	-	-	-	-	Y 1488.81	S 1828.86
17	-	-	-	-	-	P 1925.91

Table 2.10: Protein J peptide amino acid sequences.
The y-ion series obtained from tandem MS fragmentation of trypsin digested protein J (Figure 2.11) and the deduced amino acid sequence of each tryptic peptide. The monoisotopic mass difference between individual y-ions is the monoisotopic residue mass of an amino acid



.Figure 2.12: Protein K tandem MS fragmentation data.
Tryptic peptides from protein K (Figure 2.1, *C. lawsoniana*) were introduced into the Q-STAR*i* quadropole time-of-flight mass spectrometer (Applied Biosystems) by nanospray electrospray ionization (ESI). Data were managed with Peaks (BSI) and Bioanalyst Software (PE-SCIEX).

	Peptide *m/z* (z = 2)			
y ions	578.27	620.30	791.90	827.49
	R	K	R	K
1	175.12	147.11	175.12	147.11
	F	G	P	S
2	322.19	204.13	272.17	234.14
	T	Y	E	G
3	423.24	367.20	401.21	291.17
	E	V	N	G
4	552.28	466.27	515.26	348.19
	W	E	L	G
5	738.36	595.31	628.34	405.21
	G	L	L	D
6	795.38	708.39	741.43	520.24
	S	Q	E	E
7	882.41	836.45	870.47	649.28
	A	A	L	A
8	953.45	907.49	983.55	720.32
		Q	G	V
9	-	1035.55	1040.57	819.38
		G	L	L
10	-	1092.57	1153.66	932.47
			L	Y
11	-	-	1266.74	1095.53
			S	T
12	-	-	1353.77	1196.58
				G
13	-	-	-	1253.60
				L
14	-	-	-	1366.68
				T
15	-	-	-	1467.73

Table 2.11: Protein K peptide amino acid sequences.
The y-ion series obtained from tandem MS fragmentation of trypsin digested protein J (Figure 2.12) and the deduced amino acid sequence of each tryptic peptide. The monoisotopic mass difference between individual y-ions is the monoisotopic residue mass of an amino acid

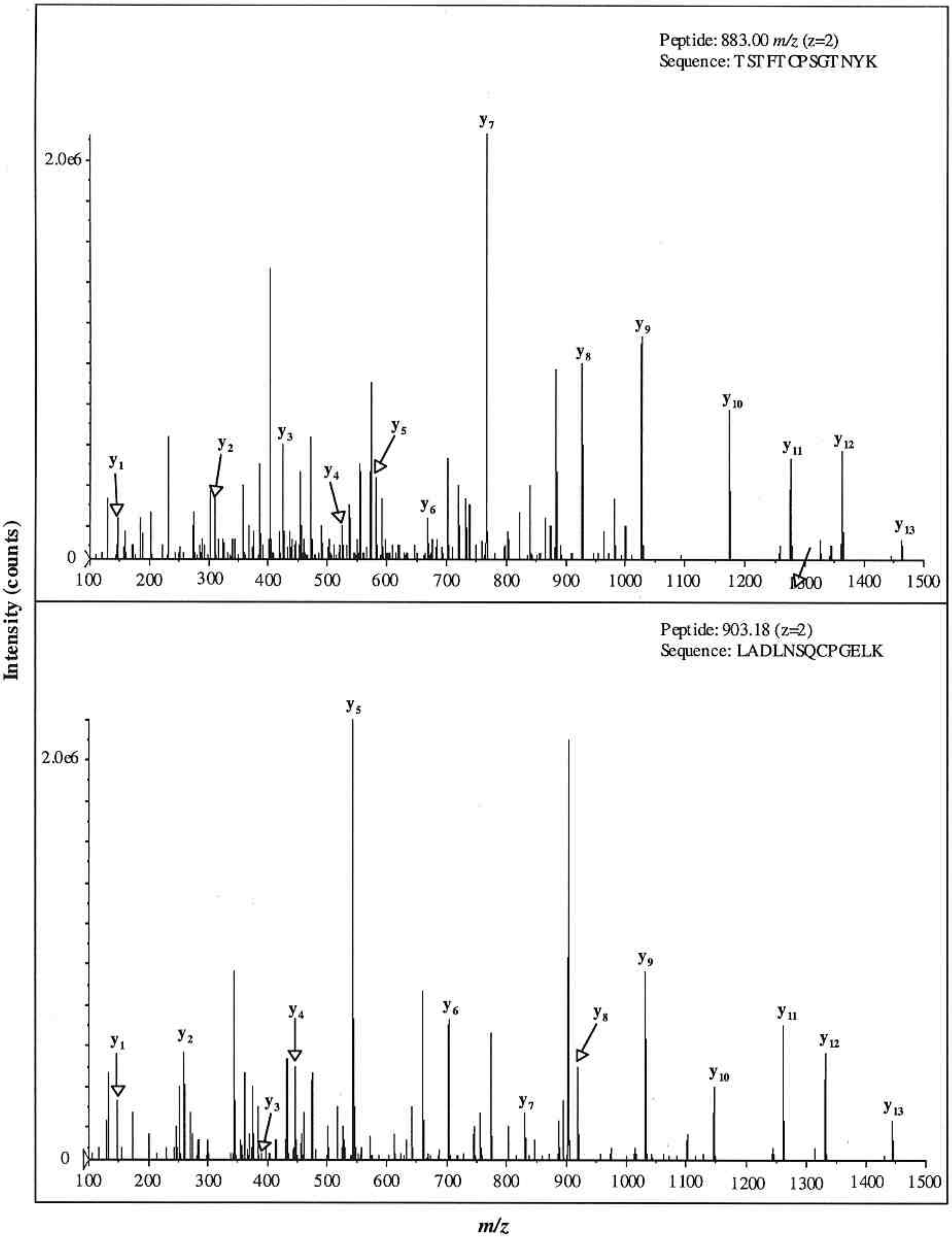


Figure 2.13: Protein L tandem MS fragmentation data.
Tryptic peptides from protein L (Figure 2.1, *C. lawsoniana*) were introduced into the Q-TRAP quadrupole ion-trap mass spectrometer (Applied Biosystems) by nanospray electrospray ionization (ESI). Data were managed with Peaks (BSI) and Bioanalyst Software (PE-SCIEX).

	Peptide *m/z* (z = 2)	
y ions	883.00	903.18
	K	K
1	147.11	147.11
	Y	L
2	310.18	260.20
	N	E
3	424.22	389.24
	T	G
4	525.27	446.26
	G	P
5	582.29	543.31
	S	C
6	669.32	703.34
	P	Q
7	766.37	831.40
	C	S
8	926.40	918.43
	T	N
9	1027.45	1032.48
	F	L
10	1174.52	1145.56
	T	D
11	1275.57	1260.59
	S	A
12	1362.60	1331.63
	T	L
13	1463.65	1444.71

Table 2.12: Protein L peptide amino acid sequences.
The y-ion series obtained from tandem MS fragmentation of trypsin digested protein K (Figure 2.13) and the deduced amino acid sequence of each tryptic peptide. The monoisotopic mass difference between individual y-ions is the monoisotopic residue mass of an amino acid

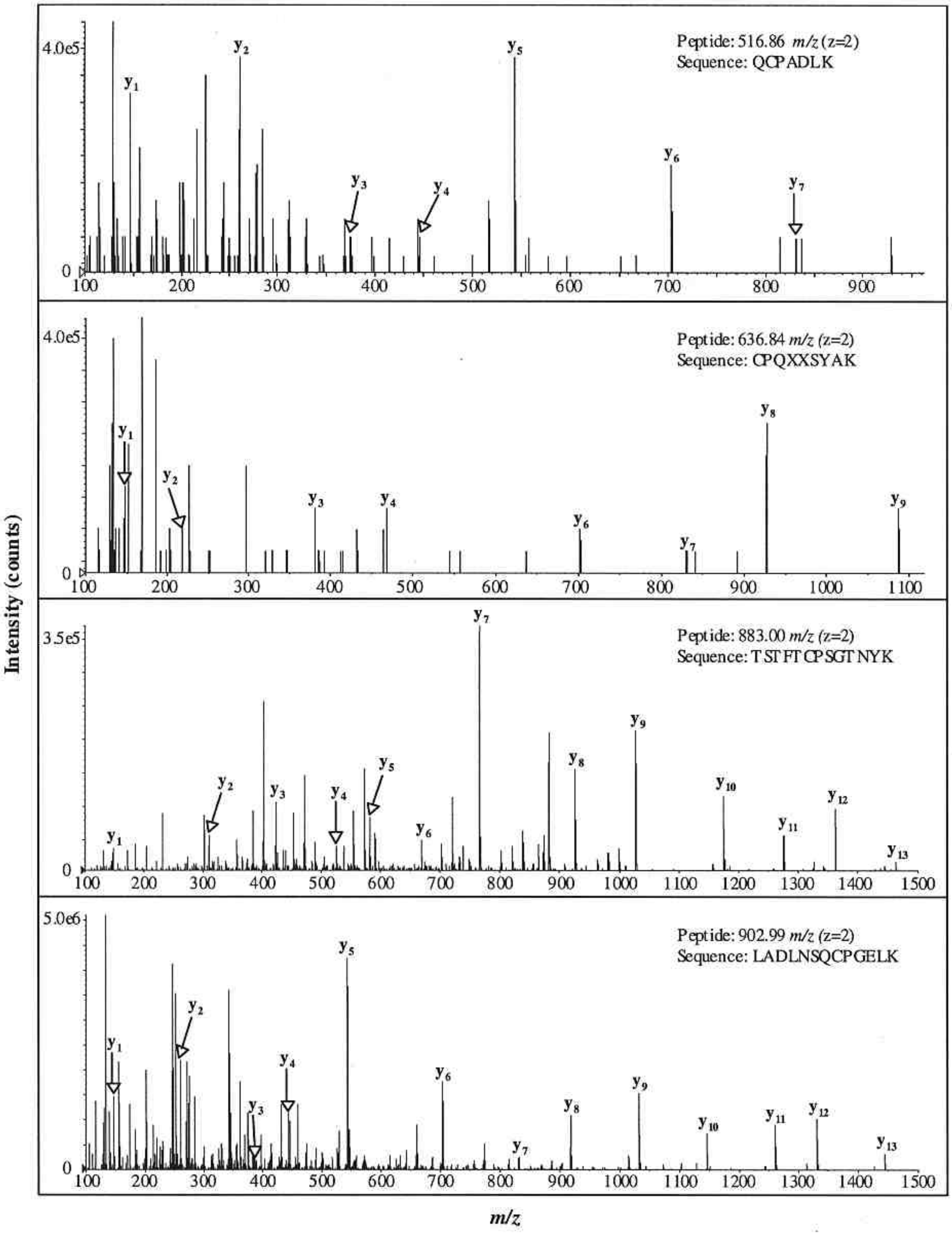


Figure 2.14: Protein M tandem MS fragmentation data.
Tryptic peptides from protein M (Figure 2.1, *C. lawsoniana*) were introduced into the Q-TRAP quadrupole ion-trap mass spectrometer (Applied Biosystems) by nanospray electrospray ionization (ESI). Data were managed with Peaks (BSI) and Bioanalyst Software (PE-SCIEX).

	Peptide m/z (z = 2)			
y ions	516.86	636.84	883.00	902.99
	K	K	K	K
1	147.11	147.11	147.11	147.11
	L	A	Y	L
2	260.20	218.15	310.18	260.20
	D	Y	N	E
3	375.22	381.21	424.22	389.24
	A	S	T	G
4	446.26	468.25	525.27	446.26
	P	X	G	P
5	543.31	-	582.29	543.31
	C	X	S	C
6	703.34	702.35	669.32	703.34
	Q	Q	P	Q
7	831.44	830.40	766.37	831.40
		P	C	S
8	-	927.46	926.40	918.43
		C	T	N
9	-	1087.49	1027.45	1032.48
			F	L
10	-	-	1174.52	1145.56
			T	D
11	-	-	1275.57	1260.59
			S	A
12	-	-	1362.60	1331.63
			T	L
13	-	-	1463.65	1444.71

Table 2.13: Protein M peptide amino acid sequences.
The y-ion series obtained from tandem MS fragmentation of trypsin digested protein M (Figure 2.14) and the deduced amino acid sequence of each tryptic peptide. The monoisotopic mass difference between individual y-ions is the monoisotopic residue mass of an amino acid.

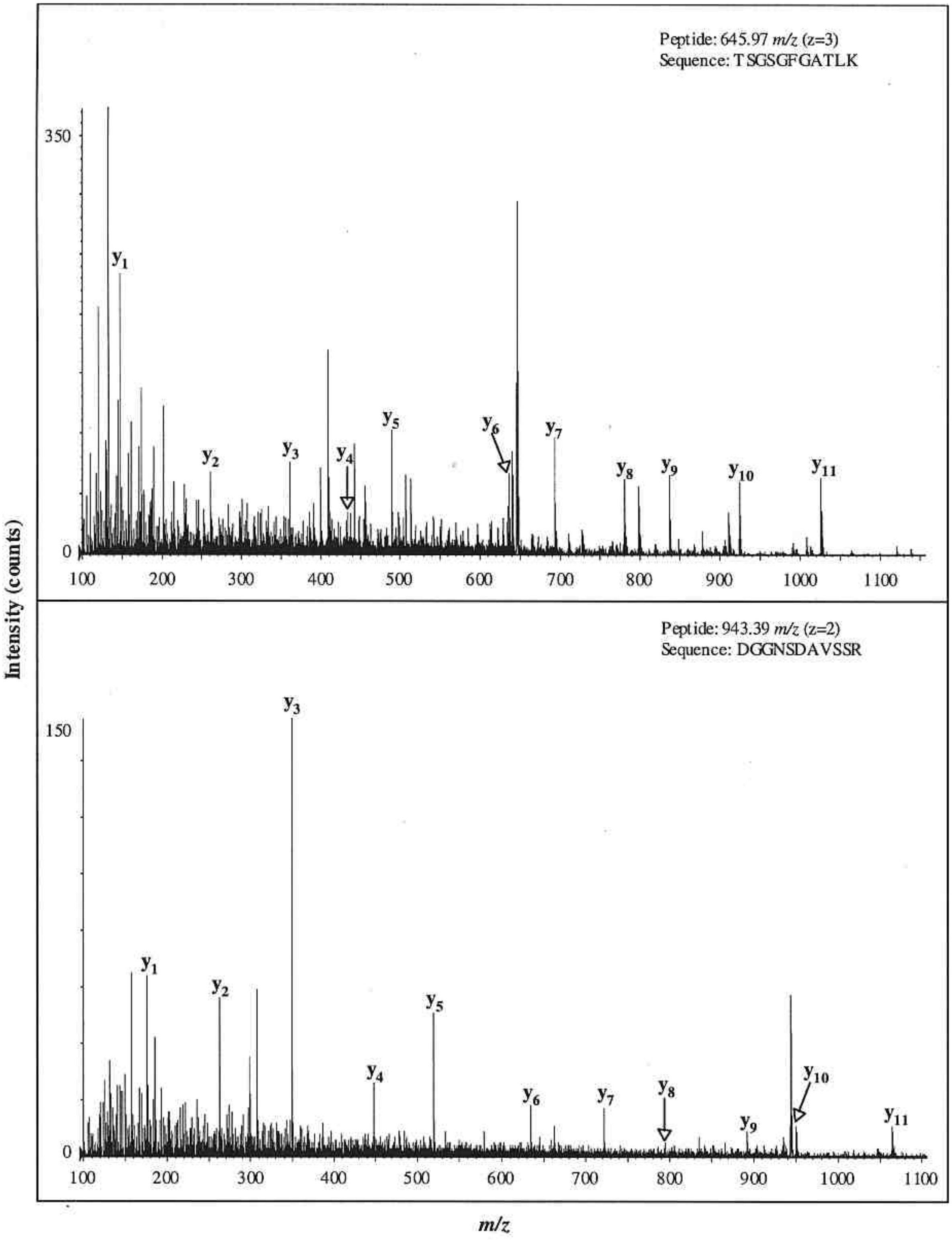


Figure 2.15: Protein N tandem MS fragmentation data.
Tryptic peptides from protein N (Figure 2.1, *W. mirablis*) were introduced into the Q-STAR*i* quadropole time-of-flight mass spectrometer (Applied Biosystems) by nanospray electrospray ionization (ESI). Data were managed with Peaks (BSI) and Bioanalyst Software (PE-SCIEX).

	Peptide *m/z* (z = 2 or 3)	
y ions	**645.97**	**943.39**
	K	**R**
1	147.11	175.12
	L	**S**
2	260.20	262.15
	T	**S**
3	361.24	349.18
	A	**V**
4	432.28	448.25
	G	**A**
5	489.30	519.29
	F	**D**
6	636.37	634.32
	G	**S**
7	693.39	721.35
	S	**N**
8	780.43	835.39
	G	**G**
9	837.45	892.41
	S	**G**
10	924.48	949.43
	T	**D**
11	1025.53	1064.46

Table 2.14: Protein N peptide amino acid sequences.
The y-ion series obtained from tandem MS fragmentation of trypsin digested protein N (Figure 2.15) and the deduced amino acid sequence of each tryptic peptide. The monoisotopic mass difference between individual y-ions is the monoisotopic residue mass of an amino acid.

***J. communis* pollination drop protein (Figure 1)**	**MW (kDa)**	**MS/MS Sequence**	**Protein Identification by MSBlast**
A (Figure 2 & Table 1)	~ 83	PAPLVASFSSR LVSNSAGNSGPSR	*Lycopersicon esculentum* (Tomato) Subtilisin-like proteinase (O65836)
B (Figure 3 & Table 2)	~ 62	AGQVVSTEAR QLVNAASAVPR ETPGEDPVLTGK QDLEDTFNPPFK	*Oryza sativa* (Rice) Glycosyl hydrolase (Q7XPG1)
C (Figure 4 & Table 3)	~ 47.5	GQAQLDVYGK APGNTDGDEHSGTR LLGLELLNEPR HYYNLFDDSK TLGTYLVAEDGGGSK	*Oryza sativa* (Rice) Glucan 1,3-β-glucosidase precursor (Q8RU51)
D (Figure 5 & Table 4)	~ 30	FAGFGTTGSVDVR FYTYDGFLSAAK QQLNVDPGSNLR LTWNYNYGAAGK SNPDLVAQQADVAFK	*Cryptomeria japonica* (Japanese cedar) Chitinase (Q5NTA4)
E (Figure 6 & Table 5)	~ 25	GCSFDNSR WAAASPGGGR TCLSDLNSK CPQAYSYAK TWTLQVAAGTTQGR TSTFTCPSGTNYK	*Cryptomeria japonica* (Japanese cedar) Thaumatin-like protein Q8H995

Table 2.15: Summary of *J. communis* PDPs A, B, C, D, and E.
J. communis PDPs were isolated by 1D SDS PAGE (Figure 2.1) and analyzed by tandem mass spectrometry (Figures 2.2-2.6). Peptide amino acid sequences were determined using Peaks (BSI) and Bioanalyst Software (PE-SCIEX) (Tables 2.1-2.5). Sequence searching was performed using Bork Group's MS Blast search at EMBL on November 1, 2006. L/I determination was based on alignment results. Molecular weights were approximated by their position on the 1D gel.

***J. oxycedrus* pollination drop protein (Figure 1)**	**MW (kDa)**	**MS/MS Sequence**	**Protein Identification by MSBlast**
F (Figure 7 & Table 6)	~ 32.5	FGLFETNK STPHAATVLSK YLFALFQDNK GWPSAGTSVATVDNAF	*Thuja occidentalis* (Eastern white cedar) Glucanase-like protein (Q5RZ68)
G (Figure 8 & Table 7)	~ 30	DLVAQQADVAFK FYTYDGFLSAAK QQLNVDPGSNLR QLTWNYNYGAAGK	*Cryptomeria japonica* (Japanese cedar) Chitinase (Q5NTA4)
H (Figure 9 & Table 8)	~ 25	GCSFDNSR WAAASPGGGR TCLSDLNSK CPQAYSYAK WAAASPGGGR TLQVAAGTTQGR STFTCPSGTNYK	*Cryptomeria japonica* (Japanese cedar) Thaumatin-like protein (Q8H995)

Table 2.16: Summary of *J. oxycedrus* PDPs F, G, and H.
J. oxycedrus PDPs were isolated by 1D SDS PAGE (Figure 2.1) and analyzed by tandem mass spectrometry (Figures 2.7-2.9). Peptide amino acid sequences were determined using Peaks (BSI) and Bioanalyst Software (PE-SCIEX) (Tables 2.6-2.8). Sequence searching was performed using Bork Group's MS Blast search at EMBL on November 1, 2006. L/I determination was based on alignment results. Molecular weights were approximated by their position on the 1D gel.

C. lawsoniana pollination drop protein (Figure 1)	MW (kDa)	MS/MS Sequence	Protein Identification by MSBlast
I (Figure 10 & Table 9)	~ 83	APLVASFSSR PDWSPAALK SLYGLAQGESR EPDTLSFSSLNQR	*Lycopersicon esculentum* (Tomato) Subtilisin-like proteinase Q9LWA4
J (Figure 11 & Table 10)	~ 62	GAATALELR GQMTQLER DQLPMNVGDK VLSDWQAVDR YYLGSVLGGGSVPAPK PSTEVVYEQSPDANYVK	*Nicotiana tabacum* (Tobacco) β-D-glucan exohydrolase (O82151)
K (Figure 12 & Table 11)	~47.5	ASGWETFR GQAQLEVYGK SLLGLELLNEPR TLGTYLVAEDGGGSK	*Oryza sativa* (Rice) Glucan 1,3-β-glucosidase (Q8RU51)
L (Figure 13 & Table 12)	~ 25	TSTFTCPSGTNYK LADLNSQCPGELK	*Cryptomeria japonica* (Japanese cedar) Thaumatin-like protein (Q8H994)
M (Figure 14 & Table 13)	< 25	QCPADLK CPQXXSYAK TSTFTCPSGTNYK LADLNSQCPGELK	*Cryptomeria japonica* (Japanese cedar) Thaumatin-like protein Q8H994

Table 2.17: Summary of C. lawsoniana PDPs I, J, K, L, and M.
C. lawsoniana PDPs were isolated by 1D SDS PAGE (Figure 2.1) and analyzed by tandem mass spectrometry (Figures 2.10-2.14). Peptide amino acid sequences were determined using Peaks (BSI) and Bioanalyst Software (PE-SCIEX) (Tables 2.9-2.13). Sequence searching was performed using Bork Group's MS Blast search at EMBL on November 1, 2006. L/I determination was based on alignment results. Molecular weights were approximated by their position on the 1D gel.

W. mirabilis pollination drop protein (Figure 1)	MW (kDa)	MS/MS Sequence	Protein Identification by MSBlast
N (Figure 15 & Table 14)	~ 25	TSGSGFGATLK DGGNSDAVSSR	*Picea abies* (Norway spruce) Chitinase (Q6WSS0)

Table 2.18: Summary of *W. mirabilis* PDP N.
W. mirablis PDPs were isolated by 1D SDS PAGE (Figure 2.1) and analyzed by tandem mass spectrometry (Figure 2.15). Peptide amino acid sequences were determined using Peaks (BSI) and Bioanalyst Software (PE-SCIEX) (Table 2.14). Sequence searching was performed using Bork Group's MS Blast search at EMBL on November 1, 2006. L/I determination was based on alignment results. Molecular weights were approximated by their position on the 1D gel.

PDP profile comparison

PDP profiles from all study species were compared based on the following criteria: 1) number of SDS PAGE bands that yielded similar data, 2) peptide *m/z* values, 3) peptide amino acid sequences, and 4) protein identifications including accession numbers (Table 2.19).

Comparing the juniper species, there were two pairs of SDS PAGE bands that yielded similar data (e.g. peptides with the same *m/z* value) (Table 2.19). The *J. communis* band D and the *J. oxycedrus* band G shared four peptides with identical *m/z* values (error tolerance ± 0.1 Da). Both peptides in a matched pair had identical amino acid sequences, with the exception of the N-terminal amino acid of the 877.00/876.94 pair. Similarly, six peptides with identical *m/z* values and amino acid sequences were shared between the *J. communis* band E and the *J. oxycedrus* band H. From a BLAST search in the non-redundant NCBI database, bands D and G were matched to the same chitinase protein (based on accession number), and bands E and H to the same thaumatin-like protein.

Between *J. communis* and *C. lawsoniana*, there were more matching bands. However, each match was based on fewer peptides. Two bands from *J. communis* matched two bands from *C. lawsoniana.* The A/I match was based on two peptides with identical *m/z* values and amino acid sequences (with the exception of the N-terminal amino acid). Bands A and I were identified as non-identical subtilisin-like proteinases (based on two different accession numbers). Three pairs of peptides matched between bands C and K: two pairs were identical in terms of *m/z* value and sequence (with the exception of the N-terminal amino acid), and one pair differed by a single amino acid.

	J. communis				*J. oxycedrus*			
	Band	Peptide	Sequence	Identification	Band	Peptide	Sequence	Identification
J. oxycedrus	D/G	793.40/793.30	FAGFGTTGSVDVR FAGFGTTGSVDVR	Chitinase (Q5NTA4)/ Chitinase (Q5NTA4)				
		820.90/820.89	FYTYDGFLSAAK FYTYDGFLSAAK					
		824.40/824.40	QQLNVDPGSNLR QQLNVDPGSNLR					
		877.00/876.94	XLTWNYNYGAAGK QLTWNYNYGAAGK					
	E/H	522.20/522.20	GCSFDNSR GCSFDNSR	Thaumatin-like protein (Q8H995)/ Thaumatin-like protein (Q8H995)				
		550.28/550.29	WAAASPGGGR WAAASPGGGR					
		575.77/575.79	TCLSDLNSK TCLSDLNSK					
		636.79/636.79	CPQAYSYAK CPQAYSYAK					
		837.90/837.94	TWTLQVAAGTTQGR TWTLQVAAGTTQGR					
		882.80/882.88	TSTFTCPSGTNYK TSTFTCPSGTNYK					
C. lawsoniana	A/I	601.86/601.84	PAPLVASFSSR XAPLVASFSSR	Subtilisin-like proteinase (Q9LWA4) Subtilisin-like proteinase (O65836)	H/L	882.88/883.00	TSTFTCPSGTNYK TSTFTCPSGTNYK	Thaumatin-like protein (Q8H995) Thaumatin-like protein (Q8H995)
	C/K	613.30/620.30	GQAQLDVYGK GQAQLEVYGK	Glucan 1,3-β-glucosidase (Q8RU51) Glucan 1,3-β-glucosidase (Q8RU51)	H/M	636.79/636.84	CPQAYSYAK CPQXXSYAK	Thaumatin-like protein (Q8H995) Thaumatin-like protein (Q8H995)
		791.90/791.90	XLLGLELLNEPR SLLGLELLNEPR			882.88/883.00	TSTFTCPSGTNYK TSTFTCPSGTNYK	
		827.49/827.49	TLGTYLVAEDGGGSK TLGTYLVAEDGGGSK					
	E/L	882.80/883.00	TSTFTCPSGTNYK TSTFTCPSGTNYK	Thaumatin-like protein (Q8H995)/ Thaumatin-like protein (Q8H995)				
	E/M	636.79/636.84	CPQAYSYAK CPQXXSYAK	Thaumatin-like protein (Q8H995)/ Thaumatin-like protein (Q8H994)				
		882.80/883.00	TSTFTCPSGTNYK TSTFTCPSGTNYK					
W. mirabilis		N/A	N/A	Chitinase (Q5NTA4)/ Chitinase (Q6WSS0)		N/A	N/A	Chitinase (Q5NTA4) Chitinase (Q6WSS0)

Table 2.19: Comparison of *J. communis* (black), *J. oxycedrus* (blue), *C. lawsoniana* (red), and *W. mirabilis* (green) PDP profiles. Comparison was made interms of number of SDS PAGE bands (Figure 2.1) that yielded similar data, peptide m/z values (Figures 2.2-2.15), peptide amino acid sequences (Tables 2.1-2.14), and protein identifications (Tables 2.15-2.18).

Bands C and K were identified as the same glucan 1,3-β-D-glucosidase. Data from *J. communis* band E matched data from both *C. lawsoniana* bands L and M, and all three bands were identified as thaumatin-like proteins. A peptide of *m/z* value 883 (error tolerance ± 0.2 Da) was common to all three bands; it was also the only peptide shared by bands E and L. Bands E and M match shared an additional peptide, with identical *m/z* values and amino acid sequences (excluding two unidentified amino acids in the *C. lawsoniana* sequence). Bands E and L were identified as the same thaumatin-like protein, and bands E and M were identified as non-identical thaumatin-like proteins.

J. oxycedrus and *C. lawsoniana* had the lowest number of matching bands, with each match supported by the least peptides. *J. oxycedrus* band H corresponded to *J. communis* band E; therefore, peptides from *C. lawsoniana* bands L and M also matched peptides from band H. However, bands H, L, and M were identified as the same thaumatin-like protein, unlike bands E, L, and M.

W. mirablis had no comparable bands or peptides to any other study species. However, chitinases were identified in *W. mirablis* and both juniper species. The *W. mirabilis* chitinase was non-identical to the juniper chitinase.

Discussion

PDPs and phylogeny

One of our main hypotheses was to investigate whether variation in gymnosperm PDP profile has a phylogenetic component. Using a proteomics approach to characterize PDP profiles in four gymnosperm species, we were able to make comparisons at the genus and family levels, and within gymnosperms as a whole. It must be noted that we sampled a limited number of gymnosperm taxa, and complete PDP profiles were not obtained for each species. Therefore, comparisons were limited. However, our study represents the first investigation of the possible relationship between PDP profile and gymnosperm phylogeny, and further research could be based on our preliminary finding. It must be also noted that our study was based on gymnosperm taxonomic divisions from current gymnosperm phylogenies. The assumed monophyletic status of the gymnosperms is supported by various studies (Bowe et al. 2000; Burleigh and Mathews 2004; Soltis and Soltis 2004).

In a genus-level comparison of PDP profiles, we observed similarities on three levels. Firstly, analysis of *J. communis* and *J. oxycedrus* pollination drops by SDS-PAGE showed a similar pattern of protein bands. Specifically, there were a similar number of bands in each species, and isobaric bands in both the high and low molecular weight range. Secondly, mass spectrometric analyses yielded identical data for each of two pairs of isobaric bands: nearly all identified peptides in each species had an identical match in the other species (Table 2.20). The third similarity between *J. communis* and *J. oxycedrus* pollination drop was the presence of two proteins identified as chitinase and thaumatin-like protein, both of which had the same accession number in each species.

The same types of similarities were found at the family level. There was an overall similarity in banding pattern for all species in the Cupressaceae, but to a lesser degree than species within *Juniperus*. For example, SDS-PAGE results showed that *C. lawsoniana* pollination drop has isobaric bands to both *Juniperus* species, and additional bands not present in either *Juniperus* species. In mass spectrometric analyses

A. Genus-level comparison

	J. communis					
	# Bands identifed	# Matched bands	# Peptides identifed		# Matched peptides	Sequence / Functional ID / Accession #
J. oxycedrus	5/3	2 →	D/G	5/5	4	+ / + / +§
			E/H	6/7	6	+ / + / +

B. Family-level comparison

	C. lawsoniana					
	# Bands identifed	# Matched bands	# Peptides identifed		# Matched peptides	Sequence / Functional ID / Accession #
J. communis	5/5	4*→	A/I	2/4	1	+ / + / -
			C/K	5/4	3	- / + / +
			E/M	6/2	1	+ / + / +
			E/L	6/4	2	+ / + / -
J. oxycedrus	5/3	2*→	H/L	7/2	1	+ / + / +
			H/M	7/4	2	- / + / +

C. Gymnosperm clade-level comparison

	W. mirabilis				
	# Bands identifed	# Matched bands	# Peptides identifed	# Matched peptides	Sequence / Functional ID / Accession #
J. communis	5/1	0	0	0	- / + / -
J. oxycedrus	3/1	0	0	0	- / + / -

* indicates comparison in which a single band yielded similar data to two other bands

§ **+** indicates that data from both matched bands yielded **identical** sequences, functional ID or accession #'s

- indicates that data from both matched bands yielded **non-identical** sequences etc.

Table 2.20: Comparison of gymnosperm PDP profiles at three levels: genus, family, and gymnosperm clade.

and resulting protein identifications, there were similarities between each *Juniperus* species and *C. lawsoniana* (Table 2.20). In some cases, however, each species yielded similar but non-identical data. For example, a peptide shared by *J. communis* and *C. lawsoniana* differed in sequence by one amino acid. A single isobaric band from both *Juniperus* species yielded similar data to two bands from *C. lawsoniana*: an indication of multiple protein forms in the latter species. Furthermore, there were identifications of non-identical subtilisin-like proteinases from an isobaric band in each species. However, the latter comparison was limited by the small amount of data (two peptides) from *C. lawsoniana*. Beyond the differences between the *Juniperus* and *Chamaecyparis* genera, it was impossible to tease out the evolutionary proximity of each *Juniperus* species to *C. lawsoniana*. As with the genus-level comparison, a more complete PDP profile for each species would have provided more clarity.

Assuming that gymnosperms are monophyletic, our comparison of *W. mirabilis* to all other study species represents a comparison within gymnosperms as a whole. Interestingly, current molecular phylogenies suggest that the Gnetales are close relatives of the conifers (Bowe et al. 2000). Regardless, *W. mirabilis* is not in the Cupressaceae, and we can therefore make comparisons within a higher-level clade than family. There were no obvious similarities between *W. mirabilis* and all other study species in terms of SDS-PAGE patterns and mass spectrometric results. However, proteins identified as chitinases were common to *W. mirabilis* and both *Juniperus* species. More than with any other study species, we were limited in our analysis of *W. mirabilis*. Sample volume was very low; consequently, sufficient data for identification were obtained from only one band.

Given the nature of our results and the current lack of an outgroup species, it is impossible to quantify the evolutionary proximities of all study species. However, our study gives preliminary findings for phylogenetic studies utilizing a proteomics approach. Such a study can be approached in a variety of ways: one common method is to compare 2D SDS-PAGE protein profiles. In the last 20 years in plant biology, experiments have been performed at various taxonomic levels to assess genetic differences using 2D SDS-PAGE protein profiles (Thiellement et al. 2002). Given the similarities and differences we were able to observe using 1D SDS-PAGE, the use of 2D SDS-PAGE could be even more informative.

Beyond spot comparison, mass spectrometry is useful in generating data for comparative studies. For typical bioinformatic analyses of complete proteomes, peptide mass fingerprinting (PMF) is the method of choice (Lester and Hubbard 2002). However, PMF is limited when analyzing divergent species (i.e. when protein similarity is less than 70% identity). We identified *m/z* values for each protein band cored, and these data were of limited use in making comparisons. Again, we would need to perform 2D SDS-PAGE to obtain a complete protein profile for PMF analysis. A drawback to PMF is the necessity for complete databases with sequences for all analyzed proteins—a luxury not afforded by those studying gymnosperms.

Mass spectrometry can also be used to obtain amino acid sequence information, which is very useful in comparative analyses. In a study by Brown et al. (2001), amino acid sequence alignments from 23 orthologous proteins conserved across 45 species were used to construct phylogenetic trees. It was the largest protein data set used for universal tree reconstruction, and results supported the three domain universal tree based on smallsubunit ribosomal RNA (Woese et al. 1990). However, protein sequence alignments are often based on *in silico* translations of DNA sequences, as it is difficult to obtain uninterrupted amino acid sequences using mass spectrometric data. Sequence data were useful in our analyses: we could detect a single amino acid change between a comparable sequence in *C. lawsoniana* and *J. communis*. Unfortunately, we didn't have enough sequence data to perform alignments. Assuming that PDPs are produced by the nucellus, it would be useful to develop gymnosperm EST libraries of nucellar tissue during drop production. With EST libraries, PDPs could be identified using peptide sequences obtained from mass spectrometric data, and orthologous PDPs could be aligned to infer species relationships.

Another method of analysis suggested by Navas and Albar (2004) also concerns orthologous proteins: traditional cladistics combined with proteomics could analyze conserved proteins as characters. In our study, we observed conservative proteins in our study species: similar functional identifications were present in all species. However, we didn't obtain a complete protein profile for each species. Furthermore, the validity of functional identification depends on the completeness of sequence databases, and gymnosperms are not well represented. The development of gymnosperm nucellar EST libraries could also assist in this area of investigation.

In conclusion, analysis of PDP variation may be predictive of phylogenetic relationships among gymnosperms: our results indicated varying degrees of relatedness among the four study species. It is important to use a data set with high resolving power, because obtaining a reliable phylogenetic signal (i.e. that part of the character variation that is potentially informative about the evolutionary history) is key to resolving phylogenetic relationships (Hillis and Huelsenbeck 1992). Specifically, the data shouldn't have too much variation (synapomorphies may be masked), or too much conservation (there may not be enough variation to distinguish groups). In an investigation of the optimal evolutionary rate in DNA sequence for phylogenetic inference, Yang (1998) found that slow sites have little phylogenetic information, intermediate sites have the most, and amount of information in fastest sites may decrease slightly due to heterogeneity in the phylogenetic signal. Given that pollination drops are conservative, they may contain conservative proteins—an idea that is supported by our results. Furthermore, these conservative proteins could provide data containing an accurate phylogenetic signal. Analysis of PDP variation may prove to be a useful tool in the extraordinarily difficult problem of resolving phylogenetic relationships among the gymnosperms.

Functionality of PDPs

Our second objective was to investigate the functionality of PDPs. Recent studies indicate that conifer ovular secretions play a larger role in reproduction than capturing and/or transporting pollen. The identification of various proteins in the ovular secretion of *Pseudotsuga menziesii*, potentially functioning in pollen selection and development, supports this idea (O'Leary et al. 2004; Poulis et al. 2005). The identification of pollination drop proteins in *J. communis*, *J. oxycedrus*, *C. lawsoniana*, and *W. mirabilis* continues this research. The identified proteins and their possible functions will be discussed in two categories: pollen development, and pathogen defense.

Proteins related to pollen development

One of the proteins isolated from *J. communis* pollination drop was a glucan-1,3 β-glucosidase (also referred to as a glucan 1,3-β-D-glucosidase). It is well established that glucan 1,3-β-D-glucosidases affect the plasticity of the pollen tube tip wall, and thereby influence pollen tube elongation (Roggen and Stanley 1969). In the experiments of Roggen and Stanley (1969), the addition of glucan 1,3-β-D-glucosidase to in vitro pear pollen tubes caused an increase in the length of the pollen tubes. Extrapolating these results, we can postulate that the *J. communis* pollination drop glucan 1,3-β-D-glucosidase facilitates pollen tube growth. Furthermore, the glucanase-like protein and β-D-glucan exohydrolase identified from *J. oxycedrus* and *C. lawsoniana* (respectively) may also influence pollen tube growth by similar action to glucan 1,3-β-D-glucosidase.

A glycosyl hydrolase was identified in *J. communis* pollination drop. Given that glycoside hydrolases are classed into 60 families based on amino acid sequences (Henrissat 1991), this identification doesn't indicate any specific function. However, the second highest scoring hit in the MS Blast search at EMBL was a xylosidase (results not shown). Xyloglucan and xylan are the main hemicellulosic polysaccharides in the primary cell walls of plants, and current models assume that hemicelluloses cross-link the cellulose microfibrils of the cell wall and act as the major factor controlling the rate of cell wall expansion (Fry 1989). Xylanases hydrolyze xyloglucans and xylans, and are implicated as one of many enzymes involved in the biosynthesis and degradation of the plant cell wall (Hruba et al. 2005). The isolation of a xylosidase from *Nicotiana tabacum* microspores (Hruba et al. 2005) indicates that xylosidase plays a role in pollen development, and is therefore relevant to our study. Hruba et al. (2005) found four homologous xylosidase genes in *A. thaliana*, with two genes expressed in early pollen, and two genes expressed in mature pollen. The authors postulated that the "early" xylosidase genes are involved in cell expansion during pollen development, and the "late" xylosidase genes are involved in cell expansion during pollen germination. If xylosidases in pollination drop function in the same way as xylosidases within pollen cell walls, they may function similarly to glucanase in aiding in cell wall expansion and pollen tube growth. Therefore, the glycosyl hydrolase in *J. communis* pollination drop may influence pollen tube growth and increase reproductive success.

Beyond successful germination, pollen tubes require nutrients for growth. In an in vitro study of *J. communis* pollen tubes, it was found that growth improved when the same major amino acids as those found in the ovules were added to the medium (Duhoux and Pham Thi 1980). A subtilisin-like proteinase (subtilase) identified in both *J. oxycedrus* and *C. lawsoniana* pollination drop may mobilize amino acids for pollen tube metabolism. Subtilases are a group of serine proteases with amino acid sequence similarity to subtilisin (a serine protease isolated from *Bacillus subtilis*) and a serine residue in their active site (Antão and Malcata 2005). Plant subtilases have been associated with various physiological processes, the most common being general protein turnover (Schaller 2004). In angiosperms, serine carboxypeptidase–like proteins have been implicated in the transference of solutes from nucellar cells to the developing endosperm (Cercós et al. 2003). Therefore, the subtilases in gymnosperm pollination drops may be providing solutes to the developing pollen during tube elongation.

Proteins related to pathogen defense

Plant pathogenesis related (PR) proteins are divided into 17 families based on structural and functional properties. Potential PR proteins identified in *J. communis*, *J. oxycedrus*, *C. lawsoniana*, and *W. mirabilis* pollination drop are glucan-β-1,3-glucosidases (or glucan 1,3-β-D-glucosidases), chitinases, and thaumatin-like proteins (TLPs). The classes of PR proteins relevant to this discussion are the PR-2 including the glucan 1,3-β-D-glucosidases; the PR-3 including the chitinases; and the PR-5 including the thaumatin like proteins (TLP's) (van Loon 1985). It should be noted that PR proteins are further classified into acidic and basic subclasses, which refer to the specific location of the protein (i.e. vacuolar or extracellular), and may refer to the specific function of the protein in pathogen defense (Selitrennikoff 2001). The relative acidity or basicity of each identified PDP was not determined in this study; therefore, discussion of function for each identified protein is limited in this extent.

Chitinases are grouped together based on their ability to cleave β-(1,4)-glycoside bonds in biopolymers of N-acetylglucosamine, including chitin (Kasprzewska 2003). Further classification is based on the reaction mechanism and location of the cleaved glycosidic bond within the biopolymer. Plants are currently known to synthesize seven different classes of chitinases, which differ in structure and substrate specificity. In conifers, chitinases have been identified in various tissues, including pollen (Fujimura et al. 2005), roots (Ekramoddoullah 2004), and megagametophytic callus cell culture (Pirttilä et al. 2002). In angiosperms, chitinases have been isolated in seeds (Regalado et al. 2000), and shown to be active during flower development (Lawton et al. 1994).

Based on the wide diversity in chitinase structure and location, can any generalization be made regarding function? Given that chitinases catalyze the hydrolytic cleavage of chitin—a major constituent of fungal cell walls—and fungal cells lyse upon cell wall degradation, the main function attributed to chitinases is defense against pathogenic fungi (Kasprzewska 2003). Many studies of both angiosperms and gymnosperms support the role of chitinases as PR proteins, citing numerous in vitro enzymatic and antifungal assays (Selitrennikoff 2001). Ekramoddoulah (2004) showed that an acidic chitinase was up-regulated in *Pseudotsuga menziesii* roots following infection by the fungal root pathogen *Phellinus weirii* (Ekramoddoulah 2004). In vitro assays confirmed the protein as a chitinase (Ekramoddoulah 2004). A study by Pirttilä et al. (2002) demonstrated both high levels of chitinase production and the presence of endophytic fungi in both somatic and reproductive tissue cultures of *Pinus sylvestris.* Interestingly, endophytes grew uncontrollably in tissue culture despite the high levels of chitinase (Pirttilä et al. 2002). Results such these have led many researchers to question whether plant chitinases are solely involved in pathogen defense (Kasprzewska 2003). Research by van Hengel et al. (2002) strongly suggests that plant chitinases are involved in the catalytic decompostion of arabinogalactan proteins (AGPs), and dos Santos et al. (2006) have suggested that the released carbohydrate side chains may be active as signaling molecules. AGPs have been identified in the pollination drops of *Taxus* x *media* (O'Leary et al. 2005). Similar studies are investigating the possibility that plant chitinases have endogenous substrates and are involved in development and growth processes (Kasprzewska 2003). The role of chitinases in pollination drops may be more complex than pathogen defense.

TLP's are a group of proteins sharing amino acid homology with the sweet tasting thaumatin protein isolated from katemfe fruit *(Thaumatococcus daniellii)* (Selitrennikoff 2001). The majority of plant TLP's are approximately 22 kDa; they have 8 disulfide bonds stabilizing the tertiary structure (Selitrennikoff 2001). Smaller, more uncommon TLP's have molecular masses of 15-18 kDa with only 5 disulfide bonds (Ekramoddoullah 2004). TLP's can be further classified into basic vacuolar and acidic extracellular forms (Ekramoddoullah 2004).

Although their exact mode of action is unknown, TLP's are assumed to be antifungal proteins. In studies of *Cronaritum ribicola* infection of *Pinus monticola,* a TLP was shown to accumulate in the canker margins of infected seedlings (Piggott et al. 2004). Similarly, Zamani et al. (2004) demonstrated up-regulation of TLP in *Psedotsuga menziesii* roots infected with *Phellinius weirii.* Based on x-ray crystallography studies of TLP's, an acidic cleft formed by conserved amino acid residues is assumed to confer antifungal activity (Koiwa et al. 1999). Proposed mechanisms of TLP function range from interference with fungal cell wall assembly (Yun et al. 1997), to permeabilzation of fungal plasma membranes by either pore formation (Roberts and Selitrennikoff 1990) or modulation of osmoregulators (Batalia et al. 1996). Regardless of the specific mode of action, TLP's are fungicidal against a wide variety of plant pathogens (Selitrennikoff 2001).

TLP's may have functions other than pathogen defense, including anti-freeze activity (Hon et al. 1995). Some studies suggest that TLPs are associated with male and female reproduction: Cheong et al. (1997) isolated an antifungal TLP from non-stressed, female reproductive tissue (*Brassica campestris* flower buds), and Walden et al. (1999)

observed TLP gene expression in developing male cones of *Pinus radiata* immediately before and after microspore formation (Walden et al. 1999). Cheong et al. (1997) proposed that TLP genes may have originally been involved in reproduction, but evolved an additional function in pathogen defense.

Glucan 1,3-β-D-glucosidases can be grouped into three classes based on amino acid sequence homology (Selitrennikoff 2001). Class I glucan 1,3-β-D-glucosidases are basic, restricted to the vacuole and and approximately 33 kDa in molecular weight; and Classes II and III are acidic, extracellular and approximately 36 kDa in molecular weight (Selitrennikoff 2001). Glucan 1,3-β-D-glucosidases are widely distributed among seed plants (Selitrennikoff 2001).

As PR proteins, glucan 1,3-β-D-glucosidases hydrolyze structural β-(1,3)-glucans in fungal cell walls and cause lysis (Selitrennikoff 2001). Not surprisingly, glucan 1,3-β-D-glucosidases work well in conjunction with chitinases to hydrolyze fungal cell walls in vitro (Mauch et al. 1988). Cheong et al. (2000) showed that a glucan 1,3-β-D-glucosidase gene was upregulated in *Glycine max* cotyledons in response to *Phytophthora parasitica* elicitors. In a study of *Nicotiana sylvestris* infected by *Cercóspora nicotianae*, Beffa and Meins (1996) found that antisense mutation of an *N. sylvestris* Class I glucan 1,3-β-D-glucosidase had no consistent effect on the serverity of disease symptoms. Based on their results, Beffa and Meins (1996) concluded that there was compensation by other classes of glucan 1,3-β-D-glucosidases, and that this functional redundancy was a strong indication of the importance of glucan 1,3-β-D-glucosidases in pathogen defense. Glucan 1,3-β-D-glucosidases are known to be involved in diverse physiological and developmental processes. In the same study, Cheong et al. (2000) determined that glucan 1,3-β-D-glucosidase was involved in both pathogen defense and seedling development.

Though functional identifications of PDPs based on similarity searches can give some indication of pollination drop function, functional studies are necessary before making any strong conclusions. The following study of hydrogen peroxides and peroxidases attempts to demonstrate one aspect of ovular secretion function.

Chapter 3: Hydrogen peroxide and peroxidase activity in gymnosperm ovular secretions

Introduction

In 1956, Denham Harman described free radicals as a "Pandora's box of evils" responsible for gross cellular damage, mutagenesis, and cancer. However, the last 50 years have proven that reactive chemical species are important in the physiological control of cell function (Dröge 2002). Reactive oxygen intermediates (ROIs) including hydrogen peroxide (H_2O_2) are derived from triplet-state molecular oxygen ($^3O_2$) and involved in a diversity of processes, from cell adhesion to programmed cell death. Enzymes regulate levels of ROIs: Peroxidases can generate hydrogen peroxide as well as catalyze its breakdown to yield highly oxidizing intermediates (McInnis et al. 2006).

Plant peroxidases (class III peroxidases) mediate the involvement of ROIs in various cellular processes. Characteristically, class III peroxidases are active at acidic pH, and either secreted or located within the cell wall (Welinder 1992; Do et al. 2003). Class III peroxidases have a broad range of substrate preferences and lack of tissue specificity (McInnis et al. 2006). This diversity reflects their ubiquity: class III peroxidases are involved in lignification, suberization, auxin metabolism, wound healing and defense mechanisms against pathogen infection (Passardi et al. 2005). Plant peroxidases may mediate certain types of growth, as non-specific cleavage of cell wall polysaccharides by ROIs is related to cell wall loosening and cell elongation (Kawano 2003; Schopfer 2001). In studies of angiosperm reproduction, researchers have postulated the involvement of peroxidases in pollination, particularly in self-incompatibility (SI), based on the high levels of peroxidase activity and hydrogen peroxide in mature receptive stigmas (McInnis et al. 2006; Carraro et al. 1986). Research has indicated that peroxidases are involved in both pollen-stigma interactions and pathogen defense of reproductive tissue (McInnis et al. 2006). Future research may indicate a similar functional complexity in gymnosperm reproduction, given the recent identification of a peroxidase in the ovular secretion of *Pseudotsuga menziesii* (Douglas fir) (Poulis et al. 2005).

In our study, we attempted to quantify both hydrogen peroxide and peroxidase activity in the ovular secretions of *Juniperus communis*, *Chamaecyparis lawsoniana*, *Pseudotsuga menziesii*, and *Larix* x *marschlinsii* (Coaz). By demonstrating the presence of hydrogen peroxide and peroxidase activity, we hope to gain a better understanding of ovular secretion function.

Methods and Materials

Plant material

Pollination drop samples were collected from *J. communis* and *C. lawsoniana* trees as described in Chapter 2. Ovular secretions of *P. menziesii* and *L.* x *marschlinsii* were collected from trees at the University of Victoria in July 2005. Female cones from *P. menziesii*, and *L.* x *marschlinsii* were collected, and ovuliferous scales were dissected and placed in humid chambers (petri dishes lined with wetted filter paper) to encourage drop formation. The secretion that formed has been shown to include the liquid contents of the micropylar canal (von Aderkas and Leary 1999). Collection was done with a 10 µL micropipette tip. Daily collections were pooled into 1.5 mL storage tubes and frozen in liquid nitrogen. The frozen samples were stored at 20°C until analysis. Before analysis, all ovular secretion samples were centrifuged at 14,000 rpm for 5 min to remove any microaggregates.

Hydrogen peroxide assay

Hydrogen peroxide (H_2O_2) was quantified using the Amplex Red assay (Molecular Probes): detection is based on the enzymatic reduction of H_2O_2 by horseradish peroxidase coupled with oxidation of the fluorogenic indicator Amplex Red (N-acetyl-3,7-dihydroxyphenoxazine). Several stock solutions were prepared according to the assay kit instructions. A 10 mM stock solution of Amplex Red was prepared by dissolving Amplex Red reagent in dimethyl sulfoxide (DMSO). Reaction buffer (0.05 M sodium phosphate (pH 7.4)) was prepared from a 5X concentrate. Horseradish peroxidase (HRP) stock solution (10 U/mL) was prepared by dissolving HRP powder in 1X reaction buffer. One unit (U) is defined as the amount of enzyme that will form 1.0 mg of purpurogallin from pyrogallol in 20 seconds at pH 6.0 and 20°C. Using the supplied ~3% H_2O_2 stabilized solution, a 20 mM working solution was prepared with 1X reaction buffer. Using 1X reaction buffer as the diluent, a series of dilutions of H_2O_2 (0 to 20 μM) were prepared for standard curve measurements. Experimental samples were prepared using 2 μL of each ovular secretion diluted in 48 μL of 1X reaction buffer. All samples were prepared in triplicate, and pipetted in 50 μL aliquots into a 96 well microtiter plate (Nunc). A working solution containing 100 μM Amplex Red and 0.2 U/mL HRP was prepared immediately before use from the stock solutions. Reactions were initiated by the addition of 50 μL of the Amplex Red/HRP working solution. The reaction was protected from light, and incubated for 30 min at ambient temperature. Fluorescence measurements were made using a Synergy HT Multi-Detection Microplate

Reader with a 530 nm excitation and 590 nm emission wavelength filter and KC4 software (Bio-Tek). Background fluorescence determined from the no-H_2O_2 control reaction was subtracted from each value. A standard curve of fluorescence and H_2O_2 concentration was plotted. H_2O_2 concentrations of ovular secretions were interpolated from the curve.

Peroxidase assay

A protocol from Khare et al. (2006) was modified for detection of peroxidase activity in ovular secretions. The basis of detection was the brown precipitate formed upon oxidation of diaminobenzidine (DAB) by peroxidase in the presence of H_2O_2 (Herzog and Fahimi 1973). A PVDF membrane was immersed in 100 % methanol for 2 sec, followed by 1 min in 5 % (v/v) methanol in dH_20 and 5 min in dH_20. The following quantities of ovular secretion and control samples were adsorbed onto the membrane: 15 µL *J. communis*, 15 µL *C. lawsoniana*, 5 µL *P. menziesii*, 5 µL *L.* x *marschlinsii*, 5 µL HRP/positive control (10 U/mL), and 5 µL BSA/negative control (5 mg/mL). The membrane was washed six times for 15 min each in 0.5 M Tris-HCl buffer (pH 7). DAB was added to a concentration of 5 mg/mL, and the membrane was incubated in the dark for 30 min at ambient temperature. A citrate buffer was prepared using the following reagents: 1.05 g citric acid, 0.52 g NaOH, 0.2 g DAB, and 200 mL 0.5 M Tris-HCl buffer. The membrane was placed in citrate buffer, and 200 µL of 30 % H_2O_2 was added to initiate the DAB reaction. The reaction was protected from light and incubated overnight at 4 °C. The following day, the membrane was washed with dH_20. The membrane was left to develop in dH_20 at 4 °C for 3 d.

Results

Using a standard curve of fluorescence versus hydrogen peroxide concentration (Fig. 3.1), hydrogen peroxide concentrations were determined by interpolation (Table 3.1). *P. menziesii* ovular secretion had the highest concentration of hydrogen peroxide (9.29 μM), *C. lawsoniana* and *J. communis* both had midrange concentrations (6.15 and 5.03 μM, respectively), and *L.* x *marschlinsii* had the lowest concentration of hydrogen peroxide (1.74 μM) (Table 3.1).

All of the ovular secretions displayed peroxidase activity in the DAB assay (Fig. 3.2). The most intense staining was observed in *P. menziesii* ovular secretion, followed by *L.* x *marschlinsii* and *C. lawsoniana* (Fig. 3.2). The lowest intensity staining was observed in *J. communis*, and no activity was observed for the BSA negative control (Fig. 3.2).

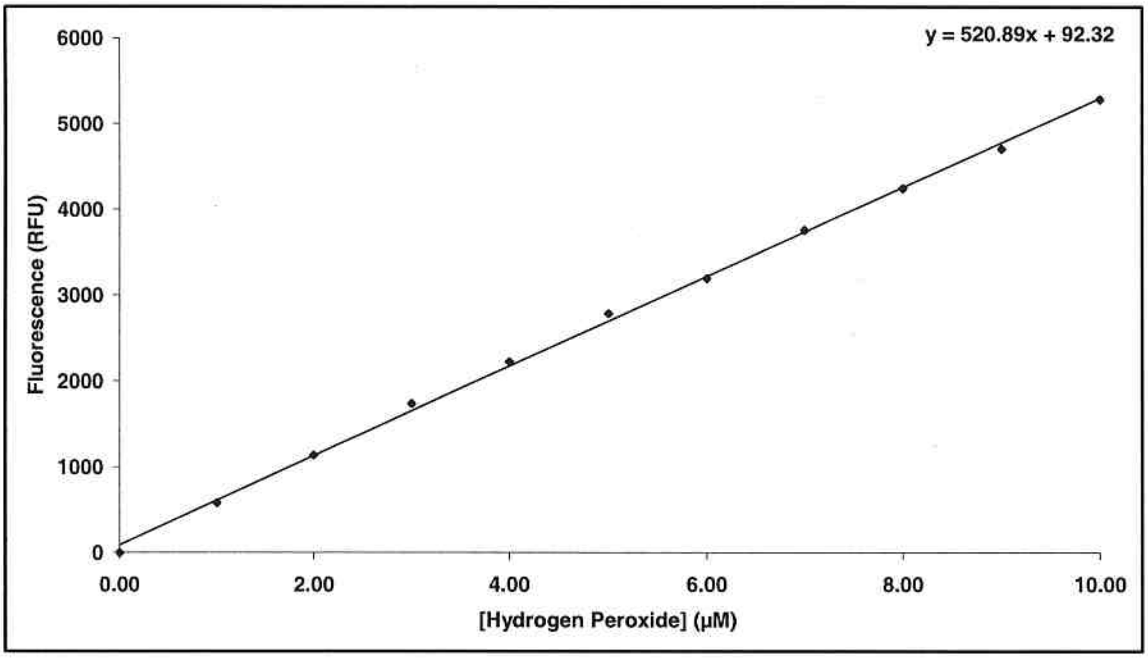


Figure 3.1: Hydrogen peroxide standard curve. Fluorescence was measured at 530 nm excitation and 590 nm emission wavelengths for hydrogen peroxide standard solutions ranging from 0 to 10.00 μM. Background fluorescence (65 arbitrary units) determined for a no-H_2O_2 control reaction, has been subtracted from each value.

Species	Average Fluorescence (530/590 nm)	Hydrogen peroxide concentration (μM)
J. communis	2714	5.03
C. lawsoniana	3296	6.15
P. menziesii	4929	9.29
L. x marschlinsii	999	1.74

Table 3.1: Hydrogen peroxide concentration of conifer ovular secretions. Two μL of each ovular secretion was assayed in a total reaction volume of 100 μL. The hydrogen peroxide concentration of each ovular secretion was interpolated from the hydrogen peroxide standard curve (Fig. 3.1).

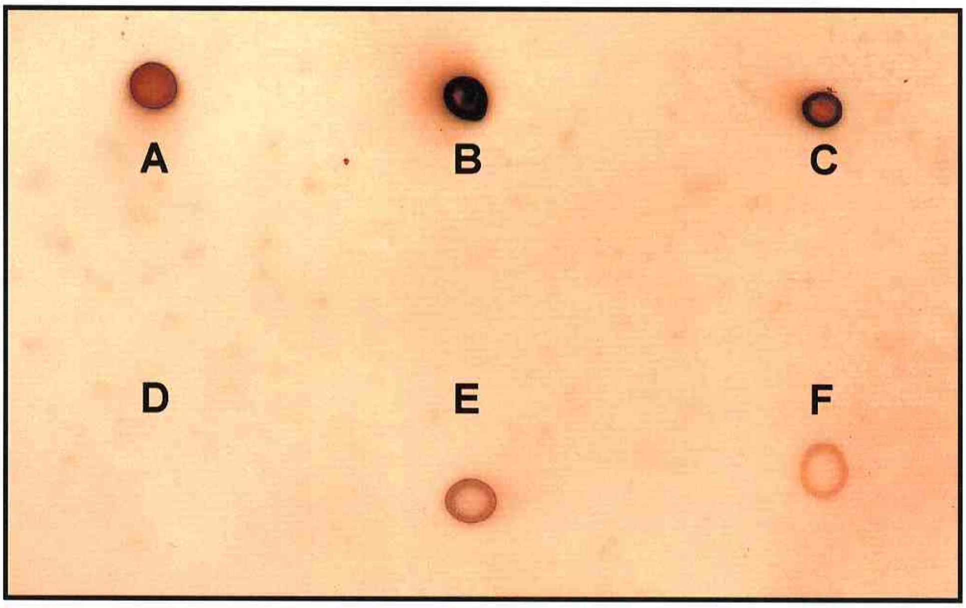


Figure 3.2: Assay for peroxidase activity in conifer ovular secretions. A spot indicates the presence of a peroxidase enzyme. A=5 µL HRPO/positive control (10 U/mL), B=5 µL *P. menziesii*, C= 5 µL *L.* x *marschlinsii*, and D=5 µL BSA/negative control (5mg/mL), E= 15 µL *C. lawsoniana*, F= 15 µL *J. communis.*

Discussion

There is a complex relationship between peroxidase activity and hydrogen peroxide levels: peroxidases catalyse a diversity of reactions in separate hydroxylic and peroxidative cycles, and both produce and consume hydrogen peroxide (Passardi et al. 2005). In the ovular secretions of our study species, we found a range in hydrogen peroxide concentration 1.74 to 9.29 μM. We also observed peroxidase activity in all ovular secretions, and a variation in level of activity. From our results, we cannot determine the relationship between hydrogen peroxide concentration and peroxidase activity without further characterization of each peroxidase. However, we can speculate about the relationship and function of peroxidase and hydrogen peroxide in conifer ovular secretions based on comparable studies in angiosperms. McInnis et al. (2006) showed that the stigma of *Senecio squalidus* Linnaeus generates hydrogen peroxide constitutively and has peroxidase activity. Because the stigma and the ovular secretion have comparable functions (e.g. pollen reception and initial interaction), ovular secretions may also constitutively produce hydrogen peroxide. Possible roles of hydrogen peroxide and peroxidase in conifer ovular secretions will be discussed.

The mechanical function of the conifer pollination drop has not been proven to be specific to conifer pollen; therefore, it must indiscriminately also capture bacterial and fungal spores. Furthermore, the nutrient rich pollination drop could provide a suitable germination medium for such pathogens. It is possible that ovular secretions have a defensive function. In many cases, plants respond to pathogens by upregulation of peroxidases and an increase in ROI—sometimes resulting in a rapid oxidative burst (Passardi et al. 2005). This strategy has been proven effective: transgenic potatoes generating high levels of hydrogen peroxide showed increased resistance to both fungal and bacterial infection (*Phytophthora infestans* and *Erwinina carotovora*, respectively) (Wu et al. 1995). In plant systems, effective pathogen defense could be directly or indirectly related to ROI production: high levels of ROI may create a cytotoxic or inhibitory environment for pathogens, and/or ROI may signal the induction of other defense genes. In the case of ovular secretions, both possibilities will be considered.

Many studies have shown that elevated levels of hydrogen peroxide are detrimental to pathogenic organisms. In Wu et al.'s (1995) study of disease resistance in transgenic potato, pathogens *E. carotovora* and *P. infestans* showed little or no growth in medium containing 100 µM hydrogen peroxide. An *in vitro* assay by Peng and Kuc (1992) showed that 26 µM of hydrogen peroxide completely inhibited germination of fungal spores of *Peronospora tabacina*, *Cladosporium cucumerinum*, and *Colletotrichum lagernarium.* In a study by Kachroo et al. (2003), bacterial growth and spore germination of *Xanthomonas oryzae* and *Magnaporthe grisea* were completely inhibited by hydrogen peroxide concentrations of 150 µM. Furthermore, Custers et al. (2004) demonstrated a 50% growth inhibition of *P. infestans* at 190 µM, and of *Pectobacterium carotovorum* ssp. *carotovorum* at 36 µM. The hydrogen peroxide concentrations that we found in conifer ovular secretions range from 1.74 to 9.29 µM, and are therefore lower than published levels of hydrogen peroxide proven to be detrimental to plant pathogens. Based on our results, we can speculate that hydrogen peroxide in conifer ovular secretions does not directly contribute to pathogen defense, or that the level of hydrogen peroxide in the samples had degraded prior to analysis. Another possibility is that hydrogen peroxide may generate other reactive free radicals that are anti-microbial, and/or stimulate the biosynthesis of antimicrobial compounds.

Hydrogen peroxide can act as an inducer and/or activator of defense genes, and is therefore indirectly involved in pathogen defense (Custers et al. 2004). Studies have also indicated that peroxidases have roles in oxidative signal transduction, leading to expression of defense-related genes (Kawano 2003). A model for plant oxidative signal transduction posits that peroxidases are transducers of external signals (e.g. plant hormones) into redox signals that stimulate defense responses (Kawano 2003). Specifically, salicylic acid (SA) signaling leads to ROI production, which eventually stimulates the intracellular Ca^{2+} signaling required for induction of defense genes. Ca^{2+} has been detected in the ovular secretions of *P. menziesii*, *L.* x *marschlinsii* and *Thuja plicata* (Gill 2002). The presence of peroxidase activity and hydrogen peroxide in conifer ovular secretions may be indicative of signal transduction stimulating a defense response. In this scenario, the defense response could be increased expression and secretion of anti-microbial proteins from the nucellus, as chitinase and TLP have been identified in ovular secretions. The exact levels of ROIs and Ca^{2+} involved in plant defense signaling cascades have not been determined; therefore, more research is necessary to determine whether the levels of hydrogen peroxide, Ca^{2+} and peroxidase activity and in ovular secretions are in accordance with Kawano's model.

The presence of peroxidases and hydrogen peroxide in conifer ovular secretions may also be related to pollen development and selection. Pollen tube formation involves growth by cell elongation, which is influenced by ROIs and peroxidase activity. Growth and elongation are both related to cell extensibility, which occurs with loosening of cell wall compounds. Peroxidases can influence elongation directly by generating hydroxyl radicals that cleave cell wall polysaccharides (Schopfer 2001), and indirectly by regulating hydrogen peroxide levels, which may limit cell wall cross-linking (Passardi et al. 2005). In Kawano et al.'s (2003) model of an extracellular signaling pathway resulting in elongation growth, the plant hormone IAA is the external signal that stimulates a peroxidase system. In a series of steps not fully characterized, the peroxidase system, in combination with other signaling paths, stimulates growth by cell wall loosening and regulation of gene expression.

Many researchers have suggested that peroxidases and ROIs are involved in pollen-stigma interactions. Carraro et al. (1986) suggested that peroxidases govern the SI response, as elevated ROI levels were found in unpollinated and self-pollinated stigmas, but not in cross-pollinated stigmas. In a study of *S. squalidus*, McInnis et al. (2006) identified a stigma specific peroxidase (*SSP*) expressed exclusively in specialized secretor cells of the stigma. Assays showed that *SSP* had a preference for diphenolic substrates, which indicated it was probably not involved in the cross-linking cell wall phenolic compounds. Furthermore, hydrogen peroxide and other ROIs were localized to same regions as *SSP*. The results of McInnis et al. (2006) help explain the high levels of hydrogen peroxide and ROIs in receptive stigmas, and may in part explain the underlying mechanism of the SI response. However, the authors highlight the fact that SI response is mediated by S-RNAses in other plants, and suggest that *SSP* may be involved in pathogen defense. This suggestion makes intuitive sense, as female reproductive tissues are most vulnerable during pollination, and increased ROI levels could increase reproductive success by preventing pathogenic attack. The same reasoning has been used to explain the release of peroxidases and ROIs by germinating *Raphanus sativus* seeds: peroxidases are secreted in the absence of pathogenic organisms, and could offer a vulnerable germinating seed a great protective advantage (Schialabba et al. 2002).

Although we detected peroxidase activity in the ovular secretions of *J. communis*, *C. lawsoniana*, *P.menziesii*, and *L.* x *marschlinsii*, mass spectrometric analyses of all four species have produced only one peroxidase identification in *P. menziesii* (Poulis et al. 2005). It is possible that peroxidases are present in all four species, and lack of identification resulted from either: 1) lack of detection in SDS-PAGE, or 2) lack of useful data generated by mass spectrometric analyses. Another possibility is that enzymes other than peroxidase are involved in ROI production in conifer ovular secretions. Nectarin 1 produces hydrogen peroxide in nectar of ornamental tobacco (*Nicotiana langsdorffii* x *N. sanderae*), and has been characterized as a germin-like superoxide dismutase enzyme (Carter and Thornburg 2000). If nectar is evolutionarily related to the ovular secretion, it is possible that a similar superoxide dismutase enzyme produces hydrogen peroxide in conifer ovular secretions. Interestingly, a BLAST search of *C. lawsoniana* data yielded a weak match to a germin-like protein (results not shown). Specific assays for superoxide dismutase activity and more thorough proteomic analysis may yield an answer.

Carter and Thornburg (2000) hypothesized that Nectarin 1 functions in pathogen defense, as it produces a high level of hydrogen peroxide (up to 4 mM) deleterious to pathogens. Following the characterization of Nectarins 1-5, Carter and Thornburg presented a model of a "nectar redox cycle" which functions in pathogen defense (Carter and Thornburg 2004). In the model, various proteins and other nectar components influence nectar ROI levels. Nectarin 1 produces high levels of hydrogen peroxide, and the antioxidant ascorbate scavenges hydroxyl free radicals formed as a result of hydrogen peroxide breakdown. Given that hydroxyl free radicals are extremely deleterious in biological systems, the nectar redox cycle may regulate the nectar environment to best protect the flower.

Conifer ovular secretions and flower nectar may be functionally related: both contain hydrogen peroxide and related enzymes, both function in reproduction, and both have defensive capabilities. However, the 1000-fold difference in hydrogen peroxide levels of nectar (4 mM) and conifer ovular secretions (1-10 μM) indicates that the two systems are not identical. Ovular secretions may be ancestral to nectar, whereas nectar is a secretion that co-evolved with insect pollination. Nectar acts as a reward for pollinators and does not directly receive pollen; therefore, increased hydrogen peroxide concentrations could help to maintain the axenic state of the nectar for pollinators (Carter and Thornburg 2004). In the future, it would be interesting to assay for hydrogen peroxide and peroxidase activity in the pollination drops of insect-pollinated cycad species.

The study of ROIs and related enzymes will be useful in determining gymnosperm ovular secretion function—whether its function lies in pollen selection and development, pathogen defense, or both. It would be beneficial to assay for more types of ROIs and related enzymes. Furthermore, enzymes need to be identified using a proteomics approach. Once enzymes have been identified, they need to be cloned and characterized. Only after characterizing enzymes can we begin to tease apart the many biological functions of ROI related enzymes in gymnosperm ovular secretions.

Chapter 4: Conclusions and Perspectives

In our study of gymnosperm pollination drops, we presented the following three hypotheses: 1) the gymnosperm pollination drop contains proteins, 2) variation in pollination drop protein (PRP) profile has a phylogenetic component, and 3) gymnosperm pollination drops may function in pathogen defense and reproduction.

In addition to findings by Poulis et al. (2005) and O'Leary (2004), we showed that the gymnosperm pollination drop contains proteins and that a proteomics approach is an effective means to this end. Analysis by SDS-PAGE indicated that the pollination drop of each species contained at least 10 to 20 proteins, and that the PDP profile of each species differed in its complexity (i.e. number of bands on gel) and complement (i.e. combination of different MW bands). Mass spectrometric analysis of Coomassie stained protein bands yielded the necessary data for protein identification. Our success highlights the sensitivity of mass spectrometry and its utility in research projects involving limited amounts of sample. With the aid of Peaks software, we were able to obtain amino acid sequences for each species. Protein identifications were made using the MSBlast search of the NCBI database. The MSBlast search algorithm was specifically designed for protein sequence data generated by mass spectrometry, and therefore very useful. The following proteins were identified (species): an 83 kDa subtilisin-like proteinase, a 62 kDa glycosyl hydrolase, a 47.5 kDa glucan 1,3-β-glucosidase precursor, a 30 kDa chitinase, and a 25 kDa thaumatin-like protein (*Juniperus communis*); a 30 kDa chitinase, a 25 kDa thaumatin-like protein, and a 32.5 kDa glucanase-like protein (*Juniperus oxycedrus*); an 83 kDa subtilisin-like proteinase, a 62 kDa β-D-glucan exohydrolase, a 47.5 kDa glucan 1,3-β-glucosidase, and two 25 kDa thaumatin-like proteins (*Chamaecyparis lawsoniana*); and a 25 kDa chitinase (*Welwitschia mirabilis*).

We conclude that pollination drop protein (PRP) profile variation does reflect gymnosperm phylogeny. Using current gymnosperm taxonomy, we compared PDP profiles at the genus, family, and gymnosperm clade levels. In each comparison, we examined variation in the following: 1) SDS-PAGE banding pattern, 2) identified peptides, 3) amino acid sequences, and 4) protein identifications based on BLAST searches of the NCBI database. We found almost identical patterns between the two species in the same genus (*J. communis* and *J. oxycedrus*). However, we observed small differences between the two species (i.e. more bands in SDS-PAGE analysis of *J. oxycedrus*). We observed similarities between the different genera in the Cupressaceae, but also noted key differences (i.e. single amino acid difference between comparable amino acid sequences of *J. communis* and *C. lawsoniana*). Furthermore, the protein identifications in different genera in the Cupressaceae were slightly different (i.e. identifications of the same types of protein but based on different accession numbers). Although minimal data were obtained for *Welwitschia mirabilis*, it was clear from the identification of a chitinase in *W. mirabilis* pollination drop that PDP profile was somewhat conservative in all study species. Beyond this similarity, *W. mirabilis* did not share any bands or peptides with any other study species. Further research will be necessary to develop PDP analysis into a tool that can be used to predict phylogenetic relationships.

Based on the types of proteins identified and the dual presence of hydrogen peroxide and peroxidase activity, we assume that the gymnosperm ovular secretion has a complex functionality. The identification of glycosyl hydrolases, 1,3-β-D-glucosidases and subtilisin-like proteinases indicates that the pollination drop may contribute to pollen development. Specifically, these proteins may encourage germination or pollen tube growth by influencing pollen cell wall structure or by mobilizing nutrients. The hydrogen peroxide and peroxidase activity found in the pollination drop may contribute to reproductive success in two ways: 1) by generating free radicals that degrade pollen cell walls and encourage growth, and 2) by mediating an SI response to foreign pollen. The identification of chitinase, thaumatin-like protein, and glucan 1,3-β-D-glucosidase in our study species indicates that the pollination drop functions in pathogen defense. Hydrogen peroxide may contribute directly to pathogen defense by killing or inhibiting growth of pathogens, or indirectly by inducing the expression of defense genes. There are other possible functional roles for pollination drops: thaumatin-like proteins have demonstrated anti-freeze activity.

Our study of gymnosperm pollination drops presented many challenges. Sample collection was difficult for all study species. In the *C. lawsoniana* collection, many trees with abundant female cones were available, but individual drops were a mere 10 nL in volume. Pollination drops of *W. mirabilis* were larger, as well as more visible and accessible, but collection was limited by the low number of female plants grown in the University of Washington greenhouse. In any case, a more thorough understanding of the biophysics of pollination drop production would be useful for future collections. Ideally, collectors would know the best conditions for drop production (i.e. relative humidity, temperature). Pressure bomb experiments in a controlled environment would help to define these relationships. Overall, the ideal species in terms of amount of sample collected was *C. lawsoniana*. With further optimization of collection procedures, *C. lawsoniana* may be used as a model species in future studies of gymnosperm pollination drops. It is unfortunate that so little sample was collectable from *W. mirabilis*: our limited analysis did not allow us to address key questions. *W. mirabilis* is the only non-conifer we have analyzed to date, and further characterization of its pollination drop will help elucidate the relationship between PDP profile and phylogeny. Interesting aspects of *W. mirabilis* biology could be investigated via pollination drop analyses: comparison of pollination drops from male and female cones could explain the presence of sterile ovules in male cones. Future research efforts should focus on other non-conifer gymnosperms such as *Ginkgo biloba* and cycad species.

We have presented the first study of PDP variation and gymnosperm phylogeny from a proteomics perspective. The development of PDP analysis into a tool used to predict phylogenetic relationships will be challenging, but some necessary adjustments to our methodology are immediately apparent. Most importantly, we need an outgroup for our phylogenetic analysis. Finding an appropriate outgroup will be difficult, as the closest ancestors to seed plants (the progymnosperms) are extinct. In fact, one of the major problems in gymnosperm molecular phylogenetic analyses is finding an outgroup that does not produce biased phylogenetic trees (i.e. long branch attraction). Although the extant seedless plants are evolutionarily distant from the gymnosperms, they face the same challenges in terms of reproduction—if not to a higher degree. Seedless plants produce eggs and sperm, and sperm is conveyed to archegonia by water. Therefore, seedless plants may face greater challenges in prevention of pathogenic attack of reproductive tissue and the coordination of male and female gametes. Perhaps an analysis of female reproductive tissues in seedless plants would uncover proteins that are comparable to gymnosperm PDPs. A wider sampling of gymnosperm taxa is also needed: analyzing species from each conifer family would be a good start. To date, we have analyzed species from the Pinaceae, Cupressaceae, and Taxaceae. A combined analysis of conifers and Gnetales would be very informative, as many consider the Gnetales to be closely related to the conifers. To further develop our methodology, we need to employ standardized techniques used in comparative proteomics (e.g. 2D gels and various tagging or chemical modification methods [i.e. ITRAQ]). The technique yielding the most useful data (i.e. best phylogenetic signal) could be determined, or a combination of analyses could be used. Finally, we need to determine how to best quantify relatedness and construct phylogenetic trees. A combination of cladistic methods and statistics used in molecular phylogenetics could be employed.

We assumed PDP function based on identifications in BLAST searches, but no definitive functional studies were performed. Fortunately, Dr. Stephen O'Leary (a past member of the von Aderkas lab) has succeeded in expressing a pollination drop TLP in *Pichia pastoris* (pers. comm.) and antimicrobial and anti-freeze activity assays are now possible. Furthermore, chitinases have been tested in Douglas fir by Brett Poulis (pers. comm.). Future research will result in recombinant expression of more ovular secretion proteins. We await the first EST databases of gymnosperm reproductive tissues to aid in protein identification.

Now that the existence of ovular secretion proteins has been established, future studies can focus on ovular secretion protein interactions. The identification of interactions between ovular secretion proteins and pollen could provide a mechanistic understanding of various phenomena.

Due to worldwide incidences of allergy, much research has been devoted to pollen characterization. Proteins on the outer surface of pollen are often the cause of allergic reactions in humans (Hoffmann-Sommergruber 2000). Two extensively studied pollen proteins are Jun a 1 and Cry j 1; respectively isolated from *Juniperus ashei* (J. Buchholz)) *Cryptomeria japonica* (Sugi) (Midoro-Horiuti et al. 1999; Yasueda et al. 1983). A study by Suárez-Cervera et al. (2003) focused on pollen protein localization and changes associated with germination. Cross-reactive antigens to Cry j 1 antibody were detected in the outer surfaces of mature pollen grains of *Cupressus arizonica* and *Cupressus sempervirens*. When pollen was immersed in a sugar solution, the intensity of Cry j 1 labeling decreased dramatically after 5 min. The authors interpreted their results as evidence of a rapid release of proteins during pollen attachment in the pollination drop (Suárez-Cervera et al. 2003). They further suggest that proteins labeled with Cry j 1 are present in the first steps of male-female gametophyte recognition and have a dynamic role in the underlying pollen/pollination drop/ovule interactions (Suárez-Cervera et al. 2003). Given that recognition and coordination of male and female gametophytes is essential to reproductive success (Herrero 2001), ovular secretion/pollen protein interactions may be the mechanism underlying these processes. It would be interesting to purify pollen and ovular secretion proteins and study their interactions (e.g. by pull-down assays).

Pollen/ovular secretion interactions may be involved in pollination drop retraction. In a 1935 study of *Pinus* spp., Doyle and O'Leary observed a complete, permanent, and rapid (10 min in duration) withdrawal of pollination drops after pollination. Tomlinson et al. (1997) reported similar findings for *Phyllocladus*, and further noted that pollination drop retraction was not induced by mechanical stimuli (i.e. disturbance with a dissecting needle, introduction of 75 μm diameter glass Balloti spheres). Pollination drop retraction has also been observed in *Callitris*, *Chamaecyparis*, *Cryptomeria*, and *Thuja* species (Baird, 1953; Owens et al. 1980; Yokoyama 1975; Owens and Molder 1980). Although some believe the phenomenon to be an active process, others see it as a cessation of secretion followed by evaporation (Owens et al. 1998). A signal is required in either scenario, and the signal could be mediated by protein interactions.

Recent evidence indicates that pollination drop retraction is both a physical and biological phenomenon. In their research of *Juniperus oxycedrus* pollination, Mugnaini et al. (2004) measured the effect of both biotic and abiotic particles on pollination drop retraction. Pollination drop volumes were calculated prior to and at various time intervals after the introduction of either viable con-specific pollen, non-viable con-specific pollen (incubated at 120°C for 1 h), or silica particles of three size ranges (10 to 14, 40 to 63, and 63 to 200 μm). Any variation in volume was interpreted as partial or total reabsorption due to recognition of pollen by pollination drop. Results showed total reabsorption following viable and non-viable pollen, partial reabsorption when smaller silica particles were used (10 to 15 and 40 to 63 μm), and no reabsorption after applying large silica particles (63 to 200 μm). Muganaini et al.'s (2005) results contradict the Tomlinson et al. (1997) assumption that retraction is not influenced by mechanical stimuli; however, the partial retraction following the 10 to 63 μm silica particles indicates that specific interactions between pollen and the pollination drop are necessary for total retraction. It is interesting that complete retraction was induced by both viable and non-viable pollen: this indicates that the biological signal for retraction (if one exists) is not actively synthesized by pollen. It is possible that a component of the pollen wall elicits the retraction response: in cupressad pollen, the outer pollen coat (exine) is cast off following imbibition of water—accompanied in some species by a rapid release of proteins (Suárez-Cervera et al. 2003). In future research, the effect of *J. oxycedrus* pollen proteins on drop retraction could be measured. For example, isolated proteins from both from the exine and inner pollen grain could be immobilized on 10 to 63 μm silica beads and introduced into pollination drops.

Our study is the third investigation of gymnosperm ovular secretions using a proteomics approach. We believe it is imperative to extend the scope of this research to include non-conifer gymnosperms, and we hope that future researchers will take up the challenge. In combination with previous studies, the identification of pollination drop proteins and hydrogen peroxide in our study species further highlights the complexity of gymnosperm ovular secretions: the possibilities for functional characterization are endless. Hopefully, the suggestion that PDP variation reflects gymnosperm evolutionary will inspire those dedicated to resolving seed plant phylogeny.

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