Proteins in the Ovular Secretions of Conifers

Stephen James Bernard O'Leary
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Supervisor: Dr. Patrick von Aderkas

Abstract

Most conifers employ a liquid secretion originating from within the ovule at some point during reproduction. Although widely known, these ovular secretions have been poorly characterized. Biochemical analyses of these liquids have been limited to reports of sugars, amino acids, organic acids, and calcium. The purpose of this study was to investigate the physiological regulation of conifer ovular secretions and to further elucidate their contents.

Postpollination droplet production was measured in three hybrid larch trees (*Larix* x *marschlinsii* Coaz) in relation to xylem water tension in the stem. Secretion production was not correlated to the predictable diurnal fluctuation of tree water status. The ovular secretions of this species were found to be independent of the physiological condition of the stem and are likely under the control of local structures such as the cones or ovules.

The concentrations of glucose, fructose, and sucrose were measured in the secretions of larch and hybrid yew (*Taxus* x *media* Rehder). In agreement with results from other conifers, the concentrations of glucose (156 mM) and fructose (145 mM) in the larch secretion were found to be higher than sucrose (108 mM). The pollination droplet of yew displayed a novel pattern. The sucrose concentration in this species (23 mM) was found to be an order of magnitude higher than either glucose (2.7 mM) or fructose (2.1 mM).

The ovular secretions of larch, yew, Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), and western red cedar (*Thuja plicata* Donn. ex D. Donn) were found to contain complex mixtures of proteins when examined by polyacrylamide gel electrophoresis or

reversed phase high performance liquid chromatography. The proteins of larch and yew were produced consistently from tree to tree and throughout the period of secretion production.

N-terminal amino acid sequencing and antibody recognition identified proteins in larch and yew samples believed to be involved in pollen germination and the promotion of pollen tube elongation. The cell wall modifying enzyme xyloglucan endotransglycosylase (XET) was identified in the larch secretion. Immunolocalization identified cells in the apical region of the larch micropyle as the site of XET production. Arabinogalactan proteins (AGPs), known to promote pollen tube growth in angiosperms, were found in the secretions of both conifer species. AGP production in the yew ovule was localized to the nucellus.

Four pathogenesis-related (PR) proteins were identified in the larch and yew ovular secretions. A lipid transfer protein (LTP) belonging to the PR-14 group was identified in the larch secretion by N-terminal amino acid sequencing. A thaumatin-like protein (TLP, PR-5) was tentatively identified in the larch sample by antibody recognition. One acidic and one basic TLP were identified in the yew secretion by tandem mass spectrometry (MS/MS) sequencing of internal peptide fragments. These proteins were named *Tx*m*TLPa and *Tx*m*TLPb respectively. MS/MS sequencing also identified a β-1,3-glucanase of the PR-2 group in the yew secretion (*Tx*m*βGlu).

The cDNA coding for *Tx*m*TLPa was sequenced and assessed for heterologous protein expression. The nucleic acid sequence predicts a preprotein of 233 amino acid residues with a 28 residue export signal. The putative mature protein has a predicted molecular weight of 21.40 kDa and pI of 4.4. The deduced protein sequence contains 16

cysteine residues conserved across TLPs, and five residues that contribute to the acidic cleft of antifungal TLPs. In order to produce *Tx*m*TLPa in sufficient quantities to perform bioassays, the mature sequence of this protein has been inserted into a plasmid vector for the expression of a *Tx*m*TLPa fusion protein.

This report contains the first simultaneous study of ovular secretion production and tree water status, the first measurements of the sugar concentrations in the ovular liquids of *L.* x *marschlinsii* and *T.* x *media*, and the first identification of proteins in the ovular secretion of any seed plant.

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AGP: arabinogalactan protein
amu: atomic mass unit
βGlu: β-1,3-glucanase
BAP: bacterial alkaline phosphatase
bp: base pairs
cDNA: complimentary deoxyribonucleic acid
dATP: deoxyadenosine triphosphase
dNTP: deoxyribonucleoside triphosphate
ECM: extracellular matrix
FITC: fluorescein isothiocyanate
GSA: goat serum albumin
IPTG: isopropylthio-β-galactoside
kDa: kiloDalton
LTP: lipid transfer protein
MALDI-TOF: matrix assisted laser desorption/ionization time of flight
MCS: multiple cloning site
mRNA: messenger ribonucleic acid
MS/MS: tandem mass spectrometry
nsLTP: non-specific lipid transfer protein
OmpA: outer membrane protein A
PCR: polymerase chain reaction
Poly-(A): polyadenylation tail
PR: pathogenesis-related
PVDF: polyvinylidene difluoride
RACE: rapid amplification of cDNA ends
RNA: ribonucleic acid
RP-HPLC: reversed phase high performance liquid chromatography
SCA: stigma/stylar cysteine-rich adhesin
SDS-PAGE: sodium dodecylsulphate polyacrylamide gel electrophoresis
TBS: Tris buffered saline
TBST: Tris buffered saline + Tween 20
TCA: trichloroacetic acid
TTS: transmitting tissue-specific
UTR: untranslated region
UV: ultraviolet
XET: xyloglucan endotransglycosylase

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Chapter 1

General Introduction

The term pollination refers to the events that occur during the transport of male gametes from their site of production to the female gametophyte. It begins with the shedding of pollen grains from a male reproductive structure and ends with fertilization of an egg cell. These events are essential to the sexual reproduction of seed producing plants. Plants are sessile organisms, and as a result an enormous amount of plant diversity is driven by the evolution of adaptations that ensure adequate delivery of appropriate pollen to the female reproductive structures. Variation in pollination mechanism is one of the key characteristics that determines the taxonomic division of extant seed plants into two major groups, the angiosperms and gymnosperms. In this overview of plant reproduction, the structures and mechanisms typical of angiosperm pollination will be discussed, and conifers will be considered representative of the gymnosperm condition.

Pollination in flowering plants

Flowering plants have evolved a multitude of strategies to ensure successful pollination. Numerous studies document the wide variety of pollination mechanisms and species interactions that lead to fertilization. Many plants flower in response to particular environmental cues, attract species-specific pollinators, or employ specialized floral morphology to ensure the timely arrival of pollen at the female receptive surface, the stigma.

The arrival of pollen at the stigma, however, does not ensure a successful fertilization event. In order for fertilization to occur, a pollen grain must germinate on the stigma and develop a pollen tube capable of penetrating the tissues of the style before finding its way to the ovules at the base of the flowering structure (Figure 1.1). During this process, the pollen encounters a number of physical and chemical barriers that are intended to weed out inferior pollen, self-pollen (from the mother sporophyte or a close relative), or pollen from the wrong species (Sage *et al.* 1994, Silva and Goring 2001, Wheeler *et al.* 2001, Takayama and Isogai 2003). Only viable pollen capable of correctly following the signals of the female reproductive structures will find itself in a position to enter the ovule and fertilize the egg within (Herrero 2001).

The stigma, style, and ovary are known collectively as the pistil. Interactions between pollen and the tissues of the stigma and style during compatible pollination events have been well studied in a number of angiosperm species (for reviews see Cheung *et al.* 2000, Franklin-Tong 2002, Johnson and Pruess 2002, Lord 2003, Wolters-Arts *et al.* 2002). In contrast, very little is known about the events that direct pollen development when the tube reaches the ovule, which is enclosed within the ovary at the base of the pistil.

Genetic studies with *Arabidopsis thaliana* have implicated the female gametophyte in the attraction of pollen tubes within the ovary to the micropyle, the entrance of the ovule (Hulskamp *et al.* 1995a, Ray *et al.* 1997). Unfortunately, these studies were not able to determine the nature of the attractant. *In vitro* experiments with excised embryo sacs of *Torenia fournieri* provided the first conclusive evidence that a

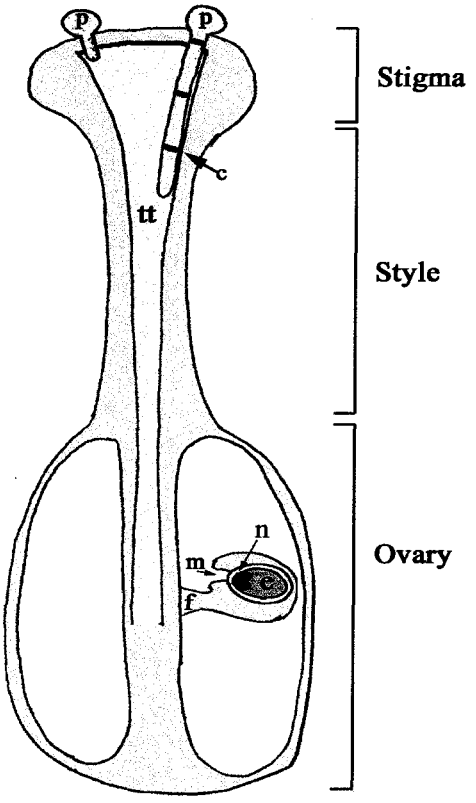


Figure 1.1. An idealized angiosperm pistil. For successful pollination to occur, the pollen grain (*p*) must first germinate on the surface of the stigma and develop a tube that passes through to the style. In the style, the pollen tube elongates through the extracellular spaces of the transmitting tract (*tt*) toward the ovary. An empty tube cut off by a callose wall (*c*) is left behind. At the base of the transmitting tract, the pollen tube enters a locule and grows along the placental surface to a funiculus (*f*), the bridge that leads to the micropylar entrance (*m*) of an ovule. After the pollen tube enters the ovule, it traverses the nucellus (n) and penetrates the embryo sac (*e*), releasing its sperm for the double-fertilization event that follows.

diffusible attractant from the female gametophyte is involved in signalling pollen (Higashiyama *et al.* 1998). Pollen tubes exiting the bottom of a cut style tracked along the surface of a solid medium and were guided to viable female gametophytes distributed on the medium, avoiding heat damaged ones. In a subsequent experiment where laser cell-ablation was used to destroy specific cells within the embryo sac, the synergid cells were identified as the source of the pollen-attracting signal (Higashiyama *et al.* 2001). The chemical attractant that directs pollen tube growth toward the synergid cells of *Torenia* remains unidentified.

The interactions between pollen and the tissues of the flower are quite complex. The most confounding issue in this field is the lack of knowledge about pollen interaction within the secluded tissues of the angiosperm ovule. As Herrero (2000) laments, "The paucity of information on the male-female interaction in the ovary may be related to the fact that this region is far more difficult to investigate since a number of concentric wrappings envelop the female gametophtye." The concentric wrappings referred to are the nucellus and integuments that house the megagametophyte and make up the ovule, as well as the tissues of the ovary that contain the ovules of angiosperms.

Pollination in conifers

A conspicuous difference between conifers and flowering plants lies in their specialized reproductive structures. Angiosperms are typified by complex flowering structures; often coloured, scented, and constructed in an ostentatious manner to attract insect pollinators. As their name implies, conifers bear cones instead of flowers. They rely on wind dispersal (anemophily) to carry pollen from male to female structures.

Like all gymnosperms, conifer ovules are not contained within tissues of an ovary, and there are is no stigma or style involved in pollination. Instead, gymnosperm ovules are naked and directly exposed to incoming pollen. During pollination, conifer ovules are typically located on the bracts of open megastrobilate cones, which can usually be recognized as pine cones, spruce cones, fir cones, and so on. In a few conifer species, such as yew and juniper, the ovules are not contained within a cone structure, but are simply attached to the vegetative branches amongst the needles.

At some point during pollination, many conifers employ a liquid secretion that originates from within the ovule and carries pollen to the nucellus where germination occurs (Gelbart and von Aderkas 2002). In some species, these liquids occur in the form of a pollination droplet (e.g., *Taxus*, *Thuja*, *Podocarpus*) that accepts pollen directly from the air (Figure 1.2). In other species, the pollen may be collected on sticky appendages at the mouth of the micropyle and brought into the ovule by a secretion that occurs at a later time. In a few cases, such as *Pseudotsuga* and *Larix*, pollen is brought into the micropyle by other mechanisms (Doyle 1945, Owens and Molder 1979, Owens *et al.* 1981). Even in these instances a secretion occurs within the ovule that transports the pollen to its final resting spot.

In general, conifer pollination may be summarized as follows: pollen is indiscriminately brought to the naked ovule where it may be collected directly into the micropyle, typically by a liquid secretion. Variations on this process will be discussed later.

Figure 1.2. Pollination droplet production from the ovules of *Taxus* x *media*. This species uses a liquid secretion at the tip of the micropyle to collect pollen from the air. Yew pollen triggers retraction of the fluid, which carries the captured grains into the ovules. Bar = 5 mm.

Studying pollen / ovule interactions in conifers

In terms of the number of tissues involved, the female reproductive structures of conifers are far simpler than those of flowering plants. The conifer ovule is directly accessible to observation and sampling. In many conifer species, the bracts of the female cone enclose the ovules after pollination, but even in these instances removal of the bracts expose the ovules without any dissection of surrounding tissues. In addition, pollen tubes are not required to interact with intervening sporophytic structures en route to the conifer ovule. For these reasons, conifers are much more amenable to the study of pollen / ovule interactions than are angiosperms. Even so, the majority of studies on conifer pollination focus solely on the timing and techniques of pollen application for commercial seed production. Of the studies that do focus on pollen germination, tube growth, and egg fertilization, most are conducted by microscopic examination of fixed tissue.

All conifer families but one (Araucariaceae) contain members that employ a liquid secretion from the ovule at some point during pollination (Gelbart and von Aderkas 2002). The ubiquity of conifer ovular secretions suggests that they play an import role(s) in pollen collection and / or germination and pollen tube development. The contents of this single liquid secretion are to the conifer pollen grain what the secretions of the stigma, style, and ovule are to angiosperm pollen. Surprisingly, there are few reports pertaining to the biochemical compositions of these conifer secretions. In a handful of studies, sugar concentrations have been measured, amino acids have been detected, and the presence of proteins has been suggested (reviewed in Chapter 4).

The ovular secretions of larch and yew

The aim of my research has been to study the ovular secretions of two conifer species, hybrid larch (*Larix* x *marschlinsii*) and hybrid yew (*Taxus* x. *media*) to determine how the contents of these liquids affect conifer reproduction. The main focus of this work is on the proteinaceous content of the ovular secretions; however, this report begins with preliminary findings about the underlying whole-tree physiology that has been linked to the production of ovular secretions in the literature.

The primary hypotheses that I address with this research are:

H_1: Conifer ovular secretions are under developmental control and are not simply produced as a result of tree water status.

H_2: The ovular secretions of conifers are complex liquids containing proteins.

H_3: Proteins present in ovular secretions play a role in facilitating pollination and ensuring reproductive success.

H_4: Elucidation of the contents of conifer ovular secretions will provide insight into the pollination mechanisms and general reproduction of all seed plants.

Chapter 2

Literature review

The phylogeny of seed plants

The first seed plants were established more than 365 million years ago (Bateman *et al.* 1998). The evolution from spore-producing plants to seed-bearing plants occurred in two important stages. The first stage was the production of two distinct haploid spore types, the male gametophyte (pollen) and the female gametophyte. In the second stage, the female gametophyte-producing structures were retained on the parent sporophyte by enclosure of the reproductive stages within leaf-like structures (integuments) to form the first ovules (Graham *et al.* 2003). Of the three major groups of seed plants known to have existed; progymnosperms, gymnosperms, and angiosperms, only the later two groups contain extant species. Present-day gymnosperms comprise four groups of plants with "naked seeds": the conifers, cycads, *Ginkgo*, and the Gnetales. The angiosperms include the flowering plants, which are typified by ovules and seeds that are enclosed within tissues of an ovary, at the base of female floral structures; typically a stigma and style.

Whether the gymnosperms form a monophyletic group is a topic of much debate (Doyle 1998, Friedman and Floyd 2001). Morphological features including the type of wood vessels, the presence of net-veined leaves (in *Gnetum*), and the unisexual flower-like structures of the reproductive organs, have resulted in many phylogenies grouping Gnetales as a sister group to the angiosperms, distinct from the other gymnosperms (Donoghue and Doyle 2000). On the other hand, most phylogenetic analysis based on molecular data group Gnetales with the other gymnosperms (Bowe *et al.* 2000, Chaw *et*

al. 2000, Soltis *et al.* 2002), forming a monophyletic group. A few molecular studies continue to group the Gnetales with the angiosperms (Stefanovic *et al.* 1998) or find the placement of this group to be ambiguous (Rydin *et al.* 2002). In any case, there is little doubt that angiosperms are the most recently diverged of the seed plants.

By studying nucleotide substitutions in the plastid gene coding for the large subunit of RUBISCO (*rbcL*) and the nuclear gene coding for the small subunit of rRNA (*Rrn18*), and considering five distinct molecular clock calibrations, Savard *et al.* (1994) have placed the divergence of the five groups of extant seed plants (angiosperms, conifers, cycads, *Ginkgo* and Gnetales) from their common ancestor at 275 – 290 million years ago. The fossil record indicates that angiosperms underwent a major diversification in the Early Cretaceous period (130 – 90 million years ago) leading to the more than 250,000 extant species of flowering plants that now dominate many terrestrial environments (Crane *et al.* 1995). Extant gymnosperms in comparison are depauperate in species number. In round figures, there are 50 genera and 550 species of conifers, 10 genera and 100 species of cycads, 70 species within the Gnetales (including *Gnetum*, *Ephedra*, and *Welwitschia*), and a single *Ginkgo* species, *Ginkgo biloba* (Mauseth 2003). The limited gymnosperm diversity of today compared to angiosperm species can be attributed to the loss of gymnosperm species over time, and the slow rate of evolution and speciation of gymnosperms compared to angiosperms, particularly annuals (Levin and Wilson 1976, Bousquet *et al.* 1992).

Although angiosperms and gymnosperms are primarily differentiated by the status of their ovules, being either enclosed or naked, these groups also differ in other aspects of

their reproduction. Reproductive structures and mechanisms germane to this thesis are reviewed below.

Pollen tube development

Upon germination, pollen grains mobilize reserves of stored RNA, proteins, lipids, sugars, and small bioactive molecules allowing for the rapid development of a pollen tube (see Mascarenhas 1993). The pollen tube can be thought of as a straw with a dome-shaped apical tip that extends from the geminated pollen grain through the intervening tissues of the mother sporophyte to the megagetophyte. Within this developing tube, the contents of the generative cell (which contains the sperm) are carried forward by networks of actin filaments and microtubules (Romagnoli *et al.* 2003). The growth of the tube is polarized, expanding only from the leading tip. As the tube lengthens, callose walls are deposited behind the migrating cytoplasm, ensuring that the volume of the active cell remains relatively constant while an empty tube is left in its wake (Taylor and Hepler 1997, Hepler *et al.* 2001). This method of cell migration allows the transfer of the male generative nuclei through the tissues of the female sporophyte to arrive at the egg cell. Here, the tip of the pollen tube bursts, releasing the paternal contribution to fertilization.

In the growing tip of an angiosperm pollen tube, a characteristic organization occurs in which cytoskeletal elements maintain the relative position of the organelles within the cell (Pierson and Cresti 1992). Endoplasmic reticulum, golgi bodies, and mitochondria are most concentrated near the tip, immediately behind an area of vigorous cytoplasmic streaming. The streaming carries vesicles containing cell wall components

to the growing surface (Pierson *et al.* 1990, Franklin-Tong 1999). Pollen tube growth in flowering plants can be extremely rapid. Pollen tubes have been demonstrated to grow at rates of 45 μm/min in the style of lily (Jauh and Lord 1995) and an astounding 180 μm/min through the silks of maize (Barnabas and Fridvalszky 1984).

Compared to angiosperms, gymnosperm pollen exhibits characteristics that may be described as primitive, including slow germination and growth (Singh 1978). Pollen tubes of *Pinus sylvestris* cultured *in vitro* have been shown to grow at a rate of about 1 μm/h (de Win 1996). In a study of pollen from 14 species of gymnosperms incubated on basal medium for 72 hours, Yatomi *et al.* (2002) reported that pollen tubes ranged in length from 60 μm (*Ginkgo biloba*) to 210 μm (*Podocarpus macrophyllus*). The relatively slow growth rate of gymnosperm pollen tubes (when compared to angiosperms) has been attributed to the lack of zonal organization of organelles in the growing tip (de Win *et al.* 1996).

A second conspicuous difference between the pollen of angiosperms and gymnosperms is the material comprising the pollen tube wall. Angiosperm pollen tubes are bilayered, with a primarily pectin-containing outer layer, and a callose-reinforced inner layer (Steer and Steer 1989, Geitmann *et al.* 1995, Hasegawa *et al.* 2000). Gynmnosperms, in contrast, have pollen tube walls that are abundant in cellulose and arabinogalactan proteins (AGPs) (Mogami *et al.* 1999, Yatomi *et al.* 2002). Pectins are rare in conifer pollen tubes, but abundant in the tubes of *Cycas revolta* (Yatomi *et al.* 2002). AGPs are present in the in the cell walls of angiosperm pollen tubes, but to a lesser extent than in gymnosperms. Immunolocalization studies have demonstrated that these glycoproteins are deposited in regular annular patterns along the length of pollen

tubes of tobacco (Li *et al.* 1992, 1995). In *Lilium loniflorum*, AGPs have been observed in secretory vesicles at the tip of pollen tubes where they were demonstrated to play a critical role in tube growth (Jauh and Lord 1996, Roy *et al.* 1998). Yatomi *et al.* (2002) have suggested an evolutionary shift from pollen tube walls containing primarily AGPs and cellulose to walls comprised mainly of pectins and callose.

Angiosperm pollination

The angiosperm flower is a complex structure typically containing both male and female reproductive organs (stamens and gynoecia) and, with some exceptions, petals and sepals (Hasebe 1999). The general pattern of a typical flower consists of the stamens positioned peripherally to the pistil, which is composed of the stigma held away from the ovary by a style though which the pollen tubes must grow. Flowers of different angiosperm groups can usually be identified by variation in colour, number, and relative position of their flowering parts. From the time of Darwin (1862, cited in Gorelick 2001), to the present day, it is widely purported that the rapid and prolific diversification of angiosperms is due to co-evolution of these flowering plants with biotic (primarily insect) pollinators (Crepet 1983, Grimaldi 1999). There are lines of argument, however, which suggest that insect pollination was neither necessary nor sufficient to generate large numbers of angiosperm species, and other mechanisms must be invoked (reviewed in Gorelick 2001).

Besides playing a role in pollination strategies, the elaborate tissues of the angiosperm flower provide effective structures for the maternal sporophyte to screen and hold back incompatible or inferior pollen (Heslop-Harrison 1983, de Nettancourt 1997,

Wheeler *et al.* 2001, Herrero 2001). Higashiyama *et al.* (1998) reported that the pollen tube of *Torenia* would not interact with receptive embryo sacs *in vitro* unless the tubes had first passed through the tissues of the style. Herrero (2001) described the angiosperm pollen tube as having to pass through a series of "gates" or checkpoints in the style before it may approach an ovule. Presumably these checkpoints serve to ensure the fitness of the pollen. Pollen has also been shown to arrest at specific locations within the pistil only to resume growth when ovular development achieves a specific stage of maturity (Arbeloa and Herrero 1987, Herrero 2000).

Pollen binding to the stigma

Interaction between the pollen grain and the sporophytic tissues of the mother plant is initiated when the pollen arrives at the stigma. Pollen is desiccated and dormant upon arrival. For germination to be successful, two events must occur: the pollen grain must adhere to the stigma, and it must hydrate. Angiosperm stigmas are classed as either wet or dry, depending on whether a secretion is present on the receptive surface at the time of pollen arrival. Wet stigmas are typical of members of the Solanaceae, Leguminosae, and Orchidaceae, while dry stigmas are found in the Brassicaceae, Gramineae, and Compostitae (Zinke and Pruess 2000). Dry stigmas present an obvious obstacle to pollination; pollen grain adhesion is not indiscriminate.

In an early study on the mechanism by which pollen adheres to the dry stigma of *Brassica oleracea*, Stead *et al.* (1980) found that treatment of the stigmatic surface with proteases greatly reduces subsequent pollen grain adherence. Stigmatic function recovered fully within 90 minutes unless cycloheximide was applied to prevent protein

synthesis. These results provided the first evidence of stigmatic proteins being involved in pollen adherence. Further investigations with pollen mutants demonstrated that the pollen coats of Brassicaceous species contain components that are involved in mediating cell-cell interactions between the pollen grain and stigma (Doughty *et al.* 1993, Pruess *et al.* 1993, Dickinson 1995). Using transmission electron microscopy, Elleman and Dickinson (1986) had previously identified a superficial layer of the pollen exine they suggested is necessary for attachment to the stigma in *Brassica*.

Lipids and pollen hydration

On the dry stigma of *Arabidopsis*, pollen binding has been observed within a second after pollen deposition, before hydration of the grains (Zinkle *et al.* 1999). Rapid binding was found to be species-specific. Pollen from other plants, including a closely related *Brassica* species, failed to readily adhere to the *Arabidopsis* stigma. In itself, strong adherence does not ensure pollen germination. Pollen from *eceriferum* (*cer*) mutants of *Arabidopsis*, deficient in several long-chain lipids in their pollen coat (Preuss *et al.* 1993), can bind to wild-type stigmas, but they will not hydrate or germinate (Zinkle *et al.* 1998, 1999). With sufficient ambient humidity, *cer* pollen can be hydrated *in vivo*, regaining fertility. The *cer* mutants appear only to lack the ability to trigger hydration from the stigma (Hulskamp *et al.* 1995b).

A second line of *Arabidopsis* male sterile mutants that fail to bind the stigma are known as *lap* mutants (*less adherent pollen*). They possess gross defects in the exine, suggesting that pollen form is also important for binding (Zinkle and Preuss 2000). However, *lap* mutants do not exhibit reduced fertility when artificially bound to the

stigma, providing further evidence that later processes such as hydration and germination are independent of pollen binding in *Arabidopsis* (Zinkle and Preuss 2000).

In contrast to dry stigmas, flowers possessing a wet stigma are characteristically unable to discriminate. Pollen adherence is general (Zinkle *et al.* 1999, Zinkle and Preuss 2000). Arriving at a so-called wet stigma does not ensure pollen hydration. Numerous studies have identified long-chain lipids necessary for regulating pollen hydration. The *pop1* line of *Arabidopsis* pollen mutants, also deficient in lipid biosynthesis, fail to initiate pollen hydration (Preuss *et al.* 1993, Hulskamp *et al.* 1995b). Wolters-Arts *et al.* (1998) have demonstrated that the application of the *cis*-unsaturated triacylglyceride trilinolein can rescue *cer* and *pop1* pollen mutants, allowing hydration on the stigma surface.

Species of *Nicotiana*, a genus possessing a wet stigma, supply the lipids necessary for pollen hydration in their stigmatic exudates (Goldman *et al.* 1994, Wolters-Arts *et al.* 1998). It has been postulated that directional germination and growth of the pollen tube towards the stigmatic papillar cells of *Nicotiana* is controlled by the directional flow of free water through the lipid matrix of the stigmatic secretion (Wolters-Arts *et al.* 1998). This theory was upheld by experimentation with an artificial stigma surface in which directional pollen tube growth occurred at the interface between various lipids and an aqueous medium (Lush *et al.* 1998, 2000). More recently, however, Wolters-Arts *et al.* (2002) were unable to detect sufficient free water in the lipid matrix of tobacco exudates to account for pollen grain hydration. The authors now suggest that water is passed by direct contact between the pollen grain and the stigma, or from pollen grain to pollen grain. Nonetheless, there is little doubt that lipids are necessary for pollen hydration to

occur, even when the grain is in contact with the stigma (Preuss *et al.* 1993, Wolters-Arts *et al.* 1998, Zinkle *et al.* 1999). It is possible that lipids trigger hydration by affecting the permeability of the pollen grain and/or the stigma cuticle (Lolle *et al.* 1997, 1998).

Non-specific lipid transfer-like proteins on the stigma

Lipids are not the only molecules found to affect pollen germination and growth on the stigma. A small (9 kDA) basic protein named SCA (for stigma/stylar cysteine-rich adhesin) is abundantly produced on the lily stigma (Park and Lord 2003). This protein was originally discovered in the extracellular matrix (ECM) of cells lining the style, where it is involved in the binding of pollen tubes (Park *et al.* 2000). The cDNA sequence of SCA shares identity with non-specific lipid transfer proteins from other plant species (Park and Lord 2003). SCA protein has been found within lily pollen tubes grown *in vivo*, but only occurs in cultured tubes when supplied exogenously (Park *et al.* 2000). SCA mRNA is not found in the pollen tube, suggesting that the protein is imported into the growing tube from the surrounding female tissues (Park and Lord 2003). Although SCA on its own plays a role in pollen tube guidance, it was also found to potentiate the ability of chemocyanin, a recently discovered chemotropic molecule, to direct pollen tube growth (Kim *et al.* 2003).

Pollen tube development in the style

After germination, the pollen tube must make its way down the style towards the ovary. Styles are of two kinds - hollow or solid. In species with hollow styles, the pollen tube travels to the centre of the stigma, enters the funnel-like aperture of the style, and

travels down the style. It grows through the ECM of secretory cells lining the hollow lumen. In species that possess a solid style, the pollen tube pushes its way between the stigmatic papillar cells and enters the transmitting tract of the style beneath (Cresti *et al.* 1986). The transmitting tract is composed of cell files that secrete ECM abundantly into the apoplast.

Whether pollen germinates on a wet or dry stigma, or traverses a solid or hollow style, all pollen growth through these tissues is extracellular (Cheung 1996). There is no penetration of the cells of the sporophyte. The angiosperm pollen tube is the most rapidly growing plant cell known (Taylor and Hepler 1997). However, it is well established that pollen tubes are not able to achieve their extraordinary growth rates on germination medium, suggesting that there is a contribution from the tissues of the style that promotes pollen tube development *in planta* (Jauh and Lord 1995, Lord 2003). There is a wide range in floral structures and pollination mechanisms and no single model can unify the various pollen-style interactions. The two main research initiatives that are providing insight into this phenomenon focus on the solid style of tobacco and the hollow style of lily.

Arabinogalactan proteins and pollen tube growth in the style of tobacco

There is growing evidence that the sporophytic tissue of the tobacco style influences pollen tube development primarily through glycoproteins found exclusively in the transmitting tract (Cheung 1996, Wu *et al.* 2000). These glycoproteins, known as transmitting tissue-specific (TTS) proteins (Cheung *et al.* 1993, Wang *et al.* 1993), are members of the more widely expressed plant arabinogalactan protein (AGP) family

(Cheung and Wu 1999, Showalter 2001). The genes that encode the two AGPs restricted to the tobacco transmitting tract (*TTS-1* and *TTS-2*) share a high degree of sequence identity (Cheung *et al.* 1993). These genes code for proteins with predicted molecular weights of approximately 28 kDa, but extensive glycosylation of the peptide backbones results in apparent molecular weights ranging from 45 – 105 kDa as determined by gel electrophoresis (Wang *et al.* 1993). When added to artificial growth medium, TTS proteins have been demonstrated to promote pollen tube growth (Cheung *et al.* 1995). In the same study, transgenic *N. tabacum* plants that had their normal TTS levels reduced showed impaired pollen tube growth rate and reduced female fertility. The authors concluded that TTS proteins are important for the maintenance of rapid pollen tube growth and delivery of the sperm cells to the waiting eggs before stylar abscission or ovular degeneration occur. This process may be mediated by pollen enzymes that deglycosylate TTS proteins (Wu *et al.* 1995). The deglycosylated proteins are then incorporated into the growing tube wall (Wu *et al.* 1995, Cheung *et al.* 1995). The bound protein, and the sugar molecules that are liberated from it, might provide a readily available source of material and metabolites for growing pollen tubes.

TTS proteins may play multiple roles in successful pollination. Wu *et al.* (1995) described an increasing gradient of TTS glycosylation from the base of the tobacco stigma to the ovary. In a separate study, pollen tubes exiting the base of a cut style onto an agarose medium were found to migrate toward plugs containing TTS protein (Cheung *et al.* 1995). Taken together, these findings led the authors to suggest that TTS proteins play a role in guiding pollen tubes to the ovule in a gradient-directed manner. By deglycosylating TTS proteins as they encounter them in the style, pollen tubes would

constantly re-sharpen the local glycosylation gradient (Cheung *et al.* 2000). TTS proteins have been identified more recently in *N. sylvestris* (Cheung and Wu 1999) and *N. alata* (Wu *et al.* 2000). In these species, they are reported to have the same effects on pollen tube growth as they do in *N. tabacum*.

SCA and pollen tube growth in the style of lily

The second model of pollen tube guidance, based on the hollow lily style, relies on matrix adhesion-driven (haptotactic) signals to guide pollen grains from the stigma to the ovary (Sanders and Lord 1992, Lord 2000). Put simply, the pollen is not guided by a chemical attractant but behaves like a locomotive barrelling down a preformed set of rails. The origin of this model was the observation that inert latex beads could be translocated down hollow styles in a manner similar to pollen tubes (Sanders and Lord 1989). However, as in the *Nicotiana* system, pollen tube growth rates for lily are greater *in planta* than they are *in vitro*, indicating that there are stylar factors interacting with the developing pollen tube and promoting elongation (Jauh and Lord 1995, 1996).

To isolate these factors, bioassays were developed. Pollen tube growth studies carried out on an artificial matrix supplemented with components of the lily stylar ECM identified two components required for proper pollen tube adhesion and growth rate; the SCA protein (discussed above) and a large molecular weight stylar pectin (Park *et al.* 2000, Mollet *et al.* 2000). Immunogold localization determined that SCA is present in the stylar epidermis bordering the transmitting tract, the ECM of the tract, and in the walls of *in planta* pollen tubes (Park *et al.* 2000). SCA is not found in pollen grown on artificial medium. SCA mRNA, which is plentiful in the tissues of the lily style, is absent

in both *in vitro* and *in vivo* grown pollen tubes (Park and Lord 2003). These findings indicate that SCA is secreted by the transmitting tract, but not pollen. SCA appears to mediate contact between the pectins of the tract ECM and the walls of pollen tubes passing by. Whether SCA only binds to the surface or enters pollen tubes *in planta* is not known. No pollen tube receptors have been identified, but researchers are searching for a lily ortholog for LePRK2, a receptor kinase found in the plasma membrane and cell wall of the tomato pollen tube (Muschietti *et al.* 1998). LePRK2 is a serine/threonine kinase with an extracellular domain containing a leucine-rich repeat, a motif that is thought to be involved in protein-protein interactions (Kobe and Deisenhofer 1994). This receptor is of particular interest because it is dephosphorylated in response to stylar extracts (Muschietti *et al.* 1998). It is also believed to interact with LAT52, a tomato protein similar in sequence to SCA (Tang *et al.* 2002, Johnson and Preuss 2003).

Pollen tube development in the angiosperm ovary

When developing pollen tubes reach the base of the style they leave the nutrient-rich transmitting tract and enter the third major zone of the pistil, the ovary. The ovary is typically hollow. The ovules within are often held away from the inner placental wall by the funiculi. A pollen tube must track the placental surface to a funiculus, and then grow across this tissue bridge to enter an ovule and deliver its sperm. Most authors agree that pollen tubes are directed in the ovary by chemotactic signals originating both from sporophytic tissues and the megagametophytes themselves (Herrero 2001, Johnson and Preuss 2002, Willemse and van Lammeren 2002, Higashiyama *et al.* 2003, Lord 2003).

Pollen tube growth in the ovaries of wild-type *Arabidopsis* displays a high degree of organization and directionality. The first pollen tubes to emerge from the transmitting tract tend to approach ovules closest to the style, while subsequent pollen tubes approach ovules that are more basally located (Hulskamp *et al.* 1995a). Roughly 40 percent of pollen tubes are found to grow towards the first available ovule, avoiding ovules that have already interacted with a pollen tube (Hulskamp *et al.* 1995a). The fact that only a single pollen tube will approach the funiculus of each ovule suggests that the ovule itself has a method of attracting one tube, while deterring subsequent ones (Shimizu and Okada 2000). The aim and timing of the pollen tube shows impeccable fidelity, always penetrating one of the two synergid cells at the micropylar end of the embryo sac immediately prior to sperm release (reviewed in Higashiyama *et al.* 2003).

Most studies of *Arabidopsis* ovaries with reproductive mutations agree that there are at least two signals (one derived from the female gametophyte and the other from the maternal sporophyte) responsible for pollen tube guidance (Hulskamp *et al.* 1995a, Ray *et al.* 1997, Baker *et al.* 1997, Shimizu and Okada 2000). This level of signalling is necessary to maximize seed set in species with multiple ovules per ovary. No candidate molecules have yet been identified to fulfill these signalling roles. Ionic calcium (Ca^{2+}), which has long been implicated in pollen tube guidance to the ovule (Mascarenhas and Machlis 1962) and specifically to the synergid cells (Jensen 1965), has been eliminated as the possible female gametophytic signal in *Torenia* (Higashiyama *et al.* 2003). Researchers are currently looking for a molecule, possibly a peptide, synthesized and secreted by the synergid cells as the agent of chemotaxis at the micropyle (Higashiyama 2002).

After passing through the hollow micropyle leading into the ovule, and prior to penetrating the embryo sac, pollen tubes traverse the final layer of sporophytic tissue, the nucellus. The nucellus is a diploid structure that gives rise to the megagametophye during ovular development, and in most cases persists as a layer of cells surrounding the mature embryo sac (Russell 2001). Except as a medium for passing products of the synergid cells into the micropyle (Tilton 1980, Franssen-Verheijen and Willemse 1993), no role is reported for the angiosperm nucellus in pollen signalling.

The events that direct pollen tube development in the angiosperm ovary continue to elude researchers.

Conifer pollination

In contrast to the angiosperms, pollen germination and pollen tube growth are, with few exceptions, entirely ovular events in conifers. Conifer species are wind pollinated and rely on massive pollen production to carry sufficient amounts to their naked ovules for satisfactory seed set (Dogra 1964). The pollen capturing mechanisms of many conifer species employ a liquid secretion that originates from within the ovule, contacts pollen grains, and transports the pollen into the micropyle for germination.

Ovular secretions are obvious in species with pollination droplets that accept pollen directly (e.g. *Taxus* and *Podocarpus*). In these trees, a liquid projects beyond the tip of the micropyle and collects pollen either from the air or from surfaces immediately adjacent to the micropyle (Anderson and Owens 2000, Tomlinson *et al.* 1991, 1997). When pollen enters the secretion, it either sinks downward or floats upward into the micropyle depending on the orientation of the ovule and whether or not the pollen is

saccate and buoyant (Tomlinson 1994, Owens *et al.* 1998, Gelbart and von Aderkas 2002). In some cases, pollen is carried into the micropyle by retraction of the droplet. Anderson and Owens (2000) reported that hand pollination of *Taxus brevifolia* resulted in droplet withdrawal within 30 minutes, with no further droplet production from pollinated ovules. A similar response to pollen was reported for the drop of *Phyllocladus*, but not for members of the Podocarpaceae, which may re-exude a pollination drop numerous times - even after initial pollen collection (Tomlinson *et al.* 1997). The active mechanism that triggers drop retraction remains unknown.

In *Pinus* and *Picea*, pollen grains are captured by sticky extensions of the integument. A pollination drop subsequently emerges from the micropyle, contacts the captured pollen and recedes, depositing the grains onto the nucellus where they germinate (Doyle and O'Leary 1935a, McWilliam 1958, Owens *et al.* 1987). Here too, retraction of the liquid may be triggered by the presence of pollen.

In *Larix* and *Pseudotsuga*, pollen grains are not taken inside the ovule by a liquid, but by mechanical force. Pollen is collected on sticky hairs borne on a large flap of the integument. This flap eventually folds inward, carrying the captured pollen into the micropyle (Doyle 1945, Owens and Molder 1979, Owens *et al.* 1981). An ovular secretion (postpollination / prefertilization drop) enters the micropylar chamber 5-6 weeks later in *Larix* (Owens *et al.* 1994), and 7-9 weeks post engulfment in *Pseudotsuga* (von Aderkas and Leary 1999a). These secretions fill the chamber, make contact with the pollen grains at the apex of the micropyle and then recede, carrying the pollen toward the nucellus (Doyle and O'Leary 1935b, Barner and Christiansen 1960, Barner and Christiansen 1962).

The pollination mechanisms of *Taxus* and *Larix* are described here in further detail, as members of these two genera are the primary focus of the following chapters.

The pollination mechanism of *Taxus*

In common with most gymnosperms, pollen enters yew ovules via pollination droplets (Gelbart and von Aderkas 2002). Pollination and early embryological events have been described in a number of *Taxus* species including: *T. baccata* L. (Pennel and Bell 1987, 1988), *T. brevifolia* Nutt. (Anderson and Owens 1999, 2000), *T. Canadensis* Marshall (Dupler 1917), *T. chinensis* Pilger (Xing *et al.* 2000), and *T. cuspidata* Siebold & Zucc. (Sterling 1948). Pollination in *T.* x *media* occurs in a manner similar to the other members of its genus. During a two-week period coincident with anthesis, ovules exude a conspicuous pollination droplet. The volume of this droplet is approximately 250 nL (Seridi-Benkaddour and Chesnoy 1988). As ovules on a branch may vary in orientation - some are upright, others point to the side or are inverted – pollen enters the micropylar chamber by sinking into the drop in the first instance, or by co-transport with the retracting fluid in the second (Anderson and Owens 2000, Xing *et al.* 2000).

In spite of the numerous studies of yew in which droplet production has been reported, neither the process nor the origin of the secretion has been established. Ziegler (1959) used metabolic toxins to kill ovular tissues, but this did not prevent droplet production. He concluded that secretion was not an active process, but a physical phenomenon driven by gradients in osmolarity and local atmospheric vapour pressure deficit. In contrast, most yew embryologists consider the pollination droplet to be a result of an active secretion. Anderson and Owens (2000) reported that individual ovules of *T.*

brevifolia produced pollination drops for up to two weeks with maximum volume observed in the early morning. Hand pollination brought about droplet retraction within 30 minutes, with no subsequent production from pollinated ovules.

A cross section of the yew ovule at the stage of pollination droplet production is depicted in Figure 2.1. During droplet secretion, the outermost nucellar cells disintegrate, forming an irregular margin where pollen grains eventually become lodged and germinate (Dupler 1917, Sterling 1948). Pollen tube growth may reach the mid-nucellar region of an ovule within ten days of germination (Dupler 1917, Anderson and Owens 1999). Having reached the middle nucellus, pollen tubes pause in their development until the megagametophytes are sufficiently mature for fertilization. Megagametophyte maturity varies widely, with the range of developmental stages including undifferentiated sporogeneous tissue, megaspore mother cells, free nuclear megagametophytes, or cellular megagaetophytes (Sterling 1948, Anderson and Owens 1999). Fertilization does not occur for at least a month following pollen arrival (Dupler 1917, Sterling 1948). The resumption of pollen growth appears to require a signal, but no analytical work has been undertaken to date.

The pollination mechanism of *Larix*

Larix species require a secretion from the ovule to complete pollination, but this secretion is developmentally delayed compared to other conifers. *Larix* ovules are not fully exposed like the ovules of *Taxus*; instead they are found within a female strobilus, or cone, which is composed of closely arranged bracts. Two ovules are present at the base of each ovuliferous scale, which is borne on a bract (Doyle and O'Leary 1935b).

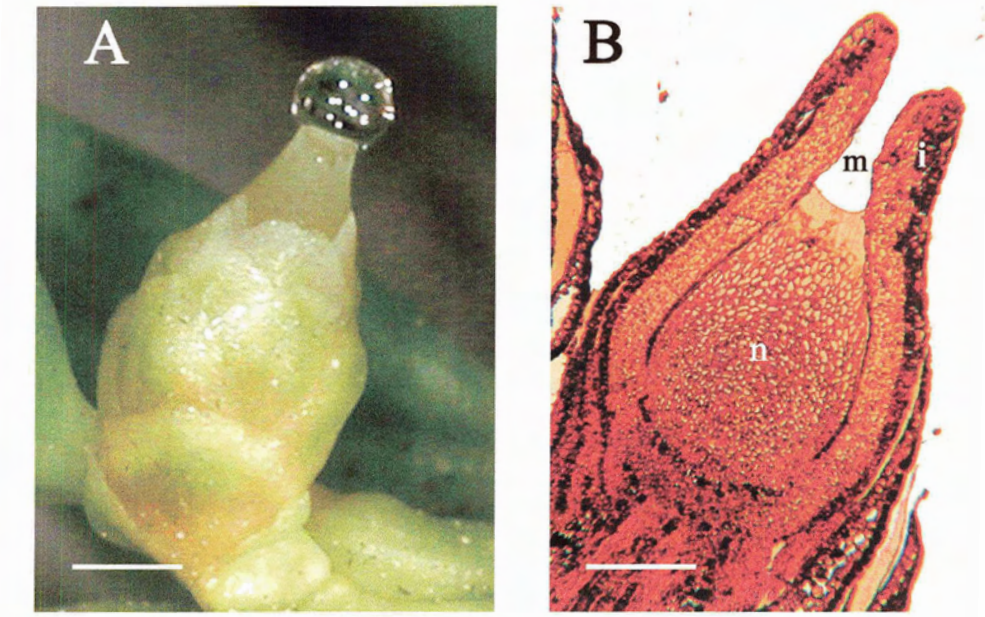


Figure 2.1. The ovule of *Taxus* x *media* during pollination droplet production.

A. A yew ovule exuding a pollination droplet. Bar = 500 μm.

B. Longitudinal section through a yew ovule fixed at the time of pollination droplet production. Section is stained with Safranin-O and post-stained with iodine/potassium iodide. The micropyle (m), undifferentiated nucellus (n), and integument (i) are indicated. Bar = 250 μm.

Larix species are monecious. Consequently, female cones may receive pollen from the same tree or from nearby trees. Aerially borne pollen sifts through the bracts of the cone toward the base of the ovuliferous scales. Here the pollen is collected by the sticky projections of a large outgrowth of integument tissue that forms an open flap near the micropyle (Doyle 1945). This flap remains receptive for several days collecting pollen grains. As the ovule continues to develop, the outer cells of the flap elongate more than the inner cells, causing the hairy tip to grow into the micropyle - effectively engulfing the pollen grains and closing the ovule (Owens *et al.* 1994). The structure of the closed *Larix* ovule is depicted in Figure 2.2. Captured pollen grains remain ungerminated until the ovule produces a secretion, 5 – 6 weeks after pollen capture (Barner and Christiansen 1960, Owens *et al.* 1994). In the sense that it occurs after pollen collection, the larch secretion is considered a post-pollination phenomenon (Villar *et al.* 1984).

The postpollination droplet of *Larix* appears only after the cone has closed. *In planta* the liquid does not exude beyond the apex of the micropyle, which is already closed by the infolded hairs (Said *et al.* 1991, von Aderkas and Leary 1999b). Dissection of the cone and placement of the ovule-bearing bracts into a humid environment prompts an exudation of liquid from within the ovule that breaches the micropyle and forms an external droplet (Barner and Christiansen 1960). This liquid has been shown to be continuous with the internal ovular secretion (von Aderkas and Leary 1999b).

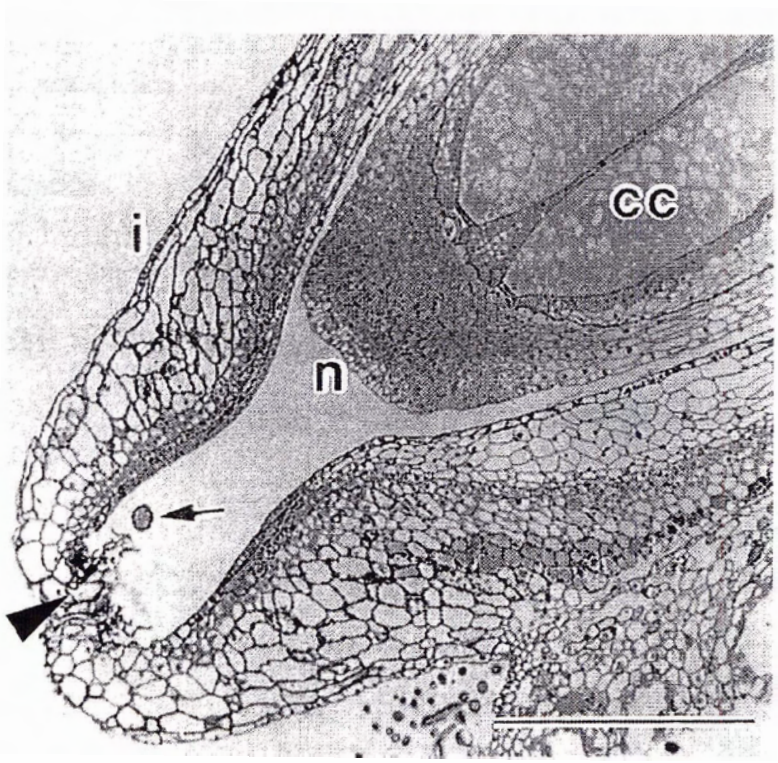


Figure 2.2. The structure of the closed *Larix* ovule. A light micrograph depicting the micropylar region of a longitudinal section through a closed ovule of *Larix* x. *eurolepis*. A central cell (cc), the integument (i) and the nucellus (n) are indicated. A pollen grain (arrow) was brought into the micropyle by the collapsed stigmatic flap (Arrowhead). Bar = 500 μm. Adapted from Takaso and Owens (1997).

In the ovule, the secretion plays an active role in the transport of pollen grains from the distal end of the micropylar canal to the surface of the nucellus (Doyle and O'Leary 1935b, Takaso and Owens 1997). Very shortly following droplet production, the pollen germinates and each grain develops a tube to penetrate the nucellus and enter an archegonium. Fertilization is complete 6 – 8 weeks after pollen collection (Owens and Molder 1979).

Besides its transport function, the postpollination droplet is also suspected of having a role in freeing pollen from the infolded sticky hairs of the integument flap (Barner and Christiansen 1960) and releasing the protective exine coat from the grains (Takaso and Owens 1994). Villar *et al.* (1984) suggest that the secretion may signal the end of pollen dormancy, coinciding with the last developmental stage of the maturing egg.

Conclusions

The transport of male gametes to the egg cells is a critically important step in the reproduction of seed plants. Coordination of the mechanisms that lead to fertilization is vital to the fitness of a plant species. In most conifers, an ovular secretion is present that plays a role in transporting pollen to the nucellus and initiating germination. There is little information about the composition of this liquid in the literature. The aim of this thesis is to provide insights into the physiology and biochemistry of these liquids. The possible functions of the constituents of these liquids will be elucidated. These findings will be placed into the context of conifer reproduction in particular, and the evolution of seed plant pollination mechanisms in general.

Chapter 3

Postpollination droplet production in *Larix* x *marschlinsii* in relation to the diurnal pattern of xylem water potential

Introduction

Larch trees require a secretion from the ovule to complete gamete delivery. This secretion is developmentally delayed compared with those of other conifers. Postpollination droplets of larch appear only after the cones have closed. *In vivo* the liquid does not exude past the apex of the micropyle, which is partially sealed by the infolded stigmatic hairs (Said *et al.* 1991, Owens *et al.* 1994, von Aderkas and Leary 1999b).

Very little is known about the environmental and physiological conditions that affect the production of ovular secretions in any conifer. Although Takaso *et al.* (1996) suggest that the origin of the *Pseudotsuga* postpollination drop may be the megagametophyte, most consider the nucellus to be the source of the ovular secretion in other species (Gelbart and von Aderkas 2002). No satisfactory mechanism has been put forward to explain how the drop is secreted. Some authors have reported that the secretions follow a diurnal pattern in which the liquid is present at night and absent during the day. McWilliam (1958) observed that the pollination drop of Austrian pine (*Pinus nigra*) was exuded at night and withdrawn during the daytime over a period of 5 days. In their study of eight *Larix* species, Barner and Christiansen (1960) found that there is no day and night mechanism *per se* in this genus, but instead hypothesized that high and low "sap pressure", respectively, cause the postpollination drop to exude and retract within the micropyle. The authors suggested that high "sap pressure" occurs when transpiration within a tree is low - at night or on rainy or humid days. Owens *et al.*

(1980) described an "approximate diurnal" rhythm of pollination droplet appearance and withdrawal for yellow cypress (*Chamaecyparis nootkatensis*). Although they did not describe a mechanism to account for the droplet exudation and retraction, the authors suggested that humidity is an important factor in this process. In contrast, Owens *et al.* (1987) found no diurnal pattern in droplet production for potted Engelmann spruce (*Picea engelmannii*) under controlled environmental conditions.

It is likely that conditions that diminish droplet formation may result in poor transfer of pollen through the micropylar chamber, decreased pollen germination, and ultimately, reduced fertilization rates. Villar *et al.* (1984) have suggested that insufficient droplet production may be partially responsible for the poor seed set often observed in *Larix* species (Owens and Molder 1979, Kosiński 1986, Owens *et al.* 1994). Identifying the conditions under which postpollination droplet production is favoured may be key to improving seed production in these trees. The objective of this study was to determine whether there is a relationship between diurnal fluctuations in xylem water potential and the appearance of the *Larix* postpollination secretion.

Methods and Materials

Plant material

Three mature hybrid larch trees (*Larix* x *marschlinsii* Coaz) growing openly on the campus of the University of Victoria (British Columbia, Canada) were used for this study. These trees were watered nightly by an automated sprinkler system and did not experience water stress.

Measurement of xylem water potential

Xylem water potential was measured hourly in 3 sample branches per tree and averaged. The branches were selected randomly at breast height from around the trees. A Scholander-type pressure chamber (Soilmoisture Equipment Corp. model 3005) supplied with nitrogen gas was used to take measurements in the field. When a branch tip (10 – 15 cm) was clipped from a tree it was immediately sealed in the airtight chamber with only the cut surface of the stem remaining outside, secured by a snug rubber grommet. The branch was pressurized until xylem sap was seen on the cut surface of the exposed stem. The pressure required to overcome the xylem tension of the branch stem was recorded.

Xylem water potential was monitored hourly over a period of 24 h at two different times of year. One monitoring period was carried out during the time of ovular secretion and measurements were taken simultaneously with droplet collection (May 12, 2000). Xylem water potential was also monitored in late summer (August 18, 1999), allowing a comparison of the diurnal patterns of the trees' water status after the reproductive period had passed.

Ovular Secretions

Three seed cones were removed from each of the three trees at the beginning of every hour over a 24 h period. The cones were selected randomly at breast height from around the trees. Within ten min of collection, the cones were taken to a nearby laboratory where they were dissected according to von Aderkas and Leary (1999b). The ovuliferous scale/bract complexes were placed in petri dishes kept humid with moistened

filter paper. Ovules (n = 100) were sampled from each tree every hour. Drops that were exuded from the tips of the micropyles were counted. The number of drops produced per 100 ovules was recorded on an hourly basis plus or minus the standard error of the proportion [s.e. = $(p*q/(n-1))^{1/2}$].

The droplets produced by the ovules of a given tree were collected at each hour with a 10 μL micropipette tip and pooled in pre-weighed centrifuge tubes. The tubes (containing the drops) were again weighed with an ATI Cahn (model C-44) microbalance and the mass of the droplets per 100 ovules was recorded to the nearest microgram. An overall mean droplet mass was calculated for each tree. This was done by summing the total mass of droplets collected from a tree over the 24 h period and dividing this by the number of droplets the tree produced over that time.

Droplet collection dates occurred during the peak production period of two years (May 25, 1999 and May 12, 2000). The second year, collection was carried out simultaneously with 24 h measurements of the xylem water potential of the three trees.

Statistics

SPSS software was used to conduct a two-way ANOVA analysis to determine if there was significant difference in droplet mass between trees and between years. Change in droplet mass between years was tested separately for each tree using independent sample t-tests. One-way ANOVA followed by a Student-Newman-Keul post hoc test was employed to test for significant droplet mass differences between the three trees in either year.

Results

On both sampling dates all three hybrid larch trees showed a typical diurnal pattern of xylem water potential (Figures 3.1a&b). Water potential began to drop around 0700 h and became increasingly negative throughout the morning. By 1100 h, water potential measurements approached their lowest values and levelled off. In the late afternoon, the water potential of the trees began to rise again. The pattern of xylem water potential was similar for the three trees between both sampling dates. Overall, water potential was consistently more negative during the 24 h period monitored in August. Mean water potential of the three trees measured at noon and 0300 h during late summer ranged from –1.40 to –1.63 MPa, and –0.65 to –0.71 MPa respectively (Figure 3.1a). Xylem water potential measured at the same times of day for the three trees during droplet production season (in May) ranged from –1.15 to –1.40 MPa, and –0.43 to –0.67 MPa (Figure 3.1b).

Patterns of droplet production were not consistent from tree to tree or from year to year (Figure 3.2). The first year (1999), tree 1 showed an erratic pattern of droplet production. The number of ovules actively secreting droplets was found to rise and fall from 22 – 77 % over the 24 h period. Tree 2 showed a diurnal pattern of droplet production, with 15 – 58 % of ovules producing droplets at night and none producing during the day. For tree 3, droplet production remained consistently high with 65 – 90 % of the ovules producing droplets throughout the 24 h period.

In the second reproductive season (2000), tree 3 again showed consistently high postpollination droplet production over the course of the 24 h monitoring period (Figure 3.2). The percent ovule production of 53 – 92 % throughout the 24 h was similar to the

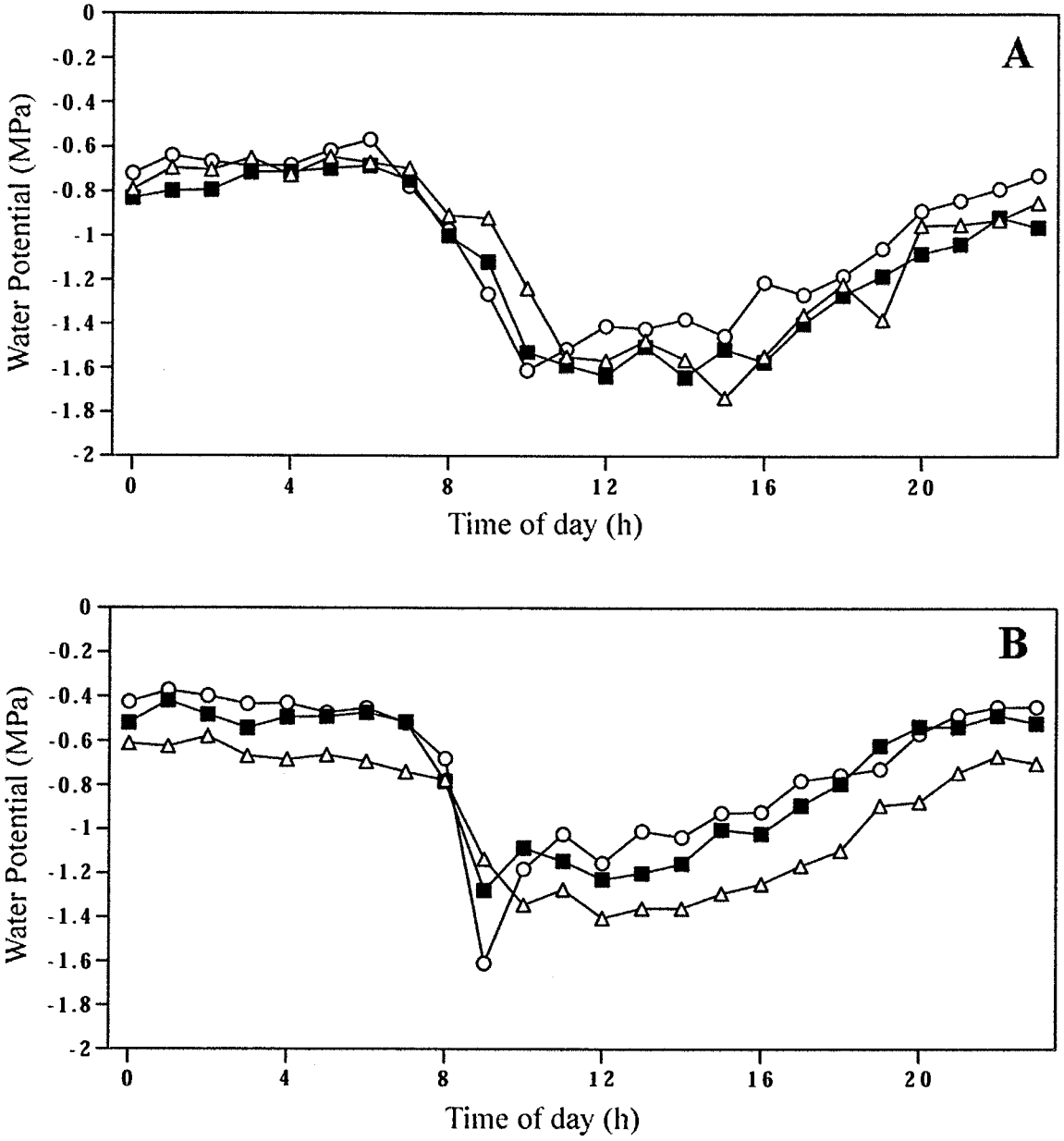


Figure 3.1. Daily course of xylem water tension measured in stem cuttings of hybrid larch trees. A. Measurements taken during late summer (August 18, 1999). B. Measurements taken during the period of postpollination droplet production (May 12, 2000). Open triangle – tree 1, filled square – tree 2, open circle – tree 3.

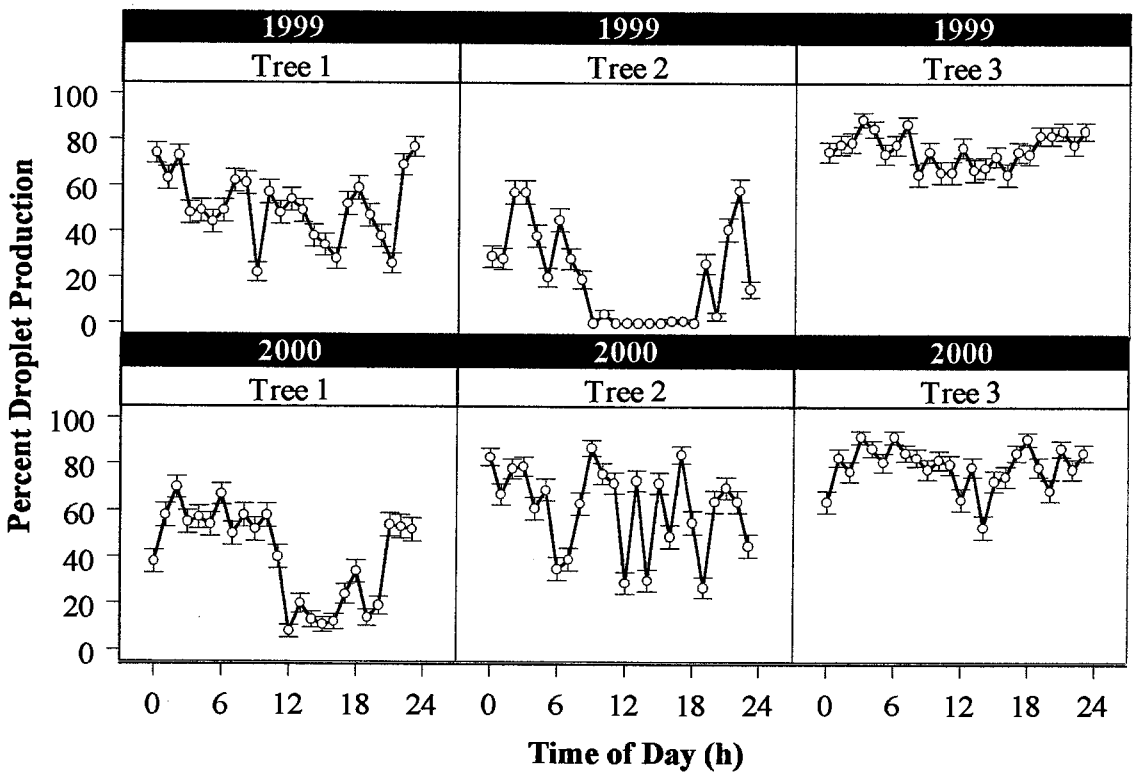


Figure 3.2. Postpollination droplet production in hybrid larch trees over a 24 h period. Production is presented as the number of ovules per 100 that produced a droplet at each hour (± the standard error of the proportion). Droplets were counted during peak production; May 25, 1999 and May 12, 2000.

range of measurements in the previous year. The patterns of droplet production in trees 1 and 2 differed form those of the previous year. In the second pollination season, tree 2 did not restrict its droplet production to the nighttime. Droplet production for this tree was much higher than it had been the previous year with 27 – 96 % of the ovules producing droplets at any given hour, and no discernable pattern of production observed. The droplet production of tree 1 was much reduced in the afternoon and evening (1200 – 2000 h) compared with the previous year.

Mean droplet mass varied between trees, and for two of the trees between years (Table 3.1). Not only did tree 3 produce more postpollination droplets per 100 ovules than the other two trees, its mean droplet mass was also significantly larger ($p<0.001$) than those of the others during both seasons. The mean droplet masses of tree 1 and tree 2 were not significantly different from each other in either year. Tree 3 produced larger droplets in the first year than in the second. Tree 2 produced larger droplets in the second year than in the first. Mean droplet mass did not change significantly between the two years for tree 1.

Table 3.1. Mean mass of postpollination droplets for hybrid larch trees during the sampling dates in 1999 and 2000. Means are reported ± standard error.

	Mean Mass (μg) of Droplet (± s.e.)	
	1999	2000
Tree 1	21.6 ± 1.2* [a]	19.9 ± 1.5* [a]
Tree 2	17.1 ± 1.6* [a]	24.5 ± 1.4* [b]
Tree 3	76.2 ± 2.0** [a]	57.4 ± 3.0** [b]

A difference in asterisk number indicates a significant difference in droplet mass between trees within a year ($p<0.001$). A difference in superscripted letter indicates a significant difference in droplet mass for a given tree between years ($p<0.001$).

Discussion

The results of this study refute the notion proposed by Barner and Christiansen (1960) that the production of the postpollination droplet of *Larix* corresponds to the "sap pressure" of the tree. In the case of these test trees, the diurnal pattern of xylem water potential is consistent; water potential falls during the day and rises at night. This is true of all three trees during the period of ovular secretion and at a point three months later in the growing season. The hourly xylem water potential measurements and the pattern of diurnal fluctuation of the experimental trees appear to be typical for larch. The measurements taken in this study agree very closely with those reported by Schulze *et al.* (1985) from hybrid larch trees growing in a mixed *Larix* forest under natural environmental conditions.

In general, droplet production showed no diurnal rhythm. Postpollination droplet production did not parallel the predictable rise and fall of xylem water potential in the three trees during either of the monitoring periods. Trees 1 and 2 appeared to follow a diurnal pattern of droplet production once each between the two years.

These findings contradict certain processes that have previously been suggested to regulate the formation of the ovular droplet. Because droplets are found in the micropyles of *Larix* at all times of the day, and the days that droplet production was monitored were sunny and hot, I must agree with Barner and Christiansen (1960) that droplet production in *Larix* is not a result of guttation, as McWilliam (1958) proposed for pine. However, Barner and Christiansen's hypothesis that it is "sap pressure" (hydraulic pressure) that governs the exudation of the postpollination droplet in *Larix* is also incorrect. If this were the case, droplet production would follow the water status of the

tree and a diurnal pattern would be evident in the data. Owens *et al.* (1980) described an "approximate diurnal" rhythm of droplet production from unpollinated ovules of *Chamaecyparis*. They reported a switch from a diurnal production of droplets to a sustained production, and then back to a diurnal pattern before cone closure. The three test *Larix* trees have not shown such a switch in pattern during a reproductive season over the five years for which droplet production has been closely monitored.

In *Larix*, regulation of the postpollination droplet may not be related to the water status of the tree. Trees that had an unpredictable pattern of secretion had a typical daily pattern of water potential (e.g., tree 1 in 1999 and tree 2 in 2000). Thus, it would appear that the ability of the ovule to secrete a liquid is not controlled by the xylem water potential of the tree branch, but is under the regulation of a more local structure such as the cone, or perhaps the ovule itself. In *Picea*, ovules were found to form droplets in an acropetal wave (Owens *et al.* 1987, Runions *et al.* 1995). In a number of species, active re-absorption of the pollination drop, or immediate cessation of droplet production has been observed upon introduction of pollen into the drop of one ovule, while the secretion of a neighbouring ovule remained unaffected (e.g. *Picea* - Owens *et al.* 1987, *Pinus* - Doyle and O'Leary 1935a, McWilliam 1958, *Chamaecyparis* - Owens *et al.* 1980, *Phyllocladus* - Tomlinson *et al.* 1997). These activities must be controlled on an ovule-to-ovule basis, strengthening my general argument that the secretion is not produced in response to the physiology of the whole tree - as is widely accepted in the literature without any measured evidence.

Humidity has been identified as an important factor influencing droplet persistence in *Pinus* (Lill and Sweet 1977) and *Picea* (Owens *et al.* 1987).

Environmental humidity, however, cannot be expected to affect the postpollination droplet of *Larix*. Under normal conditions, the ovules are partially sealed by their infolded integument and are within the already closed cone during the period of droplet secretion. Generally, the humidity inside the cone must be higher than outside. Runions and Owens (1996) reported that after cone closure in *Picea*, reduced evaporation within the enclosed spaces allowed the formation of very large pollination drops capable of scavenging pollen from the cone axis beyond the micropylar arms. Douglas-fir pollen was observed to imbibe moisture and elongate inside a closed cone, even when well away from the micropyle (Takaso *et al.* 1996).

On a comparative annual basis, variation was observed in both the volume and number of droplets produced by the three trees. Tree 3 produced more numerous and larger secretions than the other two trees during both sampling periods. Tree 3 produced larger droplets in 1999 than it did in 2000. Tree 2 did the opposite, producing larger droplets in the second year. The mean droplet mass of tree 1 did not change significantly between the two years. These data indicate that a tree may display seasonal variation in both number of droplets produced and droplet size. In the case of the three test trees, annual variation occurred on an individual basis and thus is not likely to be the result of environmental variation from season to season as each tree responded differently to common conditions.

These experiments have demonstrated that postpollination droplet production in *Larix* is not related to the water status of the tree as a whole, at least under non-stressed conditions. One interpretation of this finding is that water status may not drastically

affect the pollination success of larch trees in non-stressed conditions. Additional studies on *Larix* trees experiencing a limited watering regime would be necessary to confirm this.

The postpollination secretion of *Larix* is an active exudation. It is not the result of excessive whole-tree xylem water potential. Further work is required to establish how droplet production is regulated by structures within or near the ovule.

Chapter 4

The contents of conifer ovular secretions

Introduction

Thorough analysis of the contents of conifer ovular secretions has been hampered by the lack of techniques to analyze the minute drops, which often measure in nanolitres (Seridi-Benkaddour and Chesnoy 1988, von Aderkas and Leary 1999a,b). The inventory of compounds within these liquids has so far has been limited to sugars, amino acids, and other small organic compounds (Table 4.1). These molecules are amenable to detection by the methods typically employed – paper chromatography, colour response to various stains (e.g., ninhydrin for amino acids, p-anisidine for carbohydrates), and HPLC analysis.

Sucrose, the typical transport sugar in conifers, is consistently found in less abundance than the other sugars measured in ovular secretions. McWilliam (1958) reported the concentration of sucrose in the micropylar extract of *Pinus nigra* to be 2.5 mM, while D-glucose and D-fructose were present at 33 mM and 40 mM, respectively. In C*ephalotaxus drupacea*, Seridi-Benkaddour and Chesnoy (1988) found that fructose was the dominant sugar (77% of sugars detected), glucose was present in a relatively small amount (2.4 %), and sucrose was not detected at all. Sucrose was also absent in the pollination droplet of *Picea engelmannii*, where glucose (4.3 % w/v) and fructose (3.8 %) were present in similar amounts (Owens *et al.*, 1987). Sugar concentrations in the ovular secretions of *Larix* x *marschlinsii* and *Taxus* x *media* have not previously been reported.

Amino acids are also known to be present in conifer ovular secretions (Table 4.1). These may represent free amino acids present in the liquid, or they may be the result of

Table 4.1. Compounds identified in the ovular secretions of conifers.

Species	Compounds	Reference
Pinus nigra	D-fructose, D-glucose, sucrose	McWilliam 1958
Cephalotaxus drupacea	Fructose, glucose	Seridi-Benkaddour and Chesnoy 1988
	Proline, asparagine, glutamic acid, alanine, serine, leucine[a], isoleucine[a], threonine[a], glutamine[a], aspartic acid[a]	
	Galacturonic acid	
	Polymer comprised of galactose, arabinose, glucose, rhamnose, mannose, and unidentified phenolic compounds	
Thuja orientalis	Fructose	Seridi-Benkaddour and Chesnoy 1988
	Serine, glycine, alanine, glutamic acid, phenylalanine[a], tyrosine[a], leucine[a], isoleucine[a], threonine[a], aspartic acid[a]	
	Galacturonic acid	
Taxus baccata	Fructose, glucose, sucrose	Seridi-Benkaddour and Chesnoy 1988
	Galacturonic acid	
	Glutamic acid, proline, alanine, glutamine, lysine[a], tryptophan[a], valine[a]	Zeigler 1959
	Phosphate, malic acid, citric acid	
	Glucose, calcium	Fujii 1903
Pinus engelmannii	Fructose, glucose	Owens *et al.* 1987

[a] present in trace amounts

protein hydrolysis (Ziegler 1959). Prior to the present study, the only conclusive evidence of proteins in the ovular secretion of any conifer is an acrylamide gel separation of the ovular secretion of *Larix leptolepis* in the Ph.D. thesis of Said (1988). Carafa *et al.* (1992) reported acid phosphatase activity in the micropylar drop of *Welwitschia mirabilis*. This is the only tentative identification of a protein in the ovular secretion of any gymnosperm.

Pettitt (1977) suggested that the fertilization fluid of cycads, a micropylar secretion into which cycad pollen tubes release their zooidogamous sperm, contains proteins and glycoproteins likely to play a significant role during reproduction. He was able to demonstrate the presence of proteins in the material that breaks down to form the fertilization fluid, but was unable to sample the fluid itself. Unfortunately, this work was not furthered.

This chapter contains the results of my investigation into the biochemical composition of the ovular secretions of *L.* x *marschlinsii* and *T.* x *media*. The concentrations of glucose, fructose, and sucrose were measured in the ovular secretions of these species and are reported here for the first time. The protein compositions of the secretions were examined by 1- and 2-dimensional polyacrylamide gel electrophoresis, and also, in the case of larch, by RP-HPLC. Comparisons of the protein content between species, between individuals within a species, and over the course of the secretion period of each species are reported herein. Also discussed is a preliminary analysis of the protein content of the ovular secretions of *Pseudotsuga menziesii* and *Thuja plicata*.

Methods and Materials

Plant material

All trees studied in this analysis were found on the campus of the University of Victoria (British Columbia, Canada). Over 5 years (1999 – 2003), secretions were collected from the cones of three *Larix* x *marschlinsii* Coaz (hybrid larch) trees and more than 30 *Pseudotsuga menziesii* (Mirb.) Franco (Douglas-fir) trees. The postpollination droplets of both species were collected according to von Aderkas and Leary (1999a,b). Cones were removed from the trees and brought into the laboratory where bracts containing the ovuliferous scales were dissected and placed in Petri dishes kept humid with wetted filter paper. As droplets formed and extended past the apex of the micropyle (5 – 30 minutes after cone dissection) they were collected by micropipette tip with the aid of a dissecting microscope.

Pollination droplets were collected from more than 30 female *Taxus* x *media* Rehder (hybrid yew) trees during three seasons (2001 – 2003). Yew branches (10 – 20 cm) bearing droplet-producing ovules were clipped from the trees and placed in plastic containers kept humid with moistened paper towel. Droplets were collected by micropipette tip 1 – 3 h after branch harvesting. *Thuja plicata* Donn. ex D. Donn (western red cedar) pollination droplets were collected by Karen Gill from approximately 2500 seed cones from more than 10 trees in the spring of 2002. Branches bearing first-year seed cones were sealed in plastic containers with moistened paper towel for up to four days. Ovules were dissected with the aid of a dissecting microscope and pollination drops were collected by 5 μL glass capillary tubes pulled to a fine point over flame.

Ovular secretions harvested from all species were frozen in liquid nitrogen immediately after collection and stored at – 20 °C.

Determination of sugar concentrations in the ovular secretions of larch and yew

D-glucose and D-fructose concentrations were measured in the ovular secretions of larch and yew with a Boehringer Mannhein glucose/fructose determination kit (Roche) according to the manufacturer's directions. This method measures the concentrations of the two monosaccharides sequentially by the enzymatic generation of a quantitive amount of a UV product. A Phillips UV / Visible light spectrophotometer (model PU8620) was used to measure the absorbance of the product. Seven samples of larch ovular secretion collected throughout the 2003 production season (May 7 – 17) were pooled for analysis. Five samples of yew pollination droplet collected throughout the peak of the 2003 pollination season (Febuary 26 – March 3) were pooled for monosaccharide analysis of this species. Five aliquots of the pooled samples were analyzed separately for each species and the average measurements are reported.

The pooled samples used for glucose and fructose determination were also analyzed for sucrose concentration with a Sigma Sucrose Assay Kit (Sigma) according to the manufacturer's directions. This assay generates a coloured compound in direct proportion to the amount of sucrose present in the analyte. Concentration of the coloured product was determined by absorbance at 340 nm with the Phillips UV/Vis spectrophotometer. Five aliquots of each pooled sample were analyzed separately for sucrose concentration and the averages of these measurements are reported.

Sample preparation for gel electrophoresis

The secretions of larch, Douglas-fir, and western red cedar required no purification prior to gel electrophoresis. The yew pollination droplet was found to be poorly resolved by 2D electrophoresis unless first purified by trichloroacetic acid (TCA) / acetone precipitation to separate the proteins from the confounding compound(s). This was accomplished by centrifugation of whole yew pollination droplet at 16,000 x g for 5 min to pellet cellular debris and transfer of the supernatant into a 10 x volume of TCA in ice-cold acetone (10 % TCA w/v). The mixture was vortex-mixed and stored at –20 °C overnight. The following day, the sample was centrifuged at 16,000 x g for 10 min to pellet the precipitated protein. The supernatant was removed, and the pellet was washed in an equal volume of ice-cold acetone, then returned to –20 °C for 30 min. The sample was again centrifuged at 16,000 x g for 10 min, the acetone removed, and the pellet air-dried prior to re-suspension in the appropriate sample buffer for electrophoresis.

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

All buffers for SDS-PAGE were made up according to Laemmli (1970). SDS-PAGE Gels (70 mm x 100 mm x 0.75 mm) were typically 7.5 or 12 % acrylamide with a 4 % stacking gel. Electrophoresis was carried out at 20 mA per gel for approximately 60 min in a Mini-Protean II Cell electrophoresis unit (BioRad). Samples were boiled for 5 min in sample buffer prior to loading. After electrophoresis, gels were stained by silver, Gelcode staining reagent (Pierce), or by the colloidal Coomassie method of Neuhoff *et al.* (1988).

2-Dimensional gel electrophoresis

2D gel electrophoresis was performed at the University of Victoria - Genome British Columbia Proteomics Centre with the Zoom IPGRunner System (Invitrogen) for small gells (80 x 70 x 1 mm), or the Ettan Dalt II (Amersham Biosciences) system for larger gels (240 x 200 x 1.5 mm). In both cases, isoelectric focusing was carried out over an immobilized pH gradient of 3 – 10. Separation in the second dimension was by SDS-PAGE with a linear gradient of 4 – 12 % acrylamide. Separated proteins were detected either by Sypro Ruby protein stain (Molecular Probes), or colloidal Coomassie staining.

Both 1- and 2D electrophoresis of larch, yew, and Douglas-fir secretions were carried out many dozens of times. The resultant banding or spotting patterns were highly reproducible. 1D SDS-PAGE of the western red cedar pollination droplet was only performed twice due to limited sample. Brett Poulis kindly shared his results from 2D gel separations of Douglas-fir.

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) of protein spots

In-gel trypsin digestion of Coomassie-stained protein spots separated by 2D gel electrophoresis was carried out according to Kinter and Sherman (2000). Spots were excised from the gels by pipette tips cut to the appropriate diameter and used as a coring tool. Protein samples were reduced with 10 mM dithiothreitol and alkylated by 100 mM iodoacetamide, both in 100 mM ammonium bicarbonate. Tryptic digestion was carried out over night at 37 °C with 20 ng/μL sequencing-grade modified porcine trypsin (Sigma). Digested peptides were eluted into 100 mM sodium carbonate (pH 10). Eluted

peptides were desalted on a C18 ZipTip (Millipore) with the final elution in 5 µL 70 % acetonitrile / 0.1 % formic acid. 1 µL of the eluted peptides was combined with 1 µL of a saturated solution of α-cyano-4-hydroxycinnamic acid (Aldrich) in 70 % acetonitrile / 0.1 % formic acid and applied to a MALDI-TOF sample plate. After drying, the sample spectra were acquired with an ABI Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems) in reflectron mode with a delayed extraction of 80 ns. External calibration was done with protein standards of known molecular weight, including angiotensin I, ACTH (clip 1-17), ACTH (clip 18-39), ACTH (clip 7-38), and bovine insulin (Sigma). One hundred spectra were averaged to yield a representative spectrum.

Reversed phase high performance liquid chromatography (RP-HPLC)

RP-HPLC was carried out on a BrownLee narrow-bore C8 column (Perkin Elmer) attached to a Beckman System Gold HPLC and UV detector. Whole larch ovular secretion was injected off-line into a 100 µL injection loop. The sample was brought on-line and loaded onto the column in 0.1 % trifluoroacetic acid (TFA) in HPLC grade water for 2 minutes at a flow rate of 0.25 mL/min. Fractions were eluted in a linear gradient of 0.1 % TFA in water to 90 % acetonitrile (containing 0.075 % TFA) over 90 min at a flow rate of 0.25 mL/min. UV absorbance of the eluent was monitored at 220 nm. Fractions were eluted into 1.5 mL centrifuge tubes and the solvent removed by evacuated centrifugation (Jouan RC 10.22). Dried fractions were later re-suspended in 10 µL SDS-PAGE sample buffer for electrophoretic separation.

Results

Concentration of sugars in the ovular secretions of larch and yew

The disaccharide sucrose and the monosaccharides glucose and fructose were present in measurable quantities in the postpollination droplet of larch and in the pollination droplet of yew (Table 4.2). Overall sugar concentration was higher in the larch secretion. Larch samples contained roughly 75 X more glucose and fructose than the yew samples, but only 5 X more sucrose. The relative amounts of glucose to fructose were similar for both species, but the proportion of the monosaccharides to sucrose was not. Both fructose and glucose were more abundant than sucrose in the larch secretion. In yew, the concentration of sucrose was an order of magnitude higher than that of either monosaccharide.

Protein detection by electrophoresis

The secretions of all four conifers tested – larch, yew, Douglas-fir, and western red cedar - showed complex mixtures of proteins that could be separated by electrophoretic methods.

1D SDS-PAGE of the postpollination droplets of larch and Douglas-fir resolved upwards of 50 proteins from both secretions (Figure 4.1). The protein banding patterns from these closely related species showed some similarity, but there were a significant number of bands unique to one species or the other. For example, there was a very noticeable difference in protein banding pattern of larch and Douglas-fir in the area of the gel corresponding to approximately 29 kDa. In this region, the larch secretion displayed two darkly stained protein bands that differed by 1 kDa. In contrast, the Douglas-fir

Table 4.2. The concentration of glucose, fructose, and sucrose in the ovular secretion of *Larix* x *marschlinsii* and the pollination droplet of *Taxus* x *media*.

Species	[glucose] mM	[fructose] mM	[sucrose] mM
Larix x *marschlinsii*	156	145	108
Taxus x *media*	2.7	2.1	23

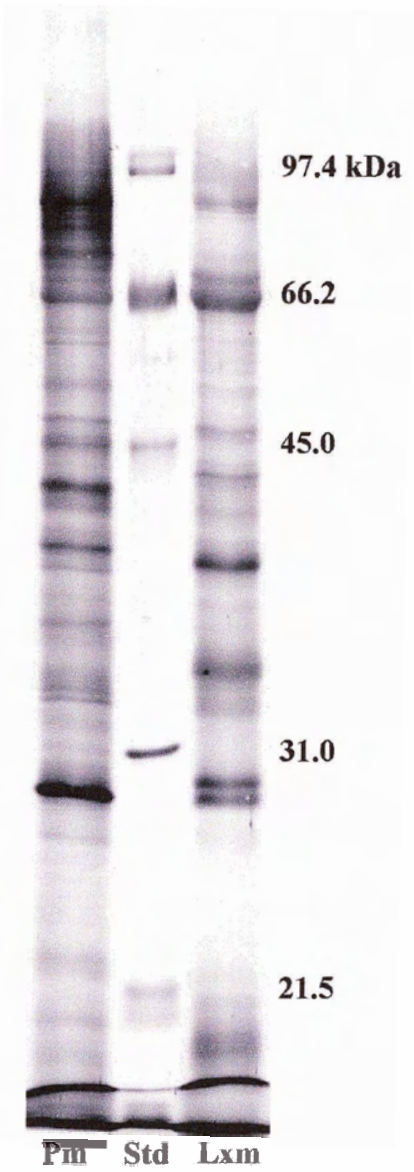


Figure 4.1. 1D SDS-PAGE (12% acrylamide) separation of the ovular secretions of *Pseudotsuga menziesii* (Pm) and *Larix* x *marschlinsii* (Lxm). 3 µL of whole secretion was loaded into the indicated lanes. Molecular weights of BioRad medium range protein standards (Std) are indicated at right. Protein bands were stained with silver.

secretion contained only a single darkly stained protein band with a mass intermediate to the two larch proteins.

The protein banding patterns of yew and western red cedar indicated that the pollination droplets of these species contained fewer proteins than the ovular secretions of the two pineaceous species (Figure 4.2). Both pollination droplets contained fewer than ten protein bands that were clearly resolvable by 1D SDS-PAGE. A number of proteins in the pollination droplets of yew and western red cedar had similar weights (Figure 4.2). There was, however, no obvious overall similarity in the pattern of protein banding. The pollination droplets of both species contained proteins that were all smaller than 100 kDa. The yew secretion generated a broad band of darkly staining protein from 10 – 15 kDa that was not present in the western red cedar sample.

Further resolution of the protein complement of the conifer ovular secretions was obtained by 2D gel electrophoresis. Separation by both isoelectric point and mass indicated that there were no fewer than 100 protein spots resolvable by this method in the postpollination droplets of larch (Figure 4.3) and Douglas-fir (Brett Poulis pers. comm.). Protein spots from the larch secretion were detectable over the entire range of isoelectric focusing (pH 3 – 10) and were separated in the second dimension into a range of molecular weights from 5 – 100 kDa. This range of protein masses in the 2D separation of larch agreed with the masses of the protein bands determined by 1D SDS-PAGE. A number of spots in the 2D gel of the larch secretion differed in pI but not in mass. An obvious example is a train of large, darkly stained spots that occurred over a range of pI from 4.5 – 6, all with a molecular weight of approximately 60 kDa. It is unlikely that these spots represent entirely different proteins. The similarity in molecular weights and

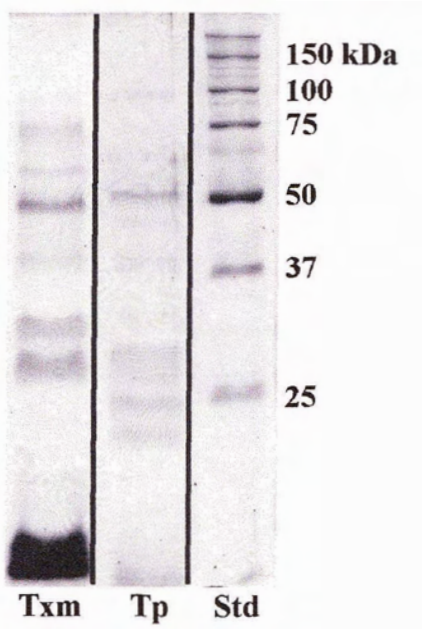


Figure 4.2. 1D SDS-PAGE (12 % acrylamide) separation of the pollination droplets of *Taxus* x *media* (Txm) and *Thuja plicata* (Tp). 5 μL of whole secretion was loaded into the indicated lanes. Molecular weights of BioRad Precision broad range protein standards (Std) are indicated at right. Bands were stained with Gelcode protein staining solution.

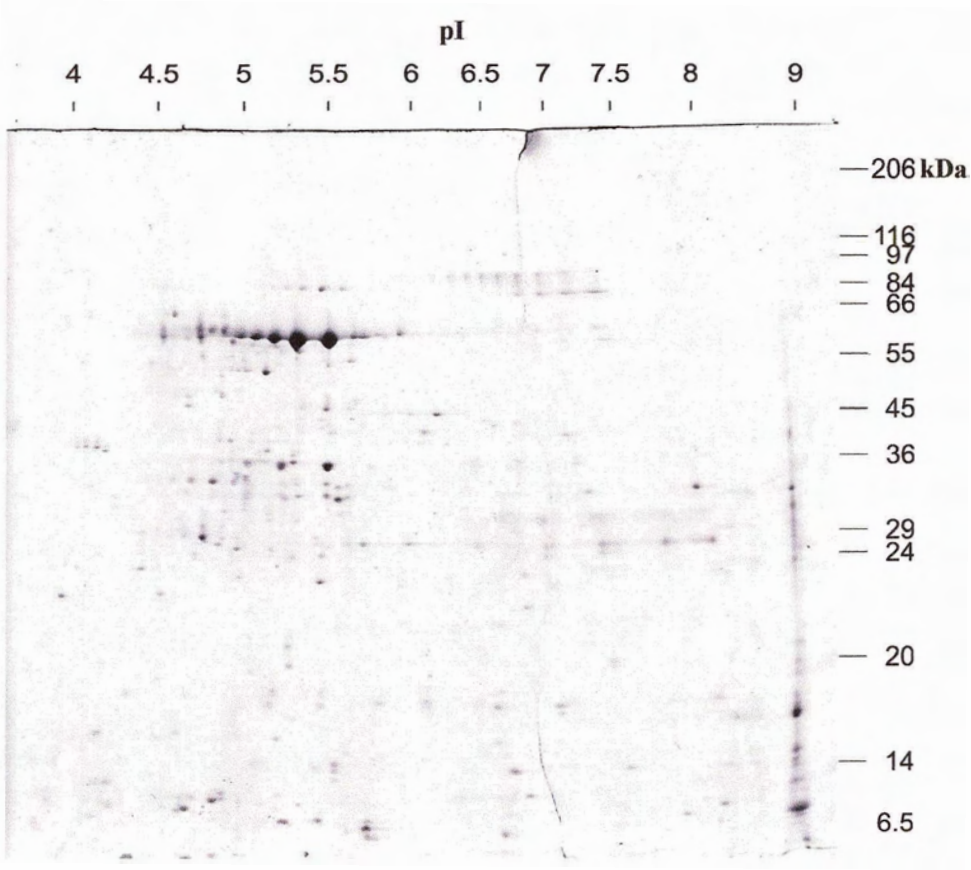


Figure 4.3. 2D electrophoretic separation of whole ovular secretion (45 µL) of *Larix* x *marschlinsii*. Isoelectric focusing was carried out over an IEF strip with an immobilized linear pH gradient from 3 – 10. Mass separation in the second dimension was through a gradient of 4 – 12 % acrylamide. Isoelectric point markers were determined from the overlaid IEF strip. Indicated masses were determined by comparison with a gel loaded with protein standards and run in parallel. Protein spots were stained with silver.

the regular spacing of the spots in the first dimension suggest that this spotting pattern, and ones similar to it, represent single proteins that either occur naturally in a number of differently charged forms or have been modified by charged molecules during isoelectric focusing.

Two-dimensional separation of the yew pollination droplet followed by Sypro Ruby staining revealed approximately 20 well-resolved protein spots (Figure 4.4). Like the larch secretion, more protein spots were resolved by 2D separation than could be resolved by mass separation alone. As predicted by 1D SDS-PAGE, all of the yew proteins resolved in the 2D gel had molecular masses greater than 10 kDa and less than 80 kDa. These proteins had isoelectric points spanning the entire range of separation (pH 3 - 10). Most occured in the acidic region of the gel, a few were clearly basic, and only one appeared to have a pI near neutrality.

For the most part, the protein spots of the yew secretion were well resolved and discrete. One exception is the large spot that resolved on the basic edge of the gel with a molecular weight of approximately 25 kDa (Figure 4.4). The size and shape of this spot made it difficult to determine whether it represented a single protein or several overlapping proteins of similar molecular weight and pI. To resolve the question of uniformity, three samples were excised from this spot (Figure 4.5). They were subjected to trypsin digestion and analyzed by MALDI-TOF MS. The mass spectra of the peptide fragments from all three samples were identical, indicating that the protein composition of the entire spot was homogenous (Figure 4.5). Additionally, the identity of the protein responsible for this spot was determined, and 2D immunoblot analysis confirmed the spot was uniform throughout (data presented in Chapter 6).

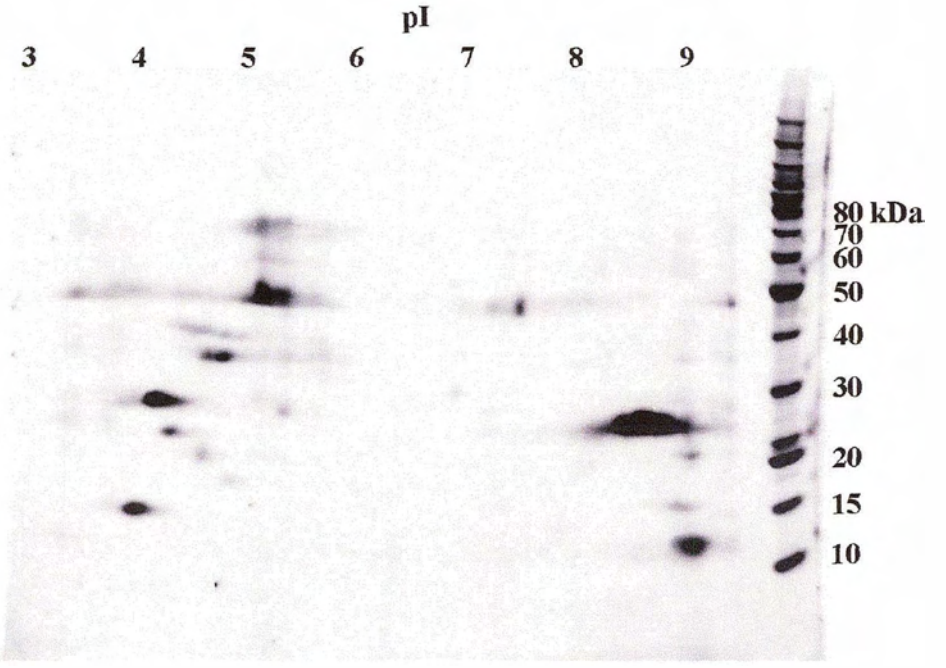


Figure 4.4. 2D electrophoretic separation of the pollination droplet of *Taxus* x *media*. Acetone / TCA was used to precipitate the protein component of 60 µL of *Taxus* pollination droplet for isoelectric focusing over an IEF strip with an immobilized linear pH gradient of 3 – 10. Mass separation in the second dimension was through a gradient of 4 – 12 % acrylamide. Isoelectric point markers were determined from the overlaid IEF strip. Molecular weights of protein standards run with the sample in the second dimension are indicated on the right. Protein staining was carried out with Sypro Ruby protein specific dye. The gel image displayed is digitally colour-reversed.

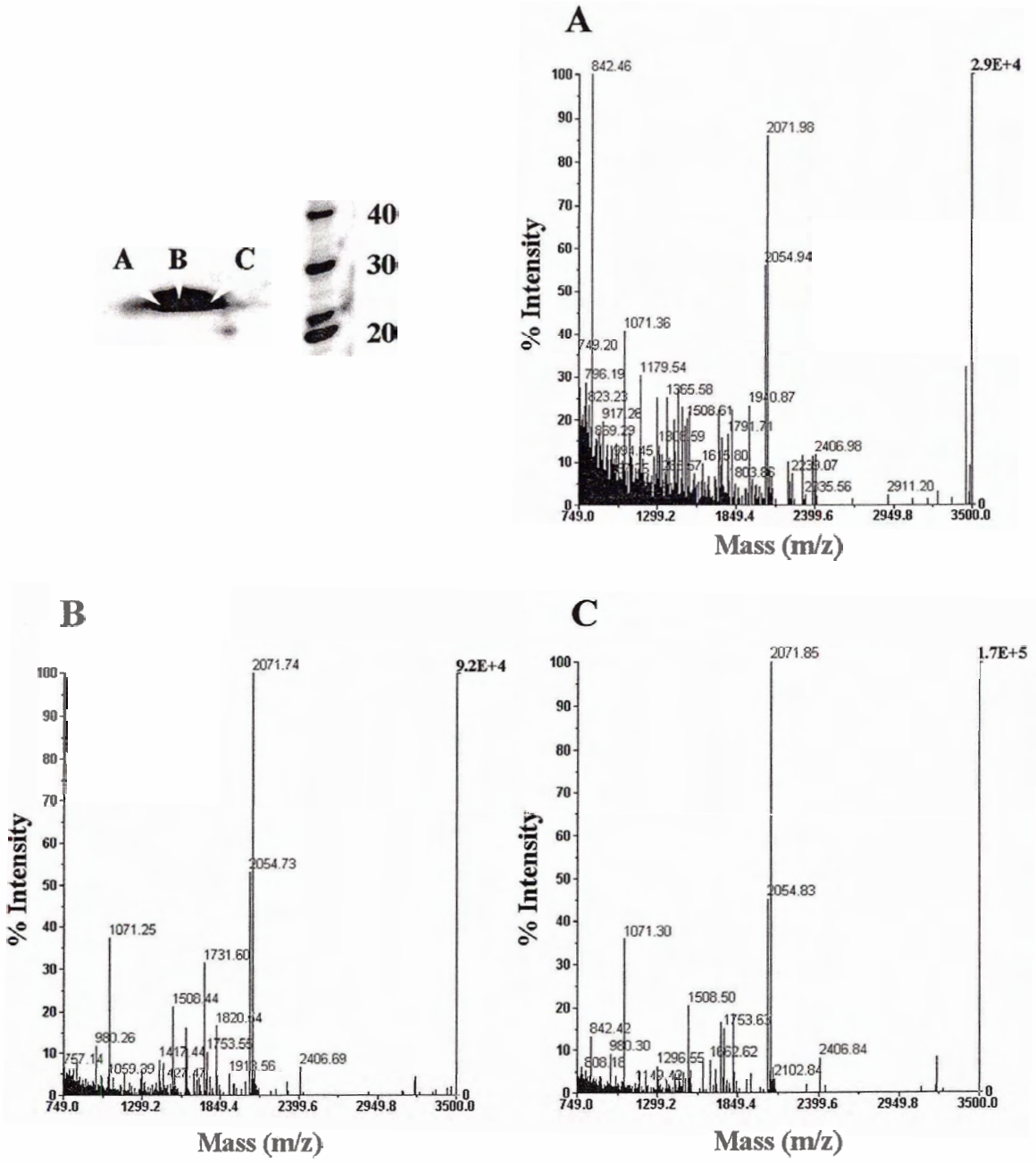


Figure 4.5. Mass spectra of peptides generated by trypsin digestion of three samples from a 25 kDa protein of the *Taxus* x *media* pollination droplet. The protein was spot resolved by 2D gel electrophoresis. The peptide ions with masses (m+H) of 1071.25, 1508.44, 2071.74, 2054.73, and 2406.69 amu shared by all three samples indicate that the spectra were generated from the same protein. Spectra were obtained with an ABI Voyager DE-STR MALDI-TOF mass spectrometer in reflectron mode with a delayed extraction of 80 ns.

Protein production between individuals and collection dates

Ovular secretions prepared for electrophoretic separation typically contained pooled samples of liquid collected from a number of trees on a given day, or from a number of days in a given year. In order to pool samples for analysis, it was first necessary to determine whether the protein composition of the ovular secretion of a given species varied from tree to tree or throughout the reproductive season. 1D SDS-PAGE of larch ovular secretions collected separately from each of the three sample trees demonstrated nearly identical protein compositions (Figure 4.6a). The only noticeable difference between the three individuals was a protein band of approximately 31 kDa that appeared to be more abundant in the tree 2 sample compared to the other trees. Larch ovular secretions collected from a single tree over the duration of the droplet production season yielded identical profiles by 1D SDS-PAGE (Figure 4.6b).

The hedged yew trees sampled for this research did not carry enough ovules to generate sufficient pollination droplet to compare protein profiles between individuals. Instead, samples of yew pollination droplets from two spatially separated groups were compared. No difference could be detected in the protein banding profiles of the two groups (Figure 4.7a). Like the larch secretion, the protein composition of the yew pollination drop also remained consistent throughout the pollination season (Figure 4.7b).

RP-HPLC of the larch ovular secretion

RP-HPLC was also used to analyze the contents of the larch ovular secretion. Constituents of the whole secretion were separated by relative hydrophobicity over a C8 column in an increasing gradient of acetonitrile. During a typical separation, 34 – 36

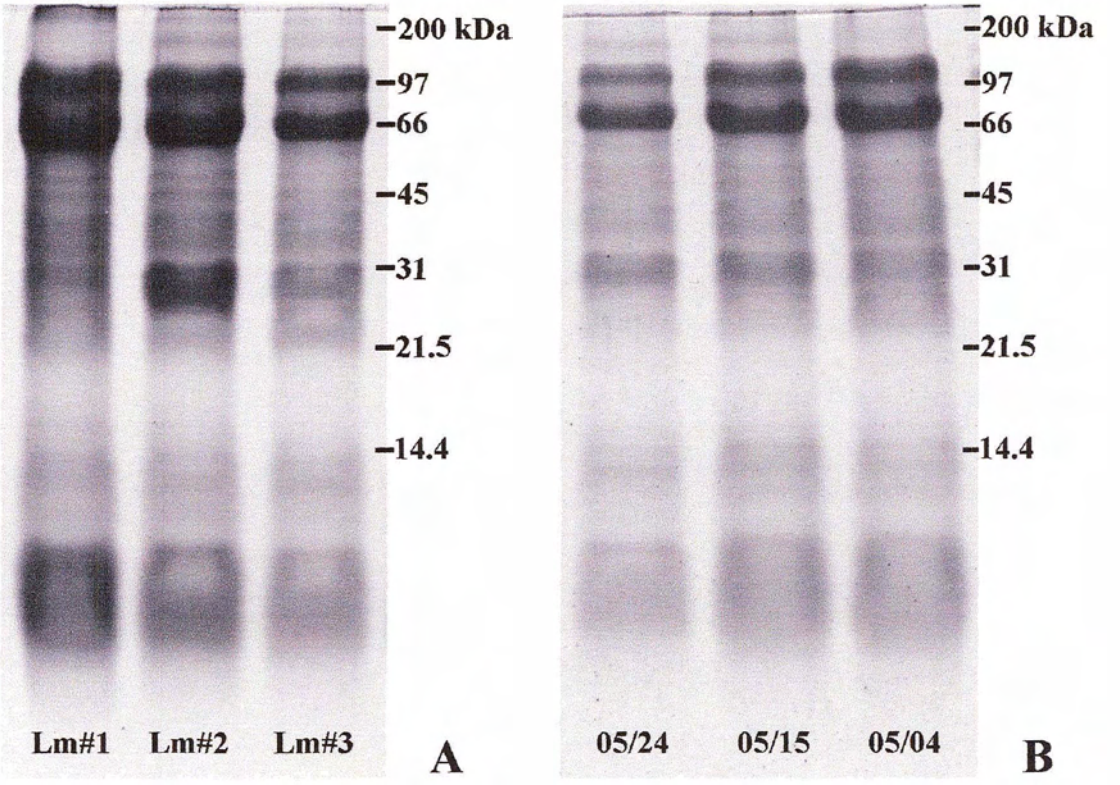


Figure 4.6. 1D SDS-PAGE (12% acrylamide) separation of the ovular secretions from (**A**) three *Larix* x *marschlinsii* (Lxm) trees collected on the same date and (**B**) one *L.* x *marschlinsii* tree (Lxm #3) collected early (05/04), in the middle (05/15), and near the end (05/24) of the 2000 pollination season. 5 µL of whole secretion was loaded into the indicated lanes. Molecular weights of BioRad protein standards separated in an adjacent lane are indicated at the right of each gel. Protein bands were stained with Gelcode reagent.

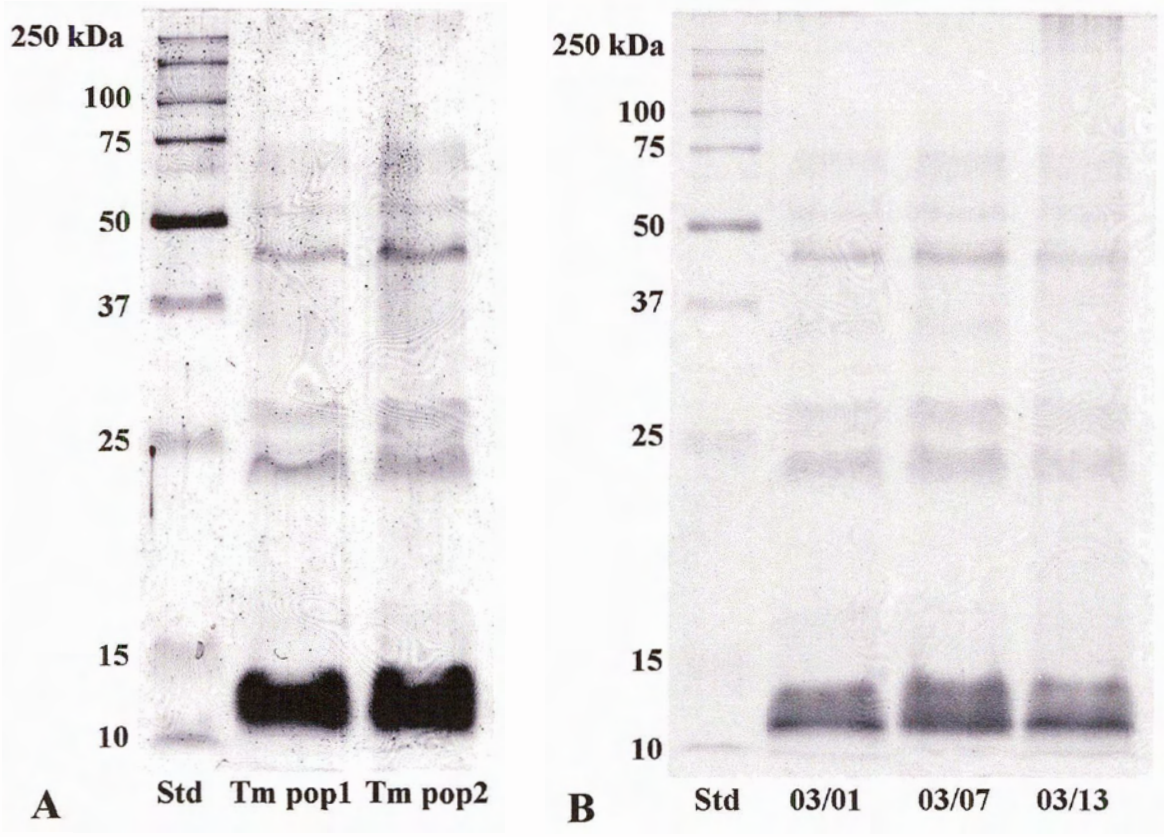


Figure 4.7. 1D SDS-PAGE (12% acrylamide) separation of (**A**) the pollination droplets from two spatially separated populations of *Taxus* x *media* collected on the same date and (**B**) one population of *T.* x *media* collected early (03/01), in the middle (03/07), and near the end (03/13) of the 2003 pollination season. 5 μL of whole droplet was loaded into the indicated lanes. Molecular weights of BioRad Precision protein standards (Std) are indicated at the left of the gel. Protein bands were stained with Gelcode reagent.

fractions representing distinct absorbance peaks (at 220 nm) were collected (Figure 4.8). 1D SDS-PAGE separation followed by Gelcode staining of each fraction confirmed the presence of proteins in 18 of the fractions (Figure 4.8).

RP-HPLC separation profiles of the larch postpollination droplet were consistent for samples collected at different points during the period of secretion production, and between samples collected in different years (Figure 4.8).

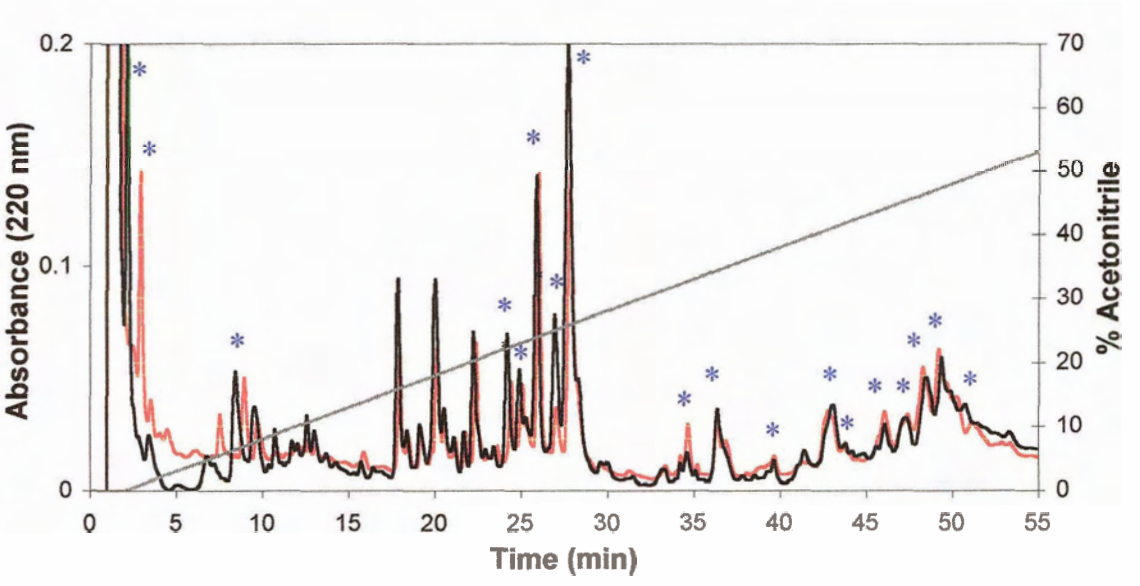


Figure 4.8. RP-HPLC profiles of two *Larix* x *marschlinsii* ovular secretion samples. One sample was collected at the beginning of the secretion period (red trace) and the other collected seven days later (black trace). In each experiment 20 µL of whole sample was loaded onto a C8 column and separation occurred in a linear gradient of increasing acetonitrile concentration. UV absorbance of eluent was monitored at 220 nm. Asterisks denote fractions shown by SDS-PAGE to contain protein.

Discussion

Sugar composition of conifer ovular secretions

At the very least, the ovular secretion is a nutritive medium for developing pollen (Tison 1911). The relative abundance of fructose to other sugars reported in the contents of conifer ovular secretions (McWilliam 1958, Owens *et al.* 1987, Seridi-Benkaddour and Chesnoy 1988) suggests that this sugar may be an important carbon source for pollen metabolism. In carbohydrate uptake experiments performed with suspension cultures of *Pinus mungo* pollen, exogeneous fructose was found to be more rapidly metabolized than glucose, and was the preferred source for starch synthesis during germination (Nygaard 1977).

The ovular secretions of yew and larch contained very different relative amounts of sucrose to glucose and fructose. In this study, the glucose and fructose concentrations of the larch ovular secretion were measured to be 156 mM and 145 mM, respectively. The concentration of sucrose in this liquid was 108 mM, just over two-thirds the concentration of either of the monosaccharides. In the yew pollination drop, the sucrose concentration of 23 mM was ten times that of both glucose (2.7 mM) and fructose (2.1 mM). If sugars occur in conifer ovular secretions for the benefit of incoming pollen, these results suggest that the metabolic requirements of larch and yew pollen vary significantly.

The greater abundance of glucose and fructose to sucrose in the larch droplet agrees with the measurements taken from the secretions of *Pinus* (McWilliam 1958), *Picea* (Owens et al. 1987) and *Cephalotaxus* (Seridi-Benkaddour and Chesnoy 1988). In these species, sucrose may be cleaved into its constituents (glucose and fructose) to meet

the specific requirements of the pollen. The postpollination droplet of Douglas-fir contains an invertase enzyme that may be responsible for sucrose cleavage (B. Poulis pers. comm.). *Larix*, *Pinus*, *Picea*, and *Cephalotaxus* may regulate the sugar concentration of their ovular secretions using similar sugar-modifying enzymes.

Taxus x *media* is the first conifer species for which a higher concentration of sucrose to glucose and fructose has been reported. The unique sugar composition of the secretion of this species may represent a difference in the preference of exogenous carbon source for yew pollen compared to the other conifers studied. The yew pollination droplets sampled did not contain pollen at the time of collection; otherwise, they would have been retracted and unavailable (Anderson and Owens 2000). It is possible that yew pollen grains possess their own suite of sugar-cleaving enzymes that may modify the composition of the liquid by their presence. Germinating petunia pollen has been demonstrated to completely convert sucrose medium into equimolar amounts glucose and fructose monosaccharides (Ylstra *et al.* 1998). It was proposed that in petunia, sucrose is transported from source tissues to the apoplast of the transmitting tract where it is hydrolysed by pollen wall-bound invertases and then transported into the pollen tube by monosaccharide transporters (Ylstra *et al.* 1998). Yew pollen may employ a similar strategy in the sucrose-rich pollination droplet.

Proteins in the ovular secretions of conifers

This study shows that there is a complement of proteins in the secretions of all four conifers examined. The protein composition is distinct from species to species, although a subset of proteins may be present in two or more species. Areas of similarity

in the protein banding patterns of larch and Douglas-fir suggest that these species share some constituents of their postpollination secretions; however, the total protein profiles are unique to each species. The pollination droplets of yew and western red cedar contain far fewer proteins than either of the species that employ a delayed secretion. Though these protein profiles are simpler, they nevertheless show species-specificity.

The diversity in sugar content and protein composition among conifers may constitute an interspecific breeding barrier. Successful pollen germination and development is likely to be impacted by the nutrients and enzymes present in a given ovular secretion. Pollen is most likely to be adapted to the conditions of the ovular secretion of its own species.

Nucellar tissues of *Larix occidentalis* and Douglas-fir have been reported to degenerate at the time of ovular secretion (Owens *et al.* 1994, Takaso and Owens 1996). Some of the proteins found in the secretions of larch and Douglas-fir may occur in the liquid as a result of this breakdown. Regardless of the source of the proteins in these postpollination droplets - whether actively secreted by healthy cells, or passively washed out of ruptured cells - pollen grains always encounter the same complex chemical milieu when the secretion occurs. Although a minor amount of nucellar breakdown has been reported with the production of the pollination droplet of *Taxus* (Dupler 1917, Xing *et al.* 2000), the small number of proteins found in the yew secretion suggests that their presence was not due to the leakage of ruptured cells.

The composition of proteins in the ovular secretions of larch and yew is consistent from tree to tree, day to day, and year to year in either species. This is evidenced by the consistent electrophoresis banding patterns between trees and collection dates for both

larch and yew, and repeatable RP-HPLC profiles between samples of the larch secretion. The only exception is the tendency of one of the three larch trees (tree 2) to produce a higher abundance of a 31 kDa protein compared to the other sample trees. The identity of this differently expressed protein has not been determined. These findings indicate that the proteins of the ovular secretions of larch and yew occur consistently in all individuals and are therefore encountered routinely by pollen *in planta.*

RP-HPLC analysis indicates that non-proteinaceous compounds that absorb UV light at 220 nm are also consistently present in the ovular secretion of larch. These compounds are likely to be polysaccharides, various carbohydrates, phenolics, and other products of conifer secondary metabolism. Further analysis of these components of the secretion is necessary to elucidate their contributions to reproduction.

The roles of ovular secretions in conifers

The complex mixture of compounds found in conifer ovular secretions implies that these liquids do more than simply transport pollen. A number of roles have already been suggested for these liquids in the literature. Barner and Christiansen (1960, 1962) proposed that the postpollination secretions of larch and Douglas-fir are involved in the loosening of pollen from the infolded sticky hairs of the closed ovule. In Douglas-fir, the secretion may also be responsible for the dissolution of the outer intine prior to pollen germination (Takaso and Owens 1994). Ovular secretions are thought to induce pollen tube development in larch species (Barner and Christiansen 1960, Said *et al.* 1991) and Douglas-fir (Barner and Christiansen 1962, Takaso and Owens 1996, Takaso *et al.* 1996).

Villar *et al.* (1984) further proposed that the secretion in ovules of larch coordinates pollen germination with the maturation of the egg cells.

In contrast to activities that promote pollen germination, Takaso *et al.* (1996) found that the ovular secretion of Douglas-fir causes distortion and lysis in as many as 84 % of elongated pollen grains within the ovule. These authors suggested that the secretion may be a mechanism of pre-zygotic selection by the megagametophyte. It had previously been reported that cross-pollinated grains were less distorted than self-pollinated grains in the ovules of Douglas-fir (Takaso and Owens 1994).

The findings summarized above are the results of microscopic examination of pollinated ovules, or *in vitro* observations made of pollen grains exposed to tissue homogenates containing ovular secretions. No mechanisms have been put forward to explain how ovular secretions carry out the various actions attributed to them. Better identification of the contents of these liquids is key to understanding what roles they play in conifer reproduction, and the manner in which they operate. The discovery of proteins in the ovular secretions of conifers introduces a promising line of investigation into the function(s) of these liquids.

The consistent nature of the production of ovular secretions and the compounds that they contain provides strong evidence that these liquids play an important role during reproduction. Identification of some of the proteins present in the secretions of larch and yew and their potential functions are reported in the chapters that follow.

Chapter 5

Proteins in the ovular secretions of *Larix* x *marschlinsii* and *Taxus* x *media* involved in pollen germination and pollen tube elongation

(Portions of this chapter pertaining to AGP localization in the yew ovule have been published separately as: O'Leary SJB, Joseph C, von Aderkas P. 2004. Origin of arabinogalactan proteins in the pollination drop of *Taxus* x *media*. Austrian Journal of Forest Science 121: 35-46.)

Introduction

Pollen grains of most conifers are bathed in a liquid secretion at the time of germination and during pollen tube elongation. In larch, yew, Douglas-fir, and western red cedar, these secretions are rich in proteins, sugars, and other compounds. There can be little doubt that at least a fraction of the compounds found in these liquids influence the development of pollen. This chapter pertains to two proteins that are likely to affect pollen behaviour in the ovules larch and yew, xyloglucan endotransglycosylase (XET) and arabinogalactan protein (AGP).

Pollen tube extension requires both cell well degradation and cell well synthesis. Before a pollen tube is produced, pollen grains typically hydrate and shed their outer exine. After tube initiation, continued growth requires loosening of the cell wall at the tip, followed by cellular expansion and reinforcement of the wall behind the growing front (Taylor and Hepler 1997, Cosgrove 1997). These processes depend on coordinated enzymatic modifications of the existing wall and incorporation of new material.

Controlled, irreversible extension of plant cells involves the breakage and reformation of linkages between the molecules that comprise the rigid wall (Tiaz 1984). Xyloglucans are the major polysaccharide constituent in the cell walls of gymnosperms (Acebes *et al.* 1993, Barrachina and Lorences 1998). The cross-linking of these

polysaccharides forms a matrix that provides the principle tension-bearing structure in the longitudinal axis of an elongating plant cell. The xyloglucan components of this matrix may be cleaved and rejoined, allowing the temporary softening of cell walls and subsequent reinforcement (Fry *et al.* 1992, Fry 1995). This controlled plasticity of the wall facilitates cell elongation in response to turgor-driven expansive forces (Cosgrove 1997).

The enzymes responsible for the reversible cleavage of xyloglucan molecules in the plant cell wall are known as xyloglucan endotransglycosylases (XETs) (Fry *et al.* 1992, Fry 1995). Besides restructuring the existing cell wall, XETs have also been demonstrated to incorporate newly synthesized xyloglucan molecules into regions of cellular expansion (Thompson *et al.* 1997, Thompson and Fry 2001). By catalyzing both the integration of new xyloglucan into the plant cell wall and the active restructuring of existing xyloglucan in the growing areas, XETs play an important role in enabling cell extension with minimal loss of wall strength (Fry 2004).

In addition to xyloglucans, plant cell walls contain a collection of glycoproteins that includes arbinogalactan proteins (AGPs). AGPs are a family of highly glycosylated proteins expressed throughout the plant kingdom, typically at cell surfaces where they are involved in cell growth and development (Showalter 2001). AGPs are typified by large oligosaccharide branches dominated by arabinose and galactose residues. These sugar moieties are covalently linked to a small protein backbone. The majority of AGPs are more than 90% carbohydrate and less than 10% polypeptide in composition (Du *et al.* 1996). The protein backbone is typically rich in hydroxyproline, alanine, threonine, glycine, and serine (Clarke *et al.* 1979). The carbohydrate branches are usually attached

to the peptide backbone by *O*-glycosylation to serine and hydroxyproline residues. In principle, the extent of glycosylation and variation in the branching patterns of the oligosaccharide chains is unlimited. Consequently, there is little data about the precise structure of the oligosaccharide elaboration of any AGP (Cassab 1998). Despite variation in the protein core and oligosaccharide chains of different AGPs, a unifying characteristic of these molecules is that they may be specifically bound by β-glucosyl Yariv reagent (Yariv *et al.* 1962).

An approximately 33 kDa protein isolated from the ovular secretion of *L.* x *marschlinsii* has been identified as an XET and is likely to operate on larch pollen during germination and pollen tube development. AGPs have been detected in the secretions of both larch and yew and are a possible source of cell wall material for growing pollen tubes.

A polyclonal antibody (anti-*Pse m* I) created to recognize a Douglas-fir pathogenesis-related protein has been found to cross-react with the larch XET in a specific manner. This antibody was used to localize the production of XET within the larch ovule. Similarly, the site of AGP production within the yew ovule has been determined by immunocytochemistry. The localization of XET and AGP provides insight into the functions of these proteins during pollination and identifies tissues contributing to the ovular secretions of larch and yew.

Methods and Materials

Plant material

All samples of larch (*Larix* x *marschlinsii*) and yew (*Taxus* x. *media*) were collected from the same trees and in the same manner described in Chapter 4. Larch ovules exuding droplets were excised from a sampling of cones throughout the period of droplet production and fixed in paraformaldehyde for later use in immunolocalization studies. Yew ovules were excised from trees in the weeks prior to droplet secretion, during droplet secretion, and after secretion had ceased. These ovules were also fixed in paraformaldehyde for later use.

Gel electrophoresis of ovular secretions

The preparation of the yew ovular secretion for gel electrophoresis, and 1D SDS-PAGE of larch and yew ovular secretions were conducted according to the methods described in Chapter 4.

Immunoblotting

Electro-transfer of proteins from acrylamide gels onto Hybond-P polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences) was performed with the Mini Trans-Blot Cell system (BioRad) according to manufacturer's directions. Transfers were conducted at 90 V for 30 min. Immunodetection was carried out on the membrane as follows: 20 min rinse in TBST (1 % Tween 20 in 150 mM NaCl, 10 mM Tris, pH 7.5), 2 h incubation in blocking solution (5 % skim milk powder in TBST), and three 10 min rinses in TBST. The membranes were then incubated overnight with primary antibody

(see below) at 4°C. The following day the primary antibody was removed with three 10 min rinses in TBST prior to a 90 min incubation in secondary antibody (see below). The membranes were rinsed three times for 10 min in TBST before detection with the ECL Plus chemiluminescent system (Amersham Biosciences) or by the alkaline phosphatase colour reaction (BioRad). Membranes were rinsed with water and stained with Gelcode after immunodetection.

The primary antibody for XET binding in the larch ovular secretion, anti-*Pse m* I, was kindly supplied by Dr. Abul Ekramoddoullah (Canadian Forest Service). It was diluted 1/400 in blocking solution for immunoblot detection. The secondary antibody for these experiments was goat anti-rabbit I_gG conjugated to alkaline phosphatase (diluted 1/3000 in blocking solution). The rat monoclonal antibodies, Jim13, LM2, and Mac207, used to detect AGPs in the yew ovular secretion, were generously supplied by Dr. Paul Knox (University of Leeds). LM2 and Mac207 were used in a working dilution of 1/500, while Jim13 was diluted 1/2000 in blocking solution. The secondary antibody for AGP western blot detection was goat anti-rat I_gG conjugated to horseradish peroxidase (diluted 1/2000 in blocking solution).

N-terminal sequencing of larch XET

A ~ 33 kDA band isolated by SDS-PAGE and electroblotted onto PVDF membrane was subjected to N-terminal amino acid sequence analysis at the University of Victoria Protein Chemistry Centre (now the University of Victoria - Genome BC Proteomics Centre) using an Applied Biosystems Inc. automated sequencer (Model

470A). Protein identity was determined by alignment with known sequences from the GenBank database using the pBLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/).

Reversed phase high performance liquid chromatography (RP-HPLC) of the larch ovular secretion

Samples (20 μL) of larch secretion were separated using a Brownlee Aquapore reverse phase C-8 column connected to a Beckman System Gold HPLC according to the method described in Chapter 4.

Detection of AGPs by the Yariv Reagent

Ovular secretions separated by gel electrophoresis were stained overnight with β-glucosyl Yariv reagent (Biosupplies Australia) 0.0005% in 1% NaCl. Gum arabic (2 mg/mL) was used as a positive control. After staining, gels were washed in 1 % NaCl. Yariv reagent binds in-gel with arabinogalactan proteins and forms an orange precipitate (van Holst and Clarke 1985).

Preparation of Ovules for Sectioning

Larch ovules were fixed for no less than 1 d and yew ovules no less than 5 d with 4 % paraformaldehyde in 0.035 M Sorenson's phosphate buffer, pH 7.0. Ovules were then washed in six changes of Sorenson's buffer over 3 d and dehydrated in a graded ethanol series (30, 50, 70, 95, 100 %). Dehydrated material was processed for embedding in glycol methacrylate (Technovit 7100, Kulzer) according to the manufacturer's instructions. Due to the thickness of the specimens, pre-infiltration was

carried out overnight and infiltration was allowed to proceed for 24 h before addition of the hardening catalyst.

Immunofluorescence microscopy

Glycol methacrylate-embedded ovules were sectioned to 5 μm thickness with a Leica sledge microtome (model SM2400) equipped with a tungsten carbide blade. Sections were dried onto SuperFrost Plus slides (Fisher Scientific), rinsed for 3 min with TBS, washed twice for 15 min with blocking solution (0.05 % Tween 20, 1.0 % heat-inactivated goat serum albumin (GSA) in TBS), rinsed twice for 10 min with TBS, and incubated with primary antibody (see below) overnight at 4 °C. The following day, the sections were rinsed twice for 10 min with blocking solution, twice for 10 min with TBS, then incubated for 90 min with the secondary antibody (see below). From this point onward the slides were kept in the dark to prevent photo-destruction of the fluorochromes bound to the secondary antibodies. Unbound secondary antibodies were removed with two rinses of 0.05 % Tween 20 in TBS and two 10 min washes in TBS. Sections were immediately covered and observed by epifluorescence microscopy with a Zeiss AxioPhot microscope equipped for FITC imaging.

For immunolocalizaton of XET in larch ovules, anti-*Pse m* I diluted 1/100 with 1 % GSA in TBS was used as the primary antibody. The secondary antibody was goat anti-rabbit IgG conjugated to Alexa 488 (Molecular Probes) diluted 1/400 with 1 % GSA in TBS. Controls included absence of primary antibody, absence of secondary antibody, and substitution of a chicken anti-α8 integrin (1/100 in GSA/TBS) as the primary antibody.

LM2 and Jim13 antibodies diluted 1/250 with 1.0 % GSA in TBS were used to localize AGP production in yew ovules. The secondary antibody was goat anti-rat IgG conjugated to Alexa 488 (Molecular Probes) diluted 1/500 with GSA/TBS. Controls for this experiment included absence of primary antibody, absence of secondary antibody, and the use of Mac207, which is non-reactive with gymnosperm AGPs (Pennel *et al.* 1991), as the primary antibody.

Results

Identification of XET in the ovular secretion of larch

Upon separation by 1D SDS-PAGE, numerous protein bands were resolved in the ovular secretion of larch, including a conspicuous band approximately 33 kDa in molecular weight (Figure 5.1). The N-terminal amino acid residues of this protein were sequenced and determined to be KPVDVPFQRNYVPRW. This sequence was queried against the GenBank protein database to identify nearly identical matches to known sequences. The twenty closest matches were XET proteins from various plant species. The larch protein has been identified as larch XET based on these findings.

XET sequences that aligned with the larch protein contained predicted N-terminal export sequences (Figure 5.2). Export sequences are cleaved from the N-termini of preproteins as they are secreted from the cells that produce them; these signals are not present in mature proteins. The alignment of the larch XET sequence began near the first amino acid of the predicted mature orthologous proteins. The molecular weight of XET from the larch ovular secretion was similar to the molecular weights of the mature XETs of other species (Figure 5.2).

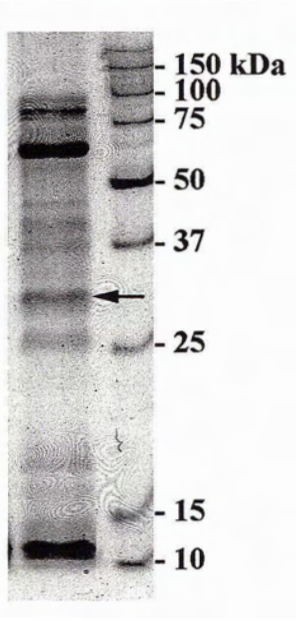


Figure 5.1. 1D SDS-PAGE (12 % acrylamide) separation of the ovular secretion of *Larix* x *marschlinsii* (5 µL). Arrow indicates 33 kDa protein (larch XET). Molecular weights of BioRad Precision protein standards are indicated. Bands were stained with Gelcode protein staining solution.

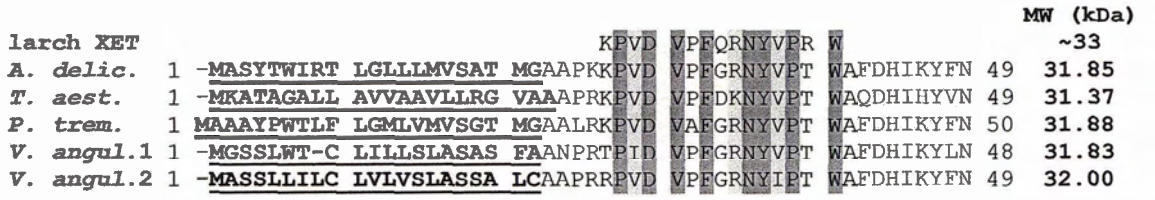


Figure 5.2. Multiple sequence alignment of the N-terminal amino acid sequence of larch XET with known XET sequences from the GenBank database. The N-terminal sequences of XET precursors from *Actinidia deliciosa* (Acc. no. AAC09388.1), *Triticum aestivum* (Q41542), *Populus tremula* x *P. tremuloides* (AAN87142.1), *Vigna angularis* 1 (Q41638), and *V. angularis* 2 (Q8LNZ5) are shown. Putative N-terminal export signals are bold and underlined. Amino acid residues identical across all sequences are shaded dark gray. Residues representing conservative replacement across all sequences are shaded light gray. The predicted molecular weights (from amino acid sequence) of the mature proteins are presented on the right. The molecular weight of larch XET was deduced from 1D SDS-PAGE.

Detection of larch XET by anti-*Pse m* I

Larch XET was strongly bound by the polyclonal antibody anti-*Pse m* I (Figure 5.3). This antibody recognized no other proteins from the larch ovular secretion. Anti-*Pse m* I was raised against a synthetic peptide corresponding to a 20 amino acid conserved sequence from a pathogenesis-related protein (PR-10) of Douglas-fir (Ekramoddoullah *et al.* 2000). It is not known why anti-*Pse m* I cross-reacted with larch XET, but the binding was found to be consistent in all immunoblots performed, and the antibody did not bind any other proteins in the larch secretion.

To ensure that anti-*Pse m* I was binding XET and not another 33 kDa protein that co-migrates with it, XET was partially purified by RP-HPLC prior to immunoblot detection and N-terminal amino acid sequencing (Figure 5.4).

Immunolocalization of XET production in the larch ovule

Anti-*Pse m* I and a secondary antibody conjugated to the Alexa 488 fluorochrome were used to identify XET producing cells in 5 μm sections of larch ovules. Epifluorescence microscopy indicates that XET production was restricted to a single tier of cells lining the micropyle in the vicinity of the in-folded stigmatic flap (Figure 5.5). All other tissues of the larch ovule, including other regions of the integument, the nucellus, and the megagametophyte lacked detectable XET production. Control sections, in which either the primary or secondary antibodies were omitted or a different primary antibody was used (anti-α8-integrin) yielded no Alexa 488 fluorescence.

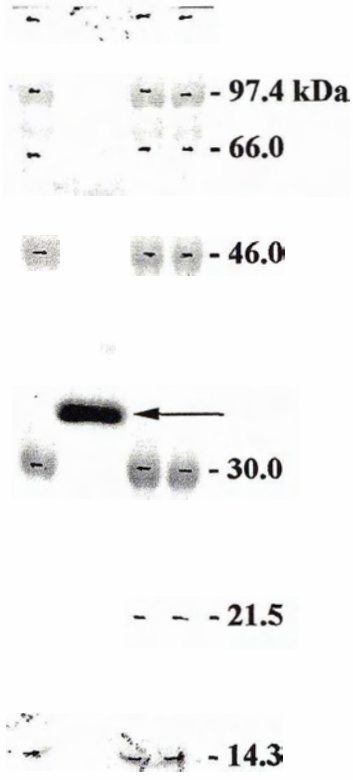


Figure 5.3. Immunodetection of larch XET (arrow) from the ovular secretion of *Larix* x *marschlinsii* (40 µL) by anti-*Pse m* I. Proteins were separated by 1D SDS-PAGE through a 12 % acrylamide gel (200 mm) prior to electro-transfer onto PVDF membrane. Anti-*Pse m* I binding was visualized with the Alkaline Phosphatase Conjugate Substrate Kit (BioRad). Molecular weights of Amersham Rainbow protein standards are indicated.

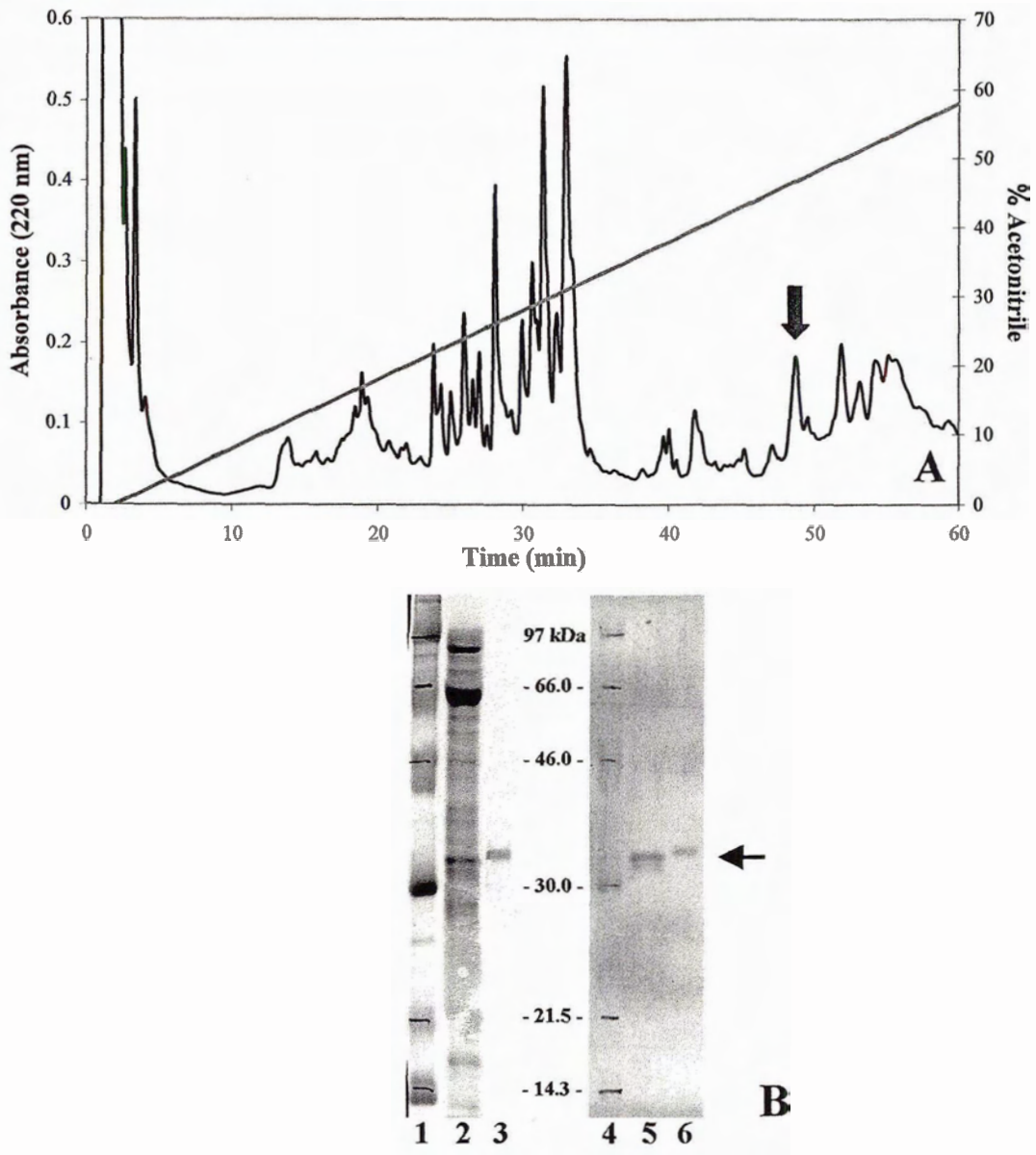


Figure 5.4. Isolation and immunodetection of larch XET.
A. RP-HPLC profile of whole ovular secretion from *Larix* x *marschlinsii* (20 µL) separated over a C-8 column in an increasing gradient of acetonitrile. Absorbance of eluent was monitored at 220 nm. Arrow indicates the fraction containing larch XET.
B. 1D SDS-PAGE (lanes 1-3) and immunodetection (lanes 4-6) of larch XET (arrow) from whole ovular secretion of *L.* x *marschlinsii* (lanes 2 and 5, 10 µL each) and from an RP-HPLC fraction containing larch XET (lanes 3 and 6, 12.5 µL each). Proteins were separated by 1D SDS-PAGE through a 12 % acrylamide gel prior to staining or electro-transfer onto PVDF. Immunodetection was carried out with anti-*Pse m* I and the Alkaline Phosphatase Conjugate Substrate Kit (BioRad). Protein staining was performed with Gelcode staining reagent. Molecular weights of Amersham Rainbow protein standards (lanes 1 and 4) are indicated between gels.

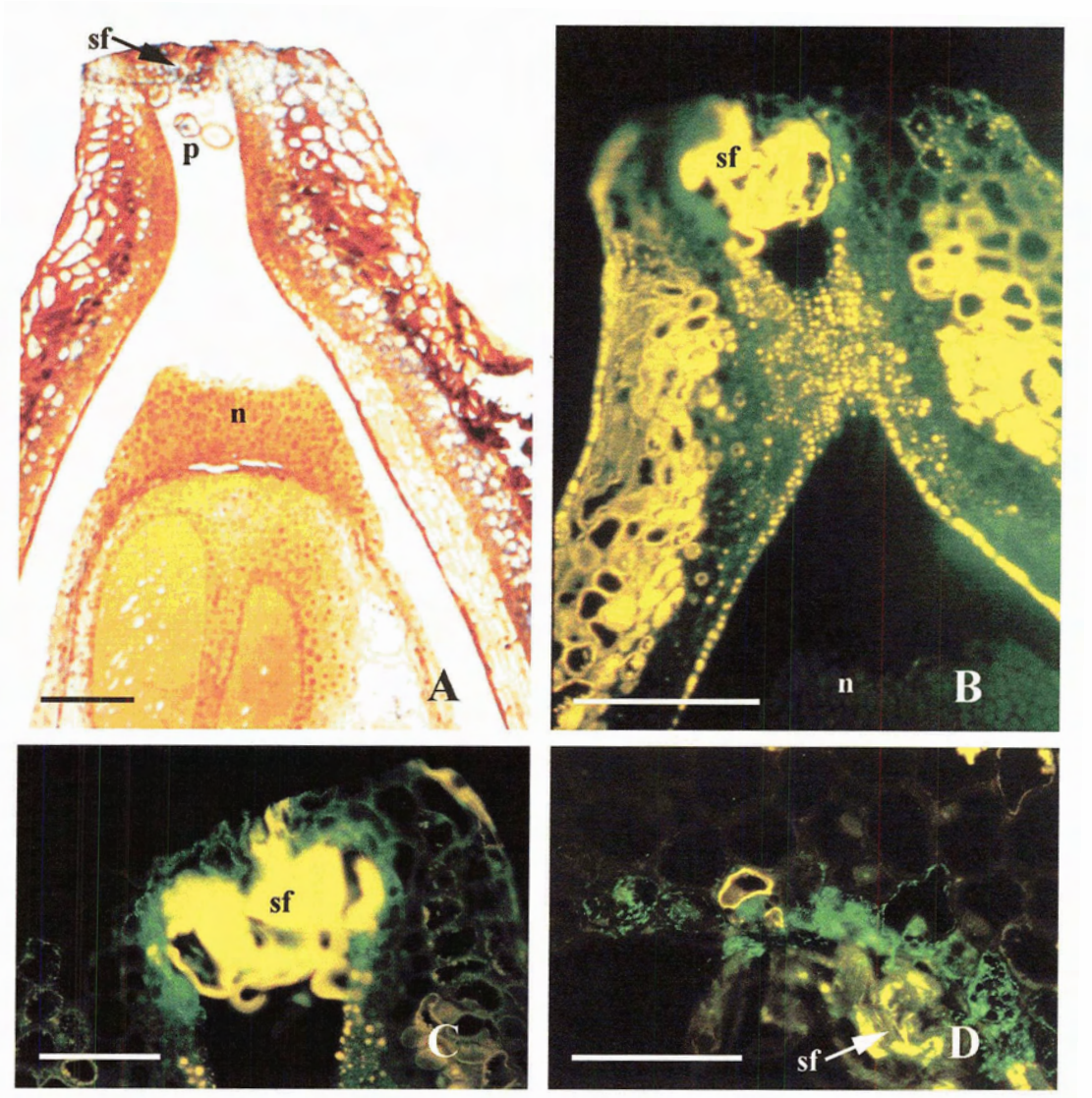


Figure 5.5. Light and fluorescence micrographs of sections (5 μm thickness) of glycol methacrylate-embedded ovules of *Larix* x *marschlinsii*. **A.** Light micrograph of a longitudinal section (l.s.) through the micropylar end of an ovule stained with Safranin-O and post-stained with I/KI. The infolded stigmatic flap (sf), nucellus (n), and pollen grains (p) within the micropyle are indicated. **B.** Fluorescence micrograph of a l.s. through the micropylar end of an ovule. XET detection is indicated by green fluorescence of the Alexa 488 fluorochrome in cells of the micropyle adjacent to the stigmatic flap. **C, D.** Fluorescence micrographs of a l.s. (C) and a cross section (D) through the micropyle of a larch ovule. Cells producing XET are labelled green. Bar = 200 μm (A and B), 50 μm (C and D).

Detection of AGPs in yew and larch ovular secretions by the Yariv reagent

A positive reaction with the Yariv reagent indicated that both yew and larch ovular secretions contained proteins belonging to the family of glycoproteins collectively named arabinogalactan proteins (AGPs). The positive Yariv reaction was manifested as a smear of orange precipitate ranging from 50 to 250 kDa after SDS-PAGE separation of the secretion of either species (Figure 5.6).

Immunolocalization of AGPs in yew ovules

Three anti-AGP monoclonal antibodies, Jim13, LM2, and Mac207, were used to detect the presence of AGPs in the yew pollination droplet by immunoblot analysis. Both Jim13 and LM2 generated a smear of antibody labelling. The Jim 13 smear ran from approximately 30 to 250 kDa and the LM2 smear ran from 75 to 250 kDa (Figure 5.7). The Mac207 antibody did not bind to AGPs in the yew sample.

The localization of AGP production within the yew ovule was determined by immuncytochemical study of 5 μm sections with Jim13 and LM2 antibodies. Most ovules fixed at the time of pollination droplet production contained undifferentiated sporogenous tissue (Figure 5.8a). Jim13 and LM2 were both found to label ovular tissues (Figure 5.8b,c). In sections from ovules sampled during pollination, LM2 bound to the tissue of the micropylar end of the nucellus. Labelling was observed between cells, within cell walls, and in intracellular spaces (Figure 5.8b). Jim13 showed a similar labelling pattern in the nucellus (5.7c), and also labelled non-reproductive tissues in the sterile bracts exterior to the ovule (not shown). Controls lacking primary antibody application showed some auto-fluorescence of ovular tissue, but none of the green

Figure 5.6. Yariv staining of AGPs. Indicated lanes were loaded with 10 µL of gum arabic (GA) (2 mg/ml), 10 µL of ovular secretion from *Larix* x *marschlinsii* (Lxm), or 10 µL of pollination droplet from *Taxus* x *media* (Txm). Samples were separated by 1D SDS-PAGE through a 7.5 % acrylamide gel prior to addition of the Yariv reagent. A blank lane (-) was loaded with 10 µL of water as negative control for Yariv staining. Note: the lane containing the secretion of *L.* x *marschlinsii* (Lxm) was added to this figure from a comparable gel.

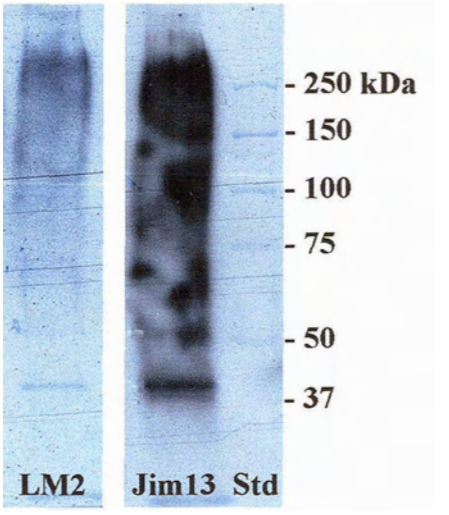


Figure 5.7. Immunodetection of AGPs in pollination droplet of *Taxus* x. *media* (10 µL) by the monoclonal antibodies LM2 and Jim13. Samples were separated by 1D SDS-PAGE through a 7.5 % acrylamide gel prior to electro-transfer onto PVDF membrane. Membranes were stained with Gelcode reagent after immunodetection was completed. Molecular weights of BioRad Precision protein standards (Std) are indicated.

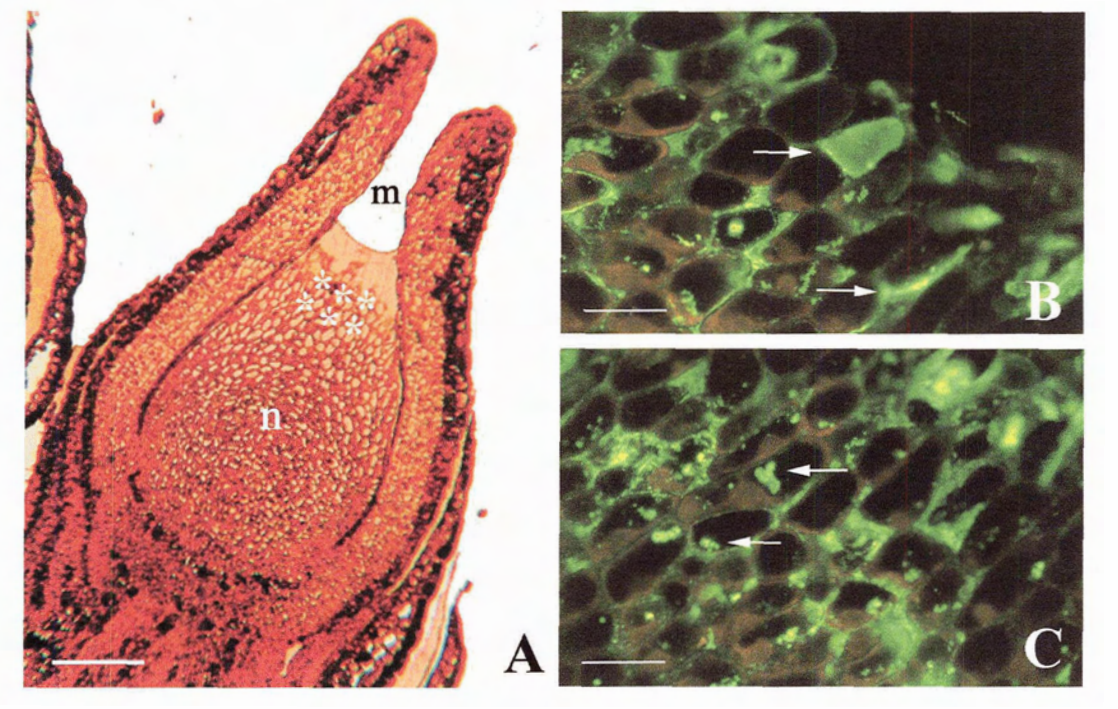


Figure 5.8. Light and fluorescence micrographs of sections (5 μm thickness) of glycol methacrylate-embedded ovules of *Taxus* x *media*. **A.** Light micrograph of a l.s. through an ovule stained with Safranin-O and post-stained with I/KI. The micropyle (m) and nucellus (n) are indicated. The area of AGP localization in the neighbouring micrographs is indicated with asterisks. **B.** Fluorescence micrograph of a l.s. through the nucellar tissue of an ovule fixed during the period of pollination droplet secretion. LM2 antibody against AGP was used. Intercellular deposits are indicated with arrows.
C. Fluorescence micrograph of a l.s. through the nucellar tissue of an ovule fixed during the period of pollination droplet secretion. Jim13 antibody against AGP was used. Intracellular aggregations are indicted with arrows.
Bar = 250 μm (A), 30 μm (B and C)

fluorescence characteristic of Alexa 488, indicating an absence of non-specific binding of the secondary antibody. Mac207 did not label any ovular tissues; sections labelled with this antibody were indistinguishable from control sections lacking primary antibody.

Stages of ovule development before and after pollination droplet production showed either an absence or weak concentration of AGP epitopes. Neither Jim13 nor LM2 labelled nucellar tissue in immature ovules (ovules fixed prior to droplet production). After pollination droplet production had ceased, ovules showed a reduced immunolabelling pattern compared with those actively producing pollination droplets. In the more mature ovules, Jim13 and LM2 labelling in the nucellus was much less intense, suggesting a reduced production of AGPs. At this advanced stage, labelling in the nucellus was solely intercellular. The fluorescing intracellular regions observed in droplet-producing ovules were no longer present, suggesting that AGP production had ceased.

Discussion

XET in the larch ovule

XET activity is typically found in areas of cell elongation and growth (de Silva *et al.* 1994). The growth-promoting effect of XET was demonstrated in gymnosperms for the first time in expanding pine hypocotyls (Barrachina and Lorences 1998). In a later study of 61 plant species from 33 families representing club-mosses, ferns, gymnosperms, and angiosperms, all species showed high levels of XET activity during root hair development, and all but one exhibited higher XET activity in the root elongation zone compared to non-elongating zones (Vissenberg *et al.* 2003). Members of

the XET gene family are also active during other aspects of plant development requiring cell wall modification, including leaf and flower abscission, fruit ripening, and processes requiring the detachment of neighbouring cells (reviewed in Campbell and Braam 1999). Surprisingly, nothing is reported in the literature relating XET activity directly to pollen germination or pollen tube elongation.

A number of physical characteristics of the 33 kDa protein of the larch ovular secretion support its identification as a XET. The most convincing evidence is the sequence of the 15 N-terminal amino acid residues, which share significant identity with XET proteins previously described in the GenBank database. The exact match of 13 of the 15 amino acids with sequences of other plant XETs is sufficient for a positive identification. In addition to this, the molecular weight of the larch XET is very similar to the predicted molecular weights of mature XET proteins from other plant species (Figure 5.2). Also, it should be noted that the larch N-terminal sequence aligns to the N-termini of the mature sequences of its XET orthologs, leaving open the possibility that larch XET may be the product of a precursor protein containing a peptide export signal. This would be consistent with the presence of larch XET in an extracellular secretion.

The anti-*Pse m* I polyclonal antibody binds to larch XET in a specific but unknown manner. There is no significant sequence similarity between the 20 amino acid peptide that this antibody was raised against and known XET amino acid sequences. A possible explanation is that a portion of the antibodies contained in the polyclonal mixture bind a structural epitope of larch XET. Regardless of the reason for the cross-reaction of this antibody and larch XET, immunoblot analysis indicated that anti-*Pse m* I did not bind other proteins present in the larch ovular secretion. Likewise,

immunolocalization demonstrated that anti-*Pse m* I did not bind indiscriminately to proteins in larch ovules.

Based on microscopic examination of fixed ovules of *Pseudotsuga menziesii*, Takaso and Owens (1994) suggested that a secretion originating in the integuments contains substances that cause cell wall dissolution. The authors later reported that the proposed secretion appeared to originate in close proximity to the engulfed flap, and suggested an involvement in the release of pollen from the stigmatic hairs (Takaso and Owens 1996). A similar secretion was hypothesized to occur in *Larix occidentalis*; in this case from a distinct layer of cells lining the tip of the micropylar canal (Owens *et al.* 1994). This secretion was also proposed to occur coincidently with pollen hydration, swelling, and release from the stigmatic hairs. The location of the hypothesized integumentary secretions of Douglas-fir and larch correspond to the cells identified as the source of larch XET production in this study. Taken together, the immunolocalization results of the present study and the observations of Takaso and Owens (1994, 1996) and Owens *et al.* (1994) suggest that the function of larch XET during reproduction may be to loosen pollen from the sticky hairs of the engulfed flap. This enzyme may also play a role in the removal of the exine coat from pollen grains and loosening of the intine wall for hydration.

XET is relatively abundant compared to other proteins in the larch ovular secretion. It may also be active in areas of the ovule away from the site of its production. This enzyme might be capable of loosening the attachments between cells of the nucellus, reducing resistance to pollen tube ingression. Since the larch ovular secretion bathes

pollen grains during their development, XET found in this liquid might also promote pollen tube growth directly.

The localization of XET production in the tissues near the stigmatic flap does not prove that the entire larch postpollination drop originates from this source. It is difficult to imagine that the total volume of the ovular secretion comes from such a small area at the tip of the micropyle. The bulk of the fluid may come from another tissue and pick up XET as the integument secretes it.

AGPs in the ovular secretions of larch and yew

As a group, AGPs are widely distributed throughout all plant tissues. Many individual AGPs, however, demonstrate specific developmental and spatial patterns of expression in reproductive tissues (Du *et al.* 1994 and 1996, Coimbra and Salema 1997, Qiu *et al.* 1997, Mogami *et al.* 1999). AGPs are a predominant component of angiosperm pistil exudates and are known to interact with pollen during germination and tube elongation (reviewed in Cheung and Wu 1999). The current literature contains no reports on the distribution of AGPs in the female reproductive tissues of gymnosperms, even though these molecules are a major component of the pollen tube wall in this group of plants (Mogami *et al.* 1999, Yatomi *et al.* 2002).

The results of this study demonstrate that AGPs are a significant ingredient of the larch ovular secretion and the yew pollination droplet. This is shown by the extensive staining of AGPs in the liquids of these species by the Yariv reagent, as well as antibody detection of AGPs in the secretion of yew.

Immunodetection of AGPs in the yew pollination droplet was carried out with monoclonal antibodies previously reported to bind AGP epitopes expressed in reproductive tissues of angiosperms (Pennel and Roberts 1990, Li *et al.* 1995, Jauh and Lord 1996, Coimbra and Salema 1997). The difference in AGP binding of the three antibodies, illustrated by the differing patterns of immunoblot detection, was due to the specificity of each antibody for unique carbohydrate epitopes (Yates *et al.* 1996). Jim13 detected a broader range of AGPs (30 – 250 kDa) from the yew pollination droplet than LM2 (75 – 250 kDa). Mac207 failed to bind any AGPs from the yew secretion. This finding is in agreement with Pennel *et al.* (1989), who reported that the Mac207 epitope is not present in the AGPs of gymnosperms.

The difference in epitope recognition of the three antibodies was also reflected in their localization patterns within the tissues of sectioned yew ovules. Jim13 and LM2 both localized in nuccelar tissue immediately adjacent to the micropylar cavity. Jim13 also localized in tissue of the sterile bracts that subtend the yew ovule. LM2 did not. Consistent with the immunoblotting results, Mac207 did not bind to any cells in the yew ovule.

The results of this immunolocalization study provide some insight into the origin of the yew pollination droplet, and perhaps the ovular secretions of other conifers. Various origins have been conjectured for conifer ovular liquids, including the vascular system of the tree, the integuments of the ovule, the megagametophyte, and the nucellus. This study demonstrated that the production of the AGPs present in the yew pollination droplet is restricted to the cells of the nucellus. These findings are consistent with those of Fujii (1903) and Tison (1911) who proposed that the nucellus is the source of the

pollination droplet of yew. This hypothesis is reinforced by the observation in this study that AGP production in the nucellar tissue was absent before droplet production, peaked with the production of the liquid, and was severely reduced after the period of pollen collection. In short, AGP production in the nucellus and the appearance of the pollination droplet were contemporaneous.

There is an abundance of AGPs present in the micropylar region of the nucellus at the time of pollen receptivity (= period of pollination droplet production). AGPs in the intercellular spaces of this region lie in the path of the pollen tube during its growth through the nucellus. This intercellular localization also provides a source for the AGP component of the pollination droplet, as the liquid is continuous with the intercellular space. These observations are comparable to those from a study of AGP localization within the ovules of the angiosperm *Amaranthus hypochondriacus* (Coimbra and Salema 1997). In that species, AGP epitopes recognized by Mac207 and Jim8 localized specifically to nucellar cells between the micropyle and the embryo sac.

In terms of temporal expression of AGPs, *A. hypochondriacus* ovules differed from yew ovules in that antibody binding was present prior to pollination and continued to be present post-fertilization. Expression of AGPs in yew ovules appeared to be of shorter duration and more specific to the period of pollen receptivity. In addition, the ovule of *A. hypochondriacus* contained a different suite of AGP epitopes than the yew ovule; the antibodies that bound AGPs in the angiosperm (Mac 207 and Jim8) differed from those that were active in the gymnosperm (LM2 and Jim13, but not Mac 207).

The Jim8 antibody has also been used to localize AGP production in the ovules of *Brassica napus* (Pennel *et al.* 1991). In this species, expression of nucellar AGPs began

at the micropylar end of the tissue and extended throughout the nucellar epidermis during ovule maturation. As in yew and *Amaranthus*, the production of AGPs in the ovules of *Brassica* appears to define the path of pollen tube elongation.

In angiosperms, AGPs have been implicated in the development (Cheung *et al.* 1995, Wu *et al.* 2000), nutrition (Wu *et al.* 1995), and guidance (Wu *et al.* 1995, Cheung *et al.* 2000) of the pollen tube. The AGPs present in the larch and yew ovular secretions may play similar roles in conifer pollen development. This proposal is consistent with the localization of AGPs to the ECM of cells that lie in the path of pollen tube growth. AGPs may be supplied to the developing yew pollen tube exogeneously by a process that parallels the deglycosylation and import of TTS proteins (AGPs) by tobacco pollen tubes. (Wu *et al.* 1995, Cheung *et al.* 1995).

Conclusions

This work demonstrates that the ovular secretions of larch and yew contain proteins known to be important for cell elongation and pollen tube development in other plant species. Larch XET is likely involved in the release of pollen from the sticky hairs of the integument, and the preparation of pollen grains for germination. The abundance of this protein in the larch secretion may be sufficient to act upon the cells of the nucellus, or on the pollen tubes themselves, facilitating tube elongation.

Exogenous AGPs almost certainly have a direct influence on pollen tube development. Dumont-Béboux *et al.* (1998) identified the nucellus as a likely source of regulators affecting temporal delays in conifer pollen tube development. AGPs produced in these tissues may be one such regulator. These glycoproteins are strong candidates for

sporophytically-derived cell wall materials that may be supplied to pollen at developmentally defined stages of reproduction.

This report describes the first examination of XET and AGP production in the ovules of conifers. The localization of the production sites of these proteins identifies specific cells of the integument as contributors to the ovular secretion of larch, and the nucellus as a probable source of the yew pollination droplet. XET is known to play a key role in plant cell wall modification and cell elongation. AGPs are important components of plant cell walls and are known to influence pollen tube development in angiosperms. The prominence of these proteins in the ovular secretions, and the locations of their production, suggest that they play critical functions in the reproduction of larch and yew.

Chapter 6

Pathogenesis-related proteins in the ovular secretions of *Larix* x *marschlinsii* and *Taxus* x *media*

Introduction

Plants live in communities that harbour potential pathogens. As a result, plant species have evolved a number of strategies to protect themselves from the constant threat of invasion from bacteria, fungi, and viruses. In a broad sense, there are three reasons that a given plant does not succumb to pathogenic attack: (1) the plant may not support the nutritive requirements or growth habit of a potential pathogen, making it a non-host; (2) the plant may possess pre-formed structures such as waxy cuticles, reinforced cell walls, or toxic compounds that deter all but specially adapted pathogens; (3) upon recognition of an attacking pathogen, the plant mobilizes defence responses that sequester or destroy the invader. These three cases result in plant/pathogen interactions that are said to be incompatible - the pathogen is not able to exploit the plant. Compatible reactions, in which pathogen infection and disease are successful, occur when a plant does not posses pre-formed defences, fails to detect an invading pathogen, or fails to activate an effect defensive response.

A response to pathogen invasion commonly observed in plants is the production of pathogenesis-related (PR) proteins. The production of protein in response to pathogen invasion was described for the first time by Van Loon and Van Kammen (1970) as well as Gianinazzi *et al.* (1970). Both groups observed the response of tobacco leaves to tobacco mosaic virus (TMV) infection. Extracts of the plants responding to invasion were found to contain four proteins not present in the tissues of unchallenged plants.

These four proteins, isolated by electrophoretic separation, were originally identified as bands I, II, III, and IV (Van Loon and Van Kammen 1970). In the following 10 years, a growing number of research groups reported the discovery of plant proteins that were induced in response to pathogen attack. The term pathogenesis-related proteins (PR proteins) was introduced for proteins with this expression pattern (Antoniw *et al.* 1980). The proteins responsible for the bands IV, III, II, and I previously described by Van Loon and Van Kammen (1970) were renamed PR-1a, -1b, -1c, and –2a respectively (Antoniw *et al.* 1980). The three PR-1 variants were grouped together based on similarities in their electrophoretic mobility and amino acid composition. The fourth protein was found to differ significantly from the other three and was put in a second group, PR-2.

As more classes of plant proteins were found to be responsive to pathogen attack it became necessary to increase the number PR groups. By the late 1980s, 10 PR proteins were known from tobacco. Van Loon *et al.* (1987) placed these into five groups, PR-1 to 5, based on similarities in molecular weight, amino acid composition, serological properties, and amino acid or nucleic acid sequence when available.

In the mid-1990's, a nomenclature applicable to all plant species was proposed that divided PR proteins into groups based primarily on amino acid sequence and biological activity (Van Loon *et al.* 1994). To accommodate new PR proteins identified in tobacco and other species, the original five PR groups was increased by six (PR-6 to 11). Many proteins known to possess antimicrobial activity are now placed within PR groups even if their expression is not strictly dictated by pathogen invasion. Today, as many as 14 groups of PR proteins are widely recognized in the literature (Table 6.1).

Table 6.1. The groups of pathogenesis-related (PR) proteins[a].

Group	Properties	Type member
PR-1	unknown	Tobacco PR-1a
PR-2	β-1,3-glucanase	Tobacco PR-2
PR-3	chitinase type I, II, IV, V, VI, VII	Tobacco P, Q
PR-4	chintase type I, II	Tobacco R
PR-5	thaumatin-like	Tobacco S
PR-6	proteinase-inhibitor	Tomato Inhibitor I
PR-7	endoproteinase	Tomato P_{69}
PR-8	chitinase type III	Cucumber chitinase
PR-9	peroxidase	Tobacco lignin-forming peroxidase
PR-10	ribonuclease-like	Parsley 'PR1'
PR-11	chitinase type I	Tobacco class V chitinase
PR-12	defensin	Radish Rs-AFP3
PR-13	thionin	*Arabidopsis* THI2.1
PR-14	lipid transfer protein	Barley LTP4

[a] Adapted from Van Loon and Van Strien (1999)

In this study, four new PR proteins: *Lxm*LTP, *Txm*TLPa, *Txm*TLPb, and *Txm*βGlu were discovered in the ovular secretions of larch and yew. *Lxm*LTP, a lipid transfer protein (LTP) belonging to the PR-14 group, was identified in the ovular secretion of larch. *Txm*TLPa and *Txm*TLPb are acidic and basic thaumatin-like proteins (TLPs), respectively. These members of the PR-5 group were identified in the yew pollination droplet by internal amino acid sequencing and were subsequently visualized in the yew secretion by immunodetection. Antibody binding also indicates the presence of a TLP in the larch secretion. *Txm*βGlu, a member of the PR-2 group of β-1,3-glucanase (βGlu) enzymes, was also identified in the yew pollination droplet.

These proteins are significant contributors to the total protein composition of the ovular secretions of larch and yew. The discovery of a suite of PR proteins in these liquids implies a defensive function. This report provides the first evidence that the protein composition of conifer ovular secretions plays a role in pathogen defence during reproduction.

Methods and Materials

Plant material

Larix x *marschlinsii* and *Taxus* x *media* samples used in this study were collected from the same trees and in the same manner described in Chapter 4.

Electrophoresis of ovular secretions

The preparation of yew ovular secretion for electrophoretic separation and the conditions used for 1 and 2D electrophoresis of larch and yew samples are described in Chapter 4.

RP-HPLC isolation of LxmLTP

*Lxm*LTP was isolated from the larch ovular secretion by the RP-HPLC procedure described in Chapter 4. Subsequent 1D SDS-PAGE of the fraction containing *Lxm*LTP was performed to determine the purity of the isolation.

N-terminal sequencing of LxmLTP

Purified *Lxm*LTP was electro-transferred onto PVDF membrane, stained with Coomassie blue, then excised for N-terminal amino acid sequencing. Sequencing was performed at the University Of Victoria Protein Chemistry Centre (now the University of Victoria - Genome British Columbia Proteomics Centre) using an Applied Biosystems Inc. automated sequencer (model 470A). Protein identity was determined by alignment with known sequences from the GenBank database using the pBLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/).

Amino acid sequencing of internal peptides from yew proteins by tandem mass spectrometry (MS/MS)

MS/MS sequencing was carried out on protein spots excised from 2D gel separations of the yew pollination droplet. The proteins were excised in a gel plug, reduced, alkylated, and enzymatically digested by modified porcine trypsin (Promega) according to Kinter and Sherman (2000). Digested peptides were eluted from the gel into 100 mM sodium carbonate (pH 10). Desalting of the peptides and mass spectrometry were carried out at the Genome British Columbia / University of Victoria Proteomics Centre. Peptides were introduced into a Q-STARi quadrupole time-of-flight mass spectrometer (Applied Biosystems) by electrospray ionization.

Mass spectra were analyzed with Data Analyst software (Applied Biosystems). Amino acid sequences of peptides were determined by the interpretation of spectra from collisionally induced product ions following the strategy outlined by Kinter and Sherman (2000). Sequenced peptides were aligned with known protein sequences using the Bork's Group BLAST2 database (http://dove.embl-heidelberg.de/Blast2/).

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) of yew TLPs

Trypsin digestion of yew TLP samples and subsequent MALDI-TOF MS analysis were carried out according the protocol outlined in Chapter 4.

Immunodetection of TLPs in larch and yew ovular secretions

Electro-transfer of proteins from 1D separations of yew and larch ovular secretions onto PVDF membrane is described in Chapter 5. Electro-transfer of yew sample separated in two dimensions was accomplished by transferring proteins resolved by the Zoom IPGRunner System (Chapter 4) onto PVDF membrane under the same conditions employed for a 1D gel.

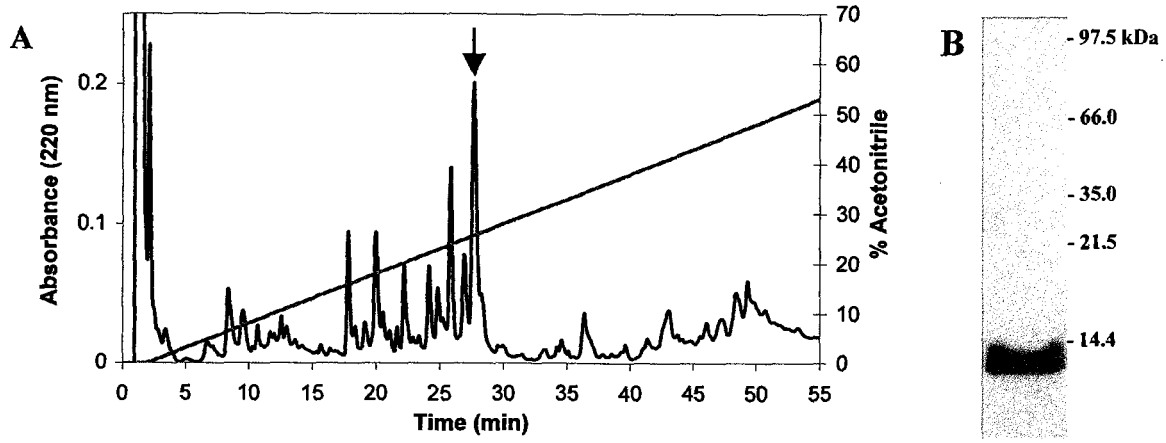
Antibody probing of immobilized proteins and chemiluminescent detection was performed according to the protocol outlined in Chapter 5. TLPs were detected by two antibodies: BARPERM1 and anti-*Pm*TLP. BARPERM1 was produced in rabbit against a barley permatin (Skadsen *et al.* 2000) and was kindly supplied by Ron Skadsen (United States Department of Agriculture). Anti-*Pm*TLP was also raised in rabbit against the synthetic peptide AAGSPGGGKQLGQGETWSFDVAADTTGGR, which corresponds to a conserved sequence in TLPs. This antibody was generously supplied by Abul Ekramoddoullah (Canadian Forest Service).

For immunodetection, BARPERM1 was diluted 1/1,000 in 5 % skim milk powder in TBST. The anti-*Pm*TLP antibody was used at a dilution of 1/500 in the same solution. In both cases, the secondary antibody was goat anti-rabbit IgG conjugated to horseradish peroxidase (Cedar Lane) diluted 1/50,000 in 5 % skim milk powder in TBST.

Results

Isolation and identification of *Lxm*LTP from the larch ovular secretion

RP-HPLC separation of the larch ovular secretion typically yielded 34 or 35 recognizable UV absorbance peaks (Figure 6.1a). 1D SDS-PAGE of the eluent fraction corresponding to the largest absorbance peak indicated that this fraction contained a predominant protein with a molecular weight of approximately 12 kDa (Figure 6.1b). The N-terminal amino acid sequence of the protein isolated in this fraction was determined to be AISXNQVVTAXTPXASYLI. A malfunction of the automated sequencer with a duplicate sample resulted in extensive hydrolysis of the protein prior to the initial sequencing step and generated an additional internal amino acid sequence of PDRQAV. Sequence alignment with a non-specific lipid transfer protein (nsLTP) of *Pinus taeda* indicated that the larch protein is a member of this group (Figure 6.1c). The larch LTP was named *Lxm*LTP. Comparison with the *P. taeda* sequence suggests that the first and third unassigned amino acids (designated by X) from the N-terminal sequence of *Lxm*LTP represent cysteine residues, and the second may be an oxidized methionine (Figure 6.1c). The N-terminus of *Lxm*LTP aligns with the *P. taeda* nsLTP sequence at a position six amino acid residues behind the putative export signal of that protein (Figure 6.1c)



C

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                                                          *       †   *
LxmLTP                                                    AISXNQVVTAXTPXASYLI
P.taeda nsLTP 1  MAVKKMVEAVFVVGLVVTMMNVWGAVPVEGAISCNQVVSAMTPCATYLIG 50

LxmLTP                                                PDRQAV
P.taeda nsLTP 51 NAATPAATCCPSIRGLDSQVKATPDRQAVCNCLKTQAKSYGVKLGKAANL 100

P. taeda nsLTP 101 PGLCKVTDLNVPISPNVDCSKVH 123
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Figure 6.1. Isolation and identification of *Lxm*LTP.

A. RP-HPLC profile of the ovular secretion of *Larix* x *marschlinsii* (20 µL) separated over a C-8 column in a linear gradient of acetonitrile. UV absorbance of the eluent was monitored at 220 nm. Arrow indicates the fraction containing *Lxm*LTP.

B. 1D SDS-PAGE (12 % acrylamide) of RP-HPLC fraction containing *Lxm*LTP. Protein staining was carried out with Gelcode staining reagent. Molecular weights of BioRad protein standards resolved in an adjacent lane are indicated at right.

C. Alignment of the N-terminal amino acid sequence of *Lxm*LTP with the deduced amino acid sequence of nsLTP from *Pinus taeda* (GenBank Accesion number Q41073, Kinlaw *et al.* 1994). The second sequence fragment of larch LTP was generated from a sample subjected to excessive hydrolysis after a failed injection by the sequencer. Amino acids representing a conservative replacement are shaded. The putative export signal of *P. taeda* nsLTP is underlined. Asterisks (*) indicate amino acids with unassigned identity that most likely represent Cys residues conserved across LTPs (Cys residues are not recovered in N-terminal amino acid sequencing). Cross (†) indicates an unassigned residue that may be the result of an oxidized Met.

Identification of *Txm*βGlu, *Txm*TLPa, and *Txm*TLPb from the yew secretion

Three of the major proteins from the pollination droplet of yew resolvable by 2D electrophoresis were subjected to sequencing by tandem mass spectrometry (MS/MS) (Figure 6.2). The largest of the protein spots (spot 3, Figure 6.2) occupied an area of the gel corresponding to a molecular weight of approximately 25 kDa and spread over a range of pI from 8 – 9. This spot was previously determined to represent a single protein by MALDI-TOF MS (Chapter 4). The second largest protein spot (spot 1) had a molecular weight of approximately 50 kDa and a pI of 5.3. The final spot (spot 2) had an apparent molecular weight of 28 kDa and an acidic pI of 4.2.

MS/MS analysis of six peptides generated from spot 1 identified this protein as a βGlu (Table 6.2). It was named *Txm*βGlu. Four peptides from spot 2 and seven from spot 3 identified both of these spots as TLPs (Table 6.2). MALDI-TOF MS analysis of the peptides generated by trypsin digestion of spots 2 and 3 produced different peptide mass profiles, indicating that the two yew TLPs were not identical (Figure 6.3). Accordingly, the acidic TLP was named *Txm*TLPa and the basic TLP, *Txm*TLPb.

Immunodetection of TLPs in the ovular secretions of yew and larch

The antibodies anti-*Pm*TLP and BARPERM1, were used to detect TLPs in 1D separated samples of yew and larch ovular secretions (Figure 6.4). Anti-*Pm*TLP bound a single protein band with an apparent molecular weight just below the 25 kDa protein standard marker in the yew secretion, and a band slightly above 25 kDa marker in the larch sample (Figure 6.4a). BARPERM 1 also bound a single protein band from the yew secretion, but not the same band that was recognized by anti-*Pm*TLP (Figure 6.4b).

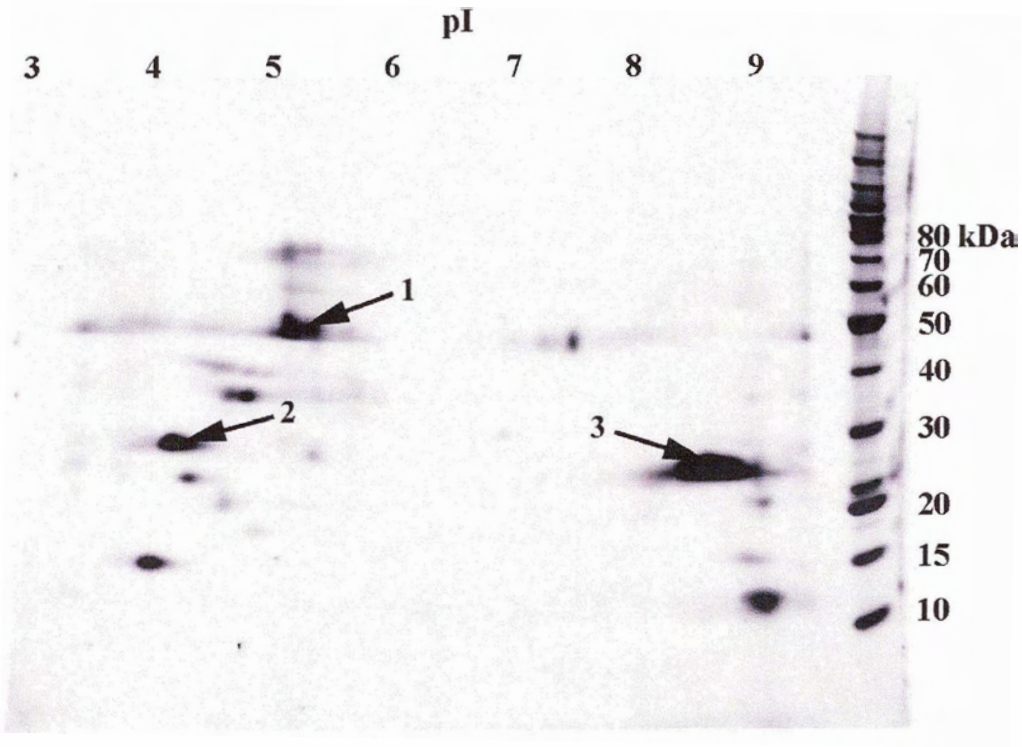


Figure 6.2. 2D electrophoretic separation of proteins in the pollination droplet of *Taxus* x *media*. The gel shown is representative of gels from which protein spots (arrows 1 – 3) were excised for MS/MS sequencing.

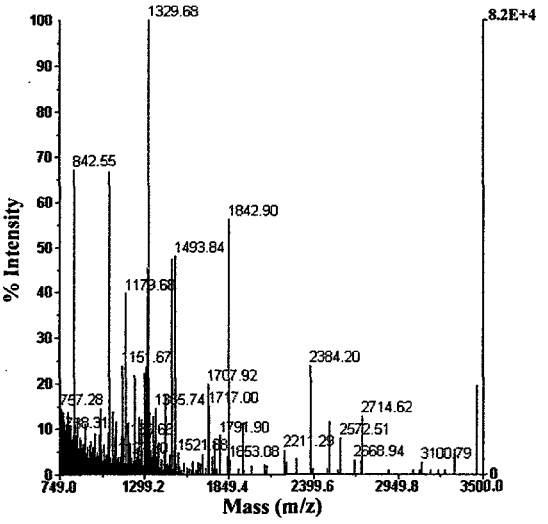
Table 6.2. Amino acid sequence and protein identification of trypsin generated peptides from three yew pollination droplet proteins isolated by 2D gel electrophoresis.

Spot	Peptide m/z (z=2)	MS/MS Sequence[a]	Identity[b]
1	604.3	SVIDFLASR	β-1,3-Glucanase
(*Txm*βGlu)	620.3	FGEAQLQVYGK	
	624.3	FDGAQLQLK	closest alignment from
	669.4	LGIELLNEPR	*Oryza sativa* AC107314
	683.8	GTYLVAEDGGGSK	
	879.4	FSFISQDGLSAVR	
2	536.2	GCSFDGNGR	Thaumatin-like protein
(*Txm*TLPa)	658.8	INAVCPAELK	closest alignment from
	665.3	QCPQAYSYAK	*Cryptomeria japonica*
	921.8	SSTFTCSSGTTDYK	AB081305
3	536.3	TGCSFDGNGR	Thaumatin-like protein
(*Txm*TLPb)	575.8	TCLSDINSK	
	850.3	TGDCDGVLECK	closest alignment from
	877.3	SYTVWAAASPGNK	*Cryptomeria japonica*
	956.3	PQYCCTGAYNATK	AB081305
	1036.5	AQGQSWSIQV	
	1204.0	DDATSTFTCPATSGSNYK	

[a] MS/MS sequencing data is presented in Appendix A. L/I determination based on alignment results.

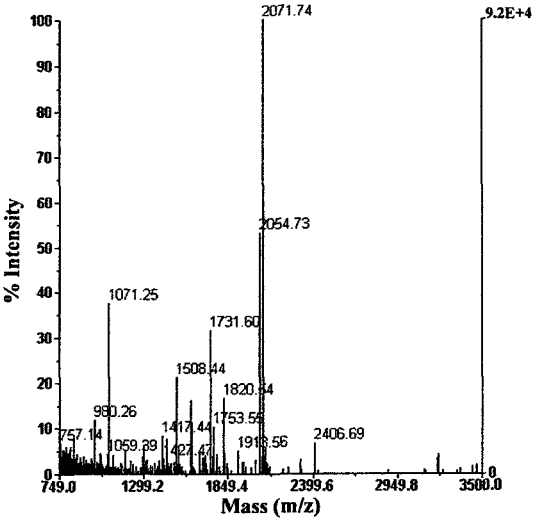
[b] Protein identity is based on most significant alignment by BLASTp2 search of EMBL sequence database. BLAST results are presented in Appendix B.

TxmTLPa (spot 2)



Peptide ions (m+H) unique to *Txm*TLPa	
1151.67	1842.90
1316.75	2384.20
13.29.68	2714.62

TxmTLPb (spot 3)



Peptide ions (m+H) unique to *Txm*TLPb	
980.26	1820.54
1071.25	2054.73
1508.44	2071.74
1731.60	2406.69

Figure 6.3. Peptide mass profiles of *Txm*TLPa and *Txm*TLPb from the pollination droplet of *Taxus* x *media*. Proteins were separated by 2D gel electrophoresis and subjected to trypsin digestion prior to mass spectrometer analysis. Unique ions for each spot indicated that the two spots represent different proteins. Spectra were obtained with an ABI Voyager DE-STR MALDI-TOF mass spectrometer in reflectron mode with a delayed extraction of 80 ns. Data managed with Data Explorer (v.4.0) software (Applied Biosystems Inc.).

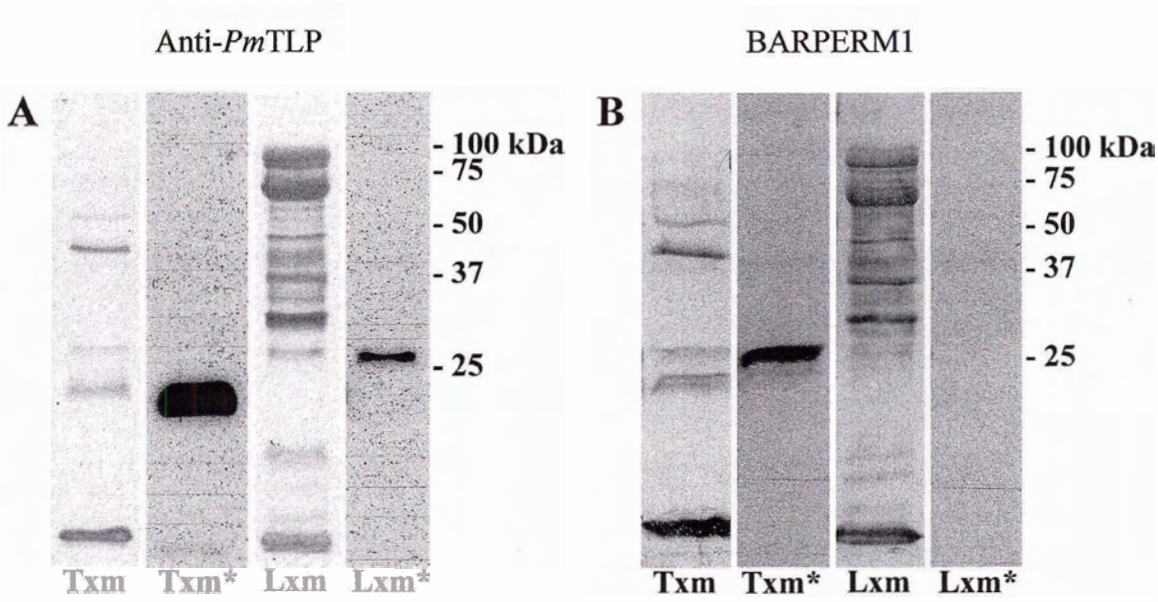


Figure 6.4. Immunodetection of TLPs in the ovular secretions of *Taxus* x *media* (Txm) and *Larix* x *marschlinsii* (Lxm) by the polyclonal antibodies anti-*Pm*TLP (A) and BARPERM1 (B). Samples (10 µL) were separated by 1D SDS-PAGE through a 12 % acrylamide gel prior to electro-transfer onto PVDF membrane. Membranes were stained with Gelcode reagent when immunodetection was complete. Detection of bound antibodies was performed with the ECL chemiluminescent system and Kodak Biomax film. Membrane sections containing Gelcode-stained proteins from *Taxus* and *Larix* samples are labelled Txm and Lxm, respectively. Exposed film segments corresponding to sample lanes on the blot are labelled with asterisks (*). Molecular weights of BioRad protein standards separated and blotted in adjacent lanes (not shown) are indicated.

The protein bound by BARPERM1 had a molecular weight of approximately 27 kDa as determined by 1D SDS-PAGE. BARPERM1 did not bind to any proteins in the larch ovular secretion. Neither anti-*Pm*TLP nor BARPERM1 displayed any non-specific binding patterns.

Based on the relative molecular weights of the different protein bands detected by Anti-*Pm*TLP and BARPERM1, it appears that Anti-*Pm*TLP binds the lighter *Tx*m*TLPb while BARPERM1 binds *Tx*m*TLPa. Recognition of *Tx*m*TLPb from the yew ovular secretion by anti-*Pm*TLP was confirmed by immunodetection after 2D separation of the yew sample (Figure 6.5).

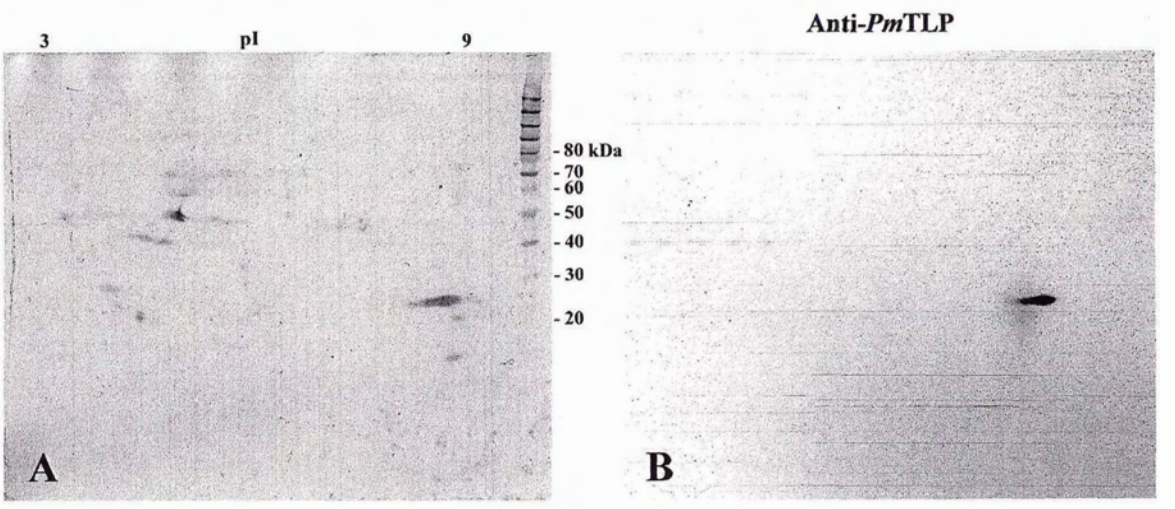


Figure 6.5. Immunodetection of *Txm*TLPb in a 2D separation of *Taxus* x *media* pollination droplet by the polyclonal antibody anti-*Pm*TLP. The protein component (after acetone / TCA precipitation) of 60 µL of *Taxus* pollination droplet was separated by 2D electrophoresis prior to electro-transfer onto PVDF membrane. The membrane was stained with Gelcode reagent when immunodetection was complete (A). Detection of bound anti-*Pm*TLP was performed with the ECL chemiluminescent system and Kodak Biomax film (B). Isolelectric point markers were determined from the overlaid IEF strip. Molecular weights of protein standards run with the sample in the second dimension are indicated.

Discussion

Conifers are particularly vulnerable to bacterial and fungal infection during the period of pollen receptivity. Even in species that employ a seed cone, conifer ovules are by necessity open to the environment during pollen shed. Pollen is accepted directly into the micropyle by movement through a fluid (e.g. *Taxus*, *Thuja*), by mechanical transference (e.g. *Larix*, *Pseudotsuga*), or by an elaborate outgrowth of the nucellus and extreme siphonogamy (e.g. *Araucaria*). Windborne pollen is accompanied by bacteria and fungal spores, which are abundant in the air. If allowed to infect the naked reproductive tissues of the ovule, pathogenic microbes possess the potential to devastate a conifer's fitness.

Conifer ovules lack physical barriers to opportunistic microbes; the nucellus has no waxy cuticle or thick epidermal covering (Takaso and Owens 1997). The interior of the conifer ovule is put at further risk by its humid state and the sugar-rich composition of the ovular secretion. These conditions provide a favourable environment for microbial growth. However, in hundreds of ovules of *Larix*, *Pseudotsuga*, *Pinus*, *Picea*, and *Taxus* dissected by myself and other members of the von Aderkas lab, not once has pathogenic infection been observed. Conifer ovules must make use of an unreported mechanism or mechanisms to maintain asepsis during pollen collection, germination, and fertilization.

In this study, PR proteins have been found to comprise some of the most abundant proteins present ovular secretions of larch and yew. The presence of these antimicrobial compounds provides the first explanation of the remarkable resistance of conifer ovules to pathogen establishment. The larch ovular secretion appears to contain at least two PR proteins that are likely to possess antibacterial and antifungal activity, while the yew

pollination droplet has a minimum of three antifungal PR proteins. A discussion of each of the three major classes of PR proteins identified in this study follows.

_Lxm_LTP (PR-14) in the ovular secretion of larch

LTPs are small proteins typically composed of 90 – 95 amino acid residues and found in all plant species (Kader 1997, Broekaert *et al.* 1997). Amino acid sequence can vary widely between LTPs of different plants, with only about 30 % of the residues appearing to be conserved (Broekaert *et al.* 1997). Conserved regions include 12 positions invariably occupied by hydrophobic residues and eight cysteine residues that form four disulphide bonds. These residues define the tertiary structure of the protein, which includes a central tunnel-like hydrophobic cavity capable of housing a fatty acyl chain (Shin *et al.* 1995, Gomar *et al.* 1996).

As their name implies, LTPs were first described for their ability to shuttle lipids between donor and acceptor membranes (Kader *et al.* 1984). With few exceptions (Phillippe *et al.* 1995), LTPs from all plant species are able to transfer an array of phospholipids (e.g., phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol) between membranes *in vitro*. Most LTPs lack specificity for a particular phospholipid and are referred to as non-specific lipid transfer proteins (nsLTPs). These proteins were first proposed to mediate intracellular trafficking of lipids from the endoplasmic reticulum to other cellular organelles (Arondel and Kader 1990), but this activity has never been demonstrated *in vivo*.

LTP gene expression occurs most actively in the epidermal cells of aerial plant tissues, including leaves (Gausing *et al.* 1994), stems (Clark and Bohnert 1993), flowers

(Suelves and Puigdomenech 1997), and fruit (Botton *et al.* 2002). Immunolocalization studies in *Arabidopsis* and *Brassica* have revealed that LTPs are located outside the plasma membrane, within the cell wall (Thoma *et al.* 1993, Pyee *et al.* 1994). These findings are in agreement with the observation that LTP cDNA sequences code for amino acid export signals for cellular secretion (Bernhard *et al.* 1991, Tchang *et al.* 1998). The majority of the literature on LTPs supports the hypothesis put forward by Sterk *et al.* (1991) that the normal role of LTP is not intracellular lipid trafficking, but is the transport of cutin monomers through the ECM of plant cells to the sites of wax synthesis. Indeed, WAX9, an LTP identified in broccoli, comprises more than 90 % of the total proteins associated with the surface wax layer of this plant (Pyee *et al.* 1994).

LTP expression has been demonstrated to rise in response to fungal and bacterial pathogens (Molina and García-Olmedo 1993, Jung *et al.* 2003), as well as fungal elicitors (Gomès *et al.* 2003). These findings prompted the classification of this group as PR proteins. Antifungal and/or antibacterial activity has been reported for all LTPs tested, although the relative activities of different LTPs vary between pathogens. This suggests that some pathogens possess a degree of resistance to specific LTPs (García-Olmedo *et al.* 1995,1998, Broekaert *et al.* 1997). The mechanism by which LTPs act against pathogens has not yet been determined. It has been suggested that the physical properties of these molecules (hydrophobic domains, small size, basic pI) may affect the integrity of fungal and bacterial plasma membranes (Gomès *et al.* 2003).

The abundance of LTPs in the outermost tissues of plants, their ability to respond to pathogen invasion, and their demonstrated antimicrobial properties led García-Olmedo *et al.* (1995) to propose that these proteins provide a "defense-protein shield" at the plant

surface. The finding of *Lxm*LTP in the ovular secretion of larch is consistent with this hypothesis. Everything that is collected by the stigmatic flap of the larch ovule from the outside environment is pushed into the micropylar cavity; the ovular secretion, in essence, occurs on an external surface. Other than the flap itself, the ovular secretion represents the only opportunity for the larch ovule to mount an active defence against pathogens before they reach the nucellus. If LTPs are present in the secretions of species that collect pollen directly by a pollination droplet, they may truly represent a first line defence against incoming pathogens in these instances.

The RP-HPLC fraction containing *Lxm*LTP dominated the UV spectrum of ovular secretion proteins separated by the column. The relative size of peak for the *Lxm*LTP fraction compared with other protein fractions suggests that *Lxm*LTP is a major component of the larch secretion.

*Lxm*LTP may play multiple roles in the larch ovule. Besides a defensive role in the secretion, this abundant protein may be involved in the deposition of wax on the inside surface of the larch integument. Takaso and Owens (1997) have proposed that the cuticle lining the larch micropyle facilitates pollen transportation to the nucellus by the receding ovular secretion. *Lxm*LTP in the larch secretion may also transport lipids produced by the ovule into the liquid medium for uptake by germinating pollen. Lipid shuttling and defensive roles for *Lxm*LTP are not mutually exclusive (García-Olmedo *et al.* 1995).

_Txm_βGlu (PR-2) in the yew pollination drop

Internal amino acid sequencing identified a protein from the yew pollination droplet as a member of the βGlu family. Endoglucanases from this group are able to catalyze the hydrolysis of β-1,3-glucan into oligomers of two to six glucose units (Boller 1993). Across plant species, βGlu exist in multiple forms that differ in size, pI, primary structure, and regulation (Leubner-Metzger and Meins 1999).

Three major classes of βGlu have been established based on amino acid sequence and cellular localization. Class I βGlu are basic proteins typically 33 kDa in size and localized in the cell vacuole (Van den Bulcke *et al.* 1989). In contrast to βGlu I, βGlu proteins of class II and III are acidic in nature and are secreted into the apoplast (Leubner-Metzger and Meins 1999). Members of class II and III tend to have molecular weights ranging from 34 – 35 kDa, but larger variants are known (Ori *et al.* 1990). Due to its acidic nature (pI of 5.3) and extracellular location, *Txm*βGlu is most likely a class II or III βGlu.

There is a substantial literature pertaining to the role of βGlu in response to pathogenic fungi. These PR proteins have been reported to operate in at least two different ways. They may act against a fungal pathogen directly by degrading the cell wall (Schlumbaum *et al.* 1986, Sela-Buurlage *et al.* 1993, Selitrennikoff 2001, Zareie *et al.* 2002), or indirectly by promoting the release of fungal cell wall materials that elicit further defence reactions in the host plant (Lamb *et al.* 1989, Boller 1995, Okinaka 1995). Along with chitin, β-1,3-glucan is a major component of the cell walls of basidiomycetes, ascomycetes, and to a lesser extent oomyctes (Wessels 1993). These three groups

represent a large proportion of potentially pathogenic fungi that may be susceptible to *Txm*βGlu activity.

TLPs (PR-5) in the ovular secretions of yew and larch

The PR-5 proteins are grouped together based on strong amino acid similarity with thaumatin, an intensely sweet-tasting protein isolated from the berries of the South African ketemfe bush *Thaumatococcus daniellii* Benth (Van der Wel and Loeve 1972). Consequently, many members of the PR-5 group are referred to as thaumatin-like proteins (TLPs), though none have been found to share the sweet taste of thaumatin (Velazhahan *et al.* 1999). Other members of the PR-5 group that share amino acid sequence and antifungal activity with TLPs are known as permatins (Skadsen *et al.* 2000) and osmotins (Anžolvar and Dermastia 2003).

Antifungal activity of a TLP was first reported in 1990 by Roberts and Selitrennikoff. They established that zeamatin, a 22 kDa protein isolated from maize seeds, inhibited the growth of the plant fungal pathogens *Neurospora crassa* and *Trichoderma reesei* as well as the human pathogen *Candida albicans*. The next year, it was determined that zeamatin shared significant amino acid sequence with proteins from oats, sorghum, wheat, and a PR-5 protein from tobacco (Vigers *et al.* 1991). All of these proteins are now included in the PR-5 group. In the years that followed, antifungal activity was demonstrated for both acidic and basic TLPs isolated from a variety of plant species, including tobacco (Vigers *et al.* 1992, Abad *et al.* 1996), tomato (Rodrigo et al. 1993), *Arabidopsis thaliana* (Hu and Reddy 1997), *Brassica campestris* (Cheong *et al.* 1997), grape (Salzman *et al.* 1998), and apple (Krebitz *et al.* 2003). The fungicidal

action of TLPs typically results in the lysis of fungal hyphae or spores, or the inhibition of spore germination.

The major protein resolved from the yew pollination droplet by 2D gel electrophoresis is the basic TLP *Tx*m*TLPb. Positive identification of this protein has been determined by internal amino acid sequence and specific recognition of the protein by the anti-*Pm*TLP antibody. The basic pI of *Tx*m*TLPb (8-9) and its molecular weight (approximately 25 kDa) fall within the ranges reported for basic TLPs from other plant species (Anžlovar and Dermastia 2003).

A second major protein isolated from the yew pollination droplet is *Tx*m*TLPa, an acidic member of the PR-5 group. Migration of this protein during 2D gel electrophoresis indicates that it has a very low pI (approximately 4.2) and a slightly larger molecular weight (approximately 28 kDa) than *Tx*m*TLPb. *Tx*m*TLPa appears to be the less abundant of the two forms of TLP in the yew pollination droplet, but still comprises the third largest protein spot evident by 2D gel separation after *Tx*m*TLPb and *Tx*m*βGlu. *Tx*m*TLPa was originally identified by amino acid sequence identity to known TLPs and a positive interaction with the anti-BARPERM1 antibody. The nucleic acid sequence of the cDNA coding for *Tx*m*TLPa has since been determined and confirms its identity as an acidic TLP (Chapter 7).

Anti-*Pm*TLP binds a single band after 1D SDS-PAGE separation of the larch ovular secretion. This larch protein has not yet been subjected to amino acid sequencing, nor has it been determined whether it represents an acidic or a basic protein. Nonetheless, the presence at least one TLP in the larch ovular secretion appears likely.

Are the ovular secretions of conifers PR protein cocktails?

As discussed above, members of the PR-2, PR-5, and PR-14 groups isolated from a variety of plant species have demonstrated activity against pathogenic fungi, and also bacteria in the case of LTPs. The defensive properties of these proteins have been further verified by the production of transgenic plants that over-express one or more PR protein. The barley LTP2 gene, when constitutively expressed in the tissues of transgenic tobacco or *Arabidopsis* conferred an elevated level of resistance to the bacterial pathogens *Pseudomonas syringae* pv. *tabacci* 153 and *P. syringae* pv. tomato DC3000, respectively (Molina and García-Olmedo 1997). A variety of crop species engineered to over-express TLPs possess increased resistance to their major fungal pathogens (e.g., potato - Zhu *et al.* 1996, rice - Datta *et al.* 1999, and orange - Fagoaga *et al.* 2001). By itself, βGlu has conferred resistance in tobacco to the pathogenic fungi *Peronospora tabaccina* and *Phytophthora parasitica* var. *nicotiana* (Lusso and Kuć 1996). Transformants expressing βGlu and a chitinase (of the PR-3,4,8, or 11 groups) show a much stronger resistance to a wider variety of fungal pathogens than plants transformed with either PR protein alone (Van den Elzen *et al.* 1993, Zhu *et al.* 1994, Jongedijk *et al.* 1995).

The example of βGlu and chitinase possessing enhanced antifungal activity when applied in combination is not unique among PR proteins. Antimicrobial proteins, including chitinases, βGlus, LTPs, TLPs, thionins, and other PR proteins are reported to be most efficacious when applied in combinations of two or more different types (Roberts and Selitrennikoff 1990, Sela-Buurlage *et al.* 1993, Molina *et al.* 1993, Jach *et al.* 1995, Punja 2001, Zareie *et al.* 2002). These finding are not surprising; sets of multiple PR proteins have been known to respond to pathogenic attack from the very first

reports of this phenomenon (Van Loon and Van Kammen 1970, Gianinazzi *et al.* 1970). Most authors agree that different PR proteins operate synergistically to provide enhanced resistance to a wider variety of pathogens.

An example of multiple PR proteins being produced in a developmentally coordinated manner for the protection of reproductive tissues is reported in grape. A TLP, a LTP, and two chitinases (PR-3) have been found to accumulate specifically in ripening grape berries concurrently with sugar accumulation (Salzman *et al.* 1998). The presence of these proteins exhibited growth-inhibiting activity against the important grape pathogens *Guignardia bidwellii* and *Botrytis cinerea*. Antifungal activity was enhanced by as much as 70 % in the presence of 1 M glucose (the physiological concentration of hexoses in the berries). In a later study, grape plants selected for anthracnose resistance have been shown to produce increased levels of LTP and two TLPs compared to non-resistant plants (Jayasankar et al. 2003).

The pollination droplet of yew, and most likely the ovular secretion of larch contain mixtures of PR proteins. In the case of yew, *Txm*TLPb, *Txm*βGlu, and *Txm*TLPa, are the three major proteins found in the secretion. The other proteins in the pollination droplet have yet to be identified; some may well be other PR proteins. The larch ovular secretion contains *Lxm*LTP, and dozens of unidentified proteins of which one is almost certainly a TLP. The presence of a mixture of PR proteins and their relative abundance in the ovular secretions of yew and larch strongly suggests that these liquids effectively rinse incoming pollen grains with a broadly-acting cocktail of antimicrobial compounds as they are transported to the nucellus. This phenomenon may account for the surprising asepsis of conifer ovules during reproduction.

Chapter 7

Molecular characterization and heterologous expression of *Txm*TLPa

Introduction

Thaumatin-like proteins (TLPs) have attracted much interest from many research groups. Most studies pertain to the antifungal activity of these proteins. A survey of the GenBank database yields TLP sequences isolated from a wide variety of plant species, including tobacco, *Arabidopsis*, lily, *Brassica*, tomato, wheat, barley, oats, rice, maize, sorghum, cherry, banana, grape, strawberry, juniper, cypress, Douglas-fir, and many others.

All of these may be placed into two distinct size classes. The more abundant group comprises TLPs ranging in length from 201 – 229 amino acid residues, with molecular weights between 22 and 26 kDa (Velazhahan *et al.* 1999). Members of this group contain 16 conserved cysteine residues that participate in the formation of eight disulphide bonds (Ogata *et al.* 1992, Batalia *et al.* 1996, Koiwa *et al.* 1999). These bonds give properly folded TLPs a highly stabilized structure that is believed to be responsible for a general resistance to proteolytic degradation and pH- or heat-induced denaturation (Roberts and Selitrennikoff 1990, Velazhahan *et al.* 1999, Selitrennikoff 2001). Native conformation is required for TLPs to demonstrate antifungal activity (Roberts and Selitrennikoff 1990, Vigers *et al.* 1992).

The second class of TLPs consists of shorter proteins that range in length from 148 – 151 amino acid residues (typically 16 kDa). This is due to an internal deletion of 58 residues. So far, these smaller TLPs have only been reported in monocots, including barley (Bryngelsson *et al.* 1989), wheat (Rebmann *et al.* 1991), maize (Frendo *et al.*

1992), and rice (Reimann and Duddler 1993). Included in the internal deletion defining this group are six cysteine residues. It has not been determined whether or not these truncated TLPs possess antifungal activity.

Antifungal action of TLPs is a result of their ability to disrupt membranes. Roberts and Selitrennikoff (1990) found that zeamatin, a permatin isolated from corn, induced rupture of the hyphae of *Candida albicans* and *Neurospora crassa* resulting in the release of cytoplasmic contents. Similarly, the lysis of fungal membranes has been reported for permatins isolated from wheat, oat, and sorghum (Vigers *et al.* 1991), and for tobacco osmotin (Woloshuk *et al.* 1991, Vigers *et al.* 1992). When the effect of tobacco osmotin was tested on 31 fungal pathogens, the ability of the protein to inhibit fungal growth was directly correlated with the extent to which the protein could permeabilize the plasma membrane of a given species (Abad *et al.* 1996). Varying results between fungal species suggested that the antifungal activity of osmotin might depend on the composition of the fungal cell wall.

The mechanism(s) by which TLPs disrupt fungal membranes are not well understood. Lacking any known enzymatic activity, it was first hypothesized that zeamatin and related TLPs caused membrane leakage by direct insertion into the lipid bilayer (Roberts and Selitrennikoff 1990, Vigers *et al.* 1991). Later determination of the 3-dimensional structures of TLPs (including zeamatin), however, showed none of the features typical of membrane pore-forming proteins (Batalia *et al.* 1996, Koiwa *et al.* 1999). It has been noted that Roberts and Selitrennikoff (1990) demonstrated zeamatin activity at 4 °C, a temperature at which the lipid bilayer would be expected to be sufficiently crystalline to resist the incorporation of pore-forming compounds (Batalia *et*

al. 1996). More recently, it was reported that some TLPs are able to bind β-1,3-glucans (Trudel *et al.* 1998, Osmond *et al.* 2001), a major component of fungal cell walls (Wessels 1993). Trudel *et al.* (1998) have suggested a two-step mechanism for TLP action involving an initial binding of the fungal cell wall followed by insertion into the membrane.

To gain further insight into TLP activity, Koiwa *et al.* (1999) examined the crystal structure of tobacco PR-5d and compared it to the previously elucidated structures of the antifungal zeamatin protein (Batalia *et al.* 1996) and non-antifungal thaumatin (Ogata *et al.* 1992). The three proteins share over 50 % amino acid sequence identity and were found to fold into similar 3-dimensional structures with three domains determined by the conserved disulphide bonds (Koiwa *et al.* 1999). The authors identified a cleft in the surface of these proteins that appears to be important for antifungal activity. Comparison of the tertiary and primary structures of anti-fungal TLPs demonstrated the presence of five charged residues that contributed to an acidic environment in the cleft of these proteins, while non-antifungal thaumatin possessed a cleft much more basic in nature.

Heterologously expressed TLPs have been used to confer increased fungal pathogen resistance in a number of plant species (Punja 2001). In a few cases, recombinant TLPs successfully expressed in plant (Krebitz *et al.* 2003) or bacterial systems (Hu and Reddy 1997) have been isolated, purified and shown to possess antifungal activity.

This report describes the characterization of the complete cDNA for *Txm*TLPa, the acidic TLP identified in the pollination droplet of hybrid yew (*Taxus* x. *media*). The deduced amino acid sequence of this protein has the hallmarks of an antifungal TLP as

predicted by Koiwa *et al.* (1999). The sequence pertaining to the mature chain of *Txm*TLPa was cloned into a plasmid vector to drive the expression of a FLAG-*Txm*TLPa fusion protein in *E. coli*. The ultimate aim of this research is to produce and purify recombinant *Txm*TLPa in order to assess its efficacy as an antifungal agent. The preliminary results of the heterologous expression of the fusion protein are presented below.

Methods and Materials

Isolation of mRNA from yew ovules

Yew ovules from six trees were excised from their branches over dry ice at the time of pollination droplet production, flash frozen in liquid nitrogen, and stored at – 80 °C. Total RNA was later extracted from the frozen tissue using the extraction protocol of Chang *et al.* (1993), with the addition of one phenol and one phenol/chloroform extraction step after LiCl precipitation. Poly-adenylated (Poly-(A)) mRNA was isolated from total RNA with the PolyATtract mRNA Isolation System (Promega).

Cloning and sequence determination of TxmTLPa cDNA

5' and 3' Rapid Amplification of cDNA Ends (RACE) was used to generate full-length cDNA coding for *Txm*TLPa. Reverse-transcription of mRNA extracted from yew ovules and ligation of 5' and 3' RACE adapters was performed with the FirstChoice RLM-RACE Kit (Ambion).

Polymerase Chain Reaction (PCR), cloning, and nucleic acid sequencing

An Eppendorf Mastercyler Gradient thermocycler was used for all PCR reactions. *Taq* DNA polymerase, PCR buffer, and dNTPs were supplied by Invitrogen. The sequences of oligonucleotide primers generated during this study and the PCR conditions used for specific primer pairs are listed in Table 7.1.

PCR products were assessed by agarose electrophoresis and ethidium bromide visualization. Amplified products of the expected size were purified with the QIAquick PCR Purification Kit (Qiagen) prior to T/A ligation into the pGEM-T vector (Promega). The resulting vectors were tranfected into competent XL2-Blue *E.coli* (Stratagene) by heat shock according to manufacturer's directions. Blue/white screening was used to identify colonies carrying pGEM-T with a PCR insert for overnight liquid culture. Plasmids were harvested from liquid cultures with the QIAprep Miniprep kit (Qiagen). Double digestion of harvested plasmids with *Not* I / *Nco* I (NEB) restriction enzymes followed by agarose electrophoresis of the digest products was performed to confirm insert size. Plasmid construction, *E.coli* manipulation and cloning were conducted according to standard methods (Sambrook *et al.* 1989).

Nucleic acid sequencing was performed from plasmids submitted to the Centre for Biomedical Research (CBR) DNA Sequencing Facility (University Of Victoria) with a LI-COR 4200 IR^2 DNA sequencer and fluorescently labelled M13 primers. To ensure sequence fidelity, sequencing was carried out in both directions from multiple clones for each PCR product.

Sequence analysis and characterization of the physical properties of TxmTLPa

Sequences were aligned with the Clustal W program (Thomspon *et al.* 1994). Sequence analysis and generation of deduced amino acid sequence was performed with the BioEdit software package (Hall 1999). Molecular weight and pI from deduced amino acid sequence was calculated using the ExPASy Molecular Biology Server (http://ca.expasy.org/). Hydropathy analysis for N-terminal sequence prediction was done with the iPSORT program (http://hypothesiscreator.net/iPSORT/). Searches against the GenBank (nr) database (http:///www.ncbi.nlm.nih.gov/BLAST/) were conducted with the BLASTp algorithm (Altschul *et al.* 1997)

Heterologous expression of TxmTLPa

Tailed PCR was used to amplify the full-length sequence coding for the mature *Txm*TLPa protein from cDNA. Primer tails were designed to add a *Hind* III restriction site to the 5'end and a *Bgl* II restriction site to the 3' end of the product. Platinum *Pfx* DNA polymerase (Invitrogen) was used for this PCR reaction to limit amplification error. The PCR products were incubated with *Taq* DNA polymerase and 0.2 mM dATP for 30 min at 70 ºC to introduce 3'-A overhangs (A-tailing) for T/A cloning into pGEM-T to produce pGEM*Txm*TLPa. After sequence confirmation, pGEM*Txm*TLPa was harvested from overnight cultures and purified. Double digestion with *Hind* III and *Bgl* II was used to excise the *Txm*TLPa fragment from pGEM*Txm*TLPa and insert it into the expression plasmid pFLAG-ATS (Sigma) to create pFLAG-*Txm*TLPa. Nucleic acid sequencing with the N-26 / C-24 primer set supplied with pFLAG-ATS confirmed the proper insertion of the *Txm*TLPa sequence.

Heat shock was used to transform Rosetta-gami competent cells (Novagen) with pFLAG-*Tx*m*TLPa. This *E. coli* strain was selected for heterologous protein expression because it harbours mutations in the thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes allowing for enhanced disulphide bond formation in the cytoplasm. Additionally, this strain carries genes for the production of rare bacterial tRNAs for the codons AUA, AGG, AGA, CUA, CCC, and CGA, making it amenable to eukaryotic protein expression.

Transformed cells were grown in LB medium + 0.4 % glucose containing kanamycin (15 μg/mL), chloramphenicol (34 μg/mL), tetracycline (12.5 μg/ mL), and ampicillin (50 μg/mL) to select for the Rosetta-gami traits and pFLAG-*Tx*m*TLPa. When the cells entered exponential growth phase (OD_{600} = 0.200) expression of the FLAG-*Tx*m*TLPa fusion protein was induced by addition of isopropylthio-β-galactoside (IPTG - final concentration 0.5 mM) to the growth medium. Cells were harvested 2 h after IPTG induction.

Immunodetection of recombinant TxmTLPa

The protocol for immunodetection of proteins immobilized on PVDF membrane is described in Chapter 5. The detection of the *E. coli*-expressed FLAG-*Tx*m*TLPa fusion protein was performed with BARPERM1 (Chapter 6) and anti-FLAG M2 (supplied with pFLAG-ATS expression kit, Sigma). BARPERM1 binds to *Tx*m*TLPa, while anti-FLAG M2 binds the FLAG peptide (DYKDDDDK) at the N-terminus of the fusion protein. *E. coli*-expressed FLAG-BAP (bacterial alkaline phosphatase) was used as a control.

BARPERM1 was used in a 1/1,000 dilution in 5 % skim milk powder in TBST. Anti-FLAG M2 was reconstituted in the same solution to a working concentration of 10 μg / mL. Goat anti-rabbit IgG conjugated to horseradish peroxidase (Cedar Lane) diluted 1/50,000 in 5 % skim milk powder in TBST served as the secondary antibody for BARPPERM1 detection. Similarly diluted goat anti-mouse IgG conjugated to horseradish peroxidase (Cedar Lane) was the secondary antibody for anti-FLAG M2 detection.

Results

Cloning and sequence determination of *Txm*TLPa cDNA

Three partially degenerate PCR primers (two forward and one reverse) were designed to anneal to conserved regions of TLP based on the cDNA sequences of three conifers: *Pseudotsuga menziesii* (Accession no. AJ131731), *Cupressus arizonica* (AJ294411), and *Juniperus ashei* (AF121776). The sequences of these primers (TLPfor1, TLPfor2, and TLPrev1) and all other primers generated for this study can be found in Table 7.1. A half-nested PCR amplification of the cDNA pool generated from droplet-producing yew ovules was performed with TLPfor1 / TLPrev1 (1^{st} round) and TLPfor2 / TLPrev1 (2^{nd} round). The second round of PCR amplified a single product 359 bp in length (excluding the degenerate primers). Alignment of the nucleic acid sequence of this product with sequences in the GenBank database confirmed it was a fragment of a TLP cDNA. From this sequence, two non-degenerate yew-specific primers (yewTLPrev1 and yewTLPrev2) were designed to carry out 5' RACE from the

Table 7.1. Primer sequences and PCR conditions for the amplification of *Txm*TLPa cDNA and the construction of pFLAG-*Txm*TLPa.

Primer Pair (Forward / Reverse)	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
TLPfor1 / TLPrev1	TGCGGGTACACWGTCTGGGCAGC	CCTTMGCATAGCTRTAAGCCTGAGGGCACTG
TLPfor2 / TLPrev1	GGACGTACGGGCTGCACTTTCGA	CCTTMGCATAGCTRTAAGCCTGAGGGCACTG
5' RACE outer / yewTLPrev1	GCTGATGGCGATGAATGAACACTG	CAGTTGTCGGTATTTGCACCTGTGC
5' RACE inner / yewTLPrev2	CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG	CACTGTTGCAACCTCCAGACACCTTC
yewTLPfor1 / 3' RACE outer	CTGTCTCGCTTGCAGAGTATTCCC	GCGAGCACAGAATTAATACGACT
yewTLPfor2 / 3' RACE inner	GACATTTCCCTCGTCGACGGCTTC	CGCGGATCCGAATTAATACGACTCACTATAGG
yewTLP5utr / yewTLP3utr	TCAAACACCCAAATATAATGGCAACA	TTATTGCAGGATGATTATTCGGAGCA
XTxmTLPaF1 / XTxmTLPaR1	TTCCAAGGAAGCTTGCAGCAAAGTTCGAGATAACAAACC	TTTGGGAAAGATCTTTATTGCAGGATGATTATTCGGAGCA

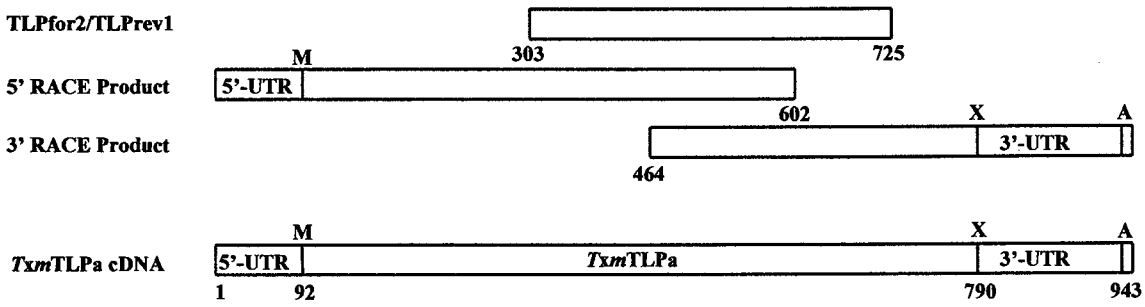
Primer Pair (Forward / Reverse)	PCR Conditions
TLPfor1 / TLPrev1	94 °C (3 min), 30 X [94 °C (30 s), 45 °C (30 s), 72 °C (30 s)], 72 °C (7 min)
TLPfor2 / TLPrev1	"
5' RACE outer / yewTLPrev1	94 °C (3 min), 35 X [94 °C (30 s), 62 °C (30 s), 72 °C (30 s)], 72 °C (7 min)
5' RACE inner / yewTLPrev2	"
yewTLPfor1 / 3' RACE outer	94 °C (3 min), 30 X [94 °C (30 s), 65 °C (30 s), 72 °C (30 s)], 72 °C (7 min)
yewTLPfor2 / 3' RACE inner	"
yewTLP5utr / yewTLP3utr	94 °C (3 min), 30 X [94 °C (30 s), 55 °C (30 s), 72 °C (60 s)], 72 °C (7 min)
XTxmTLPaF1 / XTxmTLPaR1	94 °C (3 min), 30 X [94 °C (30 s), 55 °C (30 s), 68 °C (60 s)], 68 °C (7 min)

W = A/T, M = A/C, R= A/G. *Hind* III restriction site introduced by XTxmTLPaF1 is underlined. *Bgl* II restriction site introduced by XTxmTLPaR1 is double underlined.

yew ovular cDNA pool. The second round of amplification resulted in a single product that comprised the 5' end of the cDNA coding for *Tx*m*TLPa, including the first 511 nucleic acids from the translation start codon and 91 nucleic acids of the 5'-untranslated region (5'-UTR). The non-degenerate primers TLPfor1 and TLPfor2 were then designed to carry out 3' RACE from the same cDNA pool. The major 3' RACE product consisted of the 3' end of the *Tx*m*TLPa cDNA, including the last 327 bp of protein coding sequence, the translation termination codon, and 165 bp of 3'UTR with the poly-(A) tail. The 5' and 3' RACE products have an exact 139 bp overlap. The product from the initial TLPfor2 / TLPrev1 PCR reaction overlaps with both RACE products. When assembled, these three nucleic acid sequences provide the complete 955 bp cDNA sequence of *Tx*m*TLPa (Figure 7.1).

Primers designed from 5' and 3'-UTR sequences (yewTLP5utr and yewTLP3utr, respectively) were used to amplify the coding region of *Tx*m*TLPa as a single fragment. Five different *Tx*m*TLPa cDNAs were identified from 10 clones (sequences in Appendix C). The deduced amino acid sequences of the cDNAs differ from each other by no more than four residues (Figure 7.2). The amino acid sequence that was predicted by four of the ten cDNA clones (*Tx*m*TLPa1, Figure 7.2) was the same amino acid sequence predicted by the original full-length cDNA (Figure 7.1). *Tx*m*TLPa1 was the sequence that all further analysis was based on. Significant alignment of the deduced amino acid sequence of *Tx*m*TLPa with TLPs from the GenBank database confirmed membership in this protein group. Among the highest scoring alignments were TLPs expressed in pollen of *Cryptomeria japonica* and *Juniperus ashei*, seed tissues of *Pseudotsuga menziesii*, and floral buds of *Vitis riparia* (Table 7.2).

A



B

TxmTLPa cDNA

```
1   CGCGGATCCG AACACTGCGT TTGCTGGCTT TGATGAAAGT ATATCTATTC  50
51  ATCTTCATTC AATCTCTTGG TTATTCAGAC ACCCAGATAT AATGGCAACA 100
101 ATATCAGTAT CAGGTCTTGG GCATCTTGTC CTTCTGCTTG GAATCGCCGT 150
151 ATCTGTATAT GTGCAAGAGG CTGGAGCAGC AAAGTTCGAG ATAACAAACC 200
201 AGTGCTCGTA CACCGTTTGG GCCGCAGGAA CCCCTGGCGG AGGGCAGCAA 250
251 CTCCAGACCG GCGAGACCTG GAGCGTAGAC GTTGCAGCGG GGACAGCAGC 300
301 GGGGAGATTC TGGGGCCGAA CAGGCTGCTC TTTCGATGGA AATGGGAGAG 350
351 GGAGCTGCCA AACTGGCGAC TGTGGTGGCT TACTCAGCTG CCAAGCCTCT 400
401 GGAGCAGTCC CTGTCTCGCT TGCAGAGTAT TCCCTCAACC AAGATCAGAA 450
451 CAAGGATTTC TATGACATTT CCCTCGTCGA CGGCTTCAAC GTTCCAATTT 500
501 CTCTCGCTCC TACCAACGGG CAGTGCACCA CCACTAGCTG CAAAAGTGAC 550
551 ATTAATGCAG TTTGCCCTGC AGAATTGAAG GTGTCTGGAG GTTGCAACAG 600
601 TGCCTGTGCT GCCTTTCACA CTGACCAGTA TTGCTGCACA GGTGCAAATA 650
651 CCGACAACTG CGCTCCCACC AATTACTCCA TGTTCTTCAA GAACCAGTGT 700
701 CCCCAGGCGT ATAGTTATGC AAAGGACGAT GCCTCCAGCA CTTTCACCTG 750
751 CTCCTCTGGT ACCACTGACT ACAAAATTGT ATTCTGTCCT TAAACACATA 800
801 CATATACTTT TACAGAGAAT AATATGCTCC GAATAATCAT CCTGCAATAA 850
851 TCTATACACA TAGCTGTATA GCCTGTATAC ATATATTCTG CAATGGAATA 900
901 ATATACCATG GAGTCATTTC TTGTGACTTT CCACTTCTAC CTNAAAAAAA 950
951 AAAAA 955
```

Figure 7.1. Assembly of the full-length cDNA sequence of *Txm*TLPa.

A. Schematic representation of the assembly of the full-length *Txm*TLPa cDNA sequence from the TLPfor2/TLPrev1 PCR, 5'RACE, and 3'RACE products. Position of 5'-UTR, 3'-UTR, putative translation start codon (M), translation stop codon (X), and poly-(A) tail (A) are indicated. Numbers indicate position from the first base of the cDNA sequence.

B. Full-length cDNA sequence of *Txm*TLPa. Putative ATG translation start codon is indicated by bold type, TAA stop codon is underlined, poly-(A) tail is in italics. The identity of bp 943 (*N*) and the length of the poly-(A) sequence are artefacts of the 3'RACE adapter and cannot be unambiguously assigned.

```
TxmTLPa1 (4)   1   MATISVSGLG HLVLLLGIAV SVYVQEAGAA KFEITNQCSY TVWAAGTPGG  50
TxmTLPa2 (3)   1   .......... .......... .......... .......... ..........  50
TxmTLPa3 (1)   1   .......... .......V.. .......... .......... ..........  50
TxmTLPa4 (1)   1   .......... .......... ....K..... .......... ..........  50
TxmTLPa5 (1)   1   .......... .......... .......... .......... ..........  50

TxmTLPa1 (4)  51   GQQLQTGETW SVDVAAGTAA GRFWGRTGCS FDGNGRGSCQ TGDCGGLLSC  100
TxmTLPa2 (3)  51   .......... .......... .......... .......... ..........  100
TxmTLPa3 (1)  51   .......... .......... .......... .......... ..........  100
TxmTLPa4 (1)  51   .......... .......... .......... .......... ..........  100
TxmTLPa5 (1)  51   .......... .......... .......... .......... ..........  100

TxmTLPa1 (4)  101  QASGAVPVSL AEYSLNQDQN KDFYDISLVD GFNVPISLAP TNGQCTTTSC  150
TxmTLPa2 (3)  101  .......A.. .......... .......... .......... .......S..  150
TxmTLPa3 (1)  101  .......A.. .......... .......... .......... .....S....  150
TxmTLPa4 (1)  101  .......A.. .......... .......... .......... ..........  150
TxmTLPa5 (1)  101  .......S.. .......... .......... .......... ..........  150

TxmTLPa1 (4)  151  KSDINAVCPA ELKVSGGCNS ACAAFHTDQY CCTGANTDNC APTNYSMFFK  200
TxmTLPa2 (3)  151  .......... .......... .......... .......... ..........  200
TxmTLPa3 (1)  151  .......... .......... .....R.... .......... ...S......  200
TxmTLPa4 (1)  151  .......... .......... .......... .......... ..........  200
TxmTLPa5 (1)  151  .......... ....P..... .......... .......... ..........  200

TxmTLPa1 (4)  201  NQCPQAYSYA KDDASSTFTC SSGTTDYKIV FCP 233
TxmTLPa2 (3)  201  .......... .......... .......... ... 233
TxmTLPa3 (1)  201  .......... .......... .......... ... 233
TxmTLPa4 (1)  201  .......... .......... .......... ... 233
TxmTLPa5 (1)  201  .......... .......... .......... ... 233
```

Figure 7.2. Alignment of deduced amino acid sequences from five *Txm*TLPa cDNA variants. The number of times each sequence was represented in 10 randomly selected clones is presented in brackets. Amino acid residues identical to the *Txm*TLPa1 sequence are represented by dots (.). Uppercase letters represent amino acid replacements. Putative N-terminal export signal is underlined. Areas corresponding to peptide sequences of *Txm*TLPa generated by MS/MS (Table 6.2) are bold and double-underlined.

Table 7.2. Results of the alignment of the deduced amino acid sequence of *Txm*TLPa with sequences in the GenBank database. The top five species showing similarity with *Txm*TLPa are listed.

Protein and Species	Identical Residues	Conserved Residues	Tissue of Expression	GenBank Accession no.
Jun a 3 (TLP) *Juniperus ashei*	137/214 (64 %)	165/214 (77 %)	pollen	P81295
TLP *Crpytomeria japonica*[a]	140/218 (64 %)	164/218 (75 %)	pollen	BAC15614.1
TLP *Pseudotsuga menziesii*	129/215 (60 %)	156/215 (72 %)	seed	CAA10492.1
Cup a 3 *Cupressus arizonica*	127/203 (62 %)	155/203 (76 %)	pollen	CAC05258.1
TLP *Vitis riparia*	115/211 (54 %)	142/211 (67 %)	floral bud	AAD55090.1

[a] Three nearly identical *C. japonica* sequences (BAC 15614.1, BAC15615.1, BAC15616.1) aligned with *Txm*TLPa.

Alignment with other TLPs and hydropathy analysis predict a 28-residue N-terminal export signal in the *Tx*m*TLPa protein precursor (Figure 7.2). The deduced amino acid sequence of mature *Tx*m*TLPa cDNA predicts a protein 205 amino acids in length with a predicted molecular weight of 21.40 kDa and pI of 4.4. Identity of this cDNA sequence as *Tx*m*TLPa was confirmed by the alignment of the deduced amino acid sequence with the four previously determined peptide sequences of *Tx*m*TLPa (Chapter 6) isolated from the yew pollination droplet (Figure 7.2).

In order to assess the antifungal potential of *Tx*m*TLPa, the deduced amino acid sequence of the mature protein was compared with the sequences of the known antifungal TLPs PR-5d, zeamatin, and NP24 (Figure 7.3). *Tx*m*TLPa shares considerable amino acid identity with these proteins, including all 16 conserved cysteine residues and the five charged residues identified in the acidic cleft of antifungal TLPs (Koiwa *et al.* 1999).

Expression of recombinant *Tx*m*TLPa

The construction of the pFLAG-*Tx*m*TLPa vector designed for the expression of the FLAG-*Tx*m*TLPa fusion protein is outlined in Figure 7.4.

Expression of FLAG-*Tx*m*TLPa was carried out in the Rosetta-gami strain of *E.coli*. This strain was selected for its enhanced ability to express eukaryotic proteins with the properly formed disulphide bridges necessary for TLP activity (see methods and materials). Cells were grown in liquid culture into the exponential growth phase, at which point recombinant *Tx*m*TLPa production was induced with IPTG.

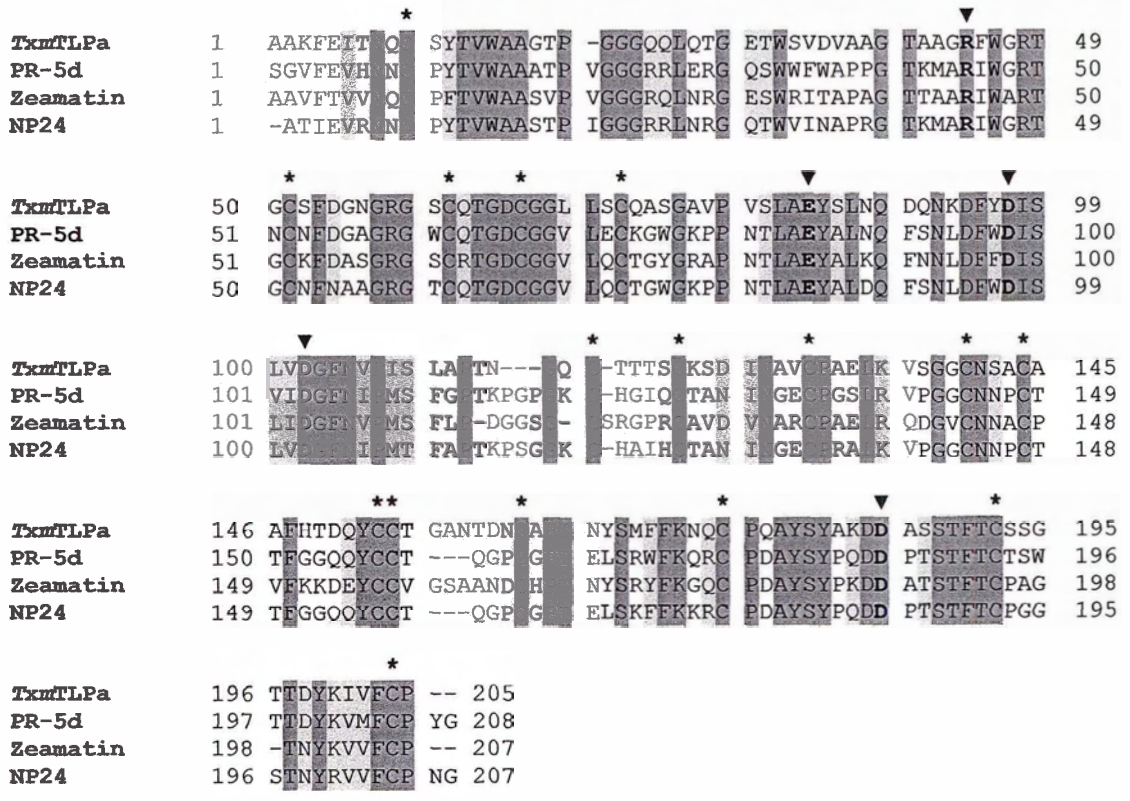


Figure 7.3. Multiple sequence alignment of the deduced amino acid sequences of mature *Txm*TLPa and the antifungal TLPs PR-5d (*Nicotiana tabacum*, Acc. no. P25871), zeamatin (*Zea mays* Acc. no. P33679), and NP24 (*Lycopersicon esculentum* Acc. no. S07406). Conserved amino acids are shaded dark gray, conservative replacements light gray. Conserved cysteine residues are indicated by asterisks (*). Residues contributing to the acidic cleft of antifungal TLPs (Koiwa *et al.* 1999) are bold and indicated by arrowheads (▼).

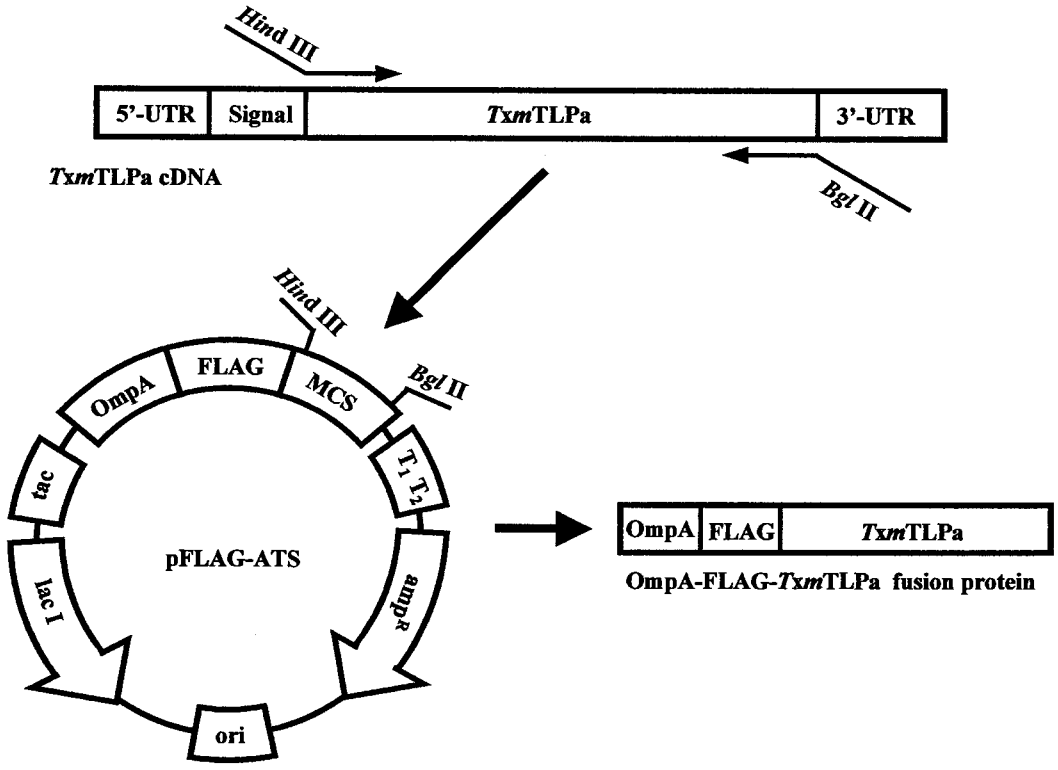


Figure 7.4. Plasmid construction for the expression of recombinant *Txm*TLPa. Tailed PCR with the primer set XTxmTLPaF1 / XTxmTLPaR1 was used to amplify the mature sequence of *Txm*TLPa from cDNA and introduce unique *Hind* III and *Bgl* II restriction sites at the 5' and 3' ends, respectively. This product was inserted directionally into the multiple cloning site (MCS) of the pFLAG-ATS expression plasmid by *Hind* III / *Bgl* II double digestion. Sequencing from the N-26 and C-24 primer sites (not shown) confirmed the proper insertion of the *Txm*TLPa sequence into the correct reading frame. The resulting plasmid, pFLAG-*Txm*TLPa, coded for the expression of an OmpA-FLAG-*Txm*TLPa fusion protein under the control of a *lacI* repressed *tac* promoter. The OmpA (outer membrane protein A) signal peptide directs the export of the fusion protein into the periplasmic space. This peptide is cleaved during secretion leaving the FLAG-*Txm*TLPa protein. The FLAG octapeptide (DYKDDDDK) fused to the N-terminus of *Txm*TLPa allows for the immunodetection and immunopurification of the fusion protein. It may be removed from *Txm*TLPa by enterokinase cleavage. Other important features of pFLAG-*Txm*TLPa include a T_1T_2 ribosomal DNA operon compound terminator downstream of the MCS, a pBR322 origin of replication (ori) for double strand replication of the plasmid, an ampicillin resistance cassette (amp^R) for selection of the plasmid, and the coding sequence for the lacI repressor protein required for repression of the *tac* promoter. Repression is relieved by the addition of IPTG.

Immunoblot detection with both the anti-FLAG M2 antibody and BARPERM1 confirmed the presence of a FLAG-*Tx*m*TLPa protein of roughly 28 kDa in the lysate of induced transformants (Figure 7.5). Under the expression conditions employed (37 °C, 0.5 mM IPTG induction), FLAG-*Tx*m*TLPa was retained with the intracellular insoluble protein fraction and was not recoverable by osmotic disruption of the ECM. It is likely that FLAG-*Tx*m*TLPa has been sequestered in inclusion bodies, from which it cannot be recovered without employing denaturing conditions (detergents or urea) that may diminish or destroy its activity.

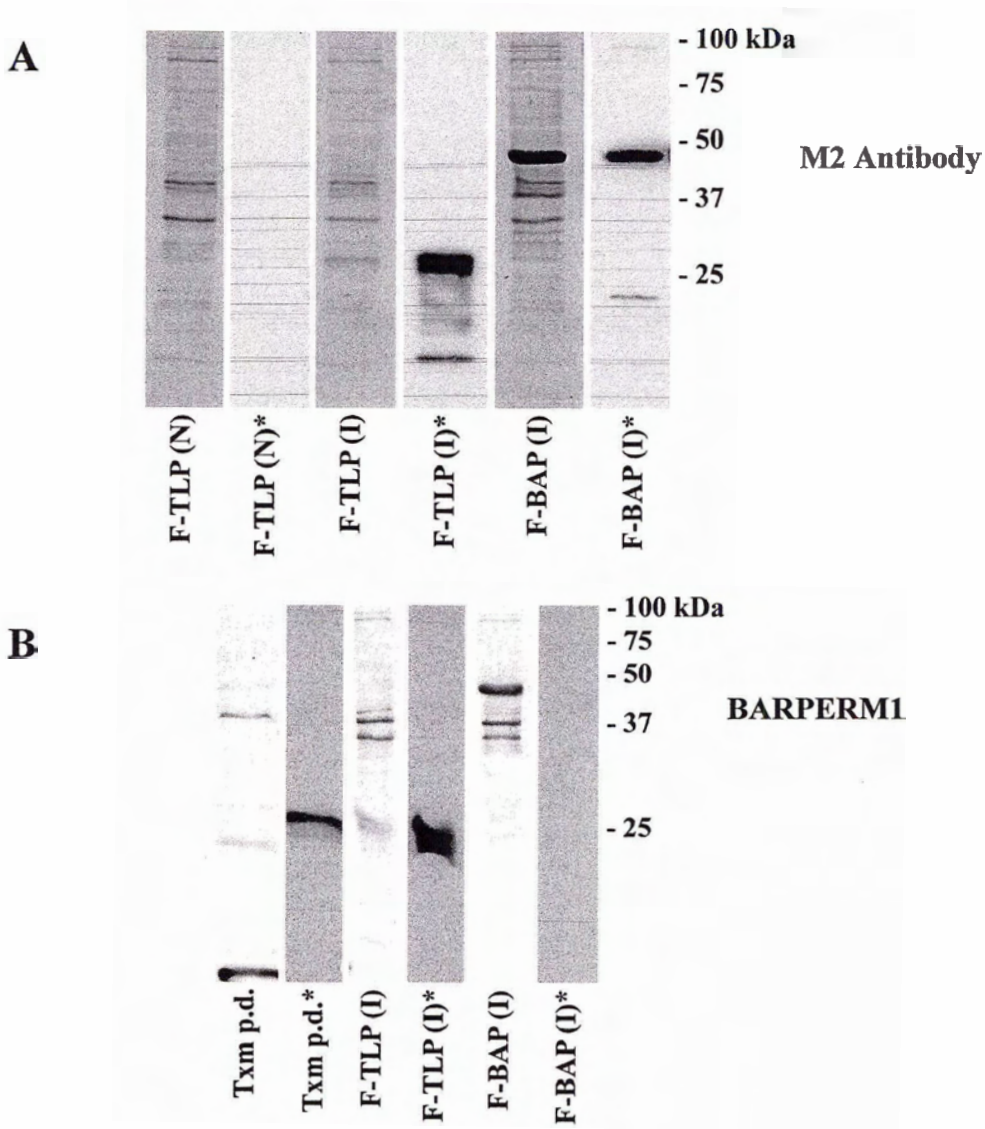


Figure 7.5. Immunodetection of the FLAG-*Txm*TLPa fusion protein by the anti-FLAG M2 (A) and BARPERM1 (B) antibodies. *E.coli* transformed for FLAG-*Txm*TLPa (F-TLP) or FLAG-BAP (F-BAP) expression were harvested from liquid cultures two hours after IPTG induction (I). Cultures grown in parallel, but not induced, are labelled (N). Cells pelleted from 0.5 mL of culture were lysed in 100 µL of SDS-PAGE sample buffer. 5 µL of lysate was loaded into the indicated lanes for 1D SDS-PAGE prior to electro-transfer onto PVDF membrane. Membranes were stained with Gelcode reagent when immunodetection was complete. Detection of bound antibodies was performed with the ECL chemiluminescent system and Kodak Biomax film. Exposed film segments corresponding to sample lanes on the blot are labelled with asterisks (*). Cultures expressing FLAG-BAP, and cultures containing pFLAG-*Txm*TLPa but not induced with IPTG, are included as positive and negative controls, respectively, for anti-FLAG M2 binding. Whole *T.* x *media* pollination droplet (Txm p.d., 5 µL), and cultures expressing FLAG-BAP, were included as positive and negative controls, respectively, for BARPERM1 binding.

Discussion

*Tx*m*TLPa in the yew ovular secretion is likely to play a role in pathogen defence. Collecting enough of this protein in a purified form to perform antifungal bioassays, however, presents a daunting prospect. Isolating *Tx*m*TLPa from the other proteins present in the pollination droplet without destroying activity may be possible with the sequential application of gentle purification techniques, including size exclusion or ion exhchange chromatography and non-denaturing electrophoresis. Unfortunately, this approach is not practical; the yield would be severely limited by the small volumes of total secretion that can be collected annually (a few mL). In order to overcome sample limitations, the cDNA sequence coding for *Tx*m*TLPa has been determined and the deduced amino acid sequence assessed for heterologous expression in a prokaryotic system.

PCR-based cloning of *Tx*m*TLPa cDNA yielded five unique but highly similar sequences from 10 separate clones. These results suggest that *Tx*m*TLPa may be a member of a multigene family that has undergone very limited divergence. TLP gene families have been identified in other plant species (Lin *et al.* 1996, Velazhahan *et al.* 1998) and a multigene family has been reported for another PR protein (PR-10) in a conifer (Liu *et al.* 2003). It must be noted, however, that yew ovules were harvested and pooled from a small population (n = 6) of trees for mRNA extraction; the cDNA sequences generated may represent allelic variation of a single locus. Further sequencing of a greater number of clones and Southern blot analysis should be carried out to determine the copy number of the *Tx*m*TLPa gene or genes in the yew genome.

The nucleic acid sequence most frequently represented in the selected cDNA clones predicts a TLP precursor that is 233 amino acid residues in length with a 28-residue export signal at the N-terminus. *Txm*TLPa is found in an extracellular secretion, so the presence of an N-terminal signal was expected. A translation stop codon immediately following the residue Pro233 precludes the presence of a C-terminal signal characteristic of TLPs targeted for vacuolar inclusion (Anžlovar and Dermastia 2003). The predicted molecular weight of 21.40 kDa and pI of 4.4 for the mature protein are similar to the values determined by gel electrophoresis for *Txm*TLPa isolated from the yew pollination droplet (Chapter 6), and are typical for acidic TLPs (Velazhanan *et al.* 1999). It has been observed that the calculated molecular weights of TLPs from amino acid sequence often differs slightly from those deduced by SDS-PAGE (Anžlovar and Dermastia 2003). The most convincing evidence that the cDNA sequence recovered corresponds to the *Txm*TLPa of the yew pollination droplet is that the four internal peptides sequenced from *Txm*TLPa isolated by 2D SDS-PAGE are found in the deduced amino acid sequence.

The primary structure of *Txm*TLPa suggests that this protein is likely to possess antifungal properties. Comparison of the deduced amino acid sequence of *Txm*TLPa with previously described TLPs indicates that this protein contains the 16 conserved cysteine residues that stabilize the tertiary structure characteristic of the PR-5 group (Battalia *et al.* 1996, Koiwa *et al.* 1999). *Txm*TLPa also possesses the five charged residues reported to play a role in the acidic cleft of TLPs having demonstrated antifungal activity (Koiwa et al. 1999). Based on these observations, *Txm*TLPa was deemed to be a

good candidate for heterologous expression and subsequent determination of antifungal activity.

Certain factors must be considered when selecting a protein expression system. TLPs are not glycosylated (Velazhanan *et al.* 1999) and thus are suitable for heterologous expression in prokaryotes. Hu and Reddy (1997) reported the expression of two TLPs of *Arabidopsis* from the transformed *E. coli* stain BL21 (DE3). They encountered solubility problems with both heterologously expressed proteins; however, they were able to re-solubilize one of the two and demonstrate normal antifungal activity.

The Rosetta-gami strain of *E. coli* is optimized for the expression of eukaryotic proteins with the proper formation of disulphide bonds. The Origami strain, a progenitor of Rosetta-gami that shares the genetic background for enhanced disulphide bond formation, has recently been employed for the production of properly folded wheat nsLTP1 (Elmorjani *et al.* 2004).

Mature *Tx*m*TLPa was expressed as a fusion protein with the bacterial OmpA signal for periplasmic targeting and the FLAG peptide for purification by an anti-FLAG affinity column. Transformed cells harvested 2 hours after IPTG induction appear to contain the full-length fusion protein. Immunodetection with the M2 antibody against the FLAG peptide labels a protein of approximately 28 kDa in pFLAG-*Tx*m*TLPa transformed cells that is not present in controls. Unfortunately, this protein was sequestered in the intracellular insoluble protein fraction of the cells, and the labelled band likely represents the FLAG-*T*mxTLPa fusion protein with the unprocessed OmpA signal still attached. Binding of this same protein by the BARPERM1 antibody further confirms that the fusion protein contains both the FLAG peptide and *Tx*m*TLPa.

In order to recover recombinant *Txm*TLPa in an active form, it is preferable to recover this protein from the periplasmic space of the bacterial cells with a mild procedure that does not require denaturing conditions (such as osmotic shock). To this end, further optimization of the growth and expression conditions for this system will be attempted. It is hoped that by testing various temperature regimes and IPTG concentrations, the FLAG-*Txm*TLPa fusion protein can be targeted to the periplasmic space in its proper conformation. If this proves impossible, it will be necessary to express the FLAG-*Txm*TLPa fusion without the OmpA signal, and re-solubilize it from inclusion bodies. This strategy has proven successful with *E. coli*-expressed ATLP-3 (*Arabidopsis* TLP-3) (Hu and Reddy 1997) and thaumatin II (Daniell *et al.* 2000).

In conclusion, with some modifications of protocols, it may be possible to express proteins of the yew ovular secretion in an *E. coli* system and generate sufficient quantities to carry out bioassay experiments.

Chapter 8

General discussion and concluding remarks

This study has confirmed that the ovular secretions of conifers are more than a simple solution of sugar and water. Consequently, it is necessary to re-evaluate the biology of these liquids. It has been proposed that conifer secretions are a derived feature. Owens *et al.* (1998) contend that early conifers did not possess these liquids, and instead depended on rainwater to deliver pollen into the ovule. From this hypothesis, it follows that ovular secretions are a later adaptation that allowed conifers to provide their own water to facilitate pollen scavenging (Runions and Owens 1996).

Two lines of evidence contradict this position. The first argument that the ovular secretion is an ancestral feature of conifers is a taxonomic one. In a recent review of conifer pollination mechanisms, Gelbart and von Aderkas (2002) clearly demonstrated that liquids, in the form of pollination droplets or post-pollination secretions, are employed by the majority of conifer families, and by groups basal to the conifers (Cycadaceae and Ginkgoaceae). The second indication that the ovular secretion is not simply a replacement for rainwater is the biochemistry of the liquid itself. Previous authors have reported the presence of sugars, amino acids, organic acids, and inorganic compounds in the ovular secretions of a variety of conifers (reviewed in Table 4.1). This study established the presence of proteins in the ovular secretions of larch, yew, Douglas-fir, and western red cedar, thus revealing a previously unknown level of complexity in these liquids.

It is clear in at least some conifer species that the production of the ovular secretion is actively regulated (Tomlinson *et al.* 1997, Anderson and Owens 2000, this

study). This dissertation has demonstrated that the secretions of at least two conifers contain a consistent complement of proteins. Given these observations, and the prevalence of this pollination mechanism among conifers, there can be little doubt that ovular liquids play an important role in the reproduction of these plants. The presence of ovular exudates in all other groups of extant seed plants - Cycadales (Pettitt 1977), Gnetales (Carafa *et al.* 1992, Endress 1996), *Ginkgo* (Friedman 1987), and angiosperms (Tilton 1980, Franssen-Verheijen and Willemse 1993, Herrero 2000), suggests that a secretion in the micropyle may be as old, and almost as conserved, as the ovule itself.

In conifers, ovular secretions have been implicated in pollen signalling (Villar *et al.* 1984), pollen germination and tube development (Barner and Christiansen 1960, 1962, Said *et al.* 1991, Takaso and Owens 1996, Takaso *et al.* 1996) and pollen selection (Takaso *et al.* 1996). Secretions in the angiosperm ovule have been similarly implicated in pollen signalling (Herrero and Arbeloa 1989, Herrero 2001), tube development (Franssen-Verheijen and Willemse 1993, Herrero 2000) and pollen selection (Sage *et al.* 1994). Given the importance of the roles attributed to these liquids, it is astounding how little is known about their biochemistry. Our ignorance of their compositions can be explained by the methods of study employed. Most investigations of ovular events have been conducted by microscopic examination of fixed tissues. Molecular studies are typically restricted to model angiosperms, and primarily involve analysis of more-or-less randomly generated reproductive mutants. Biochemical analysis of ovular liquids is lacking. The minute size of the secretions and the inaccessibility of angiosperm ovules are likely to blame for this shortcoming.

The advent of new analytical technologies, particularly in the area of proteomics, coupled with a judicious selection of study organisms provides an unprecedented opportunity to break new ground in this field. Gymnosperms, with their "naked ovules", present themselves as an obvious choice for studies of pollen-ovule interactions. Conifers are well studied among the gymnosperms and there is a rich literature describing the reproductive phenology of many species in this group. Many conifers produce conspicuous ovular secretions, which, with some patience and care, may be collected *in situ*. What better place to start this research?

An SDS-PAGE gel in the Ph.D. dissertation of Said (1988) showed protein bands in the postpollination drop of *Larix leptolepis*, providing the first confirmation of proteins in a conifer ovular secretion. This work was not continued, and no protein identifications were made. Four years later, Carafa *et al.* (1992) reported trace amounts of acid phosphatase activity in the micropylar drop of another gymnosperm, *Welwitschia mirabilis*, but no proteins were isolated. Again, this observation was not furthered.

In the present study, a predictable protein profile has been identified for two conifer species. AGPs have been detected in the secretions of both larch and yew by binding with the Yariv reagent in both species, and recognition by AGP-specific antibodies in yew. N-terminal amino acid sequencing and antibody detection has established the presence of XET, LTP, and most likely a TLP in the ovular secretion of larch. Proteomic methods, immunodetection, and molecular techniques have been used to identify two TLPs and a βGlu in the pollination droplet of yew. To my knowledge, these are the first positive identifications of any protein from the ovular secretion of any plant.

These findings provide new insight into the function of conifer ovular secretions. These liquids appear to be involved in both pollen development and pathogen defence within the ovule. A role in pollen development was anticipated, the discovery of PR proteins in the secretions was not. In retrospect, it would have been reasonable to expect that conifers possess a mechanism to protect these otherwise vulnerable tissues.

The involvement of XET and AGPs in plant cell growth has been well established (Fry *et al.* 1992, Campbell and Braam 1999, Fry 2004, Willats and Knox 1996, Majewska-Sawka and Nothnagel 2000, Showalter 2001). AGPs are known to influence pollen tube development in angiosperms (Cheung and Wu 1999, Cheung *et al.* 2000). These molecules have been localized to the transmitting tract of the style (Wang *et al.* 1993, Juah and Lord 1996, Cheung 1996), where they have been implicated in directing pollen tube growth (Cheung *et al.* 1995, Wu *et al.* 1995, Wu *et al.* 2000). AGPs have also been found in angiosperm nucellar tissues (Pennel *et al.* 1991, Coimbra and Salema 1997), and this study contains the first report of AGPs in a conifer nucellus (O'Leary *et al.* 2004). Considering the pollen-influencing properties of AGPs, their developmentally regulated production in the nucellar tissues of the species examined, and their presence within the liquid secretion of the yew and larch micropyle, these glycoproteins should be seriously considered as candidates for pollen signalling compounds within the ovule.

Many functions are attributed to LTPs in angiosperms. Among these are extracellular export of lipophilic molecules (Pyee *et al.* 1994), pathogen defence (Molina *et al.* 1993, García-Olmedo *et al.* 1995), pollen tube adhesion (Park *et al.* 2000) and pollen tube guidance (Kim *et al.* 2003). In the larch ovule, this protein may serve any combination of these functions.

The PR proteins described in the ovular secretions of larch and yew are likely to play a critical role in pathogen defence. The importance of their presence during reproduction is evidenced by the relative abundance of *Tx*mβGlu, *Tx*mTLPa and *Tx*mTLPb in the yew pollination droplet, and LTP in the larch secretion. Heterologous expression of these proteins and functional bioassays will ascertain the ability of each to inhibit pathogen establishment within the ovule. Defensive proteins from conifer ovular secretions may show promise as candidates for the genetic modification of crop plants for increased pathogen resistance (Melchers and Stuiver 2000, Punja 2001), or as a biocontrol agent applied in lieu of chemical fungicides (Karasuda *et al.* 2003).

PR proteins are not unknown in the floral tissues of angiosperms. As mentioned previously, proteins related to LTPs have been identified in the stigmatic and stylar exudates of lily, and have been implicated in pollen tube binding and guidance (Park *et al.* 2000, Park and Lord 2003, Kim *et al.* 2003). LTPs are also specifically expressed in pistil tissues of peach (Botton *et al.* 2002) and almond (Suelves and Puigdomenech 1997). Park *et al.* (2000) speculated that LTPs performing a defensive function in the angiosperm pistil might have been recruited evolutionarily as pollen tube adhesion and guidance molecules.

βGlus and TLPs have also been identified in flowers. The expression of certain βGlu genes is under developmental control in floral tissues of tobacco (Coté *et al.* 1994) and peach (Ko *et al.* 2003). Pistil-specific TLP cDNA has been isolated in tobacco (Kuboyama 1998) and tomato (Chen *et al.* 1996). A TLP with demonstrated antifungal activity, which is predominately expressed in the floral bud, has been isolated in *Brassica campestris* (Cheong *et al.* 1997). Another TLP is a major protein in the style of Japanese

pear (Sassa and Hirano 1998). Other than a possible antifungal function (Cheong *et al.* 1997), the role that these PR proteins play in the pistil is unclear.

Defensive proteins have recently been identified in the sugar-rich nectar of an angiosperm. In a preliminary study, Thornburg *et al.* (2003) screened a cDNA library prepared from mature nectary tissue of ornamental tobacco and found that 21 % of the randomly selected ESTs coded for defensive genes. In a second experiment, an array-based analysis demonstrated that mRNA sequences coding for six defence-related genes were up-regulated in floral nectaries compared to leaves (Thornburg *et al.* 2003). Chief among these was a PR-5 mRNA that had an expression level in developing nectary glands roughly 25 X higher than in leaves. The authors concluded that a major, but previously unrecognized function of the floral nectary and its secretion is to protect the gynoecium from microbial attack.

It has been hypothesized that some of the PR protein groups, including TLPs and βGlus, arose from genes originally involved in the normal processes of reproduction that were subsequently adapted for additional roles in plant defence (Lotan *et al.* 1989, Cheong *et al.* 1997). I would suggest the opposite. PR proteins in the ovular secretions of plants that possess the more primitive condition of a naked ovule, such as conifers, almost certainly play a role in pathogen defence. If some of these proteins are also involved in pollen tube development within the elaborated reproductive tissues of angiosperms, then surely this is a later adaptation.

In conclusion, there is much to be gained by studying the ovular secretions of conifers and other seed plants. The results of this study provide new information that

answers a number of questions, but also opens the door to many more. Of the numerous avenues of research that may now be undertaken, three are most prominent:

First of all, it would be logical to characterize the complete protein complement of the ovular secretion of at least one conifer species. For this work, I would recommend a species that employs a pollination droplet, such as *Taxus* or *Thuja* species. The protein composition of this type of ovular secretion appears to be less complicated than those of species employing a delayed drop (such as *Larix* and *Pseudotsuga*). A comparison of the biochemistry of ovular secretions from a variety of conifers will provide us with more information about the taxonomic relationships between members of this group.

Secondly, a thorough analysis of the genes coding for the proteins of ovular secretions would be beneficial. Expression studies would shed light on the sort of regulatory control and tissue specificity that these proteins are subject to. The structure of promoter regions, and any regulatory elements they may contain, will provide new insight into the genetic controls of plant reproductive development.

Finally, with a solid understanding of the ovular secretions of conifers, researchers can turn their focus to the secretions of other gymnosperms and the exudates of angiosperms. There is good evidence to suggest that the reproductive processes in gymnosperms and angiosperms were inherited from a common ancestor (see Walden *et al.* 1999, and references therein). A thorough understanding of the workings of the gymnosperm ovule will provide the molecular tools required to uncover the secrets of its secluded angiosperm counterpart. By characterizing the secretions from a range of seed plants, it will be possible to identify elements common to many pollination mechanisms.

With this information, we will be able to infer the ancestral condition of ovular liquids and their evolutionary history.

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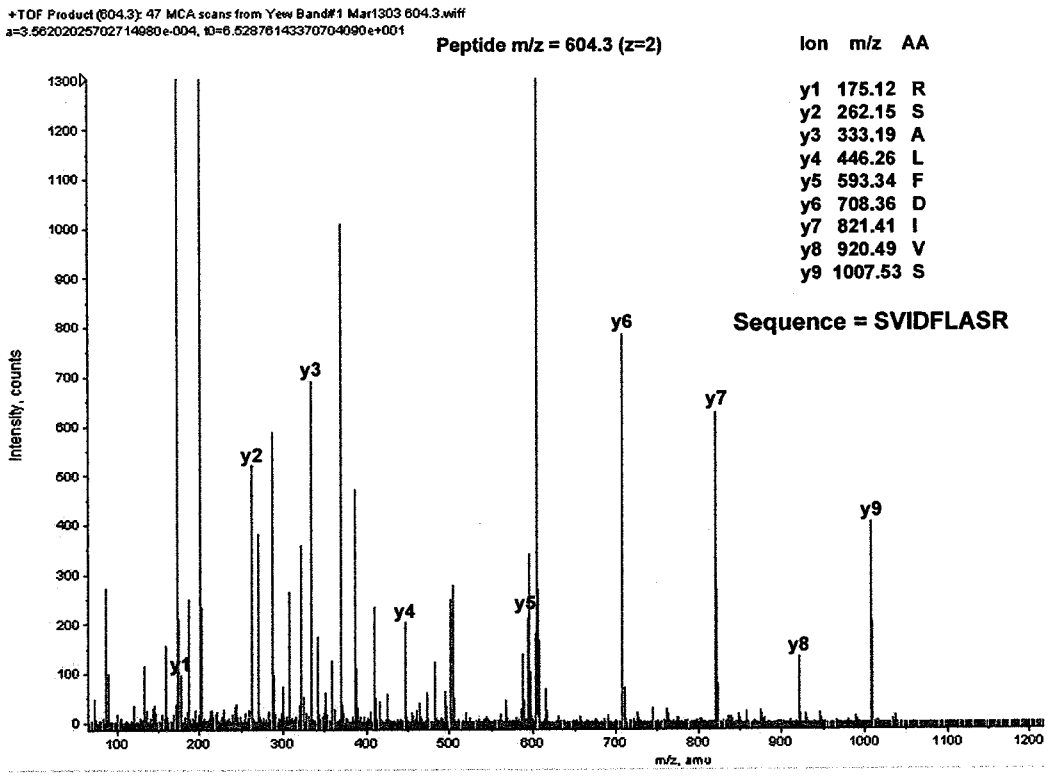
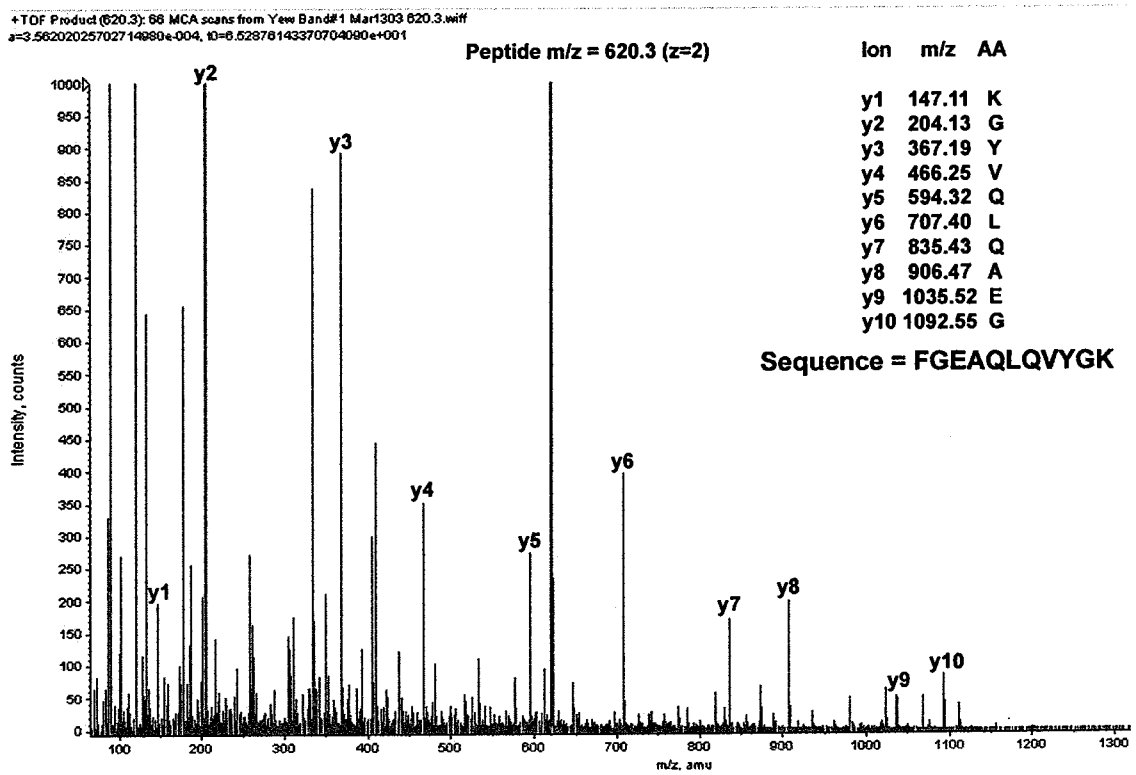
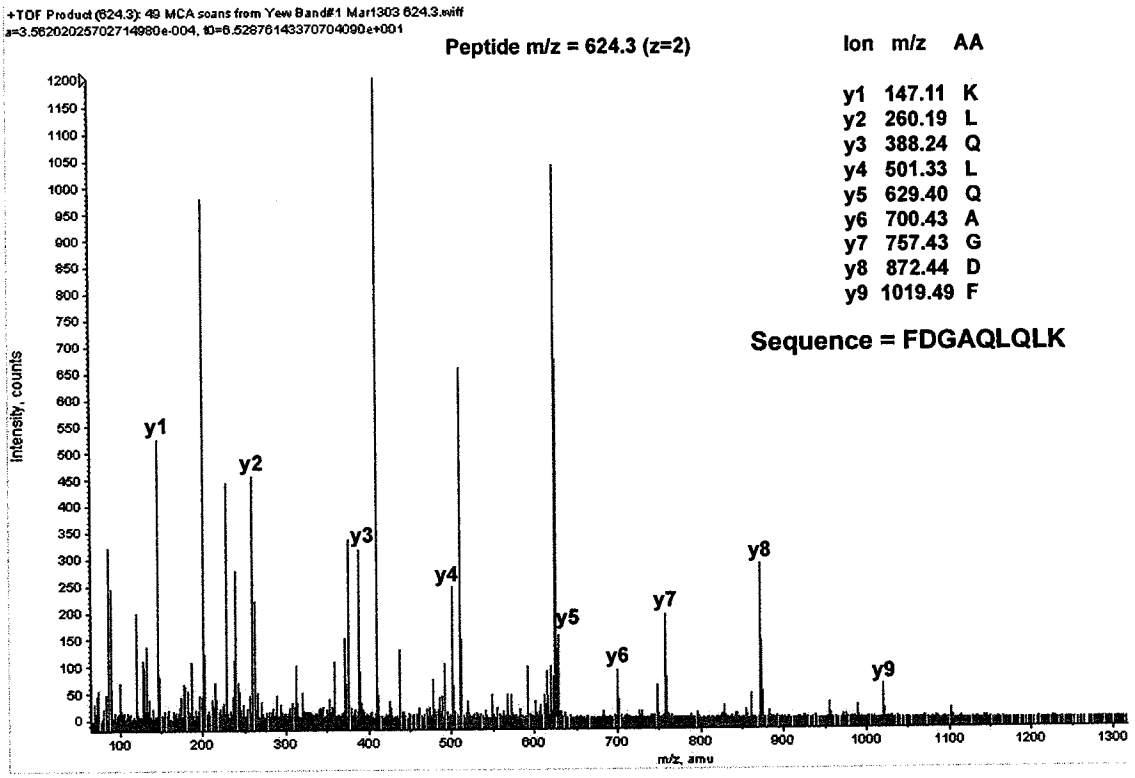
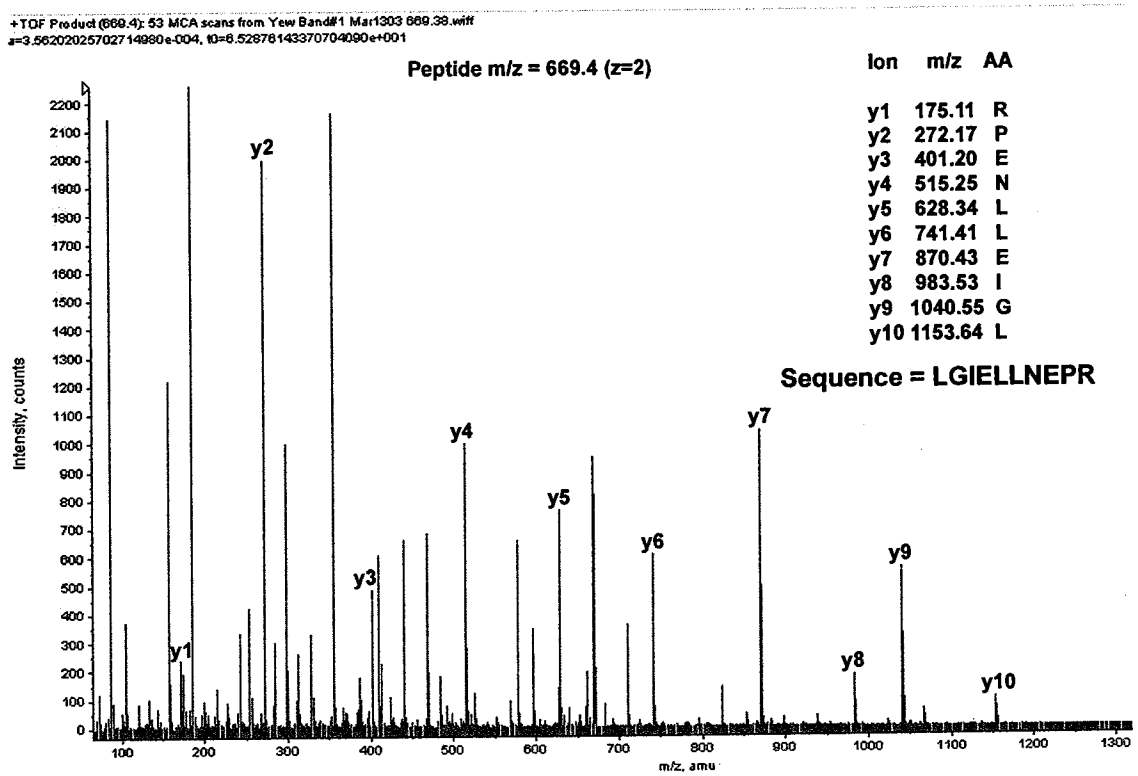
Appendix A: MS/MS Sequencing of *Txm*βGlu Internal Peptides



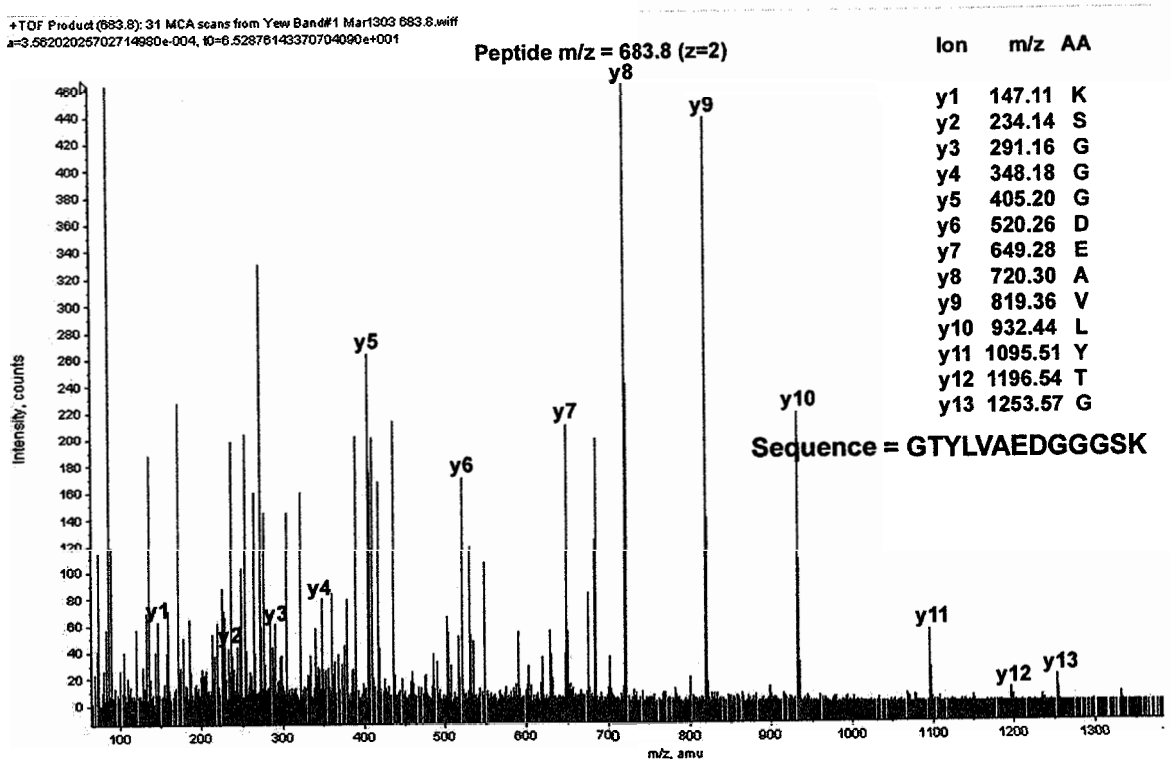


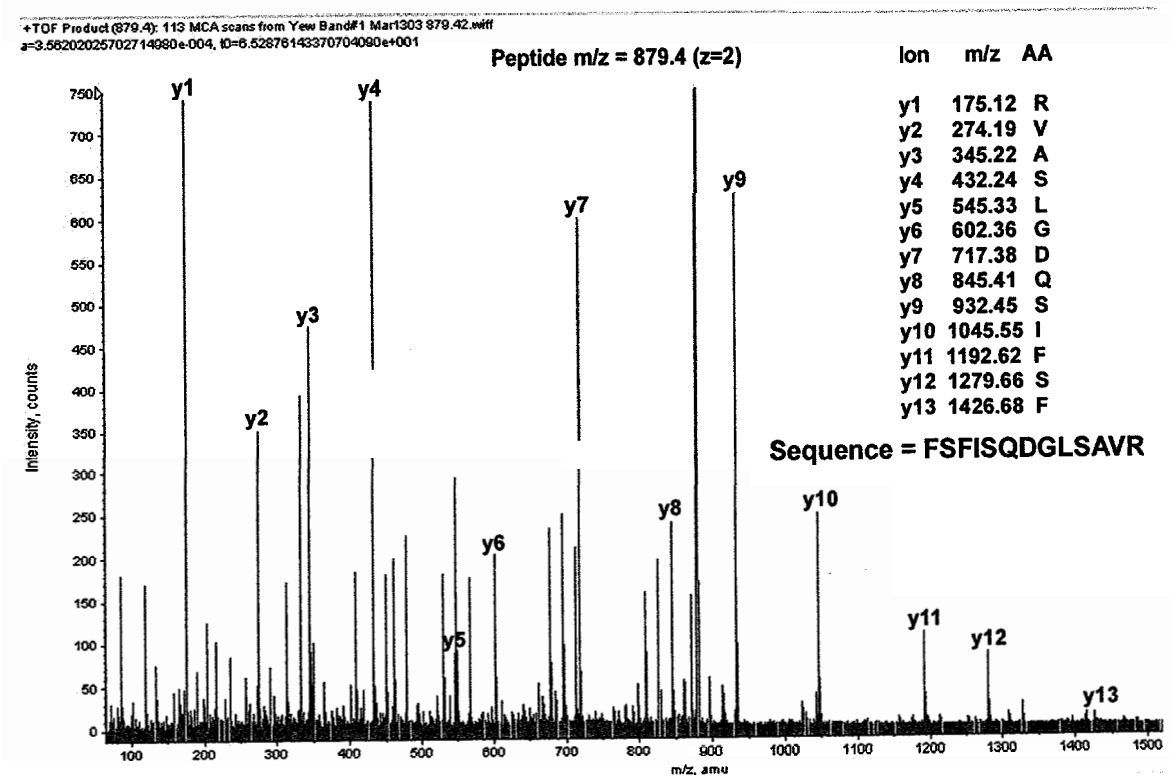
Appendix A: MS/MS Sequencing of *Tx*mβGlu Internal Peptides



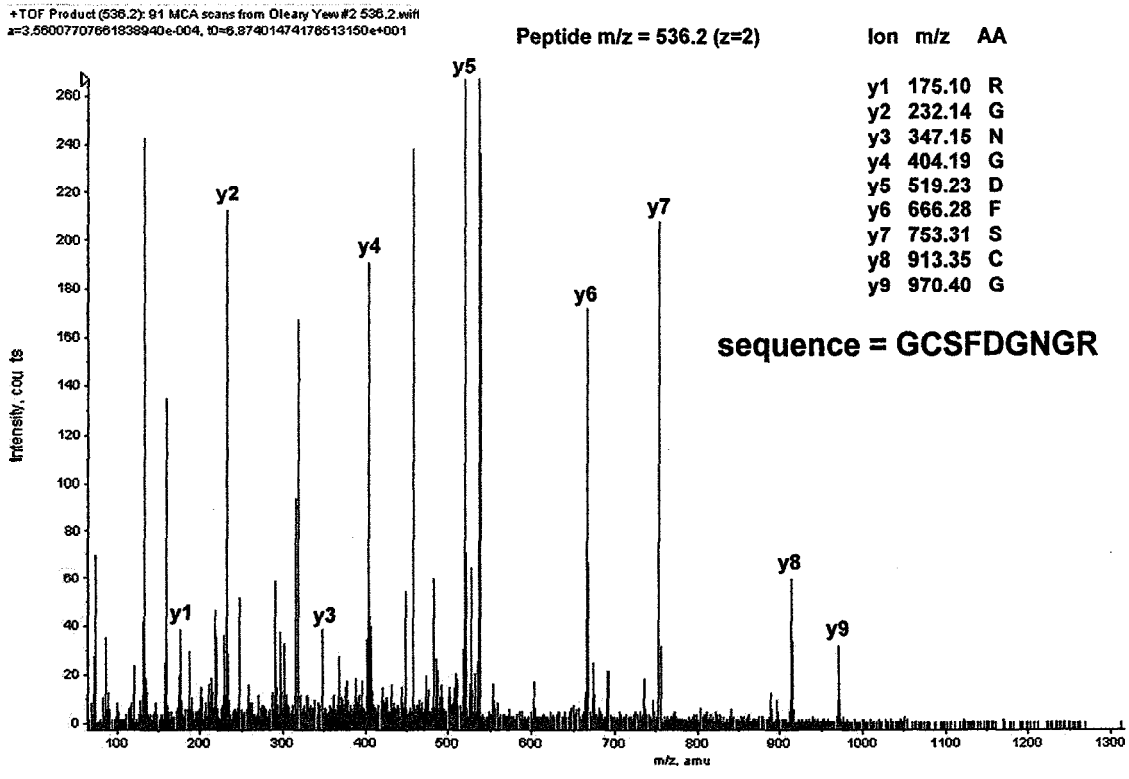


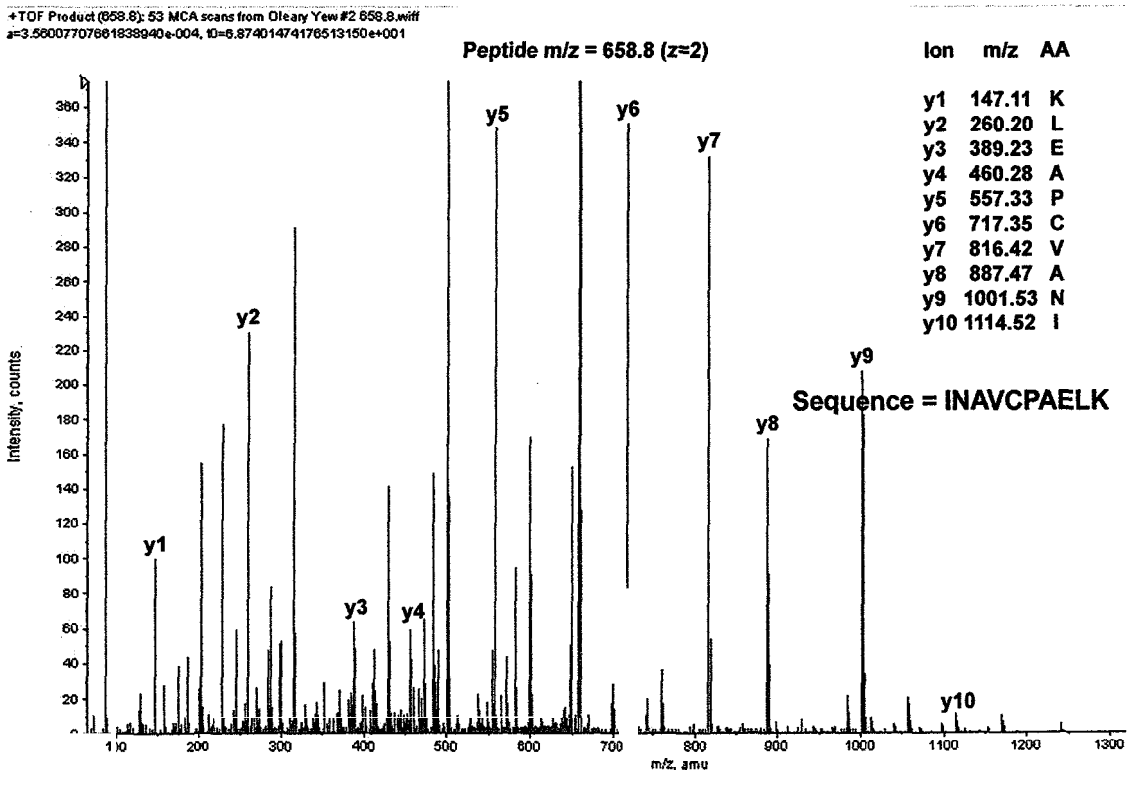
Appendix A: MS/MS Sequencing of *Txm*βGlu Internal Peptides



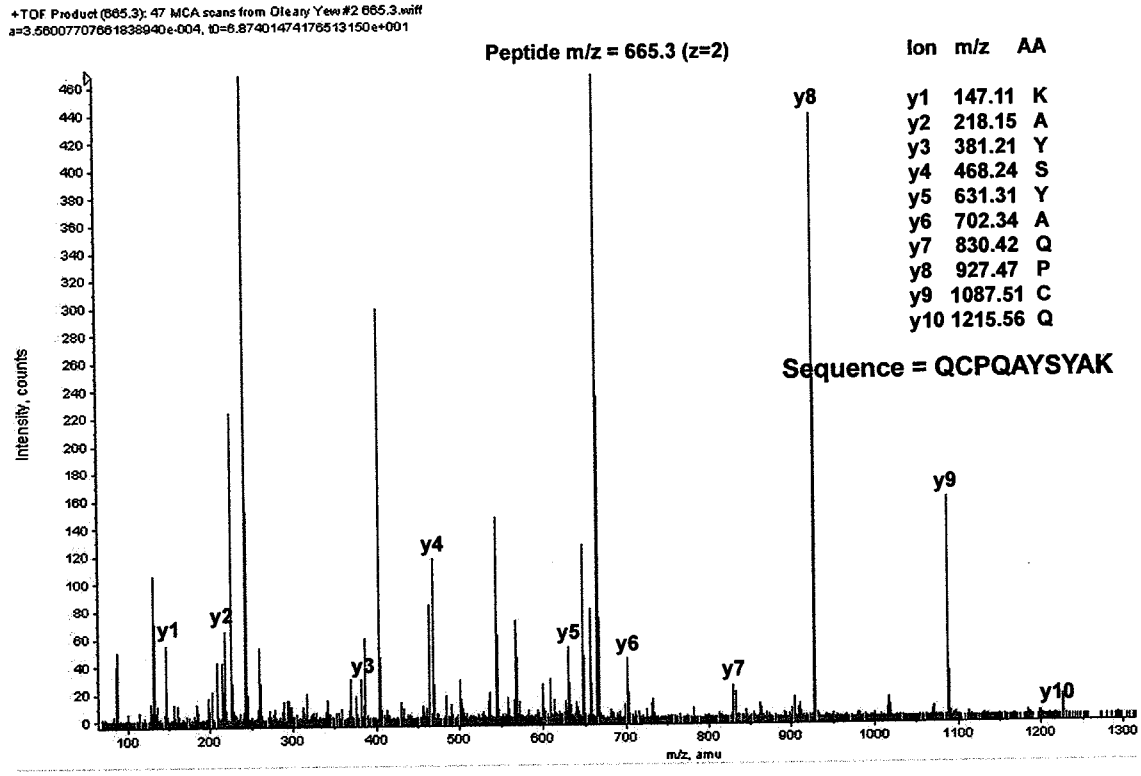


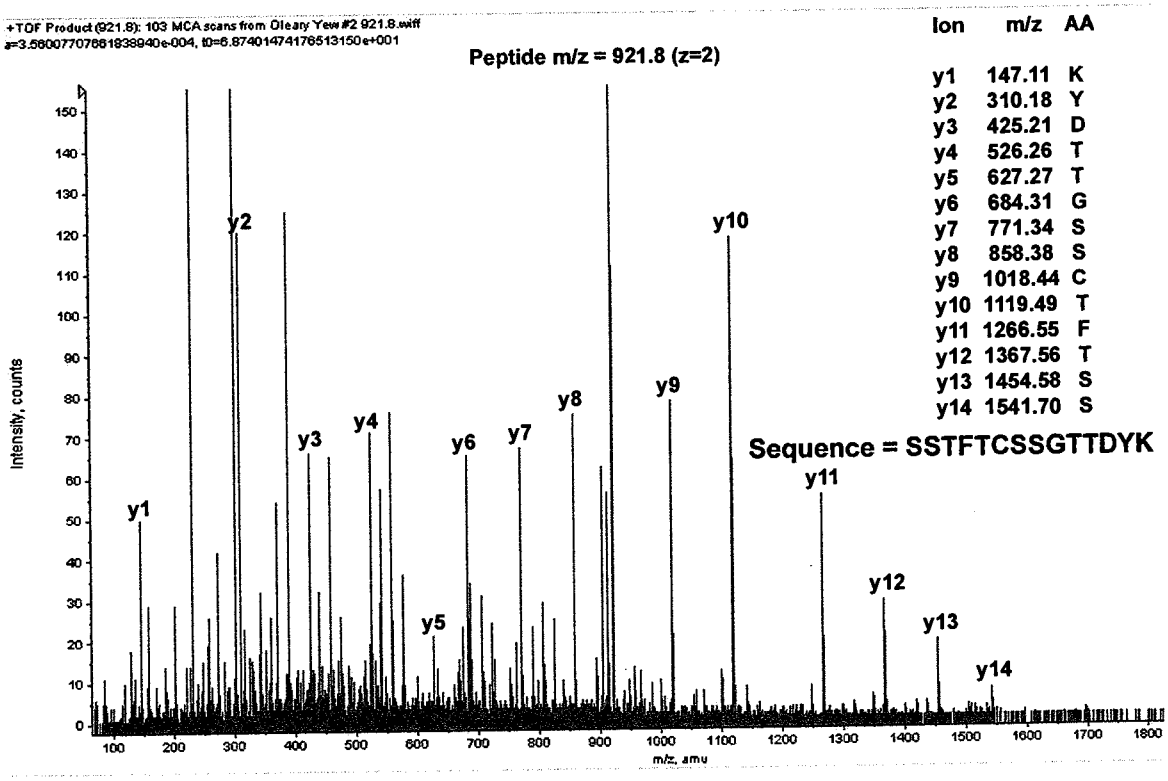
Appendix A: MS/MS Sequencing of *Txm*TLPa Internal Peptides



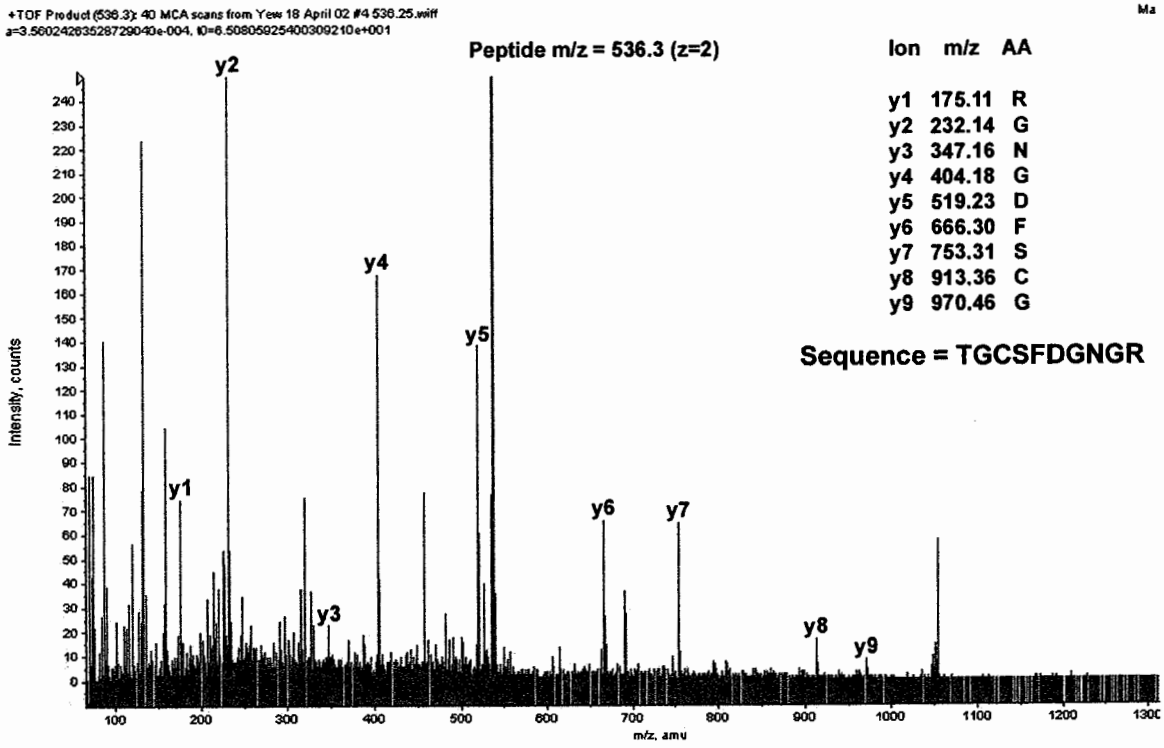


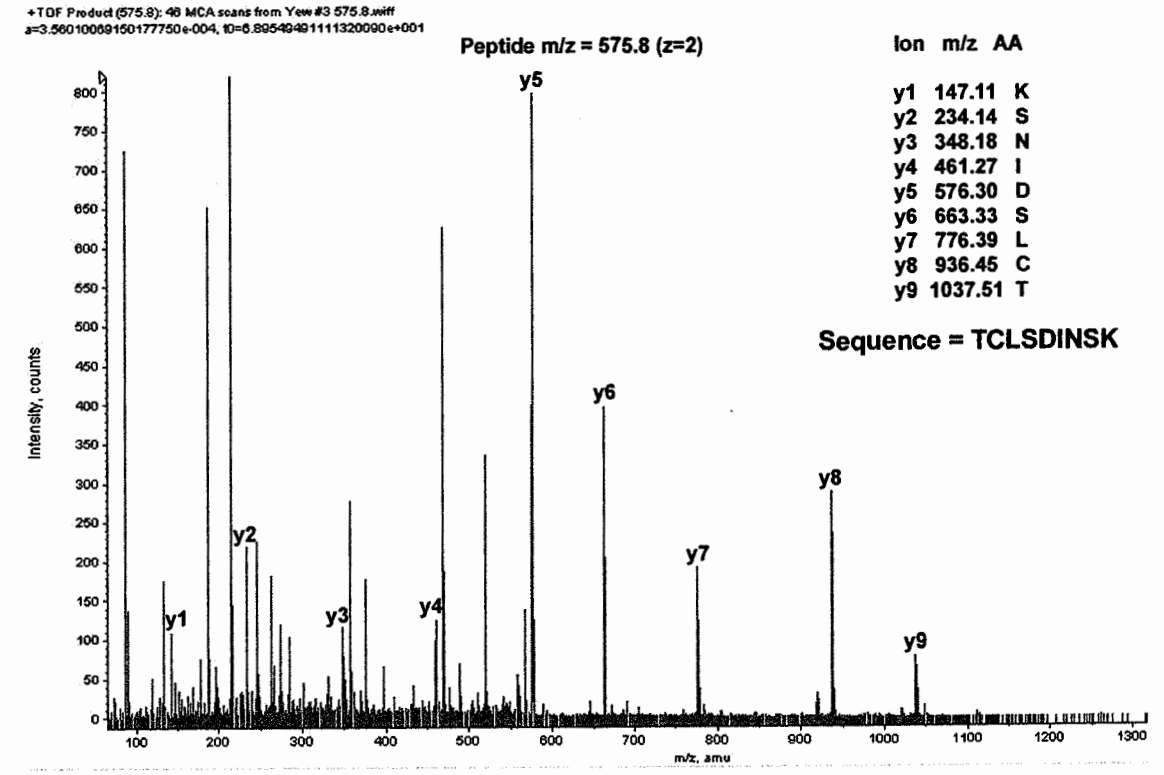
Appendix A: MS/MS Sequencing of *Txm*TLPa Internal Peptides



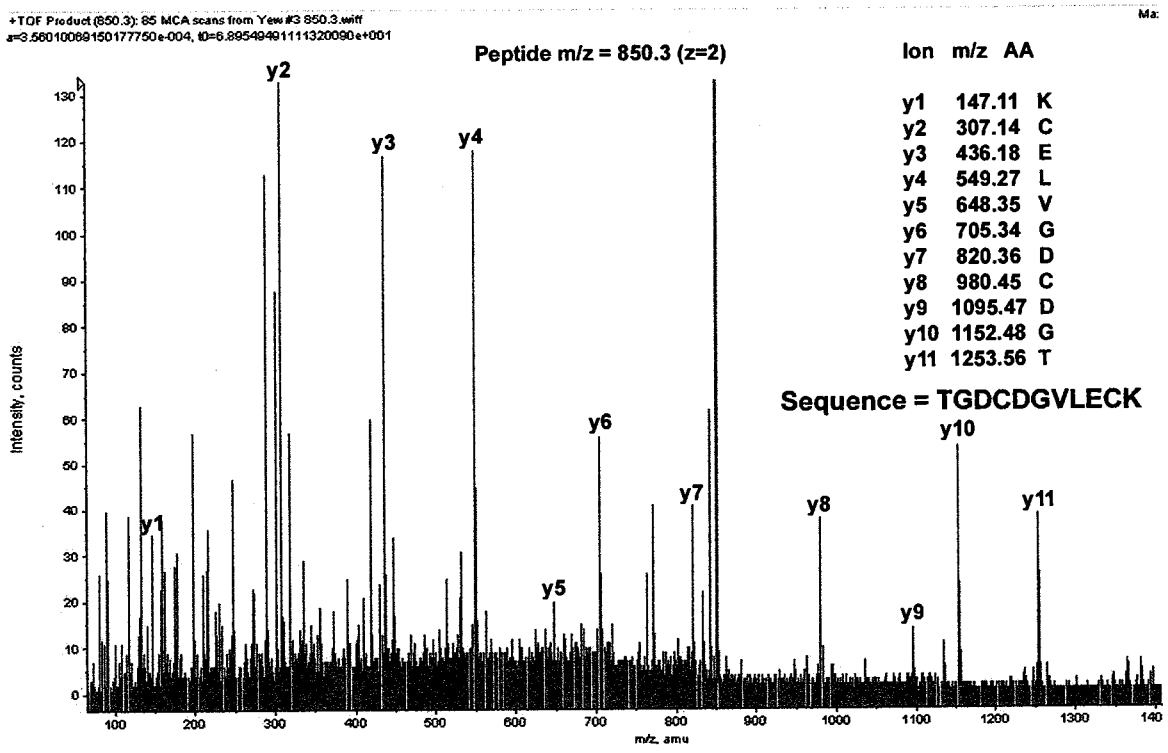


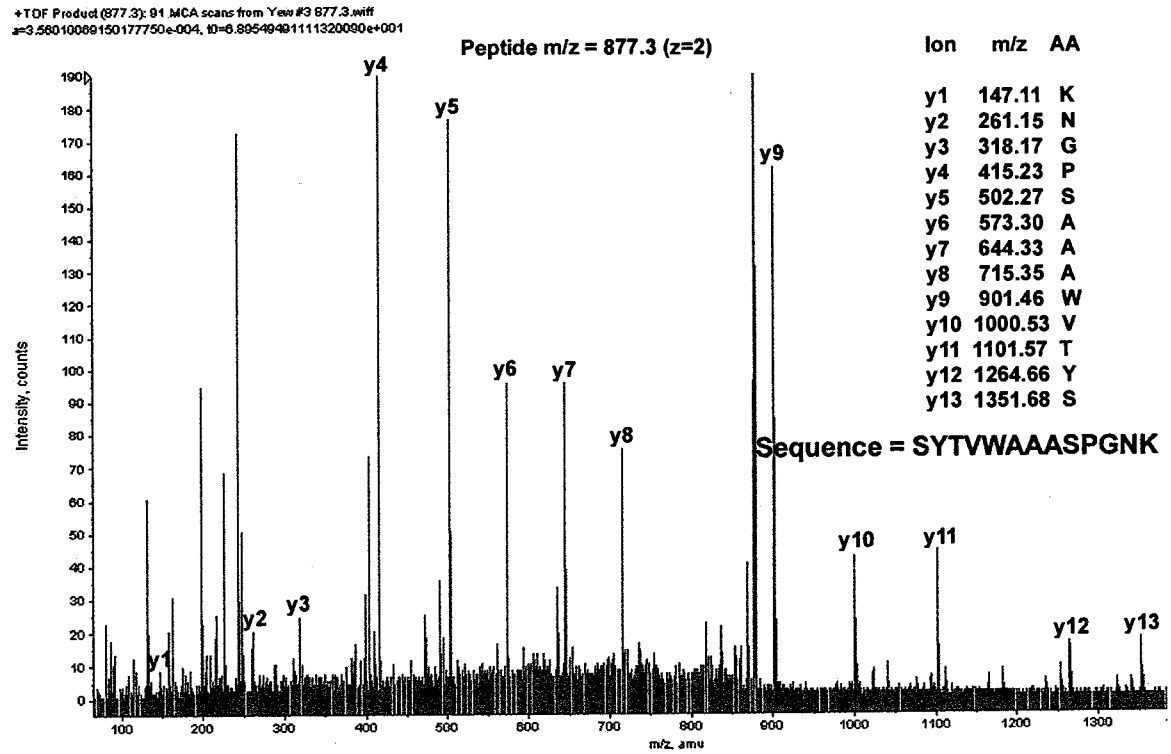
Appendix A: MS/MS Sequencing of *Txm*TLPb Internal Peptides



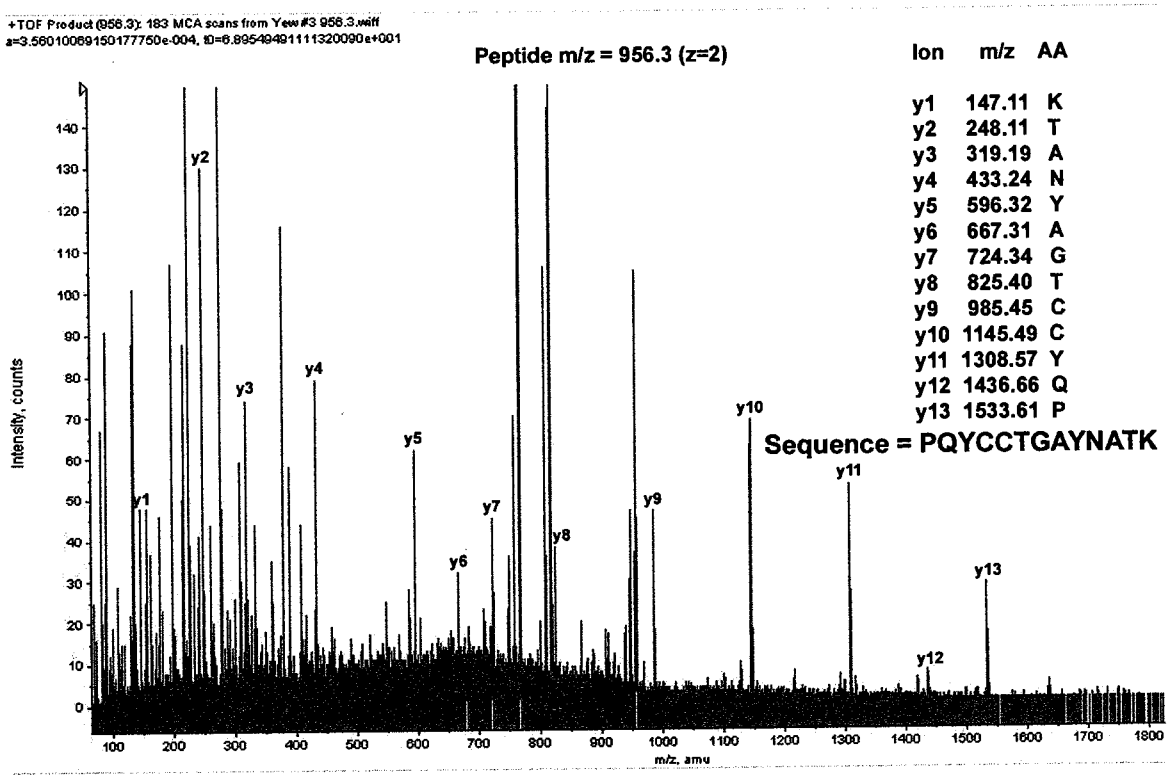


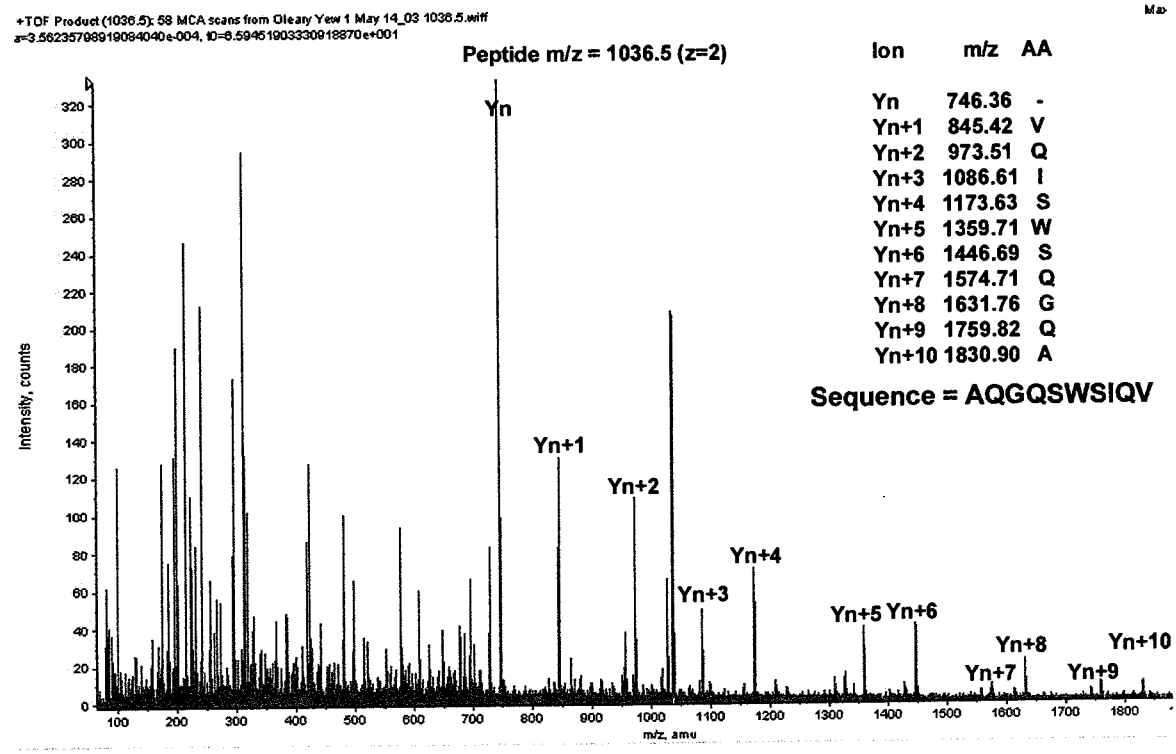
Appendix A: MS/MS Sequencing of *Txm*TLPb Internal Peptides



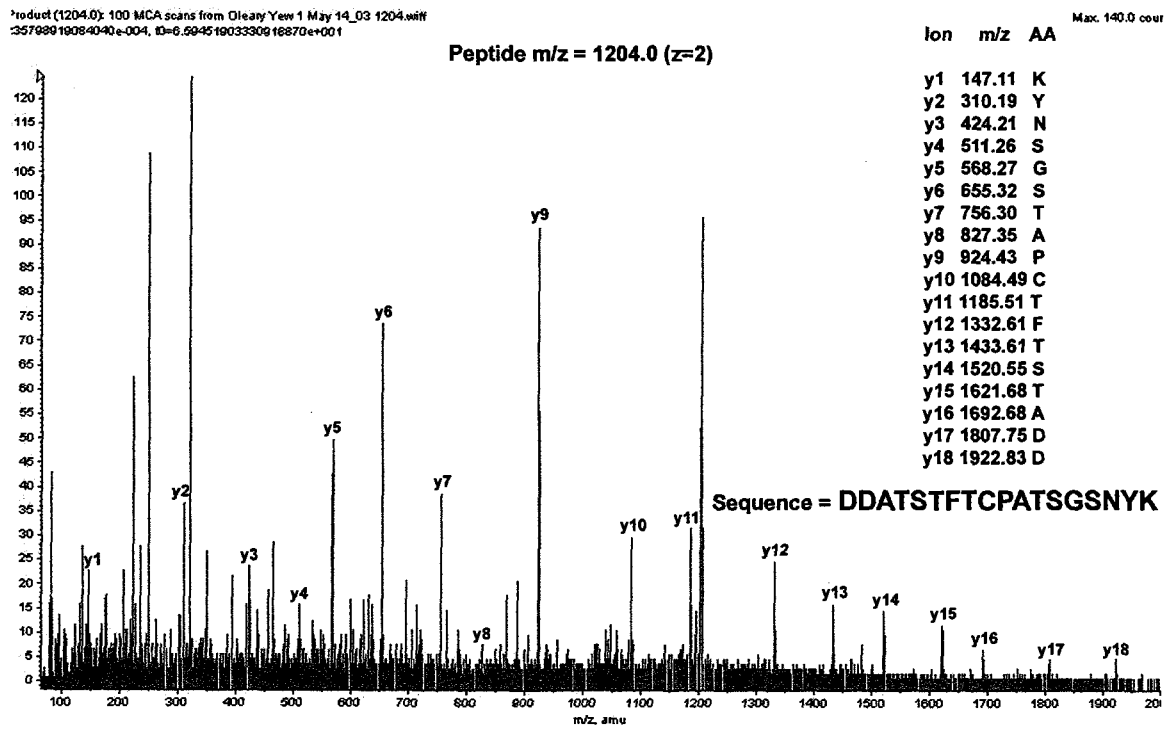


Appendix A: MS/MS Sequencing of *Tx*mTLPb Internal Peptides





Appendix A: MS/MS Sequencing of *Txm*TLPb Internal Peptides



Appendix B: BLASTp2 results from *Txm*βGlu

BLASTP2 Result of Bork Groups's Advanced BLAST2 Search Service at EMBL

BLASTP 2.0MP-WashU [16-Dec-1999] [irix6-r10k-L64 23:35:48 16-Dec-1999]

Reference: **1.** Gish, W. (1996-1999) http://blast.wustl.edu

2. Shevchenko A, Sunyaev S, Loboda A, Shevchenko A, Bork P, Ens W, Standing KG. (2001), Anal Chem 73(9),1917-26: Charting the proteomes of organisms with unsequenced genomes
by MALDI-quadrupole time-of-flight mass spectrometry and BLAST homology searching
Abstract

Query= query_sequence
(70 letters)

Database: nrdb-95%
942,687 sequences; 310,100,202 total letters.
Searching....10....20....30....40....50....60....70....80....90....100% done

Summary:

Sequences producing High-scoring Segment Pairs:	High Score	Total Score
/:sptrembl|Q8RU51|Q8RU51 Putative glucan 1,3-beta-glucosi...	57	350

Alignments:

^ = sptrembl|Q8RU51|Q8RU51 Putative glucan 1,3-beta-glucosidase.//:trembl|AC107314|AC107314_11 gene: "OJ1208D02.11"; product: "Putative Glucan 1,3-beta-glucosidase precursor"; Oryza sativa (japonica cultivar-group) cultivar Nipponbare clone OJ1208D02 from chromosome 10, complete sequence. //:trembl|AE017085|AE017085_2 product: "putative Glucan 1,3-beta-glucosidase precursor"; Oryza sativa (japonica cultivar-group) chromosome 10, section 39 of 77 of the complete sequence. //:gp|AE017085|31431641 putative Glucan 1,3-beta-glucosidase precursor [Oryza sativa (japonica cultivar-group)]//:gp|AC107314|19920188 Putative Glucan 1,3-beta-glucosidase precursor [Oryza sativa (japonica cultivar-group)]
Length = 1449

Total Score: 350

Appendix B: BLASTp2 results from *Txm*βGlu

```
                      0         290        580        870       1160
                      |          |          |          |          |
sptrembl|Q8RU51|Q8RU5  |
_____________________________________________________________________
Local hits (HSPs)     |   _      _   _       _ _          _     _
```

```
 Score = 57 (29.9 bits)
 Identities = 8/8 (100%), Positives = 8/8 (100%)

Query:     2 VIDFLASR 9
             VIDFLASR
Sbjct:   304 VIDFLASR 311

 Score = 57 (29.9 bits)
 Identities = 9/13 (69%), Positives = 10/13 (76%)

Query:    58 FSFISQDGLSAVR 70
             F FIS +GL AVR
Sbjct:   214 FKFISSNGLNAVR 226

 Score = 53 (28.0 bits)
 Identities = 8/9 (88%), Positives = 8/9 (88%)

Query:    33 LGIELLNEP 41
             L IELLNEP
Sbjct:   797 LAIELLNEP 805

 Score = 53 (28.0 bits)
 Identities = 8/10 (80%), Positives = 9/10 (90%)

Query:    46 YLVAEDGGGS 55
             YL AE+GGGS
Sbjct:   539 YLAAENGGGS 548

 Score = 48 (25.5 bits)
 Identities = 7/11 (63%), Positives = 8/11 (72%)

Query:    11 FGEAQLQVYGK 21
             F +AQL  YGK
Sbjct:   925 FAQAQLDLYGK 935

 Score = 44 (23.5 bits)
 Identities = 8/10 (80%), Positives = 8/10 (80%)

Query:    45 TYLVAEDGGG 54
             TYL AE GGG
Sbjct:    57 TYLCAEHGGG 66

 Score = 38 (20.5 bits)
 Identities = 6/8 (75%), Positives = 6/8 (75%)

Query:    24 DGAQLQLK 31
             DG QLQ K
Sbjct:   525 DGTQLQFK 532
```

Appendix B: BLASTp2 results from *Txm*TLPa

BLASTP2 Result of Bork Group Advanced BLAST2 Search Service at EMBL.

BLASTP 2.0MP-WashU [16-Dec-1999] [irix6-r10k-L64 23:35:48 16-Dec-1999]

Reference: 1. Gish, W. (1996-1999) http://blast.wustl.edu

2. Shevchenko A, Sunyaev S, Loboda A, Shevchenko A, Bork P, Ens W, Standing KG. (2001), Anal Chem 73(9),1917-26: Charting the proteomes of organisms with unsequenced genomes by MALDI-quadrupole time-of-flight mass spectrometry and BLAST homology searching
Abstract

Query= query_sequence
(46 letters)

Database: nrdb-95%
942,687 sequences; 310,100,202 total letters.
Searching....10....20....30....40....50....60....70....80....90....100% done

Summary:

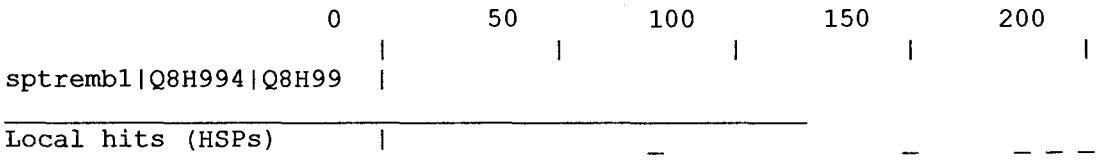
Sequences producing High-scoring Segment Pairs:	High Score	Total Score
/:sptrembl|Q8H994|Q8H994 Thaumatin-like protein (Fragment...	79	262

Alignments:

^ = sptrembl|Q8H994|Q8H994 Thaumatin-like protein (Fragment).//:trembl|AB081305|AB081305_1 gene: "Cry j 3.3"; product: "thaumatin-like protein"; Cryptomeria japonica Cry j 3.3 mRNA for thaumatin-like protein, complete cds. //:gp|AB081305|22830597 thaumatin-like protein [Cryptomeria japonica]
Length = 230

Total Score: 262

Appendix B: BLASTp2 results from *Txm*TLPa




```
 Score = 79 (41.9 bits)
 Identities = 10/10 (100%), Positives = 10/10 (100%)

Query:    22 QCPQAYSYAK 31
             QCPQAYSYAK
Sbjct:   200 QCPQAYSYAK 209

 Score = 59 (31.8 bits)
 Identities = 8/9 (88%), Positives = 8/9 (88%)

Query:     1 GCSFDGNGR 9
             GCSFDG GR
Sbjct:    73 GCSFDGSGR 81

 Score = 56 (30.2 bits)
 Identities = 8/9 (88%), Positives = 8/9 (88%)

Query:    34 STFTCSSGT 42
             STFTC SGT
Sbjct:   214 STFTCPSGT 222

 Score = 41 (22.6 bits)
 Identities = 6/10 (60%), Positives = 7/10 (70%)

Query:    11 INAVCPAELK 20
             IN  CP +LK
Sbjct:   152 INSQCPSDLK 161

 Score = 27 (15.5 bits)
 Identities = 4/5 (80%), Positives = 4/5 (80%)

Query:    41 GTTDY 45
             G TDY
Sbjct:   189 GPTDY 193
```

Appendix B: BLASTp2 results from *Txm*TLPb

BLASTP2 Resultof Bork Group's Advanced BLAST2 Search Service at EMBL.

BLASTP 2.0MP-WashU [16-Dec-1999] [irix6-r10k-L64 23:35:48 16-Dec-1999]

Reference: 1. Gish, W. (1996-1999) http://blast.wustl.edu

2. Shevchenko A, Sunyaev S, Loboda A, Shevchenko A, Bork P, Ens W, Standing KG. (2001), Anal Chem 73(9),1917-26: Charting the proteomes of organisms with unsequenced genomes
by MALDI-quadrupole time-of-flight mass spectrometry and BLAST homology searching
Abstract

Query= query_sequence
(90 letters)

Database: nrdb-95%
942,687 sequences; 310,100,202 total letters.
Searching....10....20....30....40....50....60....70....80....90....100% done

Summary:

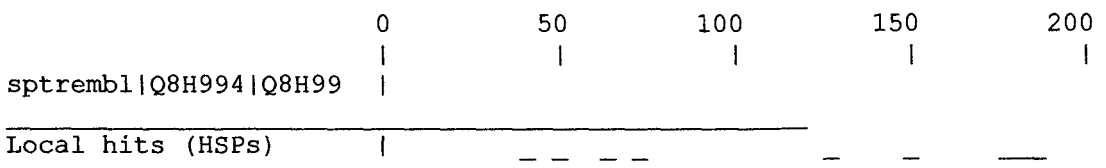
Sequences producing High-scoring Segment Pairs:	High Score	Total Score
/:sptrembl|Q8H994|Q8H994 Thaumatin-like protein (Fragment...	76	466

Alignments:

^ = sptrembl|Q8H994|Q8H994 Thaumatin-like protein (Fragment).//:trembl|AB081305|AB081305_1 gene: "Cry j 3.3"; product: "thaumatin-like protein"; Cryptomeria japonica Cry j 3.3 mRNA for thaumatin-like protein, complete cds. //:gp|AB081305|22830597 thaumatin-like protein [Cryptomeria japonica]
Length = 230

Total Score: 466

Appendix B: BLASTp2 results from *Txm*TLPb




```
 Score = 76 (40.2 bits)
 Identities = 10/10 (100%), Positives = 10/10 (100%)

Query:    73 DDATSTFTCP 82
             DDATSTFTCP
Sbjct:   210 DDATSTFTCP 219

 Score = 75 (39.7 bits)
 Identities = 10/10 (100%), Positives = 10/10 (100%)

Query:    35 YTVWAAASPG 44
             YTVWAAASPG
Sbjct:    35 YTVWAAASPG 44

 Score = 62 (33.2 bits)
 Identities = 9/10 (90%), Positives = 9/10 (90%)

Query:     1 TGCSFDGDGR 10
             TGCSFDG GR
Sbjct:    72 TGCSFDGSGR 81

 Score = 58 (31.1 bits)
 Identities = 7/8 (87%), Positives = 8/8 (100%)

Query:    48 PQYCCTGA 55
             P+YCCTGA
Sbjct:   176 PEYCCTGA 183

 Score = 58 (31.1 bits)
 Identities = 7/9 (77%), Positives = 7/9 (77%)

Query:    63 QGQSWSIQV 71
             QGQ W IQV
Sbjct:    51 QGQTWTIQV 59

 Score = 51 (27.6 bits)
 Identities = 7/7 (100%), Positives = 7/7 (100%)

Query:    13 CLSDINS 19
             CLSDINS
Sbjct:   148 CLSDINS 154

 Score = 49 (26.6 bits)
 Identities = 7/10 (70%), Positives = 8/10 (80%)

Query:    22 TGDCDGVLEC 31
             TGDC+G L C
Sbjct:    86 TGDCNGMLSC 95

 Score = 37 (20.5 bits)
 Identities = 5/6 (83%), Positives = 5/6 (83%)

Query:    85 SGSNYK 90
             SG NYK
Sbjct:   220 SGTNYK 225
```

Appendix C: Nucleic acid sequences of *Txm*TLPa cDNA clones

Bracketed numbers indicate number of times the sequence was represented in 10 clones

```
TxmTLPa1 (4)   1   TCAAACACCC AAATATAATG GCAACAATAT CAGTATCAGG TCTTGGGCAT  50
TxmTLPa2 (3)   1   TCAAACACCC AAATATAATG GCAACAATAT CAGTATCAGG TCTTGGGCAT  50
TxmTLPa3 (1)   1   TCAAACACCC AAATATAATG GCAACAATAT CAGTATCAGG TCTTGGGCAT  50
TxmTLPa4 (1)   1   TCAAACACCC AAATATAATG GCAACAATAT CAGTATCAGG TCTTGGGCAT  50
TxmTLPa5 (1)   1   TCAAACACCC AAATATAATG GCAACAATAT CAGTATCAGG TCTTGGGCAT  50

TxmTLPa1 (4)  51  CTTGTCCTTC TGCTTGGAAT CGCCGTATCT GTATATGTGC AAGAGGCTGG 100
TxmTLPa2 (3)  51  CTTGTCCTTC TGCTTGGAAT CGCCGTATCT GTATATGTGC AAGAGGCTGG 100
TxmTLPa3 (1)  51  CTCGTCCTTC TGCTTGGAGT CGCCGTATCT GTATATGTAC AAGAGGCTGG 100
TxmTLPa4 (1)  51  CTTGTCCTTC TGCTTGGAAT CGCCGTATCT GTATATGTGA AAGAGGCTGG 100
TxmTLPa5 (1)  51  CTTGTCCTTC TGCTTGGAAT CGCCGTATCT GTGTATGTGC AAGAGGCTGG 100

TxmTLPa1 (4) 101 AGCAGCAAAG TTCGAGATAA CAAACCAGTG CTCGTACACC GTTTGGGCCG 150
TxmTLPa2 (3) 101 AGCAGCAAAG TTCGAGATAA CAAACCAGTG CTCGTATACC GTTTGGGCTG 150
TxmTLPa3 (1) 101 AGCAGCAAAG TTCGAGATAA CAAACCAGTG CTCGTACACC GTTTGGGCCG 150
TxmTLPa4 (1) 101 AGCAGCAAAG TTCGAGATAA CAAACCAGTG CTCGTACACC GTTTGGGCCG 150
TxmTLPa5 (1) 101 AGCAGCAAAG TTCGAGATAA CAAACCAGTG CTCGTACACC GTTTGGGCCG 150

TxmTLPa1 (4) 151 CAGGAACCCC TGGCGGAGGG CAGCAACTCC AGACCGGCGA GACCTGGAGC 200
TxmTLPa2 (3) 151 CAGGAACCCC TGGCGGAGGG CAGCAACTCC AGACCGGCGA GACCTGGAGC 200
TxmTLPa3 (1) 151 CAGGAACCCC TGGCGGAGGG CAGCAACTCC AGACCGGCGA GACCTGGAGC 200
TxmTLPa4 (1) 151 CAGGAACCCC TGGCGGAGGG CAGCAACTCC AGACCGGCGA GACCTGGAGC 200
TxmTLPa5 (1) 151 CAGGAACCCC TGGCGGAGGG CAGCAACTCC AGACCGGCGA GACCTGGAGC 200

TxmTLPa1 (4) 201 GTAGACGTTG CAGCGGGGAC AGCAGCGGGG AGATTCTGGG GCCGAACAGG 250
TxmTLPa2 (3) 201 GTAGACGTTG CAGCGGGGAC AGCAGCGGGG AGATTCTGGG GTCGAACAGG 250
TxmTLPa3 (1) 201 GTAGACGTTG CAGCGGGGAC AGCAGCGGGG AGATTCTGGG GTCGAACAGG 250
TxmTLPa4 (1) 201 GTTGACGTTG CAGCGGGGAC AGCAGCGGGG AGATTCTGGG GTCGAACAGG 250
TxmTLPa5 (1) 201 GTAGACGTTG CAGCGGGGAC AGCAGCGGGG AGATTCTGGG GTCGAACAGG 250

TxmTLPa1 (4) 251 CTGCTCTTTC GATGGAAATG GGAGAGGGAG CTGCCAAACT GGCGACTGTG 300
TxmTLPa2 (3) 251 CTGCTCTTTC GATGGAAATG GGAGAGGGAG CTGCCAAACT GGCGACTGTG 300
TxmTLPa3 (1) 251 CTGCTCTTTC GATGGAAATG GGAGAGGGAG CTGCCAAACT GGCGACTGTG 300
TxmTLPa4 (1) 251 CTGCTCTTTC GATGGAAATG GGAGAGGGAG CTGCCAAACT GGCGACTGTG 300
TxmTLPa5 (1) 251 CTGCTCTTTC GATGGAAATG GGAGAGGGAG CTGCCAAACT GGCGACTGTG 300

TxmTLPa1 (4) 301 GTGGCTTACT CAGCTGCCAA GCCTCTGGAG CAGTCCCTGT CTCGCTTGCA 350
TxmTLPa2 (3) 301 GTGGCTTACT CAGCTGCCAA GCCTCTGGAG CAGTCCCTGC CTCGCTTGCA 350
TxmTLPa3 (1) 301 GTGGCTTACT CAGCTGCCAA GCCTCTGGAG CAGTCCCTGC CTCGCTTGCA 350
TxmTLPa4 (1) 301 GTGGCTTACT CAGCTGCCAA GCCTCTGGAG CAGTCCCTGC CTCGCTTGCA 350
TxmTLPa5 (1) 301 GTGGCTTACT CAGCTGCCAA GCCTCTGGAG CAGTCCCTTC CTCGCTTGCA 350

TxmTLPa1 (4) 351 GAGTATTCCC TCAACCAAGA TCAGAACAAG GATTTCTATG ACATTTCCCT 400
TxmTLPa2 (3) 351 GAGTATTCCC TCAACCAAGA TCAGAACAAG GATTTCTATG ACATTTCCCT 400
TxmTLPa3 (1) 351 GAGTATTCCC TCAACCAAGA TCAGAACAAG GATTTCTATG ACATTTCCCT 400
TxmTLPa4 (1) 351 GAGTATTCCC TCAACCAAGA TCAGAACAAG GATTTCTATG ACATTTCCCT 400
TxmTLPa5 (1) 351 GAGTATTCCC TCAACCAAGA TCAGAACAAG GATTTCTATG ACATTTCCCT 400

TxmTLPa1 (4) 401 CGTCGACGGC TTCAACGTTC CAATTTCTCT CGCTCCTACC AACGGGCAGT 450
TxmTLPa2 (3) 401 CGTCGACGGC TTCAACGTTC CAATTTCTCT CGCTCCTACC AACGGGCAGT 450
TxmTLPa3 (1) 401 CGTCGACGGC TTCAACGTTC CAATTTCTCT CGCTCCTACC AACGGGCAGT 450
TxmTLPa4 (1) 401 CGTCGACGGC TTTAACGTTC CAATTTCTCT CGCTCCTACC AACGGGCAGT 450
TxmTLPa5 (1) 401 CGTCGACGGC TTCAACGTTC CAATTTCTCT CGCTCCTACC AACGGGCAGT 450

TxmTLPa1 (4) 451 GCACCACCAC TAGCTGCAAA AGTGACATTA ATGCAGTTTG CCCTGCAGAA 500
TxmTLPa2 (3) 451 GCACCACCTC TAGCTGCAAA AGTGACATTA ATGCAGTTTG CCCTGCAGAA 500
TxmTLPa3 (1) 451 GCTCCACCAC TAGCTGCAAA AGTGACATTA ATGCAGTTTG CCCTGCAGAA 500
TxmTLPa4 (1) 451 GCACCACCAC TAGCTGCAAA AGTGACATTA ATGCAGTTTG CCCTGCAGAA 500
TxmTLPa5 (1) 451 GCACCACCAC TAGCTGCAAA AGTGACATTA ATGCAGTTTG CCCTGCAGAA 500
```

```
TxmTLPa1 (4)   501 TTGAAGGTGT CTGGAGGTTG CAACAGTGCC TGTGCTGCCT TTCACACTGA  550
TxmTLPa2 (3)   501 TTGAAGGTGT CTGGAGGTTG CAACAGTGCC TGTGCTGCCT TTCACACTGA  550
TxmTLPa3 (1)   501 TTGAAGGTGT CTGGAGGTTG CAACAGTGCC TGTGCTGCCT TTCGCACTGA  550
TxmTLPa4 (1)   501 TTGAAGGTGT CTGGAGGTTG CAACAGTGCC TGTGCTGCCT TTCACACTGA  550
TxmTLPa5 (1)   501 TTGAAGGTGC CTGGAGGTTG CAACAGTGCC TGTGCTGCGT TTCACACTGA  550

TxmTLPa1 (4)   551 CCAGTATTGC TGCACAGGTG CAAATACCGA CAACTGCGCT CCCACCAATT  600
TxmTLPa2 (3)   551 CCAGTATTGC TGCACAGGTG CAAATACCGA CAACTGCGCT CCCACCAATT  600
TxmTLPa3 (1)   551 CCAGTATTGC TGCACCGGTG CAAATACCGA CAACTGCGCT CCCACCAGTT  600
TxmTLPa4 (1)   551 CCAGTATTGC TGCACAGGTG CAAATACCGA CAACTGCGCT CCCACCAATT  600
TxmTLPa5 (1)   551 CCAGTATTGC TGCACAGGTG CAAATACCGA CAACTGCGCT CCCACCAATT  600

TxmTLPa1 (4)   601 ACTCCATGTT CTTCAAGAAC CAGTGTCCCC AGGCGTATAG TTATGCAAAG  650
TxmTLPa2 (3)   601 ACTCCATGTT CTTCAAGAAC CAGTGTCCCC AGGCGTATAG TTATGCAAAG  650
TxmTLPa3 (1)   601 ACTCCATGTT CTTCAAGAAC CAGTGCCCCC AGGCGTATAG TTATGCAAAG  650
TxmTLPa4 (1)   601 ACTCCATGTT CTTCAAGAAC CAGTGTCCCC AAGCGTATAG TTATGCAAAG  650
TxmTLPa5 (1)   601 ACTCCATGTT CTTCAAGAAC CAGTGTCCCC AGGCGTATAG TTATGCAAAG  650

TxmTLPa1 (4)   651 GACGATGCCT CCAGCACATT CACCTGCTCC TCTGGTACCA CCGACTACAA  700
TxmTLPa2 (3)   651 GACGATGCCT CCAGCACTTT CACCTGCTCC TCTGGTACCA CTGACTACAA  700
TxmTLPa3 (1)   651 GACGATGCCT CCAGCACTTT CACCTGCTCC TCTGGTACCA CTGACTACAA  700
TxmTLPa4 (1)   651 GACGATGCCT CCAGCACTTT CACCTGCTCC TCTGGTACGA CTGACTACAA  700
TxmTLPa5 (1)   651 GACGATGCCT CCAGCACTTT CACCTGCTCC TCTGGTACCA CTGACTACAA  700

TxmTLPa1 (4)   701 AATTGTATTC TGTCCTTAAA CACATACATA TACTTTTACA AAGAATAATA  750
TxmTLPa2 (3)   701 AATTGTATTC TGTCCTTAAA CACATACATA TACTTTTACA GAGAATAATA  750
TxmTLPa3 (1)   701 AATTGTATTC TGTCCTTAAA CACATACATA TACTTTTACA GAGAATAATA  750
TxmTLPa4 (1)   701 AATTGTATTC TGTCCTTAAA CACATACATA TACTTTTACA GAGAATAATA  750
TxmTLPa5 (1)   701 AATTGTATTC TGTCCTTAAA CACATACATA TACTTTTACA GAGAATAATA  750

TxmTLPa1 (4)   751 TGCTCCGAAT AATCATCCTG CAATAA 776
TxmTLPa2 (3)   751 TGCTCCGAAT AATCATCCTG CAATAA 776
TxmTLPa3 (1)   751 TGCTCCGAAT AATCATCCTG CAATAA 776
TxmTLPa4 (1)   751 TGCTCCGAAT AATCATCCTG CAATAA 776
TxmTLPa5 (1)   751 TGCTCCGAAT AATCATCCTG CAATAA 776
```