Identification and Characterization of Phloem and Xylem Sap Proteins in *Populus trichocarpa* x *P. deltoides*

by

Nicole Dafoe
B.Sc., Grand Valley State University, 2003

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of the Requirements for the Degree of

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in the Department of Biology

Supervisory Committee

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Abstract

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Hundreds of proteins have been detected in phloem and xylem sap, even though the cells involved in long distance phloem and xylem transport are incapable of protein synthesis at maturity. We are now beginning to learn the identity and function of these proteins, but this knowledge is generally limited to annual plants. The first objective of this study was to identify phloem and xylem sap proteins in the perennial, poplar. Using LC-MS/MS, 48 proteins were identified in poplar phloem exudate and 98 proteins were identified in xylem sap. A large number of phloem exudate proteins are insect defense proteins that include protease inhibitors and polyphenol oxidase and also members of the pop3/SP1 gene family, whereas a number of xylem sap proteins were pathogenesis-related proteins such as thaumatin-like proteins (TLPs) and chitinases that function in pathogen defense. The importance of xylem sap proteins in pathogen defense has been previously demonstrated, however, the role of phloem proteins in insect defense is currently unknown. A major question to be addressed in order to understand the function

of phloem proteins is if they are differentially regulated in response to insect herbivory. The second objective of this study was to identify poplar phloem proteins differentially regulated in response to simulated insect feeding. Using two-dimensional gel electrophoresis, two proteins, PtTLP1 and pop3.1, were consistently upregulated 24 hours post-wounding. The third objective of this study was to produce antibodies against these proteins to use to further characterize their expression and localization patterns. Antibodies were also produced against another phloem exudate protein, pop3.4, which is 40% similar to pop3.1. The origin of all three proteins inside sieve elements was confirmed with immunolocalization. PtTLP1, pop3.1 and pop3.4 antisera labelled organelle-like structures in sieve elements and also phloem parenchyma cells. For PtTLP1, these structures were identified as starch and starch containing plastids. All three antisera also labelled cell wall proteins in different cell types. Overall, this study represents the first large-scale analysis of phloem and xylem sap proteomes from a perennial and describes the first observation of wound-inducible phloem sap proteins.

Table of Contents

List of Tables

List of Figures

List of Supplementary Tables and Figures (Appendix 1)

List of Abbreviations

1D SDS-PAGE:	one-dimensional SDS-PAGE
2-DE:	two-dimensional gel electrophoresis
AOC:	allene oxide cyclase
AOS:	allene oxide synthase
ATP:	adenosine triphosphate
BCIP:	5-bromo-4-chloro-3'-indolyphosphate *p*-toluidine salt
BLAST:	basic local alignment search tool
cDNA:	complementary deoxyribonucleic acid
CHAPS:	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
DAB:	3,3′ diaminobenzidine tetrahydrochloride
Dabb:	dimeric alpha-beta barrel
DTT:	dithiothreitol
DUF:	domain of unknown function
EDTA:	ethylenediaminetetraacetic acid
EST:	expressed sequence tag
FT:	flowering locus T
GFP:	green fluorescent protein
Glu:	glutamic acid
GUS:	β-glucuronidase
His:	histidine
JA:	jasmonic acid
JGI:	Joint Genome Institute
LC-MS/MS:	liquid chromatography-tandem mass spectrometry
LOX:	lipoxygenase
LPI:	leaf plastochron index
Lys:	lysine
MS:	mass spectrometry
MW:	molecular weight
NAD:	nicotinamide adenine dinucleotide

NBT:	nitro-blue tetrazolium chloride
PBS:	phosphate buffered saline
PCMBS:	p-chloromercuribenzenesulfonic acid
PCR:	polymerase chain reaction
PI:	proteinase inhibitor
PPO:	polyphenol oxidase
PR:	pathogenesis-related
P-value:	probability value
PVDF:	polyvinylidene difluoride
RNA:	ribonucleic acid
RuBisCO:	ribulose-1,5-bisphosphate carboxylase/ oxygenase
SDS:	sodium dodecyl sulfate
SDS-PAGE:	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA:	short interferring RNA
SP1:	stable protein 1
TAIR:	The Arabidopsis Information Resource
TI:	trypsin inhibitor
TLP:	thaumatin-like protein
Tyr:	tyrosine

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1 Introduction

1.1 Overview of plant vascular tissues

Land plants have evolved a non-circulatory vascular system that functions in the long distance transport of nutrients and other compounds throughout the plant (Lough and Lucas, 2006). The vascular tissues consist of phloem, which is principally involved in transporting sugars and small organic compounds from source to sink tissues, and xylem that transports water and minerals absorbed from the soil to the shoot (Evert, 2006). These tissues transport numerous other compounds throughout the plant including hormones, amino acids, lipids, and various RNA species (Hoad, 1995; van Bel, 2003; Lough and Lucas, 2006; Satoh, 2006). In addition, phloem and xylem sap also contain a large number of proteins (Lough and Lucas, 2006; Satoh, 2006), which are the focus of this thesis.

There are two types of phloem and xylem, primary and secondary (Evert, 2006). The primary tissues are produced by apical meristems, whereas secondary phloem and xylem is derived from the vascular cambium. The apical meristems are located at plant apices and the vascular cambium is a lateral meristem that is arranged vertically along the axis of roots and shoots. Not all plants undergo secondary growth, but in plants that do, primary phloem and xylem only remain functional until the production of secondary vascular tissues.

Vascular tissues form a continuous system throughout the entire plant body (Evert, 2006). They are embedded in ground tissue, a simple tissue composed of parenchyma cells. These tissues are in turn surrounded by the protective, dermal tissue

system consisting of epidermal cells. In plants that undergo extensive secondary growth, the epidermis is replaced by another protective tissue called the periderm.

In this chapter, I will begin by describing phloem and xylem and how long-distance transport occurs in these tissues. I will briefly discuss what is currently known regarding phloem and xylem sap proteins before introducing poplars, the model perennial plant species that I will use to identify phloem and xylem sap proteins. I will also give a brief introduction into the active defense response that occurs in poplar and other plants in response to insect and simulated insect feeding. I will then describe how phloem and phloem sap proteins appear to have a role in this response.

1.2 Phloem

Phloem is the "food-conducting" tissue that it is composed of several cell types with different functions within the plant (Evert, 2006). Phloem fibres, for example, are highly lignified cells that provide structural support, and phloem parenchyma cells generally function in storage, although some of these cells are also involved in radial transport. The cells involved in long-distance transport, however, are sieve elements. In angiosperms, the sieve elements are called sieve-tube elements and these cells are arranged longitudinally to form sieve tubes. Sieve-tube elements are connected by large pores that occur in the sieve plate region of the cell wall. Sieve-tube elements are typically associated with companion cells, specialized phloem parenchyma cells that are derived from the same mother cell as the sieve tube element. Companion cells function in maintaining mature sieve-tube elements as the latter no longer have a vacuole, nucleus, or functioning ribosomes at maturity. Although mature sieve-tube elements are living, they

only retain their plasma membrane, endoplasmic reticulum, mitochondria, and specialized plastids. It has been suggested that companion cells provide sieve-tube elements with proteins and energy in the form of ATP (van Bel, 1996).

Phloem transport occurs from carbon exporting or source tissues to carbon importing or sink tissues (Taiz and Zeiger, 2006). Source tissues are generally mature, photosynthetic leaves and sinks include roots and actively growing leaves, tissues that cannot meet their own carbon demand. In source leaves, carbon is loaded into sieve elements apoplastically or symplastically, depending on the species, and this decreases the water potential of the cells. As a result, water passively moves into the sieve elements, increasing the hydrostatic pressure and creating a pressure gradient between source and sink leaves, resulting in mass flow of sieve element contents. In the sink tissues, the carbohydrates are unloaded from sieve elements, thus increasing the water potential and causing water to leave the cells resulting in decreased hydrostatic pressure. This helps to maintain the pressure gradient between source and sink tissues. This widely accepted model for phloem transport is based on a model originally described in 1930 by Edward Münch (Oparka and Cruz, 2000; Taiz and Zeiger, 2006).

The major constituent of the translocation stream is sugar (100-300 g/L) (Hopkins, 1995; Buchanan et al., 2000). The primary sugar that is transported in phloem is sucrose; however, other non-reducing sugars such as sugar-alcohols and galactosyl-oligosaccharides are also transported in certain plant species (Taiz and Zeiger, 2006). Originally it was hypothesized that only non-reducing sugars are transported in phloem, but recently it was discovered that hexoses are also transported (van Bel and Hess, 2008).

As determined by the analysis of phloem sap, a variety of additional compounds

are also transported in phloem. These compounds include amino acids (Fife et al., 1962; Weibull et al., 1990; Girousse et al., 1991; Vanhelden et al., 1994; Amiard et al., 2004; Gessler et al., 2004; Nakamura et al., 2004), plant hormones (auxins, gibberellins, cytokinins, and abscisic acid) (Hoad, 1995), lipids (Madey et al., 2002), minerals (Fife et al., 1962; Hu et al., 1997; Nakamura et al., 2004), and secondary metabolites such as alkaloids (Wink and Witte, 1984), glucosinolates (Chen and Andreasson, 2001), and phenolics (Gould et al., 2007). In addition, a complex array of proteins and RNA have been detected in phloem sap (Lough and Lucas, 2006).

At maturity, sieve elements do not have a nucleus or functioning ribosomes, and therefore it is generally accepted that these cells do not synthesize their own RNA or proteins (Oparka and Cruz, 2000). Instead, these macromolecules are transported from companion cells via specialized plasmodesmata called pore-plasmodesma units (van Bel, 2003). The trafficking of RNA into sieve elements is highly regulated and is mediated by RNA binding proteins (Lough and Lucas, 2006). Even single-stranded RNAs that are only 21 to 25 nucleotides appear to require protein binding for entry into sieve elements; they do not simply diffuse into cells (Yoo et al., 2004). For proteins, trafficking from companion cells to sieve elements can be selective as has been described for RNA and small RNA, but it can also occur by diffusion (Lough and Lucas, 2006). It has been estimated that proteins with a molecular weight of up to 50 kDa can easily diffuse through the pore-plasmodesma unit connecting companion cells and sieve elements (Lough and Lucas, 2006).

1.2.1 Phloem sap proteins

Once in sieve elements, some proteins are transported thoughout the plant, a phenomenon that has been elegantly illustrated using various grafting combinations (Golecki et al., 1998; Gomez et al., 2005). For example, when *Cucumis sativus* scions are grafted onto *Cucurbita* rootstocks, phloem proteins from the *Cucurbita* rootstocks are detected in the phloem sap collected from the scion (Golecki et al., 1998). Long distance phloem transport has also been observed for plants engineered to specifically express the green fluorescent protein (GFP) in companion cells (Imlau et al., 1999; Stadler et al., 2005). When wild-type tobacco scions were grafted onto GFP transgenic rootstocks, GFP was detected in the scions (Imlau et al., 1999). In another study, biotinylated pumpkin phloem sap proteins were introduced into rice sieve elements via cut aphid stylets and they were detected in distant tissues (Aoki et al., 2005). Interestingly, in this study it was observed that long distance transport of proteins within sieve elements is very controlled. In general, acropetal transport appears to occur by mass flow, but basipetal transport is very selective and not all proteins are transported into roots (Aoki et al., 2005). It is important to note that not all proteins in sieve elements are transported long-distances. Some proteins appear to be immobile and remain closely associated with the plasma membrane (van Bel, 2003). It is only when sieve elements are wounded that these immobile proteins are released and this appears to be an important mechanism for sealing damaged sieve elements (van Bel et al., 2002).

Some sieve element proteins have been identified by immunolocalization. In poplar, this technique was used to localize a peroxiredoxin in sieve elements (Rouhier et al., 2001). This protein is the only poplar sieve element protein identified to date. Other proteins localized to sieve elements include enzymes involved in the biosynthesis of

jasmonic acid (JA); allene oxide cyclase (AOC), allene oxide synthase (AOS), and lipoxygenase (LOX) (Avdiushko et al., 1994; Hause et al., 2003), and proteinase inhibitors (PIs) (Dannenhoffer et al., 2001; Xu et al., 2001).

The majority of sieve element proteins have been identified by analyzing phloem sap proteins with mass spectrometry (MS). Originally, protein identification by MS was limited by a lack of plant genomic sequence. With advances in genomics and proteomics, however, it is now possible to identify a much larger number of proteins. While relatively pure phloem sap can be easily collected from Cucurbits in quantities large enough for analysis with MS, originally there was very little genomic sequence data available for these species, which is required for protein identification. In a study published in 2004, only16 and 29 phloem proteins could be identified from cucumber and pumpkin, respectively when using MS even though the phloem sap of both species was estimated to have approximatey 400 proteins (Walz et al., 2004). More recently, 80 proteins were identified in rice phloem sap (Aki et al., 2008) and 140 phloem sap proteins were successfully identified in *Brassica napus* (Giavalisco et al., 2006). Both plants have well-developed EST and genomic resources. To date, phloem sap proteomes have been characterized for *Cucumis sativus*, *Cucurbita maximus*, *Ricinus communis*, *Oryza sativa*, and *B. napus* (Walz et al., 2002; Barnes et al., 2004; Walz et al., 2004; Giavalisco et al., 2006; Aki et al., 2008). These are all herbaceous annual plant species, which is one of the motivations for undertaking the current study in the perennial, poplar.

Using activity assays, we know that some of the proteins collected in phloem sap are functional. Kinase activity has been detected in phloem exudate collected from rice and squash (Nakamura et al., 1995; Yoo et al., 2002) and antioxidant enzymes such as

superoxide dismutase and peroxidase are active in phloem exudate collected for various Cucurbit species (Walz et al., 2002). The function of these and other proteins within sieve elements, however, is not completely understood. It has been proposed that these proteins may have a role in maintaining sieve elements or they may serve as long-distance signaling molecules (Chen and Kim, 2006). It has also been suggested that phloem sap proteins may be involved in plant stress responses, given that a large number of stress-inducible proteins, including the antioxidant proteins described above, are commonly detected in phloem sap. Other stress-inducible proteins include insect defense proteins such as lectins and proteinase inhibitors (PIs) (Haebel and Kehr, 2001; Barnes et al., 2004; Walz et al., 2004; Giavalisco et al., 2006; Aki et al., 2008). The importance of these phloem sap proteins in plant defense, however, has not been studied (Kehr, 2006). Although we now know the identity of many phloem proteins (see Chapter 2 Introduction), further research is still needed to understand their function in sieve elements and the whole plant.

1.3 Xylem

Xylem functions in the unidirectional transport of water and minerals (Evert, 2006; Taiz and Zeiger, 2006). Similar to phloem, xylem is composed of fibres and parenchyma cells, but the cells involved in long-distance transport are tracheary elements (Evert, 2006). Unlike sieve elements, these cells are dead at maturity. There are two types of tracheary elements, tracheids and vessel elements, both of which have lignified secondary cell walls. Vessel elements evolved from tracheids and they are found primarily in angiosperms. Overall, these cells are shorter than tracheids and they have a

much wider diameter, which makes them more efficient in the transport of water and other nutrients. They also have perforation plates, regions of the cell wall in which the primary and secondary cells walls have been completely degraded. These are found at the ends of vessel elements and it is the region that vessel elements are connected to form vessels.

Water is absorbed by roots and transported to shoots via the xylem (Taiz and Zeiger, 2006). In the shoot, the majority of water diffuses into the atmosphere by a process called transpiration. Transpiration occurs primarily through stomata found on the abaxial side of leaves and it creates a large negative pressure in cell walls of the leaves. Essentially, it is this negative pressure or tension that results in xylem transport; the tension pulls water through tracheary elements and this is facilitated by the cohesive nature of water. This mechanism of water transport in xylem is called the cohesion-tension theory of sap ascent.

In xylem, positive pressure in xylem can only be detected at night or during periods of little to no transpiration (Taiz and Zeiger, 2006). In this case, the ions absorbed from the soil accumulate in xylem decreasing its water potential. As a result, water is absorbed and the hydrostatic pressure is increased. This positive pressure alone cannot account for xylem transport beyond a short distance, but if the shoot is decapitated during this period, xylem sap readily exudes from the severed shoot.

As expected, xylem sap is composed primarily of water and inorganic molecules such as phosphate and nitrate (Satoh, 2006). Surprisingly, organic compounds have also been detected in xylem sap (Satoh, 2006). These compounds include amino acids such as glutamine, serine, and alanine (Dickson, 1979; Lopez-Millan et al., 2000; Alvarez et al.,

2008) and sugars including sucrose, fructose, and glucose (Satoh et al., 1992; Lopez-Millan et al., 2000; Escher et al., 2004; Alvarez et al., 2008). Proteins have also been identified in xylem sap (Satoh, 2006).

1.3.1 Xylem sap proteins

Similar to sieve elements, functioning tracheary elements are incapable of protein synthesis, so presumably xylem sap proteins are expressed in surrounding cells and then secreted into the apoplast. In fact, the majority of xylem sap proteins identified to date have an N-terminal signal peptide targeting them to the secretory pathway, which supports this hypothesis (Buhtz et al., 2004; Kehr et al., 2005; Alvarez et al., 2006; Djordjevic et al., 2007). Currently, however, there are very few studies addressing the origin of these proteins. In cucumber, glycine-rich proteins, a lectin, and a chitinase were all detected in xylem sap and the corresponding genes were subsequently shown to be expressed in roots (Sakuta et al., 1998; Masuda et al., 1999; Sakuta and Satoh, 2000; Masuda et al., 2001). Using *in situ* hybridization, it was shown that the genes encoding the glycine-rich proteins were specifically expressed in parenchyma cells surrounding xylem vessels (Sakuta and Satoh, 2000). Based on these results, it appears that some xylem sap proteins are expressed in the roots, secreted into the transpiration steam, and then transported to the shoots. However, a more recent study provides evidence that contradicts this model. The gene expression of 17 xylem sap proteins were analyzed in soybean and only four of the genes were predominately expressed in roots (Djordjevic et al., 2007). These results suggest that possibly not all xylem sap proteins are transported

long distances from the roots. Additional research is needed concerning the synthesis of xylem sap proteins and their long distance transport.

Xylem sap contains hundreds of proteins (Kehr et al., 2005; Alvarez et al., 2006). Xylem sap proteomes have been characterized for *B. napus*, *B. oleracea*, *Cucurbita maxima*, *Cucumis sativus*, *Z. mays*, *Lycoperscion esculentum*, *Glycine max*, and *Oryza sativa* (Rep et al., 2002; Buhtz et al., 2004; Kehr et al., 2005; Alvarez et al., 2006; Djordjevic et al., 2007; Aki et al., 2008). Of the proteins identified, a number are consistently detected in xylem sap. These proteins include peroxidases, proteases, and pathogenesis-related proteins (Rep et al., 2002; Buhtz et al., 2004; Kehr et al., 2005; Alvarez et al., 2006; Djordjevic et al., 2007; Aki et al., 2008).These results suggest that xylem sap proteins carry out conserved functions within xylem, but there have been no studies of perennial xylem sap proteomes to confirm this hypothesis. Additional details regarding the identity and function of xylem sap proteins are presented in the introduction to Chapter 5.

1.4 Poplar as a model plant species

A model plant species is one that has a wide genetic diversity, is fast growing, has a short life cycle, can easily be bred, is capable of being transformed, and has a characterized genome (Taylor, 2002). Poplars have many of these traits and are therefore considered an excellent model species for studying woody plants (Bradshaw et al., 2000; Taylor, 2002). They grow quickly, can be vegetatively propagated, provide large quantities of sample tissue, and most importantly, the entire genome of *Populus trichocarpa* has been sequenced (Tuskan et al., 2006). In fact, it was the first perennial

genome to be completely sequenced. For these reasons, poplar was the perennial chosen to study phloem and xylem sap proteins. Although these proteins have been studied in annual plants, it is also important to study them in perennials, because annuals and perennials are inherently different due to their growth habits. For example, perennials like poplar have more extensive formation of secondary xylem, they undergo dormancy and bud-break, and they flower at different ages as compared to annuals (Bradshaw et al., 2000; Taylor, 2002). Therefore not all aspects of annual plant biology can be directly applied to perennials.

Poplars are also important both ecologically and economically. There are 29 species including poplars, cottonwoods, and aspens (Stettler et al., 1996), and these rapidly growing trees can be found throughout North America as well as other parts of the world. They are keystone species in riparian forests (Stettler et al., 1996; Dickmann et al., 2001). Their wood has many uses, including the production of pulp and paper and composite products (Stettler et al., 1996; Dickmann et al., 2001). Poplars are also planted to provide windbreaks and prevent erosion and they have great potential for use in phytoremediation (Dickmann et al., 2001).

1.5 Poplars and plant defense against insect herbivory

Like many plants, poplars are capable of producing an arsenal of repellent, anti-nutritive, and even toxic chemicals and proteins in response to insect herbivory (Constabel and Major, 2005; Philippe and Bohlmann, 2007). When poplars are wounded by insects or mechanical damage or if caterpillar regurgitant is applied to leaves, hundreds of genes are differentially regulated (Lawrence et al., 2006; Major and

Constabel, 2006; Ralph et al., 2006; Ralph et al., 2008). While some photosynthetic genes are down-regulated, many potential defensive genes are upregulated (Major and Constabel, 2006; Ralph et al., 2006), reprogramming the plant from growth and development to defense. Strongly upregulated defense genes include chitinases, trypsin inhibitors, and polyphenol oxidases (Christopher et al., 2004; Lawrence et al., 2006; Major and Constabel, 2006; Ralph et al., 2006). There is direct evidence that these proteins negatively impact insect feeding. Although the mechanism is not understood, a poplar chitinase (WIN6) overexpressed in tomato plants exhibited insecticidal activity against the Colorado potato beetle (Lawrence and Novak, 2006). Similarly, a poplar trypsin inhibitor (WIN3), which functions by inhibiting proteases in insect guts, was overexpressed in tobacco and negatively impacted the growth of tobacco budworm (Lawrence and Novak, 2001). Like protease inhibitors, polyphenol oxidases are anti-nutritive proteins. These proteins produce highly reactive *ortho*-quinones, which are capable of cross-linking proteins essentially decreasing their digestibility (Constabel and Barbehenn, 2008). When fed poplar leaves that were engineered to overexpress polyphenol oxidase, forest tent caterpillars (*Malacosoma disstria*) hatched from aged egg bands exhibited slower growth and had an overall higher mortality rate than caterpillars feeding on non-transgenic plant tissue (Wang and Constabel, 2004).

Defense genes are not only upregulated locally at the site of wounding, but also systemically, in unwounded tissue on wounded plants possibly as a preventative measure against further insect attack. This systemic defense response was originally described in tomato by Green and Ryan (1972) who suggested that the systemic induction of protease inhibitors was dependent on the transport of a mobile signal from the wounded leaf to

unwounded leaves. It has since become apparent that the mobile signal is transported in the phloem, since the greatest induction of defense genes occurs in leaves that have the strongest vascular connections with the wounded leaf (Davis et al., 1991; Orians et al., 2000; Schittko and Baldwin, 2003; Viswanathan and Thaler, 2004). Girdling tomato plants, thereby destroying phloem transport while leaving xylem intact, resulted in an absence of the systemic induction of defense genes, providing strong evidence that the signal moves within the phloem (Nelson et al., 1983; Rhodes et al., 1999). Furthermore, when an inhibitor of active apoplastic phloem loading, *p*-chloromercuribenzenesulfonic acid (PCMBS), was added to tomato after wounding, the systemic wound response was inhibited (Narvaez-Vasquez et al., 1994). Studies in tomato, potato, tobacco, and poplar provide evidence that that the signal moves bidirectionally, at least in some plants, which is also in agreement with phloem transport pathways (Pena-Cortes et al., 1988; Schittko and Baldwin, 2003; Major and Constabel, 2007).

The importance of vascular connections in plant defense has been extensively studied in poplar. As described above, the strongest induction of defense genes occurs in sink leaves with the strongest vascular connections to the wounded leaf (Davis et al., 1991). Defense genes are also induced in roots (Major and Constabel, 2007). Furthermore, the synthesis of phenolics is increased in wounded or JA-treated sink tissues (Arnold and Schultz, 2002; Arnold et al., 2004), and this increase is positively correlated with an increase in carbohydrates imported from source leaves. JA-treatment has also been shown to increase carbohydrate transport to stems and roots (Babst et al., 2005), possibly to sequester nutrients from leaf-chewing insects. Overall, the systemic

defense response in poplar has been shown to effectively reduce subsequent feeding by insect herbivores (Jones et al., 1993).

Altogether, phloem transport has an important role in the plant defense response, but it is not known if phloem proteins play a role in this process. Interestingly, enzymes involved in the biosynthesis of the phloem mobile defense signal, likely JA or a JA-like molecule (Schilmiller and Howe, 2005), have been localized to sieve elements (see section 1.2.1) (Hause et al., 2003). In addition, defense proteins including PIs, have been identified in phloem sap (Haebel and Kehr, 2001; Barnes et al., 2004; Walz et al., 2004; Giavalisco et al., 2006; Aki et al., 2008). Currently, it is not know if these proteins function in plant defense (Kehr, 2006), but there is indirect evidence that some proteins in phloem tissues may be differentially expressed in response to wounding. Based on transgenic *Arabidopsis* plants in which a sucrose transporter promoter was fused to GUS, staining repeatedly increased in phloem cells when the stems and leaves of plants were cut suggesting that it is also wound inducible (Meyer et al., 2004). The same methods were used with a ribonuclease LE gene localized to phloem tissue in tomato, and again staining increased in response to wounding (Kock et al., 2004). However, there have been no studies to specifically identify phloem sap proteins that are differentially expressed in response to insect attack or wounding. Like the inducible defense proteins described above, such proteins may also have a role in plant defense.

1.6 Objectives and rationale

Prior to this work, our knowledge of phloem and xylem sap proteins was limited to annuals. The first objective of this study was to use MS to identify phloem and xylem

sap proteins from the perennial, *P. trichocarpa* x *P. deltoides* (Chapters 2 and 5, respectively). These proteins can then be compared to phloem and xylem sap proteins from annual plants in order to identify proteins that may have particularly important functions. In addition, novel phloem and xylem sap proteins may be identified. Poplar is the model perennial choosen because it is ecologically and economically signficant and the *P. trichocarpa* genome has been sequenced. A completed genome will be indispensable in facilitating the identification of proteins. The second objective was to use comparative proteomics to identify phloem exudate proteins that are induced in response to insect feeding; as such proteins may have a role in plant defense (Chapter 2). The wound-inducibility of phloem sap proteins has not been previously studied. After identifying proteins of interest, the third objective was to produce antibodies as tools for studying these proteins (Chapters 3 and 4). These antibodies will be used to further characterize the expression and localization of these proteins, which may help to elucidate their function within poplar.

2 Analysis of the poplar phloem proteome and its response to leaf wounding

2.1 Introduction

Phloem is a complex tissue with multiple roles in vascular plants. Its primary function in transport of sugars and organic compounds from mature leaves to sink tissues via the process of mass flow is well established (Taiz and Zeiger, 2006). More recently, it has become apparent that phloem has essential roles in other plant processes such as long-distance signalling. A large number of RNAs and proteins are present in phloem sap (Lough and Lucas, 2006). This has led to the idea that macromolecules in the phloem may play vital roles in plant development and environmental adaptation. For example, the anti-viral defense known as post-transcriptional gene silencing appears to spread systemically through phloem-mobile short interfering RNA (siRNA) (Lough and Lucas, 2006) and the elusive phloem-mobile 'florigen' which induces flowering in day-length sensitive plants appears to be the FT protein (Lin et al., 2007). Clearly, understanding many whole-plant processes will require a detailed knowledge of phloem contents including proteins.

In flowering plants, the conducting tissue consists of sieve tubes, which are composed of sieve-tube elements connected by cytoplasmic strands that pass through pores in sieve plates (Taiz and Zeiger, 2006). Phloem sap is under positive pressure which drives mass flow; therefore, sieve elements typically respond to damage by rapidly sealing the pores at the sieve plates with phloem-specific proteins and callose. This makes it difficult to collect phloem sap in large quantities for analysis, and thus the identity of phloem proteins and other macromolecules has remained relatively obscure. In

some plants belonging to the Cucurbitaceae, as well as *Ricinus communis* and *Brassica napus*, relatively uncontaminated phloem exudate can be collected from incisions in the stem (Milburn, 1970; van Bel, 2003; Giavalisco et al., 2006); in most other species, phloem exudate can only be collected by adding the Ca^{2+} chelator EDTA, which is thought to prevent the sealing of sieve elements (King and Zeevaart, 1974).

Based on two-dimensional gel electrophoresis (2-DE) analyses, phloem exudate has been estimated to contain hundreds of proteins (Fisher et al., 1992; Walz et al., 2004; Giavalisco et al., 2006). At maturity, sieve elements lack a nucleus and functioning ribosomes; thus the phloem exudate proteins are likely synthesized in companion cells. To date, proteomic studies of phloem contents have been reported for annual plants such as *Cucumis sativus*, *Cucurbita maximus*, *Ricinus communis*, *Oryza sativa*, and *B. napus* (Walz et al., 2002; Barnes et al., 2004; Walz et al., 2004; Giavalisco et al., 2006; Aki et al., 2008). Phloem proteins identified in these studies are involved in metabolism, signaling, and transport (Barnes et al., 2004; Walz et al., 2004; Giavalisco et al., 2006; Aki et al., 2008). A large number of stress response proteins were also identified in phloem, in particular, antioxidant enzymes such as superoxide dismutase, peroxidase and dehydroascorbate reductase (Walz et al., 2002). In addition, some herbivore defense proteins such as proteinase inhibitors (PIs) and lectins were present in phloem exudate of several species (Dannenhoffer et al., 2001; Barnes et al., 2004; Walz et al., 2004; Giavalisco et al., 2006). The presence of both antioxidant and defense proteins in several species suggests conservation of function of phloem proteins (Kehr, 2006); however, almost no functional studies on phloem proteins have been reported (Lough and Lucas, 2006).

The presence of herbivore defense proteins in phloem is intriguing, especially because one of the best examples of long-range signalling in plants is the systemic induction of herbivore defenses. Following localized insect feeding, defense proteins such as the PIs accumulate rapidly in both wounded and unwounded leaves (Schilmiller and Howe, 2005). This systemic induction in the unwounded leaves occurs preferentially in leaves with the strongest vascular connections to the wounded leaf, suggesting signal transmission via the phloem (Davis et al., 1991; Orians et al., 2000). The induction of defense genes after leaf damage is not limited to leaves, but also occurs in roots (Major and Constabel, 2007). The importance of phloem in herbivore defense signaling is further emphasized by immuno-detection of jasmonic acid (JA) biosynthesis enzymes in sieve elements (Hause et al., 2003), as JA or a JA-like molecule is considered to be a primary systemic signal for herbivore defense activation (Schilmiller and Howe, 2005).

Despite the clear importance of phloem in adaptation to herbivory, no studies have addressed changes in phloem sap proteins in response to insect attack or wounding. While phloem feeding insects are known to induce a large number of genes in infested tissues, it is not known if the gene products accumulate in phloem sieve elements (Kehr, 2006; Thompson and Goggin, 2006). To date, the only analysis of stress responses in phloem exudate was carried out with *C. sativus* and *C. maxima* following drought treatment. This stress induced an increase of superoxide dismutase and dehydroascorbate reductase activity, but it is not clear if this resulted from an increase in the abundance of the proteins (Walz et al., 2002).

Here we report the first analysis of the poplar phloem exudate proteome. We optimized a method to collect poplar phloem exudate from excised stems segments using

EDTA, and successfully identified 48 proteins using partial amino acid sequences obtained from LC-MS/MS. In addition, we used comparative 2-DE to detect changes in phloem protein profiles 24 h after wounding, and identified two proteins, pop3 and a thaumatin-like protein (TLP), that are upregulated in phloem exudate after this treatment.

2.2 Materials and Methods

Plant materials

Poplar hybrid H11-11 (*Populus trichocarpa* x *P. deltoides*) saplings obtained from the University of Washington/Washington State University Poplar Research Program were propagated and grown in 2.5 L pots as described previously (Major and Constabel, 2006). All plants were maintained at the University of Victoria, in the Bev Glover greenhouse with a 16 h photoperiod. The temperature was maintained at 25/18 °C (day/night) and plants were watered daily with 0.1 g/L 20-20-20 PlantProd fertilizer (Plant Products, Brampton, ON, Canada).

Phloem exudate and bark sampling

To collect phloem exudate, 10 cm stem sections between leaf plastochron index (LPI) 5-16 (Larson and Isebrands, 1971) were excised from three-month-old *P. trichocarpa* x *P. deltoides* saplings and the leaves were removed. In order to prevent contamination from xylem sap, a section of bark was removed from the basal end and the exposed wood was sealed using a 90% lanolin / 10% paraffin wax mixture. The cut and sealed end was washed, blotted dry, and placed into 50 mL Falcon tubes containing 300 µL of 50 mM Tris (pH 8.0) with 2 mM EDTA. The tubes were sealed and phloem exudate collection proceeded for 2 h at room temperature. Protein samples were dialyzed

overnight (Spectra/Por, 23 mm, MWCO: 6,000-8,000, Spectrum Laboratories, RanchoDominquez, CA, USA) at 4 °C against 200 vol. deionized water, with one change of H_2O. Samples were then lyophilized, and resuspended in resolubilization buffer (8 M urea, 65mM DTT, and 16 mM CHAPS) for 30 min at room temperature prior to protein quantification using a modified Bradford assay (Ramagli and Rodriguez, 1985).

For analysis of 'bark' protein, green stem tissue including phloem, cortical cells, and epidermis, was peeled from the surrounding lignified xylem cells. The tissue was then ground to a fine powder in liquid nitrogen-chilled mortars, and proteins were extracted as previously described (Haruta et al., 2001). Soluble protein was precipitated using 2 vol. cold acetone for at least 1 h at -20 °C. After centrifugation (15 min, 16,000 x g) at 4 °C, the resulting pellet was washed twice with 80% cold acetone, dried, and resuspended in resolubilization buffer. Proteins were quantified as described above.

Gel electrophoresis and immunoblotting

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 10 µg of protein were separated on 12% polyacrylamide gels under a constant voltage (100 V) in a Mini PROTEAN II system (BioRad, Hercules, CA, USA). For 2-DE, 4 µg of protein was diluted with rehydration buffer (8 M urea, 32 mM CHAPS, 20 mM DTT, and 0.5% v/v IPG buffer, pH 3-10 (Amersham Biosciences, Piscataway, NJ, USA) to a total volume of 125 µL. The sample was then used to rehydrate a 7 cm Immobiline Drystrip (Amersham) with a linear pH range of 3-10. Isoelectric focusing of the sample was carried out for a total of 5.7 kVh with an IPGphor apparatus (Amersham). Prior to running the second dimension, the proteins were reduced with 65 mM DTT followed by

alkylation with 135 mM iodoacetamide, both in equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, and trace bromophenol blue) for 15 min. The gel was then fixed onto a 12% acrylamide gel (BioRad) using 0.5% (w/v) agarose. The second dimension was completed as decribed for SDS-PAGE. Proteins were visualized using either Bio-Safe Coomassie stain (BioRad) for 1D SDS-PAGE or Sypro Ruby (BioRad) for 2-DE and gels were scanned using the ProXPRESS Proteomic Imaging System (Perkin Elmer, Waltham, MA, USA).

For immunoblotting, after SDS-PAGE, proteins were electro-transferred to PVDF membrane (Pierce, Brockville, ON, Canada) and proteins were detected using standard protocols (Harlow and Lane, 1999). The anti-lsRuBisCO primary antibody (Agrisera, Vännäs, Sweden) was detected using a horse radish peroxidase conjugated secondary antibody and 3,3′ diaminobenzidine tetrahydrochloride (DAB) as the substrate.

Immunolocalization

A gene encoding a thaumatin-like protein (TLP, JGI acc. no. 828883) was PCR-amplified using 5'-GGGGGTCGACTGGACAAAAGGTTATCAAAT-3' as the forward primer and 5'-TTTTGGATCCCAATCTGTGACTTTCGACTT-3' as the reverse primer. The PCR products were cloned into the BamHI and Sal1 restriction sites of the pQE30 expression vector (Qiagen, Mississauga, ON, Canada) in frame with the N-terminal His-tag. The recombinant protein was produced and purified as previously described (Haruta et al., 2001). Anti-TLP antibodies were produced in rabbits using standard procedures at Cocalico Biologicals (Reamstown, PA, USA). The TLP antiserum specifically labelled proteins of the expected size in bark and phloem exudate samples, whereas the

preimmune serum and pre-adsorbed TLP antiserum did not react with any proteins in these samples (see Chapter 3).

For immunolocalization of TLPs, stem segments (near LPI 11) were fixed and embedded in BMM resin mixture (4 parts *n*-butyl methacrylate to 1 part methyl methacrylate) by Brent Gowen as previously described (Chaffey et al., 1997). Stem cross sections (6 μm thick) were cut and mounted on positive-charged glass slides. Prior to labelling, the plastic was partially removed by soaking the sections in acetone for 20 min. The sections were labelled with an anti-TLP antibody using standard protocols (Harlow and Lane, 1999) and the antibody was detected with an Alexafluor 568 goat anti-rabbit antibody (Molecular Probes, Eugene, OR, USA). Controls included omitting the primary antibody or incubating sections with preimmune serum. Labelling was visualized using a Zeiss Universal epifluorescence microscope equipped with a digital camera and a fluorescein isothiocyanate filter (excitation at 495 nm and emission at 519 nm).

Identification of proteins with LC-MS/MS

Total phloem proteins were first separated using 1D SDS-PAGE and the unstained lane containing the phloem proteins was excised and sliced into 15 gel segments corresponding to different size ranges. The proteins were digested as previously described (Poulis et al., 2005). After washing gel slices, the proteins were reduced with 50 mM DTT (Amersham) for 30 min at 56 °C and then alkylated with 100 mM iodoacetamide (Sigma, Oakville, ON, Canada) for 30 min at 45 °C in the dark. After dehydrating the gel pieces, the proteins were digested overnight with trypsin (20 ng/μL, Promega) at 37 °C and then extracted with 100 mM Na_2CO_3 for 1 h at 37 °C.

Peptides were analyzed at the UVic Genome BC Proteomics Centre using a Q TRAP hybrid triple quadrupole/linear ion trap MS/MS Mass Spectrometer equipped with a nano-electrospray ionization source (Applied Biosystems/MDS Sciex) as previously described (Wagner et al., 2007). MS data was processed with Analyst 1.4.1 software and a built in Mascot script (1.6b16 ABI – Matrix Science Limited, Boston, MA, USA). Trypsin was selected as the digest enzyme and up to one missed cleavage was allowed. Carbamidomethyl cysteine was set as a fixed modification and variable modifications included oxidation of methionine and deamidation of asparagine and glutamine. Peptide tolerance and MS/MS tolerance were set at 0.5 Da and 0.3 Da, respectively. Parameters also permitted one missed cleavage and were limited to 2+ and 3+ charged peptides.

A custom poplar database was developed and provided by the Treenomix project (http://www.treenomix.ca/), University of British Columbia, comprising both EST and genomic data was used for protein identification. Poplar ESTs (369,674 sequences) were obtained from GenBank (http://www.ncbi.nlm.nih.gov/) and clustered using CAP3 to produce 44,700 contigs and 52,316 singlets. Each sequence was translated in six reading frames that were included as separate database entries. An additional 58,036 gene models were included from version 1.1 of the *P. trichocarpa* genome provided by the Joint Genome Institute (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html), as well as 1,818 poplar proteins present in GenBank. All unigene proteins from the *Arabidopsis thaliana* (http://www.arabidopsis.org/) and *Oryza sativa* (http://rice.plantbiology.msu.edu) genomes were also included. All sequence sources were the most up-to-date available as of October 2006. Protein identification with Mascot software was considered correct if the match had a score greater than 52, which is

indicative of identity or significant ($P<0.05$) similarity, and had at least two peptide matches in which no other peptide sequence in the database gave a better score for that spectrum (Perkins et al., 1999). In some cases, single peptide matches were deemed correct if an antibody could be used to verify the presence of the protein in phloem exudate samples using western blots (data not shown). SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the presence of N-terminal signal peptides (Nielsen et al., 1997; Bendtsen et al., 2004).

Analysis of differentially regulated proteins using 2D gel electrophoresis

For wounding, the leaves of LPI 11-16 were crushed with pliers until approximately two-thirds of the leaf margins were damaged. The remaining one-third of the margins were wounded 1.5 h after the initial treatment. Phloem samples were individually collected from six control and six wounded trees 24h after wounding and pooled prior to analysis as described above. Samples from three biological replicates were analyzed by 2-DE. Each sample was analyzed by three separate gels in order to account for technical variation. The gels were stained, scanned, and analyzed with Progenesis Workstation version 2003.02 (Nonlinear Dynamics, Newcastle upon Tyne, UK). Comparative gel analysis was performed as described in Lippert et al. (2007). Background subtraction was performed using the "mode of nonspot" method and normalization was based on the "total spot volume" method. Two-factor ANOVA tests were used to compare protein expression levels between the control and wounded samples and protein spots with a significant ($P<0.05$) induction were excised from 2-DE gels and sequenced as described above.

Carbohydrate analysis of phloem exudate

Sucrose, glucose, and fructose concentrations were quantified as previously described (Campbell et al., 1999). Briefly, the sugars were enzymatically converted to glucose 6-phosphate and carbohydrate concentrations were quantified colormetrically by the production of NADH from NAD in conversion of glucose 6-phosphate to gluconate 6-phosphate using glucose 6-phosphate dehydrogenase (Sigma). For the sucrose assay, the following modifications were made: 0.01% (w/v) bovine serum albumin was added to the reagent mixture and a total of 50 U of sucrose phosphorylase (Sigma) was used for the reactions for one 96-well microtitre plate. [Assays carried out by Dr. Lynn Yip]

2.3 Results

Collection of Populus phloem exudate

To facilitate the analysis of poplar phloem proteins, we tested several phloem collection methods using an EDTA exudation buffer (King and Zeevaart, 1974; Marentes and Grusak, 1998; Hoffmann-Benning et al., 2002). The samples were checked for the absence of RuBisCO, an approach used previously to verify the purity of phloem samples (Schobert et al., 1995; Marentes and Grusak, 1998; Giavalisco et al., 2006). On western blots, commercial antibodies directed against the RuBisCO large subunit recognized the corresponding poplar polypeptide, which was highly abundant in leaf and green stem tissue (i.e., 'bark') of poplar saplings at approximately 55kDa (Fig. 2-1b). We note that at the stage used for experiments, these saplings have not yet developed true bark with cork cambium, but have soft green photosynthetic stems; for simplicity, we refer to the green

outer tissues as 'bark'. A second protein, approximately 33 kDa, was also detected in both tissues after extended periods of staining, but appears to be due to non-specific binding.

We first tested the method of Herschbach et al. (1998) for collection of poplar phloem proteins with an EDTA solution. This method resulted in a relatively high protein yield; however RuBisCO was detected in these samples (data not shown); therefore we modified the technique to limit contamination from surrounding cells. Significant amounts of protein were recovered in exudate collected using this modified method (Fig. 2-1a). If stems were incubated without EDTA or in the presence of excess $CaCl_2$, protein yields were strongly reduced, confirming that this method promotes exudation of phloem (data not shown). No RuBisCO could be detected in these exudate preparations, indicating that contamination by other proteins is low (Fig. 2-1b).

To confirm the purity of the exudate obtained, sucrose, glucose, and fructose concentrations of the exudate were measured (Campbell et al., 1999). Sucrose was the most abundant carbohydrate, consisting of 90% of the total sugars measured (Fig. 2-1c). The average ratio of sucrose: glucose and sucrose: fructose was 12 and 30, respectively. Since sucrose is typically the primary form of sugar transported in phloem, these ratios confirm that our samples contain predominantly phloem sap. To further corroborate this, we also used 2-DE to compare protein profiles from bark tissue and phloem exudate (Fig. 2-2a). In this analysis, approximately 60 phloem proteins were visible, but up to 100 proteins could be detected when larger quantities of protein were loaded. The phloem sample contained many proteins in the pH 3-7 range that were smaller than 70 kDa (Fig. 2-2b). Phloem exudate exhibited a protein profile clearly distinct from that of bark tissue, suggesting that contamination from surrounding tissues was minimal.

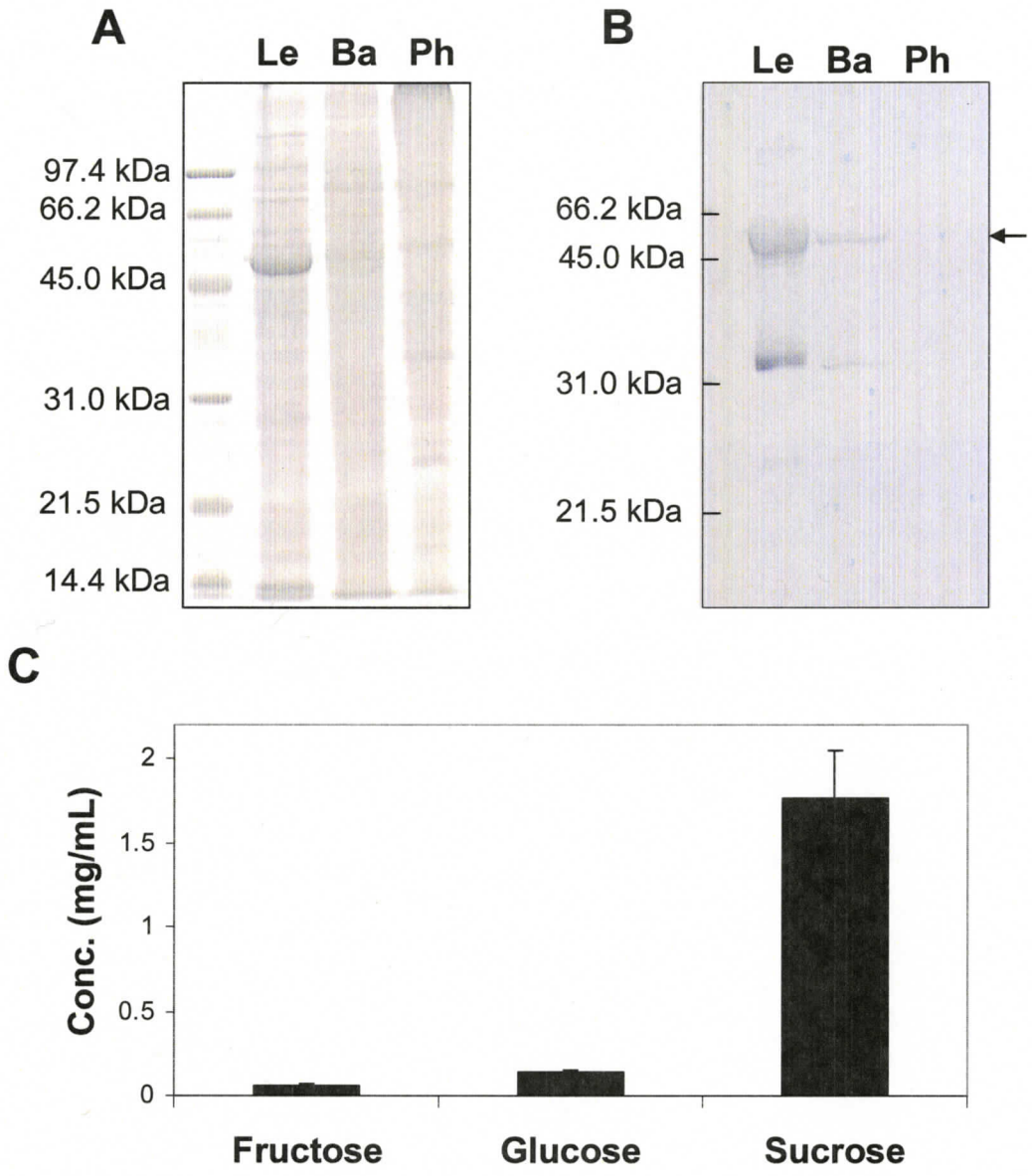


Figure 2-1. Analysis of hybrid poplar phloem exudate.

Comparison of poplar phloem exudate (Ph) to leaf (Le) and bark (Ba) proteins using (A) Coomassie stained SDS-PAGE gel and (B) western blot with antibodies to RuBisCO (large subunit). The arrow indicates the position of the RuBisCO protein. For both Coomassie and western blot analyses, 10 μg of protein were loaded. C. Analysis of sugars in phloem exudate as determined by enzymatic assays. Means and standard errors are shown (n=6).

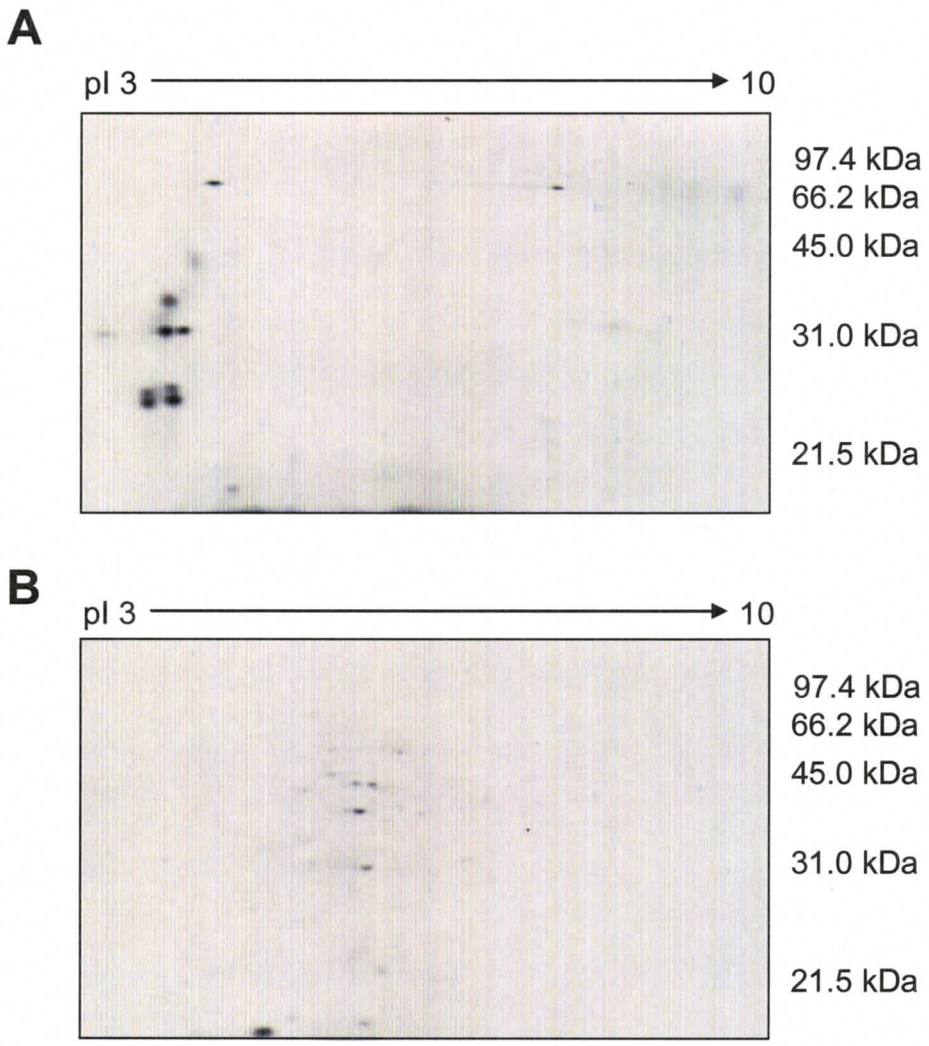


Figure 2-2. Comparison of proteins from phloem exudate (A) and bark (B).
Proteins (4 μg) were separated by 2-DE and stained with Sypro Ruby.

Characterization of the Populus phloem exudate proteome

To identify proteins in poplar phloem exudate, polypeptides were first separated by 1D SDS-PAGE prior to analysis with LC-MS/MS. We obtained positive identification for 48 proteins (Table 2-1). Comparisons with the *Arabidopsis* genome using BLAST identified *Arabidopsis* homologs for most of the poplar proteins. Based on the *Arabidopsis* gene ontology annotations, the phloem proteins were grouped into one of six putative functional categories comprising energy/metabolism, amino acid metabolism, signaling, stress response, structure, and unknown (Table 2-1). In a few cases, genes known to be upregulated in poplar in response to environmental stresses were classified with the stress response proteins (Major and Constabel, 2006; Ralph et al., 2006). Genes within the stress category accounted for almost one-half of all proteins identified, and included both oxidative stress response and defense-related proteins (Table 2-1). Antioxidant proteins identified include glutathione S-transferase and 1,4-benzoquinone reductase-like proteins. Several additional proteins with putative functional annotation as oxidative stress proteins were also identified; however these hits were based on single peptide matches and although the peptide scores were greater than 52 indicating homology, they were excluded from the final list (Table 2-1). These proteins included superoxide dismutase and peroxidase. A single peptide also matched a peroxiredoxin. Defense-related proteins identified include a Kunitz trypsin inhibitor (TI), polyphenol oxidase, and a pop3-/SP1-like protein, as well as the pathogenesis-related (PR) proteins chitinase, ß-1,3-glucanase and thaumatin-like protein (TLP). Thus, both herbivore and pathogen defense proteins appeared to be present in the poplar phloem exudate.

Table 2-1. Poplar phloem exudate proteins identified by LC-MS/MS.

Identification	JGI Protein ID[a]	Theoretical MW	Theoretical pI	Mascot Score	No. of Matched Peptides[b]	Function[c]	SignalP[d]
dTDP-glucose 4-6-dehydratase/UDP-glucuronic acid decarboxylase	832538	39104	6.13	105	2	ME	
Enolase	575698	47924	5.67	159	3	ME	
Glyceraldehyde 3-phosphate dehydrogenase	728998	36724	6.36	219	5	ME	
Glycosyltransferase	826368	92152	6.18	598	14	ME	
Glucose-6-phosphate 1-dehydrogenase	736146	59156	6.29	112	2	ME	
Malate dehydrogenase	564942	35714	6.13	219	4	ME	
Triosephosphate isomerase	724697	27397	6.51	210	3	ME	
Carbonic anhydrase	417951	25504	9.4	190	3	ME	
Ribulose bisphosphate carboxylase, large chain	279035	21534	6.47	88	2	ME	
Lipolytic enzyme, G-D-S-L	758353	32714	8.82	124	3	ME	y
Lipolytic enzyme, G-D-S-L	580490	42332	5.09	137	4	ME	y
Phospholipase D1	829577	91816	5.03	134	2	ME	
Glycine/serine hydroxymethyltransferase	829808	51907	7.26	138	3	AA	
S-adenosyl-L-homocysteine hydrolase	597072	53195	5.75	289	6	AA	
Vitamin-B12 independent methionine synthase	679841	84592	6.27	791	17	AA	
Annexin	818283	36018	6.2	152	3	SI	
Annexin	643752	35965	6.17	102	3	SI	
Cyclophilin type peptidyl-prolyl cis-trans isomerase	813818	18094	8.72	216	4	SI	
Multifunctional chaperone (14-3-3 family)	711617	29378	4.68	173	4	SI	
1,4-benzoquinone reductase-like	726993	21671	5.97	94	3	SR	
Glutathione S-transferase	243514	24195	5.73	207	5	SR	
Bark storage protein B precursor (nucleoside phosphorylase)	740814	35860	6.24	69	1	SR	y
Vegetative storage protein win4.5	596927	35306	5.62	61	1	SR	y
Nucleoside phosphatase (apyrase 2)	573883	49760	5.31	116	2	SR	y
Polyphenol oxidase (PPO5)	674097	64884	6.31	79	2	SR	y
Pop3-/SP1-like	822230	12976	5.73	72	2	SR	
Kunitz trypsin inhibitor, miraculin-like	763975	20273	5.01	183	3	SR	

(Table continues on following page.)

Table 2-1. Continued

Identification	JGI Protein ID[a]	Theoretical MW	Theoretical pI	Mascot Score	No. of Matched Peptides[b]	Function[c]	SignalP[d]
Caffeic acid 3-O-methyltransferase	834247	39732	5.48	150	5	SR	
Phenylcoumaran benzylic ether reductase 1	830063	33977	5.52	206	3	SR	
β-1,3-glucanase (glycoside hydrolase, family 17)	652688	31638	5.52	545	17	SR	y
β-1,3-glucanase (glycoside hydrolase, family 17)	751998	36564	4.78	228	5	SR	y
β-glucosidase (glycoside hydrolase, family 1)	294573	55629	5.85	94	2	SR	
Chitinase (glycosyl hydrolase family 18)	717157	40233	7.82	100	2	SR	
Chitinase (glycosyl hydrolase family 18)	746640	30847	4.42	164	5	SR	y
Chitinase (glycoside hydrolase, family 19)	826290	29337	7.91	408	6	SR	y
Chitin-binding (hevein-like)	571046	20789	8.37	298	4	SR	y
Thaumatin, pathogenesis-related, PR-5	828883	25856	4.9	62	1	SR	y
Thaumatin, pathogenesis-related, PR-5	669475	24731	7.89	332	5	SR	y
Defense-related protein containing SCP/PR-1 domain	595857	17257	8.54	302	5	SR	y
Molecular chaperone (HSP90)	582316	79950	4.94	135	2	SR	
Plant basic secretory protein (BSP) family protein	549955	24995	7.18	162	2	SR	y
Tubulin alpha-2 chain	831020	49777	4.92	154	4	ST	
Beta tubulin	203493	49713	4.78	126	2	ST	
Beta-Ig-H3/fasciclin	728480	28208	7.95	122	3	ST	
Fasciclin-like arabinogalactan-protein 10	730906	43247	5.37	120	2	ST	y
Unknown (desiccation-associated)	642406	32846	4.83	181	2	UN	y
Unknown (DUF1278)	672963	11071	9.03	215	4	UN	
Unknown (DUF26)	718495	26450	5.78	216	4	UN	

[a] Protein identification number was identified using the DOE Joint Genome Institute *P. trichocarpa* genome v1.1.

[b] The peptide sequences used for identification, in addition to their molecular weight, charge, and specific Mascot score can be accessed in Supplementary Table 2-1 found in Appendix 1.

[c] Abbreviations used for assigned functions are ME, metabolism; AA, amino acid metabolism; SI, signalling; SR stress response; ST, structure, UN, unknown.

[d] Proteins predicted to have an N-terminal signal peptide are designated with a y. SignalP probability scores can be accessed in Supplementary Table 2-1.

Energy/metabolism and protein/amino acid metabolism accounted for the majority of the remaining proteins identified. Several proteins involved in glycolysis were identified in poplar phloem exudate. Enzymes involved in amino acid metabolism including glycine/serine hydroxymethyltransferase, S-adenosyl-L-homocysteine hydrolase, and methionine synthase were also identified. Overall, our proteomic experiments led to the identification of a broad range of proteins, with strong representation by defense proteins.

Differential expression of poplar phloem proteins in response to wounding

To characterize systemic changes in the phloem protein profile triggered by simulated leaf herbivory or wounding, comparative 2-DE was used to compare protein profiles of phloem exudate collected from stem sections of control and wounded plants 24 h after wounding leaf margins. Visual inspection of the gels and mean spot volumes indicated that the wound treatment did not lead to highly dramatic changes in the overall protein profile. However, a systematic statistical analysis of all replicates determined that two proteins were consistently and significantly ($P < 0.05$) upregulated in response to the wound treatment (Fig. 2-3; Supplementary Fig. 2-1 and Supplementary Table 2-2). Both protein spots were excised and subjected to LC-MS/MS, which identified them as pop3 protein and thaumatin-like protein (TLP) (Table 2-2). Pop3 has no similarity to proteins of known function, but it is orthologous (99.1% amino acid identity) to the *P. tremula* stable protein 1 (SP1). TLPs are similar to thaumatin, a sweet-tasting protein first

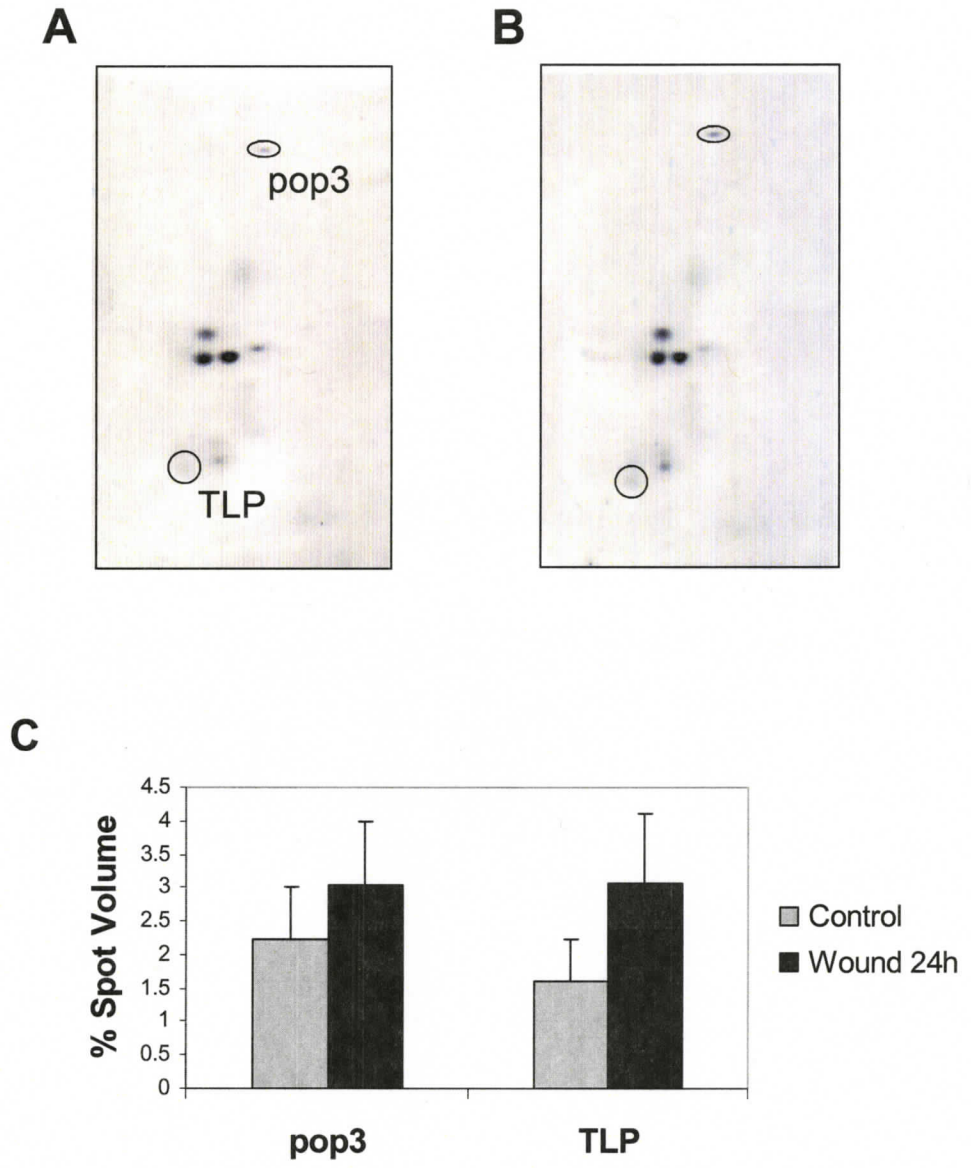


Figure 2-3. Changes in hybrid poplar phloem exudate protein profiles following simulated insect herbivory by leaf wounding.

The acidic portions of representative 2-DE gels used to compare protein abundance in phloem exudate from control (A) and wounded trees (B) are shown. Circles indicate the two proteins, pop3 and a thaumatin-like protein (TLP), that were significantly upregulated in response to wounding ($P<0.05$). (C) Mean spot volumes and standard errors for differentially regulated proteins, as calculated from three independent experiments, each with three technical replicates.

Table 2-2. Identification of two poplar phloem exudate proteins induced 24h after wounding.

Protein	JGI Protein ID	Theoretical MW	pI	Mascot Score	No. of Matched Peptides	Sequence Matches	Charge
pop3	723969	12425	4.87	230	3	GTDLGMESAELNR	2
						GYTHAFESTFESK	2
						SGLQEYLDSAALAAFAEGFLPTLSQR	3
TLP	828883	25856	4.9	68	2	QQCPQAYSYAYDDK	2
						SSTFTCPSGGNYLITFCP	2

identified in the African shrub *Thaumatococcus danielli,* and also belong to the PR-5 family of pathogenesis-related proteins (Shatters et al., 2006; van Loon et al., 2006). In response to wounding, pop3/SP1 and TLP increased in the phloem exudate by 1.3-fold and 1.9-fold, respectively (Fig. 2-3c). Both proteins have an acidic isoelectric point (Fig. 2-3a,b). TLP migrated at 23 kDa (Fig. 2-3a,b), similar to its predicted MW of 24 kDa (Table 2-2). Pop3/SP1 had an observed MW of approximately 100 kDa (Fig. 2-3a,b), but its predicted MW is only 12.4 kDa (Table 2-2).

We could not rule out minor contamination of phloem exudate by some apoplast proteins. Therefore, to independently confirm the presence of a PR protein within sieve elements, we carried out immunolocalization with an antibody raised against the TLP identified by our wounding experiment. In immunofluorescence experiments using poplar stem cross-sections, TLP antiserum specifically labelled organelle-like structures within large phloem cells, identified as sieve elements (Fig. 2-4f). When sections were treated with preimmune serum or no primary antibody, no fluorescence could be detected, verifying that this signal is specific for TLP (Fig. 2-4d, e). These experiments confirm the sieve element origin of TLP in our exudate samples.

2.4 Discussion

Phloem exudates contain hundreds of proteins that are now being identified using sensitive mass spectrometry techniques (Walz et al., 2002; Barnes et al., 2004; Walz et al., 2004; Giavalisco et al., 2006). Here we describe the first analysis of phloem exudate proteins in hybrid poplar. Of more than 100 proteins spots visualized by 2-DE, we identified 48 proteins, including several defense- and pathogenesis-related proteins. The

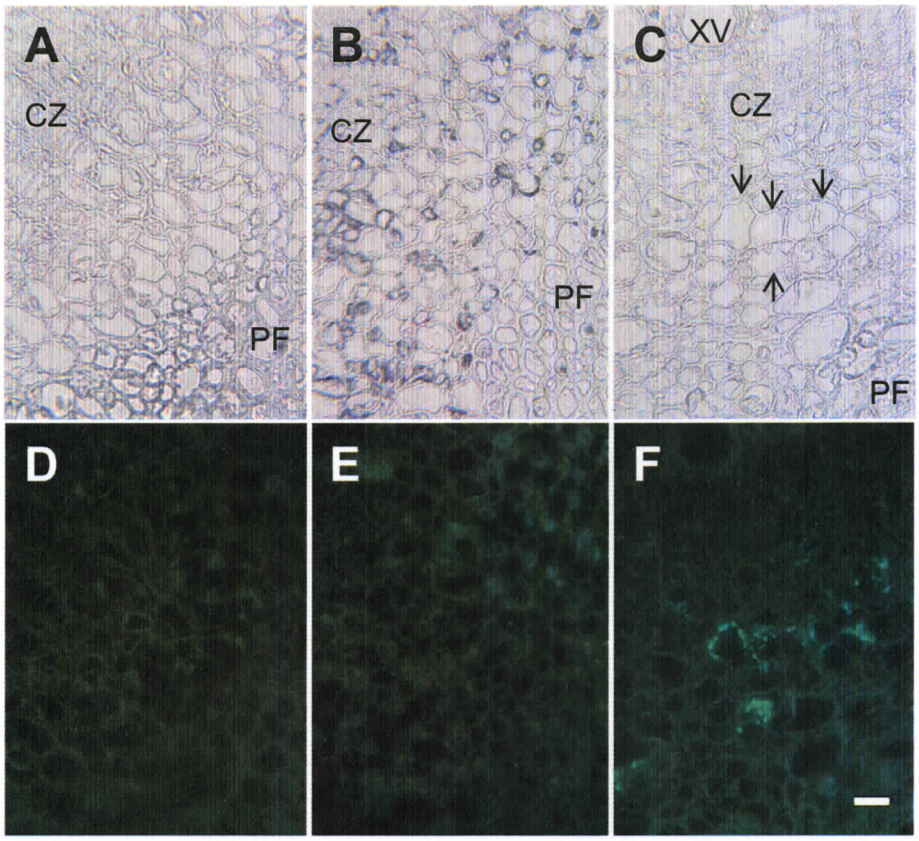


Figure 2-4. Localization of thaumatin-like proteins (TLP) in phloem cells of stem cross sections.

Panels A-C show bright field images and panels D-F the corresponding immunofluorescent images of stem cross sections. Panel D was treated without a primary antibody and panel E was treated with preimmune serum, while panel F was treated with TLP antiserum. CZ, cambial zone; PF, phloem fibres; and XV, xylem vessels. Arrows indicate labelled sieve elements. Scale bar = 20 μm.

majority of phloem protein spots did not change in abundance 24 h after leaf wounding; however, two proteins, pop3/SP1 and TLP, were significantly upregulated by wounding.

Poplar phloem exudate can be collected using EDTA

We tested and optimized a phloem collection technique that allowed us to collect phloem exudate with minimal contamination by surrounding tissues, as visualized by the absence of RuBisCO on western blots. Nevertheless, the RuBisCO large subunit was detected by LC-MS/MS, a much more sensitive technique. Previously, the RuBisCO large and small subunits were also identified in *B. napus* phloem sap collected via small incisions into the stem, despite controls which indicated minimal contamination from surrounding tissues (Giavalisco et al., 2006). Our controls also indicate that we have collected phloem exudate of high purity based on the following observations. First, 2-DE indicates that the pattern of phloem proteins is distinct compared to soluble bark proteins, suggesting very little protein contamination. Second, many proteins identified in our phloem exudate are known to be present in phloem of other plants. Third, sucrose was the most abundant sugar analyzed in our exudate, present at 12- and 30-fold higher levels than the hexoses glucose and fructose, which is consistent with the role of sucrose as the primary sugar transported by the phloem (Oparka and Cruz, 2000). The ratio of sucrose: glucose and fructose in our exudate preparations was four times greater than that reported by Herschbach et al. (1998), though lower than reported for exudate collected from *B. napus* (Giavalisco et al., 2006). In general, sucrose accounts for 95% of the total sugars in phloem, consistent with our data. Our immunolocalization of TLP further confirms that the phloem exudate collected contains proteins that are localized to phloem cells.

Therefore, we conclude that EDTA provides a useful means to study phloem exudate in poplar, yielding suitable protein quantities needed for comparative proteomic studies.

The poplar phloem proteome contains many stress response proteins

The suite of proteins identified in our analysis of poplar phloem exudate is generally consistent with reports from other species. For example, enzymes for amino acid metabolism and glycolysis are also found in phloem sap of various annual plant species, and signaling proteins such as the annexins identified here were identified in the phloem sap of *B. napus* and *R. communis* (Kennecke et al., 1971; Geigenberger et al., 1993; Schobert et al., 1995; Schobert et al., 1998; Hoffmann-Benning et al., 2002; Barnes et al., 2004; Walz et al., 2004; Giavalisco et al., 2006). Likewise, the structural proteins, α- and ß-tubulin were localized in sieve elements of horse-chestnut and cotton (Thorsch and Esau, 1982; Chaffey et al., 2000). The presence of antioxidant proteins is also consistently noted in studies of phloem (Walz et al., 2002; Aki et al., 2008). A major component of the stress response proteins in our poplar phloem samples was found to be the herbivore and pathogen defense-related category. Protease inhibitors (PIs) are well-known anti-herbivore defense proteins (Ryan, 1990), and PIs predicted to inhibit serine, cysteine, and aspartic proteases were previously identified in phloem of other plant species (Dannenhoffer et al., 2001; Walz et al., 2004; Giavalisco et al., 2006). In poplar phloem, we detected a miraculin-like Kunitz trypsin inhibitor by LC-MS/MS, and the TI3 trypsin inhibitor using western analysis (data not shown). PIs reduce insect performance by inhibition of gut proteases, and their role against leaf-eating insects has been well established; in addition, they appear to affect phloem-feeding insects (Kehr, 2006). We

also identified other known herbivore defense-associated proteins in poplar phloem exudate, including PPO, a pop3/SP1-like protein, and vegetative storage proteins, which are all consistently upregulated in poplar leaves by simulated herbivory (Davis et al., 1993; Major and Constabel, 2006; Ralph et al., 2006). Our analysis thus extends the list of defense-related proteins described from phloem; however, the function of such proteins in defense against phloem-feeding insects remains to be tested directly. To date, only in one report has the protective effect of a plant protein (a lectin) against a phloem-feeding insect has been directly demonstrated using transgenic plants (Gatehouse et al., 1996). Interestingly, a hevein-like lectin was recently found to be induced in wheat by a sucking insect, the Hessian fly (Giovanini et al., 2007). We note that a protein with strong similarity to this lectin was present in poplar phloem.

Several pathogenesis-related (PR) proteins, including TLPs, chitinases, and ß-1,3-glucanases were identified in poplar phloem exudate. These types of proteins have predicted N-terminal signal peptides and they are commonly detected in apoplastic fluids (Buhtz et al., 2004; Kehr et al., 2005; Alvarez et al., 2006), however, similar proteins have also been detected in phloem sap. For example, a ß-glucosidase was detected in phloem sap of *C. maxima* (Walz et al., 2004) and several PR proteins, including a β-1,3-glucanase, were recently identified in *O. sativa* phloem sap (Aki et al., 2008). Since we were not able to completely rule out contamination from cell wall proteins using our collection method and controls, we independently confirmed the sieve element localization of one of the PR proteins, TLP, using immunofluorescence. This experiment clearly demonstrated the intracellular localization of TLP within phloem cells, in

particular in sieve elements. Several recent reports also localized TLP or PR-5 transcripts to phloem tissue using in situ hybridization (Hong et al., 2004; Kavroulakis et al., 2006).

It is interesting to note that many PR proteins including TLPs, ß-1,3-glucanases, and chitinases are strongly induced in leaf tissue infested by various phloem-feeding insects (Moran and Thompson, 2001; Gao et al., 2007; Kempema et al., 2007; Zarate et al., 2007). It is not known whether the PR proteins induced by phloem-feeders accumulate in phloem, but one can speculate that PRs in phloem may help defend against such pest insects. This idea is indirectly supported from experiments in tomato, where disruption of salicylic acid signaling, important for induction of PR proteins, resulted in enhanced performance of aphids (Moran and Thompson, 2001).

Leaf wounding alters the abundance of specific phloem proteins

In our experiments, the global protein profile did not differ strongly between control and wounded trees 24 h after wounding; we only detected two proteins that were differentially regulated in response to wounding. By contrast, in whole leaf extracts, up to 90 protein spots can be easily found to be differentially regulated by wounding or elicitor treatment (Giri et al., 2006). The process of collecting poplar phloem exudate involves wounding stem tissue and therefore, it is possible that more phloem proteins are differentially regulated, but their induction is masked by our collection technique. As described above, a large number of stress-inducible proteins were identified in poplar phloem exudate collected from healthy, unstressed poplar saplings; however, these types of proteins have previously been shown to be constitutively expressed in phloem tissues (Le Hir et al., 2008). In addition, temporal analyses indicates that the transcription of

defense genes such as trypsin inhibitors and chitinases does not occur until two to six hours after wounding (Haruta et al., 2001; Lawrence et al., 2006) and the transcription of PR proteins, including PR-1 and β-1,3-glucanases, does not proceed until 20 to 40 hours after pathogen infection (Friedrich et al., 1991; Ward et al., 1991; Vega-Sanchez et al., 2005).

The two poplar phloem exudate proteins that were consistently and significantly induced in poplar phloem by leaf wounding were identified as pop3/SP1 and thaumatin-like protein (TLP). Neither protein was previously reported from phloem sap of other plants, but both are known to be upregulated by biotic and abiotic stress (Wang et al., 2002; Gu et al., 2004; Major and Constabel, 2006; Ralph et al., 2006; van Loon et al., 2006). While the *P. tremula* SP1 gene was originally identified as a boiling stable protein, it was also shown to be induced in response to salt, cold, drought, and freezing stress treatments as well as herbivory (Wang et al., 2002; Gu et al., 2004; Renaut et al., 2005; Ferreira et al., 2006; Ralph et al., 2006). The predicted protein does not have an obvious biochemical function, but *P. tremula* SP1 has been shown to assemble into a dodecamer that may function as a protein chaperone (Wang et al., 2002; Wang et al., 2003; Dgany et al., 2004). Likewise, the pop3/SP1 protein identified in this study also appears to form a large multimeric complex, based on its migration in 2-DE, but a possible function will require further testing. Interestingly, chaperones such as heat shock proteins are prominent in phloem sap and they may function in facilitating protein folding as well as protein transport (Schobert et al., 1998; Walz et al., 2004; Giovanini et al., 2007). By contrast, TLPs are often studied in the context of plant pathogen defense based on their similarity to PR-5 proteins. However, TLP genes can also respond to other

stresses including wounding and infestation by phloem feeding insects (Frendo et al., 1992; Piggott et al., 2004; Gao et al., 2007; Kempema et al., 2007; Zarate et al., 2007). TLPs can exhibit a wide range of biochemical activities including ß-1,3-glucan binding and/or endo-ß-1,3-glucanase activity, and ß-amylase and trypsin inhibitor activity (Trudel et al., 1998; Grenier et al., 1999; Schimoler-O'Rourke et al., 2001; Menu-Bouaouiche et al., 2003). Further research is needed to determine if this phloem-inducible poplar TLP exhibits any such biological activity or deters insect feeding.

To summarize, we have undertaken the first characterization of phloem proteins from a perennial plant. At least two of these proteins, pop3/SP1 and TLP, were induced by leaf wounding, suggesting that they may have a role in plant defense. Further research is needed to determine how these proteins function, where they originate, and if they are mobile throughout the plant.

3 Expression of thaumatin-like proteins in phloem tissues of *Populus trichocarpa* x *P. deltoides*

3.1 Introduction

In angiosperms, the sieve elements involved in long-distance phloem transport are sieve-tube elements (Evert, 2006). Sieve-tube elements undergo selective autophagy and at maturity, they no longer have a vacuole, nucleus, or functioning ribosomes, but they do retain their plasma membrane, mitochondria, endoplasmic reticulum, and specialized plastids (Evert, 2006). At maturity, a large number of proteins, most likely originating from closely associated companion cells, are also present in sieve elements (Lough and Lucas, 2006). Analysis of *Populus trichocarpa* x *P. deltoides* phloem exudate resulted in the identification of two thaumatin-like proteins (TLPs) (Chapter 2), proteins with sequence similarity to the sweet tasting thaumatin protein discovered in *Thaumatococcus daniellii* Benth (Selitrennikoff, 2001). One of these proteins, PtTLP1, was induced 24 hours after leaves were mechanically wounded (Chapter 2). Even though TLPs and closely related genes have been detected in phloem tissues (Hong et al., 2004; Kavroulakis et al., 2006), these proteins have not been previously reported in phloem sap. The presence of TLPs in poplar sieve-tube elements, however, was confirmed with immunofluorescence using an antibody produced against recombinant PtTLP1 (Chapter 2). When stem cross sections were labelled with PtTLP1 antiserum, the fluorescent label in sieve elements was punctate and appeared to be associated with organelle-like structures, but the nature of these structures was not identified (Chapter 2).

Although some TLPs are constitutively expressed in flowers and fruits (Fils-Lycaon et al., 1996; Clendennen and May, 1997; Salzman et al., 1998; Sassa and Hirano,

1998; Ruperti et al., 2002; Van Damme et al., 2002; Gao et al., 2005), many TLPs are known to be expressed in response pathogen infection and are therefore considered pathogenesis-related (PR) proteins (van Loon et al., 2006). Currently PR proteins are classified into 17 families and TLPs belong to the PR-5 protein family (van Loon et al., 2006), which also includes permeatins that are found in cereal seeds and osmotins, a protein first identified in salt-adapted tobacco cells (Anzlovar and Dermastia, 2003). Typically, osmotins are basic PR-5 proteins that are targeted to vacuoles whereas other PR-5 proteins, including TLPs, are acidic, extracellular proteins (Anzlovar and Dermastia, 2003). Even with these differences, the sequence and structure of all PR-5 proteins are homologous to thaumatin (Anzlovar and Dermastia, 2003) and it has been suggested that these proteins have not diverged enough to warrant the use of different nomenclature (Shatters et al., 2006).

Proteins of the PR-5 family have had many types of biological and biochemical functions attributed to them. Some TLPs are known to exhibit antifungal activity (Grenier et al., 1999; Van Damme et al., 2002) and they function by permeabalizing fungal membranes possibly by interacting with water or ion channels of fungal hyphae (Batalia et al., 1996). Various TLPs have also been shown to bind (Trudel et al., 1998; Osmond et al., 2001) and some even hydrolyze β-1,3-glucans (Grenier et al., 1999; Menu-Bouaouiche et al., 2003) whereas other have been shown to inhibit xylanases (Fierens et al., 2007) or α-amylases (Schimoler-O'Rourke et al., 2001). Currently, there is very little known about the activity of PtTLP1 or any other TLP in poplar, even though these genes are overrepresented in the *P. trichocarpa* genome as compared to the *Arabidopsis* genome (Tuskan et al., 2006). A total of 57 genes are annotated as TLPs in the poplar

genome and this large number may indicate that these proteins have evolved multiple functions within poplars.

Here, we use the PtTLP1 antiserum, which appears to recognize multiple TLP isoforms, to further characterize the expression of TLPs in poplar sapling tissues. We use immunofluorescence to expand our knowledge concerning the cellular expression of TLPs within stem segments and compare this pattern to other tissues. We also use immunogold labelling and electron microscopy to characterize their subcellular localization.

3.2 Materials and Methods

Plant materials

Poplar hybrid H11-11 (*Populus trichocarpa* x *P. deltoides*) saplings obtained from the University of Washington/Washington State University Poplar Research Program. These saplings were propagated and grown in 2.5 L pots as described previously (Major and Constabel, 2006). All plants were maintained at the University of Victoria, in the Bev Glover greenhouse with a 16 h photoperiod. The temperature was maintained at 25 °C during the day and 18 °C at night. Plants were watered daily with 0.1 g/L 20-20-20 PlantProd fertilizer (Plant Products, Brampton, ON, Canada).

Tissue sampling and protein extraction

Samples were collected from the apex and petioles and blades corresponding to leaf plastochron index (LPI) 3-5, 9-11, 15-17 (Larson and Isebrands, 1971). Midveins were dissected and analyzed for only LPI 9-11 and LPI 15-17. Insufficient quantities of

protein were extracted from midveins of LPI 3-5 for western analysis. Bark and wood samples were collected from LPI 9-11. As described in Chapter 2, bark samples consisted of green stem tissue peeled from the wood or lignified secondary xylem. Root samples were collected from areas of new root growth (young root) and areas near the base of the stem (old root). Plant tissue was frozen and ground in liquid N_2 in a pre-cooled mortar and pestle. Total soluble proteins were extracted using sodium phosphate buffer (100 mM NaPO4, pH 7.0 containing 0.1% (v/v) Triton X-100, and 2% (v/v) β-mercaptoethanol) as previously described (Haruta et al., 2001). Phloem exudate was collected as previously described in Chapter 2. Briefly, stem sections, approximately 10 cm in length, were excised between LPI 5-16 and a section of bark was removed from the cut end exposing wood which was then sealed using a 90% lanolin, 10% paraffin wax mixture. The cut and sealed end was washed, blotted dry, and then placed into exudation buffer (50 mM Tris, pH 8.0, 2 mM EDTA) for 2 h at room temperature. Tissue survey samples and phloem exudate samples were acetone precipitated with 2 vol. cold acetone for at least one hour at -20 °C. After 15 min of centrifugation at 16,000 x g at 4 °C, the resulting pellet was dried and resuspended in Laemmli buffer (Laemmli, 1970) and quantified using a RC DC protein assay (BioRad, Hercules, CA, USA).

Gel electrophoresis and immunoblotting

Two-dimensional gel electrophoresis (2-DE) was completed as described in Chapter 2. Briefly, 50 µg of phloem exudate protein diluted with rehydration buffer (8 M urea, 32 mM CHAPS, 20 mM DTT, and 0.5% (v/v) IPG buffer, pH 3-10 (Amersham Biosciences, Piscataway, NJ, USA) was used to rehydrate a 7 cm Immobiline Drystrip

(Amersham) with a linear pH range of 3-10. Isoelectric focusing of the sample was carried out for a total of 5.7 kVh with an IPGphor apparatus (Amersham). Proteins were then reduced and alkylated prior to separation with SDS-PAGE. Proteins were visualized using Bio-Safe Coomassie stain (BioRad) and gels were scanned using the ProXPRESS Proteomic Imaging System (Perkin Elmer, Waltham, MA, USA).

For immunoblotting, proteins (10 μg) were first separated with 15% polyacrylamide gels as described above and then electro-transferred to PVDF membrane (Pierce, Brockville, ON, Canada). To detect TLP, blots were labelled with a polyclonal antibody generated against recombinant PtTLP1 protein (Chapter 2) and then visualized with an alkaline phosphatase conjugated secondary antibody using 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP) and nitro-blue tetrazolium chloride (NBT) as substrates. The specificity of the PtTLP1 antiserum was tested by labelling blots with preimmune serum or pre-adsorbed PtTLP1 antiserum. To pre-adsorb antiserum, 30 μL of PtTLP1 antiserum (total protein 1200 μg) was diluted 1:5 with deionized H_2O and then incubated with an equal quantity of denatured recombinant PtTLP1 protein for 24 h at 4 °C with constant shaking.

Light microscopy and immunofluorescence

Petioles and midveins corresponding to LPI 3 and LPI 11 and stem segments near LPI 11 were excised, then fixed and embedded in BMM resin mixture (4 parts n-butyl methacrylate to 1 part methyl methacrylate) as previously described (Chaffey et al., 1997). Stem cross sections (6 μm thick) were cut and mounted on positive-charged glass slides. Prior to labelling, the plastic was partially removed by soaking the sections in

acetone for 20 min. The sections were labelled with a PtTLP1 antiserum using standard protocols (Harlow and Lane, 1999) and the antibody was detected with an Alexafluor 568 goat anti-rabbit antibody (Molecular Probes, Eugene, OR, USA). Controls included omitting the primary antibody or incubating sections with preimmune serum. Fluorescent labelling was visualized using a Zeiss Universal epifluorescence microscope equipped with a digital camera and a fluorescein isothiocyanate filter (excitation at 495 nm and emission at 519 nm). [Samples for immunolocalization were prepared by Brent Gowen]

Electron microscopy

Stem sections near LPI 11 were collected and immediately placed in freshly prepared fixative buffer (sodium cacodylate buffer containing 3% (v/v) glutaraldehyde and 3% (v/v) formaldehyde (Karnovsky, 1965)) for 1.5 h at room temperature with constant rotation. Samples were washed thoroughly with fixative buffer prior to dehydration with a graded ethanol series (50, 70, 80, 90, 95, and 2 X 100% (v/v) ethanol for 10 min each). After dehydration, samples were infiltrated with a mixture of ethanol and LR White (Electron Microscopy Sciences, Hatfield, PA, USA) at ratios of 1:1 and 1:3 for 30 min each, followed by two incubations with pure LR White for 30 min each. Samples were placed into individual gelatin capsules and then polymerized at 50 °C for 24 h. Ultrathin sections were cut and mounted on formvar/ carbon coated nickel grids (Electron Microscopy Sciences). Sections were first pre-treated with saturated sodium metaperiodate for 10 min and then incubated with PBS/ ovalbumin (1% (w/v) ovalbumin (Sigma-Aldrich, Oakville, ONT, Canada) in PBS) for 10 min prior to labelling with PtTLP1 antiserum for 1 h. Sections were then washed three times, 5 min each, with PBS/

ovalbumin and incubated with the secondary antibody (12 nm Colloidal Gold-AffiniPure Goat Anti-rabbit IgG (H+L), Jackson Immunoreseach Inc., West Grove, PA, USA) for 1 h. Sections were washed three times as described above and then washed four times, 1 min each, with deionized H_2O. Prior to viewing, sections were stained with 5% (w/v) uranyl acetate in 50% (v/v) ethanol for 10 min, washed four times with deionized H_2O, incubated with 5% (w/v) lead citrate for 1 min and washed an additional four times with deionized H_2O. Sections were viewed with a Hitachi H-7000 transmission electron microscope and images were taken with a digital camera (Advanced Microscopy Technique Corp., Danvers, MA, USA). To verify the specificity of PtTLP1 antiserum, sections were also incubated with PBS/ ovalbumin (no antibody), preimmune serum, or pre-adsorbed PtTLP1 antiserum (see gel electrophoresis and immunoblotting in the materials and methods above). For the cytosol, and various components of sieve elements, phloem parenchyma cells, and phloem fibres, the density of gold labelling for each treatment was calculated by averaging the number of gold particles/ μm^2 from five replicate areas in three separate stem tissue samples.

3.3 Results

Characterization of PtTLP1 protein

The PtTLP1 protein was compared with translated plant nucleotide sequences in the National Center for Biotechnology Information database using tblastn. The top non-poplar matches were CHTL, Mal d 2, PsTL1, and PpAz8, TLPs identified in cherry fruit, apple fruit, pear styles, and peach flowers, respectively. These TLPs share greater than 60% amino acid identity with PtTLP1. Similar to these proteins, PtTLP1 has a predicted signal peptide targeting it to the secretory pathway. Analysis by SignalP (Nielsen et al.,

1997; Bendtsen et al., 2004), a program that predicts N-terminal signal peptide cleavage sites, suggests that PtTLP1 has a predicted cleavage site between amino acids 26 and 27. The predicted molecular weight of the mature, processed protein is therefore approximately 23 kDa, which is consistent with the molecular weight observed in previous work (Chapter 2).

TLPs can be classified into two categories, large or small. PtTLP1 is an example of a large TLP based on the conservation of 16 cysteine residues that are found in other large TLPs (Fig. 3-1). In addition to conserved cysteines, all PR-5 proteins have an acidic cleft formed by five negatively charged amino acids (Batalia et al., 1996; Koiwa et al., 1999; Min et al., 2004; Leone et al., 2006), which are conserved in PtTLP1 (Fig. 3-1).

Western blot analysis of TLPs in poplar

The PtTLP1 antiserum detected a 23 kDa as well as a 31 kDa protein in bark and phloem exudate (Fig.3- 2a). Based on its predicted molecular weight (23 kDa), the 23 kDa band corresponds to PtTLP1. This 23 kDa band is also consistent with the migration of the protein spot from which PtTLP1 was sequenced when proteins were separated by 2-DE (Chapter 2). The antiserum also strongly detected a 31 kDa protein in both samples (Fig. 3-2a). No proteins were labelled when western blots were incubated with preimmune serum or pre-adsorbed PtTLP1 antiserum, indicating that the PtTLP1 antiserum is specific to both proteins (Fig. 3-2b, c).

Based on these observations, it appears that the PtTLP1 antiserum cross-reacts with one or more TLP isoforms. We observed that in addition to PtTLP1, two other TLPs (JGI protein IDs 669475 and 583370) were identified in poplar phloem exudate. TLP

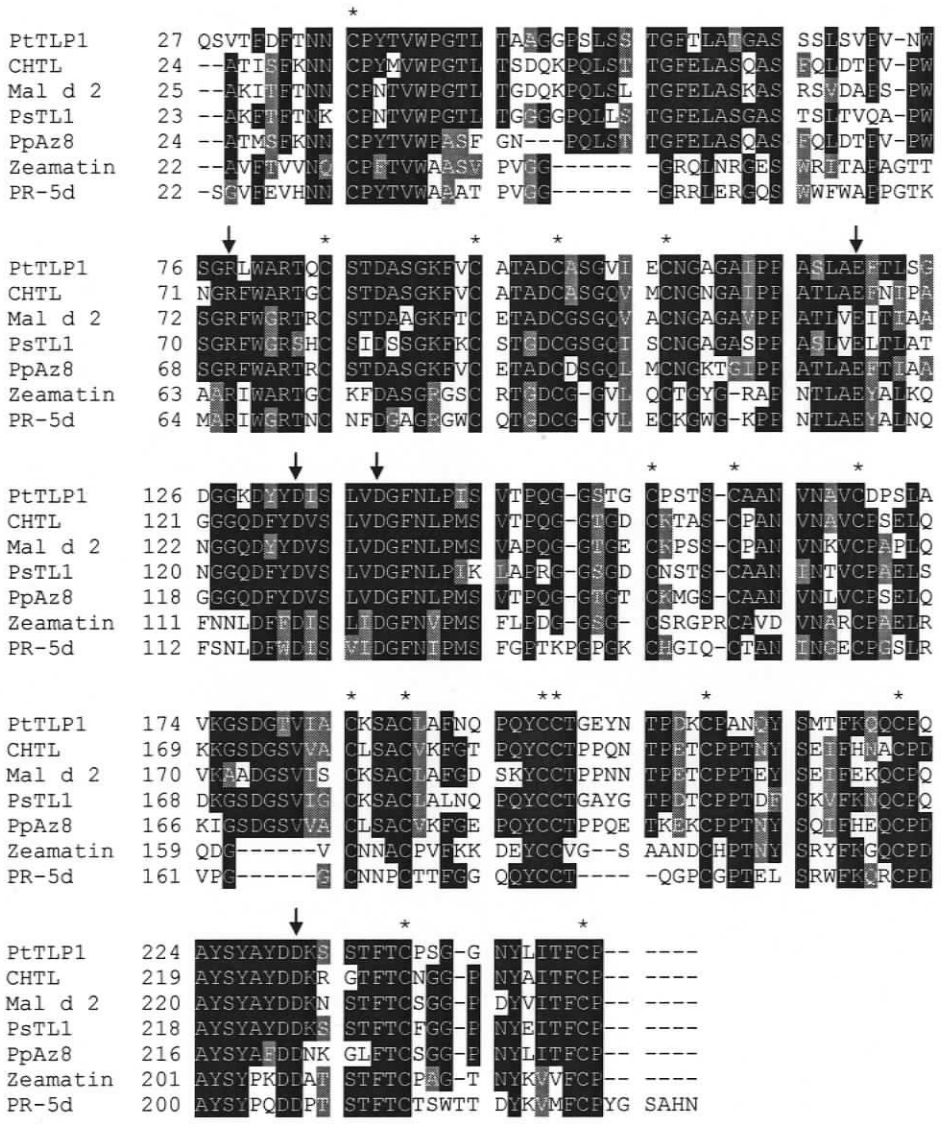


Figure 3-1. Alignment of the processed PtTLP1 protein sequence (JGI protein ID 828883) with the processed sequences of previously characterized TLPs.

Sequences were retrieved from the National Center for Biotechnology Information with the following accession numbers: CHTL (AAB38064), Mal d 2 (AAX19846), PsTL1 (BAA28872), PpAz8 (AAM00215), Zeamatin (P33679), and PR-5d (P258871). Black boxes indicate areas of identity and grey boxes represent amino acid changes that are conserved. Asterisks (*) indicate the position of conserved cysteine residues and arrows mark the position of the residues that contribute to the formation of an acidic cleft in TLPs.

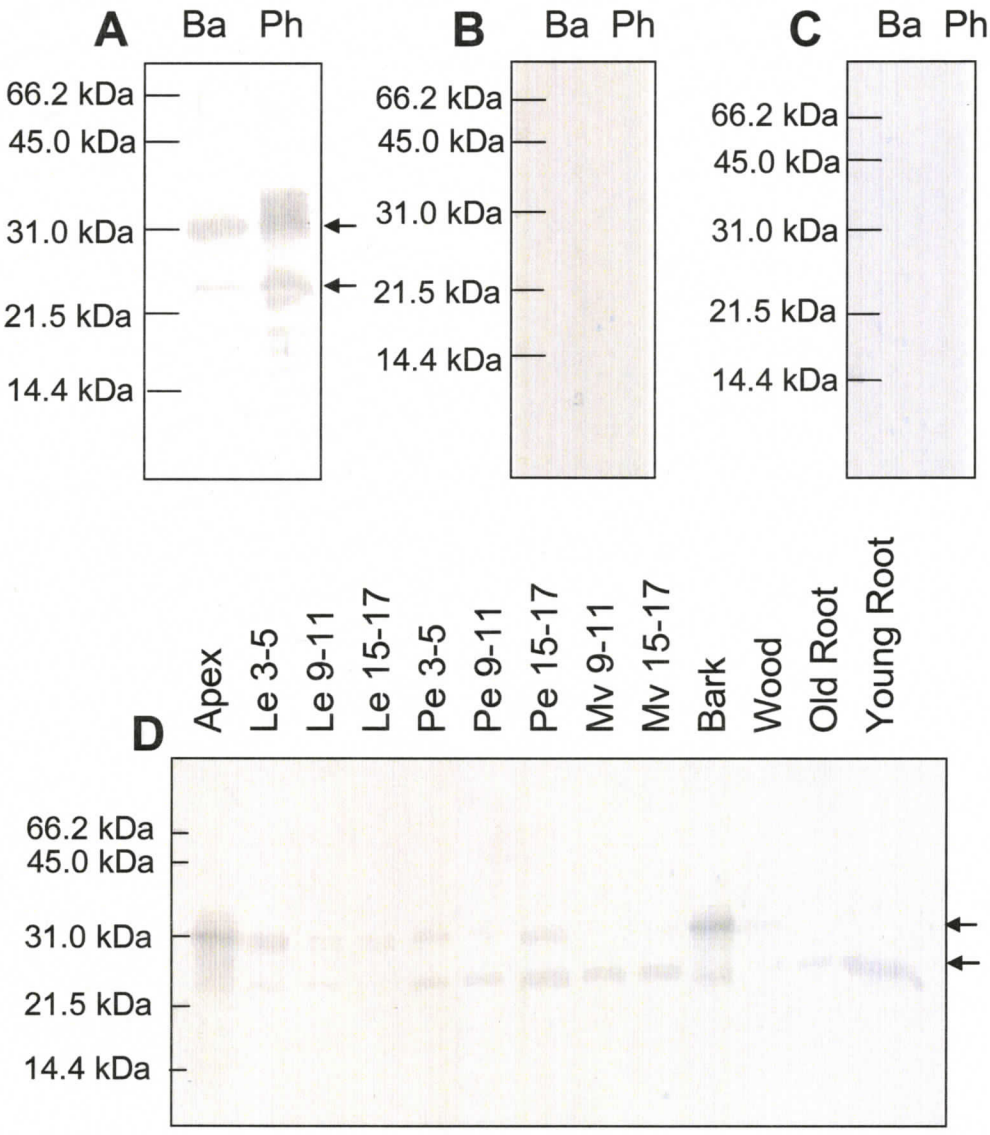


Figure 3-2. Expression PtTLP1 and closely related TLPs in *P. trichocarpa* x *P. deltoides* saplings.

Western blots of bark (Ba) and phloem exudate (Ph) proteins labelled with A) PtTLP1 antiserum, B) preimmune serum and C) pre-adsorbed PtTLP1 antiserum. D) Survey of poplar tissues by western blot labelled with PtTLP1 antiserum. Samples were collected from the apex, leaves (Le) and petioles (Pe) from positions LPI 3-5, 9-11, and 15-17, midveins (MV) from LPI 9-11 and 15-17, bark and wood collected from LPI 9-11 and old and young root samples. Arrows indicated the position of the 23 kDa and 31 kDa proteins detected by anti-PtTLP1.

669475 (Chapter 2) is only 49% identical to PtTLP1, but TLP 583370 (Supplementary Table 3-1) is 88% identical to PtTLP1 and it has a predicted molecular weight of 31 kDa (Fig. 3-3), which is consistent with the molecular weight of the second protein recognized by the PtTLP1 antiserum in poplar phloem exudate. TLP 583370 is one of ten TLPs in the *P. trichocarpa* genome that share greater than 80% sequence identity with PtTLP1 at the amino acid level. Each of these proteins have several long stretches of amino acids (>10 amino acids) that are identical to PtTLP1 (data not shown).

Both the 23 kDa and 31 kDa TLPs exhibit very different tissue-specific expression patterns as visualized with western blots (Fig. 3-2). The 23 kDa protein was most abundant in petioles, midveins, bark, and roots; all tissues with prominent vascular systems. However, it was only weakly detected in the shoot apex and young leaves (Fig. 3-2d). In contrast, the 31 kDa TLP was very abundant in the shoot apex and young leaves. It was also strongly labelled in bark samples. The 31 kDa protein was not labelled in midveins or roots and was only faintly detected in young and old petioles, tissues where the 23 kDa band was clearly visible. Little or no label was detected for either protein in older leaf and wood samples.

Immunolocalization of TLPs

Preliminary observations suggest that PtTLP1 is located inside sieve elements of stem sections (Chapter 2). Further, the fluorescent labelling observed with the PtTLP1 antiserum was punctate, suggesting that the PtTLP1 protein was associated with organelle-like structures. In young and old petiole and midvein sections a similar punctate labelling pattern was detected (Fig. 3-4b, d, f, h). In stem and young petiole

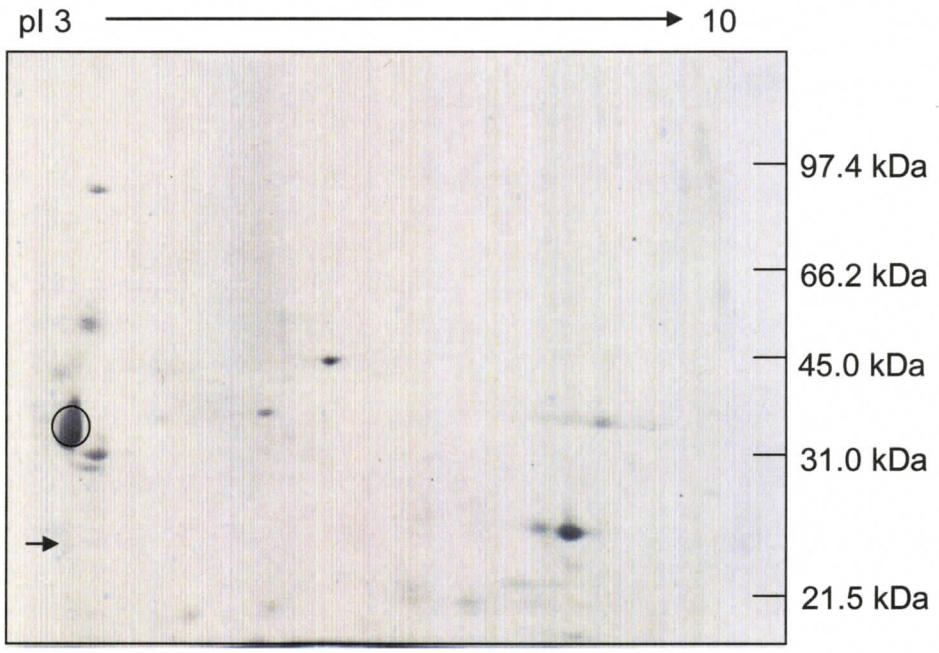


Figure 3-3. 2-DE of poplar phloem exudate proteins (50µg) to show relative positions of PtTLP1 and second TLP (JGI protein ID 583370) sequenced from phloem exudate.

The 31 kDa TLP protein (JGI protein ID 583370) sequenced by LC-MS/MS is circled and the position of PtTLP1 (see Chapter 2) is indicated by an arrow (→).

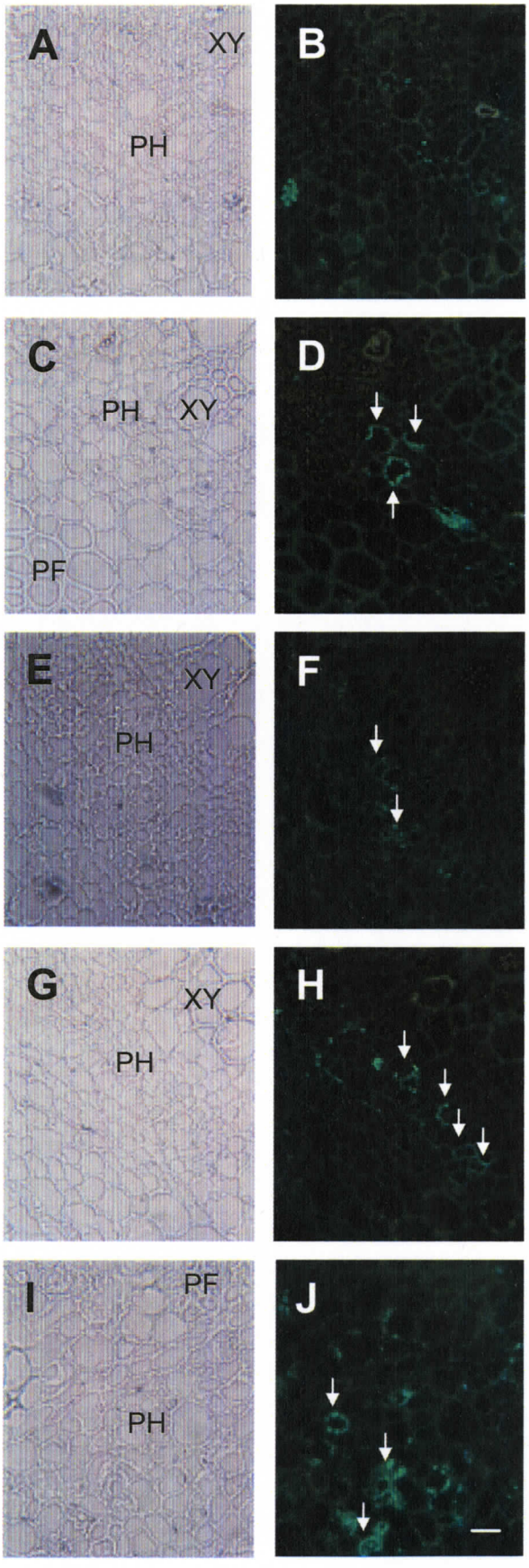
cross sections, intracellular labelling was also detected in a second phloem cell type; phloem parenchyma cells typically adjacent to phloem fibres (Fig. 3-4b, j). Again, this immunofluorescence labelling was punctate suggesting that the PtTLP1 antiserum was labelling another intracellular, organelle-like structure. In addition, labelling was also detected in cell walls surrounding sieve elements specifically in stem, and old petiole and midvein sections (Fig. 3-4d, h, j). In contrast, no fluorescence could be detected when sections were labelled with PBS/ ovalbumin (no primary antibody) or with TLP preimmune serum (Chapter 2, data not shown).

The subcellular localization of PtTLP1 was characterized with immunogold labelling and electron microscopy. The PtTLP1 antiserum clearly labelled plastids inside phloem parenchyma cells in sections of mature stem (Fig. 3-5b). These plastids had very large starch granules and label was associated with these and the regions around the plastid membranes (Table 3-1). The PtTLP1 antiserum also labelled starch granules in sieve elements of mature stem (Fig. 3-6b).

In addition, immunogold labelling was also detected in the cell walls, but only in the cell walls of sieve elements and phloem fibres (Fig. 3-6c, 3-7b). In sieve elements, 84% of the gold label was found in the inner cell wall and very strong labelling was also observed in the callose deposited in the sieve pores of sieve plates (Fig. 3-6b). In adjacent phloem fibres, labelling was concentrated in the pectin-rich cell corners found between neighbouring fibres (Fig. 3-7b), but nowhere else.

To determine the specificity of immunogold labelling with PtTLP1 antiserum, stem sections were labelled with PBS/ ovalbumin (no primary antibody), preimmune serum, or pre-adsorbed PtTLP1 antiserum (data not shown). No label was detected when

Figure 3-4. Immunofluorescence of TLPs in the phloem of diverse poplar tissues.
A), C), E), G), and I) show bright field images and B), D), F), H), and J) show the corresponding immunofluorscent images of young petiole (LPI 3), old petiole (LPI 11), young midvein (LPI 3), old midvein (LPI 11), and stem (LPI 11) cross-sections labelled with PtTLP1 antiserum, respectively. PH, phloem; PF, phloem fibres; and XV, xylem vessels. Arrows indicate labelled sieve elements. Scale bar = 20 μm.


A
XY
PH
B
C
PH
XY
PF
D
E
XY
PH
F
G
XY
PH
H
I
PF
PH
J

sections were labelled with PBS/ ovalbumin, but a low level of gold was detected with the preimmune serum and pre-adsorbed PtTLP1 antiserum in the cell wall and starch granules of sieve elements, the starch containing plastids in phloem parenchyma, and the pectin-rich cell wall joints of phloem fibres. In addition, these two controls strongly labelled callose found in the sieve plates of sieve elements. On western blots, however, neither the preimmune nor pre-adsorbed antisera detected any proteins (Fig. 3-2b, c); these results suggest that components of the sera may be non-specifically labelling these cellular components. We therefore calculated the average density of labelling for regions of interest in stem sections treated with PtTLP1 antiserum, preimmune serum, and pre-adsorbed PtTLP1 antiserum. The density of labelling was also calculated for the cytosol as a background control for each treatment (Table 3-1). With the exception of the cytosol, the density of labelling was consistently and significantly (Student's t-test, $P<0.05$) higher in sections labelled with PtTLP1 antiserum compared to either control treatments. This suggests that despite some non-specific background, the PtTLP1 antiserum is specifically labelling TLPs in the cell wall and starch granules of sieve elements, the starch containing plastids in phloem parenchyma, and the pectin-rich cell wall joints of phloem fibres. For callose of sieve elements, however, the density of non-specific labelling of callose was particularly high in both control treatments. Thus in this region, the density of gold label in the callose region was only 1.6 times greater when treated with PtTLP1 antiserum as compared to the preimmune serum (Table 3-1).

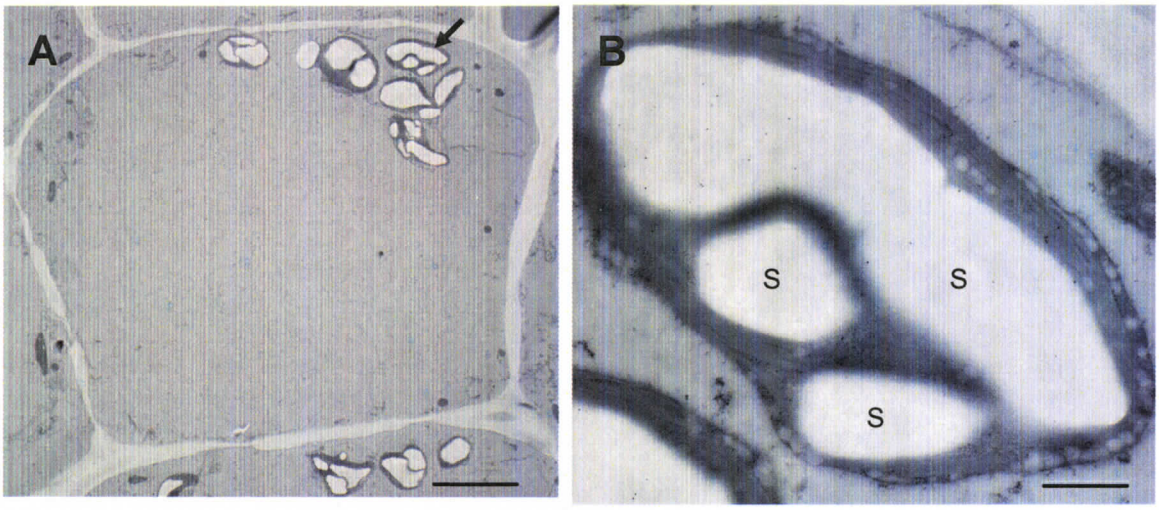


Figure 3-5. Immunogold labelling of a phloem parenchyma cell in mature stem with PtTLP1 antiserum.

A) Low magnification image of a phloem parenchyma cell. Scale bar = 5 μm. Arrow indicates position of magnified B) starch (S)-containing plastid. Scale bar = 500 nm.

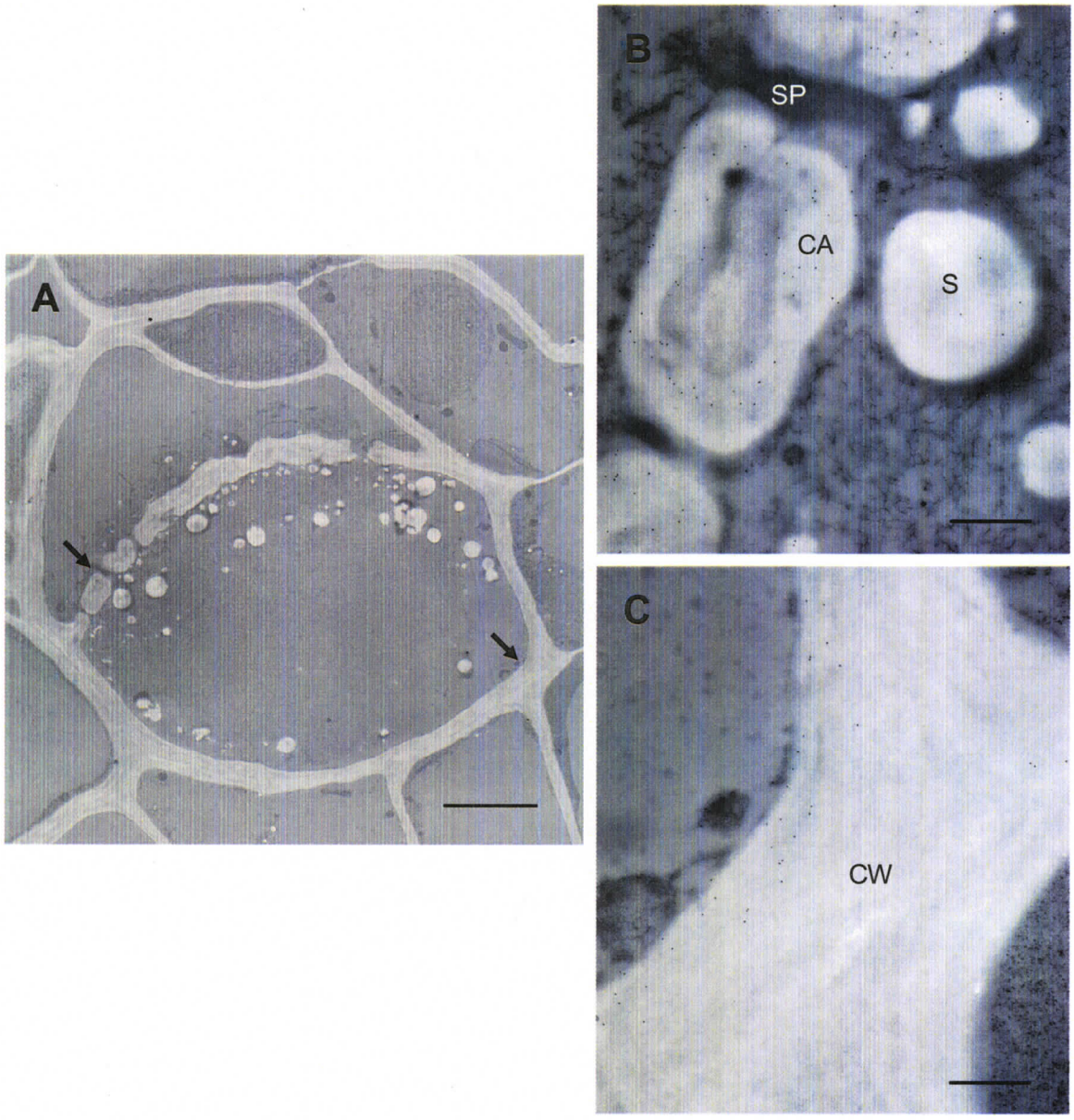


Figure 3-6. Immunogold labelling of a sieve element in mature stem with PtTLP1 antiserum.

A) Low magnification image of sieve element. Scale bar = 5 µm. Arrows indicate position of magnified B) sieve plate and C) cell wall. CA, callose; CW, cell wall; S, starch; SP, sieve pore. Scale bars = 500 nm.

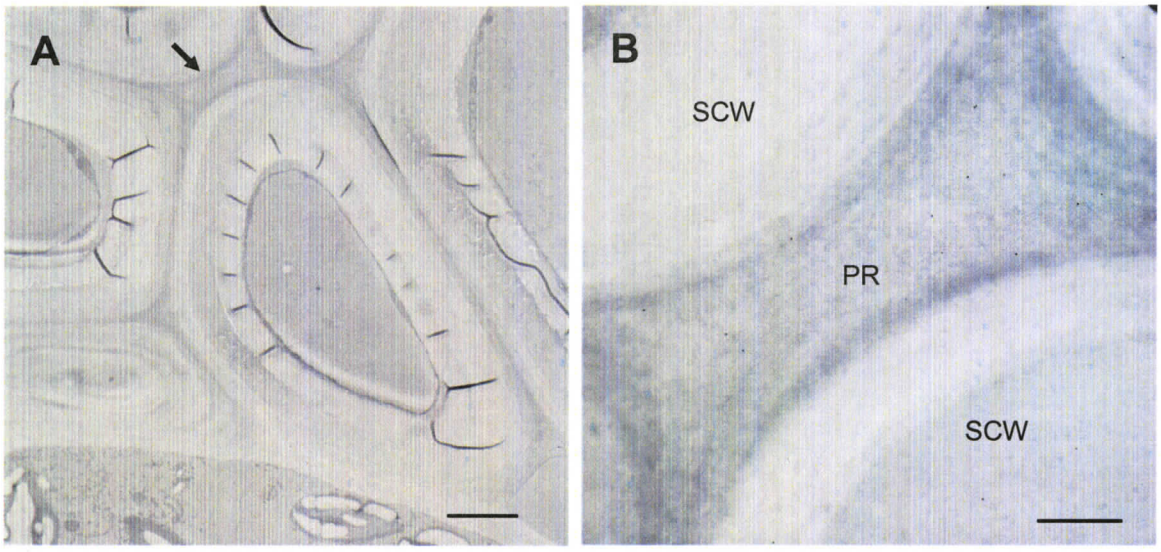


Figure 3-7. Immunogold labelling of a phloem fibre in mature stem with PtTLP1 antiserum. A) Low magnification image of phloem fibres with thick secondary cell walls. Scale bar = 2 μm. Arrow indicates position of magnified B) cell wall joint. PR, pectin-rich region; SCW, secondary cell wall. Scale bar = 500 nm.

Table 3-1. Average density[a] of preimmune serum, pre-adsorbed PtTLP1 antiserum, and PtTLP1 antiserum labelling.

	Preimmune Serum	Pre-adsorbed PtTLP1 antiserum	PtTLP1 antiserum
Sieve Element			
Cell Wall	0.498 (± 0.120)	1.372 (± 0.177)	5.345 (± 0.685)
Callose	22.369 (± 2.379)	9.992 (± 0.926)	35.100 (± 3.466)
Starch Granules	1.765 (± 0.361)	1.132 (± 0.403)	11.620 (± 1.920)
Phloem Parenchyma			
Plastids containing Starch	1.463 (± 0.176)	0.887 (± 0.220)	4.461 (± 0.993)
Phloem Fibres			
Pectin-rich region	0.743 (± 0.130)	0.350 (±0.105)	2.413 (± 0.316)
Cytosol	0.141 (±0.038)	0.205 (±0.094)	0.239 (±0.081)

[a] Average number of gold particles/ μm^2 ± SE, n=15

3.4 Discussion

In the current report, we used the PtTLP1 antiserum for immunogold labelling and electron microscopy to confirm the presence of TLPs inside sieve elements and determine that the organelle-like structures observed by immunofluorescence are starch granules. Furthermore, the PtTLP1 antiserum labelled proteins within starch-containing plastids in phloem parenchyma cells. In addition to intracellular labelling, the PtTLP1 antiserum detected proteins in the cell walls of sieve elements and phloem fibres, and labelled the callose deposited around sieve pores of sieve elements. With the exception of older leaves and wood, TLPs appear to be constitutively expressed throughout poplar saplings, but the cellular expression of TLPs is not consistent among all tissues.

Multiplicity and diversity of TLPs in poplar

Prior to using the PtTLP1 antiserum to study the expression of TLPs, we used western blots to characterize the labelling observed with the antibody and verify its specificity for TLPs. In phloem exudate and bark proteins, two samples in which PtTLP1 had been previously detected (Chapter 2), the PtTLP1 antibody labelled two proteins, a 23 kDa protein and a 31 kDa protein. This labelling appeared to be specific to TLPs given that both controls, the preimmune serum and pre-adsorbed PtTLP1 antiserum, did not label any proteins on westerns. The size of the 23 kDa band is consistent with the predicted and observed molecular weight of the processed, mature PtTLP1 protein indicating that this protein is most likely PtTLP1 (Chapter 2). The 31 kDa protein, however, is likely to be a second closely related TLP that is cross-reacting with the PtTLP1 antiserum. In addition to the 23 kDa PtTLP1 protein that was originally detected in poplar phloem exudate, a second TLP (JGI protein ID 583370) migrating at 31 kDa

was also identified in phloem exudate. Since this protein is 88% identical to PtTLP1 it is conceivable that it corresponds to the 31 kDa protein recognized by the PtTLP1 antiserum on western blots. The difference in expression profiles of the 23 kDa and 31 kDa proteins provide further evidence that the PtTLP1 antiserum recognized two distinct proteins.

Localization of TLPs in phloem tissue

When stem cross sections were labelled with PtTLP1 antiserum, fluorescent label appeared to be specifically associated with organelle-like structures inside sieve elements. Using immunogold labelling and electron microscopy, these organelle-like structures inside sieve elements were identified as starch granules, apparently remnants of specialized starch containing plastids present in sieve elements (van Bel et al., 2002; Wise, 2006). There are two types of plastids in sieve elements, S- type plastids that only contain starch and P- type plastids that contain proteins and in some cases starch. The function of these plastids is currently unknown (Wise, 2006), but it is known that they are very sensitive to wounding and they rupture even upon inserting microcapillaries into sieve tubes (van Bel et al., 2002). When these plastids rupture, the plastid membrane remains attached to the plasma membrane and its contents are released (Knoblauch and van Bel, 1998). It is therefore possible that the starch granules were released during the fixation process. Interestingly, other PR-5 proteins that have also been localized to plastids (Jeun and Buchenauer, 2001; Liljeroth et al., 2005; Sturrock et al., 2007). The PtTLP1 antiserum also labelled intact plastids in phloem parenchyma cells.

The plastids labelled in phloem parenchyma cells are starch-containing plastids. The immunogold labelling was detected in the membrane region and it was also associated with the starch. The labelling of starch in sieve elements and phloem parenchyma cells is similar to a previous study in which a tomato osmotin (AP24) was specifically localized to starch granules found in chloroplasts (Jeun and Buchenauer, 2001).

TLPs have also been previously localized to cell walls and extracellular vesicles (Dore et al., 1991; Melchers et al., 1993; Sturrock et al., 2007) and they have been detected in apoplastic fluids (Rep et al., 2002; Buhtz et al., 2004; Piggott et al., 2004; Alvarez et al., 2006; O'Leary et al., 2007). Consistent with these reports, the PtTLP1 antiserum labelled proteins in the apoplast, specifically in pectin-rich cell wall areas of phloem fibres and in cell walls and the callose surrounding sieve pores of sieve elements.

Expression of TLPs in poplar

Fluorescent label was detected in sieve elements for all of the tissues analyzed, but labelling of phloem parenchyma cells was limited to young petioles (LPI 3) and bark (LPI 11). A similar trend was observed for the expression of the two proteins detected by PtTLP1 antiserum with western blots. Thus, the 31 kDa protein was also strongly detected in the same tissues in which phloem parenchyma cells were labelled, in particular bark (LPI 11) and petioles. The 23 kDa protein had a more ubiquitous expression pattern and was detected in every tissue analyzed with the exception of old leaves and wood. Generally, the 23 kDa protein was most abundant in tissues that have a well-developed vascular system, indicating that this protein may be correlated to the

PtTLP1 labelling detected in cell walls and callose surrounding sieve pores of sieve elements. By contrast, the 31 kDa TLP may be specific to starch and starch containing plastids found in parenchyma cells. This hypothesis is further supported by the observation that PtTLP1 is only weakly detected in the shoot apex, a tissue that does not have extensive vascular development. The 31 kDa protein, however, was very abundant in the apex and when sections of shoot apex were labelled with PtTLP1 antiserum, intracellular organelle-like structures, similar to those observed in phloem parenchyma, were strongly labelled (data not shown). Overall there is a good correlation between protein abundance observed in western blots and immunolocalization patterns.

Potential functions of constitutively expressed TLPs

Currently, the precise biochemical and physiological functions of TLPs in poplar is unknown. Many TLPs identified in other plant species appear to be involved in pathogen defense (van Loon et al., 2006) and the transcription of PtTLP1 is increased in poplar leaves in response to *Melampsora* infection (Miranda et al., 2007). PtTLP1, however, is also expressed in the absence of pathogen infection suggesting it has additional functions within poplar. Interestingly, compared to other plant protein sequences, it was most similar to developmentally regulated TLPs. For example, PsTL1 which accumulates in styles of Japanese pear (Sassa and Hirano, 1998), is 67% identical to PtTLP1; CHTL, a TLP identified in ripe cherry fruit (Fils-Lycaon et al., 1996) is 61% identical; and Mal d 2, a known allergen found in apple fruit (Krebitz et al., 2003), and PpAz8, a TLP expressed in peach flowers (Ruperti et al., 2002), are both 60% identical to PtTLP1. Of these proteins, the activities of CHTL and Mal d 2 have been characterized

and only Mal d 2 is known to exhibit antifungal activity. CHTL has no known antifungal activity, but it has been shown to bind and hydrolyze β-1,3-glucans. Interestingly, callose is a β-1,3-glucan and PtTLP1 appears to be localized to callose. The site of β-1,3-glucan binding appears to be the acidic cleft that is formed by five charged amino acids between domains I and II of TLP proteins (Osmond et al., 2001; Menu-Bouaouiche et al., 2003; Reiss et al., 2006). These amino acids are conserved in PtTLP1 and it is therefore possible that PtTLP1 binds and possibly hydrolyzes callose.

The localization of TLPs to sieve elements may also indicate a possible role in defense against insects, specifically phloem-feeding insects. TLPs and other PR-5 genes are known to be induced in leaves in response to various phloem-feeding insect (Gao et al., 2007; Kempema et al., 2007; Zarate et al., 2007) and the PtTLP1 protein was induced in phloem exudate in response to simulated insect feeding (Chapter 2). The exact function of TLPs in insect defense is currently unknown, but they may negatively impact insect feeding or they may be involved in signaling. For example, the PR-5 protein, zeamatin, is capable of inhibiting α-amylase activity extracted from larval insect guts (Schimoler-O'Rourke et al., 2001), but TLPs have also been shown to hydrolyze β-1,3-glucans into smaller β-1,3-glucans that can elicit a defense response in tobacco cell cultures (Klarzynski et al., 2000). Therefore, in order to fully comprehend the role of phloem exudate TLPs in plant defense, activity assays are needed to determine their biochemical function.

4 Two poplar pop3 proteins identified in poplar phloem exudate exhibit distinct expression patterns and biochemical properties

4.1 Introduction

Two proteins with sequence similarity to pop3, a functionally uncharacterized, stress-inducible protein, were identified in *Populus trichocarpa* x *P. deltoides* phloem exudate collected from stems (Chapter 2). These two proteins were designated pop3.1 and pop3.4. Based on comparative two-dimensional gel electrophoresis (2-DE), one of these proteins is induced in phloem exudate 24 h after leaf wounding (Chapter 2). Prior to this study, pop3 proteins had been detected in extracted leaf proteins (Wang et al., 2002; Renaut et al., 2005; Ferreira et al., 2006; Lee et al., 2008), but they have not been previously reported in phloem exudate.

Pop3 was originally identified in *P. trichocarpa* x *P. deltoides* and it is orthologous (>99% identical) to stable protein 1 (SP1), a protein identified by immunoscreening a *P. tremula* expression library with antibodies produced against a 66 kDa protein named BspA (Wang et al., 2002). BspA is a boiling stable proteins that is expressed in water stressed *P. tremula* leaves. Its N-terminal sequence is similar to wheat germins (Pelah et al., 1995), but its biochemical function is not known. Surprisingly, there were no sequence similarities between the N-terminal protein sequences of BspA and SP1 (Wang et al., 2002), but SP1 is very similar to BspA in that it remains soluble after boiling and is induced in response to environmental stresses (Pelah et al., 1995; Wang et al., 2002). In addition to water stress, pop3/ SP1 transcripts are induced in response to cold, heat, and salt stress (Wang et al., 2002; Gu et al., 2004; Renaut et al., 2005; Ferreira et al., 2006). They are also known to be induced during insect and

pathogen defense responses (Lawrence et al., 2006; Major and Constabel, 2006; Ralph et al., 2006; Park et al., 2007; Lee et al., 2008).

Pop3/ SP1 has no homology to proteins with known functions, therefore the structure of *P. tremula* SP1 was analyzed by X-ray crystallography in an effort to identify a biochemical function (Wang et al., 2003; Dgany et al., 2004). It was observed that SP1 has a ferredoxin-like fold consisting of three α-helices and a β-sheet (Dgany et al., 2004). Two SP1 monomers dimerize via the β-sheet to form a dimeric alpha-beta barrel (Dgany et al., 2004). This dimeric alpha-beta barrel (Dabb) domain (pfam O7876) is conserved in a diverse range of proteins that also includes two pop3/ SP1 proteins from *Arabidopsis* (Cornilescu et al., 2004; Lytle et al., 2004), a hypothetical protein (TT1380) from *Thermus thermophilus* (Wada et al., 2004), a monooxygenase (ActVA-Orf6) from *Streptomyces coelicolor* (Sciara et al., 2003), and a rhamnose mutarotase from *Escherichia coli* (Ryu et al., 2005). These proteins may share a conserved domain, but it does not appear that they have a conserved function. For example, the amino acids in the active site of ActVA-Orf6 are not conserved in pop3/ SP1 proteins suggesting that these proteins do not function as monooxygenases (Cornilescu et al., 2004; Dgany et al., 2004; Lytle et al., 2004). In fact, it has been suggested that the dimer is not the biologically active unit of SP1 (Dgany et al., 2004). Structural analysis of SP1 indicates that six SP1 dimers stack together to form a dodecamer that may function as a protein chaperone (Wang et al., 2003; Dgany et al., 2004). The *P. tremula* SP1 dodecamer is a highly stable structure. It does not dissociate when treated with detergents such as sodium dodecyl sulfate (SDS) (Wang et al., 2002). It is also resistant to organic solvents including

methanol and hexane, and it does not degrade when treated with proteases (Wang et al., 2006).

The pop3/ SP1 protein family is much larger in poplar than in *Oryza sativa* and *Arabidopsis*. In poplar, nine other proteins have the conserved Dabb domain and share sequence similarity to pop3/SP1 whereas only two pop3/SP1 proteins were identified in the rice and *Arabidopsis* genomes. Currently, we know that several of these poplar pop3/ SP1 proteins are induced in response to environmental stresses (Lawrence et al., 2006; Major and Constabel, 2006; Ralph et al., 2006) and that two of these proteins, pop3.1 and pop3.4, are present in phloem exudate (Chapter 2). In the current study, we produced antibodies against pop3.1 and pop3.4. We analyzed various biochemical properties of both proteins including their boiling stability and ability to form SDS-resistant oligomers. Using immunofluorescence, we determined the cellular localization of these proteins and demonstrated their presence inside sieve elements, the specialized phloem cells involved in long-distance transport (Lough and Lucas, 2006). We also analyzed the expression of both proteins in different tissues collected from poplar saplings.

4.2 Materials and Methods

Bioinformatic analysis of pop3/ SP1-like proteins

Using tblastn with the pop3 protein from *P. trichocarpa* x *P. deltoides* (GenBank acc. no. AAC26526), nine additional pop3/SP1 homologues were identified in the DOE Joint Genome Institute *Populus trichocarpa* genome v1.1 (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html). The pop3 protein was also used to query the *Oryza sativa* (http://rice.plantbiology.msu.edu/) and *Arabidopsis thaliana*

(http://www.arabidopsis.org/) genomes. A total of 15 amino acid sequences were first aligned with ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) (Larkin et al., 2007) using default parameters and then edited with MEGA 4 (http://www.megasoftware.net/). A neighbor-joining phylogenetic tree was created with MEGA 4 using p-distance and pairwise deletion of gaps. ClustalW2 was also used to compare the amino acid sequences of the *P. trichocarpa* pop3/SP1 proteins to *P. tremula* SP1 (GenBank acc. no. CAC34953).

The digital expression profiles of pop3.1 and pop3.4 were created as previously described (Major and Constabel, 2008). Briefly, the pop3.1 and pop3.4 nucleotide sequences were queried against the PopulusDB EST database (http://www.populus.db.umu.se/index.html). The number of ESTs (Sterky et al., 2004) for the corresponding gene clusters were used to construct a digital expression profile.

Plant materials

Poplar hybrid H11-11 (*Populus trichocarpa* X *P. deltoides*) saplings obtained from the University of Washington/Washington State University Poplar Research Program were propagated and grown as previously described (Major and Constabel, 2006). Saplings were grown in 0.25 L pots containing Sunshine Mix #4 (Sungro, Seba Beach, AB, Canada) supplemented with slow-release nutrients (8.9 g/L controlled release 8-6-12 NPK plus micronutrients (Acer, Delta, BC, Canada), 0.458 g/L superphosphate 0-20-0 (Green Valley, Surrey, BC, Canada), 1.21 g/L Micromax Micronutrients (Scotts-Sierra, Marysville, OH, USA), and 4.75 g/L Dolomite lime (IMASCO, Surrey, BC, Canada)) (2006). All plants were maintained at the University of Victoria, BC, in the

Bev Glover greenhouse with 16 h photoperiod. The temperature was maintained at 25 °C/18 °C (day/ night) and plants were watered daily with 0.1 g/L 20-20-20 PlantProd fertilizer (Plant Products, Brampton, ON, Canada).

Tissue sampling and protein extraction

The boiling stability of pop3 proteins were tested as previously described (Pelah et al., 1995). Leaf samples were collected from leaf plastochron index (LPI) 9-11 (Larson and Isebrands, 1971) and proteins were extracted in 50 mM Tris-HCl, pH 7.0 containing 1% (w/v) polyvinylpolypyrrolidine. Crude extracts were centrifuged and a 400 µL aliquot of total soluble protein was boiled for 10 min. Boiled samples were centrifuged to sediment precipitated proteins and the boiling stable proteins were precipitated with 3 vol cold acetone for at least 1 h at -20 °C. Samples were then centrifuged (15 min, 16,000 x g, 4 °C) and the resulting pellet was dried and resuspended in Laemmli buffer (Laemmli, 1970). The remaining total soluble proteins were further separated into two aliquots of equal volumes. Both samples were diluted to 1 mg/mL with Laemmli buffer (containing 2% (w/v) SDS), but only one aliquot was boiled prior to analysis with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The total soluble proteins and the boiling stable proteins were quantified using the Bradford protein assay (Bradford, 1976).

For the tissue survey, samples were collected from the apex and petioles and blades corresponding to LPI 3-5, 9-11, 15-17. Midveins were dissected and analyzed for LPI 9-11 and LPI 15-17. Bark (green stem tissue consisting of phloem and epidermal cells) and wood samples were collected between LPI 9-11. Root samples were collected

from areas of new root growth (young root) and areas near the base of the stem (old root). Plant tissue was frozen and ground in liquid N_2 in a pre-cooled mortar and pestle and total soluble proteins were extracted using sodium phosphate buffer (100 mM NaPO4, pH 7.0 containing 0.1% (v/v) Triton X-100, and 2% (v/v) β-mercaptoethanol) (Haruta et al., 2001). Proteins were concentrated by acetone precipitation as described above, resuspended in Laemmli buffer, and quantified using the RC DC protein assay (BioRad, Hercules, CA, USA).

Expression and purification of recombinant pop3.1-pop3.3

Pop3.1 (JGI gene model estExt_Genewise1_v1.C_LG_X0701) and pop3.4 (estExt_fgenesh4_pg.C_LG_X1353) were cloned using a previously constructed *P. trichocarpa* x *P. deltoides* cDNA library (Constabel et al., 2000). For PCR, the following primers were used for their respective protein: pop3.1 (5'-GGGGGGATCCATGGCAACCAGAACTCCAAA; 3'-GGGGGTCGACGTAGAGAAAGTAGTCTATCA) and pop3.4 (5'-GGGATCCATGGCCTCGAAGAAATCTGC; 3'-CAAGCTTGAAGAGATAGTAGTCCATGA). The amplified cDNA was cloned into either the BamHI and Sal1 restriction sites for pop3.1 or the BamHI and HindIII restriction sites for pop3.4 of the pQE30 bacterial protein expression vector (Qiagen, Mississauga, ON, Canada). Each full-length gene was cloned in frame with the N-terminal His-tag of the pQE30 vector and the genes were sequenced to verify that the *P. trichocarpa* x *P. deltoides* pop3 genes were identical to the *P. trichocarpa* genes. The recombinant proteins, which were detected as monomers, were expressed, harvested, and

purified as previously described (Haruta et al., 2001). After purification with Ni-NTA resin (Novagen, San Diego, CA, USA), fractions containing the respective pop3 proteins were dialyzed three times against 100 vol of deionized H_2O to remove urea prior to lyophilization. [Dr. Ian Major and Eric Bol cloned, expressed and purified pop3.4]

Production of pop3.1 and pop3.4 antibodies

Approximately 250 μg of purified, lyophilized recombinant protein were used to produce polyclonal antibodies for pop3.1 and pop3.4 at Cocalico Biologicals, Inc (Reamstown, PA, USA). Rabbits were initially immunized with 100 μg of protein emulsified with Complete Freund's Adjuvant followed by three boosters of 50 μg of protein emulsified with Incomplete Freund's Adjuvant.

Western blot analysis

Proteins were first separated using 15% polyacrylamide gels under a constant voltage (200 V) in a Mini PROTEAN II system (BioRad, Hercules, CA, USA) and then electro-transferred to PVDF membrane (Pierce, Brockville, ON, Canada) prior to detection using standard protocols (Harlow and Lane, 1999). Pop3 proteins were detected using the primary antibodies described above, and visualized with alkaline phosphatase-conjugated secondary antibodies and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP) and nitro-blue tetrazolium chloride (NBT) as substrates.

The specificity of the pop3.1 and pop3.4 antisera was tested by labelling blots with preimmune serum or pre-adsorbed antisera. To pre-adsorb antiserum, 30 μL of the pop3.1 and pop3.4 antisera (total protein 1200 μg) were diluted 1:5 with deionized H_2O

and then incubated with an equal quantity of their respective recombinant protein for 36 h at 4 °C with constant shaking.

Light microscopy and immunofluorescence

Immunofluorescence of pop3.1 and pop3.4 was carried out as described in Chapters 2 and 3. The pop3.1 and pop3.4 antisera were used to label stem cross sections (6 μm thick) mounted on positive-charged glass slides. Prior to labelling, the plastic was partially removed by soaking the sections in acetone for 20 min. Antibodies were detected with an Alexafluor 568 goat anti-rabbit antibody (Molecular Probes, Eugene, OR, USA). Controls included omitting the primary antibody or incubating sections with preimmune serum. Labelling was visualized using a Zeiss Universal epifluorescence microscope equipped with a digital camera and a fluorescein isothiocyanate filter (excitation at 495 nm and emission at 519 nm). [Samples were prepared by Brent Gowen]

4.3 Results

Poplar genome contains 10 pop3/SP1-like proteins

Using a tblastn search with the protein sequence encoded by the original pop3 cDNA sequence (GenBank acc. no. AAC26526), a total of ten pop3 genes were identified in the DOE Joint Genome Institute *Populus trichocarpa* genome v1.1. The original pop3 was identical to JGI protein ID 723969 and was named pop3.1. The other nine homologues are herein listed as pop3.2 (566885), pop3.3 (814158), pop3.4 (822230), pop3.5 (566884), pop3.6 (833676), pop3.7 (565393), pop3.8 (657714), pop3.9 (649096), and pop3.10 (676240). As determined by the National Centre for Biotechnology

Information conserved domain search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), all ten proteins are predicted to have the conserved Dabb domain (pfam 07876).

SP1, identified in *P. tremula*, is most likely orthologous to pop3.1; they differ by a single amino acid (Fig. 4-1). When comparing *P. tremula* SP1 to the other pop3 proteins, the amino acids forming β-strands, B1, B2, and B3, and α-helix, H2a, are strongly conserved (Fig.4-1). Glu-68 (indicated by ▲) is required for dimer formation (Dgany et al., 2004) and it is conserved in all pop3 proteins, except pop3.9 and pop3.10 (Fig. 4-1). Weaker conservation was observed in the regions forming B4, H1, and H2b, and in the loop connecting B1 and B2. The N-terminal tails are also not strongly conserved. Pop3.4 has a six amino acid insertion in its N-terminus.

The pop3/ SP1 protein family is expanded in poplar when compared to the rice and *Arabidopsis* genomes, which each only have two pop3/ SP1 genes. Phylogenetic analyses indicate that the pop3/ SP1 proteins of these three plant species separate into two distinct clades (Fig. 4-2). Rice and *Arabidopsis* each have one member in each clade, but of the ten poplar pop3/SP1 proteins, eight group into one clade. This clade is further separated into two major branches (Fig. 4-2). Interestingly, one of these branches is composed solely of poplar pop3 proteins. This branch includes pop3.2, pop3.3, and the two phloem exudate proteins, pop3.1 and pop3.4. *P. tremula* SP1 also clusters with these four proteins.

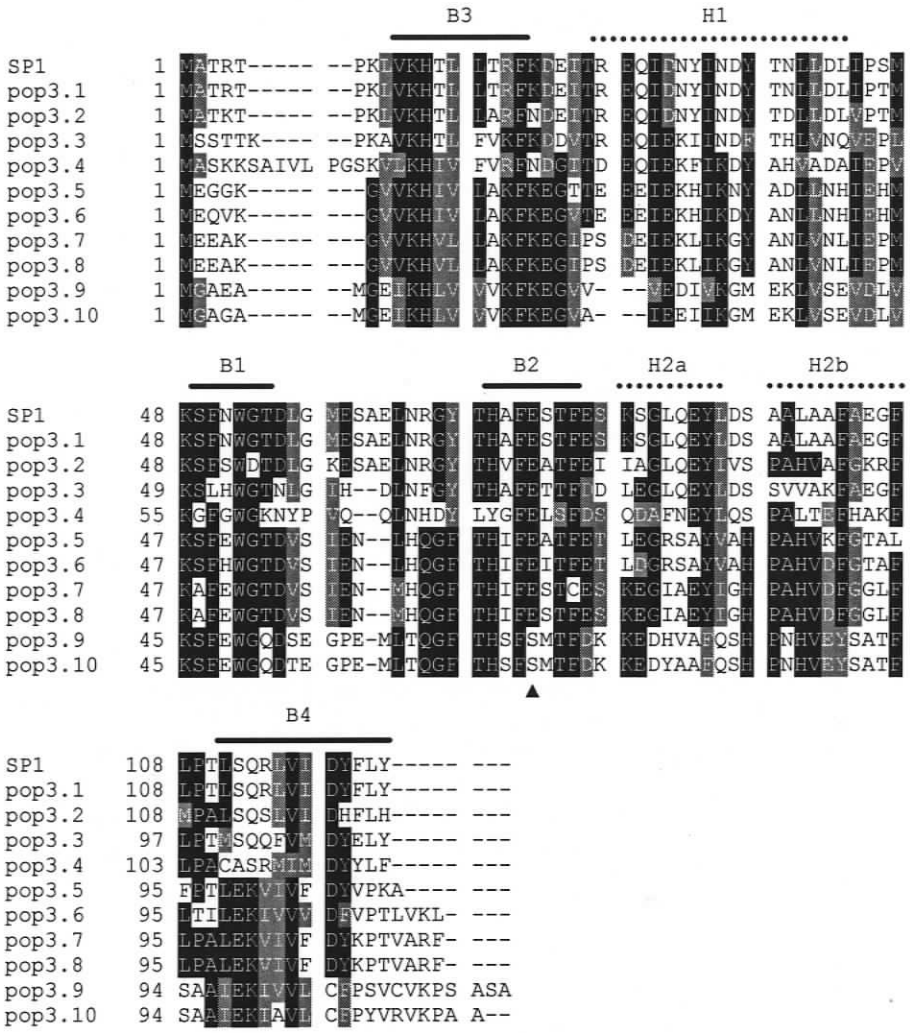


Figure 4-1. Multiple sequence alignment of SP1 (Genbank protein ID CAC34953) and the ten pop3 proteins identified in the *P. trichocarpa* genome.

Black boxes indicate areas of identity and grey boxes represent amino acid changes that are conserved. Solid lines are the region of SP1 that forms β-strands and dashed lines represent α-helices (Dgany et al., 2004). The arrow (▲) indicates the position of SP1 Glu-68.

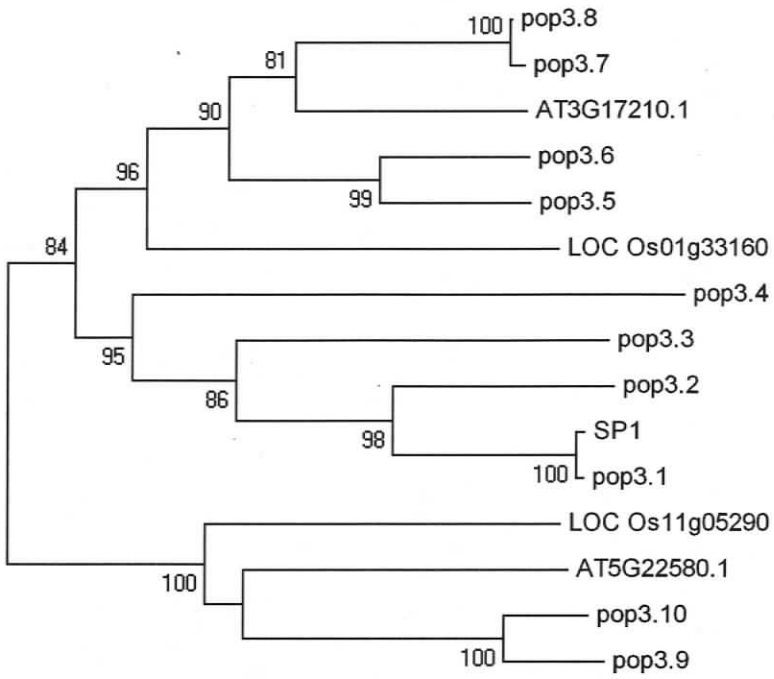


Figure 4-2. Phylogenetic tree of poplar, rice, and *Arabidopsis* pop3/SP1 proteins created by neighbor joining of p distance.

The tree includes *P. tremula* SP1 and the 10 pop3 homologues identified in the genome of *P. trichocarpa*. The tree also includes the gene names of pop3/SP1 proteins identified in the rice (http://rice.plantbiology.msu.edu/) and *Arabidopsis* (http://www.arabidopsis.org/) genomes. Numbers at branches represent bootstrap support from 2000 replicates (only values > 80% are shown).

Generation of pop3.1 and pop3.4 antibodies

The full length cDNAs for pop3.1 and pop3.4 were cloned in frame with an N-terminal His-tag and the recombinant proteins were expressed in *E. coli*. These recombinant proteins were detected on SDS-PAGE as polypeptides of the expected molecular weight. These proteins were affinity purified by means of the His-tag and then used to produce polyclonal antibodies.

The pop.1 and pop3.4 antisera effectively labelled their respective recombinant protein on western blots of crude extracts of overexpressing *E.coli* cells (Fig. 4-3a, e). Both labelled proteins were approximately 13 kDa, which is consistent with the predicted molecular weights of 12.4 kDa and 12.9 kDa for pop3.1 and pop3.4, respectively. Higher molecular weight proteins were also detected and these appear to be the result of the antisera cross-reacting with *E. coli* proteins. Both antisera also cross-reacted with the other pop3 recombinant protein (data not shown), however, in plant extracts, each antiserum detected distinct proteins. The pop3.1 antiserum labelled a 66 kDa protein in leaf and phloem exudate samples (Fig. 4-3b, upper arrow), whereas the pop3.4 antiserum only labelled a 12 kDa protein (Fig. 4-3f).

The 66 kDa protein detected by the pop3.1 antiserum is much larger than its predicted molecular weight and therefore it is likely an oligomeric form of pop3.1. Pop3.1 was previously observed to migrate at a much higher molecular weight (approximately 100 kDa) than predicted (Chapter 2). Note that the pop3.1 antiserum also detected a second, 55 kDa protein in leaf samples (Fig. 4-3b, lower arrow). Based on controls, the pop3.1 antiserum appears to be specific to the 66 kDa protein and the 55 kDa band may represent non-specific binding to the large subunit of RuBisCO (Fig. 2-1).

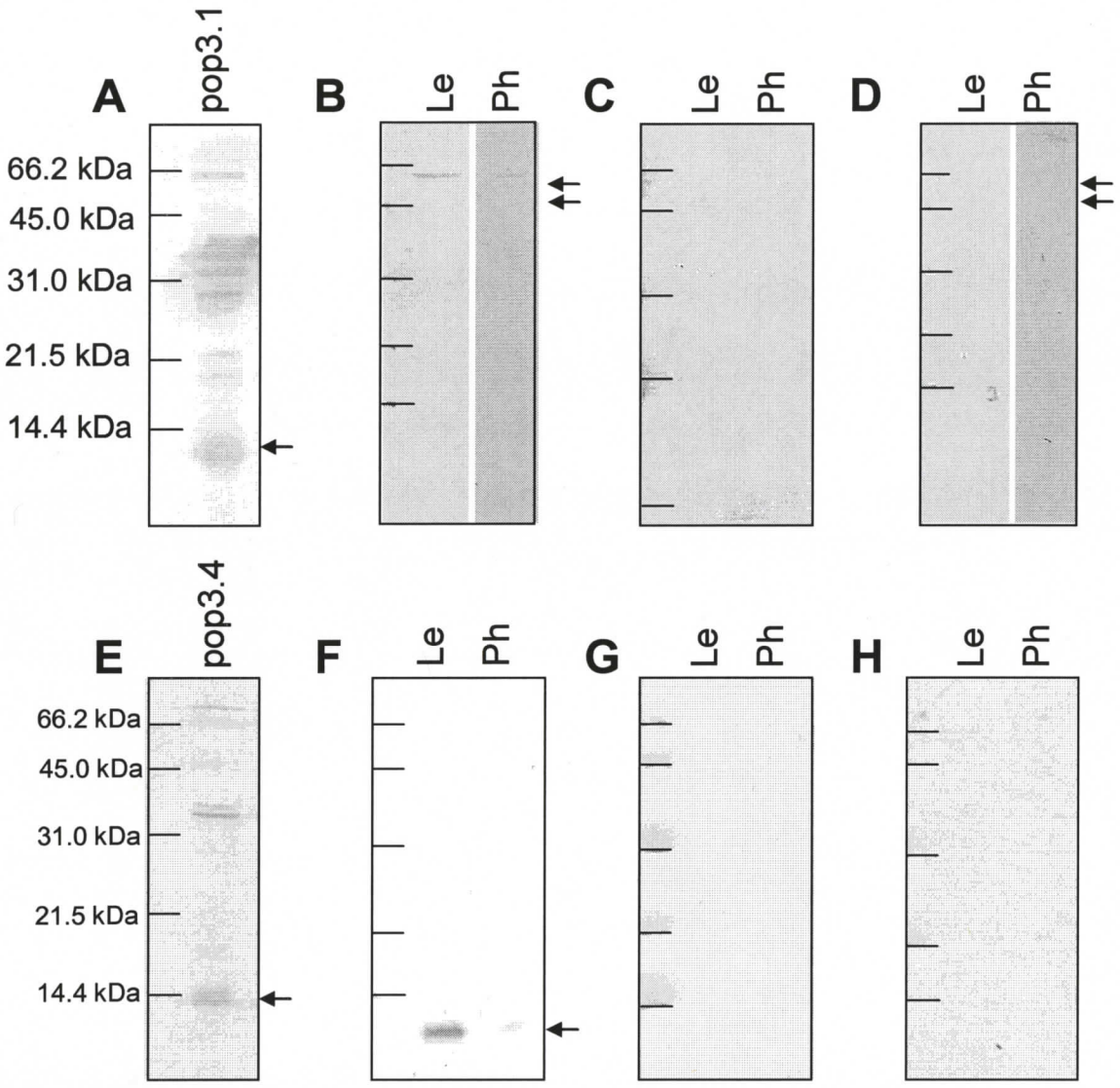


Figure 4-3. Specificity of pop3.1 and pop3.4 antisera.

A) and E) Western blots of pop3.1 and pop3.4 recombinant proteins extracted from *E. coli* cells and labelled with their respective antiserum. B), C), and D) Leaf (Le) and phloem exudate (Ph) proteins labelled with pop3.1 antiserum, preimmune serum and pre-adsorbed pop3.1 antiserum, respectively. F), G), and H) Leaf and phloem exudate proteins labelled with pop3.4 antiserum, preimmune serum and pre-adsorbed pop3.4 antiserum, respectively. Arrows indicated the position of the proteins detected by anti-pop3.1 and anti-pop3.4.

Neither protein was detected by the preimmune serum (Fig. 4-3c), but only the intensity of the 66 kDa band significantly decreased when labelled with pre-adsorbed antiserum, whereas the intensity of the 55 kDa band remained unchanged (Fig. 4-3d). In addition, the 55 kDa protein was only detected in leaves and midveins, samples that have high concentrations of RuBisCO (Fig. 4-3b, 4-6a).

Unlike the pop3.1 antiserum, the pop3.4 antiserum detected a 12 kDa protein in leaf and phloem exudate samples (Fig. 4-3f), which is consistent with its predicted molecular weight. Based on controls, this labelling appears to be very specific. No proteins were detected on western blots labelled with pop3.4 preimmune serum (Fig. 4-3g) or pre-adsorbed pop3.4 antiserum (Fig. 4-3h).

Analysis of pop3.1 and pop3.4 by western blots

Pop3.1 appears to form an SDS-resistant oligomeric complex. The pop3.1 antiserum detected a 66 kDa protein after proteins were separated by SDS-PAGE. This 66 kDa protein was also detected when samples were boiled for ten minutes in the presence of 2% (w/v) SDS prior to separation by SDS-PAGE, indicating that this structure is very stable (Fig. 4-4a, lane 2). Under these harsh conditions, the pop3.1 oligomer partially dissociated since a 12 kDa polypeptide, presumably the pop3.1 monomer, was also weakly labelled (Fig. 4-4a, lane 2).

Unlike pop3.1, pop3.4 does not appear to form an SDS-resistant oligomer. A 12 kDa protein was detected by the pop3.4 antiserum in SDS-treated samples even if they were not boiled prior to gel loading (Fig. 4-4b, lane 1 and 2). This is consistent with the predicted molecular weight of pop3.4.

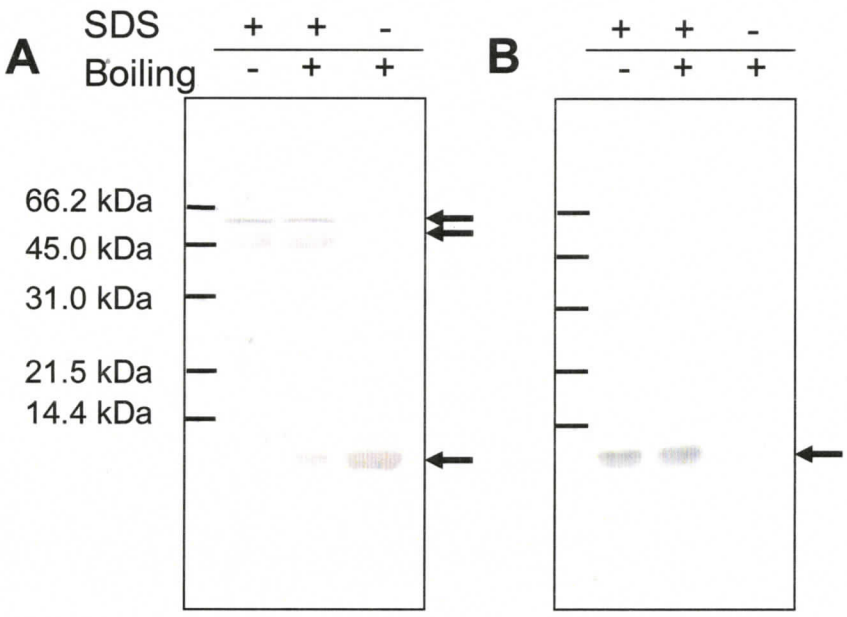


Figure 4-4. Effects of SDS and boiling on A) pop3.1 and B) pop3.4 proteins.

Protein samples were either boiled (+) or not boiled (-) in the presence (+) or absence (-) of 2% (w/v) SDS. Arrows indicate position of proteins detected by the antibodies.

To determine the thermostability of pop3.1 and pop3.4, we boiled leaf proteins extracted from *P. trichocarpa* x *P. deltoides*. After ten minutes of boiling (in the absence of SDS), only 2% of the total soluble leaf protein remained in solution. In this sample, the pop3.1 antiserum detected the 12 kDa polypeptide, while the 66 kDa protein was absent (Fig. 4-4a, lane 3). These results suggest that pop3.1 remains soluble after boiling, but it completely dissociates into its monomer form when boiled in the absence of SDS.

In contrast to pop3.1, pop3.4 does not appear to be boiling stable. The pop3.4 antiserum did not label any proteins within the boiling soluble fraction (Fig. 4-4b, lane 3); the 12 kDa protein was only detected in the denatured protein sample (data not shown). Although pop3.1 and pop3.4 proteins are 40% similar and they cluster together within the same clade, they appear to exhibit different biochemical characteristics.

Spatial expression of pop3.1 and pop3.4

Both pop3.1 and pop3.4 proteins were detected in leaf extracts and phloem exudate (Fig. 4-3), but the expression of these two proteins (or genes) in other tissues has not been studied. Based on digital expression profiles created using PopulusDB (http://www.populus.db.umu.se/), pop3.1 and pop3.4 appear to have distinct expression patterns. As seen in Fig. 4-5, there is a difference in abundance of ESTs for gene clusters corresponding to pop3.1 and pop3.4 when comparing different poplar tissues. Both transcripts were expressed in the cambial zone, petioles, bark, and apical shoot meristem, but only the pop3.1 gene cluster was expressed in young leaves and only the pop3.4 gene cluster was expressed in roots (Fig. 4-5).

In order to characterize the expression of pop3.1 and pop3.4 proteins in more detail, proteins were extracted from a variety of tissues including the shoot apex (LPI 0 and above), leaves 3-5, 9-11, and 15-17, and the corresponding petioles and midveins (except LPI 3-5). Protein levels were also assessed in bark and wood samples corresponding to LPI 9-11 and young and old root samples. Both pop3.1 and pop3.4 antisera labelled their respective proteins in all of the tissues tested (Fig. 4-6). This is not consistent with their digital expression profiles (Fig. 4-5). The pop3.1 protein was labelled in young and old roots even though there were no ESTs detected in the root cDNA library for the gene cluster corresponding to pop3.1. Likewise, there were no ESTs matching the pop3.4 gene cluster in the young leaf cDNA library, but this protein was detected in young leaves.

The abundance of the pop3.4 protein was consistent in all tissues (Fig. 4-6b), but the quantity of pop3.1 varied depending on the age and type of tissue (Fig. 4-6a). As compared to other plant tissues, pop3.1 was more abundant in leaves. The pop3.1 protein was also most strongly expressed in younger tissues including the apex and young leaves and petioles. These results demonstrate that pop3.1 and pop3.4 proteins have different expression patterns.

Immunolocalization of pop3.1 and pop3.4

Pop3.1 and pop3.4 proteins were previously identified in phloem exudate (Chapter 2) and the presence of these proteins in phloem exudate was shown with western blots (Fig. 4-3b, f). To confirm these results and demonstrate the presence of these proteins in sieve elements, the specialized cells involved in phloem transport, we

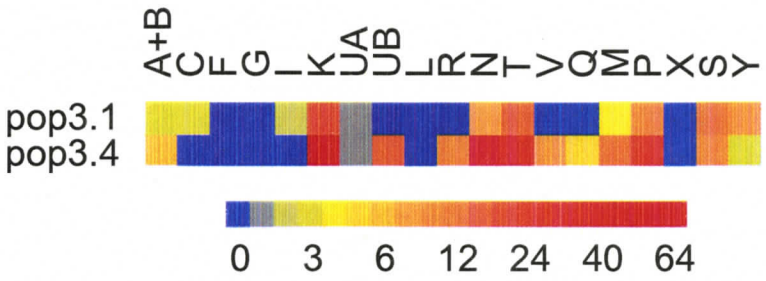


Figure 4-5. Pop3.1 and pop3.4 digital expression profiles.

Sequence clusters corresponding to the pop3.1 and pop3.4 transcripts were identified from the PopulusDB EST database (http://www.populus.db.umu.se/) and the number of ESTs for these clusters are shown for 19 cDNA libraries. A+B, cambial zone; C, young leaves; F, flower buds; G, tension wood; I, senescing leaves; K, apical shoot; UA, dormant cambium; UB, active cambium; L, cold stressed leaves; R, roots; N, bark; T, shoot meristem; V, male catkin; Q, dormant buds; M, female catkin; P, petioles; X, wood cell death; S, imbibed seeds; Y, virus/ fungus infected tissue.

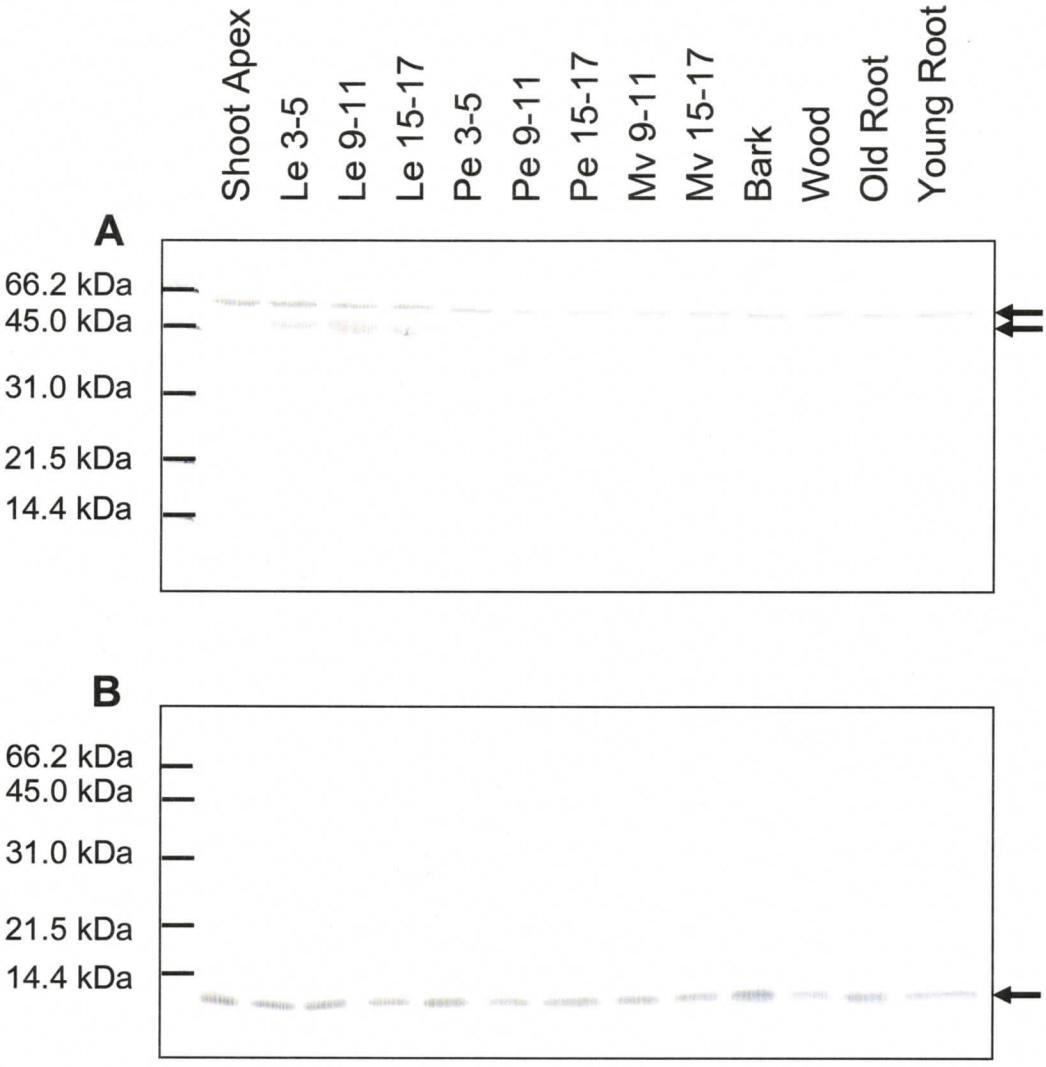


Figure 4-6. Tissue survey of *P. trichocarpa* x *P. deltoides* saplings to determine the expression patterns of A) pop3.1 and B) pop3.4.

Western blots of samples collected from the apex, leaves (Le) and petioles (Pe) from positions LPI 3-5, 9-11, and 15-17, midveins (Mv) from LPI 9-11 and 15-17, bark and wood collected from LPI 9-11 and old and young root samples. Tissues were harvested and proteins were extracted as described in the methods and arrows indicate the position of proteins detected by the antibodies.

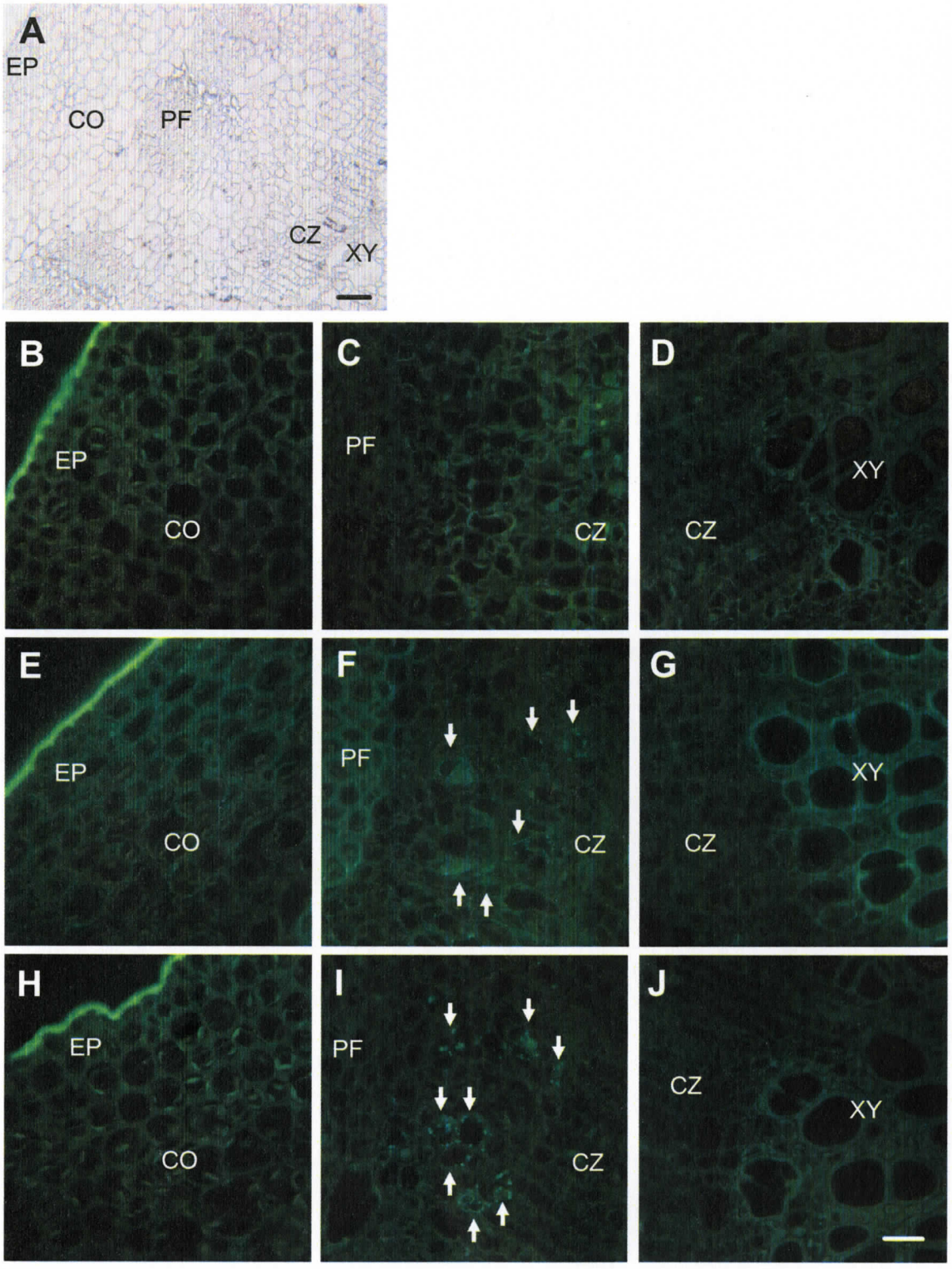
carried out immunofluorescence using pop3.1 and pop3.4 antisera. Both antisera labelled punctate, organelle-like structures inside sieve elements of mature stem cross sections (Fig. 4-7f, i; labelling indicated by arrows). This labelling appeared to be specific since no fluorescence was detected in stem sections labelled with pop3.1 and pop3.4 preimmune sera (Supplementary Figure 4-1) or if no primary antibody was used (Fig. 4-7c). Altogether, these results confirm the origin of these phloem exudate proteins within sieve elements, although the nature of these structures has not been determined.

Pop3.1 and pop3.4 proteins were also detected in other cell types. The pop3.1 antiserum labelled punctate structures inside phloem parenchyma cells surrounding phloem fibres (Fig. 4-8e). The pop3.1 antiserum also strongly labelled the cell walls of epidermal and cortical cells (Fig. 4-7e), phloem fibres adjacent to sieve elements (Fig. 4-7f), and the secondary cell wall thickenings of xylem vessels (Fig. 4-7g, 4-9e). The presence of pop3.1 in xylem vessels is consistent with its detection in extracted wood proteins (Fig. 4-6a) and in xylem exudate.

Similar to pop3.1, pop3.4 antiserum also labelled punctate, organelle-like structures in phloem parenchyma cells (Fig. 4-8f). Unlike pop3.1, the pop3.4 antiserum only weakly labelled the cell walls of epidermal and cortical cells (Fig. 4-7h). It also appeared to weakly label xylem vessels in stem cross sections (Fig. 4-7j), but the signal was not much stronger than the autofluorescence detected in the no primary antibody control (Fig. 4-7d). Furthermore, no fluorescence was detected when longitudinal sections of xylem vessels were labelled with pop3.4 antiserum (Fig. 4-9f) indicating that unlike pop3.1, pop3.4 is not localized to xylem vessels. Likewise, the pop3.4 antiserum

Figure 4-7. Immunofluorescence of mature stem cross-sections labelled with pop3.1 (E-G) and pop3.4 (H-J) antisera.

Panel A is a bright-field image of a stem cross-section. On the far left, epidermal cells (EP) surround the cortical cells (CO). Directly to the right of the cortical cells are phloem cells, including phloem fibres (PF). The cambial zone and xylem cells including xylem vessles (XY) are on the far right adjacent to the phloem. Panels B-D are images of sections labelled with no primary antibody. Arrows indicate position of sieve elements. Scale bar for panel A equals 50 μm and scale bar for panels B-J equals 20 μm.


A
EP
CO
PF
CZ
XY
B
EP
CO
C
PF
CZ
D
XY
CZ
E
EP
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J
CZ
XY

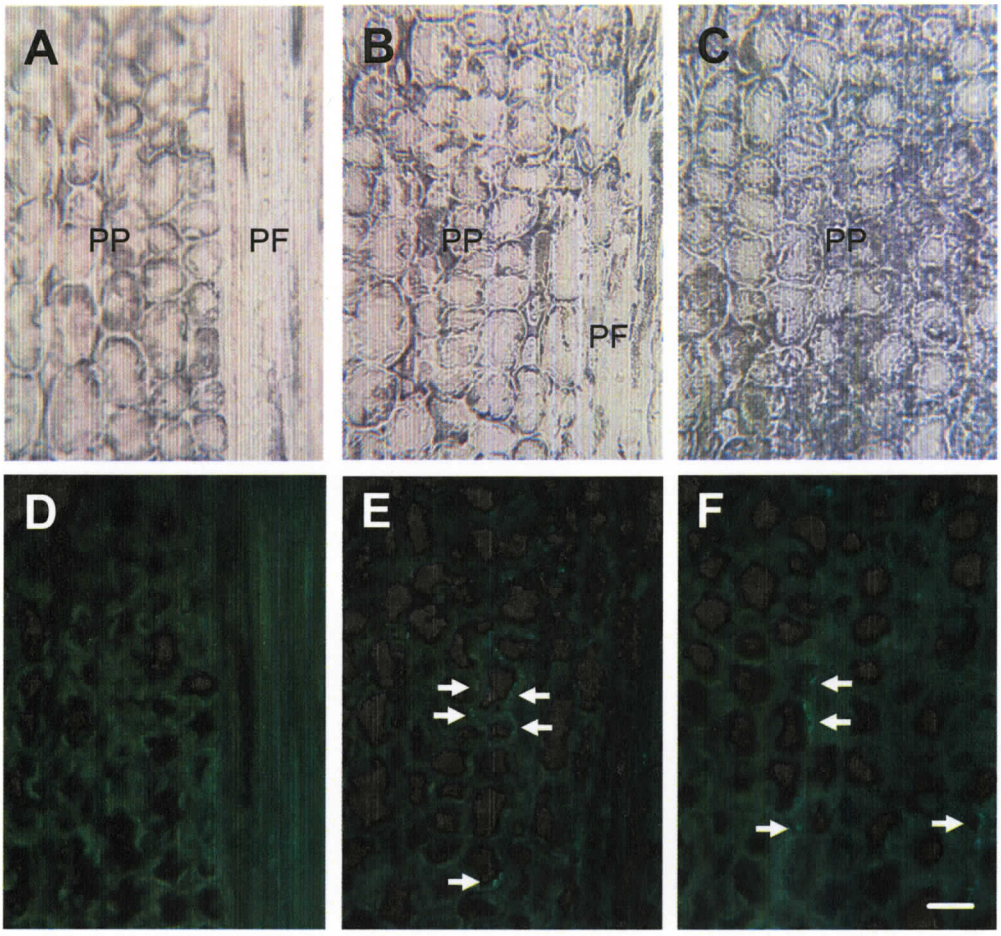


Figure 4-8. Longitudinal sections of phloem parenchyma cells in mature stem labelled with pop3.1 (B,E) and pop3.4 (C,F) antisera.

Panels A-C show bright field images and panels D-F the corresponding immunofluorescent images. Panel D was treated without a primary antibody, while sections E and F were treated with pop3.1 and pop3.4 antiserum, respectively. PP, phloem parenchyma cells; and PF, phloem fibres. Arrows indicate position of labelled phloem parenchyma cells. Scale bar = 20μm.

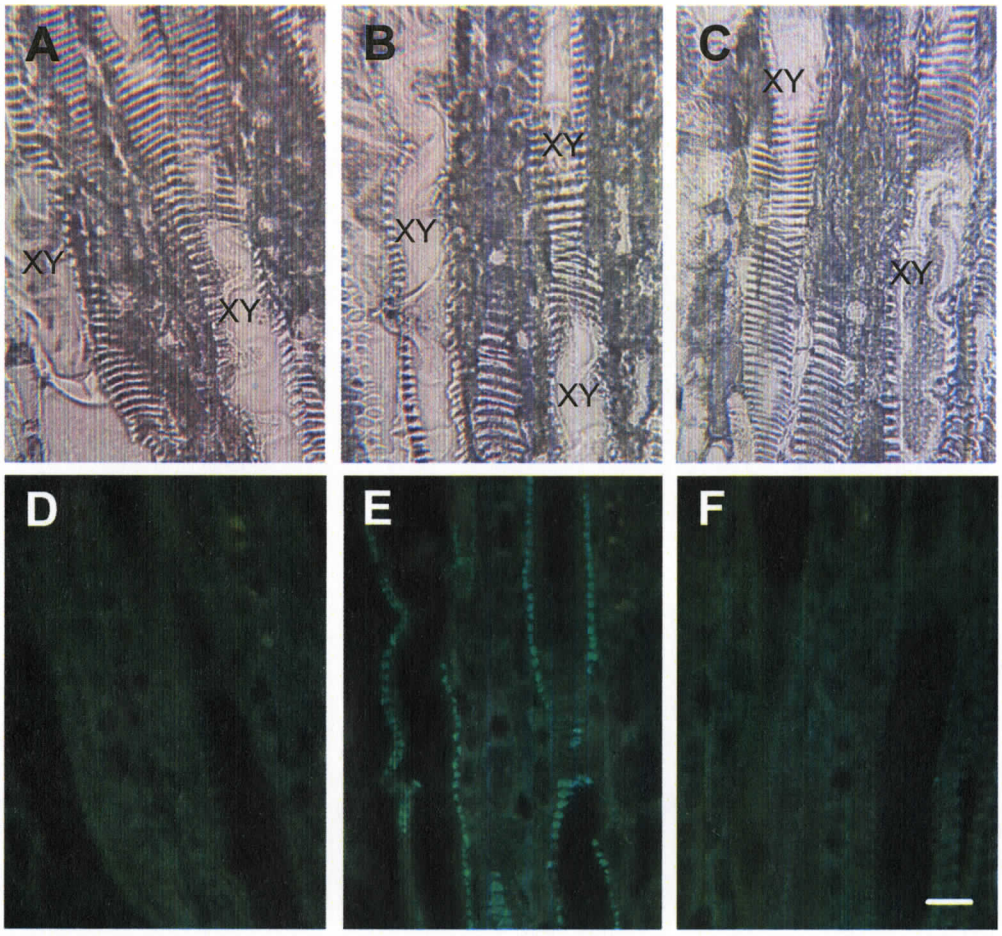


Figure 4-9. Longitudinal sections of xylem vessels in mature stem labelled with pop3.1 (B,E) and pop3.4 (C,F) antisera.

Panels A-C show bright field images and panels D-F the corresponding immunofluorescent images. Panel D was treated without a primary antibody, while sections E and F were treated with pop3.1 and pop3.4 antiserum, respectively. XY, xylem vessels. Scale bar = 20μm.

did not label phloem fibres (Fig. 4-7i). Plastids in epidermal and cortical cells also fluoresced in stem cross sections labelled with pop3.4 antiserum (Fig.4-7h), but this appears to be due to autofluorescence because plastids fluoresced in stem cross sections incubated with PBS and no primary antibody (Fig. 4-7b). Autofluorescence was also detected in the cuticle covering epidermal cells. This area strongly fluoresced in control treated stem sections (Fig. 4-7b) as well as stem sections incubated with pop3.1 and pop3.4 antisera and preimmune sera (Fig. 4-7e, h, Supplementary Fig. 4-1).

4.4 Discussion

In the *P. trichocarpa* genome, a total of ten pop3 homologues were identified, one of which, pop3.1, corresponded to the original pop3 protein and was 99.1% identical to the *P. tremula* pop3 orthologue, SP1. Pop3.1 and a second pop3 homologue, pop3.4, were recently identified in phloem exudated collected from *P. trichocarpa* x *P. deltoides* (Chapter 2). Pop3.1 was also induced in phloem exudate 24 h after leaves were mechanically wounded (Chapter 2). Pop3.1 and pop3.4 proteins are 40% similar, but each appears to be biochemically and biologically distinct. Pop3.1 appears to be an SDS-resistant oligomer that remains soluble after boiling whereas pop3.4 was detected as a monomer that precipitated when boiled. Pop3.1 and pop3.4 proteins were ubiquitously expressed throughout the plant, but upon closer inspection with immunofluorescence, these two proteins exhibit somewhat different localization patterns. Both proteins were detected in sieve elements and phloem parenchyma, but only the pop3.1 antiserum strongly labelled the cell walls of epidermal and cortical cells, phloem fibres and xylem vessels.

Oligomer formation and boiling stability of pop3.1-pop3.4

In order to characterize the two pop3 homologues identified in poplar phloem exudate, pop3.1 and pop3.4 were overexpressed in *E. coli* and the purified recombinant proteins were used to produce polyclonal antibodies. Using these antibodies, both proteins were tested for oligomer formation, SDS-resistance, and boiling stability; all features observed in SP1, the well characterized pop3 orthologue from *P. tremula* (Wang et al., 2002; Wang et al., 2003; Dgany et al., 2004; Wang et al., 2006).

Under native conditions *P. tremula* SP1 forms a dodecamer and this complex is resistant to dissociation by treatment with SDS (Wang et al., 2002; Dgany et al., 2004; Wang et al., 2006). SDS-resistance is generally uncommon for protein oligomers, although it has been reported for some protein complexes (Weisz et al., 1993; Natarajan et al., 1999; Timmins et al., 2003; Kubista et al., 2004; Cacciapuoti et al., 2005; Cacciapuoti et al., 2007). Pop3.1 also appeared to form an SDS-resistant oligomer. The 66 kDa protein detected by the pop3.1 antiserum is much larger than the predicted molecular weight of pop3.1 monomer, but it is consistent with the molecular weight observed for recombinant SP1, which assembles into an SDS-resistant hexamer (Wang et al., 2003; Wang et al., 2006). A 66 kDa protein, however, is considerably smaller than the 116 kDa protein reported for native SP1 (Wang et al., 2002; Dgany et al., 2004). The difference in molecular weight may thus be due to analytical conditions. The 116 kDa SP1 form is observed when leaf proteins were separated by tricine-SDS-PAGE (Wang et al., 2002), whereas in the current study, the pop3.1 antiserum detected a 66 kDa protein when proteins were separated by glycine-SDS-PAGE. Tricine gels are typically used to separate small proteins (<30 kDa) and glycine gels are used to separate larger proteins

(>30 kDa) (Schagger, 2006). When hybrid aspen (*P. tremula* x *P.alba*) soluble leaf proteins were separated using glycine-SDS-PAGE and analyzed by western blots, the pop3.1 antiserum labelled the 66 kDa protein (data not shown). This protein is presumably SP1, which would confirm that the differences between our data in *P. trichocarpa* x *P. deltoides* with pop3.1 and the published data on SP1 in *P. tremula* are due to different protein separation techniques.

The 66 kDa oligomeric complex formed by pop3.1 remains stable even when boiled in the presence of SDS. Unlike SDS-treated *P. tremula* SP1 that completely dissociates when heated above 95°C (Wang et al., 2002; Wang et al., 2006), the 66 kDa pop3.1 protein was still detected in boiled, SDS-treated samples. This protein complex, however, does appear to partially dissociate since a 12 kDa protein, presumably the pop3.1 monomer, was also weakly detected in these samples. Surprisingly, the pop3.1 oligomer only completely dissociated when boiled in the absence of SDS. Similar to SP1 (Wang et al., 2002), pop3.1 remained soluble after boiling, but only as the 12 kDa form. These results are unexpected given that the thermostability of other SDS-resistant proteins has been shown to decrease when treated with SDS (Arakawa et al., 1992; Cacciapuoti et al., 2005; Cacciapuoti et al., 2007).

In contrast to pop3.1, the observed molecular weight of pop3.4, in the presence of SDS, is consistent with its predicted molecular weight indicating that this protein does not form an SDS-resistant oligomer. A 12 kDa protein was labelled by the pop3.4 antiserum in SDS-treated samples, independent of boiling. It is possible that pop3.4 forms an oligomeric complex that is susceptible to dissociation by detergents. When comparing the amino acid sequences of pop3.4 and SP1, the glutamic acid residue that is

important for the formation of an *P. tremula* SP1 dimer (Dgany et al., 2004) is conserved in pop3.4. However, the β-strand, B4, is not strongly conserved in pop3.4 and this is an important contact region for *P. tremula* SP1 dimer formation (Dgany et al., 2004). In addition, SP1 dimers connect along B1, H1, and N-terminal tails to form a dodecamer (Dgany et al., 2004). H1 is not strongly conserved in pop3.4 and the six amino acid insertion in its N-terminus may hinder the formation of a higher order oligomer. Pop3.4 does not form an SDS-resistant oligomer, but further research is needed to determine if it assembles into an oligomer under native conditions.

Unlike *P. tremula* SP1 and pop3.1, and other plant boiling stable proteins such as dehydrins (Close et al., 1989), a superoxide dismutase (Khanna-Chopra and Sabarinath, 2004), and a cyclophilin (Sharma et al., 2006), pop3.4 did not remain soluble after boiling. The basis for boiling stability appears to be dependent on all aspects of protein structure, from primary to quaternary structure (Sterner and Liebl, 2001). For SP1, boiling stability has been attributed to two structural features that are also conserved in a hypothetical protein from the extremely thermophilic bacterium, *Thermus thermophilus* (Wada et al., 2004); a conserved ferredoxin-like fold and the ability to form stable dimers via hydrogen bonds (Dgany et al., 2004). Both pop3.1 and pop3.4 appear to have this conserved ferredoxin-like fold, but an oligomeric complex has only been detected for pop3.1 and only pop3.1 was detected in the boiling soluble proteins. In order to fully comprehend why pop3.4 does not remain soluble after boiling, it will be important to elucidate the three-dimensional structure of pop3.4 and compare this to *P. tremula* SP1 and other heat stable proteins.

Localization and possible functions of pop3.1 and pop3.4

Both pop3.1 and pop3.4 were previously identified in *P. trichocarpa* x *P. deltoides* phloem exudate (Chapter 2) and immunofluorescence confirms that these two proteins are present inside sieve elements. Inside sieve elements and phloem parenchyma cells, the pop3.1 and pop3.4 antisera labelled organelle-like structures and preliminary observations indicate that these structures are starch granules and starch-containing plastids as was observed for PtTLP1 and closely related TLPs (Chapter 3). Both proteins are similar to *P. tremula* SP1 and therefore they may also function as protein chaperones (Wang et al., 2003; Dgany et al., 2004). Protein chaperones, including heat shock proteins, have been previously identified in phloem sap collected from a variety of different plant species (Schobert et al., 1995; Barnes et al., 2004; Giavalisco et al., 2006; Aki et al., 2008). The presence of pop3.1 and pop3.4 in organelle-like structures is also consistent with the localization of heat shock proteins in organelles such as chloroplasts and mitochondria (Wang et al., 2004). It has been suggested, however, that the oligomer is the biologically active unit of *P. tremula* SP1 (Dgany et al., 2004) and an oligomer was only detected for pop3.1. Therefore, only pop3.1 may function as a protein chaperone.

The molecular function of pop3 proteins may not be completely understood, but based on expression patterns; some of these proteins may have a role in plant defense against abiotic and biotic stresses. For example, transcription and translation of pop3/SP1 proteins have been shown to be induced in response to salt, cold, heat, and drought stress (Wang et al., 2002; Gu et al., 2004; Renaut et al., 2005; Ferreira et al., 2006) and when *Arabidopsis* plants were transformed *P. tremula* SP1, these plants were more heat-tolerant than control plants (Zhu et al., 2008). In addition, pop3/SP1 transcripts are

induced in response to wounding and insect feeding (Major and Constabel, 2006; Ralph et al., 2006).

Previous studies confirm that pop3.1 and pop3.4 are stress inducible. Pop3.1 and pop3.4 were among the pop3/SP1 transcripts induced in response mechanical wounding and treatment with caterpillar regurgitant (Major and Constabel, 2006) and the pop3.1 protein was one of two proteins that were induced in phloem exudate 24h after wounding leaves (Chapter 2). This provides indirect evidence that these proteins have roles in plant insect defense, which possibly includes defense against phloem-feeding insects since both proteins were localized to sieve elements. Based on digital expression profiles, both pop3.1 and pop3.4 transcripts were also expressed in pathogen infected tissues. These results are particularly intriguing because both proteins were detected in cell walls and many known pathogenesis-related proteins are localized extracellularly where they serve as a first line of defense against invading pathogens (van Loon et al., 2006). Two pop3/SP1 proteins in *Arabidopsis* were also recently shown to exhibit antifungal activity (Park et al., 2007; Lee et al., 2008). Biologically, these two proteins appear to be involved in plant defense responses, but how they may function requires additional research. Given the biological and biochemical differences observed for pop3.1 and pop3.4, these two proteins may have very different roles in *P. trichocarpa* x *P. deltoides*.

5 Characterization of the poplar xylem sap proteome

5.1 Introduction

The primary function of xylem is traditionally thought to be the transport of water and minerals (Satoh, 2006); however, xylem sap also contains a diversity of organic compounds including carbohydrates, amino acids, and even proteins (Dickson, 1979; Vogelmann et al., 1985; Biles and Abeles, 1991; Vancleve et al., 1991; Satoh et al., 1992; Lopez-Millan et al., 2000; Rep et al., 2002; Buhtz et al., 2004; Escher et al., 2004; Kehr et al., 2005; Alvarez et al., 2006; Djordjevic et al., 2007; Aki et al., 2008). Similar to phloem sap, the presence of proteins in xylem sap is surprising. Tracheary elements, the specialized cells involved in xylem transport, are dead at maturity and therefore are incapable for protein synthesis. Until recently, very little was known about the identity of xylem sap proteins. With the availability of sensitive proteomic techniques (Domon and Aebersold, 2006), a large number of xylem sap proteins have been identified, but mostly from annual species.

Overall, proteins are present at very low concentrations (10-300 μg/mL) in xylem sap (Biles and Abeles, 1991; Satoh et al., 1992; Buhtz et al., 2004; Alvarez et al., 2006), but nevertheless on two-dimensional gel electrophoresis (2-DE), hundreds of proteins can be detected in xylem sap from *Brassica napus* and *Zea mays* (Kehr et al., 2005; Alvarez et al., 2006). Currently, xylem sap proteins have been studied in *B. napus*, *B. oleracea*, *Cucurbita maxima*, *Cucumis sativus*, *Z. mays*, *Lycopersicon esculentum*, *Glycine max*, and *Oryza sativa* (Rep et al., 2002; Buhtz et al., 2004; Kehr et al., 2005; Alvarez et al., 2006; Djordjevic et al., 2007; Aki et al., 2008). In the xylem sap proteomes analyzed to

date, peroxidases, as well as pathogenesis-related (PR) proteins and proteases, have been consistently detected (Rep et al., 2002; Buhtz et al., 2004; Kehr et al., 2005; Alvarez et al., 2006; Djordjevic et al., 2007; Aki et al., 2008). PR proteins are typically expressed in response to pathogen infection and are classified into 17 families (van Loon et al., 2006). PR-1, β-1,3-glucanases, chitinases, and thaumatin-like proteins (TLPs) are among the PR proteins previously identified in xylem sap (Rep et al., 2002; Buhtz et al., 2004; Kehr et al., 2005; Alvarez et al., 2006; Djordjevic et al., 2007; Aki et al., 2008).

Other regular constituents of xylem sap include cell wall proteins and cell wall metabolic enzymes (Buhtz et al., 2004; Kehr et al., 2005; Alvarez et al., 2006; Djordjevic et al., 2007; Aki et al., 2008; Alvarez et al., 2008). In addition to peroxidases, which are likely involved in the final stage of lignin polymerization (Hiraga et al., 2001), glycoside hydrolases and polygalacturonases have also been detected in xylem sap from various plant species (Buhtz et al., 2004; Kehr et al., 2005; Alvarez et al., 2006; Djordjevic et al., 2007; Aki et al., 2008; Alvarez et al., 2008). These proteins are typically involved in degrading primary cell walls, a process that occurs in developing tracheary elements during cell death (Turner et al., 2007). Cell wall localized glycine-rich proteins and arabinogalactan-rich proteins have been identified in xylem sap of several species (Buhtz et al., 2004; Kehr et al., 2005; Alvarez et al., 2006; Djordjevic et al., 2007; Aki et al., 2008; Alvarez et al., 2008). In general, there are a number of proteins that are consistently found in xylem sap, suggesting that these proteins have an important role within the xylem.

There is indirect evidence that xylem sap proteins may be involved in protecting plants against environmental stresses. Several PR proteins were upregulated in tomato

xylem sap after infection by the pathogen, *Fusarium oxysporum* (Rep et al., 2002). Furthermore, maize xylem sap inhibited fungal growth and this activity was abolished when xylem sap was pre-treated with proteases suggesting that the antifungal activity is specific to xylem sap proteins (Alvarez et al., 2006). In addition to biotic stresses, xylem sap proteins may play an important role in abiotic stress responses; in maize, 39 xylem sap proteins were differentially regulated in response to water stress (Alvarez et al., 2008). Many of the upregulated proteins were cell wall metabolic enzymes. These enzymes may function by reinforcing the secondary cell walls of xylem vessels during periods of drought.

To date, xylem sap proteins have been studied and appear to have important roles in annual plants. However, very little is known regarding such proteins in longer-living, perennial and woody plants, which usually encounter many diverse stresses in their life times. Therefore, we undertook a study of xylem sap proteins in hybrid poplar (*P. trichocarpa* x *P. deltoides*). We used root pressure to collect xylem sap for proteomic analysis.

5.2 Materials and Methods

Plant Material

Poplar hybrid H11-11 (*Populus trichocarpa* x *P. deltoides*) saplings (University of Washington/Washington State University Poplar Research Program) were propagated and grown in 0.25 L pots containing Sunshine Mix #4 (Sungro, Seba Beach, AB, Canada) supplemented with slow-release nutrients (Major and Constabel, 2006). All plants were maintained under controlled conditions (16 h photoperiod, 25 °C day, 18 °C night) in the

Bev Glover greenhouse at the University of Victoria. The plants were watered hourly with 0.1 g/L 20-20-20 PlantProd fertilizer (Plant Products, Brampton, ON, Canada).

Sample Preparation

To collect xylem sap, shoots of three-month-old saplings were severed 5 cm above the soil and 1 cm of bark was stripped back from the cut surface to expose the wood. The cut surface was rinsed with distilled water and blotted dry before a Tygon tube was fitted over the wood. This was used to help minimize sample contamination from surrounding bark tissue. The xylem sap generated by root pressure was collected from the tube over a 30 min interval and placed directly on ice. Using this technique, approximately 2-4 mL of xylem sap could be collected. Proteins were precipitated using 2 vol. cold acetone for at least one hour at -20 °C. After 15 min of centrifugation at 16,000 x g at 4 °C, the resulting pellet was dried and resuspended in Laemmli sample buffer (Laemmli, 1970) for SDS-PAGE or resolubilization buffer (8 M urea, 65 mM DTT, and 16 mM CHAPS) for 2-DE. Protein samples for SDS-PAGE were quantified using a RC DC protein assay (BioRad, Hercules, CA, USA) because the concentration was too low to be quantified using standard Bradford protein assays (Bradford, 1976). Samples for 2-DE were quantified using a modified Bradford assay (Ramagli and Rodriguez, 1985).

Phloem exudate was collected as previously described (Chapter 2). Briefly, stem sections, approximately 10 cm in length, were excised between leaf plastochron index (LPI) 5-16 (Larson and Isebrands, 1971) and a section of bark was removed from the cut end exposing wood which was then sealed using a 90% lanolin, 10% paraffin wax

mixture. The cut and sealed end was washed, blotted dry, and then placed into exudation buffer (50 mM Tris, pH 8.0, 2 mM EDTA) for 2 h at room temperature. Phloem exudate proteins were acetone precipitated and quantified as described above.

Bark proteins were extracted as previously described (Chapter 2). The tissue was ground in liquid nitrogen and proteins were extracted in a sodium phosphate buffer (100 mM, pH 7.0) containing 0.1% Triton X-100 and 0.1% v/v β-mercaptoethanol (Haruta et al., 2001). Proteins were acetone precipitated and quantified as described above.

Gel Electrophoresis and Immunoblotting

For SDS-PAGE, 10 µg of protein were separated on 12% or 15% polyacrylamide gels under a constant voltage of 100 in a mini PROTEAN II system (BioRad). The gels were not stained prior to excising gel segments for sequencing.

For 2-DE, 4 µg of xylem sap protein was diluted with rehydration buffer (8 M urea, 32 mM CHAPS, 20 mM DTT, and 0.5% v/v IPG buffer, pH 3-10 (Amersham Biosciences, Piscataway, NJ, USA) to a total volume of 125 µL. The sample was then used to rehydrate a 7 cm Immobiline Drystrip (Amersham Biosciences) with a linear pH range of 3-10. Isoelectric focusing of the sample was carried out for a total of 5.7 kVh with an IPGphor apparatus (Amersham Biosciences). Prior to running the second dimension, the proteins were reduced with 65 mM DTT followed by alkylation with 135 mM iodoacetamide both in equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, and trace bromophenol blue) for 15 min. The gel was then fixed onto a 12% acrylamide gel (BioRad) using 0.5% (w/v) agarose. The second dimension was completed as decribed for SDS-PAGE. Proteins were visualized using Sypro Ruby

(BioRad) and gels were scanned using ProXPRESS Proteomic Imaging System (Perkin Elmer, Waltham, MA, USA).

For immunoblotting, proteins were separated by SDS-PAGE and then electro-transferred to PVDF membrane (Pierce, Brockville, ON, Canada). The detection of a thaumatin-like protein (PtTLP1), WIN4, and polyphenol oxidase (PPO) with their respective antiserums was carried out as previously described (Lawrence et al., 1997; Wang and Constabel, 2003) (Chapter 3). WIN4 and polyphenol oxidase (PPO) are known to be present in phloem exudate and bark tissue, but not expected to be in xylem sap. WIN4 is a vegetative storage protein that has been localized to cells surrounding the vasculature (Lawrence et al., 1997). Although a similar 32 kDa storage protein has been previously identified in xylem sap, it was only found in xylem sap collected from dormant trees or trees undergoing bud break (Vancleve et al., 1991). PPO, is a plastidal protein (Marusek et al., 2006). The PtTLP1 antiserum (Chapter 2) was used to check for the presence of thaumatin-like proteins (TLPs), which are known to be present in xylem sap (Rep et al., 2002; Kehr et al., 2005; Alvarez et al., 2006).

Protein Identification using Mass Spectrometry

Total xylem sap proteins were separated using SDS-PAGE and the lane containing the xylem sap proteins was excised and sliced into 15 gel segments. The proteins were digested overnight with trypsin and the resulting peptides were analyzed at the UVic Genome BC Proteomics Centre using a Q TRAP hybrid triple quadrupole/linear ion trap MS/MS Mass Spectrometer equipped with a nano-electrospray ionization source (Applied Biosystems/MDS Sciex) as previously described (Chapter 2). MS data was

processed with Analyst 1.4.1 software and integratedMascot script (1.6b16 ABI – Matrix Science Limited, Boston, MA, USA). Trypsin was selected as the digest enzyme and up to one missed cleavage was allowed. Carbamidomethyl cysteine was set as a fixed modification and variable modifications included oxidation of methionine and deamidation of asparagine and glutamine. Peptide tolerance and MS/MS tolerance were set at 0.5 Da and 0.3 Da, respectively. Parameters also permitted one missed cleavage and were limited to 2+ and 3+ charged peptides. A custom poplar database (Treenomix project, University of British Columbia) comprising both EST and genomic data was used for protein identification (Chapter 2). The sequence sources for the database were the most up-to-date as of October 2006. Protein identification with Mascot software was considered correct if the match had a score greater than 52, which is indicative of identity or significant ($p<0.05$) similarity, and had at least two peptide matches in which no other peptide sequence in the database gave a better score for that spectrum (Perkins et al., 1999). For each protein identified, SignalP 3.0 server was used to predict the presence of signal peptides (Nielsen et al., 1997; Bendtsen et al., 2004).

5.3 Results

Collection of xylem sap proteins

Xylem sap was collected using root pressure. The protein concentration of poplar xylem sap was estimated to be approximately 6 μg/mL. To further investigate the xylem sap proteins, they were separated using 2-DE (Fig. 5-1). The xylem sap protein pattern

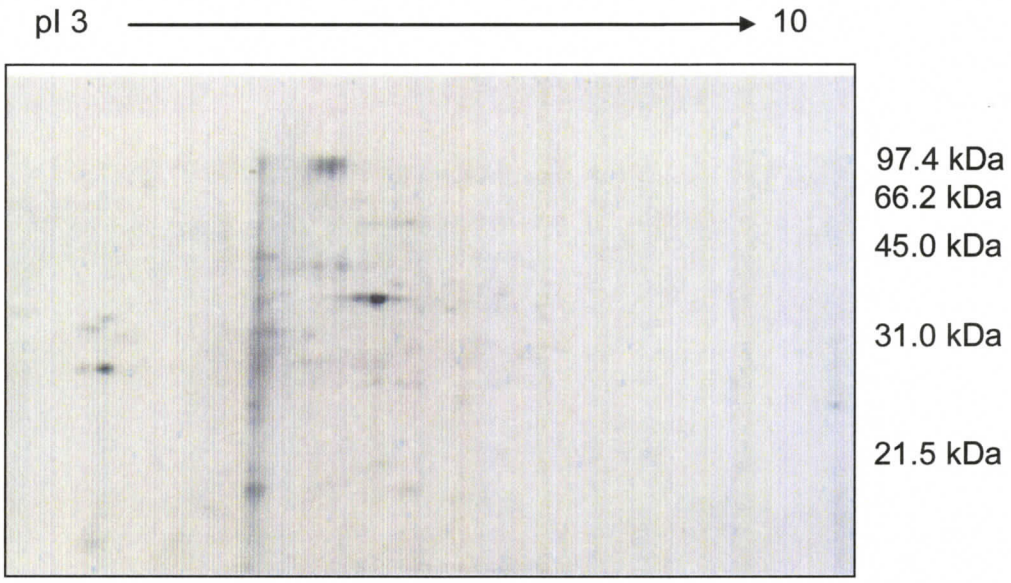


Figure 5-1. Characterization of the hybrid poplar xylem sap proteome using 2-DE.
Xylem sap proteins (4 μg) were separated by 2-DE and stained with Sypro Ruby.

was distinct from that of phloem exudate (Chapter 2). The phloem exudate proteins were acidic and smaller than 70 kDa whereas the xylem sap proteins were concentrated in the pH 4-8 range and their molecular weights were more evenly distributed from 10-100 kDa. In total, 130 xylem sap proteins could be visualized by 2-DE.

To verify the purity of our poplar xylem sap preparations and test for contamination, we used western blots to compare specific bark, phloem, and xylem sap proteins (Fig. 5-2). The vegetative storage protein, WIN4, was not detected in xylem sap, but a 35 kDa band corresponding to this protein could be detected in bark and phloem exudate (Fig. 5-2a). In contrast, the TLP and PPO antisera detected protein bands in all three samples (Fig. 5-2b, c). However, in xylem sap, proteins detected by anti-PPO and anti-TLP migrated at different molecular weights than the proteins recognized in phloem exudate and bark. As previously described in Chapter 3, the PtTLP1 antiserum recognized two proteins, a 23 kDa protein and 31 kDa protein, in bark and phloem exudate (Fig. 5-2c). The 23 kDa protein, which appears to be specific to cell walls (Chapter 3), was also labelled in xylem sap along with a xylem-specific 18 kDa protein. Likewise, the PPO antiserum recognized a 66 kDa protein in bark and phloem exudate likely corresponding to the PPO-2 protein previously described from stem, leaf and midveins (Wang and Constabel, 2003). However, a slightly larger, 68 kDa protein was detected in xylem sap and the 66 kDa band was absent (Fig. 5-2b). With all three antibodies, the major immunoreactive bands of phloem exudate and bark proteins are not found in the xylem sap preparations, and vice versa. These results are consistent with the distinct 2-DE protein profiles observed for all three samples. Together, this clearly

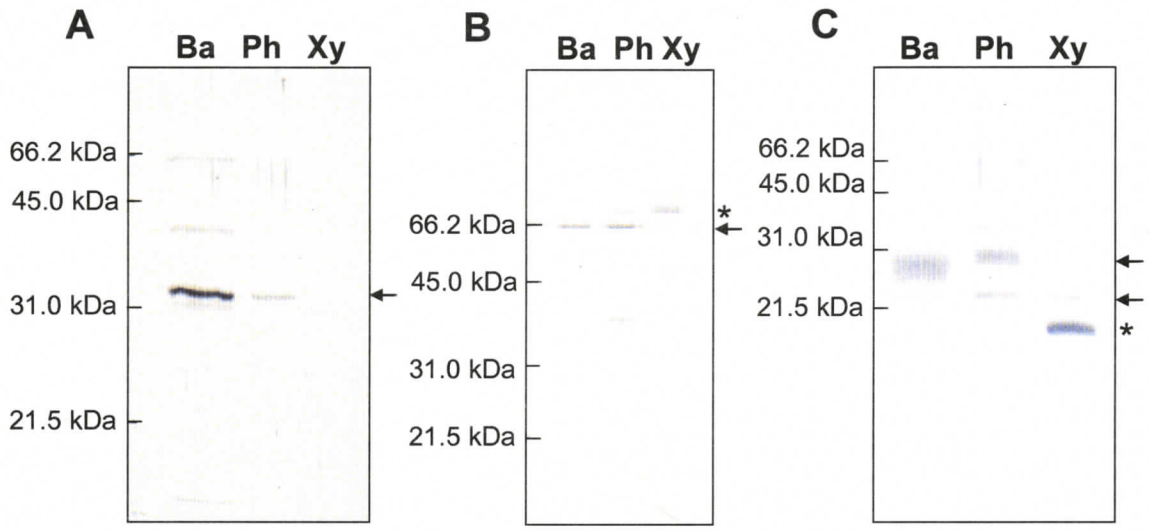


Figure 5-2. Comparison of bark, phloem exudate, and xylem sap proteins using western blots.

Comparison of xylem sap (Xy) to bark (Ba) and phloem exudate (Ph) proteins using western blots labelled with antibodies to A) WIN4, B) PPO, and C) TLP. For each sample, 10 μg of protein was loaded. Arrows indicate position of respective proteins and asterisks indicate the presence of xylem specific protein bands.

indicates that that poplar xylem sap was not contaminated from surrounding tissues, but likely contains a distinct set of proteins.

Identification of xylem sap proteins

To identify the xylem sap proteins, proteins were first separated by SDS-PAGE prior to digestion and subsequent analysis with LC-MS/MS. A total of 97 proteins were identified, 77 of which were unique (Table 5-1). With SignalP (Nielsen et al., 1997; Bendtsen et al., 2004), 33 of the 97 proteins were predicted to have a clear N-terminal signal peptide that targets them to the secretory pathway (Table 5-1).

By comparing xylem sap proteins to the translated *Arabidopsis* genome, corresponding *Arabidopsis* homologs were identified. Based on the Arabidopsis Information Resource (TAIR, www.arabidopsis.org), the gene ontology for these homologs was used to group the xylem sap proteins into 11 functional categories including cell wall, cytoskeleton, metabolism, signalling, stress response, and transport. Enzymes for primary metabolism represented over one-half of the proteins identified, which were further grouped into four separate categories: general metabolism, amino acid metabolism, proteolysis, and translation. The metabolic proteins included various proteases and several non-cell wall related glycoside hydrolases. Ten ribosomal proteins were also identified as well as several glycolytic enzymes. Other types of proteins identified include plastocyanin-like proteins, arabinogalactan-rich proteins, peroxidases, lipid transfer proteins, and several pathogenesis-related (PR) proteins including PR-1, chitinases, and TLPs. In summary, poplar xylem sap contains a diverse number of proteins, many of which do not have signal peptides.

Table 5-1. List of xylem sap proteins identified in *P. trichocarpa* x *P. deltoides*.

Identification	JGI Protein ID[a]	Theoretical MW	Theoretical pI	Mascot Score	No. of Matched Peptides[b]	Function[c]	SignalP[d]
Beta-Ig-H3/fasciclin	728480	28208	7.95	334	5	CW	y
Fasciclin-like arabinogalactan protein 10 precursor	730906	43247	5.37	160	3	CW	y
Caffeic acid 3-O-methyltransferase	834247	39732	5.48	375	12	CW	
Phenylcoumaran benzylic ether reductase 1	830063	33977	5.52	400	10	CW	
Multicopper oxidase	827894	66097	8.78	88	2	CW	y
Plant peroxidase	817694	37228	4.47	60	2	CW	y
Plant peroxidase	800693	34634	5.53	484	7	CW	y
Plant peroxidase	589413	36915	5.74	213	5	CW	y
Plant peroxidase	208491	32612	5.17	336	7	CW	
Plant peroxidase	267768	31983	6.97	141	2	CW	y
Plant peroxidase	767526	32575	6.37	157	3	CW	
UDP-glucose/GDP-mannose dehydrogenase	835719	52964	5.71	449	9	CW	
β-1,3-glucanase (glycoside hydrolase, family 17)	769807	38883	7.14	292	7	CW	y
Polygalacturonase (glycoside hydrolase, family 28)	644497	51463	5.88	711	13	CW	y
Actin and related proteins	813612	41693	5.31	240	5	CY	
Actin depolymerizing factor	829643	16019	5.95	107	2	CY	
Beta tubulin	180424	49470	4.8	518	10	CY	
Tubulin alpha-3 chain	707456	49668	4.93	324	8	CY	
3-phosphoglycerate kinase	659332	42677	5.7	590	11	ME	
Enolase	575698	47924	5.67	786	13	ME	
Fructose-biphosphate aldolase	578574	38382	6.69	542	11	ME	
Glyceraldehyde 3-phosphate dehydrogenase	575307	36768	7.16	560	11	ME	
Phosphoglucomutase	832763	63053	5.49	121	3	ME	
Triosephosphate isomerase	656103	27258	6.02	450	7	ME	
Triosephosphate isomerase	713199	27297	4.99	179	4	ME	
Zinc-containing alcohol dehydrogenase	421020	33585	6.04	549	10	ME	
6-phosphogluconate dehydrogenase	837012	53739	6.26	98	2	ME	y
Transaldolase	646955	47491	6.44	423	9	ME	
Aconitate hydratase	593790	98208	5.92	108	3	ME	
Malate dehydrogenase	564942	35714	6.13	350	8	ME	
NAD-dependent malate dehydrogenase	822907	35586	8.72	336	7	ME	

(Table continues on following page.)

Table 5-1. Continued

Identification	JGI Protein ID[a]	Theoretical MW	Theoretical pI	Mascot Score	No. of Matched Peptides[b]	Function[c]	SignalP[d]
NADP-dependent isocitrate dehydrogenase	678577	46391	6.31	255	6	ME	
dTDP-glucose 4-6-dehydratase/UDP-glucuronic acid decarboxylase	822488	39025	7.25	614	12	ME	
Fructokinase	741475	35270	4.99	261	5	ME	
Sucrose synthase	826368	92152	6.18	343	8	ME	
UDP-glucose pyrophosphorylase	592888	51588	5.79	270	7	ME	
Plastocyanin-like	669166	23233	9.26	92	4	ME	y
Plastocyanin-like	816369	18790	5.65	152	3	ME	y
D-arabinono-1, 4-lactone oxidase	809065	61700	7.6	210	5	ME	
F1 ATP synthase beta subunit	822103	59880	5.97	342	8	ME	
Alpha-L-arabinofuranosidase	255102	73315	5.46	319	6	ME	y
β-glucosidase (glycoside hydrolase, family 1)	181149	54812	5.6	329	6	ME	y
β-glucosidase (glycoside hydrolase, family 3)	799561	83410	5.74	119	3	ME	y
Glutamate decarboxylase/sphingosine phosphate lyase	648236	56332	5.79	243	6	AA	
Adenosylhomocysteinase	707148	53431	5.6	509	10	AA	
Glycine/serine hydroxymethyltransferase	829808	51907	7.26	223	4	AA	
Methylenetetrahydrofolate reductase	828552	67491	6.01	167	4	AA	
Vitamin-B12 independent methionine synthase	679841	84592	6.27	1304	25	AA	
Aspartyl protease	828515	55382	5.57	139	5	PR	y
Aspartyl protease	261465	46401	8.55	197	3	PR	y
Aspartyl protease	261478	46401	8.55	177	4	PR	y
Subtilisin-related protease/Vacuolar protease B	551801	82091	6.57	632	11	PR	y
Tripeptidyl peptidase II	559867	82272	8.4	66	2	PR	y
Plant basic secretory protein	549955	24995	7.18	418	8	PR	y
40S ribosomal protein S11	677637	17916	10.66	89	2	TL	
40S ribosomal protein S13	666912	17113	10.34	210	5	TL	
40S ribosomal protein S20	824872	13681	9.65	127	2	TL	
40S ribosomal protein S3	835769	26133	9.59	153	3	TL	
40S ribosomal protein S4	831930	29761	10.28	138	3	TL	
60S ribosomal protein L11	819383	20657	9.94	168	4	TL	
60S ribosomal protein L4	735444	44965	10.3	316	7	TL	
60S ribosomal protein L7	579008	33339	9.78	158	3	TL	
60S ribosomal protein L9	829935	21919	9.5	202	5	TL	

(Table continues on following page.)

Table 5-1. Continued

Identification	JGI Protein ID[a]	Theoretical MW	Theoretical pI	Mascot Score	No. of Matched Peptides[b]	Function[c]	SignalP[d]
Ribosomal protein S7	256059	22594	9.75	210	4	TL	
Seryl-tRNA synthetase	716082	51596	5.93	94	2	TL	
Endoribonuclease	829920	21680	8.76	135	3	TL	
Glycine-rich RNA binding protein	712972	16506	5.51	96	2	TL	
Adenosine kinase	659896	37544	5.09	89	2	SI	
Serine/threonine protein kinase	662643	22656	6.94	260	6	SI	y
Acid phosphatase	711542	29086	8.25	150	5	SI	y
Annexin	818283	36018	6.2	288	7	SI	
Cyclophilin type peptidyl-prolyl cis-trans isomerase	813818	18094	8.72	334	7	SI	
Multifunctional chaperone (14-3-3 family)	651251	29298	4.67	572	11	SI	
Multifunctional chaperone (14-3-3 family)	551637	28593	4.74	161	4	SI	
1,4-benzoquinone reductase-like	727757	21691	6.11	225	5	SR	
Dehydroascorbate reductase	833836	23630	5.77	128	3	SR	
Short-chain dehydrogenase/reductase SDR	833658	28294	7.7	232	3	SR	
Thioredoxin	818765	12575	5.58	216	6	SR	
Chitinase	572334	29937	7.04	157	3	SR	y
Chitinase	270686	27161	4.37	91	2	SR	y
Thaumatin, pathogenesis-related	669475	24733	7.83	118	2	SR	y
Thaumatin, pathogenesis-related	180318	25018	5.86	305	6	SR	y
Defense-related protein containing SCP/ PR-1 domain	595857	17257	8.54	184	3	SR	y
Kunitz trypsin inhibitor	746481	22837	8.97	224	6	SR	y
pop3.1	723969	12424	4.87	149	2	SR	
pop3.6	833676	12467	5.79	191	4	SR	
Molecular chaperones HSP70	832542	71169	5.09	588	12	SR	
Plant lipid transfer protein/Par allergen	414321	8435	6.27	100	2	TR	
Plant lipid transfer/seed storage/trypsin-alpha-amylase inhibitor	825296	11902	8.16	334	8	TR	y
Plant lipid transfer/seed storage/trypsin-alpha-amylase inhibitor	820375	12545	8.61	375	10	TR	y
Allene oxide cyclase	198984	26604	9.05	90	2	OT	
Cupin	591472	23999	7.13	246	5	OT	y

(Table continues on following page.)

Table 5-1. Continued

Identification	JGI Protein ID[a]	Theoretical MW	pI	Mascot Score	No. of Matched Peptides[b]	Function[c]	SignalP[d]
GTP-binding ADP-ribosylation factor Arf1	744433	20651	6.49	188	4	OT	
Translationally controlled tumor protein	725698	19066	4.46	110	2	OT	
Unknown	573059	18523	3.98	196	3	UN	
Unknown	565652	7377	9.3	200	4	UN	
Unknown (DUF 26)	562320	26390	5.78	229	5	UN	y

[a] Protein identification number was identified using the DOE Joint Genome Institute *P. trichocarpa* genome v1.1.

[b] The peptide sequences used for identification, in addition to their molecular weight, charge, and specific Mascot score can be accessed in Supplementary Table 5-1 found in Appendix 1.

[c] Abbreviations used for assigned functions are CW, cell wall; CY, cytoskeleton; ME, metabolism; AA, amino acid metabolism; PR, proteolysis; TL, translation; SI, signalling; SR, stress response; TR, transport; OT, other; and UN, unknown.

[d] Proteins predicted to have an N-terminal signal peptide are designated with a y. SignalP probability scores can be accessed in Supplementary Table 5-1.

5.4 Discussion

In this study, we carried out the first analysis of proteins in xylem sap of hybrid poplar. Over 100 xylem sap proteins were visualized with 2-DE and 97 of these proteins were identified using LC-MS/MS. Of the proteins identified, many are metabolic enzymes involved in a diverse array of processes including translation and proteolysis. In contrast to previous literature on other plant species, most poplar xylem sap proteins did not have an N-terminal signal peptide. These are not likely the result of contamination by surrounding damaged cells based on western blots confirming the absence of phloem exudate and bark proteins in our xylem sap sample. These proteins could, however, be due to cell death that occurs during tracheary element differentiation.

Xylem sap proteins identified in poplar are similar to those in annual plants

Three proteins, peroxidases, chitinases, and proteases, have been consistently reported in the xylem proteomes of annual plants (Buhtz et al., 2004; Kehr et al., 2005; Alvarez et al., 2006). Here, multiple isoforms for each of these proteins were found in poplar xylem sap. A total of six peroxidases, three aspartic proteases, and two chitinases were identified. Not all of these proteins had predicted N-terminal signal peptides; only three peroxidases and one aspartic protease and chitinase.

Peroxidases have a large number of potential biological functions (Hiraga et al., 2001). In xylem, the presence of peroxidases could be related to lignin biosynthesis. Lignin is an important component of the secondary cell walls that surround xylem tracheary elements (Plomion et al., 2001) and in poplar, several peroxidases that oxidize lignin monomers in the final stages of lignin biosynthesis have been identified (Christensen et al., 1998; Sasaki et al., 2006). The synthesis of lignin is not only essential

for xylem function, but it may serve as a physical barrier to invading pathogens (Hiraga et al., 2001). In response to pathogens, xylem peroxidases may also promote the synthesis of reactive oxygen species and phytoalexins (Hiraga et al., 2001). The remaining two types of xylem sap proteins, chitinases and proteases, may also function in plant defense. Chitinases break down chitin, a component of fungal cell walls (van Loon et al., 2006). The importance of xylem chitinases in plant defense is supported by a study in which a chitinase was induced in xylem sap of tomato plants infected by the vascular wilt fungus, *Fusarium oxysporum* (Rep et al., 2002). Aspartic proteases are also known to be induced after pathogen infection (Guevara et al., 2002; Xia et al., 2004; Guevara et al., 2005). These may have a direct inhibitory effect on pathogens (Guevara et al., 2002), but they may also be involved in producing a peptide signal that activates the plant defense response (Xia et al., 2004). Proteases are also known to accumulate in the vacuoles of tracheary elements prior to being released during programmed cell death (Turner et al., 2007). Upon release, these enzymes are involved destroying cell contents of developing tracheary elements. Several different types of proteases have been implicated in the development of tracheary elements, including an aspartic protease (Runeberg-Roos and Saarma, 1998); however, there is currently no evidence that these proteins are involved in programmed cell death.

A number of additional proteins, which have been previously identified in the xylem sap of annual plant species, were also identified in the current study. These include several additional defense proteins, including the PR proteins PR-1 and TLPs, and also cell wall proteins (arabinogalactan-rich proteins) and enzymes such as glycoside

hydrolases and polygalacturonase. Overall, the presence of these proteins in both annual and perennial plants suggests that they are important for functional tracheary elements.

Poplar xylem sap proteins and plant defense

One challenge that plants face is pathogen infection. Pathogen defense proteins have been previously detected in annual xylem sap and they were also identified in poplar xylem sap. The transcription of several of these poplar xylem sap proteins are known to increase in response to environmental stresses. For example, the PR-1 protein and both TLPs are known to be induced in *P. trichocarpa* x *P. deltoides* leaves infected with *Melampsora medusae* (Miranda et al., 2007; Rinaldi et al., 2007). In addition, the pop3/SP1 proteins and trypsin inhibitor are strongly induced in leaves in response to insect feeding (Major and Constabel, 2006; Ralph et al., 2006). Currently these studies have focused on transcription in leaves and therefore it will be interesting to determine if these proteins are differentially regulated in xylem sap, as has been shown for a number of xylem sap proteins in tomato and maize (Rep et al., 2002; Alvarez et al., 2008). It will also be important to determine if these proteins are active against pathogens like maize xylem sap proteins (Alvarez et al., 2006).

Poplar xylem sap proteins and tracheary element differentiation

One common feature shared by xylem sap proteins that have been previously identified is the presence of a N-terminal signal peptide that targets them to the secretory pathway (Buhtz et al., 2004; Kehr et al., 2005; Alvarez et al., 2006; Djordjevic et al., 2007). To date, only a few xylem sap proteins without signal peptides have been

identified; these proteins were considered either contamination from surrounding bark tissue or the result of proteins being released immediately after tracheary element cell death. Surprisingly, 64 of the poplar xylem sap proteins identified in this study did not have predicted signal peptides. If these proteins are not due to contamination as argued above, we propose that they are remnants of tracheary element differentiation, which is an idea that was previously proposed by Kehr et al. (Kehr et al., 2005). When the poplar shoot is severed, the positive root pressure may flush proteins from recently matured xylem vessels. This effect may be particularly exaggerated for perennial plants that undergo extensive secondary xylem growth, including in roots. The potential number of tracheary elements undergoing maturation is much greater compared to annual plants and this may explain the different set of proteins identified in poplar xylem sap as compared to annual plants.

There are several stages in tracheary element differentiation, cell division followed by cell expansion and elongation, formation of the secondary cell wall, and finally maturation involving programmed cell death (Plomion et al., 2001). In poplar, the tissues corresponding to these various stages have been dissected and analyzed for gene expression (Sterky et al., 1998; Hertzberg et al., 2001; Dejardin et al., 2004). Based on these studies, protein biosynthetic enzymes, including ribosomal proteins, are strongly expressed during cell division and cell expansion. Although transcription of ribosomal proteins decreases during the later stages of tracheary element differentiation (Hertzberg et al., 2001; Dejardin et al., 2004), it appears that active transcription and translation is still occuring and it is required for secondary cell wall thickening and the disruption of the cell vacuole. Ten different ribosomal proteins were identified in the current study.

After cell expansion is complete, developing tracheary elements begin to form secondary cell walls (Hertzberg et al., 2001), which consists of cellulose, hemicellulose, and lignin (Plomion et al., 2001). The formation of the secondary cell wall during the later stages of tracheary element differentiation is correlated to an increase in the expression of cell wall biosynthetic enzymes (Allona et al., 1998; Sterky et al., 1998; Hertzberg et al., 2001; Yang et al., 2004; Juan et al., 2006). Some of these enzymes are involved in sugar metabolism such as sucrose synthase, UDP-glucose pyrophosphorylase, and UDP-glucose dehydrogenase (Robertson et al., 1996; Hertzberg et al., 2001; Johansson et al., 2002; Meng et al., 2007). Sucrose synthase and UDP-glucose pyrophosphorylase are both capable of producing UDP-glucose, a precursor for cellulose synthesis (Hertzberg et al., 2001; Meng et al., 2007) and UDP-glucose dehydrogenase is involved in the synthesis of hemicellulose and pectin (Hertzberg et al., 2001; Johansson et al., 2002). All three proteins were identified in poplar xylem sap. Tubulin and actin were also identified in poplar xylem sap and these cytoskeletal proteins are known to have an important role in secondary cell wall formation. In poplar, tubulin has been localized to xylem vessels undergoing secondary cell wall thickening (Chaffey et al., 2002; Oakley et al., 2007) and these proteins appear to be involved in regulating secondary cell wall deposition, whereas actin is involved in transporting cell wall components (Turner et al., 2007).

Glycolytic enzymes represent the majority of the remaining poplar xylem sap proteins without a signal peptide. Glycolysis is important for producing ATP and carbon compounds (Plaxton, 1996) that may be incorporated into tracheary element cell walls. Interestingly, glycolytic enzymes, including triose phosphate isomerase and

glyceraldehyde phosphate dehydrogenase, have also been detected in rice and soybean xylem sap (Djordjevic et al., 2007; Aki et al., 2008). In addition, several of these enzymes have been localized to secondary cell wall thickenings of tracheary elements in pea leaves (Anderson and Carol, 2004). Overall, further research is needed to determine whether poplar xylem sap proteins, without signal peptides, are the just the result of incomplete degradation during development of xylem vessels or if the plant is utilizing these proteins for another purpose.

6 General discussion

The overall goal of this study was to identify phloem and xylem sap proteins in poplar. Phloem sap does not readily exude from poplars therefore a method was developed using EDTA to facilitate the collection of phloem exudate (Chapter 2). Based on 2-DE, over 100 proteins were estimated to be present in poplar phloem exudate and the identity of 48 of these proteins were determined using a combination of SDS-PAGE and LC-MS/MS. A large number of phloem exudate proteins identified were stress-responsive proteins including antioxidant enzymes, insect defense proteins such as PIs and PPO, and pathogenesis-related proteins including TLPs and chitinases.

Unlike phloem sap, xylem sap can easily be collected using root pressure (Chapter 5). Approximately 130 poplar xylem sap proteins were visualized with 2-DE and 97 were successfully identified using LC-MS/MS. Over one-half of the proteins identified were metabolic enzymes involved in various biological processes including cell wall metabolism, glycolysis, translation and proteolysis.

The second goal of this thesis was to identify and characterize phloem exudate proteins that potentially function in plant defense by screening for wound-induced phloem proteins. Poplar phloem exudate proteins were analyzed 24 h post-leaf wounding (Chapter 2). At this time point, two proteins were induced in response to wounding, providing indirect evidence that these proteins may be involved in plant defense. These proteins were subsequently identified as pop3.1 and PtTLP1. Pop3.1 is orthologous to *P. tremula* SP1 and PtTLP1 is a thaumatin-like protein. Genes encoding both proteins are known to be induced in response to abiotic and biotic stresses (Wang et al., 2002; Gu et al., 2004; Major and Constabel, 2006; Ralph et al., 2006; van Loon et al., 2006).

Antibodies were produced for PtTLP1 (Chapter 2) and pop3.1 (Chapter 4). Antibodies were also produced for another known, stress-inducible protein, pop3.4 (Chapter 3), which is 40% similar to pop3.1. Like pop3.1, pop3.4 was identified in phloem exudate, but it did not appear to be induced in the 24 h wounding experiment. All three proteins were localized to starch granules inside sieve elements of mature stem tissue confirming the origin of these phloem exudate proteins (Chapters 2-4). These granules are most likely remnants of starch-containing plastids that are typically attached to the endoplasmic reticulum of sieve elements (Knoblauch and van Bel, 1998). Since pop3.1, pop3.4, and PtTLP1 appear to be localized to these plastids, they are presumably not transported in sieve elements, however, when these cells are wounded in the exudate collection process, the starch-containing plastids will rupture releasing the proteins into the translocation stream (Knoblauch and van Bel, 1998). Hence, phloem exudate proteins actually appear to be a collection of phloem-specific soluble proteins and proteins from ruptured sieve element plastids as previously suggested (van Bel et al., 2002)

Pop3.1, pop3.4, and PtTLP1 were associated with plastids not only in sieve elements, but also in phloem parenchyma cells (Chapters 3 and 4). These results are unexpected since these proteins do not have predicted transit peptides targeting them to plastids. However, a recent analysis of the chloroplast proteome in *Arabidopsis* indicated that only 86% of plastid proteins have a predicted transit peptide (Zybailov et al., 2008). Likewise, all three proteins were also detected in cell walls of various cell types, but only PtTLP1 had an N-terminal signal peptide targeting it to the secretory pathway (Chapters 2 and 3). Pop3.1 and pop3.4 may therefore utilize alternate secretory pathways. For

example, some mammalian proteins are secreted without an N-terminal signal peptides (Bendtsen et al., 2004).

6.1 Significance

This study is significant because xylem and phloem proteomes have not been previously studied in a perennial. A number of proteins identified in this thesis have been previously identified in phloem and xylem sap of annual plants, which supports the hypothesis that these proteins have important roles in these tissues. However, many novel phloem and xylem sap proteins were also identified in poplar. Unexpectedly, pathogenesis-related (PR) proteins such as TLPs, chitinases, and β-1,3-glucanases were identified in phloem exudate. These proteins have been previously identified in xylem sap (Rep et al., 2002; Buhtz et al., 2004; Kehr et al., 2005; Alvarez et al., 2006; Djordjevic et al., 2007; Aki et al., 2008; Alvarez et al., 2008), but their presence in phloem sap is not well documented. In fact, several PR proteins were only recently detected in rice phloem sap, but not TLPs or chitinases (Aki et al., 2008). The presence of PR proteins in phloem sap has been considered a result of contamination from apoplastic fluids, but immulocalization of TLPs confirmed that at least some of these proteins are bona fide constituents of phloem sap (Chapters 2-3).

Novel xylem sap proteins consist of cytoskeleton proteins and metabolic enzymes, including several ribosomal proteins. Unlike previous studies, these poplar xylem sap proteins do not have N-terminal signal peptides targeting them to the secretory pathway. These results are significant because it raises questions regarding the origin of xylem sap proteins. Originally it was predicted that xylem sap proteins were synthesized in the roots, secreted into the apoplast, and then transported throughout the plant (Sakuta

et al., 1998; Masuda et al., 1999; Sakuta and Satoh, 2000; Masuda et al., 2001). My results, however, contradict this hypothesis. As described above, it is possible that the xylem sap proteins identified in this thesis do not use the traditional secretory pathway, but is also possible that these xylem sap proteins were flushed from recently matured xylem vessels and they are the result of incomplete proteolysis during xylogenesis. If they are an artifact of tracheary element differentiation as predicted, this raises questions regarding the function of these proteins. Under natural conditions, would these proteins normally be degraded or does the plant utilize these proteins for other purposes in mature tracheary elements?

To my knowledge, this thesis also presents the first report of wound-inducible phloem exudate proteins. The role of phloem transport in the systemic defense response have been clearly established (Davis et al., 1991; Orians et al., 2000; Schittko and Baldwin, 2003; Viswanathan and Thaler, 2004), but it is not known if phloem proteins are involved. The inducibility of two poplar phloem exudate proteins provides indirect evidence that phloem exudate proteins may also have a role in the systemic defense response.

6.2 Future directions

The results presented in this thesis represent only a first characterization of the poplar phloem and xylem sap proteomes. Although 48 and 97 proteins were successfully identified in poplar phloem and xylem sap, respectively, a much larger number of proteins were estimated to be present in both samples. Therefore additional sequencing experiments will be needed to provide a more comprehensive list of poplar phloem and

xylem proteins. In addition, since contamination from the apoplast can not be ruled out for poplar phloem exudate, one could collect poplar phloem sap from aphid stylets to avoid artifacts that are sometimes observed with EDTA (Gaupels et al., 2008). Although technically difficult, this method would provide pure phloem sap, which could then be used to verify the presence of proteins identified in this thesis.

Of the proteins that have been identified, the challenge is to characterize these proteins and identify their function within the vascular system. By identifying wound-inducible phloem exudate proteins, my research indicated that phloem exudate proteins may function in plant defense. My research, however, is limited to a single time point and a single stress response. It will be important to study phloem exudate proteins at multiple time points after mechanically wounding leaves to possibly identify additional proteins that are involved in the systemic defense response. It will also be interesting to study the expression of these proteins in response to other stresses including pathogen infection or nutrient deficiencies.

As part of this research, I began characterizing three phloem exudate proteins, pop3.1, pop3.4 and PtTLP1. This thesis provides an overview of the expression and localization of these proteins, but further research is still needed in order to fully comprehend how they may function within the phloem and in poplar. Biologically, pop3.1 and PtTLP1 appear to function in defense, but because the biochemical activity of these proteins has not yet been determined it is difficult to understand what role, if any that these proteins may have in the systemic defense response. To determine a biochemical function, these proteins could be purified and used for activity assays. In addition, these proteins could be overexpressed or knocked-out in poplar and these plants

could be used for insect-feeding assays in order to determine if they negatively affect insects.

Overall, my research provides the foundation for future studies of poplar phloem and xylem sap proteins. This is an area of research that has not been previously pursued and therefore there are limitless opportunities for additional research.

References

Aki T, Shigyo M, Nakano R, Yoneyama T, Yanagisawa S (2008) Nano scale proteomics revealed the presence of regulatory proteins including three FT-Like proteins in phloem and xylem saps from rice. Plant and Cell Physiology 49: 767-790

Allona I, Quinn M, Shoop E, Swope K, St Cyr S, Carlis J, Riedl J, Retzel E, Campbell MM, Sederoff R, Whetten RW (1998) Analysis of xylem formation in pine by cDNA sequencing. Proceedings of the National Academy of Sciences USA 95: 9693-9698

Alvarez S, Goodger JQD, Marsh EL, Chen SX, Asirvatham VS, Schachtman DP (2006) Characterization of the maize xylem sap proteome. Journal of Proteome Research 5: 963-972

Alvarez S, Marsh EL, Schroeder SG, Schachtman DP (2008) Metabolomic and proteomic changes in the xylem sap of maize under drought. Plant Cell and Environment 31: 325-340

Amiard W, Morvan-Bertrand A, Cliquet JB, Billard JP, Huault C, Sandstrom JP, Prud'homme MP (2004) Carbohydrate and amino acid composition in phloem sap of *Lolium perenne* L. before and after defoliation. Canadian Journal of Botany 82: 1594-1601

Anderson LE, Carol AA (2004) Seven enzymes of carbon metabolism, including three Calvin cycle isozymes, are present in the secondary cell wall thickenings of the developing xylem tracheary elements in pea leaves. International Journal of Plant Sciences 165: 243-256

Anzlovar S, Dermastia M (2003) The comparative analysis of osmotins and osmotin-like PR-5 proteins. Plant Biology 5: 116-124

Aoki K, Suzui N, Fujimaki S, Dohmae N, Yonekura-Sakakibara K, Fujiwara T, Hayashi H, Yamaya T, Sakakibara H (2005) Destination-selective long-distance movement of phloem proteins. Plant Cell 17: 1801-1814

Arakawa T, Hung L, Narhi LO (1992) Stability of fungal alpha-amylase in sodium dodecyl-sulfate. Journal of Protein Chemistry 11: 111-117

Arnold T, Appel H, Patel V, Stocum E, Kavalier A, Schultz J (2004) Carbohydrate translocation determines the phenolic content of *Populus* foliage: a test of the sink-source model of plant defense. New Phytologist 164: 157-164

Arnold TM, Schultz JC (2002) Induced sink strength as a prerequisite for induced tannin biosynthesis in developing leaves of *Populus*. Oecologia 130: 585-593

Avdiushko SA, Ye XS, Kuc J, Hildebrand DF (1994) Lipoxygenase is an abundant protein in cucumber exudates. Planta 193: 349-357

Babst BA, Ferrieri RA, Gray DW, Lerdau M, Schlyer DJ, Schueller M, Thorpe MR, Orians CM (2005) Jasmonic acid induces rapid changes in carbon transport and partitioning in *Populus*. New Phytologist 167: 63-72

Barnes A, Bale J, Constantinidou C, Ashton P, Jones A, Pritchard J (2004) Determining protein identity from sieve element sap in *Ricinus communis* L. by quadrupole time of flight (Q-TOF) mass spectrometry. Journal of Experimental Botany 55: 1473-1481

Batalia MA, Monzingo AF, Ernst S, Roberts W, Robertus JD (1996) The crystal structure of the antifungal protein zeamatin, a member of the thaumatin-like, PR-5 protein family. Nature Structural Biology 3: 19-23

Bendtsen JD, Jensen LJ, Blom N, von Heijne G, Brunak S (2004) Feature-based prediction of non-classical and leaderless protein secretion. Protein Engineering Design & Selection 17: 349-356

Bendtsen JD, Nielsen H, von Heijne G, Brunak S (2004) Improved prediction of signal peptides: SignalP 3.0. Journal of Molecular Biology 340: 783-795

Biles CL, Abeles FB (1991) Xylem sap proteins. Plant Physiology 96: 597-601

Bogeat-Triboulot MB, Brosche M, Renaut J, Jouve L, Le Thiec D, Fayyaz P, Vinocur B, Witters E, Laukens K, Teichmann T, Altman A, Hausman JF, Polle A, Kangasjarvi J, Dreyer E (2007) Gradual soil water depletion results in reversible changes of gene expression, protein profiles, ecophysiology, and growth performance in *Populus euphratica*, a poplar growing in arid regions. Plant Physiology 143: 876-892

Bradford MM (1976) Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Analytical Biochemistry 72: 248-254

Bradshaw HD, Ceulemans R, Davis J, Stettler R (2000) Emerging model systems in plant biology: poplar (*Populus*) as a model forest tree. Journal of Plant Growth Regulation 19: 306-313

Buchanan BB, Gruissem W, Jones RL (2000) Biochemistry & molecular biology of plants. American Society of Plant Physiologists, Rockville, Md.

Buhtz A, Kolasa A, Arlt K, Walz C, Kehr J (2004) Xylem sap protein composition is conserved among different plant species. Planta 219: 610-618

Cacciapuoti G, Forte S, Moretti MA, Brio A, Zappia V, Porcelli M (2005) A novel hyperthermostable 5 '-deoxy-5 '-methylthioadenosine phosphorylase from the archaeon *Sulfolobus solfataricus*. FEBS Journal 272: 1886-1899

Cacciapuoti G, Gorassini S, Mazzeo MF, Siciliano RA, Carbone V, Zappia V, Porcelli M (2007) Biochemical and structural characterization of mammalian-like purine nucleoside phosphorylase from the archaeon *Pyrococcus furiosus*. FEBS Journal 274: 2482-2495

Campbell JA, Hansen RW, Wilson JR (1999) Cost-effective colorimetric microtitre plate enzymatic assays for sucrose, glucose and fructose in sugarcane tissue extracts. Journal of the Science of Food and Agriculture 79: 232-236

Chaffey N, Barlow P, Barnett J (2000) Structure-function relationships during secondary phloem development in an angiosperm tree, *Aesculus hippocastanum*: microtubules and cell walls. Tree Physiology 20: 777-786

Chaffey N, Barlow P, Sundberg B (2002) Understanding the role of the cytoskeleton in wood formation in angiosperm trees: hybrid aspen (*Populus tremula* x *P. tremuloides*) as the model species. Tree Physiology 22: 239-249

Chaffey NJ, Barnett JR, Barlow PW (1997) Visualization of the cytoskeleton within the secondary vascular system of hardwood species. Journal of Microscopy 187: 77-84

Chen S, Andreasson E (2001) Update on glucosinolate metabolism and transport. Plant Physiology and Biochemistry 39: 743-758

Chen XY, Kim JY (2006) Transport of macromolecules through plasmodesmata and the phloem. Physiologia Plantarum 126: 560-571

Christensen JH, Bauw G, Welinder KG, Van Montagu M, Boerjan W (1998) Purification and characterization of peroxidases correlated with lignification in poplar xylem. Plant Physiology 118: 125-135

Christopher ME, Miranda M, Major IT, Constabel CP (2004) Gene expression profiling of systemically wound-induced defenses in hybrid poplar. Planta 219: 936-947

Clendennen SK, May GD (1997) Differential gene expression in ripening banana fruit. Plant Physiology 115: 463-469

Close TJ, Kortt AA, Chandler PM (1989) A cDNA-based comparison of dehydration-induced proteins (dehydrins) in barley and corn. Plant Molecular Biology 13: 95-108

Constabel CP, Barbehenn R (2008) Defensive roles of polyphenol oxidase in plants. *In* A Schaller, ed, Induced Plant Resistance to Herbivory. Springer Netherlands, pp 253-270

Constabel CP and Major IT (2005) Molecular biology and biochemistry of induced insect defense in *Populus*. *In* Chemical Ecology and Phytochemistry of Forest Ecosystems, Recent Advances in Phytochemistry Vol 39. Elsevier Amsterdam, pp119-143

Constabel CP, Yip L, Patton JJ, Christopher ME (2000) Polyphenol oxidase from hybrid poplar. Cloning and expression in response to wounding and herbivory. Plant Physiology 124: 285-295

Cornilescu G, Cornilescu CC, Zhao Q, Frederick RO, Peterson FC, Thao S, Markley JL (2004) Letter to the Editor: Solution structure of a homodimeric hypothetical protein, At5g22580, a structural genomics target from *Arabidopsis thaliana*. Journal of Biomolecular NMR 29: 387-390

Dannenhoffer JM, Suhr RC, Thompson GA (2001) Phloem-specific expression of the pumpkin fruit trypsin inhibitor. Planta 212: 155-162

Davis JM, Egelkrout EE, Coleman GD, Chen THH, Haissig BE, Riemenschneider DE, Gordon MP (1993) A family of wound-induced genes in *Populus* shares common features with genes encoding vegetative storage proteins. Plant Molecular Biology 23: 135-143

Davis JM, Gordon MP, Smit BA (1991) Assimilate movement dictates remote sites of wound-induced gene-expression in poplar leaves. Proceedings of the National Academy of Sciences USA 88: 2393-2396

Dejardin A, Leple JC, Lesage-Descauses MC, Costa G, Pilate G (2004) Expressed sequence tags from poplar wood tissues - a comparative analysis from multiple libraries. Plant Biology 6: 55-64

Dgany O, Gonzalez A, Sofer O, Wang WX, Zolotnitsky G, Wolf A, Shoham Y, Altman A, Wolf SG, Shoseyov O, Almog O (2004) The structural basis of the

thermostability of SP1, a novel plant (*Populus tremula*) boiling stable protein. Journal of Biological Chemistry 279: 51516-51523

Dickmann DI, Isebrands JG, Eckenwalder JE, Richardson J (2001) Poplar Culture in North America. NRC Research Press, Ottawa, Ontario, Canada

Dickson RE (1979) Xylem Translocation of amino acids from roots to shoots in cottonwood plants. Canadian Journal of Forest Research 9: 374-378

Djordjevic MA, Oakes M, Li DX, Hwang CH, Hocart CH, Gresshoff PM (2007) The *Glycine max* xylem sap and apoplast proteome. Journal of Proteome Research 6: 3771-3779

Domon B, Aebersold R (2006) Review - mass spectrometry and protein analysis. Science 312: 212-217

Dore I, Legrand M, Cornelissen BJC, Bol JF (1991) Subcellular localization of acidic and basic PR proteins in tobacco mosaic virus-infected tobacco. Archives of Virology 120: 97-107

Escher P, Eiblmeier M, Hetzger I, Rennenberg H (2004) Seasonal and spatial variation of carbohydrates in mistletoes (*Viscum album*) and the xylem sap of its hosts (*Populus x euamericana* and *Abies alba*). Physiologia Plantarum 120: 212-219

Evert RF (2006) Esau's plant anatomy: meristems, cells, and tissues of the plant body: their structure, function, and development, Ed 3rd. John Wiley & Sons, Inc., Hoboken, New Jersey

Ferreira S, Hjerno K, Larsen M, Wingsle G, Larsen P, Fey S, Roepstorff P, Pais MS (2006) Proteome profiling of *Populus euphratica* Oliv. upon heat stress. Annals of Botany 98: 361-377

Fierens E, Rombouts S, Gebruers K, Goesaert H, Brijs K, Beaugrand J, Volckaert G, Van Campenhout S, Proost P, Courtin CM, Delcour JA (2007) TLXI, a novel type of xylanase inhibitor from wheat (*Triticum aestivum*) belonging to the thaumatin family. Biochemical Journal 403: 583-591

Fife JM, Price C, Fife DC (1962) Some properties of phloem exudate collected from root of sugar beet. Plant Physiology 37: 791-792

Fils-Lycaon BR, Wiersma PA, Eastwell KC, Sautiere P (1996) A cherry protein and its gene, abundantly expressed in ripening fruit, have been identified as thaumatin-like. Plant Physiology 111: 269-273

Fisher DB, Wu Y, Ku MSB (1992) Turnover of soluble-proteins in the wheat sieve tube. Plant Physiology 100: 1433-1441

Frendo P, Didierjean L, Passelegue E, Burkard G (1992) Abiotic stresses induce a thaumatin-like protein in maize - cDNA isolation and sequence analysis. Plant Science 85: 61-69

Friedrich L, Moyer M, Ward E, Ryals J (1991) Pathogenesis-related protein 4 is structurally homologous to the carboxy-terminal domains of hevein, win-1 and win-2. Molecular & General Genetics 230: 113-119

Gao LL, Anderson JP, Klingler JP, Nair RM, Edwards OR, Singh KB (2007) Involvement of the octadecanoid pathway in bluegreen aphid resistance in *Medicago truncatula*. Molecular Plant-Microbe Interactions 20: 82-93

Gao ZS, van de Weg WE, Schaart JG, van Arkel G, Breiteneder H, Hoffmann-Sommergruber K, Gilissen L (2005) Genomic characterization and linkage mapping of the apple allergen genes Mal d 2 (thaumatin-like protein) and Mal d 4 (profilin). Theoretical and Applied Genetics 111: 1087-1097

Gatehouse AMR, Down RE, Powell KS, Sauvion N, Rahbe Y, Newell CA, Merryweather A, Hamilton WDO, Gatehouse JA (1996) Transgenic potato plants with enhanced resistance to the peach-potato aphid *Myzus persicae*. Entomologia Experimentalis Et Applicata 79: 295-307

Gaupels F, Knauer T, van Bel AJE (2008) A combinatory approach for analysis of protein sets in barley sieve-tube samples using EDTA-facilitated exudation and aphid stylectomy. Journal of Plant Physiology 165: 95-103

Geigenberger P, Langenberger S, Wilke I, Heineke D, Heldt HW, Stitt M (1993) Sucrose is metabolized by sucrose synthase and glycolysis within the phloem complex of *Ricinus communis* L seedlings. Planta 190: 446-453

Gessler A, Rennenberg H, Keitel C (2004) Stable isotope composition of organic compounds transported in the phloem of European beech - evaluation of different methods of phloem sap collection and assessment of gradients in carbon isotope composition during leaf-to-stem transport. Plant Biology 6: 721-729

Giavalisco P, Kapitza K, Kolasa A, Buhtz A, Kehr J (2006) Towards the proteome of *Brassica napus* phloem sap. Proteomics 6: 896-909

Giovanini MP, Saltzmann KD, Puthoff DP, Gonzalo M, Ohm HW, Williams CE (2007) A novel wheat gene encoding a putative chitin-binding lectin is associated with resistance against Hessian fly. Molecular Plant Pathology 8: 69-82

Giri AP, Wunsche H, Mitra S, Zavala JA, Muck A, Svatos A, Baldwin IT (2006) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. VII. Changes in the plant's proteome. Plant Physiology 142: 1621-1641

Girousse C, Bonnemain JL, Delrot S, Bournoville R (1991) Sugar and amino-acid-composition of phloem sap of *Medicago sativa* - a comparative study of 2 collecting methods. Plant Physiology and Biochemistry 29: 41-48

Golecki B, Schulz A, Carstens-Behrens U, Kollmann R (1998) Evidence for graft transmission of structural phloem proteins or their precursors in heterografts of *Cucurbitaceae*. Planta 206: 630-640

Gomez G, Torres H, Pallas V (2005) Identification of translocatable RNA-binding phloem proteins from melon, potential components of the long-distance RNA transport system. Plant Journal 41: 107-116

Gould GG, Jones CG, Rifleman P, Perez A, Coleman JS (2007) Variation in eastern cottonwood (*Populus deltoides* Bartr.) phloem sap content caused by leaf development may affect feeding site selection behavior of the aphid, *Chaitophorous populicola* Thomas (Homoptera : Aphididae). Environmental Entomology 36: 1212-1225

Green TR, Ryan CA (1972) Wound-induced proteinase inhibitor in plant leaves - possible defense mechanism against insects. Science 175: 776-777

Grenier J, Potvin C, Trudel J, Asselin A (1999) Some thaumatin-like proteins hydrolyse polymeric beta-1,3-glucans. Plant Journal 19: 473-480

Gu RS, Fonseca S, Puskas LG, Hackler L, Zvara A, Dudits D, Pais MS (2004) Transcript identification and profiling during salt stress and recovery of *Populus euphratica*. Tree Physiology 24: 265-276

Guevara MG, Almeida C, Mendieta JR, Faro CJ, Verissimo P, Pires EV, Daleo GR (2005) Molecular cloning of a potato leaf cDNA encoding an aspartic protease (StAsp) and its expression after *P. infestans* infection. Plant Physiology and Biochemistry 43: 882-889

Guevara MG, Oliva CR, Huarte M, Daleo GR (2002) An aspartic protease with antimicrobial activity is induced after infection and wounding in intercellular fluids of potato tubers. European Journal of Plant Pathology 108: 131-137

Haebel S, Kehr J (2001) Matrix-assisted laser desorption/ionization time of flight mass spectrometry peptide mass fingerprints and post source decay: a tool for the identification and analysis of phloem proteins from *Cucurbita maxima* Duch. separated by two-dimensional polyacrylamide gel electrophoresis. Planta 213: 586-593

Harlow E, Lane D (1999) Using antibodies: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Haruta M, Major IT, Christopher ME, Patton JJ, Constabel CP (2001) A Kunitz trypsin inhibitor gene family from trembling aspen (*Populus tremuloides* Michx.): cloning, functional expression, and induction by wounding and herbivory. Plant Molecular Biology 46: 347-359

Hause B, Hause G, Kutter C, Miersch O, Wasternack C (2003) Enzymes of jasmonate biosynthesis occur in tomato sieve elements. Plant and Cell Physiology 44: 643-648

Herschbach C, Jouanin L, Rennenberg H (1998) Overexpression of gamma-glutamylcysteine synthetase, but not of glutathione synthetase, elevates glutathione allocation in the phloem of transgenic poplar trees. Plant and Cell Physiology 39: 447-451

Hertzberg M, Aspeborg H, Schrader J, Andersson A, Erlandsson R, Blomqvist K, Bhalerao R, Uhlen M, Teeri TT, Lundeberg J, Sundberg B, Nilsson P, Sandberg G (2001) A transcriptional roadmap to wood formation. Proceedings of the National Academy of Sciences USA 98: 14732-14737

Hiraga S, Sasaki K, Ito H, Ohashi Y, Matsui H (2001) A large family of class III plant peroxidases. Plant and Cell Physiology 42: 462-468

Hoad GV (1995) Transport of hormones in the phloem of higher-plants. Plant Growth Regulation 16: 173-182

Hoffmann-Benning S, Gage DA, McIntosh L, Kende H, Zeevaart JAD (2002) Comparison of peptides in the phloem sap of flowering and non-flowering *Perilla* and lupine plants using microbore HPLC followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Planta 216: 140-147

Hong JK, Jung HW, Lee BK, Lee SC, Lee YK, Hwang BK (2004) An osmotin-like protein gene, CAOSM1, from pepper: differential expression and in situ localization of its mRNA during pathogen infection and abiotic stress. Physiological and Molecular Plant Pathology 64: 301-310

Hopkins WG (1995) Introduction to plant physiology. J. Wiley, New York

Hu HN, Penn SG, Lebrilla CB, Brown PH (1997) Isolation and characterization of soluble boron complexes in higher plants - the mechanism of phloem mobility of boron. Plant Physiology 113: 649-655

Imlau A, Truernit E, Sauer N (1999) Cell-to-cell and long-distance trafficking of the green fluorescent protein in the phloem and symplastic unloading of the protein into sink tissues. Plant Cell 11: 309-322

Jeun YC, Buchenauer H (2001) Infection structures and localization of the pathogenesis-related protein AP24 in leaves of tomato plants exhibiting systemic acquired resistance against *Phytophthora infestans* after pre-treatment with 3-aminobutyric acid or tobacco necrosis virus. Journal of Phytopathology 149: 141-153

Johansson H, Sterky F, Amini B, Lundeberg J, Kleczkowski LA (2002) Molecular cloning and characterization of a cDNA encoding poplar UDP-glucose dehydrogenase, a key gene of hemicellulose/pectin formation. Biochimica Et Biophysica Acta 1576: 53-58

Jones CG, Hopper RF, Coleman JS, Krischik VA (1993) Control of systemically induced herbivore resistance by plant vascular architecture. Oecologia 93: 452-456

Juan D, Hong-Li X, De-Qiang Z, Xin-Qiang H, Min-Jie W, Ying-Zhang L, Ke-Ming C, Meng-Zhu L (2006) Regeneration of the secondary vascular system in poplar as a novel system to investigate gene expression by a proteomic approach. Proteomics 6: 881-895

Karnovsky MJ (1965) A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. Journal of Cell Biology 27: 137A-138A

Kavroulakis N, Papadopoulou KK, Ntougias S, Zervakis GI, Ehaliotis C (2006) Cytological and other aspects of pathogenesis-related gene expression in tomato plants grown on a suppressive compost. Annals of Botany 98: 555-564

Kehr J (2006) Phloem sap proteins: their identities and potential roles in the interaction between plants and phloem-feeding insects. Journal of Experimental Botany 57: 767-774

Kehr J, Buhtz A, Giavalisco P (2005) Analysis of xylem sap proteins from *Brassica napus*. BMC Plant Biology 5: 11

Kempema LA, Cui XP, Holzer FM, Walling LL (2007) *Arabidopsis* transcriptome changes in response to phloem-feeding silverleaf whitefly nymphs. Similarities and distinctions in responses to aphids. Plant Physiology 143: 849-865

Kennecke M, Ziegler H, Fekete M (1971) Enzyme activities in sieve tube sap of *Robinia pseudoacacia* L and of other 3 species. Planta 98: 330-356

Khanna-Chopra R, Sabarinath S (2004) Heat-stable chloroplastic Cu/Zn superoxide dismutase in *Chenopodium murale*. Biochemical and Biophysical Research Communications 320: 1187-1192

King RW, Zeevaart JA (1974) Enhancement of phloem exudation from cut petioles by chelating-agents. Plant Physiology 53: 96-103

Klarzynski O, Plesse B, Joubert JM, Yvin JC, Kopp M, Kloareg B, Fritig B (2000) Linear beta-1,3 glucans are elicitors of defense responses in tobacco. Plant Physiology 124: 1027-1037

Knoblauch M, van Bel AJE (1998) Sieve tubes in action. Plant Cell 10: 35-50

Kock M, Gross N, Stenzel I, Hause G (2004) Phloem-specific expression of the wound-inducible ribonuclease LE from tomato (Lycopersicon esculentum cv. Lukullus). Planta 219: 233-242

Koiwa H, Kato H, Nakatsu T, Oda J, Yamada Y, Sato F (1999) Crystal structure of tobacco PR-5d protein at 1.8 angstrom resolution reveals a conserved acidic cleft structure in antifungal thaumatin-like proteins. Journal of Molecular Biology 286: 1137-1145

Krebitz M, Wagner B, Ferreira F, Peterbauer C, Campillo N, Witty M, Kolarich D, Steinkellner H, Scheiner O, Breiteneder H (2003) Plant-based heterologous expression of Mal d 2, a thaumatin-like protein and allergen of apple (*Malus domestica*), and its characterization as an antifungal protein. Journal of Molecular Biology 329: 721-730

Kubista H, Edelbauer H, Boehm S (2004) Evidence for structural and functional diversity among SDS-resistant SNARE complexes in neuroendocrine cells. Journal of Cell Science 117: 955-966

Laemmli UK (1970) Cleavage of structural proteins during assembly of head of bacteriophage-T4. Nature 227: 680-685

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) ClustalW and ClustalX version 2.0. Bioinformatics 23: 2947-2948

Larson PR, Isebrands JG (1971) The plastochron index as applied to developmental studies of cottonwood. Canadian Journal of Forest Research 1: 1-11

Lawrence SD, Dervinis C, Novak N, Davis JM (2006) Wound and insect herbivory responsive genes in poplar. Biotechnology Letters 28: 1493-1501

Lawrence SD, Greenwood JS, Korhnak TE, Davis JM (1997) A vegetative storage protein homolog is expressed in the growing shoot apex of hybrid poplar. Planta 203: 237-244

Lawrence SD, Novak NG (2001) A rapid method for the production and characterization of recombinant insecticidal proteins in plants. Molecular Breeding 8: 139-146

Lawrence SD, Novak NG (2006) Expression of poplar chitinase in tomato leads to inhibition of development in Colorado potato beetle. Biotechnology Letters 28: 593-599

Le Hir R, Beneteau J, Bellini C, Vilaine F, Dinant S (2008) Gene expression profiling: keys for investigating phloem functions. Trends in Plant Science 13: 273-280

Lee JR, Lee SS, Park S-C, Kang JS, Kim SY, Lee KO, Lee SY (2008) Functional characterization of pathogen-responsive protein AtDabb1 with an antifungal activity from *Arabidopsis thaliana*. Biochimica et Biophysica Acta doi:10.1016/j.bbapap.2008.06.023

Leone P, Menu-Bouaouiche L, Peumans WJ, Payan F, Barre A, Roussel A, Van Damme EJM, Rouge P (2006) Resolution of the structure of the allergenic and antifungal banana fruit thaumatin-like protein at 1.7-angstrom. Biochimie 88: 45-52

Liljeroth E, Marttila S, von Bothmer R (2005) Immunolocalization of defence-related proteins in the floral organs of barley (*Hordeum vulgare* L.). Journal of Phytopathology 153: 702-709

Lin MK, Belanger H, Lee YJ, Varkonyi-Gasic E, Taoka KI, Miura E, Xoconostle-Cazares B, Gendler K, Jorgensene RA, Phinney B, Lough TJ, Lucas WJ (2007)

FLOWERING LOCUS T protein may act as the long-distance florigenic signal in the cucurbits. Plant Cell 19: 1488-1506

Lippert D, Chowrira S, Ralph SG, Zhuang J, Aeschliman D, Ritland C, Ritland K, Bohlmann J (2007) Conifer defense against insects: proteome analysis of Sitka spruce (*Picea sitchensis*) bark induced by mechanical wounding or feeding by white pine weevils (*Pissodes strobi*). Proteomics 7: 248-270

Lopez-Millan AF, Morales F, Abadia A, Abadia J (2000) Effects of iron deficiency on the composition of the leaf apoplastic fluid and xylem sap in sugar beet. Implications for iron and carbon transport. Plant Physiology 124: 873-884

Lough TJ, Lucas WJ (2006) Integrative plant biology: role of phloem long-distance macromolecular trafficking. Annual Review of Plant Biology 57: 203-232

Lytle BL, Peterson FC, Kjer KL, Frederick RO, Zhao Q, Thao S, Bingman C, Johnson KA, Phillips GN, Volkman BF (2004) Letter to the Editor: Structure of the hypothetical protein At3g17210 from Arabidopsis thaliana. Journal of Biomolecular NMR 28: 397-400

Madey E, Nowack LM, Thompson JE (2002) Isolation and characterization of lipid in phloem sap of canola. Planta 214: 625-634

Major IT, Constabel CP (2006) Molecular analysis of poplar defense against herbivory: comparison of wound- and insect elicitor-induced gene expression. New Phytologist 172: 617-635

Major IT, Constabel CP (2007) Shoot-root defense signaling and activation of root defense by leaf damage in poplar. Canadian Journal of Botany 85: 1171-1181

Major IT, Constabel CP (2008) Functional analysis of the Kunitz trypsin inhibitor family in poplar reveals biochemical diversity and multiplicity in defense against herbivores. Plant Physiology 146: 888-903

Marentes E, Grusak MA (1998) Mass determination of low-molecular-weight proteins in phloem sap using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Journal of Experimental Botany 49: 903-911

Marusek CM, Trobaugh NM, Flurkey WH, Inlow JK (2006) Comparative analysis of polyphenol oxidase from plant and fungal species. Journal of Inorganic Biochemistry 100: 108-123

Masuda S, Kamada H, Satoh S (2001) Chitinase in cucumber xylem sap. Bioscience Biotechnology and Biochemistry 65: 1883-1885

Masuda S, Sakuta C, Satoh S (1999) cDNA cloning of a novel lectin-like xylem sap protein and its root-specific expression in cucumber. Plant and Cell Physiology 40: 1177-1181

Melchers LS, Selabuurlage MB, Vloemans SA, Woloshuk CP, Vanroekel JSC, Pen J, Vandenelzen PJM, Cornelissen BJC (1993) Extracellular targeting of the vacuolar tobacco proteins-AP24, chitinase and beta-1,3-glucanase in transgenic plants. Plant Molecular Biology 21: 583-593

Meng M, Geisler M, Johansson H, Mellerowicz EJ, Karpinski S, Kleczkowski LA (2007) Differential tissue/organ-dependent expression of two sucrose- and cold-responsive genes for UDP-glucose pyrophosphorylase in *Populus*. Gene 389: 186-195

Menu-Bouaouiche L, Vriet C, Peumans WJ, Barre A, Van Damme EJM, Rouge P (2003) A molecular basis for the endo-beta 1,3-glucanase activity of the thaumatin-like proteins from edible fruits. Biochimie 85: 123-131

Meyer S, Lauterbach C, Niedermeier M, Barth I, Sjolund RD, Sauer N (2004) Wounding enhances expression of AtSUC3, a sucrose transporter from *Arabidopsis* sieve elements and sink tissues. Plant Physiology 134: 684-693

Milburn JA (1970) Phloem exudation from castor bean - induction by massage. Planta 95: 272-276

Min K, Ha SC, Hasegawa PM, Bressan RA, Yun DJ, Kim KK (2004) Crystal structure of osmotin, a plant antifungal protein. Proteins-Structure Function and Genetics 54: 170-173

Miranda M, Ralph S, Mellway R, White R, MCHeath, Bohlmann J, Constabel C (2007) The transcriptional response of hybrid poplar (*Populus trichocarpa* x *P. deltoides*) to infection by *Melampsora medusae* leaf rust involves induction of flavonoid pathway genes leading to the accumulation of proanthocyanidins. Molecular Plant-Microbe Interactions 20: 816-831

Moran PJ, Thompson GA (2001) Molecular responses to aphid feeding in *Arabidopsis* in relation to plant defense pathways. Plant Physiology 125: 1074-1085

Nakamura S, Hayashi H, Mori S, Chino M (1995) Detection and characterization of protein kinases in rice phloem sap. Plant and Cell Physiology 36: 19-27

Nakamura S, Watanabe A, Chongpraditnun P, Suzui N, Hayashi H, Hattori H, Chino M (2004) Analysis of phloem exudate collected from fruit-bearing stems of coconut palm: palm trees as a source of molecules circulating in sieve tubes. Soil Science and Plant Nutrition 50: 739-745

Narvaez-Vasquez J, Orozco-Cardenas ML, Ryan CA (1994) A sulfhydryl reagent modulates systemic signaling for wound-induced and systemin-induced proteinase inhibitor synthesis. Plant Physiology 105: 725-730

Natarajan SK, Stern LJ, Sadegh-Nasseri S (1999) Sodium dodecyl sulfate stability of HLA-DR1 complexes correlates with burial of hydrophobic residues in pocket 1. Journal of Immunology 162: 3463-3470

Nelson CE, Walker-Simmons M, Makus D, Zuroske G, Graham J, Ryan CA (1983) Regulation of synthesis and accumulation of proteinase inhibitors in leaves of wounded tomato plants. ACS Symposium Series 208: 103-122

Nielsen H, Engelbrecht J, Brunak S, vonHeijne G (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Engineering 10: 1-6

Oakley RV, Wang YS, Ramakrishna W, Harding SA, Tsai CJ (2007) Differential expansion and expression of alpha- and beta-tubulin gene families in *Populus*. Plant Physiology 145: 961-973

O'Leary SJB, Poulis BAD, Von Aderkas P (2007) Identification of two thaumatin-like proteins (TLPs) in the pollination drop of hybrid yew that may play a role in pathogen defence during pollen collection. Tree Physiology 27: 1649-1659

Oparka KJ, Cruz SS (2000) The great escape: phloem transport and unloading of macromolecules. Annual Review of Plant Physiology and Plant Molecular Biology 51: 323-347

Orians C, Pomerleau J, Ricco R (2000) Vascular architecture generates fine scale variation in systemic induction of proteinase inhibitors in tomato. Journal of Chemical Ecology 26: 471-485

Osmond RIW, Hrmova M, Fontaine F, Imberty A, Fincher GB (2001) Binding interactions between barley thaumatin-like proteins and (1,3)-beta-D-glucans - kinetics, specificity, structural analysis and biological implications. European Journal of Biochemistry 268: 4190-4199

Park SC, Lee JR, Shin SO, Park Y, Lee SY, Hahin KS (2007) Characterization of a heat-stable protein with antimicrobial activity from *Arabidopsis thaliana*. Biochemical and Biophysical Research Communications 362: 562-567

Pelah D, Shoseyov O, Altman A (1995) Characterization of BspA, a major boiling-stable, water-stress-responsive protein in aspen (*Populus tremula*). Tree Physiology 15: 673-678

Pena-Cortes H, Sanchez-Serrano J, Rocha-Sosa M, Willmitzer L (1988) Systemic induction of proteinase-inhibitor-II gene expression in potato plants by wounding. Planta 174: 84-89

Perkins DN, Pappin DJC, Creasy DM, Cottrell JS (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20: 3551-3567

Philippe RN, Bohlmann J (2007) Poplar defense against insect herbivores. Canadian Journal of Botany 85: 1111-1126

Piggott N, Ekramoddoullah AKM, Liu JJ, Yu XS (2004) Gene cloning of a thaumatin-like (PR-5) protein of western white pine (*Pinus monticola* D. Don) and expression studies of members of the PR-5 group. Physiological and Molecular Plant Pathology 64: 1-8

Plaxton WC (1996) The organization and regulation of plant glycolysis. Annual Review of Plant Physiology and Plant Molecular Biology 47: 185-214

Plomion C, Leprovost G, Stokes A (2001) Wood formation in trees. Plant Physiology 127: 1513-1523

Poulis BAD, O'Leary SJB, Haddow JD, von Aderkas P (2005) Identification of proteins present in the Douglas fir ovular secretion: an insight into conifer pollen selection and development. International Journal of Plant Sciences 166: 733-739

Ralph S, Oddy C, Cooper D, Yueh H, Jancsik S, Kolosova N, Philippe RN, Aeschliman D, White R, Huber D, Ritland CE, Benoit F, Rigby T, Nantel A, Butterfield YSN, Kirkpatrick R, Chun E, Liu J, Palmquist D, Wynhoven B, Stott J, Yang G, Barber S, Holt RA, Siddiqui A, Jones SJM, Marra MA, Ellis BE, Douglas CJ, Ritland K, Bohlmann J (2006) Genomics of hybrid poplar (*Populus trichocarpa* x *deltoides*) interacting with forest tent caterpillars (*Malacosoma disstria*): normalized and full-length cDNA libraries, expressed sequence tags, and a cDNA microarray for the study of insect-induced defences in poplar. Molecular Ecology 15: 1275-1297

Ralph SG, Chun HJE, Cooper D, Kirkpatrick R, Kolosova N, Gunter L, Tuskan GA, Douglas CJ, Holt RA, Jones SJM, Marra MA, Bohlmann J (2008) Analysis of 4,664 high-quality sequence-finished poplar full-length cDNA clones and their utility for the discovery of genes responding to insect feeding. BMC Genomics 9: 57

Ramagli LS, Rodriguez LV (1985) Quantitation of microgram amounts of protein in two-dimensional polyacrylamide-gel electrophoresis sample buffer. Electrophoresis 6: 559-563

Reiss E, Schlesier B, Brandt W (2006) cDNA sequences, MALDI-TOF analyses, and molecular modelling of barley PR-5 proteins. Phytochemistry 67: 1856-1864

Renaut J, Hoffmann L, Hausman JF (2005) Biochemical and physiological mechanisms related to cold acclimation and enhanced freezing tolerance in poplar plantlets. Physiologia Plantarum 125: 82-94

Rep M, Dekker HL, Vossen JH, de Boer AD, Houterman PM, Speijer D, Back JW, de Koster CG, Cornelissen BJC (2002) Mass spectrometric identification of isoforms of PR proteins in xylem sap of fungus-infected tomato. Plant Physiology 130: 904-917

Rhodes JD, Thain JF, Wildon DC (1999) Evidence for physically distinct systemic signalling pathways in the wounded tomato plant. Annals of Botany 84: 109-116

Rinaldi C, Kohler A, Frey P, Duchaussoy F, Ningre N, Couloux A, Wincker P, Le Thiec D, Fluch S, Martin F, Duplessis S (2007) Transcript profiling of poplar leaves upon infection with compatible and incompatible strains of the foliar rust *Melampsora larici-populina*. Plant Physiology 144: 347-366

Robertson D, Smith C, Bolwell GP (1996) Inducible UDP-glucose dehydrogenase from French bean (*Phaseolus vulgaris* L) locates to vascular tissue and has alcohol dehydrogenase activity. Biochemical Journal 313: 311-317

Rouhier N, Gelhaye E, Sautiere PE, Brun A, Laurent P, Tagu D, Gerard J, de Fay E, Meyer Y, Jacquot JP (2001) Isolation and characterization of a new peroxiredoxin from poplar sieve tubes that uses either glutaredoxin or thioredoxin as a proton donor. Plant Physiology 127: 1299-1309

Runeberg-Roos P, Saarma M (1998) Phytepsin, a barley vacuolar aspartic proteinase, is highly expressed during autolysis of developing tracheary elements and sieve cells. Plant Journal 15: 139-145

Ruperti B, Cattivelli L, Pagni S, Ramina A (2002) Ethylene-responsive genes are differentially regulated during abscission, organ senescence and wounding in peach (*Prunus persica*). Journal of Experimental Botany 53: 429-437

Ryan CA (1990) Protease inhibitors in plants - genes for improving defenses against insects and pathogens. Annual Review of Phytopathology 28: 425-449

Ryu KS, Kim JI, Cho SJ, Park D, Park C, Cheong HK, Lee JO, Choi BS (2005) Structural insights into the monosaccharide specificity of *Escherichia coli* rhamnose mutarotase. Journal of Molecular Biology 349: 153-162

Sakuta C, Oda A, Yamakawa S, Satoh S (1998) Root-specific expression of genes for novel glycine-rich proteins cloned by use of an antiserum against xylem sap proteins of cucumber. Plant and Cell Physiology 39: 1330-1336

Sakuta C, Satoh S (2000) Vascular tissue-specific gene expression of xylem sap glycine-rich proteins in root and their localization in the walls of metaxylem vessels in cucumber. Plant and Cell Physiology 41: 627-638

Salzman RA, Tikhonova I, Bordelon BP, Hasegawa PM, Bressan RA (1998) Coordinate accumulation of antifungal proteins and hexoses constitutes a developmentally controlled defense response during fruit ripening in grape. Plant Physiology 117: 465-472

Sasaki S, Baba K, Nishida T, Tsutsumi Y, Kondo R (2006) The cationic cell-wall-peroxidase having oxidation ability for polymeric substrate participates in the late stage of lignification of *Populus alba* L. Plant Molecular Biology 62: 797-807

Sassa H, Hirano H (1998) Style-specific and developmentally regulated accumulation of a glycosylated thaumatin/PR5-like protein in Japanese pear (*Pyrus serotina* Rehd.). Planta 205: 514-521

Satoh S (2006) Organic substances in xylem sap delivered to above-ground organs by the roots. Journal of Plant Research 119: 179-187

Satoh S, Iizuka C, Kikuchi A, Nakamura N, Fujii T (1992) Proteins and carbohydrates in xylem sap from squash root. Plant and Cell Physiology 33: 841-847

Schagger H (2006) Tricine-SDS-PAGE. Nature Protocols 1: 16-22

Schilmiller AL, Howe GA (2005) Systemic signaling in the wound response. Current Opinion in Plant Biology 8: 369-377

Schimoler-O'Rourke R, Richardson M, Selitrennikoff CP (2001) Zeamatin inhibits trypsin and alpha-amylase activities. Applied and Environmental Microbiology 67: 2365-2366

Schittko U, Baldwin IT (2003) Constraints to herbivore-induced systemic responses: bidirectional signaling along orthostichies in *Nicotiana attenuata*. Journal of Chemical Ecology 29: 763-770

Schobert C, Baker L, Szederkenyi J, Grossmann P, Komor E, Hayashi H, Chino M, Lucas WJ (1998) Identification of immunologically related proteins in sieve-tube exudate collected from monocotyledonous and dicotyledonous plants. Planta 206: 245-252

Schobert C, Grossmann P, Gottschalk M, Komor E, Pecsvaradi A, Zurnieden U (1995) Sieve-Tube exudate from *Ricinus communis* L seedlings contains ubiquitin and chaperones. Planta 196: 205-210

Sciara G, Kendrew SG, Miele AE, Marsh NG, Federici L, Malatesta F, Schimperna G, Savino C, Vallone B (2003) The structure of ActVA-Orf6, a novel type of monooxygenase involved in actinorhodin biosynthesis. EMBO Journal 22: 205-215

Selitrennikoff CP (2001) Antifungal proteins. Applied and Environmental Microbiology 67: 2883-2894

Sharma AD, Vasudeva R, Kaur R (2006) Expression of a boiling-stable protein (BsCyp) in response to heat shock, drought and ABA treatments in *Sorghum bicolor*. Plant Growth Regulation 50: 249-254

Shatters RG, Boykin LM, Lapointe SL, Hunter WB, Weathersbee AA (2006) Phylogenetic and structural relationships of the PR5 gene family reveal an ancient multigene family conserved in plants and select animal taxa. Journal of Molecular Evolution 63: 12-29

Stadler R, Wright KM, Lauterbach C, Amon G, Gahrtz M, Feuerstein A, Oparka KJ, Sauer N (2005) Expression of GFP-fusions in *Arabidopsis* companion cells reveals non-specific protein trafficking into sieve elements and identifies a novel post-phloem domain in roots. Plant Journal 41: 319-331

Sterky F, Bhalerao RR, Unneberg P, Segerman B, Nilsson P, Brunner AM, Charbonnel-Campaa L, Lindvall JJ, Tandre K, Strauss SH, Sundberg B, Gustafsson P, Uhlen M, Bhalerao RP, Nilsson O, Sandberg G, Karlsson J, Lundeberg J, Jansson S (2004) A *Populus* EST resource for plant functional genomics. Proceedings of the National Academy of Sciences USA 101: 13951-13956

Sterky F, Regan S, Karlsson J, Hertzberg M, Rohde A, Holmberg A, Amini B, Bhalerao R, Larsson M, Villarroel R, Van Montagu M, Sandberg G, Olsson O, Teeri TT, Boerjan W, Gustafsson P, Uhlen M, Sundberg B, Lundeberg J (1998) Gene discovery in the wood-forming tissues of poplar: analysis of 5,692 expressed sequence tags. Proceedings of the National Academy of Sciences USA 95: 13330-13335

Sterner R, Liebl W (2001) Thermophilic adaptation of proteins. Critical Reviews in Biochemistry and Molecular Biology 36: 39-106

Stettler RF, Bradshaw HD, Heilman PE, Hinkley TM (1996) Biology of *Populus* and its implications for management and conservation. NRC Research Press, Ottawa, Ontario, Canada

Sturrock RN, Islam MA, Ekramoddoullah AKM (2007) Host-pathogen interactions in Douglas-fir seedlings infected by *Phellinus sulphurascens*. Phytopathology 97: 1406-1414

Taiz L, Zeiger E (2006) Plant Physiology, Ed Fourth. Sinauer Associates, Sunderland, MA

Taylor G (2002) *Populus*: Arabidopsis for forestry. Do we need a model tree? Annals of Botany 90: 681-689

Thompson GA, Goggin FL (2006) Transcriptomics and functional genomics of plant defence induction by phloem-feeding insects. Journal of Experimental Botany 57: 755-766

Thorsch J, Esau K (1982) Microtubules in differentiating sieve elements of *Gossypium hirsutum*. Journal of Ultrastructure Research 78: 73-83

Timmins J, Schoehn G, Kohlhaas C, Klenk HD, Ruigrok RWH, Weissenhorn W (2003) Oligomerization and polymerization of the filovirus matrix protein VP40. Virology 312: 359-368

Trudel J, Grenier J, Potvin C, Asselin A (1998) Several thaumatin-like proteins bind to beta-1,3-glucans. Plant Physiology 118: 1431-1438

Turner S, Gallois P, Brown D (2007) Tracheary element differentiation. Annual Review of Plant Biology 58: 407-433

Tuskan GA, DiFazio S, Jansson S, Bohlmann J, Grigoriev I, Hellsten U, Putnam N, Ralph S, Rombauts S, Salamov A, Schein J, Sterck L, Aerts A, Bhalerao RR,

Bhalerao RP, Blaudez D, Boerjan W, Brun A, Brunner A, Busov V, Campbell M, Carlson J, Chalot M, Chapman J, Chen GL, Cooper D, Coutinho PM, Couturier J, Covert S, Cronk Q, Cunningham R, Davis J, Degroeve S, Dejardin A, Depamphilis C, Detter J, Dirks B, Dubchak I, Duplessis S, Ehlting J, Ellis B, Gendler K, Goodstein D, Gribskov M, Grimwood J, Groover A, Gunter L, Hamberger B, Heinze B, Helariutta Y, Henrissat B, Holligan D, Holt R, Huang W, Islam-Faridi N, Jones S, Jones-Rhoades M, Jorgensen R, Joshi C, Kangasjarvi J, Karlsson J, Kelleher C, Kirkpatrick R, Kirst M, Kohler A, Kalluri U, Larimer F, Leebens-Mack J, Leple JC, Locascio P, Lou Y, Lucas S, Martin F, Montanini B, Napoli C, Nelson DR, Nelson C, Nieminen K, Nilsson O, Pereda V, Peter G, Philippe R, Pilate G, Poliakov A, Razumovskaya J, Richardson P, Rinaldi C, Ritland K, Rouze P, Ryaboy D, Schmutz J, Schrader J, Segerman B, Shin H, Siddiqui A, Sterky F, Terry A, Tsai CJ, Uberbacher E, Unneberg P, Vahala J, Wall K, Wessler S, Yang G, Yin T, Douglas C, Marra M, Sandberg G, de Peer YV, Rokhsar D (2006) The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). Science 313: 1596-1604

van Bel AJE (2003) The phloem, a miracle of ingenuity. Plant Cell and Environment 26: 125-149

van Bel AJE (1996) Interaction between sieve element and companion cell and the consequences for photoassimilate distribution. Two structural hardware frames with associated physiological software packages in dicotyledons. Journal of Experimental Botany 47: 1129-1140

van Bel AJE (2003) Transport phloem: low profile, high impact. Plant Physiology 131: 1509-1510

van Bel AJE, Ehlers K, Knoblauch M (2002) Sieve elements caught in the act. Trends in Plant Science 7: 126-132

van Bel AJE, Hess PH (2008) Hexoses as phloem transport sugars: the end of a dogma? Journal of Experimental Botany 59: 261-272

Van Damme EJM, Charels D, Menu-Bouaouiche L, Proost P, Barre A, Rouge P, Peumans WJ (2002) Biochemical, molecular and structural analysis of multiple thaumatin-like proteins from the elderberry tree (*Sambucus nigra* L.). Planta 214: 853-862

van Loon LC, Rep M, Pieterse CMJ (2006) Significance of inducible defense-related proteins in infected plants. Annual Review of Phytopathology 44: 135-162

Vancleve B, Just J, Sauter JJ (1991) Poplar storage protein in xylem sap. Journal of Plant Physiology 137: 763-764

Vanhelden M, Tjallingii WF, Teris A, Vanbeek TA (1994) Phloem sap collection from lettuce (*Lactuca sativa* L) - chemical comparison among collection methods. Journal of Chemical Ecology 20: 3191-3206

Vega-Sanchez ME, Redinbaugh MG, Costanzo S, Dorrance AE (2005) Spatial and temporal expression analysis of defense-related genes in soybean cultivars with different levels of partial resistance to *Phytophthora sojae*. Physiological and Molecular Plant Pathology 66: 175-182

Viswanathan DV, Thaler JS (2004) Plant vascular architecture and within-plant spatial patterns in resource quality following herbivory. Journal of Chemical Ecology 30: 531-543

Vogelmann TC, Dickson RE, Larson PR (1985) Comparative distribution and metabolism of xylem-borne amino compounds and sucrose in shoots of *Populus deltoides*. Plant Physiology 77: 418-428

Wada T, Shirouzu M, Terada T, Kamewari Y, Park KY, Tame JRH, Kuramitsu S, Yokoyama S (2004) Crystal structure of the conserved hypothetical protein TT1380 from *Thermus thermophilus* HB8. Proteins-Structure Function and Bioinformatics 55: 778-780

Wagner R, Mugnaini S, Sniezko R, Hardie D, Poulis B, Nepi M, Pacini E, von Aderkas P (2007) Gymnosperm pollination drop proteins are conservative and indicate complex function. Sexual Plant Reproduction 20:181-189

Walz C, Giavalisco P, Schad M, Juenger M, Klose J, Kehr J (2004) Proteomics of curcurbit phloem exudate reveals a network of defence proteins. Phytochemistry 65: 1795-1804

Walz C, Juenger M, Schad M, Kehr J (2002) Evidence for the presence and activity of a complete antioxidant defence system in mature sieve tubes. Plant Journal 31: 189-197

Wang JH, Constabel CP (2003) Biochemical characterization of two differentially expressed polyphenol oxidases from hybrid poplar. Phytochemistry 64: 115-121

Wang JH, Constabel CP (2004) Polyphenol oxidase overexpression in transgenic *Populus* enhances resistance to herbivory by forest tent caterpillar (*Malacosoma disstria*). Planta 220: 87-96

Wang WX, Dgany O, Dym O, Altman A, Shoseyov O, Almog O (2003) Crystallization and preliminary X-ray crystallographic analysis of SP1, a novel chaperone-like

protein. Acta Crystallographica Section D-Biological Crystallography 59: 512-514

Wang WX, Dgany O, Wolf SG, Levy I, Algom R, Pouny Y, Wolf A, Marton I, Altman A, Shoseyov O (2006) Aspen SP1, an exceptional thermal, protease and detergent-resistant self-assembled nano-particle. Biotechnology and Bioengineering 95: 161-168

Wang WX, Pelah D, Alergand T, Shoseyov O, Altman A (2002) Characterization of SP1, a stress-responsive, boiling-soluble, homo-oligomeric protein from aspen. Plant Physiology 130: 865-875

Wang WX, Vinocur B, Shoseyov O, Altman A (2004) Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends in Plant Science 9: 244-252

Ward ER, Payne GB, Moyer MB, Williams SC, Dincher SS, Sharkey KC, Beck JJ, Taylor HT, Ahlgoy P, Meins F, Ryals JA (1991) Differential regulation of beta-1,3-glucanase messenger RNAs in response to pathogen infection. Plant Physiology 96: 390-397

Weibull J, Ronquist F, Brishammar S (1990) Free amino acid composition of leaf exudates and phloem sap - a comparative study in oats and barley. Plant Physiology 92: 222-226

Weisz OA, Swift AM, Machamer CE (1993) Oligomerization of a membrane protein correlates with its retention in the golgi complex. Journal of Cell Biology 122: 1185-1196

Wink M, Witte L (1984) Turnover and transport of quinolizidine alkaloids - diurnal fluctuations of lupanine in the phloem sap, leaves and fruits of *Lupinus albus* L. Planta 161: 519-524

Wise RR (2006) The diversity of plastid form and function. *In* RR Wise, JK Hoober, eds, The Structure and Function of Plastids, Vol 23. Springer, pp 3-26

Xia YJ, Suzuki H, Borevitz J, Blount J, Guo ZJ, Patel K, Dixon RA, Lamb C (2004) An extracellular aspartic protease functions in *Arabidopsis* disease resistance signaling. EMBO Journal 23: 980-988

Xu ZF, Qi WQ, Ouyang XZ, Yeung E, Chye ML (2001) A proteinase inhibitor II of *Solanum americanum* is expressed in phloem. Plant Molecular Biology 47: 727-738

Yang SH, van Zyl L, No EG, Loopstra CA (2004) Microarray analysis of genes preferentially expressed in differentiating xylem of loblolly pine (*Pinus taeda*). Plant Science 166: 1185-1195

Yoo BC, Kragler F, Varkonyi-Gasic E, Haywood V, Archer-Evans S, Lee YM, Lough TJ, Lucas WJ (2004) A systemic small RNA signaling system in plants. Plant Cell 16: 1979-2000

Yoo BC, Lee JY, Lucas WJ (2002) Analysis of the complexity of protein kinases within the phloem sieve tube system - characterization of *Cucurbita maxima* calmodulin-like domain protein kinase 1. Journal of Biological Chemistry 277: 15325-15332

Zarate SI, Kempema LA, Walling LL (2007) Silverleaf whitefly induces salicylic acid defenses and suppresses effectual jasmonic acid defenses. Plant Physiology 143: 866-875

Zhu B, Xiong AS, Peng RH, Xu J, Zhou J, Xu JT, Jin XF, Zhang Y, Hou XL, Yao QH (2008) Heat stress protection in aspen SP1 transgenic *Arabidopsis thaliana*. BMB Reports 41: 382-387

Zybailov B, Rutschow H, Friso G, Rudella A, Emanuelsson O, Sun Q, van Wijk KJ (2008) Sorting signal, N-terminal modification and abundance of the chloroplast proteome. PLoS ONE 3: e1994

Appendix 1: Supplementary Data

The following pages contain the Supplementary Tables and Figures referred to in Chapters 2, 3, 4, and 5. A Table of Contents for the Supplemental Data is printed on preliminary page ix.

Supplementary Table 2-1. Poplar phloem exudate proteins with corresponding LC-MS/MS data including sequence and charge state of matched peptides and also the probability of an N-terminal signal peptide as determined by SignalP (Nielsen et al., 1997; Bendtsen et al. 2004)

Spot ID	Identification	JGI Acc. No. Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
ND2-p82D	Glycosyltransferase	826368 db10_38427	AT5G20830.2	92152	6.18	598	14	K.ILAGGAFSEVLR.S	2	0.000
								R.VNVQALVVEELR.V	2	
								K.YIGNGVEFLNR.H	2	
								R.ALESEMLLR.I	2	
								R.FEVWPYLETYTEDVAAEIAK.E	3	
								K.ELQGKPDLIIGNYSDGNVVASLLAHK.L	4	
								R.VVHGIDVFDPK.F	2	
								R.NKPILFTMAR.L	2	
								R.ELANLVVVGGDR.R	2	
								K.DIEEQAEMK.K	2	
								K.DIEEQAEMKK.M	2	
								R.LLTLTGVYGFWK.H	2	
								R.YLEMFYALK.Y	2	
								R.KLADSVPLTIE.-	2	
ND2-p82D	Unknown (DUF1278)	672963 db10_48883	AT5G54062.1	11071	9.03	215	4	K.EIFVAISAGTGR.I	2	1.000
								R.IAIGPACCK.V	2	
								K.VINELTDVCWAR.L	2	
								R.LFPSIPATGK.F	2	

Supplementary Table 2-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
ND6-82D	S-adenosyl-L-homocysteine hydrolase	597072	db10_867288	AT4G13940.3	53195	5.75	289	6	R.DSAAVFAWK.G	2	0.000
									K.TGVLPDPASTDNVEFQLVLTIIR.D	3	
									R.LVGVSEETTTGVK.R	2	
									R.HSLPDGLMR.A	2	
									K.DIIMVDHMR.K	2	
									R.WVFPDTNSGILVLAEGR.L	2	
ND2-p82D	Molecular chaperone (HSP90)	582316	db10_34072	AT5G56010.1	79950	4.94	135	2	K.SDLVNNLGTIAR.S	2	0.000
									K.GIVDSEDLPLNISR.E	2	
ND8-p82D	Carbonic anhydrase	417951	db10_220647	AT5G04180.1	25504	9.4	190	3	K.LQSPIDLLDQNVK.V	2	0.000
									K.YGEPDPFLSK.L	2	
									K.SLGILSPNEIVFDSR.E	2	
ND8-p82D	Beta tubulin	203493	db10_1015266	AT5G23860.1	49713	4.78	126	2	K.EVDEQMLNVQNK.N	2	0.000
									K.GHYTEGAELIDSVLDVVR.K	3	
ND8-p82D	Caffeic acid 3-O-methyltransferase	834247	db10_327940	AT5G54160.1	39732	5.48	150	5	K.NPDAPVMLDR.I	2	0.000
									K.NPDAPVMLDR.I	2	
									R.TEKEFEGLAK.G	2	
									K.TAIELDLLEIMAK.A	2	
									K.TAIELDLLEIMAK.A	2	

Supplementary Table 2-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
ND5-p82D	Vitamin-B12 independent methionine synthase	679841	db10_866864	AT3G03780.2	84592	6.27	791	17	R.GNASVPAMEMTK.W	2	0.001
									K.WFDTNYHYIVPELGPEVNFSYASHK.A	4	
									K.SFSLLSLIDK.I	2	
									K.ILPVYQEVLAELK.A	2	
									K.AAGASWIQFDEPK.L	2	
									K.SVTGFGFDLVR.G	2	
									K.YLFAGVVDGR.N	2	
									K.VVEVNALAK.A	2	
									K.KLNLPILPTTTIGSFPQTMDLR.R	3	
									K.IQEELDIDVLVHGEPER.N	3	
									K.AMTVFWSSMAQSMTK.R	2	
									K.GMLTGPVTILNWSFVR.N	2	
									K.GMLTGPVTILNWSFVR.N	2	
									R.FETCYQIALAIKDEVEDLEK.A	3	
									K.AGITVIQIDEAALR.E	2	
									R.IPSEEEIADR.I	2	
									R.KYAEVKPALSNMVAAAK.H	3	
ND3-p82D	Malate dehydrogenase	564942	db10_74735	AT5G56720.1	35714	6.13	219	4	K.MELVDAAFPLLK.G	2	0.454
									K.VLVVANPANTNALILK.E	2	
									R.ELVKDDEWLNAEFITTVQQR.G	3	
									K.IVQGLSIDEFSR.K	2	

Supplementary Table 2-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
ND7-p82D	Tubulin alpha-2 chain	831020	db10_985054	AT4G14960.1	49777	4.92	154	4	K.DVNAAVATIK.T	2	0.005
									R.QLFHPEQLISGK.E	2	
									R.LISQIISSLTTSLR.F	2	
									R.AIFVDLEPTVIDEVR.T	2	
ND6-p82D	Unknown (desiccation-associated)	642406	db10_597216	AT3G62730.1	32846	4.83	181	2	R.SLVASLLGVEAGQDAVIR.T	2	0.984
									R.TTSNILSADSDSLSYAR.N	2	
ND7-p82D	Ribulose bisphosphate carboxylase, large chain	279035	db10_162609	ATCG00490.1	21534	6.47	88	2	R.FLFCAEALYK.A	2	0.000
									R.DITLGFVDLLR.D	2	
ND4-p82D	Phospholipase D1	829577	db10_179	AT3G15730.1	91816	5.03	134	2	R.ILENEATNPR.W	2	0.000
									R.VLMLIWDDR.T	2	
ND4-p82D	Fasciclin-like arabinogalactan-protein 10	730906	db10_210459	AT3G60900.1	43247	5.37	120	2	K.VGFGSAAPGSK.L	2	0.999
									K.TFASLLQTSGVIK.T	2	
ND6-p82D	Beta-Ig-H3/fasciclin	728480	db10_165972	AT5G60490.1	28208	7.95	122	3	R.DGVTIFAPTDGAFSAIIK.S	2	1.000
									K.IELVQFHIIPR.I	3	
									K.VLLPLDIFAPK.L	2	
ND5-p82D	β-glucosidase (glycoside hydrolase, family 1)	294573	db10_42123	AT5G42260.1	55629	5.85	94	2	R.FSISWTR.M	2	0.000
									R.DFADLCFQSFGDR.V	2	

Supplementary Table 2-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
ND5-p82D	Polyphenol oxidase (PPO5)	674097	db10_36026		64884	6.31	79	2	K.SPLYDPLR.D	2	0.834
									R.TGGDSVTVANVK.I	2	
ND6-p82D	Enolase	575698	db10_30345	AT2G36530.1	47924	5.67	159	3	K.TLVLPVPAFNVINGGSHAGNK.L	3	0.000
									K.SCNALLLK.V	2	
									K.VNQIGSVTESIEAVK.M	2	
ND6-p82D	Glycine/serine hydroxymethyltransferase	829808	db10_1005067	AT4G13890.1	51907	7.26	138	3	K.ISATSIYFESLPYK.V	2	0.001
									K.LIICGGSAYPR.D	2	
									R.IGTPAMTSR.G	2	
ND7-p82D	Lipolytic enzyme, G-D-S-L	758353	db10_21506	AT4G18970.1	32714	8.82	124	3	R.GVNYASGSAGIR.N	2	0.723
									R.LAVGDVISLDEQLQNHR.I	3	
									R.IIISLITEALGNK.D	2	
ND8-p82D	Lipolytic enzyme, G-D-S-L	580490	db10_349235	AT1G33811.1	42332	5.09	137	4	R.DLFTMNIQLYNHK.I	2	0.999
									K.TQLEDLYSTGAR.K	2	
									R.KIAVFGLIR.V	2	
									K.DAHPYDISELVK.L	2	
ND7-p82D	Nucleoside phosphatase (apyrase 2)	573883	db10_29394	AT1G14240.2	49760	5.31	116	2	R.SYAVVFDAGSTGSR.V	2	0.992
									K.DPQAAANSLVPLLNEAESVVPEEFSPK.T	3	

Supplementary Table 2-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
ND7-p82D	Glucose-6-phosphate 1-dehydrogenase	736146	db10_57073	AT5G35790.1	59156	6.29	112	2	R.ILTTNLWSAELSK.L	2	0.002
									R.ISSVNAMSALCEATGADVAEVSYAIGK.D	3	
ND9-p82D	Glyceraldehyde 3-phosphate dehydrogenase	728998	db10_171931	AT3G04120.1	36724	6.36	219	5	K.AGIALNDNYVK.L	2	0.000
									R.AASFNIIPSSTGAAK.A	2	
									R.VPTVDVSVVDLTVR.L	2	
									K.LVAWYDNEWGYSTR.V	2	
									K.GILGYTDEDLVSTDFIGDNR.S	2	
ND8-p82D	Chitinase (glycosyl hydrolase, family 18)	717157	db10_55687	AT4G19820.1	40233	7.82	100	2	K.SFIDSSITLAR.S	2	0.941
									R.SWIQAGMSAK.K	2	
ND11-p82D	Chitinase (glycosyl hydrolase, family 19)	826290	db10_596723	AT3G54420.1	29337	7.91	408	6	K.TALWFWMNR.V	2	0.999
									R.VRPVVSQGFGATIR.A	2	
									R.NAFLSAVNSYPQFGK.L	2	
									R.SNNFDGLNNPDIVAR.D	2	
									R.GPLQLSWNYNYGPAGR.S	2	
									R.AINGIECNGGNPGAVQAR.V	2	

Supplementary Table 2-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
ND11-p82D	Chitinase (glycosyl hydrolase, family 18)	746640	db10_108094	AT5G24090.1	30847	4.42	164	5	R.RAMPILDGVDFDIEAGSGQFWDDLAR.A	3	0.996
									R.ALNGFSQQR.K	2	
									R.KVYLAAAPQCIFPDANLDTAIK.T	3	
									K.VYLAAAPQCIFPDANLDTAIK.T	2	
									K.QYDNGYSAAIK.G	2	
ND13-p82D	Chitin-binding (hevein-like)	571046	db10_128610	AT3G04720.1	20789	8.37	298	4	R.VTNTGTGAQVTVR.I	2	1.000
									K.YGWTAFCGPVGPR.G	2	
									R.KYGWTAFCGPVGPR.G	3	
									R.IVDQCSNGGLDLDAGVFR.Q	2	
ND11-p82D	dTDP-glucose 4-6-dehydratase/UDP-glucuronic acid decarboxylase	832538	db10_46234	AT3G46440.1	39104	6.13	105	2	K.APPTPSPLR.F	2	0.000
									K.TNVIGTLNMLGLAK.R	2	
ND9-p82D	Phenylcoumaran benzylic ether reductase 1	830063	db10_44210	AT1G75290.1	33977	5.52	206	3	K.ILIIGGTGYIGK.F	2	0.004
									K.TVLIKPPK.N	2	
									K.NTYSFNELIDLWEK.K	2	
ND10-p82D	β-1,3-glucanase (glycoside hydrolase, family 17)	751998	db10_4	AT4G16260.1	36564	4.78	228	5	R.GSNIEVVLGVPNDK.L	2	0.999
									K.AAGIEQHFGLFLPNKQPK.Y	3	
									R.SGQAIETYLFAMFDENLK.A	2	
									R.RSGQAIETYLFAMFDENLK.A	3	
									R.YIAVGNEVHPGDANAQSVLPAMQNIHNAIASANLQDQIK.V	4	

Supplementary Table 2-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
ND9-p82D	Annexin	643752	db10_44313		35965	6.17	102	3	K.AFSGWGTNEGLIISILGHR.N	3	0.000
									R.VLLLWTLDPAER.D	2	
									K.AYSDEELIR.I	2	
ND10-p82D	Annexin	818283	db10_45423	AT5G65020.1	36018	6.2	152	3	K.IGTDEGALTR.V	2	0.000
									K.LLVPLVSAFR.Y	2	
									R.DAYLANEATKR.F	2	
ND9-p82D	Thaumatin, pathogenesis-related, PR-5	828883	db10_104416	AT1G20030.2	25856	4.9	62	1	K.QQCPQAYSYAYDDK.S	2	0.662
ND13-p82D	Thaumatin, pathogenesis-related, PR-5	669475	db10_578146	AT1G75040.1	24731	7.89	332	5	R.CPDAYSYPK.D	2	0.999
									R.ASAGCNNPCTVFK.T	2	
									R.CAADINGQCPNQLR.A	2	
									K.DDQTSTFTCPGGTNYR.V	2	
									K.TDQYCCNSGSCGPTDYSR.F	2	
ND10-p82D	Bark storage protein B precursor	740814	db10_133398	AT4G24340.1	35860	6.24	69	1	R.LGLVFTSDNNER.A	2	0.998
ND10-p82D	Vegetative storage protein win4.5	596927	db10_139788	AT4G24350.1	35306	5.62	61	1	R.LGLVLTSDTNEK.A	2	0.991

Supplementary Table 2-1. Continued

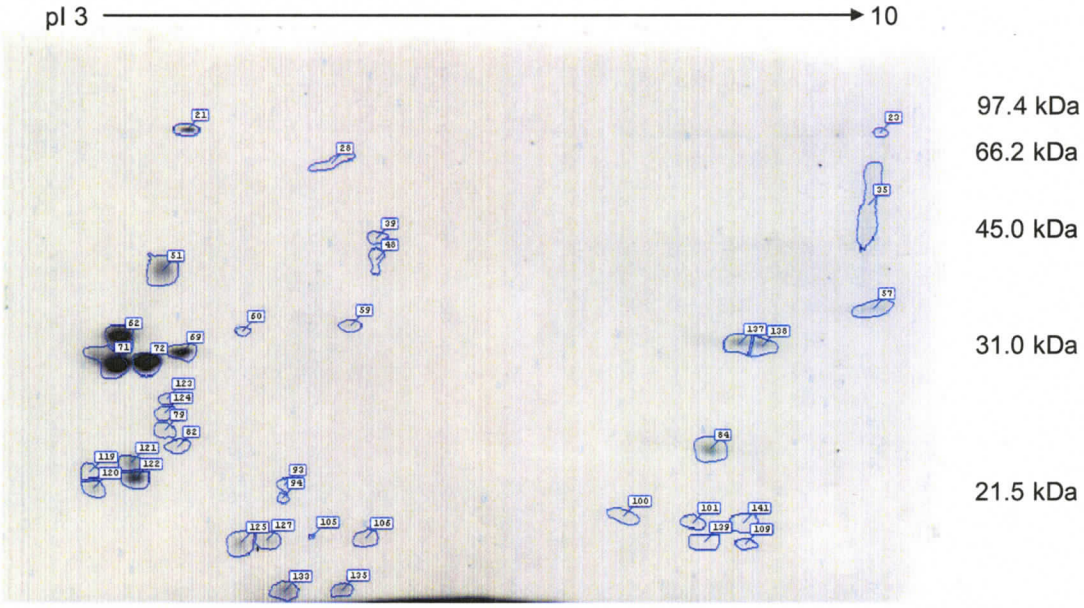
Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
ND11-p82D	Unknown (DUF26)	718495	db10_887142	AT3G22060.1	26450	5.78	216	4	K.AAIIWYDNCLLK.Y	2	1.000
									K.VVSEPVTFNGK.T	2	
									K.ELLTQLADKVEATPK.L	2	
									K.LYGLVQCTR.D	2	
ND11-p82D	Triosephosphate isomerase	724697	db10_170454	AT2G21170.1	27397	6.51	210	3	R.SLLNESNEFVGDK.V	2	0.000
									R.VSNWADVVLAYEPVWAIGTGK.V	2	
									R.IIYGGSVNGANCK.E	2	
ND11-p82D	Multifunctional chaperone (14-3-3 family)	711617	db10_20039	AT4G09000.1	29378	4.68	173	4	K.VSASLENEELTVEER.N	2	0.000
									K.IETELSSICDGILK.L	2	
									R.LIPTASAGDSK.V	2	
									K.DSTLIMQLLR.D	2	
ND13-p82D	Kunitz trypsin inhibitor, miraculin-like	763975	db10_273935	AT1G73260.1	20273	5.01	183	3	R.YILAERDPIPSSNAWHFNIVK.N	3	0.000
									K.NDQGLYNFQWCPNR.L	2	
									R.LLVLDGPAFPFIFR.R	2	
ND12-p82D	Plant basic secretory protein (BSP) family protein	549955	db10_19466	AT2G15220.1	24995	7.18	162	2	K.QTLASATDFIWR.T	2	1.000
									K.TGYSAQYFVDLLGK.T	2	

Supplementary Table 2-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
ND12-p82D	Glutathione S-transferase	243514	db10_104275	AT2G02930.1	24195	5.73	207	5	K.LHGSVLSTNTQR.V	2	0.018
									K.QEPHISLNPFGLVPAAVDGDLK.L	3	
									R.AISQYVAHQYASK.G	3	
									VTLGK.V	3	
									K.VLDVYEAR.L	2	
ND12-p82D	1,4-benzoquinone reductase-like	726993	db10_121955	AT4G27270.1	21671	5.97	94	3	R.FGMMAAQFK.A	2	0.000
									K.AFLDATGGLWK.T	2	
									K.LWQVPEILPEEVLGK.M	2	
ND14-p82D	Cyclophilin type peptidyl-prolyl cis-trans isomerase	813818	db10_22018	AT2G21130.1	18094	8.72	216	4	K.VFFDMTVGGQSVGR.I	2	0.005
									R.IVMELFADIVPR.T	2	
									K.FADENFVK.K	2	
									R.TSKPVVVADCGQLS.-	2	
ND15-p82D	Defense-related protein containing SCP domain	595857	db10_74455	AT2G14580.1	17257	8.54	302	5	R.AQVGVGNIVWDTNVAAYASDYVK.R	3	0.991
									R.LVHSGGPYGENLAGGSGDLTGSAAVK.L	3	
									K.LWVDEKPK.Y	2	
									K.YDYNSNSCVGGECR.H	2	
									R.HYTQVVWR.N	2	
ND15-p82D	Pop3-/SP1-like	822230	db10_724301	AT3G17210.1	12976	5.73	72	2	R.IREGIFLGEK.L	2	0.000
									R.FNDGITDEQIEK.F	2	

Supplementary Table 2-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
ND10-p82D	β-1,3-glucanase (glycoside hydrolase, family 17)	652688	db10_868066	AT3G57260.1	31638	5.52	545	17	R.LYDPNQDALR.A	2	0.999
									R.LYDPNQDALR.A	2	
									R.ALQGTNIELMLGLPNPDLQR.I	2	
									R.ALQGTNIELMLGLPNPDLQR.I	3	
									R.IASSQANANAWVQSNVK.F	2	
									R.YIAVGNEVKPSDSFAQFLVPAMQNIR.N	3	
									R.YIAVGNEVKPSDSFAQFLVPAMQNIR.N	4	
									R.YIAVGNEVKPSDSFAQFLVPAMQNIR.N	3	
									R.NALNSAGLGSIK.V	2	
									K.VSTAIDNGVIADGSFPPSK.G	2	
									R.TYNNNLVQHVK.R	2	
									R.TYNNNLVQHVK.R	3	
									K.RPGKPIETYIFAMFDESNKNPELEK.H	4	
									K.RPGKPIETYIFAMFDESNKNPELEK.H	4	
									K.HWGLFSPNK.Q	2	
									K.HWGLFSPNKQPK.Y	2	
									K.HWGLFSPNKQPK.Y	3	



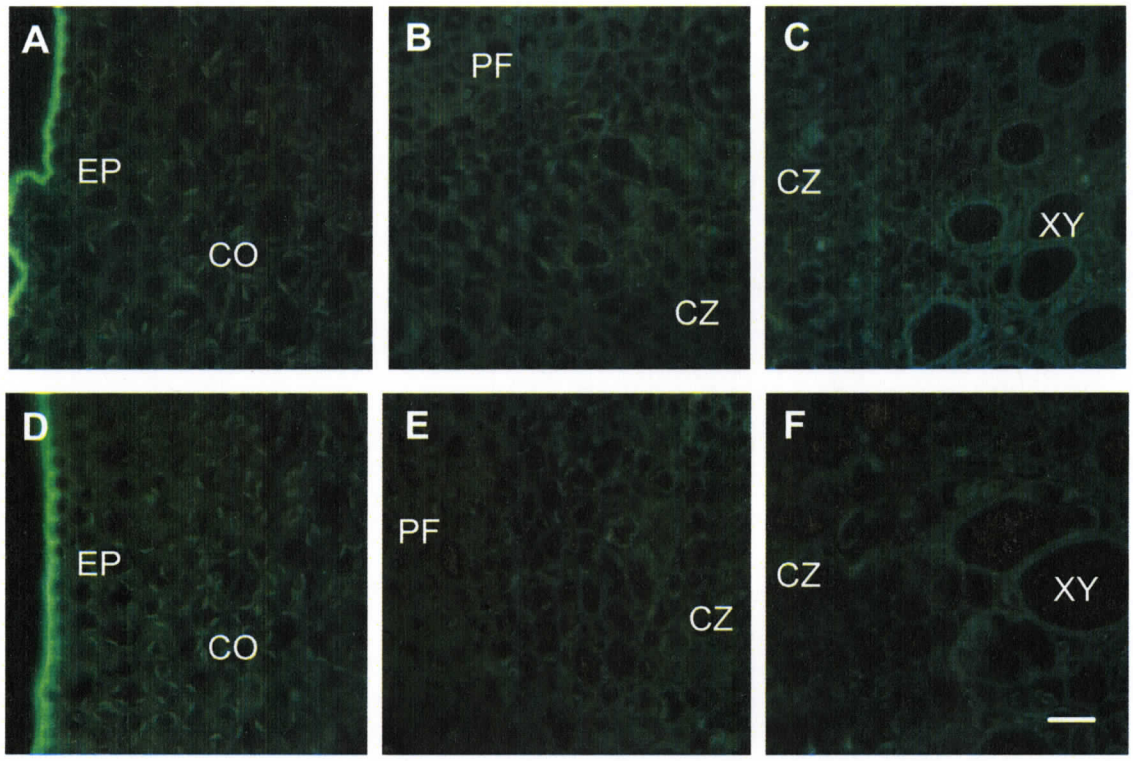
Supplementary Figure 2-1. Reference 2-DE gel used for the analysis of phloem exudate proteins collected from control and wounded poplar saplings. Protein spots are circled and numbered and the corresponding expression ratio, standard error for control and wound treatments, and P-value for each protein spot are listed in Supplementary Table 2-2.

Supplementary Table 2-2. Statistical analysis of poplar phloem exudate proteins from 24h wound experiment. See Supplementary Fig. 2-1 to view corresponding spot #s.

Protein Match	Spot #	Expression Ratio	Control Samples Standard Error	Wound Samples Standard Error	P-Value
pop3/SP1	21	1.358	0.444	0.559	0.032
	23	1.886	0.589	2.102	0.526
	28	0.403	0.108	0.044	0.028
	51	0.901	0.572	0.359	0.150
	57	0.570	1.768	0.781	0.494
	62	1.040	0.674	0.621	0.698
	69	0.840	0.485	0.383	0.292
	71	0.893	1.317	0.787	0.062
	72	0.976	1.123	1.418	0.807
	79	0.961	0.171	0.227	0.773
	84	0.892	0.327	0.458	0.478
	100	1.089	0.065	0.226	0.530
	101	1.643	0.075	0.085	0.106
	106	1.332	0.048	0.042	0.251
	109	0.949	0.089	0.085	0.908
	119	0.769	0.185	0.320	0.527
TLP	120	1.903	0.284	0.605	0.034
	121	0.973	0.508	0.641	0.790
	122	1.178	0.209	1.034	0.393
	123	0.858	0.073	0.055	0.643
	124	0.912	0.131	0.192	0.496
	125	1.028	0.040	0.209	0.873
	127	0.899	0.097	0.260	0.630
	137	0.813	0.539	0.204	0.455
	138	1.487	0.212	0.194	0.214

Supplementary Table 3-1. LC-MS/MS data for the 31 kDa TLP sequenced from poplar phloem exudate (Fig. 3-3) which includes the protein's predicted MW and pI and the sequence and charge of the matched peptides and also the probability of of an N-terminal signal peptide as determined by Signal P (Nielson et al., 1997; Bendtsen et al., 2004)

Identification	JGI Acc. No.	Theoretical MW	pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
TLP	583370	24200	5.47	146	2	QQCPQAYSYAYDDK	2	0.017
						SSTFTCPSGGNYLITFCP	2	



Supplementary Figure 4-1. Immunofluorescence of poplar stem cross-sections labelled with pop3.1 (A-C) and pop3.4 (D-F) preimmune sera. CO, cortical cells; CZ, cambial zone; EP, epidermal cells; PF, phloem fibres; and XV, xylem vessels. Scale bar = 20 μm.

Supplementary Table 5-1. Poplar xylem sap proteins with corresponding LC-MS/MS data including sequence and charge state of matched peptides and also the probability of an N-terminal secretion signal peptide as determined by SignalP (Nielsen et al., 1997;

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of Matched Peptides	Sequence Matches	Charge	SignalP Prob.
6	Enolase	575698	db10_30345	AT2G36530.1	47924.21	5.7	786	13	R.GNPTVEADILLSDGSYAR.A	2	0.000
									R.AAVPSGASTGVYEALELR.D	2	
									K.AVGNVNSIIGPALIGK.D	2	
									K.KIPLYQHIANLAGNK.T	3	
									K.TLVLPVPAFNVINGGSHAGNK.L	3	
									K.LAMQEFMILPVGASSFK.E	2	
									K.MGVEVYHHLK.S	2	
									K.VVIGMDVAASEFYNDKDK.T	3	
									K.TYDLNFKEENNDGSQK.I	3	
									K.MTGEVGEQVQIVGDDLLVTNPK.R	2	
									K.VNQIGSVTESIEAVK.M	2	
									R.SGETEDTFIADLSVGLSTGQIK.T	2	
									R.IEEELGSAAVYAGAK.F	2	
6	Beta tubulin	180424	db10_1032081	AT5G23860.1	49470.47	4.8	518	10	R.VNVYYNEASCGR.F	2	0.000
									R.AVLMDLEPGTMDSVR.S	2	
									K.GHYTEGAELIDSVLDVVR.K	3	
									R.MMLTFSVFPSPK.V	2	
									R.FPGQLNSDLR.K	2	
									K.LAVNLIPFPR.L	2	
									R.YLTASAMFR.G	2	
									K.EVDEQMINVQNK.N	2	
									K.NSSYFVEWIPNNVK.S	2	
									R.VSEQFTAMFR.R	2	

Supplementary Table 5-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
6	Adenosylhomocysteinase	707148	db10_19423	AT3G23810.1	53431.14	5.6	509	10	R.LEIELAEVEMPGLMSCR.T	2	0.000
									R.TEFGPSQPFK.G	2	
									K.GETLQEYWWCTER.A	2	
									R.LVGVSEETTTGVK.R	2	
									R.LVGVSEETTTGVKR.L	3	
									K.SKFDNLYGCR.H	2	
									R.HSLPDGLMR.A	2	
									K.VAVVCGYGDVGK.G	2	
									K.DIIMVDHMR.K	2	
									R.WVFPDTNSGIIVLAEGR.L	2	
6	UDP-glucose/GDP-mannose dehydrogenase	835719	db10_57093	AT3G29360.1	52963.63	5.7	449	9	K.AADLTYWESAAR.T	2	0.192
									K.FQILSNPEFLAEGTAIEDLFQPDR.V	3	
									K.SVYAHWVPEDR.I	2	
									K.LAANAFLAQR.I	2	
									K.FLSSSVGFGGSCFQK.D	2	
									R.VVSSMFNTVSQK.K	2	
									K.IAILGFAFK.K	2	
									K.AILNIYDPQVTEDQIQR.D	2	
									K.QVTVTWDAYEATK.G	2	

Supplementary Table 5-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
6	F1 ATP synthase beta subunit	822103	db10_51712	ATCG00480.1	59880.21	6	342	8	R.TIAMDGTEGLVR.G	2	0.232
									R.GQPVLNTGSPITVPVGR.A	2	
									K.VVDLLAPYQR.G	2	
									K.TVLIMELINNVAK.A	2	
									R.EMIESGVIK.L	2	
									R.DAEGQDVLLFIDNIFR.F	2	
									R.FTQANSEVSALLGR.I	2	
									K.NLQDIIAILGMDELSEDDKLTVAR.A	3	
6	Tubulin alpha-3 chain	707456	db10_1028494	AT4G14960.1	49668.36	4.9	324	8	K.TVGGGDDAFNTFFSETGAGK.H	2	0.006
									R.AVFVDLEPTVIDEVR.T	2	
									R.QLFHPEQLISGK.E	2	
									K.EIVDLCLDR.I	2	
									R.SLNIERPTYTNLNR.L	3	
									R.LVSQVISSLTASLR.F	2	
									R.IHFMLSSYAPVISAEK.A	3	
									K.CGINYQPPTVVPGGDLAK.V	2	
6	UDP-glucose pyrophosphorylase	592888	db10_40605	AT5G17310.2	51588.36	5.8	270	7	K.SAVANLNQISESEK.T	2	0.000
									K.TGFVNLVSR.Y	2	
									R.YLSGEAQQVEWSK.I	2	
									R.LVVDDFVPLPSK.G	2	
									K.GHTDKDGWYPPGHGDVFPSLK.N	4	
									K.VQLLEIAQVPDQHVNEFK.S	3	
									K.VLQLETAAGAAIK.F	2	

Supplementary Table 5-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
6	Glutamate decarboxylase/sphingosine phosphate lyase	648236	db10_45045	AT3G17760.1	56332.06	5.8	243	6	K.TASESDVSVHSTFASR.Y	3	0.004
									K.EAAFQIINDELMLDGNPR.L	2	
									R.YFEVELK.E	2	
									K.LSDGYYVMDPEK.A	2	
									R.FGWIVPAYTMPPDAQHVTVLR.V	3	
									K.VLHELETLPSR.I	2	
6	Glycine/serine hydroxymethyltransferase	829808	db10_112535	AT4G13890.1	51906.85	7.3	223	4	K.NAVFGDSSALAPGGVR.I	2	0.001
									R.GLVEKDFEQIGEFLHR.A	3	
									K.GLVNNKDIEALK.A	2	
									K.DIEALKADVEK.F	2	
6	Aspartyl protease	828515	db10_47591	AT4G04460.1	55381.53	5.6	139	5	K.TGEVFTMASHEPK.W	2	0.986
									K.TGEVFTMASHEPK.W	2	
									K.WWLGEPIWETVAK.Q	2	
									R.IPPIIGMIDEGFKVEQK.R	3	
									R.TIFIGHGPQFAR.G	2	
8	Aspartyl protease	261465	db10_57158	AT5G10760.1	46400.53	8.6	197	3	R.LPPAAYSALR.A	2	0.194
									K.TLEVFYDGSAGK.V	2	
									K.DGSTVGSGNYIVTVGLGTPK.K	2	
8	Aspartyl protease	261478	db10_31726	AT5G10760.1	46400.53	8.6	177	4	K.GYLSLGGQVSK.S	2	0.002
									K.LFSYCLPASSSSK.G	2	
									K.GGVEMDIDVSGILYPVNGLK.K	2	
									K.FTPLSADFDSTPFYGLDITGLSVGGR.K	3	

Supplementary Table 5-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
5	Beta-Ig-H3/fasciclin	728480	db10_165972	AT5G60490.1	28208.26	8	334	5	R.DGVTIFAPTDGAFSAIIK.S	2	1.000
									K.SGVLNSLSDHQK.I	2	
									K.IELVQFHIIPR.I	2	
									R.IITTANFQTVSNPITTLAGSGSR.F	2	
									K.VLLPLDIFAPK.L	2	
6	Seryl-tRNA synthetase	716082	db10_52869	AT5G27470.1	51595.56	5.9	94	2	R.LNQALINFGLEFLEK.R	2	0.000
									R.ELVSCSNCTDYQSR.R	2	
5	Polygalacturonase (glycoside hydrolase, family 28)	644497	db10_20267	AT3G61490.1	51463.2	5.9	711	12	R.AHSASLTDFGGVGDGTTSNTK.A	3	1.000
									K.AFKDAIDHLSQFSSDGGSQLFVPAGK.W	3	
									K.DAIDHLSQFSSDGGSQLFVPAGK.W	3	
									K.DAVLLASQDMQEWPVMKPLPSYGR.G	3	
									R.DILVQGITIIAPISSPNTDGINPDSCTNTK.I	3	
									K.IEDCYIVSGDDCVAVK.S	2	
									K.SGWDEYGIAFGMPTK.Q	2	
									R.AEDITAIHTESGVR.I	2	
									R.GGYVKDIYVK.R	2	
									K.NYDPNALPLIQGINYR.D	2	
									R.LEGIAGDPFK.E	2	
									K.KVPWTCTEIEGMTSGVSPRPCDLLPDQGPEK.I	4	
									K.ITSCDFPPENIPIDLVQFK.M	2	

Supplementary Table 5-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
7	Plant peroxidase	817694	db10_278159	AT5G06720.1	33407.29	5.8	431	8	K.FALPNINSAR.G	2	0.001
									R.GFEVVDAIK.T	2	
									K.TAVESQCSGVVSCADILAIAAR.D	2	
									K.LPSPFEDVDTIINK.F	2	
									K.LPSPFEDVDTIINK.F	2	
									K.LPSPFEDVDTIINK.F	2	
									K.FAAVGLNIIDVVALSGAHTIGQAR.C	3	
									K.ALVQTYSTNQNLFLNDFANSMIK.M	3	
9	Plant peroxidase	800693	db10_3026	AT4G33420.1	34634.11	5.5	484	7	R.STVSSALQSDPTLAAALVR.M	2	0.933
									K.DNTAEKDSPGNLSVR.G	3	
									R.GFELIDDVKEQLENQCPGVVSCADIVAMAA R.E	3	
									R.EAVSWSGGPVYDIPK.G	2	
									R.LSDPVDPTMDSDFSK.A	2	
									K.TCSGGDNAEQSFDVTR.N	2	
									R.NNFDSFYFQALQR.K	2	
8	Plant peroxidase	267768	db10_123557	AT3G28200.1	31982.7	7	141	2	K.ACPSTVSCVDILALAAR.E	2	0.001
									R.TGLLESDQALMGDSK.T	2	
9	Plant peroxidase	589413	db10_38459	AT2G37130.1	36914.92	5.7	213	5	R.AEEIIKEQVIK.L	2	0.847
									K.SCDASLLLETVNGIESEK.A	2	
									R.IYPTVDPTMDPDYAEYLK.G	2	
									K.MAADNGYFHDQFSR.A	3	
									R.AVVLLSENNPLTGNQGEIR.K	2	

Supplementary Table 5-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
9	Plant peroxidase	767526	db10_4126	AT5G06720.1	32574.53	6.4	157	3	K.YGVWAAFK.N	2	0.037
									R.ESVLLSGGPYYPLSFGR.R	2	
									K.DASNSNLAPLDYASTYR.F	2	
4	β-glucosidase (glycoside hydrolase, family 1)	181149	db10_117575	AT5G44640.1	54811.8	5.6	329	6	R.DYADLCFQK.F	2	0.006
									K.YWITLNEPQK.F	2	
									K.FSSAGYDSGDFAPGR.C	2	
									R.YQTDSNAIIK.A	2	
									R.ITFHYDHLQNVLNSINDYK.V	3	
									R.SATWFSECLLK.N	2	
5	D-arabinono-1, 4-lactone oxidase	809065	db10_7244	AT5G11540.1	61699.94	7.6	210	5	K.VSLGMLGVISK.V	2	0.000
									K.MQTSGSCLYSTK.I	2	
									R.IGGLFFYETTAIFPALK.F	2	
									R.LNQDVWEEVEQMAFFK.Y	2	
									K.ADGCALEGQCICSEDIHCSPK.K	3	
14	Plant lipid transfer/seed storage/trypsin-alpha-amylase inhibitor	825296	db10_30979	AT3G53980.1	11902.3	8.2	334	8	K.IETAITIPK.R	2	0.999
									K.IETAITIPK.R	2	
									R.CNIANRPVGYK.C	2	
									K.AAVSDSCCLQVK.R	2	
									K.RCNIANRPVGYK.C	3	
									R.MGQKPSCLCAVMLSDTAK.A	3	
									R.MGQKPSCLCAVMLSDTAK.A	3	
									K.RMGQKPSCLCAVMLSDTAK.A	3	

Supplementary Table 2-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
14	Plant lipid transfer/seed storage/trypsin-alpha-amylase inhibitor	820375	db10_715197	AT5G05960.1	12544.96	8.6	375	10	K.NTTPDMEAFK.M	2	0.972
									R.CNIADRPVGYK.X	2	
									K.RCNIADRPVGYK.X	3	
									K.SSGIKPEIAMTIPK.R	2	
									K.SSGIKPEIAMTIPK.R	2	
									K.SSGIKPEIAMTIPK.R	2	
									K.IGQNPACLCAVMLSNTAK.S	2	
									K.KIGQNPACLCAVMLSNTAK.S	3	
									K.MAPCASAAKDENSSVSSQCCAR.V	3	
									K.MAPCASAARDENSSVSSQCCAR.V	3	
2	Plant lipid transfer protein/Par allergen	414321	db10_274395	AT5G64080.1	8435.41	6.3	100	2	K.TVLGTDAECLCEAFK.S	2	0.296
									K.ALALPSACK.I	2	
2	Plastocyanin-like	669166	db10_48123	AT2G25060.1	23233.14	9.3	92	4	K.YDGQKDSVLQVTK.E	3	0.684
									K.SGPFYFISGAEGHCEK.G	3	
									K.SGPFYFISGAEGHCEK.G	2	
									K.IVVVVLSQK.H	2	
12	Plastocyanin-like	816369	db10_102405	AT1G72230.1	18789.67	5.7	152	3	K.TAGTHYFICGVPGHCGSGMK.V	3	0.966
									K.TFSVGDSLVFNYGGGHTVDEVR.A	3	
									R.ASDYSTCTTGNAITSDSSGATTIALK.T	4	

Supplementary Table 5-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
4	Subtilisin-related protease/Vacuolar protease B	551801	db10_57379	AT5G67360.1	82090.8	6.6	632	11	K.LIGAQYFSK.G	2	0.622
									R.TLTNVGVPATYK.L	2	
									K.SALMTTAYATYK.N	2	
									K.ILVEPESLSFAK.E	2	
									K.LGVEPSPVLAAFSSR.G	2	
									K.SFDDTGLGPIPSTWK.G	2	
									K.GYEAAFGPIDETMESK.S	2	
									K.NYAFSDPKPMATIASGGTK.L	3	
									K.VCWLGGCFSSDILAAMEK.A	2	
									K.YSLGDLNYPSFSVPLETASGK.G	2	
									K.SDAVPPASASMSEVIVGVLDTGVWPEIK.S	3	
4	Tripeptidyl peptidase II	559867	db10_23234	AT5G67360.1	82271.72	8.4	66	2	K.IVICDNDEDINSYYK.M	2	0.956
									R.GYDVIIGILDTGIWPESK.S	2	
3	Aconitate hydratase	593790	db10_41185	AT4G35830.1	98208.38	5.9	108	3	K.IIDWENTAPK.L	2	0.000
									R.SDETISMIESYLR.A	2	
									R.MFVDYSEPQIER.M	2	
11	Triosephosphate isomerase	713199	db10_55405	AT2G21170.1	27297.22	5	179	4	K.GGAFTGEISVEQLK.D	2	0.001
									K.GPEFATIVNSVTSK.K	2	
									K.QEDIDGFLVGGASLK.G	2	
									R.IIYGGSVNGGNCAELAK.Q	2	
4	Cyclin-dependent kinase inhibitor	730906	db10_56725	AT3G60900.1	43247.28	5.4	160	3	K.NALSLLVVLDYYDPTK.L	2	0.999
									K.TFASLLQTSGVIK.T	2	
									K.LTNAEIVSLLQYHATATYSPFGSLK.T	3	

Supplementary Table 5-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
5	β-glucosidase (glycoside hydrolase, family 3)	799561	db10_142263	AT5G09730.1	83409.74	5.7	119	3	K.NTAGSLPLSPTAIK.N	2	0.999
									K.DVCTAENQELAR.E	2	
									R.GQETPGEDPLLSSK.Y	2	
7	3-phosphoglycerate kinase	659332	db10_46738	AT1G79550.2	42676.99	5.7	590	11	R.VDLNVPLDDNFNITDDTR.I	2	0.000
									K.YSLKPLVPR.L	2	
									R.LSELLGVEVK.I	2	
									K.IANDCIGEEVEK.L	2	
									K.LVAEIPGGGVLLLENVR.F	2	
									K.ELDYLVGAVANPK.K	2	
									K.TFSEALDTTK.T	2	
									K.TIIWNGPMGVFEFEK.F	2	
									K.FAAGTEAIAWK.L	2	
									K.GVTTIIGGGDSVAAVEK.V	2	
									K.MSHISTGGGASLELLEGKPLPGVLALDDA.-	3	
7	60S ribosomal protein L4	735444	db10_30847	AT3G09630.1	44965.45	10	316	7	K.EIGAFPDAEK.A	2	0.375
									K.LDSIYGSFEK.C	2	
									R.NIPGVEVANVER.L	2	
									R.KGPLIVYGTEGAK.M	2	
									K.ASIRPDIVNYVHSNISK.N	3	
									K.TMISDSDYTEFENFTK.W	2	
									R.VESVPEMPLIISDSAESIEK.T	2	

Supplementary Table 5-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
10	60S ribosomal protein L7	579008	db10_32052	AT2G01250.1	33338.57	9.8	158	3	K.EANNFLWPFQLK.A	2	0.025
									R.VEPYVTFGYPNLK.S	2	
									R.IPLTDNSIVEQGLGK.H	2	
13	60S ribosomal protein L11	819383	db10_397256	AT4G18730.1	20657.43	9.9	168	4	R.SHTVQVPK.Y	2	0.001
									K.AMQLLESGLK.V	2	
									K.LVLNISVGESGDR.L	2	
									K.VLEQLSGQTPVFSK.A	2	
10	40S ribosomal protein S3	835769	db10_1019421	AT2G31610.1	26132.87	9.6	153	3	R.GLCAIAQAESLR.Y	2	0.001
									R.ELAEDGYSGVEVR.V	2	
									K.FVADGVFYAELNEVLTR.E	2	
10	40S ribosomal protein S4	831930	db10_18532	AT5G07090.2	29761.01	10	138	3	R.LGNVFTIGK.G	2	0.011
									K.FDVGNVVMVTGGR.N	2	
									K.GSFETIHVQDATGHEFATR.L	3	
12	Ribosomal protein S7	256059	db10_1001090	AT3G11940.2	22594.41	9.8	210	4	R.QAVDISPLR.R	2	0.000
									R.VNQAIYLLTTGAR.E	2	
									R.RVNQAIYLLTTGAR.E	2	
									K.TIAECLADELINAAK.G	2	

Supplementary Table 5-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
13	40S ribosomal protein S13	666912	db10_261287	AT4G00100.1	17112.96	10	210	5	K.GISASALPYK.R	2	0.000
									K.GISASALPYKR.T	2	
									K.GLTPSQIGVILR.D	2	
									K.KGLTPSQIGVILR.D	2	
									K.ISAQDVEDNICK.F	2	
14	40S ribosomal protein S20	824872	db10_129505	AT5G62300.1	13681.32	9.7	127	2	R.VIDLFSSADVVK.Q	2	0.000
									K.AQKPGLEEAQEQIHK.I	3	
12	60S ribosomal protein L9	829935	db10_63983	AT4G10450.1	21919.22	9.5	202	5	K.FLDGIYVSEK.G	2	0.000
									R.KFLDGIYVSEK.G	2	
									K.IGLRVLGQLVNR.E	2	
									K.TILSSETMDIPDGVK.I	2	
									K.VKDELVLDGNDIELVSR.S	3	
13	40S ribosomal protein S11	677637	db10_102278	AT3G48930.1	17915.9	11	89	2	K.CPFTGTVSIR.G	2	0.000
									R.DAIEGTYIDKK.C	2	
7	NADP-dependent isocitrate dehydrogenase	678577	db10_50192	AT1G65930.1	46390.67	6.3	255	6	K.VANPIVEMDGDEMTR.V	2	0.000
									K.KWPLYLSTK.N	2	
									K.SKYEAAGIWYEHR.L	2	
									R.LIDDMVAYALK.S	2	
									K.TIEAEAAHGTVTR.H	2	
									R.DQYLNTEEFIDAVAVELK.A	2	

Supplementary Table 5-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
8	dTDP-glucose 4-6-dehydratase/UDP-glucuronic acid decarboxylase	822488	db10_56295	AT3G46440.1	39025.24	7.3	614	12	R.LMENEKNEVIVADNYFTGSK.D	3	0.000
									R.HDVTEPLLVEVDQIYHLACPASPIFYK.Y	3	
									K.TNVIGTLNMLGLAK.R	2	
									R.VAETLMFDYHR.Q	3	
									R.IFNTYGPR.M	2	
									R.VVSNFIAQAIR.N	2	
									R.NEPLTVQAPGTQTR.S	2	
									R.SFCYVSDMVDGLIR.L	2	
									R.LMEGENTGPINIGNPGEFTMIELAENVK.E	3	
									K.IISVENTPDDPR.Q	2	
									K.AKELLGWEPK.I	2	
									R.DGLPLMEEDFR.Q	2	
8	Glyceraldehyde 3-phosphate dehydrogenase	575307	db10_30130	AT3G04120.1	36767.53	7.2	560	11	K.IGINGFGR.I	2	0.000
									R.DDVELVAVNDPFITTEYMTYMFK.Y	3	
									K.YDTVHGPWK.H	2	
									K.TLLFGERPVTVFGSR.N	2	
									R.NPEEIPWGETGAEYIVESTGVFTDKEK.A	3	
									K.DAPMFVMGVNEK.S	2	
									R.AASFNIIPSSTGAAK.A	2	
									R.VPTVDVSVVDLTVR.L	2	
									K.AGIALNDNYVK.L	2	
									K.LVAWYDNEWGYSTR.V	2	
									R.VVDLICYIASVSS.-	2	

Supplementary Table 5-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
8	Zinc-containing alcohol dehydrogenase	421020	db10_71698	AT1G23740.1	33585.19	6	549	10	K.AWVYGEYGNVSNVLK.L	2	0.000
									K.LDSNVTVPQVK.E	2	
									K.VVAASINPVDAK.R	2	
									K.VSDSPVPTVPGYDVAGVVVK.V	2	
									R.LKVGDEVYGDINEK.A	2	
									R.FGSLAEYTAVEENLLSLKPK.N	3	
									K.NLSFAEAASLPLVIETAHEGLER.T	3	
									K.HVFGASTVAATSSTSK.L	2	
									K.SLGADLAIDYTK.E	2	
									K.GPFPFSQTAEAFSYLETSR.A	2	
12	1,4-benzoquinone reductase-like	727757	db10_26499	AT4G27270.1	21691.41	6.1	225	5	R.FGMMAAQFK.A	2	0.002
									K.GADTVEGVEIK.L	2	
									K.GGSPYGAGTFAGDGTR.Q	2	
									K.LWQVPETLPEEVLGK.M	2	
									K.SDVPIIKPNDLTEADGVLFGFPTR.F	3	
8	Malate dehydrogenase	564942	db10_25279	AT5G56720.1	35714.05	6.1	350	8	R.LSVQVCDVK.N	2	0.454
									K.MELVDAAFPLLK.G	2	
									K.IVQGLSIDEFSR.K	2	
									K.LSSALSAASSACDHIR.D	3	
									K.VLVVANPANTNALILK.E	2	
									R.KLSSALSAASSACDHIR.D	3	
									R.ELVKDDEWLNAEFITTVQQR.G	3	
									R.GVMLGPDQPVILHMLDIPPAAEALNGVK.M	3	

Supplementary Table 5-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
7	Actin and related proteins	813612	db10_44008	AT3G18780.2	41692.64	5.3	240	5	R.GYMFTTTAER.E	2	0.000
									K.GEYDESGPSIVHR.K	2	
									K.LAYVALDYEQELETAK.S	2	
									K.YPIEHGIVSNWDDMEK.I	3	
									R.VAPEEHPVLLTEAPLNPK.A	3	
8	Caffeic acid 3-O-methyltransferase	834247	db10_327940	AT5G54160.1	39731.52	5.5	375	12	R.LYGLAPVCK.F	2	0.000
									K.NPDAPVMLDR.I	2	
									K.DAILDGGIPFNK.A	2	
									K.NCYDALPENGK.V	2	
									K.VLMESWYYLK.D	2	
									K.TAIELDLLEIMAK.A	2	
									K.AYGMTAFEYHGTDPR.F	3	
									K.WICHDWSDAHCLK.F	3	
									K.NEDGVSVSPLCLMNQDK.V	2	
									K.VILVECILPVAPDTSLATK.G	2	
									K.GFEGLTSLVDVGGGTGAVVNTIVSK.Y	2	
									K.GINFDLPHVIEDAPSYPGVEHVGGDMFVSVPK.A	4	
7	6-phosphogluconate dehydrogenase	837012	db10_175586	AT3G02360.2	53738.95	6.3	98	2	R.FLSGLKEER.V	2	0.837
									R.LPANLVQAQR.D	2	

Supplementary Table 5-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
8	NAD-dependent malate dehydrogenase	822907	db10_56526	AT3G47520.1	35585.76	8.7	336	7	R.LFGVTTLDVVR.A	2	0.050
									K.LKPELQSSIEK.G	2	
									R.DDLFNINAGIVK.G	2	
									K.ANLSDAEITALTK.R	2	
									R.SEVSGYSGEAELGK.A	2	
									K.ALEGADVVIIPAGVPR.K	2	
									K.VAVLGAAGGIGQPLALLMK.L	2	
8	Fructokinase	741475	db10_596340	AT1G06030.1	35270.39	5	261	5	K.LGDDEFGNMLAGILK.E	2	0.014
									R.NPSADMLLRPEELNLELIR.S	3	
									R.LPLWPSAEEAR.E	2	
									R.EQILSIWDEADVVK.V	2	
									K.IVDDQSVLEDEPR.L	2	
9	Annexin	818283	db10_867365	AT5G65020.1	36018.08	6.2	288	7	K.VPASVPPPYEDAEQLNK.A	2	0.000
									R.QVYAEAYGQDLLK.D	2	
									R.VVLLWTLDLAER.D	2	
									R.FTSSNWVLMEIACTR.S	2	
									K.LLVPLVSAFR.Y	2	
									K.IGTDEWALTR.V	2	
									R.MLLALIGHGDA.-	2	
9	Unknown	565652	db10_876224	AT1G57590.1	7376.7	9.3	200	4	K.FYTEGFGMK.L	2	0.001
									K.DPDGYIFELIQR.G	2	
									K.GNAYAQVAIGTDDVYK.S	2	
									K.SGEVVNLVIQELGGK.I	2	

Supplementary Table 5-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
9	Unknown	573059	db10_874318		18523.2	4	196	3	-.ITVEVVSAQNALVEEK.N	2	0.007
									K.VAASEEPVAPEPEAPAEAETK.E	2	
									K.IAAEEPTVLEK.T	2	
10	Multifunctional chaperone (14-3-3 family)	651251	db10_870256	AT4G09000.1	29298.35	4.7	572	11	R.NLLSVAYK.N	2	0.000
									K.EAAESTLTAYK.S	2	
									R.KEAAESTLTAYK.S	2	
									R.YEEMVEYMEK.V	2	
									R.IISSIEQKEESR.G	2	
									K.IETELSSICDGILK.L	2	
									K.VSASIDNEELTVEER.N	2	
									K.SAQDIAPAELAPTHPIR.L	3	
									K.SAQDIAPAELAPTHPIR.L	3	
									K.QAFDEAIAELDTLGEESYK.D	2	
									K.LAEQAERYEEMVEYMEK.V	3	
14	Multifunctional chaperone (14-3-3 family)	551637	db10_79598	AT5G38480.1	28592.94	4.7	161	4	K.IMDIASLEK.F	2	0.000
									K.AGALGDTVTVTR.E	2	
									K.ITVTSDSNFSKR.Y	2	
									K.LAEQAERYEEMVQFMEK.L	3	
9	Predicted chitinase	270686	db10_191946	AT3G54420.1	27161.24	4.4	91	2	R.DAFLEALNSYSR.F	2	0.009
									R.DYCDEGNTQYPCNPDK.G	2	

Supplementary Table 5-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
10	Chitinase	572334	db10_569640	AT5G24090.1	29937.15	7	157	3	R.GVAEYLWNNFLGGR.S	2	0.987
									R.GVAEYLWNNFLGGR.S	2	
									K.VMLSIGGGTSGYTLTSDAEAR.G	2	
10	Short-chain dehydrogenase/reductase SDR	833658	db10_56226	AT2G29350.1	28293.62	7.7	232	3	K.VSGSVCDVSSQADR.E	2	0.060
									R.WSLQGTTALVTGGTK.G	2	
									K.VSGSVCDVSSQADREK.L	2	
10	Unknown (DUF 26)	562320	db10_24310	AT3G22060.1	26389.77	5.8	229	5	K.TCVVEAGGEIR.K	2	1.000
									K.AAIIWYDNCLVK.Y	2	
									K.YSNNGFFGQIDNGNK.F	2	
									K.VVSEPVTFNGK.T	2	
									K.ELLTQLANK.V	2	
11	Serine/threonine protein kinase	662643	db10_47218	AT3G53840.1	22656.28	6.9	260	6	K.LVIRPPLIGK.D	2	1.000
									K.GTLCCHFLK.D	2	
									R.LDENLPFNISVR.N	2	
									K.DTCYSSDLAVGGLR.L	2	
									R.IYCNNGALEFLSAQGLYYR.I	3	
									R.IYCNNGALEFLSAQGLYYR.I	2	
11	Cupin	591472	db10_29346	AT5G39130.1	23999.16	7.1	246	5	K.AFHLDIK.I	2	1.000
									K.LATANDFSFSGLNIPR.D	2	
									K.LATANDFSFSGLNIPR.D	2	
									R.IDYAPNGGLNPPHTHPR.A	3	
									K.TPAVVFAGLSSQNPGTITIANAVFGSDPLINPDVLAK.A	3	

Supplementary Table 5-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
11	Triosephosphate isomerase	656103	db10_46242	AT2G21170.1	27257.62	6	450	7	R.SLLNESNEFVGDK.V	2	0.000
									K.VAYALSQGLK.V	2	
									R.EAGSTVEVVAAQTK.A	2	
									R.VSNWADVVLAYEPVWAIGTGK.V	2	
									K.VATPAQAQEVHYELR.K	3	
									K.WLQENTSPEVAATTR.I	2	
									R.IIYGGSVSGANCK.E	2	
7	Transaldolase	646955	db10_44849	AT5G13420.1	47490.6	6.4	423	9	K.AAVAQAALAYK.L	2	0.083
									K.IGTPEALDLR.G	2	
									R.TTLHDLYER.E	2	
									R.GVTSNPAIFQK.A	2	
									K.SFDSLLDTLQEK.A	2	
									K.DIETAYWELVVK.D	2	
									K.AISSSNAYNDQFR.E	2	
									R.TIDSNVSEAEGIYNALEK.L	2	
									K.LGIDWGYVGNQLEVEGVDSFKK.S	3	
11	Translationally controlled tumor protein	725698	db10_344936	AT3G16640.1	19066.27	4.5	110	2	K.VVDIVDTFR.L	2	0.000
									K.EGATDPTFLYFAPSLK.E	2	
11	Adenosine kinase	659896	db10_119814	AT3G09820.1	37544.29	5.1	89	2	K.AGCYAANVIIQR.S	2	0.013
									K.LVDTNGAGDAFVGGFLSQLVQEKPIEDCVK.A	3	

Supplementary Table 5-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
12	Thaumatin, pathogenesis-related	180318	db10_19067	AT4G11650.1	25018.1	5.9	305	6	R.NNCPYTVWAAASPGGGR.R	2	0.952
									R.GQTWYLNVPAGTSMAR.I	2	
									R.GQTWYLNVPAGTSMAR.I	2	
									K.CQALLCTADINGQCPNELR.A	2	
									R.APGGCNNPCTVFK.T	2	
									K.TNEYCCTNGQGSCGPTYFSR.F	2	
12	Thaumatin, pathogenesis-related	669475	db10_67753	AT1G75040.1	24732.83	7.8	118	2	R.ASGGCNNPCTVFK.T	2	0.999
									R.GQSWTITANAGTTQAR.I	2	
12	Unknown	746481	db10_57732	AT1G17860.1	22837.37	9	224	6	K.YYILPVFR.G	2	1.000
									K.TSCPLAVVQDRLEVSK.G	3	
									K.GVILVSTDLNIK.F	2	
									K.TTCPQSTVWK.I	2	
									K.VQWFVSTGGVEGNPGFNTVTNWFQIEK.A	3	
									R.DIGIYIEDNGTR.T	2	
12	Allene oxide cyclase	198984	db10_21835	AT1G13280.1	26603.78	9.1	90	2	K.ADLPEELLGK.A	2	0.110
									K.AVEPSPAVEPSPAAK.A	2	
13	GTP-binding ADP-ribosylation factor Arf1	744433	db10_1008331	AT1G10630.1	20651.46	6.5	188	4	R.DAVLLVFANK.Q	2	0.007
									K.NISFTVWDVGGQDK.I	2	
									K.QDLPNAMNAAEITDK.L	2	
									K.LGEIVTTIPTIGFNVETVEYK.N	2	

Supplementary Table 5-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
13	Endoribonulease	829920	db10_283722	AT3G20390.1	21679.77	8.8	135	7	K.APAALGPYSQAIK.S	2	0.028
									R.STYQVAALPLDAK.I	2	
									K.FVSENVEDQTEQLLK.N	2	
									M.APIAVGDVLPDGK.L	2	
									K.VILFGVPGAFTPTCSLK.H	2	
									K.AANIEGGGEFTVSSADDILK.D	2	
									K.AANIEGGGEFTVSSADDILKDL.-	2	
13	Actin depolymerizing factor	829643	db10_916787	AT5G59880.2	16018.98	6	107	2	R.IFFIAWSPDTSR.V	2	0.000
									M.ANAISGMAVHDDCK.L	2	
14	Thioredoxin	818765	db10_677567	AT1G45145.1	12575.17	5.6	216	6	K.KFPNVTFLK.V	2	0.000
									K.MIAPIFAELAK.K	2	
									K.MIAPIFAELAK.K	2	
									K.MIAPIFAELAK.K	2	
									K.TVGADKDGLPTLVAK.H	2	
									K.AVAEEWNVEAMPTFIFLK.D	2	
14	pop3-/SP1 like	833676	db10_25946	AT3G17210.1	12466.77	5.8	191	4	K.IVVVDFVPTLVK.L	2	0.000
									K.FKEGVTEEEIEK.H	2	
									K.IVVVDFVPTLVKL.-	2	
									R.SAYVAHPAHVDFGTAFLTILEK.I	3	
14	pop3 peptide	723969	db10_111914	AT3G17210.1	12423.96	4.9	149	2	R.GYTHAFESTFESK.S	2	0.000
									K.SFNWGTDLGMESAELNR.G	2	

Supplementary Table 5-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
14	Defense-related protein containing SCP/ PR-1 domain	595857	db10_110108	AT2G14580.1	17256.72	8.5	184	3	R.HYTQVVWR.N	2	0.991
									K.YDYNSNSCVGGECR.H	2	
									R.LVHSGGPYGENLAGGSGDLTGSAAVK.L	3	
14	Glycine-rich RNA-binding protein	712972	db10_103614		16505.71	5.5	96	2	R.GFGFVTFNNEK.A	2	0.085
									R.DAIDGMNGQDLDGR.N	2	
5	Alpha-L-arabinofuranosidase	255102	db10_30539	AT5G26120.1	73315.47	5.5	319	6	R.NELIEMIDDIKPQFVR.F	3	0.998
									R.NELIEMIDDIKPQFVR.F	2	
									R.FPGGCFVEGEWLR.N	2	
									R.AAMGHPEPFDLK.Y	2	
									K.VSIDGLGLSSQLSGSTK.T	2	
									K.TVLTSSNLMDENSFANPK.K	2	
5	Methylenetetrahydrofolate reductase	828552	db10_54632	AT2G44160.1	67491.47	6	167	4	K.LLEEWAAPLK.S	2	0.000
									R.GWASLYPEGDPSR.T	2	
									K.SSPWSELDGLQPETK.I	2	
									K.SALAILMNLGLIEESK.I	2	
7	Plant peroxidase	208491	db10_22808	AT4G36430.1	32611.68	5.2	336	7	K.FPYLFSK.D	2	0.000
									K.FDNLYYK.L	2	
									R.GFEVIDDIK.A	2	
									K.MANIGVLTGQNGEIRK.N	3	
									R.ACPATVSCTDILTLAAR.E	2	
									R.EAVYLSGGPYWFLPLGR.R	2	
									R.LHFHDCFVNGCDGSLLLDGGEK.N	3	

Supplementary Table 5-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
9	Phenylcoumaran benzylic ether reductase 1	830063	db10_44210	AT1G75290.1	33976.77	5.5	400	10	K.ITILGDGNAK.L	2	0.004
									K.AGHPTFALVR.E	2	
									K.EDDIGTYTIK.A	2	
									K.ILIIGGTGYIGK.F	2	
									R.DKITILGDGNAK.L	2	
									K.YTTVEEYLDQFV.-	2	
									K.LVFNKEDDIGTYTIK.A	3	
									K.NTYSFNELIDLWEK.K	2	
									K.NLGVTLIHGDVDGHDNLVK.A	3	
									R.FFPSEFGMDVDHVNAVEPAK.T	3	
11	Plant basic secretory protein	549955	db10_66788	AT2G15220.1	24994.71	7.2	418	8	R.NGFVAELNK.K	2	1.000
									R.NGFVAELNKK.M	2	
									K.FLDYCNGLR.N	2	
									R.WDQGYDVTAK.F	2	
									K.QTLASATDFIWR.T	2	
									K.TGYSAQYFVDLLGK.T	2	
									K.ANYAPSHWVQAGQGDR.W	3	
									K.ANYAPSHWVQAGQGDRWDQGYDVTAK.F	4	
11	Acid phosphatase	711542	db10_55238	AT1G04040.1	29085.92	8.3	150	5	R.LYLSSCCTLEGDGK.D	2	0.998
									K.HYMTSSQYTADSER.A	2	
									K.HYMTSSQYTADSER.A	3	
									R.IWGIIGDQWSSVEGLPGAK.R	2	
									R.INVELNNIKDFEVVPQECVDHVK.H	3	

Supplementary Table 5-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
4	Glycosyltransferase	826368	db10_38427	AT5G20830.2	92152.07	6.2	343	8	R.VNVQALVVEELR.V	2	0.000
									K.YIGNGVEFLNR.H	2	
									R.LLPDAVGTTCGQR.L	2	
									K.LGVTECTIAHALEK.T	2	
									R.VVHGIDVFDPK.F	2	
									R.NKPILFTMAR.L	2	
									R.ELANLVVVGGDR.R	2	
									R.LLTLTGVYGFWK.H	2	
5	Molecular chaperones HSP70	832542	db10_53323	AT1G16030.1	71169.19	5.1	588	12	R.TTPSYVAFTDTER.L	2	0.002
									R.YSDASVQSDIK.L	2	
									K.QFAAEEISSMVLIK.M	2	
									R.EIAEAYLGTTIK.N	2	
									K.NAVVTVPAYFNDSQR.Q	2	
									K.DAGVIAGLNVMR.I	2	
									R.IINEPTAAAIAYGLDK.K	2	
									R.IINEPTAAAIAYGLDKK.A	2	
									K.ATAGDTHLGGEDFDNR.M	2	
									K.STVHDAVLVGGSTR.I	3	
									K.VQQLLQDFFNGK.E	2	
									K.ELESICNPIIAK.M	3	
4	Multicopper oxidase	827894	db10_54539	AT4G12420.1	66097.28	8.8	88	2	R.VHNVGTSTSLNFR.I	2	0.999
									R.ATLSGISFVNPTTPIR.L	2	

Supplementary Table 5-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
4	Vitamin-B12 independent methionine synthase	679841	db10_866864	AT3G03780.2	84592	6.3	1304	25	K.YLFAGVVDGR.N	2	0.001
									K.SFSLLSLIDK.I	2	
									R.IPSEEEIADR.I	2	
									K.SVTGFGFDLVR.G	2	
									R.GNASVPAMEMTK.W	2	
									K.AAGASWIQFDEPK.L	2	
									K.AGITVIQIDEAALR.E	2	
									K.SSAEDLQKVAADLR.S	2	
									K.ILPVYQEVLAELK.A	2	
									R.IPSEEEIADRIEK.M	3	
									K.YGAGIGPGVYDIHSPR.I	3	
									K.AMTVFWSSMAQSMTK.R	2	
									R.CVKPPIIYGDVSRPK.A	3	
									K.GMLTGPVTILNWSFVR.N	2	
									R.KYAEVKPALSNMVAAAK.H	3	
									K.IQEELDIDVLVHGEPER.N	3	
									K.ALAGQQDEAFFSANAAAQASR.K	2	
									K.MLAVLESNILWVNPDCGLK.T	2	
									K.LVVSTSCSLLHTAVDLVNEPK.L	3	
									R.FETCYQIALAIKDEVEDLEK.A	3	
									K.ALGVDTVPVLVGPVSYLLLSKPAK.G	3	
									K.KLNLPILPTTTIGSFPQTMDLR.R	3	
									K.DKLVVSTSCSLLHTAVDLVNEPK.L	3	
									K.FIPSNTFSCYDQVLDTTAMLGAVPPR.Y	3	
									K.WFDTNYHYIVPELGPEVNFSYASHK.A	4	

Supplementary Table 5-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
8	Fructose-biphosphate aldolase	578574	db10_74220	AT3G52930.1	38381.97	6.7	542	11	K.GILAADESTGTIGK.R	2	0.000
									K.GILAADESTGTIGKR.L	2	
									R.LSSINVENVEENR.R	2	
									R.LSSINVENVEENRR.A	3	
									K.TASGKPFVDVLK.E	2	
									K.VDKGTVELAGTNGETTTQGLDGLGQR.C	3	
									K.IGANEPSELAIHENAYGLAR.Y	3	
									R.YAVICQENGLVPIVEPEILVDGSHDIEK.C	3	
									K.VAHEVIAEYTVR.A	2	
									R.TVPPAVPAIVFLSGGQSEEEATLNLNAMNK.L	3	
									K.GDATLGEGASESLHVK.D	2	
13	Cyclophilin type peptidyl-prolyl cis-trans isomerase	813818	db10_22018	AT2G21130.1	18093.85	8.7	334	7	K.FADENFVK.K	2	0.005
									K.FADENFVKK.H	2	
									R.IVMELFADIVPR.T	2	
									R.TSKPVVVADCGQLS.-	2	
									K.VFFDMTVGGQSVGR.I	2	
									K.HVVFGQVVEGMDVVK.A	2	
									R.VIPNFMCQGGDFTAGNGTGGESIYGAK.F	3	
11	Dehydroascorbate reductase	833836	db10_119461	AT5G36270.1	23630.04	5.8	128	3	K.ITAVDLSLAPK.L	2	0.008
									K.AAVGAPDILGDCPFSQR.A	2	
									K.FDDKWVSDSDVIVGILEEK.Y	3	

Supplementary Table 5-1. Continued

Spot ID	Identification	JGI Acc. No.	Poplar_db10	TAIR Acc. No.	Theoretical MW	Theoretical pI	Mascot Score	No. of matched peptides	Sequence Matches	Charge	SignalP Prob.
9	β-1,3-glucanase (glycoside hydrolase, family 17)	769807	db10_75979	AT3G57240.1	38883.13	7.1	292	7	R.LYDPNQAALNALR.G	2	0.988
									R.GSGIEVMLGVPNSDLQR.L	2	
									R.LSNPSDANSWVK.N	2	
									K.NNVLNFWPSVR.F	2	
									R.GDVISYLAPIVGHLSYAK.T	2	
									R.DISLPYALFTSPSVLVWDSGR.G	2	
									K.AIETYIFAMFDENQKQPELEK.H	3	
5	Phosphoglucomutase	832763	db10_53253	AT1G70730.1	63052.72	5.5	121	3	R.SMPTSAALDVVAK.N	2	0.000
									R.DSQDALAPLVAVALGLSK.M	2	
									K.VVHADEFEYKDPVDGSISK.H	3	