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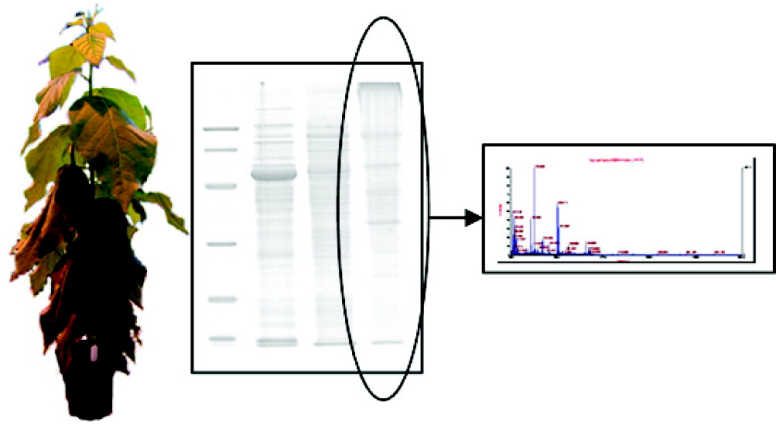

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Analysis of the Poplar Phloem Proteome and Its Response to Leaf Wounding

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Phloem exudate collected from hybrid poplar (*Populus trichocarpa* × *Populus deltoides*) was estimated to have more than 100 proteins, of which 48 were identified using LC-MS/MS. Comparative two-dimensional gel electrophoresis demonstrated that two phloem exudate proteins were significantly ($P < 0.05$) upregulated 24 h after leaf wounding. These were identified as pop3/SP1 and a thaumatin-like protein. This is the first characterization of a phloem proteome from a tree species.

Keywords: phloem exudate • herbivore defense • wounding • comparative two-dimensional gel electrophoresis

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.[1] More recently, it has become apparent that phloem has essential roles in other plant processes such as long-distance signaling. A large number of RNAs and proteins are present in phloem sap.[2] This has led to the idea that macromolecules in the phloem may play vital roles in plant development and environmental adaptation. For example, the antiviral defense known as post-transcriptional gene silencing appears to spread systemically through phloem-mobile short interfering RNA (siRNA)[2] and the elusive phloem-mobile 'florigen' which induces flowering in day-length sensitive plants appears to be the FT protein.[3] 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 elements connected by cytoplasmic strands that pass through pores in sieve plates.[1] 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.[4−7] In most other species, phloem exudate can only be collected by adding the Ca^{2+} chelator ethylenediaminetetraacetic acid (EDTA), which is thought to prevent the sealing of sieve elements.[8]

On the basis of two-dimensional gel electrophoresis (2-DE) analyses, phloem exudate has been estimated to contain hundreds of proteins.[5,6,9] 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*, *R. communis*, *Oryza sativa*, and *B. napus*.[5−7,10,11] Phloem proteins identified in these studies are involved in metabolism, signaling, and transport.[5,6,10,11] A large number of stress response proteins including antioxidant enzymes and herbivore defense proteins were also identified in phloem from several plant species.[5−7,11,12] The presence of both antioxidant and defense proteins in several species suggests conservation of function of phloem proteins;[13] however, almost no functional studies on phloem proteins have been reported.[2]

The presence of herbivore defense proteins in phloem is intriguing, especially because one of the best examples of long-range signaling in plants is the systemic induction of herbivore defenses. Following localized insect feeding, defense proteins such as the proteinase inhibitors accumulate rapidly in both wounded and unwounded leaves.[14] 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.[15,16] The induction of defense genes after leaf damage is not limited to leaves, but also occurs in roots and mature leaves.[16,17] The importance of phloem in herbivore defense signaling is further emphasized by immuno-detection of jasmonic acid (JA) biosynthesis en-

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zymes in sieve elements,[18] as JA or a JA-like molecule is considered to be a primary systemic signal for herbivore defense activation.[14]

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.[13,19] 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.[7]

The study of phloem proteins has been limited to annual plants, and therefore, very little is known regarding the identity of phloem exudate proteins from perennials. Compared to annual plants, perennials have longer lifespans, more extensive secondary growth, and undergo dormancy which involves seasonal shifts in nutrients.[20,21] Phloem in perennial plants therefore has to be adapted to these conditions and it may be reflected in the composition of phloem proteins. To test this hypothesis, we analyzed the phloem exudate proteome of a perennial, *Populus trichocarpa* × *Populus deltoides*. We optimized a method to collect phloem exudate from excised poplar stem 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. Materials and Methods

2.1. Plant Materials. Poplar hybrid H11-11 (*P. trichocarpa* × *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.[22] 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).

2.2. Phloem Exudate and Bark Sampling. To collect phloem exudate, 10 cm stem sections between leaf plastochron index (LPI) 5−16[23] were excised from 3-month-old *P. trichocarpa* × *P. deltoides* saplings and the leaves were removed. 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: 6000−8000, Spectrum Laboratories, RanchoDominquez, CA) against 200 vol. deionized water, with one change of H_2O. Samples were then lyophilized, and resuspended in resolubilization buffer (8 M urea, 65 mM dithiothreitol (DTT), and 16 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)) for 30 min at room temperature prior to protein quantification using a modified Bradford assay.[24]

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.[25] Soluble protein was precipitated using 2 vol. cold acetone for at least 1 h at −20 C. After centrifugation (15 min, 16 000*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.

2.3. Gel Electrophoresis and Immunoblotting. For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 10 μg of protein was separated on 12% polyacrylamide gels under a constant voltage (100 V) in a Mini PROTEAN II system (BioRad, Hercules, CA). 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)) 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 described for SDS-PAGE. Proteins were visualized using either Bio-Safe Coomassie stain (BioRad) for one-dimensional SDS-PAGE or Sypro Ruby (BioRad) for 2-DE and gels were scanned using the ProXPRESS Proteomic Imaging System (Perkin-Elmer, Waltham, MA).

For immunoblotting, after SDS-PAGE, proteins were electrotransferred to PVDF membrane (Pierce, Brockville, ON, Canada) and proteins were detected using standard protocols.[26] 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.

2.4. Immunolocalization. Genes encoding a thaumatin-like protein (TLP) and pop3 protein were cloned using a previously constructed *P. trichocarpa* × *P. deltoides* cDNA library.[27] The primers used for the amplification of TLP (JGI gene model estExt_fgenesh4_pg.C_6010001) were TLPs 5′-TTTTGGATCCCAATCTGTGACTTTCGACTT-3′ and TLPa 5′-GGGGGTCGACTGGACAAAAGGTTATCAAAT-3′. The primers used for pop3 (estExt_Genewise1_v1.C_LG_X0701) were pop3s 5′-GGGGGGATCCATGGCAACCAGAACTCCAAA-3′ and pop3a 5′-GGGGGTCGACGTAGAGAAAGTAGTCTATCA-3′. 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 proteins were produced and purified as previously described.[25] Anti-TLP and anti-pop3 antibodies were produced in rabbits using standard procedures at Cocalico Biologicals (Reamstown, PA). The TLP antiserum specifically labeled proteins of the expected size in bark and phloem exudate samples, whereas the preimmune serum did not label any proteins in these samples. Furthermore, TLP antiserum blocked with excess TLP antigen did not react with any plant proteins, confirming its specificity (N. Dafoe and C. P. Constabel, in preparation). The pop3 antiserum labeled a protein band corresponding to a higher molecular weight oligomeric form of the protein (N. Dafoe and C. P.

Constabel, in preparation) consistent with its observed molecular weight (Figure 3). The preimmune serum and preadsorbed pop3 antiserum did not label any plant proteins.

For immunolocalization of TLPs and pop3, stem segments (near LPI 11) were fixed and embedded in BMM resin mixture (4 parts *n*-butyl methacrylate to 1 part methyl methacrylate) as previously described.[28] Stem cross sections (6 μm thick) were cut and mounted on positive-charged glass slides. Prior to labeling, the plastic was partially removed by soaking the sections in acetone for 20 min. The sections were labeled with TLP or pop3 antisera using standard protocols[26] and the antibodies were detected with an Alexafluor 568 goat anti-rabbit antibody (Molecular Probes, Eugene, OR). Controls included omitting the primary antibody or incubating sections with the respective preimmune serum. Labeling 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).

2.5. Identification of Proteins with LC-MS/MS. Total phloem proteins were first separated using one-dimensional 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.[29] After washing the 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 using a Q TRAP hybrid triple quadrupole/linear ion trap MS/MS mass spectrometer equipped with a nanoelectrospray ionization source (Applied Biosystems/MDS Sciex) as previously described.[30] MS data was processed with Analyst 1.4.1 software and a built in Mascot script (1.6b16 ABI - Matrix Science Limited, Boston, MA). 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 and 0.3 Da, respectively. Parameters also permitted one missed cleavage and were limited to 2+ and 3+ charged peptides.

A custom poplar database, 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 1818 poplar proteins present in GenBank. All unigene proteins from the *Arabidopsis thaliana* (http://www.arabidopsis.org/) and *O. 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.[31] 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).

2.6. 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 24 h after wounding and pooled prior to analysis as described above. Samples from three biological replicates were analyzed with 2-DE. Each sample was analyzed on three separate gels and each gel was treated as a single replicate 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, U.K.). Comparative gel analysis was performed as described in Lippert et al.[32] 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.

2.7. Carbohydrate Analysis of Phloem Exudate. Sucrose, glucose, and fructose concentrations were quantified as previously described.[33] Briefly, the sugars were enzymatically converted to glucose 6-phosphate and carbohydrate concentrations were quantified colorimetrically 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 microtiter plate.

3. Results

3.1. Collection of *Populus* Phloem Exudate. To facilitate the analysis of poplar phloem proteins, we tested several phloem collection methods using an EDTA exudation buffer.[8,34,35] The samples were checked for the absence of RuBisCO, an approach used previously to verify the purity of phloem samples.[5,35,36] 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 55 kDa (Figure 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 nonspecific binding.

We first tested the method of Herschbach et al.[37] 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 (Figure 1A). If stems were incubated without EDTA or in the presence of excess $CaCl_2$, protein yields were strongly reduced,

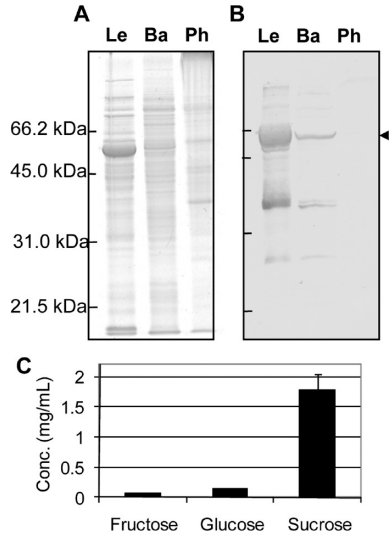

Figure 1. Analysis of hybrid poplar phloem exudate. Comparison of poplar phloem exudate (Ph) to leaf (Le) and bark (Ba) proteins using (A) Coomassie stained SDS-PAGE gel and (B) western blot with antibodies to RuBisCO (large subunit). The arrow indicates the position of the RuBisCO protein. For both Coomassie and western blot analyses, 10 μg of protein was loaded. (C) Analysis of sugars in phloem exudate as determined by enzymatic assays. Means and standard errors are shown ($n = 6$).

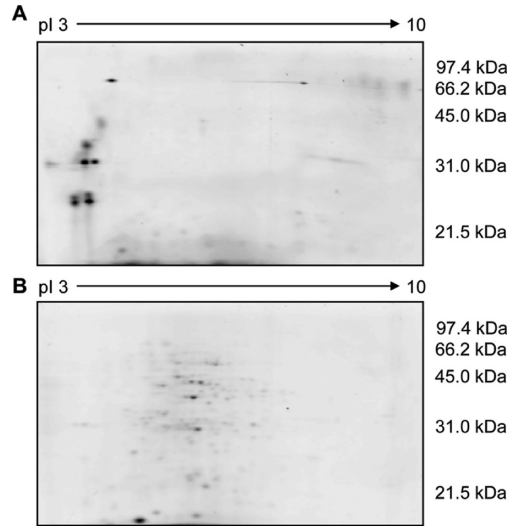

Figure 2. Comparison of proteins from phloem exudate (A) and bark (B). Proteins (4 μg) were separated by 2-DE and stained with Sypro Ruby.

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 (Figure 1B).

To confirm the purity of the exudate obtained, sucrose, glucose, and fructose concentrations of the exudate were measured. As expected, sucrose was the most abundant carbohydrate, consisting of 90% of the total sugars measured (Figure 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 (Figure 2). 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 (Figure 2A). Phloem exudate exhibited a protein profile clearly distinct from that of bark tissue (Figure 2B), suggesting that contamination from surrounding tissues was minimal and prompting us to undertake a detailed analysis of these phloem exudate proteins.

3.2. Characterization of the *Populus* Phloem Exudate Proteome. To identify proteins in poplar phloem exudate, polypeptides were first separated by one-dimensional SDS-PAGE prior to analysis with LC-MS/MS. We obtained positive identification of 48 proteins (Table 1). Comparisons with the *Arabidopsis* genome using BLAST identified *Arabidopsis* homologues for most of the poplar proteins. On the basis of 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 1). In a few cases, genes known to be upregulated in poplar in response to environmental stresses were classified with the stress response proteins.[22,38] 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 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 1). These proteins included superoxide dismutase and peroxidase, both previously identified in the phloem sap of *C. sativus* and *C. maxima*.[11] A single peptide also matched a peroxiredoxin; a protein that has been immunologically detected in sieve elements of poplar.[39] 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, β-1,3-glucanase and thaumatin-like protein (TLP). Thus, both herbivore and pathogen defense proteins appeared to be present in the poplar phloem exudate.

Energy/metabolism and protein/amino acid metabolism accounted for the majority of the remaining proteins identified. Similar to the phloem proteome of *B. napus*, several proteins involved in glycolysis were identified in poplar phloem exudate.[5] Several enzymes involved in amino acid metabolism including glycine/serine hydroxymethyltransferase, *S*-adenosyl-L-homocysteine hydrolase, and methionine synthase were identified. The presence of such proteins in phloem sap is consistent with previous reports.[5,6,11,34,40] Overall, our proteomic experiments led to the identification of a broad range of proteins, with strong representation by defense proteins.

3.3. 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

Table 1. Poplar Phloem Exudate Proteins Identified by LC-MS/MS[a]

identification	JGI protein ID	theoretical MW	theoretical p*I*	Mascot score	no. of matched peptides	functional category
dTDP-glucose 4-6-dehydratase/UDP-glucuronic acid decarboxylase	832538	39104	6.13	105	2	metabolism
Enolase	575698	47924	5.67	159	3	metabolism
Glyceraldehyde 3-phosphate dehydrogenase	728998	36724	6.36	219	5	metabolism
Glycosyltransferase	826368	92152	6.18	598	14	metabolism
Glucose-6-phosphate 1-dehydrogenase	736146	59156	6.29	112	2	metabolism
Malate dehydrogenase	564942	35714	6.13	219	4	metabolism
Triosephosphate isomerase	724697	27396	6.51	210	3	metabolism
Carbonic anhydrase	417951	25504	9.4	190	3	metabolism
Ribulose bisphosphate carboxylase, large chain	279035	21534	6.47	88	2	metabolism
Lipolytic enzyme, G-D-S-L	758353	32713	8.82	124	3	metabolism
Lipolytic enzyme, G-D-S-L	580490	42331	5.09	137	4	metabolism
Phospholipase D1	829577	91816	5.03	134	2	metabolism
Glycine/serine hydroxymethyltransferase	829808	51906	7.26	138	3	aa metabolism
S-adenosyl-L-homocysteine hydrolase	597072	53195	5.75	289	6	aa metabolism
Vitamin-B12 independent methionine synthase	679841	84592	6.27	791	17	aa metabolism
Annexin	818283	36018	6.2	152	3	signaling
Annexin	643752	35965	6.17	102	3	signaling
Cyclophilin type peptidyl-prolyl cis−trans isomerase	813818	18093	8.72	216	4	signaling
Multifunctional chaperone (14-3-3 family)	711617	29377	4.68	173	4	signaling
1,4-benzoquinone reductase-like	726993	21671	5.97	94	3	stress response
Glutathione *S*-transferase	243514	24194	5.73	207	5	stress response
Bark storage protein B precursor (nucleoside phosphorylase)	740814	35860	6.24	69	1	stress response
Vegetative storage protein win4.5	596927	35305	5.62	61	1	stress response
Nucleoside phosphatase (apyrase 2)	573883	49759	5.31	116	2	stress response
Polyphenol oxidase (PPO5)	674097	64884	6.31	79	2	stress response
Pop3-/SP1-like	822230	12975	5.73	72	2	stress response
Kunitz trypsin inhibitor, miraculin-like	763975	20273	5.01	183	3	stress response
Caffeic acid 3-*O*-methyltransferase	834247	39731	5.48	150	5	stress response
Phenylcoumaran benzylic ether reductase 1	830063	33976	5.52	206	3	stress response
β-1,3-glucanase (glycoside hydrolase, family 17)	652688	31637	5.52	545	17	stress response
β-1,3-glucanase (glycoside hydrolase, family 17)	751998	36563	4.78	228	5	stress response
β-glucosidase (glycoside hydrolase, family 1)	294573	55628	5.85	94	2	stress response
Chitinase (glycosyl hydrolase family 18)	717157	40233	7.82	100	2	stress response
Chitinase (glycosyl hydrolase family 18)	746640	30847	4.42	164	5	stress response
Chitinase (glycoside hydrolase, family 19)	826290	29337	7.91	408	6	stress response
Chitin-binding (hevein-like)	571046	20788	8.37	298	4	stress response
Thaumatin, pathogenesis-related, PR-5	828883	25856	4.9	62	1	stress response
Thaumatin, pathogenesis-related, PR-5	669475	24731	7.89	332	5	stress response
Defense-related protein containing SCP/PR-1 domain	595857	17256	8.54	302	5	stress response
Molecular chaperone (HSP90)	582316	79949	4.94	135	2	stress response
Plant basic secretory protein family protein	549955	24994	7.18	162	2	stress response
Tubulin alpha-2 chain	831020	49776	4.92	154	4	structure
Beta tubulin	203493	49712	4.78	126	2	structure
Beta-Ig-H3/fasciclin	728480	28208	7.95	122	3	structure
Fasciclin-like arabinogalactan-protein 10	730906	43247	5.37	120	2	structure
Unknown (desiccation-associated)	642406	32845	4.83	181	2	unknown
Unknown (DUF1278)	672963	11070	9.03	215	4	unknown
Unknown (DUF26)	718495	26449	5.78	216	4	unknown

[a] For each protein, a protein identification number was identified using the DOE Joint Genome Institute *P. trichocarpa* genome v1.1 (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html). The peptide sequences used for identification and their charge can be accessed in Supplementary Table 1 in Supporting Information.

Table 2. Identification of Two Poplar Phloem Exudate Proteins Induced 24 h after Wounding

protein	JGI protein ID	theoretical MW	theoretical p*I*	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

two proteins were consistently and significantly ($P < 0.05$) upregulated in response to the wound treatment (Figure 3; Supplementary Figure 1 and Supplementary Table 2 in Supporting Information). Both protein spots were excised and

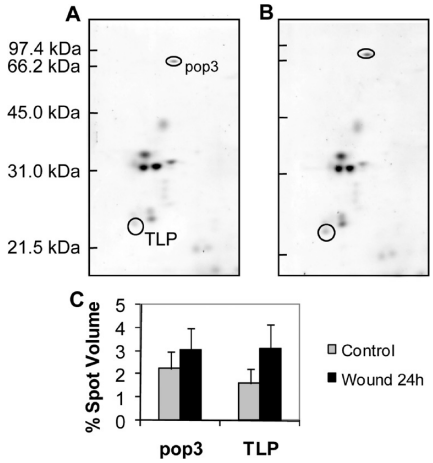

Figure 3. Changes in hybrid poplar phloem exudate protein profiles following simulated herbivory by leaf wounding. Representative acidic regions of 2-DE gels used to compare protein abundance in phloem exudate from control (A) and wounded trees (B) are shown. Circles indicate the two proteins, pop3 and 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.

subjected to LC-MS/MS, which identified them as a pop3 protein and thaumatin-like protein (TLP) (Table 2). Pop3 has no similarity to proteins of known function, but it is orthologous (99.1% amino acid identity) to the *Populus tremula* stable protein 1 (SP1).[41] TLPs are defined by their similarity to thaumatin, a sweet-tasting protein first identified in the African shrub *Thaumatococcus danielli*, and also belong to the PR-5 family of pathogenesis-related proteins.[42] In response to wounding, pop3/SP1 and TLP increased in the phloem exudate by 1.3- and 1.9-fold, respectively (Figure 3C). Both proteins have an acidic isoelectric point (Figure 3A,B). TLP migrated at 21 kDa (Figure 3A,B), similar to its predicted MW of 25 kDa (Table 2). Pop3/SP1 had an observed MW much greater than its predicted molecular weight of 12.4 kDa (compare Figure 3, Table 2). This discrepancy is presumably due to the assembly of pop3/SP1 into a stable oligomeric complex.[41,43]

Since PR proteins are known to be found in cell walls and apoplast, we could not rule out minor contamination of phloem exudate by some apoplast proteins. Therefore, to independently confirm the presence of a TLP within sieve elements, we carried out immunolocalization with an antibody raised against the TLP identified in our wounding experiment. In immunofluorescence experiments using poplar stem cross sections, TLP antiserum specifically labeled punctate, organelle-like structures within large phloem cells, identified as sieve elements (Figure 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 (Figure 4D,E). These experiments demonstrated the sieve element origin of TLP in our exudate samples. We also determined the localization of the second wound-inducible phloem exudate protein, pop3/SP1using the same technique. Pop3/SP1 was detected within sieve elements in both cross and longitudinal stem sections (Figure 5F,H), corroborating its presence in phloem exudate. Similar to the TLP antibody, the pop3 antibody labeled punctate, organelle-like structures inside sieve elements. In longitudinal sections, the antiserum also strongly reacted with the

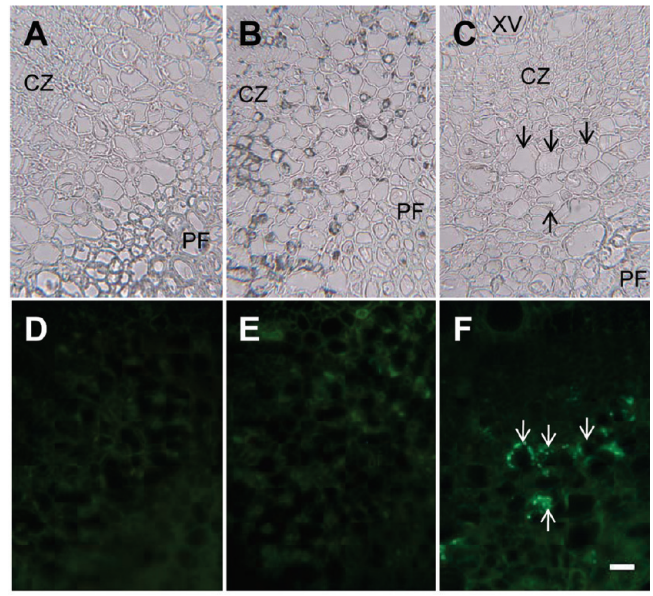

Figure 4. Localization of thaumatin-like proteins (TLP) in phloem cells in stem sections. Panels A−C show bright field images and panels D−F the corresponding immunofluorescent images of stem cross sections. Panels D and E are controls treated without the primary antibody or with preimmune serum, respectively, while F shows a section treated with TLP antiserum. CZ, cambial zone; PF, phloem fibers; and XV, xylem vessels. Arrows indicate labeled sieve elements. Scale bar = 20 μm.

sieve plates (Figure 5H). Fluorescence was also detected in the cell walls of phloem fibers (Figure 5F), and we are currently investigating this labeling using electron microscopy and immunogold-labeling. Overall, our immunofluorescence data confirms that TLP and pop3 are found in phloem and within sieve elements.

4. Discussion

Phloem exudates contain hundreds of proteins that are now being identified using sensitive mass spectrometry techniques.[5−7,10,11] Here, we describe the first analysis of phloem exudate proteins in a perennial, *P. trichocarpa* × *P. deltoides*. Poplar phloem exudate was estimated to have more than 100 proteins. We identified 48 poplar phloem exudate proteins, which included several defense- and pathogenesis-related proteins. The 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.

4.1. 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 that indicated minimal contamination from surrounding tissues.[5] 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

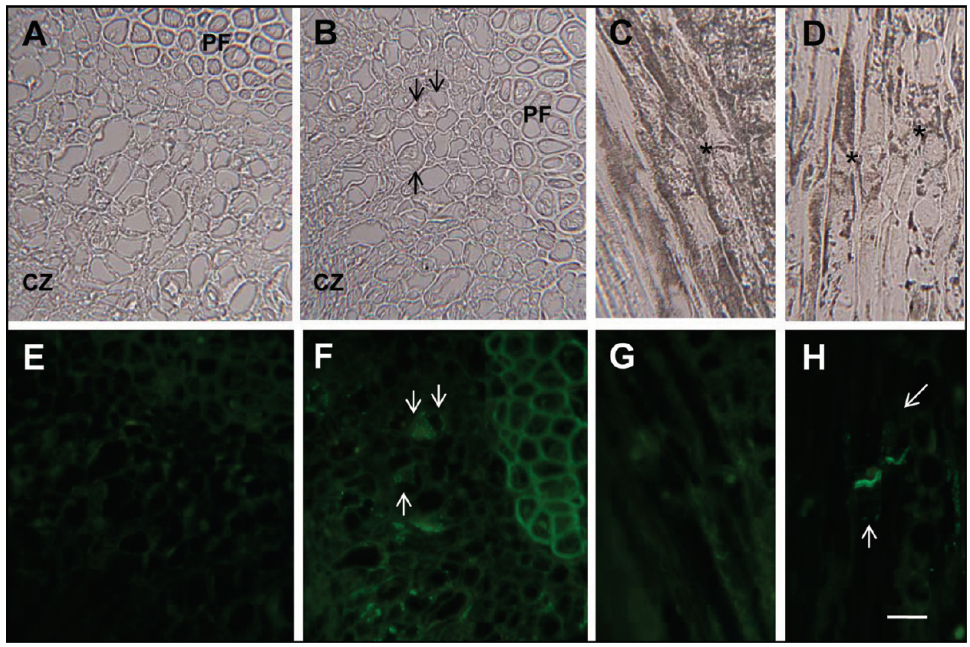

Figure 5. Localization of pop3 in phloem cells in stem sections. Panels A−D show bright field images and panels E−H the corresponding immunofluorescent images of both stem cross sections (A, B, E, F) and longitudinal sections (C, D, G, H). Panels E and G are controls treated with preimmune serum. Panels F and H are treated with pop3 antiserum. CZ, cambial zone and PF, phloem fibers. Arrows indicate labeled sieve elements and asterisks indicate position of sieve plates. Scale bar = 20 μm.

phloem exudate are known to be present in phloem of other plants (see below). There are some proteins, including phloem P-proteins and ubiquitin, that are conspicuously absent in our analysis of poplar phloem exudate. However, we were only able to unambiguously identify 48 of the estimated 100 poplar phloem exudate proteins. In fact, ubiquitin was detected in our sample, as well as several antioxidant proteins, but in these cases, only a single peptide matched, and therefore, they were excluded from the final list. 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.[44] The ratio of sucrose/glucose and fructose in our exudate preparations was four times greater than that reported by Herschbach et al.,[37] though lower than reported for exudate collected from *B. napus*.[5] In general, sucrose accounts for 95% of the total sugars in phloem, consistent with our data. Furthermore, our immunolocalization of TLP and pop3 confirms that the phloem exudate collected contains proteins that are localized to sieve elements. Therefore, we conclude that EDTA provides a useful means to study phloem exudate in poplar, yielding suitable protein quantities needed for comparative proteomic studies.

4.2. 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*.[5,6,11,34,36,45−47] However, we also found a significant number of new phloem exudate proteins, many of which are predicted to function in herbivore- and pathogen-defense. Herbivore-defense associated proteins such as proteinase inhibitors were previously reported in other phloem studies,[5,6,11,12] but in poplar, we identified additional defense proteins such as PPO, a pop3/SP1-like protein, and vegetative storage proteins. All of these proteins are consistently upregulated in poplar leaves by simulated herbivory[22,38,48] and our analysis thus extends the list of herbivore defense-related proteins described from phloem. Likewise, several pathogenesis-related (PR) proteins, including PR-1, TLPs, chitinases, and β-1,3-glucanases were identified in poplar phloem exudate. With the exception of a β-1,3-glucanase recently identified in *O. sativa* phloem sap,[10] these PR proteins have not been previously reported in phloem.

The presence of PR proteins in phloem exudate was unexpected since these types of proteins typically have predicted N-terminal signal peptides targeting them to the secretory pathway. Nevertheless, a β-glucosidase was detected in phloem sap of *C. maxima*[6] and several PR proteins were previously identified in *O. sativa* phloem sap.[10] 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. The results of these experiments confirm that TLPs are localized inside sieve elements. Other PR-5 proteins have been previously detected intracellularly, specifically in plastids of flowers and other tissues.[49−51] The TLPs in this study appeared to be associated with organelle-like structures, which could also be plastids. Sieve elements are known to contain two types of plastids, denoted as either protein- or starch-containing.[52] The function of these plastids is not known, but it is known that they rupture and release their contents when sieve elements are damaged.[53]

It is interesting to note that many PR proteins including TLPs, β-1,3-glucanases, and chitinases are strongly induced in leaf tissue infested by various phloem-feeding insects.[54−57] 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.[56]

Alternatively, it is possible that these novel defense-related phloem proteins have functions related to the perennial growth habits of poplar. β-1,3-glucanases have been shown to degrade callose and regulate transport through plasmodesmata.[58−60] Thus, it is possible that the two β-1,3-glucanases identified in this study are involved in modulating phloem transport by degrading callose surrounding sieve pores. This function would be particularly important in the spring when dormancy callose is removed from overwintering sieve elements prior to the development of new sieve elements. TLPs might also be involved in this process since some TLPs have been shown to exhibit *endo*-β-1,3-glucanase activity. Similarly, in temperate perennials, the seasonal movement and storage of nutrients is a key process involving the phloem, and we speculate that the presence of the two nitrogen storage proteins (win 4.5 and bark storage protein) in our phloem samples is related to this function. These proteins are known to be involved in N storage in actively growing poplars[61] as well as N storage during dormancy.[61,62]

4.3. 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.[63] 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.[64] In addition, temporal analyses indicates that the transcription of defense genes such as trypsin inhibitors and chitinases does not occur until 2−6 h after wounding,[25,65] and the transcription of PR proteins, including PR-1 and β-1,3-glucanases, does not proceed until 20−40 h after pathogen infection.[66−68] Because of these delays, we consider it very unlikely that rapid protein induction is significantly masking the wound response.

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.[22,38,41,69,70] While the *P. tremula* SP1 gene, which is orthologous to pop3 in *P. trichocarpa* × *P. deltoides*, 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.[7,38,69,71,72] 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.[41,43,73] Likewise, the pop3 identified in this study also appears to form a large multimeric complex based on its migration in 2-DE, but to determine 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.[5,6,45]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.[54,55,57,74,75] Further research is needed to determine the biological activity of this phloem-inducible poplar TLP or if it deters insect feeding.

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

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Supporting Information Available: Summary of poplar phloem exudate proteins with corresponding LC-MS/MS data including sequence and charge state of matched peptides (Supplementary Table 1). Statistical analysis of poplar phloem exudate proteins from 24 h wound experiment (Supplementary Table 2) and the reference gel with their corresponding spot nos. (Supplementary Figure 1). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) van Bel, A. J. E. The phloem, a miracle of ingenuity. *Plant Cell Environ.* **2003**, *26* (1), 125–149.
(2) Lough, T. J.; Lucas, W. J. Integrative plant biology: role of phloem long-distance macromolecular trafficking. *Ann. Rev. Plant Biol.* **2006**, *57*, 203–232.
(3) Lin, M. K.; Belanger, H.; Lee, Y. J.; Varkonyi-Gasic, E.; Taoka, K. I.; Miura, E.; Xoconostle-Cazares, B.; Gendler, K.; Jorgensen, R. A.; Phinney, B.; Lough, T. J.; Lucas, W. J. FLOWERING LOCUS T protein may act as the long-distance florigenic signal in the cucurbits. *Plant Cell* **2007**, *19* (5), 1488–1506.
(4) Milburn, J. A. Phloem exudation from castor bean - induction by massage. *Planta* **1970**, *95* (3), 272–276.
(5) Giavalisco, P.; Kapitza, K.; Kolasa, A.; Buhtz, A.; Kehr, J. Towards the proteome of *Brassica napus* phloem sap. *Proteomics* **2006**, *6* (3), 896–909.
(6) Walz, C.; Giavalisco, P.; Schad, M.; Juenger, M.; Klose, J.; Kehr, J. Proteomics of curcurbit phloem exudate reveals a network of defence proteins. *Phytochemistry* **2004**, *65* (12), 1795–1804.
(7) Walz, C.; Juenger, M.; Schad, M.; Kehr, J. Evidence for the presence and activity of a complete antioxidant defence system in mature sieve tubes. *Plant J.* **2002**, *31* (2), 189–197.
(8) King, R. W.; Zeevaart, J. A. Enhancement of phloem exudation from cut petioles by chelating-agents. *Plant Physiol.* **1974**, *53* (1), 96–103.
(9) Fisher, D. B.; Wu, Y.; Ku, M. S. B. Turnover of soluble-proteins in the wheat sieve tube. *Plant Physiol.* **1992**, *100* (3), 1433–1441.
(10) Aki, T.; Shigyo, M.; Nakano, R.; Yoneyama, T.; Yanagisawa, S. Nano scale proteomics revealed the presence of regulatory proteins including three FT-Like proteins in phloem and xylem saps from rice. *Plant Cell Physiol.* **2008**, *49* (5), 767–790.
(11) Barnes, A.; Bale, J.; Constantinidou, C.; Ashton, P.; Jones, A.; Pritchard, J. Determining protein identity from sieve element sap

in *Ricinus communis* L. by quadrupole time of flight (Q-TOF) mass spectrometry. *J. Exp. Botany* **2004**, *55* (402), 1473–1481.
(12) Dannenhoffer, J. M.; Suhr, R. C.; Thompson, G. A. Phloem-specific expression of the pumpkin fruit trypsin inhibitor. *Planta* **2001**, *212* (2), 155–162.
(13) Kehr, J. Phloem sap proteins: their identities and potential roles in the interaction between plants and phloem-feeding insects. *J. Exp. Bot.* **2006**, *57* (4), 767–774.
(14) Schilmiller, A. L.; Howe, G. A. Systemic signaling in the wound response. *Curr. Opin. Plant Biol.* **2005**, *8* (4), 369–377.
(15) Orians, C. M.; Pomerleau, J.; Ricco, R. Vascular architecture generates fine scale variation in systemic induction of proteinase inhibitors in tomato. *J. Chem. Ecol.* **2000**, *26* (2), 471–485.
(16) Davis, J. M.; Gordon, M. P.; Smit, B. A. Assimilate movement dictates remote sites of wound-induced gene-expression in poplar leaves. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88* (6), 2393–2396.
(17) Major, I. T.; Constabel, C. P. Shoot-root defense signaling and activation of root defense by leaf damage in poplar. *Can. J. Bot.* **2007**, *85* (12), 1171–1181.
(18) Hause, B.; Hause, G.; Kutter, C.; Miersch, O.; Wasternack, C. Enzymes of jasmonate biosynthesis occur in tomato sieve elements. *Plant Cell Physiol.* **2003**, *44* (6), 643–648.
(19) Thompson, G. A.; Goggin, F. L. Transcriptomics and functional genomics of plant defence induction by phloem-feeding insects. *J. Exp. Bot.* **2006**, *57* (4), 755–766.
(20) Bradshaw, H. D.; Ceulemans, R.; Davis, J.; Stettler, R. Emerging model systems in plant biology: poplar (*Populus*) as a model forest tree. *J. Plant Growth Reg.* **2000**, *19* (3), 306–313.
(21) Taylor, G. Populus: Arabidopsis for forestry. Do we need a model tree? *Ann. Bot.* **2002**, *90* (6), 681–689.
(22) Major, I. T.; Constabel, C. P. Molecular analysis of poplar defense against herbivory: comparison of wound- and insect elicitor-induced gene expression. *New Phytol.* **2006**, *172* (4), 617–635.
(23) Larson, P. R.; Isebrands, J. G. The plastochron index as applied to developmental studies of cottonwood. *Can. J. For. Res.* **1971**, *1* (1), 1–11.
(24) Ramagli, L. S.; Rodriguez, L. V. Quantitation of microgram amounts of protein in two-dimensional polyacrylamide gel electrophoresis sample buffer. *Electrophoresis* **1985**, *6* (11), 559–563.
(25) Haruta, M.; Major, I. T.; Christopher, M. E.; Patton, J. J.; Constabel, C. P. A Kunitz trypsin inhibitor gene family from trembling aspen (*Populus tremuloides* Michx.): cloning, functional expression, and induction by wounding and herbivory. *Plant Mol. Biol.* **2001**, *46* (3), 347–359.
(26) Harlow, E.; Lane, D. *Using Antibodies: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1999; pp 276−307.
(27) Constabel, C. P.; Yip, L.; Patton, J. J.; Christopher, M. E. Polyphenol oxidase from hybrid poplar. Cloning and expression in response to wounding and herbivory. *Plant Physiol.* **2000**, *124* (1), 285–295.
(28) Chaffey, N. J.; Barnett, J. R.; Barlow, P. W. Visualization of the cytoskeleton within the secondary vascular system of hardwood species. *J. Microsc.* **1997**, *187*, 77–84.
(29) Poulis, B. A. D.; O'Leary, S. J. B.; Haddow, J. D.; von Aderkas, P. Identification of proteins present in the Douglas fir ovular secretion: an insight into conifer pollen selection and development. *Int. J Plant Sci.* **2005**, *166* (5), 733–739.
(30) Wagner, R.; Mugnaini, S.; Sniezko, R.; Hardie, D.; Poulis, B.; Nepi, M.; Pacini, E.; von Aderkas, P. Gymnosperm pollination drop proteins are conservative and indicate complex function. *Sex. Plant Reprod.* **2007**, *20,* 181–189.
(31) Perkins, D. N.; Pappin, D. J. C.; Creasy, D. M.; Cottrell, J. S. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **1999**, *20* (18), 3551–3567.
(32) Lippert, D.; Chowrira, S.; Ralph, S. G.; Zhuang, J.; Aeschliman, D.; Ritland, C.; Ritland, K.; Bohlmann, J. 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* **2007**, *7* (2), 248–70.
(33) Campbell, J. A.; Hansen, R. W.; Wilson, J. R. Cost-effective colorimetric microtitre plate enzymatic assays for sucrose, glucose and fructose in sugarcane tissue extracts. *J. Sci. Food Agric.* **1999**, *79* (2), 232–236.
(34) Hoffmann-Benning, S.; Gage, D. A.; McIntosh, L.; Kende, H.; Zeevaart, J. A. D. 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* **2002**, *216* (1), 140–147.
(35) Marentes, E.; Grusak, M. A. Mass determination of low-molecular-weight proteins in phloem sap using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Exp. Bot.* **1998**, *49* (322), 903–911.
(36) Schobert, C.; Grossmann, P.; Gottschalk, M.; Komor, E.; Pecsvaradi, A.; Zurnieden, U. Sieve-Tube exudate from *Ricinus communis* L. seedlings contains ubiquitin and chaperones. *Planta* **1995**, *196* (2), 205–210.
(37) Herschbach, C.; Jouanin, L.; Rennenberg, H. Overexpression of gamma-glutamylcysteine synthetase, but not of glutathione synthetase, elevates glutathione allocation in the phloem of transgenic poplar trees. *Plant Cell Physiol.* **1998**, *39* (4), 447–451.
(38) Ralph, S.; Oddy, C.; Cooper, D.; Yueh, H.; Jancsik, S.; Kolosova, N.; Philippe, R. N.; Aeschliman, D.; White, R.; Huber, D.; Ritland, C. E.; Benoit, F.; Rigby, T.; Nantel, A.; Butterfield, Y. S. N.; Kirkpatrick, R.; Chun, E.; Liu, J.; Palmquist, D.; Wynhoven, B.; Stott, J.; Yang, G.; Barber, S.; Holt, R. A.; Siddiqui, A.; Jones, S. J. M.; Marra, M. A.; Ellis, B. E.; Douglas, C. J.; Ritland, K.; Bohlmann, J. 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. *Mol. Ecol.* **2006**, *15* (5), 1275–1297.
(39) Rouhier, N.; Gelhaye, E.; Sautiere, P. E.; Brun, A.; Laurent, P.; Tagu, D.; Gerard, J.; de Fay, E.; Meyer, Y.; Jacquot, J. P. Isolation and characterization of a new peroxiredoxin from poplar sieve tubes that uses either glutaredoxin or thioredoxin as a proton donor. *Plant Physiol.* **2001**, *127* (3), 1299–1309.
(40) Haebel, S.; Kehr, J. 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* **2001**, *213* (4), 586–593.
(41) Wang, W. X.; Pelah, D.; Alergand, T.; Shoseyov, O.; Altman, A. Characterization of SP1, a stress-responsive, boiling-soluble, homo-oligomeric protein from aspen. *Plant Physiol.* **2002**, *130* (2), 865–875.
(42) Shatters, R. G.; Boykin, L. M.; Lapointe, S. L.; Hunter, W. B.; Weathersbee, A. A. Phylogenetic and structural relationships of the PR5 gene family reveal an ancient multigene family conserved in plants and select animal taxa. *J. Mol. Evol.* **2006**, *63* (1), 12–29.
(43) Dgany, O.; Gonzalez, A.; Sofer, O.; Wang, W. X.; Zolotnitsky, G.; Wolf, A.; Shoham, Y.; Altman, A.; Wolf, S. G.; Shoseyov, O.; Almog, O. The structural basis of the thermostability of SP1, a novel plant (*Populus tremula*) boiling stable protein. *J. Biol. Chem.* **2004**, *279* (49), 51516–51523.
(44) Oparka, K. J.; Cruz, S. S. The great escape: phloem transport and unloading of macromolecules. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **2000**, *51*, 323–347.
(45) Schobert, C.; Baker, L.; Szederkenyi, J.; Grossmann, P.; Komor, E.; Hayashi, H.; Chino, M.; Lucas, W. J. Identification of immunologically related proteins in sieve-tube exudate collected from monocotyledonous and dicotyledonous plants. *Planta* **1998**, *206* (2), 245–252.
(46) Kennecke, M.; Ziegler, H.; Fekete, M. Enzyme activities in sieve tube sap of *Robinia pseudoacacia* L and of other 3 species. *Planta* **1971**, *98* (4), 330–356.
(47) Geigenberger, P.; Langenberger, S.; Wilke, I.; Heineke, D.; Heldt, H. W.; Stitt, M. Sucrose is metabolized by sucrose synthase and glycolysis within the phloem complex of *Ricinus communis* L. seedlings. *Planta* **1993**, *190* (4), 446–453.
(48) Davis, J. M.; Egelkrout, E. E.; Coleman, G. D.; Chen, T. H. H.; Haissig, B. E.; Riemenschneider, D. E.; Gordon, M. P. A family of wound-induced genes in *Populus* shares common features with genes encoding vegetative storage proteins. *Plant Mol. Biol.* **1993**, *23* (1), 135–143.
(49) Jeun, Y. C.; Buchenauer, H. 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. *J. Phytopathol.* **2001**, *149* (3−4), 141–153.
(50) Liljeroth, E.; Marttila, S.; von Bothmer, R. Immunolocalization of defence-related proteins in the floral organs of barley (*Hordeum vulgare* L.). *J. Phytopathol.* **2005**, *153* (11−12), 702–709.
(51) Sturrock, R. N.; Islam, M. A.; Ekramoddoullah, A. K. M. Host-pathogen interactions in Douglas-fir seedlings infected by *Phellinus sulphurascens*. *Phytopathol.* **2007**, *97* (11), 1406–1414.
(52) van Bel, A. J. E.; Ehlers, K.; Knoblauch, M. Sieve elements caught in the act. *Trends Plant Sci.* **2002**, *7* (3), 126–132.

(53) Knoblauch, M.; van Bel, A. J. E. Sieve tubes in action. *Plant Cell* **1998**, *10* (1), 35–50.
(54) Gao, L. L.; Anderson, J. P.; Klingler, J. P.; Nair, R. M.; Edwards, O. R.; Singh, K. B. Involvement of the octadecanoid pathway in bluegreen aphid resistance in *Medicago truncatula*. *Mol. Plant–Microbe Interact.* **2007**, *20* (1), 82–93.
(55) Kempema, L. A.; Cui, X. P.; Holzer, F. M.; Walling, L. L. Arabidopsis transcriptome changes in response to phloem-feeding silverleaf whitefly nymphs. Similarities and distinctions in responses to aphids. *Plant Physiol.* **2007**, *143* (2), 849–865.
(56) Moran, P. J.; Thompson, G. A. Molecular responses to aphid feeding in Arabidopsis in relation to plant defense pathways. *Plant Physiol.* **2001**, *125* (2), 1074–1085.
(57) Zarate, S. I.; Kempema, L. A.; Walling, L. L. Silverleaf whitefly induces salicylic acid defenses and suppresses effectual jasmonic acid defenses. *Plant Physiol.* **2007**, *143* (2), 866–875.
(58) Ruan, Y. L.; Xu, S. M.; White, R.; Furbank, R. T. Genotypic and developmental evidence for the role of plasmodesmatal regulation in cotton fiber elongation mediated by callose turnover. *Plant Physiol.* **2004**, *136* (4), 4104–4113.
(59) Levy, A.; Erlanger, M.; Rosenthal, M.; Epel, B. L. A plasmodesmata-associated beta-1,3-glucanase in Arabidopsis. *Plant J.* **2007**, *49* (4), 669–682.
(60) Iglesias, V. A.; Meins, F. Movement of plant viruses is delayed in a beta-1,3-glucanase-deficient mutant showing a reduced plasmodesmatal size exclusion limit and enhanced callose deposition. *Plant J.* **2000**, *21* (2), 157–166.
(61) Cooke, J. E. K.; Weih, M. Nitrogen storage and seasonal nitrogen cycling in *Populus*: bridging molecular physiology and ecophysiology. *New Phytol.* **2005**, *167* (1), 19–30.
(62) van Cleve, B.; Just, J.; Sauter, J. J. Poplar storage protein in xylem sap. *J. Plant Physiol.* **1991**, *137* (6), 763–764.
(63) Giri, A. P.; Wunsche, H.; Mitra, S.; Zavala, J. A.; Muck, A.; Svatos, A.; Baldwin, I. T. Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. VII. Changes in the plant's proteome. *Plant Physiol.* **2006**, *142* (4), 1621–1641.
(64) Le Hir, R.; Beneteau, J.; Bellini, C.; Vilaine, F.; Dinant, S. Gene expression profiling: keys for investigating phloem functions. *Trends Plant Sci.* **2008**, *13* (6), 273–280.
(65) Lawrence, S. D.; Dervinis, C.; Novak, N. G.; Davis, J. M. Wound and insect herbivory responsive genes in poplar. *Biotechnol. Lett.* **2006**, *28* (18), 1493–1501.
(66) Friedrich, L.; Moyer, M.; Ward, E.; Ryals, J. Pathogenesis-related protein 4 is structurally homologous to the carboxy terminal domains of hevein, Win-1 and Win-2. *Mol. Gen. Genetics* **1991**, *230* (1−2), 113–119.
(67) Vega-Sanchez, M. E.; Redinbaugh, M. G.; Costanzo, S.; Dorrance, A. E. Spatial and temporal expression analysis of defense-related genes in soybean cultivars with different levels of partial resistance to *Phytophthora sojae*. *Physiol. Mol. Plant Pathol.* **2005**, *66* (5), 175–182.
(68) Ward, E. R.; Payne, G. B.; Moyer, M. B.; Williams, S. C.; Dincher, S. S.; Sharkey, K. C.; Beck, J. J.; Taylor, H. T.; Ahlgoy, P.; Meins, F.; Ryals, J. A. Differential regulation of beta-1,3-glucanase messenger-RNAs in response to pathogen infection. *Plant Physiol.* **1991**, *96* (2), 390–397.
(69) Gu, R. S.; Fonseca, S.; Puskas, L. G.; Hackler, L.; Zvara, A.; Dudits, D.; Pais, M. S. Transcript identification and profiling during salt stress and recovery of *Populus euphratica*. *Tree Physiol.* **2004**, *24* (3), 265–276.
(70) van Loon, L. C.; Rep, M.; Pieterse, C. M. J. Significance of inducible defense-related proteins in infected plants. *Ann. Rev. Phytopathol.* **2006**, *44*, 135–162.
(71) Renaut, J.; Hoffmann, L.; Hausman, J. F. Biochemical and physiological mechanisms related to cold acclimation and enhanced freezing tolerance in poplar plantlets. *Physiol. Plant.* **2005**, *125* (1), 82–94.
(72) Ferreira, S.; Hjerno, K.; Larsen, M.; Wingsle, G.; Larsen, P.; Fey, S.; Roepstorff, P.; Pais, M. S. Proteome profiling of *Populus euphratica* Oliv. upon heat stress. *Ann. Bot.* **2006**, *98* (2), 361–377.
(73) Wang, W. X.; Dgany, O.; Dym, O.; Altman, A.; Shoseyov, O.; Almog, O. Crystallization and preliminary X-ray crystallographic analysis of SP1, a novel chaperone-like protein. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2003**, *59*, 512–514.
(74) Frendo, P.; Didierjean, L.; Passelegue, E.; Burkard, G. Abiotic stresses induce a thaumatin-like protein in maize - cDNA isolation and sequence analysis. *Plant Sci.* **1992**, *85* (1), 61–69.
(75) Piggott, N.; Ekramoddoullah, A. K. M.; Liu, J. J.; Yu, X. S. 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. *Physiol. Mol. Plant Pathol.* **2004**, *64* (1), 1–8.

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