

Characterization of the Maize Xylem Sap Proteome

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The xylem in plants has mainly been described as a conduit for water and minerals, but emerging evidence also indicates that the xylem contains protein. To study the proteins in xylem sap, we characterized the identity and composition of the maize xylem sap proteome. The composition of the xylem sap proteome in maize revealed proteins related to different phases of xylem differentiation including cell wall metabolism, secondary cell wall synthesis, and programmed cell death. Many proteins were found to be present as multiple isoforms and some of these isoforms are glycosylated. Proteins involved in defense mechanisms were also present in xylem sap and the sap proteins were shown to have antifungal activity in bioassays.

Keywords: xylem sap • proteome • isoform • glycoprotein • antifungal activity • maize • corn • plants

Introduction

The transport of organic and inorganic nutrients within higher plants is mediated by two specialized long-distance transport systems, the xylem and phloem. The phloem transports predominantly organic compounds from the site of synthesis in the leaves to the site of use. The xylem stream transports water and dissolved minerals entering the roots from the soil to the aerial plant parts. This important conduit is essential for transporting the raw materials that enables plants to photosynthesize and be autotrophic organisms. Xylem sap also contains dissolved substances such as hormones, other secondary metabolites, amino acids, and proteins that may play an important role in root to shoot communication. The composition of the xylem sap is modified as it is transported from the roots and throughout the plant by the addition of compounds including organic acids, plant hormones, sugars, amino acids and polypeptides to produce a relatively complex solution.^{1–4}

The complexity of xylem sap has not been well characterized, especially with respect to macromolecules such as proteins. The presence of proteins in the xylem sap of a number of plant species has been described by one-dimensional electrophoresis separation in watermelon,⁵ squash,⁶ apple, peach, and pear,⁵ cucumber,^{7–9} tomato,^{10,11} and broccoli, rape, and pumpkin.⁷ Some sap proteins have been identified, including peroxidases, pathogenesis-related (PR) proteins (e.g. chitinases and PR-5x), an aspartyl protease protein, a lectin, a lectin-like protein, a glycine-rich protein and a cysteine-rich protein.^{2,8–13} A recent study in *Brassica napus*¹⁴ characterized the protein content of xylem sap using two-dimensional electrophoresis. Peroxidases,

proteases, PR proteins, and lectins were found in sap in that study. Many of the same proteins were found at the same molecular weight, but different isoelectric points. It was suggested that these multiple isoforms were due to post-translational modifications of one single gene product.

Previous studies on plant xylem sap composition have focused on biotic stresses. Changes in xylem sap proteins occur in tomato in response to biotic infection by pathogenic fungi.^{10,11} In rice, a cationic peroxidase specifically accumulates in xylem vessels after an incompatible interaction with the vascular bacterial pathogen *Xanthomonas oryzae*.¹³

To fully understand changes in the xylem sap proteome of maize, we first characterized the identity of the more abundant proteins in the sap proteome sap using two-dimensional gel electrophoresis (2-DE). Many proteins were found that were similar to and also different from those identified in a recent study on the xylem sap proteome of *Brassica napus*.¹⁴ A specific 2-DE stain for glycoproteins revealed that many proteins in the sap were glycosylated. The presence of defense proteins in the sap led to the hypothesis that xylem sap contains antimicrobial activity. Our results show for the first time that the maize xylem sap possesses antifungal activity against *Neurospora crassa*.

Experimental Procedures

Plant Material and Growth Conditions. Single seeds of corn (*Zea mays* L. cv Fr697) were sown into 40 pots (115 mm diameter × 410 mm height, 4.26 L capacity) containing Metro-Mix 702 soil (The Scotts Company, Marysville, USA). Seeds were sown as described¹⁵ to enable the development of a long mesocotyl that aids in sap extraction. Plants were grown in a controlled-environment chamber (see ref 15 for growth conditions). Plants were watered daily with liquid fertilizer (one-eighth strength Hoagland's solution containing 7 mM nitrogen, supplied as ammonium and nitrate). An additional 70 mL of 35 mM Ferrous sulfate (QC Corporation, Cape Girardeau, USA)

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was added to each pot 11 and 16 days after sowing (DAS). Sap was extracted 23 DAS.

Xylem Sap Extraction. Forty plants were de-topped by severing the mesocotyl 5 mm below the shoot and xylem sap was collected from the cut surface of the mesocotyl under 'root pressure' as described in ref 15. Sap flowing from the tubing was collected in preweighed vials on dry ice for 210 min. Sap was pooled and Complete, EDTA-free Protease Inhibitor Cocktail (Roche Diagnostics, Penzberg, Germany) was added (one tablet per 50 mL). Sap was stored at -80°C until analyzed.

Protein Purification and Quantification. Sap was lyophilized to approximately half the original volume, thawed and filtered through $0.2\ \mu\text{m}$ cellulose acetate filters, and concentrated on Amicon 15 mL Ultra Centrifugal Filter Devices with a 5kDa MWCO (Millipore, Bedford, USA). The concentrated samples were precipitated with 10% w/v TCA and the proteins recovered by centrifugation. Protein pellets were washed three times with 100% methanol, air-dried for 5 min, and then resolubilized in the appropriate rehydration buffer (see below). Protein concentrations in sap and purified samples were determined by using either RC-DC Protein Assay (Bio-Rad, Laboratories, Hercules, USA) or NI Protein assay (Genotech, St. Louis, USA), with BSA as the standard.

Preparative 2-DE Gel for Sequence Analysis. Five hundred micrograms of protein (re-suspended in $450\ \mu\text{L}$ 8 M urea, 4% CHAPS, 20 mM DTT, 0.5% IPG Buffer pH 3–10 linear; Amersham) was applied to an IPG strip (Immobiline DryStrip, 240 mm, pH 3–10 NL; Amersham) and actively re-hydrated at 50 V for 12 h. Focusing was performed at 20°C using the IPGphor cell (Amersham) at 500 V for 20 min, 1000 V for 1 h, 2000 V for 2 h, then at 8000 V to reach a total of 79 kWh. After IEF, strips were equilibrated according to the manufacturer's procedures (Bio-Rad). Electrophoresis was performed on a 240 mm 12.5% polyacrylamide gel using the Ettan DALTsix 2D system (Amersham) at 15 W for 5 h.

Analytical 2-DE. Twenty micrograms of each protein sample (resuspended in $185\ \mu\text{L}$ Sequential Extraction Buffer 3; Biorad) was applied to an immobilized pH gradient (IPG) strip (110 mm, pH 3–10, nonlinear, Bio-Rad) and actively rehydrated. Isoelectric focusing (IEF) was performed at 20°C using a PROTEAN IEF cell (Bio-Rad) at 500 V for 1 h, 1000 V for 1 h, 2000 V for 2 h, and then at 8000 V for a total of 35 kWh. Equilibrated strips were loaded onto a gel (Criterion 8–16% gradient pre-cast 110 mm; Bio-Rad). The second-dimension electrophoresis was performed at 180 V for approximately 1.5 h.

Protein Staining. After electrophoresis, gels were fixed in 7% acetic acid, 10% methanol for 1 h, and stained overnight with SYPRO Ruby (Molecular Probes, Eugene, USA). The stained gels were washed in 7% acetic acid, 10% methanol for 1 h, rinsed with water and visualized using the TYPHOON 9410 system (Amersham Biosciences, Piscataway, USA). Specific glycoprotein staining was also used according to the manufacturer's procedures for Pro-Q Emerald 300 Glycoprotein Gel (Molecular Probes, Carlsbad, CA, USA), to determine posttranslational modifications.

Protein Identification by nanoESI–MS/MS Analysis. Gel plugs were de-stained extensively with 50% (v/v) acetonitrile and 50 mM NH_4HCO_3 , followed by dehydration in acetonitrile for 5 min. Proteins were then digested for 10 h at 37°C in $25\ \mu\text{L}$ of 50 mM NH_4HCO_3 containing $6\ \mu\text{g mL}^{-1}$ trypsin (sequencing grade, modified, Promega, Madison, USA). The digest supernatant was transferred to a clean tube. The gel pieces were

extracted twice and the pooled digest was lyophilized and re-suspended in the aqueous buffer ($10\ \mu\text{L}$ of 0.1% formic acid/5% acetonitrile) used for peptides separation by nanoflow HPLC (Ultimate, Amsterdam, NL). A $5\ \mu\text{L}$ portion of protein digest was loaded onto a C18 precolumn (LC Packing, Amsterdam, NL) for desalting and concentrating. Peptides were then eluted from the precolumn and separated on a nanoflow analytical C18 column (PepMap 75 μm I. D., LC Packing, Amsterdam, NL) at $180\ \text{nL}\cdot\text{min}^{-1}$ using a gradient. Buffers were 0.1% HCOOH/5% ACN (A) and 0.075% HCOOH/95% ACN (B). A linear gradient from 5% to 40% B for 37 min was applied. Including the regeneration step, one run was 60 min. An ABI QSTAR XL (Applied Biosystems/MDS Sciex, Foster City, USA) hybrid quadrupole TOF MS/MS system equipped with a nanoelectrospray source (Protana XYZ manipulator) was used for peptide sequence analysis. The nanoelectrospray was generated from a PicoTip needle ($10\ \mu\text{m}$ i.d., New Objectives, Wobum, USA) at a voltage of 2400 V. TOF MS spectra and product ion spectra were acquired using Analyst QS software. The peptide tandem mass spectra were searched against the MAIZE EST Database (www.maizeseq.org) using MASCOT search engine (<http://www.matrixscience.com>) with a mass tolerance of 100 ppm and one allowed trypsin miscleavage. Search parameters used the fixed cysteine carbamidomethylation and the variable methionine oxidation as modifications, and charge state from 2 to 3. Unambiguous identification was judged by the number of peptide sequence tags, sequence coverage, mowse score and the quality of tandem MS spectra.

Antifungal Assays. The antifungal activity of xylem sap proteins was measured in an in vitro assay using 96-well microtiter plates.¹⁶ Fifty microliters of *Neurospora crassa* spore suspension prepared in $2\times$ synthetic low-salt fungal medium¹⁶ at a concentration of $2000\ \text{spores mL}^{-1}$ was added to each well. A $2.65\ \mu\text{g}$ portion of xylem sap protein in $50\ \mu\text{L}$ was added to the fungal suspension. Pictures were taken using an Olympus CK40 inverted microscope at $100\times$ magnification using Kodak T-max 400 film after 16 h of growth.

Results and Discussion

Xylem sap was collected with a root pressure method described previously¹⁵ and contained very low concentrations of sucrose indicative of very low potential contamination from the phloem. The extracted xylem sap had a protein concentration of $12\ \mu\text{g mL}^{-1}$. This concentration was comparable with xylem sap extracted from squash roots which contained $19\ \mu\text{g mL}^{-1}$ total protein,⁶ but was much lower than the approximately $100\ \mu\text{g mL}^{-1}$ total protein concentration found in the xylem sap of broccoli, rape, pumpkin, and cucumber.⁷

Xylem Sap Protein Content. The proteins were separated by isoelectric focusing, using a linear pH 3–10 IPG strip as the first dimension. The second dimension gel was stained with Sypro Ruby allowing visualization of approximately 480 protein spots (Figure 1). The protein spots detected cover a range of pI's from acidic to basic and differ in size from as small as 7 kDa to over 110 kDa (Figure 1). The most abundant spots were excised from the gel, analyzed by nanoESI–MS/MS and 154 spots were identified by database searches (Table 1).

Seventy-five percent of the proteins were found to be most similar to proteins from monocot species that have been annotated in databases. The majority of annotated proteins were from *Oryza sativa* (57%) with only 3% coming from *Zea*

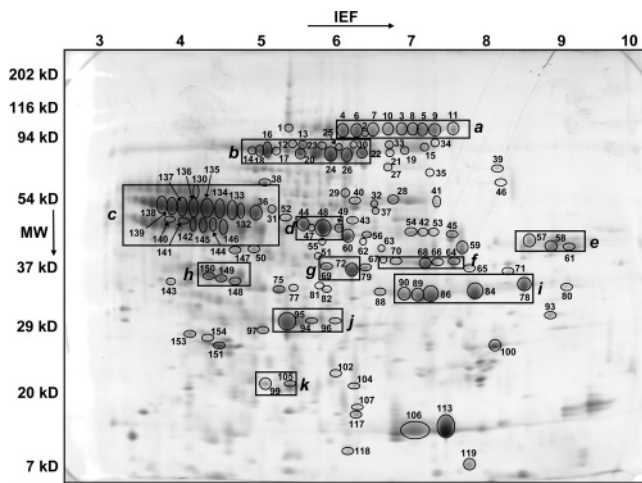


Figure 1. 2-DE of *Zea mays* xylem sap proteins. Molecular masses are indicated in kDa on the left axis and approximate pI on the top axis. Numbers correspond to spots that were identified and listed in Table 1. The squares annotated with letters indicate the protein isoforms.

mays and 15% from other monocot species. About 25% of the annotations were derived from *Arabidopsis* and other dicot species.

Many of the identified proteins were found to have experimental molecular weights that were different from the theoretical molecular weight (M_r) (Table 1). For some proteins, the experimental M_r was higher than the theoretical M_r which could be explained by the presence of a post-translational modification. When the experimental M_r is lower than the theoretical M_r , the proteins may have been degraded. The differences in expected and experimental molecular weight could also be explained by the fact that most theoretical molecular weights come from database matches to species other than *Zea mays*.

The identified proteins were classified into functional categories (Figure 2). The proteins in the xylem sap belong to three major categories: cell wall metabolism (58%), plant defense (26%), and proteins involved in proteolysis and peptidolysis (11%). One protein remains unknown, and 6 proteins with various functions were not classified in the 3 major groups.

Xylem Sap Proteins Unique to Maize and Those Common to *Brassica napus*. Very little is known about the xylem sap protein composition in plants, and in our study we identified proteins present in the xylem sap of maize that have never been reported before in xylem sap of other plant species (Table 1, see proteins designated with *). Many of these newly identified proteins are hydrolases involved in primary and secondary cell wall loosening and extension such as pectin methylesterase, beta-D-xylosidase, beta galactosidase or arabinofuranohydrolase¹⁷ (Table 1 designated with *). These proteins also included several lipases and GDSL lipases which is a subfamily of hydrolytic/lipolytic enzymes with a GDSL motif where the active site serine is located near the N-terminus.¹⁸ Other enzymes such as endonuclease (spot 62), S-like RNase (spot 151) and nucleotide pyrophosphatase (spot 32) were identified and their potential function in the xylem sap will be discussed below. Since there has only been one report of where a xylem sap was characterized using 2-DE gels and in that report only 69 proteins were identified, it is not yet possible to conclude that these newly identified proteins in maize xylem sap are specific to monocot species.

Approximately 65% of the identified proteins were similar to proteins in the xylem sap of the dicot species *Brassica napus*.¹⁴ These proteins were peroxidases, Chitinase, beta-glucanase, xyloglucan endo-transglycosylase/hydrolases (XET), polygalacturonase, polygalacturonase inhibitor (PGIP), putative disease resistance protein, putative germin A, thaumatin-like pathogenesis related proteins, and subtilisin-like serine protease and cysteine protease (see Table 1). Proteins previously reported in other dicot species might share the same function in maize which is a monocot; however, no functional information from other studies is available.

Xylem Sap Secreted Proteins. An unanswered question is whether proteins occur in xylem sap as the result of tracheid development or whether proteins are specifically secreted from adjacent xylem parenchyma cells to be transported to leaves.² Evidence suggests that at least some xylem sap proteins are actively secreted into the xylem stream. A novel lectin-like protein (XSP30) and two glycine-rich proteins were found to be actively secreted into the xylem sap by root cells of cucumber, and the corresponding genes were expressed in root vascular tissue.⁹ One of the glycine rich proteins was shown to be secreted, transported, and localized to metaxylem cells not only in roots but also in leaves and stems.¹⁹ The authors suggested that in order to get proteins into leaf xylem vessels for cell wall repair, lignification or for defense purposes, it may be necessary to send proteins from roots to the leaves.

With the use of SignalP,²⁰ a program that predicts N-terminal peptides, we determined that 97% of the identified proteins are predicted to be secreted proteins with a signal peptide at the N terminal (Table 1). The remaining 3% of the proteins were analyzed with SecretomeP,²¹ a program that predicts nonclassical, i.e., non signal peptide triggered protein secretion. The SecretomeP analysis suggests that except for the putative xylan xylanohydrolase isoenzyme (spot nos. 99 and 105), the other proteins (spot nos. 23 and 32) are nonclassical secreted proteins (Table 1). The xylan xylanohydrolase isoenzyme which catalyzes the hydrolytic cleavage of β -1,4-linked polymer of D-xylose (xylan)²² is one of the major components of hemicellulose. This protein was 19 kDa on the 2D gels, whereas the theoretical molecular weight is 82 kDa. This suggests the protein is partially degraded (Table 1) and, therefore, may be the result of protein release after the death of xylem cells during xylem differentiation.

Xylem Sap Isoform Proteins. Among the 154 proteins identified, only 59 different proteins are listed in Table 1. A large number of spots (Figure 1 and Table 1) had the same molecular weight with a variable isoelectric point, indicating the presence of multiple forms of enzymes that share common catalytic activity in the xylem sap. Approximately 42% of proteins identified (Figure 3) appear in several different spots on the gel. These include a subtilisin-like serine protease (area a, Figure 1), an arabinoxylan arabinofuranohydrolase isoenzyme AXAH-II (area b, Figure 1), a polygalacturonase-like protein (area b, Figure 1), different peroxidases (areas c, e, and g, Figure 1), a putative beta-1,3-glucanase (areas d and i, Figure 1), a putative PGIP (area c, Figure 1), a putative lipase (area f, Figure 1), the cysteine protease CP1 (area h, Figure 1), a putative germin A (area j, Figure 1), and a putative xylan xylanohydrolase isoenzyme (area k, Figure 1). A previous study showed the presence of multiple PR protein isoforms in xylem sap from fungi-infected tomato.¹¹

Many secreted proteins are known to be glycoproteins²³ and 97% of proteins in the xylem sap are predicted to be secreted

Table 1. Proteins Identified in Xylem Sap of Maize by MS/MS^a

spot no.	protein similar to	glyco protein	pI/M _r exp	pI/M _r theo	M _r exp /M _r theo no. > 18%	GI accession no.	MAS-COT score	no. of peptides matched	organism matched	secreted p-value	functional classification
1	arabinoxylan arabinofuranohydro-lase isoenzyme AXAH-II*		5.5/94	5.2/72	>	13398412	74	3	<i>Hordeum vulgare</i>	0.992	CW
2	subtilisin-like serine protease	x	9.6/90	6.2/81		21593457	352	7	<i>Arabidopsis thaliana</i>	0.968	PP
3	subtilisin-like serine protease	x	7.2/92	6.2/81		21593457	383	8	<i>Arabidopsis thaliana</i>	0.968	PP
4	subtilisin-like serine protease	x	6.3/91	6.2/81		21593457	346	7	<i>Arabidopsis thaliana</i>	0.968	PP
5	subtilisin-like serine protease	x	7.5/91	6.2/81		21593457	413	8	<i>Arabidopsis thaliana</i>	0.968	PP
6	subtilisin-like serine protease	x	6.5/90	6.2/81		21593457	358	8	<i>Arabidopsis thaliana</i>	0.968	PP
7	subtilisin-like serine protease	x	6.8/89	6.2/81		21593457	315	8	<i>Arabidopsis thaliana</i>	0.968	PP
8	subtilisin-like serine protease	x	7.3/90	6.2/81		21593457	444	8	<i>Arabidopsis thaliana</i>	0.968	PP
9	subtilisin-like serine protease	x	7.6/91	6.2/81		21593457	321	6	<i>Arabidopsis thaliana</i>	0.968	PP
10	subtilisin-like serine protease		7.0/91	6.2/81		21593457	236	7	<i>Arabidopsis thaliana</i>	0.968	PP
11	subtilisin-like serine protease	x	7.9/91	6.2/81		21593457	412	9	<i>Arabidopsis thaliana</i>	0.968	PP
12	beta-D-xylosidase*	x	5.6/83	6.8/83		18025342	349	8	<i>Hordeum vulgare</i>	1	CW
13	beta-D-xylosidase*	x	5.8/82	6.8/83		18025342	379	12	<i>Hordeum vulgare</i>	1	CW
14	arabinoxylan arabinofurano-hydro-lase isoenzyme AXAH-II*		5.0/78	5.2/72		13398412	230	7	<i>Hordeum vulgare</i>	0.992	CW
15	exhydrolase II*	x	7.5/80	6.6/68		4731111	175	3	<i>Zea mays</i>	1	CW/PD
16	arabinoxylan arabinofurano-hydro-lase isoenzyme AXAH-II*	x	5.3/77	5.2/72		13398412	310	6	<i>Hordeum vulgare</i>	0.992	CW
16	polygalacturonase-like protein	x	5.3/77	7.6/52	>	30692538	175	4	<i>Fragaria x ananassa</i>	0.998	CW/PD
17	arabinoxylan arabinofurano-hydro-lase isoenzyme AXAH-II*	x	5.4/78	5.2/72		13398412	272	6	<i>Hordeum vulgare</i>	0.992	CW
17	polygalacturonase-like protein	x	5.4/78	7.6/52	>	30692538	163	5	<i>Fragaria x ananassa</i>	0.998	CW/PD
18	arabinoxylan arabinofurano-hydro-lase isoenzyme AXAH-II*	x	5.1/78	5.2/72		13398412	309	6	<i>Hordeum vulgare</i>	0.992	CW
18	polygalacturonase-like protein	x	5.1/78	7.6/52	>	30692538	134	5	<i>Fragaria x ananassa</i>	0.998	CW/PD
19	exoglucanase precursor	x	7.2/79	7.3/67		8809764	82	2	<i>Zea mays</i>	0.999	CW
19	subtilisin-like serine protease		7.2/79	6.2/81		21593457	308	6	<i>Arabidopsis thaliana</i>	0.968	PP
20	arabinoxylan arabinofurano-hydro-lase isoenzyme AXAH-II*	x	5.7/76	5.2/72		13398412	89	2	<i>Hordeum vulgare</i>	0.992	CW
20	polygalacturonase-like protein	x	5.7/76	7.6/52	>	30692538	298	6	<i>Fragaria x ananassa</i>	0.998	CW/PD
21	subtilisin-like serine protease	x	7.0/80	6.2/81		21593457	274	5	<i>Arabidopsis thaliana</i>	0.968	PP
21	polygalacturonase-like protein	x	7.0/80	7.6/52	>	30692538	340	6	<i>Fragaria x ananassa</i>	0.998	CW
22	polygalacturonase-like protein	x	6.6/75	7.6/52	>	30692538	451	7	<i>Fragaria x ananassa</i>	0.998	CW
22	putative pectin methylesterase*	x	6.6/75	6.6/58	>	34907404	161	4	<i>Oryza sativa</i>	1	CW
23	pectin methylesterase-like protein*	x	6.0/80	7.0/63	>	9759007	211	5	<i>Arabidopsis thaliana</i>	0.03/0.692	CW
24	Arabinoxylan arabinofurano-hydro-lase isoenzyme AXAH-II*	x	6.2/74	5.2/72		13398412	96	3	<i>Hordeum vulgare</i>	0.992	CW
24	polygalacturonase-like protein	x	6.2/74	7.6/52	>	30692538	437	7	<i>Fragaria x ananassa</i>	0.998	CW/PD
25	arabinoxylan arabinofurano-hydro-lase isoenzyme AXAH-II*	x	6.377	5.2/72		13398412	138	4	<i>Hordeum vulgare</i>	0.992	CW
25	polygalacturonase-like protein	x	6.377	7.6/52	>	30692538	423	8	<i>Fragaria x ananassa</i>	0.998	CW/PD
26	arabinoxylan arabinofurano-hydro-lase isoenzyme AXAH-II*	x	6.4/75	5.2/72		13398412	183	6	<i>Hordeum vulgare</i>	0.992	CW
26	polygalacturonase-like protein	x	6.4/75	7.6/52	>	30692538	318	6	<i>Fragaria x ananassa</i>	0.998	CW/PD
26	putative pectin methylesterase*	x	6.4/75	6.6/58	>	34907404	176	4	<i>Oryza sativa</i>	1	CW
27	putative polygalacturonase		7.0/67	6.8/47	>	34912726	84	2	<i>Oryza sativa</i>	1	CW/PD
28	putative subtilisin serine protease		7.7/50	8.4/80	<	23296838	75	2	<i>Arabidopsis thaliana</i>	0.876	PP
29	putative pectinacylesterase*		6.4/52	6.1/45	>	34907550	97	4	<i>Oryza sativa</i>	0.999	CW
30	putative pectin methylesterase*	x	6.5/82	6.6/58	>	34907404	124	3	<i>Oryza sativa</i>	1	CW
31	peroxidase (EC 1.11.1.7) poxN	x	5.4/48	6.2/35	>	7433039	184	4	<i>Oryza sativa</i>	0.961	CW/PD
31	putative polygalacturonase inhibitor	x	5.4/48	6.4/36	>	50931079	109	2	<i>Oryza sativa</i>	1	CW
32	nucleotide pyrophosphatase homolog*		6.8/49	5.3/52		34903578	96	2	<i>Oryza sativa</i>	0.522/0.867	Unclassified protein
33	alpha-mannosidase*	x	6.8/69	8.3/118	<	10177130	267	6	<i>Arabidopsis thaliana</i>	0.971	CW
34	exhydrolase II*		7.4/71	6.1/68		4731111	284	6	<i>Zea mays</i>	1	CW/PD
35	putative PS60 *		7.3/59	8.8/60		52076641	106	2	<i>Oryza sativa</i>	0.983	Unclassified protein
35	putative polygalacturonase		7.3/59	5.9/51		55775064	136	3	<i>Oryza sativa</i>	0.995	CW/PD
36	peroxidase (EC 1.11.1.7) poxN [similarity]	x	5.2/47	6.2/35	>	7433039	354	6	<i>Oryza sativa</i>	0.961	CW/PD
36	putative polygalacturonase inhibitor	x	5.2/47	6.4/36	>	50931079	108	2	<i>Oryza sativa</i>	1	CW
37	beta-galactosidase*		6.8/48	7.4/91	<	14970841	149	4	<i>Fragaria x ananassa</i>	1	CW
38	OSJNBa0070C17.16*		5.5/48	6.3/51		50928681	211	5	<i>Oryza sativa</i>	0.994	Unknown protein
39	GDSL-lipase-like *		8.0/45	9.3/39	<	54291019	143	2	<i>Oryza sativa</i>	0.94	CW
39	putative beta-galactosidase*		8.0/45	7.5/78		54291174	201	5	<i>Oryza sativa</i>	1	CW

Table 1. (Continued)

spot no.	protein similar to	glyco protein	pI/M _r exp	pI/M _r theo	M _r exp /M _r theo no. > 18%	GI accession no.	MASCOT score	no. of peptides matched	organism matched	secreted p-value	functional classification
40	putative polygalacturonase		6.4/43	5.9/51		55775064	90	2	<i>Oryza sativa</i>	0.995	CW/PD
41	glycosyl hydrolase family 5 protein/cellulase family		7.7/50	9.0/63		15231513	94	2	<i>Arabidopsis thaliana</i>	0.978	CW
42	putative polygalacturonase inhibitor		7.1/42	5.9/36		50931079	119	2	<i>Oryza sativa</i>	1	CW
42	putative early nodulin 8 precursor *		7.1/42	6.9/42		50938787	75	2	<i>Oryza sativa</i>	0.995	CW
43	beta-galactosidase*		6.5/45	7.4/91	<	14970841	163	4	<i>Fragaria x ananassa</i>	1	CW
44	putative beta-1,3-glucanase		5.8/43	5.4/59	<	50938049	79	3	<i>Oryza sativa</i>	1	CW/PD
45	GDSL-lipase-like*		7.4/43	9.3/39		54291019	138	3	<i>Oryza sativa</i>	0.94	CW
46	GDSL-lipase-like*		8.0/42	9.3/39		54291019	148	3	<i>Oryza sativa</i>	0.94	CW
46	putative beta-galactosidase*		8.0/42	7.5/78	<	54291174	178	5	<i>Oryza sativa</i>	1	CW
47	putative alpha-galactosidase preproprotein*		5.9/37	8.1/46		37535646	115	2	<i>Oryza sativa</i>	0.998	CW/PD
48	putative beta-1,3-glucanase		6.1/42	5.4/59	<	50938049	161	3	<i>Oryza sativa</i>	1	CW/PD
49	putative beta-1,3-glucanase		6.3/43	5.4/59	<	50938049	187	6	<i>Oryza sativa</i>	1	CW/PD
50	putative polygalacturonase inhibitor		5.3/35	5.9/36		50931079	122	2	<i>Oryza sativa</i>	1	CW
50	peroxidase 7		5.3/35	6.0/38		57635159	212	4	<i>Triticum monococcum</i>	1	CW/PD
51	beta glucanase:ISOTYPE=II		6.0/36	8.9/35		228411	193	4	<i>Hordeum vulgare</i>	0.999	CW/PD
51	alpha-rhamnosidase-like protein*		6.0/36	5.8/47	<	50937769	184	3	<i>Oryza sativa</i>	0.614	CW
52	TPA: class III peroxidase 38 precursor		5.7/42	5.8/35		55700943	97	3	<i>Oryza sativa</i>	0.972	CW/PD
53	aldose-1-epimerase-like protein*		7.6/42	9.4/39		2739168	222	4	<i>Nicotiana tabacum</i>	0.996	CW
54	putative alpha-galactosidase preproprotein*		7.3/42	7.9/46		37535646	212	6	<i>Oryza sativa</i>	0.998	CW/PD
55	putative alpha-galactosidase preproprotein*		6.1/39	7.9/46		37535646	235	7	<i>Oryza sativa</i>	0.998	CW/PD
56	putative alpha-galactosidase preproprotein*		6.8/41	7.9/46		37535646	214	7	<i>Oryza sativa</i>	0.998	CW/PD
57	cationic peroxidase isozyme 40K precursor		9.0/39	8.7/36		575605	375	7	<i>Nicotiana tabacum</i>	0.988	CW/PD
58	cationic peroxidase isozyme 40K precursor		9.3/37	8.7/36		575605	527	9	<i>Nicotiana tabacum</i>	0.988	CW/PD
59	Peroxidase	x	8.1/37	8.5/34		28400794	138	3	<i>Asparagus officinalis</i>	0.997	CW/PD
60	putative alpha-galactosidase preproprotein*		6.4/40	7.9/46		37535646	266	8	<i>Oryza sativa</i>	0.998	CW/PD
61	cationic peroxidase isozyme 40K precursor		9.7/38	8.7/36		575605	496	9	<i>Nicotiana tabacum</i>	0.988	CW/PD
62	endonuclease*		6.6/39	5.8/34		3242447	132	4	<i>Asparagus officinalis</i>	0.867	Unclassified protein
63	Peroxidase		6.9/35	8.5/34		28400794	173	3	<i>Asparagus officinalis</i>	0.997	CW/PD
64	putative lipase*	x	7.9/35	8.8/43	<	53793018	157	4	<i>Oryza sativa</i>	0.997	CW
65	putative lipase*		8.1/31	8.2/38	<	55297457	70	2	<i>Oryza sativa</i>	0.999	CW
66	putative lipase*		7.6/35	8.8/43	<	53793018	173	5	<i>Oryza sativa</i>	0.997	CW
67	Peroxidase	x	6.9/36	8.5/34		28400794	114	2	<i>Asparagus officinalis</i>	0.997	CW/PD
67	putative lipase*	x	6.9/36	8.8/43	<	53793018	107	3	<i>Oryza sativa</i>	0.997	CW
68	putative lipase*	x	7.5/35	8.8/43	<	53793018	217	5	<i>Oryza sativa</i>	0.997	CW
69	Peroxidase	x	6.1/35	7.6/32		520570	368	5	<i>Cenchrus ciliaris</i>	1	CW/PD
70	putative lipase*		7.1/35	8.8/43	<	53793018	279	5	<i>Oryza sativa</i>	0.997	CW
71	putative bacterial-induced peroxidase precursor		7.9/34	8.1/33		50251693	133	4	<i>Oryza sativa</i>	1	PD
72	Peroxidase	x	6.5/34	7.6/32		520570	314	7	<i>Cenchrus ciliaris</i>	1	CW/PD
72	putative lipase*		6.5/34	8.8/43	<	53793018	57	2	<i>Oryza sativa</i>	0.997	CW
75	cathepsin B*		5.5/31	6.5/37		40643250	133	4	<i>Hordeum vulgare</i>	1	PP
77	beta glucanase:ISOTYPE=II		5.7/31	8.9/35		228411	321	7	<i>Hordeum vulgare</i>	0.999	CW/PD
78	putative beta-1,3-glucanase		8.9/31	5.4/59	<	50938049	209	5	<i>Oryza sativa</i>	1	CW/PD
79	xylanase inhibitor protein I*	x	6.0/34	8.5/33		20804336	396	10	<i>Triticum aestivum</i>	1	CW
80	chitinase (EC 3.2.1.14) III C10502		9.5/32	8.2/32		7451375	297	6	<i>Oryza sativa</i>	0.999	CW/PD
81	beta glucanase:ISOTYPE=II		5.9/32	8.9/35		228411	291	7	<i>Hordeum vulgare</i>	0.999	CW/PD
82	beta glucanase:ISOTYPE=II		6.1/31	8.9/35		228411	373	8	<i>Hordeum vulgare</i>	0.999	CW/PD
84	putative beta-1,3-glucanase	x	8.1/27	5.1/59	<	50938049	245	7	<i>Oryza sativa</i>	1	CW/PD
86	putative beta-1,3-glucanase		7.6/30	5.4/59	<	50938049	95	3	<i>Oryza sativa</i>	1	CW/PD
88	putative beta-1,3-glucanase		6.9/31	5.4/59	<	50938049	162	3	<i>Oryza sativa</i>	1	CW/PD
89	putative beta-1,3-glucanase		7.4/30	5.4/59	<	50938049	155	4	<i>Oryza sativa</i>	1	CW/PD
90	putative beta-1,3-glucanase		7.2/30	5.4/59	<	50938049	234	5	<i>Oryza sativa</i>	1	CW/PD
93	Chitinase		9.3/28	8.3/31		2109457	125	4	<i>Oryza sativa</i>	0.64	CW/PD
94	putative Cupin family protein		6.1/27	6.9/23		50919209	134	2	<i>Oryza sativa</i>	1	PD
95	putative germin A	x	5.6/27	6.9/24		50941859	84	3	<i>Oryza sativa</i>	1	PD
96	putative germin A	x	6.3/27	6.9/24		50941859	65	2	<i>Oryza sativa</i>	1	PD
97	putative polygalacturonase		5.2/26	6.4/51	<	55775064	111	2	<i>Oryza sativa</i>	0.995	CW/PD
99	putative xylan xylanohydrolase isoenzyme*		5.3/20	5.6/82	<	50917687	149	3	<i>Oryza sativa</i>	0/0.291	CW
100	antifungal zeamatin-like protein		8.5/24	7.6/24		7442163	75	2	<i>Zea mays</i>	0.999	PD
102	At5 g42500		5.7/18	6.5/19		62734184	138	2	<i>Oryza sativa</i>	0.999	PD
104	putative disease resistance response protein		6.6/20	6.2/23		51971026	92	2	<i>Arabidopsis thaliana</i>	0.93	PD

Table 1. (Continued)

spot no.	protein similar to	glyco protein	pI/ M_r exp	pI/ M_r theo	M_r exp / M_r theo no. > 18%	GI accession no.	MASCOT score	no. of peptides matched	organism matched	secreted p -value	functional classification
105	putative xylan xylanohydrolase isoenzyme*		5.6/19	5.6/82	<	50917687	322	7	<i>Oryza sativa</i>	0/0.291	CW
106	At3 g13650		7.0/16	6.7/18		62734201	99	2	<i>Oryza sativa</i>	1	PD
107	putative disease resistance response protein		6.6/17	6.2/23		51971026	110	3	<i>Arabidopsis thaliana</i>	0.93	PD
113	thaumatin-like pathogenesis-related protein	x	7.8/15	6.3/17		662351	149	4	<i>Avena sativa</i>	1	CW/PD
117	putative disease resistance response protein		6.6/16	6.2/23	<	51971026	138	4	<i>Arabidopsis thaliana</i>	0.93	PD
118	chitinase (EC 3.2.1.14) III C10501		6.5/12	8.2/32	<	7451375	120	2	<i>Oryza sativa</i>	0.999	CW/PD
119	putative blue copper protein*		7.4/10	8.4/19	<	51535359	126	3	<i>Oryza sativa</i>	1	unclassified protein
130	putative polygalacturonase inhibitor	x	4.5/53	6.4/36	>	50931079	73	2	<i>Oryza sativa</i>	1	CW
132	peroxidase (EC 1.11.1.7) poxN [similarity]	x	4.9/47	6.2/35	>	7433039	293	6	<i>Oryza sativa</i>	0.961	CW/PD
133	peroxidase (EC 1.11.1.7) poxN [similarity]	x	4.8/47	6.2/35	>	7433039	112	3	<i>Oryza sativa</i>	0.961	CW/PD
133	putative polygalacturonase inhibitor	x	4.8/47	6.4/36	>	50931079	92	3	<i>Oryza sativa</i>	1	CW
134	peroxidase (EC 1.11.1.7) poxN [similarity]	x	4.8/48	6.2/35	>	7433039	161	4	<i>Oryza sativa</i>	0.961	CW/PD
134	putative polygalacturonase inhibitor	x	4.8/48	6.4/36	>	50931079	90	4	<i>Oryza sativa</i>	1	CW
135	peroxidase (EC 1.11.1.7) poxN [similarity]	x	4.6/48	6.2/35	>	7433039	128	3	<i>Oryza sativa</i>	0.961	CW/PD
135	putative polygalacturonase inhibitor	x	4.6/48	6.4/36	>	50931079	164	2	<i>Oryza sativa</i>	1	CW
136	putative polygalacturonase inhibitor	x	4.6/48	6.4/36	>	50931079	134	2	<i>Oryza sativa</i>	1	CW
137	peroxidase (EC 1.11.1.7) poxN [similarity]	x	4.4/48	6.2/35	>	7433039	72	2	<i>Oryza sativa</i>	0.961	CW/PD
137	putative polygalacturonase inhibitor	x	4.4/48	6.4/36	>	50931079	155	2	<i>Oryza sativa</i>	1	CW
138	peroxidase (EC 1.11.1.7) poxN [similarity]	x	4.2/48	6.2/35	>	7433039	36	2	<i>Oryza sativa</i>	0.961	CW/PD
139	putative polygalacturonase inhibitor	x	4.3/48	8.5/43	>	50931079	55	3	<i>Oryza sativa</i>	1	CW
140	putative peroxidase	x	4.4/46	4.9/36		37530470	91	2	<i>Oryza sativa</i>	0.952	CW/PD
140	putative polygalacturonase inhibitor	x	4.4/46	6.4/36	>	50931079	116	2	<i>Oryza sativa</i>	1	CW
141	peroxidase (EC 1.11.1.7) (clone prxRPA)	x	4.5/45	4.9/36	>	7433034	145	2	<i>Oryza sativa</i>	0.952	CW/PD
141	putative polygalacturonase inhibitor	x	4.5/45	6.4/36	>	50931079	159	3	<i>Oryza sativa</i>	1	CW
142	peroxidase (EC 1.11.1.7) (clone prxRPA)	x	4.5/45	4.9/36	>	7433034	163	2	<i>Oryza sativa</i>	0.952	CW/PD
142	putative polygalacturonase inhibitor	x	4.5/45	6.4/36	>	50931079	169	3	<i>Oryza sativa</i>	1	CW
143	xyloglucan endo-transglycosylase/hydrolase		4.2/28	4.7/31		57753593	80	3	<i>Zea mays</i>	1	CW
144	peroxidase (EC 1.11.1.7) (clone prxRPA)	x	4.7/44	4.9/36	>	7433034	280	4	<i>Oryza sativa</i>	0.952	CW/PD
144	putative polygalacturonase inhibitor	x	4.7/44	6.4/36	>	50931079	139	2	<i>Oryza sativa</i>	1	CW
145	peroxidase (EC 1.11.1.7) (clone prxRPA)	x	4.7/45	4.9/36	>	7433034	165	2	<i>Oryza sativa</i>	0.952	CW/PD
145	putative polygalacturonase inhibitor	x	4.7/45	6.4/36	>	50931079	155	2	<i>Oryza sativa</i>	1	CW
146	peroxidase (EC 1.11.1.7) (clone prxRPA)	x	4.8/43	4.9/36	>	7433034	177	5	<i>Oryza sativa</i>	0.952	CW/PD
146	putative polygalacturonase inhibitor	x	4.8/43	8.5/43		50931079	118	6	<i>Oryza sativa</i>	1	CW
147	putative peroxidase 47 precursor		4.9/37	6.7/34		50940455	248	4	<i>Oryza sativa</i>	0.991	CW/PD
148	putative cysteine proteinase		4.9/32	5.4/40		34905996	70	2	<i>Oryza sativa</i>	0.999	PP
149	cysteine protease CP1	x	4.8/33	5.3/39		33151125	167	3	<i>Oryza sativa</i>	0.999	PP
150	cysteine protease CP1	x	4.7/33	5.3/39		33151125	218	4	<i>Oryza sativa</i>	0.999	PP
151	S-like RNase*		4.8/24	5.2/25		41387691	84	2	<i>Triticum aestivum</i>	1	Unclassified protein
153	secretory protein*		4.2/26	9.3/24		5669008	158	3	<i>Triticum aestivum</i>	1	Unclassified protein
154	putative beta-1,3-glucanase precursor		4.7/21	8.3/51	<	50913221	90	2	<i>Oryza sativa</i>	1	CW/PD

^a Experimental (Exp) and theoretical (Theo) M_r (kDa) and pI, the MASCOT score and the number of peptides matched are shown. The assigned protein name is given along with the organism from which the annotation was derived and its GenBank accession number. The asterisk indicates the proteins identified only in maize xylem sap and not previously identified in other species. Information about potential glycosylation and secretion (with a secreted p -value > 0.95) or non classical secretion (with a second secreted p -value > 0.6) are indicated. The M_r Exp/ M_r Theo column highlights the proteins with a M_r experimental greater than or less than 18% from M_r theoretical. The proteins have been classified in different categories according to their function in cell wall metabolism (CW), in proteolysis and peptidolysis (PP), or in plant defense mechanism (PD).

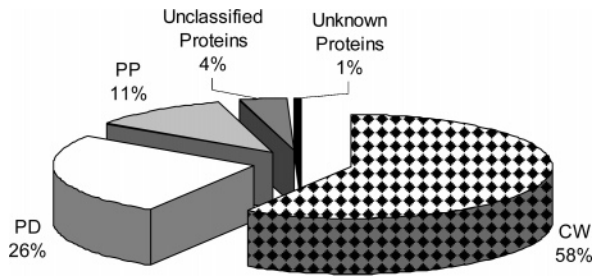


Figure 2. Functional classification of proteins identified in xylem sap. Cell wall metabolism (CW), plant defense mechanism (PD), and proteolysis and peptidolysis (PP) are the three main categories.

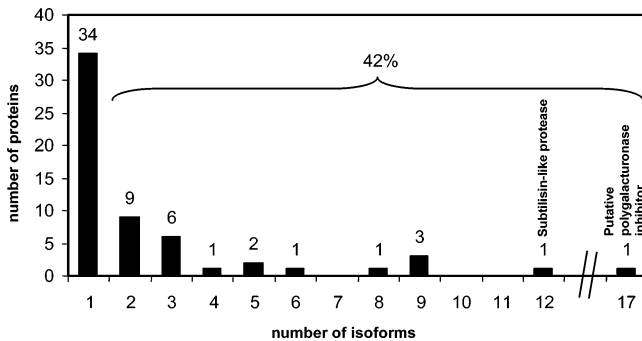


Figure 3. Number of proteins found as single proteins and as isoforms on gel from Figure 1. Also shown is the number of protein spots for proteins with multiple spots or isoforms.

proteins. In addition, the molecular weight of many proteins in the xylem sap differs from predicted molecular weights (Table 1). Therefore, an attempt was made to determine whether these proteins were glycosylated. A specific stain (ProQ-Emerald) was used to reveal the presence of glycoproteins (Figures 4A). On gels stained with ProQ-Emerald, 92 spots were recognized (Figure 4A) which were also found on a SyproRuby stained gel (Figures 4B). Fewer proteins could be visualized on the gels in Figure 4 as compared to Figure 1 because less protein and smaller gels were used for Figure 4. Fifty-seven spots corresponded to proteins that had been previously identified (Table 1). The ProQ-Emerald stain revealed that the glycosylated isoforms present in the areas a, b, c, e, g, h, and j correspond to the proteins highlighted by the glycoprotein stain (Figure 4A and B). The post-translationally modified train of spots appears to originate from the same

protein, because they match the same accession number in the NCBI database. However, this would have to be confirmed by increasing the coverage of peptides identified from each protein, to ensure they originated from a single gene and not another member of the family of genes to which they belong.

Xylem Sap Glycoproteins. Most isoforms present in the xylem sap were identified as glycoproteins based on gel staining. These include isoforms of subtilisin-like serine protease (area a), cationic peroxidase isozyme 40K precursor (area e), cysteine protease (area h), putative germin A (area j). In area b (Figure 1), two different cell wall proteins overlay each other, an arabinoxylan arabinofuranohydrolase isoenzyme AXAH-II, also called α -L-arabinofuranosidase, and a polygalacturonase-like protein. The function of α -L-arabinofuranosidase is to remove the arabinofuranosyl residues from arabinoxylans when they are deposited into maize cell walls during secondary cell wall synthesis.²⁴ This protein is previously found in germinating maize seeds with several isoforms in an isoelectric point range of 5.8–6.0.¹⁷ Polygalacturonases have been characterized in different plant physiological processes, organs and species.²⁵ Polygalacturonase function is related to cell wall expansion through the hydrolysis of the pectin matrix. The oligogalacturonides released may have a role in developmental signaling and the activation of defense responses.²⁶ Because two different proteins are present in the same spot, it is not possible to determine if one or both are glycosylated. However, some spots corresponding only to the polygalacturonase-like protein such as spot number 21 and 22 (Table 1) were isolated and detected in the glycoprotein stained gel, indicating that at least the polygalacturonase-like protein is a glycoprotein. Glycosylated forms of polygalacturonases have been found in *Aspergillus*²⁷ where glycosylation was shown to be important in the maintenance of the enzyme activity.

In another area of the gel (Figure 1, area c) there are also two different proteins identified from the same spots. These were a peroxidase and PGIP. Higher plants have a number of different isozymes of peroxidase.²⁸ Two isoforms of peroxidases were found in xylem sap (Figure 1, spot nos. 31, 36, 132, 133, 134, 135, 137, 138, 141, 142, 144, 145, and 146) that were similar to predicted peroxidase proteins in rice: *poxA* (clone *prxRPA*) and *poxN*.²⁹ The promoters of these genes in rice have been shown to contain a cis element AGCCGCC, which is highly conserved in various plant stress- and defense-related genes.³⁰ Both the rice peroxidase genes are expressed in xylem-parenchyma cells of leaf blade and sheath.³¹ This is the first time that a similar peroxidase has been found in xylem sap

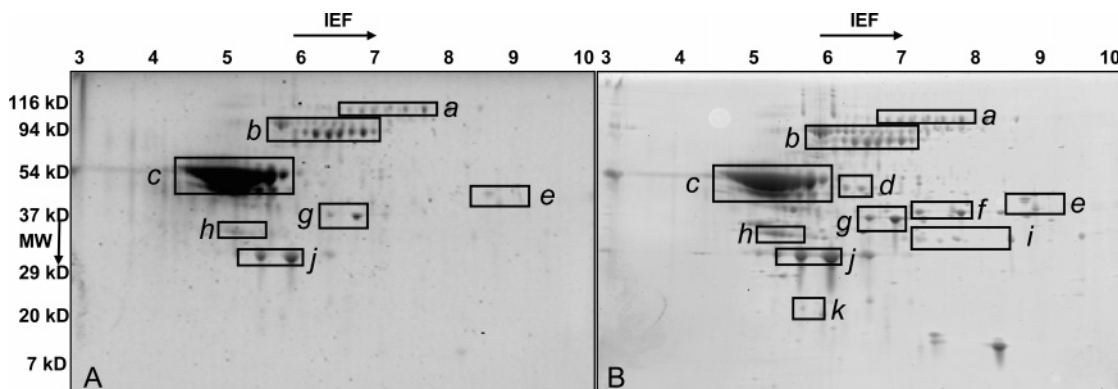


Figure 4. Glyco staining of 2-DE gels of xylem sap protein. A. Xylem sap proteins stained with ProQ Emerald which recognizes glycosylated proteins B. Xylem sap proteins stained with SYPRO Ruby which recognizes all proteins. The squares annotated with letters indicate the protein isoforms.

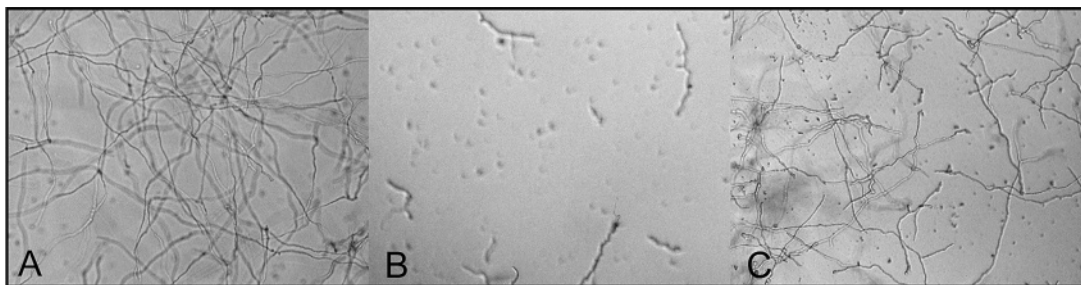


Figure 5. Antifungal assay of xylem sap proteins. A. Control (*Neurospora crassa*), B. *Neurospora crassa* + xylem sap proteins, C. *Neurospora crassa* + xylem sap proteins treated with Pronase. The pictures were taken with a 100× magnification and are all at the same scale.

which may suggest that these peroxidases are secreted into the xylem stream from the surrounding parenchyma cells. Secreted plant peroxidases are assigned to class III whose functions include lignification, suberization, cross-linking of cell wall structural proteins, auxin catabolism, defense against pathogen attack, salt tolerance, oxidative stress protection, cell elongation, and ROS generation.²⁸ Class III peroxidases are heme-containing glycoproteins encoded by a large multigene family in plants. The glycosylation of class III peroxidases is suggested by the detection of glycoproteins in area c (Figure 4A). However, this cannot be confirmed since these protein spots also contained PGIP proteins which are extracellular plant proteins typically induced by pathogen infection and stress-related signals.³²

The function of the proteins in the xylem sap is unknown; however, in the following sections we suggest how some of the proteins may be involved in xylem development and, as others have suggested, in plant defense responses.^{2,7,11}

Xylem Sap Proteins and Cell Wall Modifications. A number of the proteins identified in the maize xylem sap are functionally associated with cell wall metabolism. Many of these proteins are hydrolases involved in cell wall break down such as exohydrolases, glucanases, galactosidases, xylosidases, and lipases.^{18,33–36} Indeed secondary cell wall formation in tracheary elements is coupled with the degradation of primary cell walls which is accompanied by the activity of many cell wall degrading enzymes.³⁷ Some of these proteins, such as the beta-1,3-glucanase that we identified in maize sap, were also shown to increase in abundance in xylem sap from plants infected with fungi.¹¹ In addition, the sap contained pectin methyl-esterase and pectin acetyl-esterase associated with the demethylation and deacetylation of pectin.³⁸ The sap proteome also contained polygalacturonase and arabinoxylan arabinofuranohydrolase which hydrolyze pectin.^{24,27} The presence of these hydrolytic enzymes and the XET, a putative cell wall-loosening enzyme, in the xylem sap suggests that they may be involved in the control of cell wall extension. XET may also function to incorporate new xyloglucan during the development of the cell wall.³⁹ Cell wall degrading enzymes may also play a role in the perforation of the cell walls necessary in the formation of mature hollow tubes that will be then reinforced by secondary walls.

Xylem Sap Proteins and Lignification. The xylem of higher plants contains lignified tissue and it is the presence of lignin that facilitates xylem function and defines xylem structure.⁴⁰ Lignin is a strengthening polymer that provides structural support and also waterproofs the cell wall enabling the transport of water through xylary elements. There are three basic steps in lignin biosynthesis: monolignols are (1) synthe-

sized within cells, (2) transported to the cell wall, and (3) then polymerized. This process requires dimerization and polymerization of monolignols, and this reaction is dependent on hydrogen peroxide. In maize xylem sap, we found a large number of peroxidases that could potentially be involved in lignification. Peroxidases catalyze the polymerization of cinnamyl alcohols or aldehydes, long chain or hydroxylated fatty acids, and long chain alcohols into lignin and the related cell wall component suberin.⁴¹ The presence of peroxidases has been observed in the xylem sap and in the guttation fluid of a variety of plants including *Helianthus annuus*, grasses, broccoli, rape, pumpkin, strawberry, tomato, cucumber, apple, peach, and pear.^{2,7,13,42} The occurrence of peroxidases in xylem sap may simply be due to leakage from developing root xylem vessels, however it is possible that peroxidases are actively secreted into xylary elements and transported within the xylem stream to facilitate lignification of immature xylem vessels throughout the plant. Alternatively, peroxidases may have other functions in xylem sap such as roles in plant defense or reactive oxygen species metabolism. Several anionic and cationic peroxidases are found in maize xylem sap and therefore it is possible that the different peroxidases in xylem sap have different functions.

Xylem Sap Proteins and Programmed Cell Death. During the process of cell death leading to the differentiation of xylary elements, the primary cell wall and protoplast are completely degraded.^{43,44} The digested cell contents containing proteases and nucleases are released into a neighboring hollow tracheary element. Cysteine proteases, serine proteases, RNases, S1-type nucleases, acid phosphatases and lipases are some enzymes involved in programmed cell death (PCD).³⁷ In xylem development, cysteine and serine proteinases have been implicated in differentiation and cell death^{45,46} and both were found in the maize xylem sap (Table 1). Proteinases may be involved in this process as mediators of signal transduction or as effectors of PCD.⁴⁷ Several isoforms of subtilisin-like serine protease (Clan SB, Family S8) were identified in the xylem sap (spot nos. 2–11, 19, and 21), along with four cysteine proteases CP1 and a cathepsin B (Clan CA, Family C1). The C1 family of cathepsin proteases have been implicated in plant cell suicide programs;⁴⁷ and in mammalian systems, cathepsin B mediates cell death in non-small-cell lung cancer cells.⁴⁸ The plant cathepsin B proteins and the other proteases in the xylem sap of maize may be involved in PCD or some other aspect of xylary element differentiation required for the development of functional, conducting xylem vessels. Similarly, an endonuclease (spot 62), an S-like-RNase (spot 151), a nucleotide pyrophosphatase (spot 32) and several lipases (spot nos. 64–68, 70, and 72) in xylem

sap could also be involved in the early stages of cell content degradation during PCD and then released in the xylem stream.⁴⁹

Xylem Sap Proteins and Plant Defense Mechanisms. In contrast to these cell wall loosening enzymes, the sap also contains xylanase inhibitor protein which inhibits the activity of certain glucanases and also PGIPs that inhibit the hydrolysis of pectin. The presence of PGIPs has been postulated as a plant defense response that may inhibit a pathogen's hydrolytic activity toward the cell wall.³² A xylanase inhibitor protein was also identified in the maize xylem sap (Table 1) which has been shown to be induced by pathogens and wounding in wheat.⁵⁰ The maize sap proteome also contained a fasciclin-like arabinogalactan protein (AGP) and a putative AGP (Table 1). Arabinogalactan proteins are cell wall proteoglycans and the binding of plant surface AGPs can trigger wound-like responses such as callose synthesis.⁵¹

Besides the presence of cell wall proteins and peroxidases with potential roles in plant defense mechanisms, the maize xylem sap proteome contains numerous plant defense proteins such as thaumatin, chitinase, a zeamatin-like protein and a putative disease resistance response protein. Proteins related to plant defense have previously been identified in xylem sap from different plant species.^{2,7,10} Zeamatin is a basic thaumatin-like protein, thought to be involved in plant defense against fungal pathogens.⁵² The maize sap also contained putative germin A proteins. Germin is a protease-resistant, apoplastic glycoprotein with peroxide-generating oxalate oxidase activity.⁵³ Germin proteins are known to be involved in various processes including defense and apoptosis.⁵⁴ A re-enforcement of cell walls in response to pathogen attack⁵⁵ may occur due to the peroxide production by germins. These proteins may promote lignification and the oxidative cross-linking of the cell walls in order to limit the fungal penetration.

To determine the antifungal activity of the xylem sap and the potential function of proteins with classified roles in defense, the sap was assayed using a *Neurospora crassa* bioassay¹⁶ (Figure 5). Treatment with the protein fractions from the xylem sap caused the inhibition of fungal growth relative to the control (Figure 5A and B). To confirm the involvement of proteins in the growth inhibition of *Neurospora*, the protein fraction was treated with Pronase which is a complex mixture of proteases. Pronase treatment abolished the antifungal activity of the sap which suggests the activity was due to proteins in the sap (Figure 5C). Therefore, the xylem sap in maize contains some proteins with antifungal activity that are present under conditions when pathogens are not challenging the plant.

Conclusions

In this work, we identified many of the proteins in the maize xylem sap proteome. The xylem sap of maize contains proteins that may play a role in tracheary element differentiation with functions that include cell wall loosening, lignification, and programmed cell death. Several proteins were identified for the first time in the xylem sap such as arabinoxylan arabinofuranohydrolase, exhydrolase II and lipases. In addition many proteins play a potential role in defense and a protein component of the sap was shown for the first time to possess antifungal activity.

Abbreviations. 2-DE, two-dimensional gel electrophoresis; PCD, programmed cell death; PGIP, polygalacturonase inhibitor; XET, xyloglucan endo-transglycosylase/hydrolases.

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References

- Berger, D.; Altmann, T. *Genes Dev.* **2000**, *14*, 1119–1131.
- Biles, C. L.; Abeles, F. B. *Plant Physiol.* **1991**, *96*, 597–601.
- Else, M. A.; Davies, W. J.; Whitford, P. N.; Hall, K. C.; Jackson, M. B. *J. Exp. Bot.* **1994**, *45*, 317–323.
- Schell, J. *Tree Physiol.* **1997**, *17*, 479–483.
- Biles, C. L.; Martyn, R. D.; Wilson, H. D. *Hortsci.* **1989**, *24*, 810–812.
- Satoh, S.; Iizuka, C.; Kikuchi, A.; Nakamura, N.; Fufii, T. *Plant Cell Physiol.* **1992**, *33*, 841–847.
- Buhtz, A.; Kolasa, A.; Arlt, K.; Walz, C.; Kehr, J. *Planta* **2004**, *219*, 610–618.
- Masuda, S.; Kamada, H.; Satoh, S. *Biosci. Biotech. Biochem.* **2001**, *65*, 1883–1885.
- Masuda, S.; Sakuta, C.; Satoh, S. *Plant Cell Physiol.* **1999**, *40*, 1177–1181.
- Rep, M.; Dekker, H. L.; Vossen, J. H.; de Boer, A. D.; Houterman, P. M.; de Koster, C. G.; Cornelissen, B. J. C. *FEBS Lett.* **2003**, *534*, 82–86.
- Rep, M.; Dekker, H. L.; Vossen, J. H.; de Boer, A. D.; Houterman, P. M.; Speijer, D.; Back, J. W.; de Koster, C. G.; Cornelissen, B. J. C. *Plant Physiol.* **2002**, *130*, 904–917.
- Sakuta, C.; Oda, C.; Yamakawa, S.; Satoh, S. *Plant Cell Physiol.* **1998**, *39*, 1330–1336.
- Young, S. A.; Guo, A.; Guikema, J. A.; White, F. F.; Leach, J. E. *Plant Physiol.* **1995**, *107*, 1333–1341.
- Kehr, J.; Buhtz, A.; Giavalisco, P. *BMC Plant Biol.* **2005**, *5*, 11.
- Goodger, J. Q.; Sharp, R. E.; Marsh, E. L.; Schachtman, D. P. *J. Exp. Bot.* **2005**, *56*, 2389–2400.
- Spelbrink, R. G.; Dilmac, N.; Allen, A.; Smith, T. J.; Shah, D. M.; Hockerman, G. H. *Plant Physiol.* **2004**, *135*, 2055–2067.
- Biely, P.; Ahlgren, J. A.; Leathers, T. D.; Greene, R. V.; Cotta, M. A. *Cereal Chem.* **2003**, *80*, 144–147.
- Akoh, C. C.; Lee, G. C.; Liaw, Y. C.; Huang, T. H.; Shaw, J. F. *Prog. Lipid Res.* **2004**, *43*, 534–552.
- Sakuta, C.; Satoh, S. *Plant Cell Physiol.* **2000**, *41*, 627–638.
- Bendtsen, J. D.; Nielsen, H.; von Heijne, G.; Brunak, S. *J. Mol. Biol.* **2004**, *340*, 783–95.
- Bendtsen, J. D.; Jensen, L. J.; Blom, N.; von Heijne, G.; Brunak, S. *Protein Eng. Des. Sel.* **2004**, *17*, 349–356.
- Biely, P.; Markovic, O.; Mislovicova, D. *Anal. Biochem.* **1985**, *144*, 147–51.
- Lerouge, P.; Cabanes-Macheteau, M.; Rayon, C.; Fischette-Laine, A. C.; Gomord, V.; Faye, L. *Plant Mol. Biol.* **1998**, *38*, 31–48.
- Gibeau, D. M.; Carpita, N. C. *Plant Physiol.* **1991**, *97*, 551–561.
- Hadfield, K. A.; Bennett, A. B. *Plant Physiol.* **1998**, *117*, 337–343.
- Cote, F.; Hahn, M. G. *Plant Mol. Biol.* **1994**, *26*, 1379–1411.
- Stratilova, E.; Mislovicova, D.; Kacurakova, M.; Machova, E.; Kolarova, N.; Markovic, O.; Jornvall, H. *J. Proteom. Chem.* **1998**, *17*, 173–179.
- Hiraga, S.; Sasaki, K.; Ito, H.; Ohashi, Y.; Matsui, H. *Plant Cell Physiol.* **2001**, *42*, 462–468.
- Ito, H.; Kimizuka, F.; Ohbayashi, A.; Matsui, H.; Honma, M.; Shinmyo, A.; Ohashi, Y.; Caplan, A. B.; Rodriguez, R. L. *Plant Cell Rep.* **1994**, *13*, 361–366.
- Vogeli-Lange, R.; Frundt, C.; Hart, C. M.; Nagy, F.; Meins, F., Jr. *Plant Mol. Biol.* **1994**, *25*, 299–311.
- Ito, H.; Hiraga, S.; Tsugawa, H.; Matsui, H.; Honma, M.; Otsuki, Y.; Murakami, T.; Ohashi, Y. *Plant Sci.* **2000**, *155*, 85–100.
- de Lorenzo, G.; d'Ovidio, R.; Cervone, F. *Rev. Phytopath.* **2001**, *39*, 313–335.
- Fry, S. C. *Annu. Rev. Physiol. Plant Mol. Biol.* **1995**, *46*, 497–520.
- Inouhe, M.; Inada, G.; Thomas, B. R.; Nevins, D. J. *Int. J. Biol. Macromol.* **2000**, *27*, 151–156.
- Ross, G. S.; Wegrzyn, T.; MacRae, E. A.; Redgwell, R. J. *Plant Physiol.* **1994**, *106*, 521–528.
- Van den Ende, W.; De Coninck, B.; Van Laere, A. *Trends Plant Sci.* **2004**, *9*, 523–528.

- (37) Demura, T.; Tashiro, G.; Horiguchi, G.; Kishimoto, N.; Kubo, M.; Matsuoka, N.; Minami, A.; Nagata-Hiwatashi, M.; Nakamura, K.; Okamura, Y.; Sassa, N.; Suzuki, S.; Yazaki, J.; Kikuchi, S.; Fukuda, H. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 15794–15799.
- (38) Nari, J.; Noat, G.; Ricard, J. *Biochem. J.* **1991**, *279*, 343–350.
- (39) Cosgrove, D. J. *Plant Cell* **1997**, *9*, 1031–1041.
- (40) Boyce, C. K.; Zwieniecki, M. A.; Cody, G. D.; Jacobsen, C.; Wirick, S.; Knoll, A. H.; Holbrook, N. M. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 17555–17558.
- (41) Boerjan, W.; Ralph, J.; Baucher, M. *Annu. Rev. Plant Biol.* **2003**, *54*, 519–546.
- (42) Magwa, M. L.; Lindner, B.; Brand, J. M. *Phytochem.* **1993**, *32*, 251–253.
- (43) Roberts, K.; McCann, M. C. *Curr. Opin. Plant Biol.* **2000**, *3*, 517–522.
- (44) Turner, S. R.; Hall, M. *Plant J.* **2000**, *24*, 477–488.
- (45) Groover, A.; Jones, A. M. *Plant Physiol.* **1999**, *119*, 375–384.
- (46) Xu, F.-X.; Chye, M.-L. *Plant J.* **1999**, *17*, 321–327.
- (47) Beers, E. P.; Woffenden, B. J.; Zhao, C. *Plant Mol. Biol.* **2000**, *44*, 399–415.
- (48) Bröker, L. E.; Huisman, C.; Span, S. W.; Rodriguez, J. A.; Kruyt, F. A. E.; Giacome, G. *Cancer Res.* **2004**, *64*, 27–30.
- (49) Fukuda, H. *Plant Mol. Biol.* **2000**, *44*, 245–253.
- (50) Igawa, T.; Ochiai-Fakuda, T.; Takahashi-Ando, N.; Ohsato, S.; Shibata, T.; Yamaguchi, I.; Kimura, M. *Plant Cell Physiol.* **2004**, *45*, 1347–1360.
- (51) Guan, Y.; Nothnagel, E. A. *Plant Physiol.* **2004**, *135*, 1346–1366.
- (52) Malehorn, D. E.; Borgmeyer, J. R.; Smith, C. E.; Shah, D. M. *Plant Physiol.* **1994**, *106*, 1471–1481.
- (53) Lane, B. G. *IUBMB Life* **2002**, *53*, 67–75.
- (54) Caliskan, M. *Turk. J. Biol.* **2000**, *24*, 717–724.
- (55) Schweizer, P.; Christoffel, A.; Dudler, R. *Plant J.* **1999**, *20*, 541–552.

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