

Cell Wall Proteome in the Maize Primary Root Elongation Zone. I. Extraction and Identification of Water-Soluble and Lightly Ionically Bound Proteins¹

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Cell wall proteins (CWPs) play important roles in various processes, including cell elongation. However, relatively little is known about the composition of CWPs in growing regions. We are using a proteomics approach to gain a comprehensive understanding of the identity of CWPs in the maize (*Zea mays*) primary root elongation zone. As the first step, we examined the effectiveness of a vacuum infiltration-centrifugation technique for extracting water-soluble and loosely ionically bound (fraction 1) CWPs from the root elongation zone. The purity of the CWP extract was evaluated by comparing with total soluble proteins extracted from homogenized tissue. Several lines of evidence indicated that the vacuum infiltration-centrifugation technique effectively enriched for CWPs. Protein identification revealed that 84% of the CWPs were different from the total soluble proteins. About 40% of the fraction 1 CWPs had traditional signal peptides and 33% were predicted to be nonclassical secretory proteins, whereas only 3% and 11%, respectively, of the total soluble proteins were in these categories. Many of the CWPs have previously been shown to be involved in cell wall metabolism and cell elongation. In addition, maize has type II cell walls, and several of the CWPs identified in this study have not been identified in previous cell wall proteomics studies that have focused only on type I walls. These proteins include endo-1,3;1,4- β -D-glucanase and α -L-arabinofuranosidase, which act on the major polysaccharides only or mainly present in type II cell walls.

Plant cell wall proteins (CWPs) comprise less than 10% of cell wall dry weight (Fry, 1988), but play crucial roles in cell wall structure and architecture, cell wall metabolism, cell enlargement, signaling, responses to abiotic and biotic stresses, and many other physiological processes (Cassab and Varner, 1988; Carpita and Gibeaut, 1993; Keller, 1993; Showalter, 1993; McCann and Roberts, 1994; Cosgrove, 1999; Wu and Cosgrove, 2000; Somerville et al., 2004). However, our understanding of CWPs is still very limited. One fundamental objective to enhance our understanding of cell wall biology will be to identify the complete spectrum of proteins that are localized in the cell walls. An effort was made to estimate the number of proteins in cell walls after the Arabidopsis (*Arabidopsis thaliana*) genome was completely sequenced (Arabidopsis Genome Initiative, 2000). However, current knowledge

does not allow for accurate determination of CWPs based only on sequence information. Proteomics approaches are increasingly being used to directly identify proteins from cell walls (Robertson et al., 1997; Blee et al., 2001; Chivasa et al., 2002; Borderies et al., 2003; Watson et al., 2004; Boudart et al., 2005; Dani et al., 2005). In these studies, along with known CWPs, many new proteins, including hypothetical or putative proteins, were identified to be associated with cell walls or present in the extracellular matrix.

Previous studies with maize (*Zea mays*) primary roots have indicated that CWPs play an important role in controlling cell wall extensibility and thus cell expansion rate. Spatial analysis of the profile of elongation rate within the root elongation zone under well-watered conditions revealed that variation in cell wall extensibility is a key factor in determining the rate of elongation because turgor pressure is relatively constant along the root (Spollen and Sharp, 1991). Under water stress, elongation rates are maintained in the apical region of the elongation zone despite reduced turgor pressure (Sharp et al., 1988; Spollen and Sharp, 1991). These results suggested that water stress results in an increase in cell wall extensibility in the apical region, which was confirmed by direct measurement of acid-induced extension properties (Wu et al., 1996). Further studies demonstrated that activities of two CWPs, expansins and xyloglucan endotransglycosylase, were increased specifically in the apical few millimeters of water-stressed compared

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to well-watered roots (Wu et al., 1994, 1996), providing a biochemical basis for the increase in cell wall extensibility.

To gain a more comprehensive understanding of the involvement of CWP in controlling cell elongation in the maize primary root, we have initiated a proteomics project to identify CWPs from the root elongation zone. CWPs can be divided into three major categories based on their chemical and physical association with cell walls and available extraction methods: water-soluble and loosely ionically bound (fraction 1), tightly ionically bound (fraction 2), and covalently bound (fraction 3) CWPs. As the first step, we studied fraction 1 CWPs from the elongation zone of well-watered roots.

Fraction 1 CWP-related proteomics analysis has been performed on cultured cells of dicot species (Robertson et al., 1997; Borderies et al., 2003). The proteins were extracted by washing cultured cells with buffer or low concentrations of salt solutions. The use of cultured cells is advantageous for the convenience of protein extraction and the simplicity of protein composition from undifferentiated cells. However, root elongation depends on the coordinated regulation of growth processes in differentiated cell types within a complex of tissues. The method used for extracting fraction 1 CWPs from cultured cells is not suitable for complex tissues. A method involving vacuum infiltration with buffer containing a low concentration of salts followed by low-speed centrifugation was developed to collect CWPs from living tissues (Terry and Bonner, 1980). The vacuum infiltration-centrifugation technique has been used to isolate apoplastic fluid with little detectable cytosolic contamination (assessed from the activity of cytosolic marker enzymes) from tissues of leaves, stems, and roots (Morrow and Jones, 1986; MacAdam et al., 1992a, 1992b; Cordoba-Pedregosa et al., 1996; de Souza and MacAdam, 2001). However, previous studies of fraction 1 CWPs from tissues have mostly focused on a single protein or protein class, such as peroxidases. Two recent reports used the technique to examine the cell wall proteome of leaf tissues (Boudart et al., 2005; Dani et al., 2005), but to our knowledge no proteomics studies have focused on roots or specifically on growing regions.

In this study, we examined the effectiveness of the vacuum infiltration-centrifugation technique for extracting fraction 1 CWPs from the maize primary root elongation zone. CWPs were analyzed using a proteomics approach to identify the protein composition and also to use this information to evaluate the purity of the fraction 1 CWP population. Maize plants have type II cell walls due to a different polysaccharide composition from the walls of dicot and most monocot plants, which have type I cell walls (Fry, 1988; Carpita and Gibeau, 1993). To our knowledge, no proteomics work has been reported to address the protein composition in type II cell walls. Thus, this study also provides valuable information on the differences in CWP composition between cell wall types.

RESULTS

Optimization of the Vacuum Infiltration-Centrifugation Method for Extracting Fraction 1 CWPs from the Root Elongation Zone

To effectively extract fraction 1 CWPs from the root elongation zone, we first tested the effect of different concentrations of KCl, as an extractant, on CWP yield and cytosolic protein contamination. Because the yield of CWPs proved to be very low, the feasibility and efficiency of sequential extractions from the same tissue were examined. In these initial tests, cytosolic protein contamination in the extracts was assessed by assay of Glc-6-P dehydrogenase (G6PDH) activity. Infiltration with 0.2 M KCl yielded a similar amount of protein in each of three sequential extractions, without detectable cytosolic contamination (Table I). Decreasing the KCl concentration below 0.2 M greatly reduced the extraction efficiency (Fig. 1). Infiltration with 0.4 M KCl yielded a greater amount of protein, but cytosolic contamination was detected after the first infiltration (Table I). Further increase in KCl concentration to 1.0 or 1.5 M decreased protein yield. This may have resulted from shrinkage (due to dehydration) of the cell walls and thus a reduction in wall pore sizes that hindered protein release.

Accordingly, to obtain the maximum protein yield with minimum cytosolic protein contamination, 0.2 M KCl was chosen for extracting fraction 1 CWPs from the root elongation zone. Three sequential extractions of the same sample were combined for gel analysis and protein identification. A KCl concentration of 0.2 M was also found to be optimal for extracting apoplastic peroxidases from the elongation zone of tall fescue leaves (MacAdam et al., 1992a, 1992b).

Table I. Effect of KCl concentration and number of infiltrations on the yield of fraction 1 CWPs from the root elongation zone

Fraction 1 CWPs were extracted from elongation zone segments (approximately 100 segments per replicate) by vacuum infiltration with different concentrations of KCl followed by low-speed centrifugation. The extraction process was repeated three times with each sample. For each of the three extracts, four replicate samples were combined. G6PDH activity was assayed as an indicator of cytosolic contamination.

KCl	Infiltration	Protein Yield	G6PDH Activity
M		$\mu\text{g g}^{-1}\text{ FW}$	$\text{nmol min}^{-1}\text{ g}^{-1}\text{ FW}$
0.2	First	17.3	0.00
	Second	25.5	0.00
	Third	17.7	0.00
0.4	First	31.5	0.00
	Second	35.5	2.09
	Third	41.1	2.36
1.0	First	3.7	0.00
	Second	9.2	0.00
	Third	6.8	3.66
1.5	First	11.4	2.33
	Second	8.1	0.76
	Third	6.0	1.46

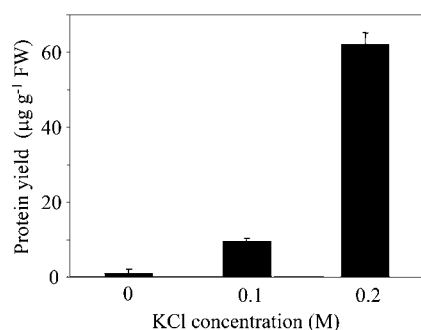


Figure 1. Effect of KCl concentration on the yield of fraction 1 CWPs from the root elongation zone. Fraction 1 CWPs were extracted from elongation zone segments (approximately 100 segments/replicate) by vacuum infiltration with different concentrations of KCl solution followed by low-speed centrifugation. Three sequential extractions of the same samples were combined. Data are means \pm SE of four replicates. No G6PDH activity, a marker of cytosolic contamination, was detected in any of the samples.

Two-Dimensional Gel Analysis and Protein Identification of Fraction 1 CWPs and Total Soluble Proteins

To analyze and identify fraction 1 CWPs, proteins were extracted from the root elongation zone using the infiltration-centrifugation method. Three replicate samples of 50- μ g CWPs (extracted from approximately 100 12-mm segments per sample) were collected in independent experiments and separated by two-dimensional electrophoresis (2-DE). One gel was stained with Coomassie Blue (Fig. 2A) and the others with SyproRuby (for increased staining sensitivity; data not shown). The total number of spots on the gels varied depending on the staining method, from 149 with Coomassie staining to 225 with SyproRuby staining. Of the 149 spots on the Coomassie-stained gel, 144 were present on both of the SyproRuby-stained gels. For comparison, three replicate samples of 50 μ g of total soluble proteins extracted from the elongation zone after tissue homogenization were separated by 2-DE and stained with Coomassie Blue. One hundred thirty-six spots were common to the three replicates; a representative 2-DE gel image is shown in Figure 2B.

The profiles of the CWP gels and the total soluble protein gels were very different. On the CWP gels, 111 of the 144 reproducible spots did not match those on the total soluble protein gels, suggesting that at least 77% of the CWPs could be different from the total soluble proteins. To further analyze the difference between the two protein profiles, the 53 most abundant reproducible spots on the Coomassie Blue-stained CWP gel (Fig. 2A) and 42 of the most abundant reproducible spots (randomly selected) on the total soluble protein gel (Fig. 2B) were excised, digested with trypsin, and analyzed by mass spectrometry (MS). Most of the digests yielded good-quality MS data. The results are shown for CWPs in Table II and for total soluble proteins in Table III; two examples of

mass spectra are shown in Figure 3. From the 53 and 42 spots used for MS analysis, 43 and 36 spots matched proteins in databases, representing 81% and 86% identification rates for CWPs and total soluble proteins, respectively. All protein identifications reported in Table II had Mascot scores greater than or equal to two times the accepted significance threshold (at the $P < 0.05$ level) calculated by Mascot. Mascot software incorporates a probability-based implementation of the Mowse algorithm (Pappin et al., 1996). For matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) peptide mass fingerprint data shown in Table III, an arbitrary peptide mass fingerprint data quality (PMFQ) score was used to quantify the quality of the peptide fingerprint. The PMFQ scores (1–5, with 5 being the best) were assigned to each peptide mass fingerprint based on the relative number of analyte peptides observed and their relative intensities (Watson et al., 2003). Protein identifications were based on multiple peptide matches. In addition, in some cases the same proteins were identified from multiple spots (e.g. spots 2 and 3, spots 10–13, spots 25 and 26, spots t2 and t3), indicating that posttranslational modifications may have occurred for those proteins or that they may have come from multigene families. It should be noted that some of the protein spots (e.g. spots 6 and 20 in Table II) showed a large discrepancy between the observed and the expected molecular weight (MW; in kD). There are several possibilities that may account for these discrepancies. First, there are no full-length cDNAs available for these proteins because of an incomplete genome sequence, and these protein spots may have high similarity to other proteins in the database; second, some of the sequences in the database are probably alternate splicing products or degradation products; and third, protein posttranslational modifications can sometimes alter MW greatly (Sun et al., 2005).

Based on the proteins identified, it is clear that the fraction 1 CWP and total soluble protein populations were very different. From the 43 proteins identified from the CWP gel, only seven had the same identification as proteins from the total soluble protein gel (italicized in Tables II and III). Moreover, all of the common protein identifications between the two gels had different accession numbers and were from unmatched spots, indicating they may be different members of the same gene families. The CWP gel (Table II) contained a large number of proteins related to cell wall metabolism and structural modification, including one β -xylosidase, one β -D-glucan exohydrolase, one α -L-arabinofuranosidase, five β -glucosidases, two β -galactosidases, one α -galactosidase, one endoxyloglucan transferase, one chitinase, one endo-1,3;1,4- β -D-glucanase, and four peroxidases (Fry, 1988). None of these proteins were found in the identified proteins from the total soluble protein gel (Table III).

The difference between the two profiles was also obvious when the number of proteins carrying predicted signal peptides that lead to protein targeting

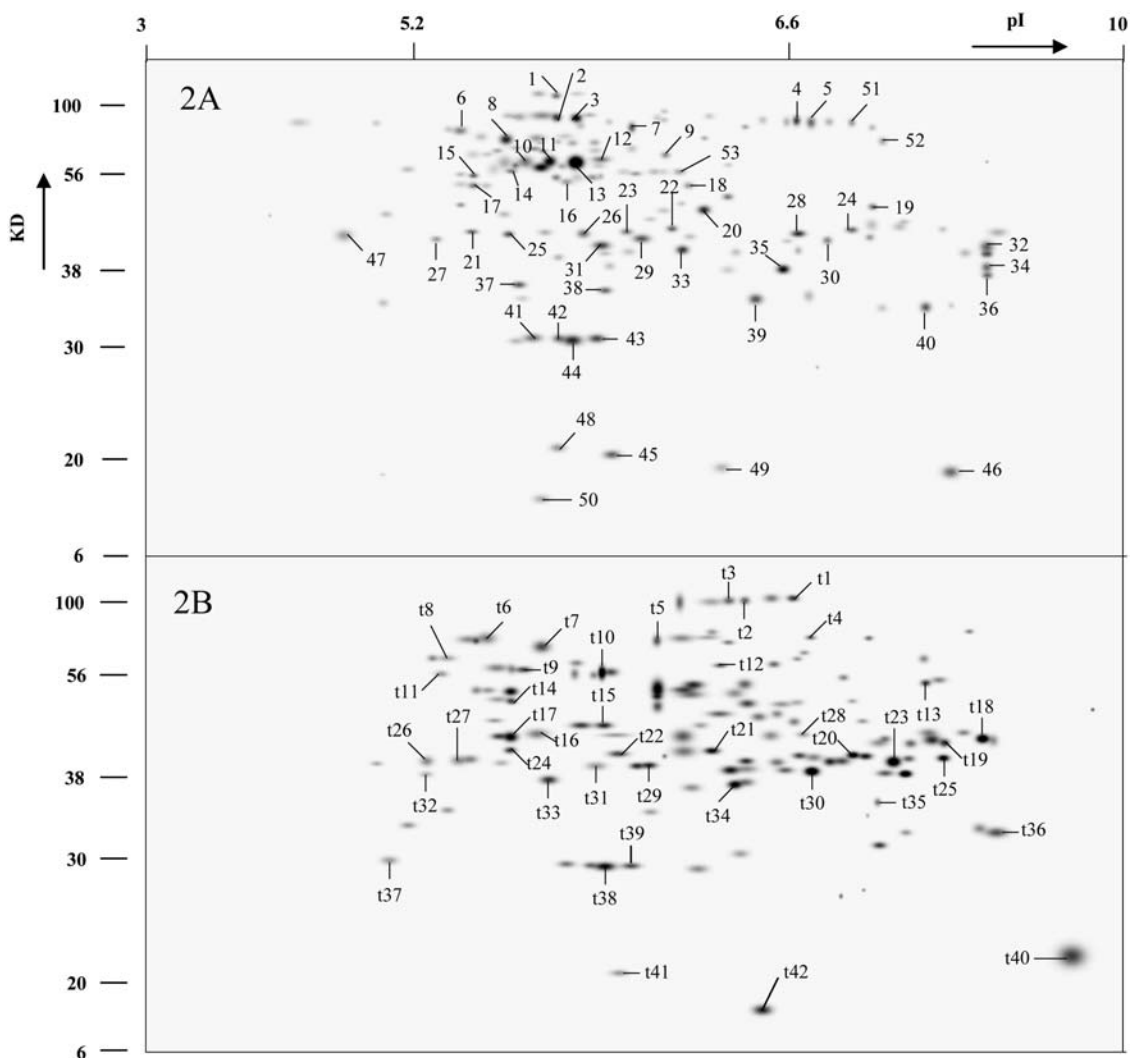


Figure 2. Images of 2-DE gels for fraction 1 CWP (A) and total soluble proteins (B). Fifty micrograms of fraction 1 CWPs or total soluble proteins from the root elongation zone were separated on each gel. Gels were stained with Coomassie Blue and processed by PDQuest 7.2 (Bio-Rad). The 53 most abundant spots in A and 42 of the most abundant spots (randomly selected) in B were labeled and picked for MS analysis.

into the secretory pathway was analyzed (Nielsen et al., 1997). Seventeen of 43 protein identifications from fraction 1 CWPs had signal peptides (Table II), whereas only one protein from the total soluble protein gel had a signal peptide (Table III). Increasing evidence indicates that many proteins lacking signal peptides can be secreted into the extracellular matrix through an unknown mechanism. A program was recently developed to predict these so-called nonclassical secretory proteins for the mammalian system (Bendtsen et al., 2004). We explored the use of this program to analyze plant proteins. Fourteen of 26 proteins from the CWP gel that did not contain signal peptides were predicted to be nonclassical secretory proteins (Table II), whereas only four of 35 proteins from the total soluble protein gel were identified as nonclassical secretory proteins (Table III), further illustrating that these two protein populations were different.

To identify CWP of lower abundance, 150 μg of fraction 1 CWPs (combined from three independent experiments) were extracted from the root elongation zone and analyzed by 2-DE (stained with Coomassie Blue; Fig. 4). Image comparison between the 50- and the 150- μg CWP gels showed that all 53 of the spots on the 50- μg gel that were selected for protein identification matched the spots on the 150- μg gel. Because of the high reproducibility of the CWP gel pattern and the large amount of work involved in collecting CWPs, the 150- μg CWP gel was only performed once. Thirty-five protein spots with reasonable staining intensity that were not identified from the 50- μg CWP gel were excised for MS analysis, resulting in 31 protein identifications with high confidence (Table IV). All of these spots were also present on both of the 50- μg SyproRuby-stained CWP gels (note that because of the lower sensitivity of protein staining with Coomassie Blue, not all

Table II. Identities of protein spots from the 2-DE gel of 50- μ g fraction 1 CWPs

Spots are listed corresponding to the numbers on the gel image in Figure 2A, which were identified by electrospray ionization-MS/MS analysis. SP refers to the presence of a signal peptide sequence predicted by SignalP (version 3) with a P-value threshold > 0.900. NSP indicates nonclassical secretory proteins predicted by SecretomeP server 1.0 with an NN score > 0.600. Identifications and accession numbers (nucleotide sequence GI no.) are from the National Center for Biotechnology Information (NCBI) database. The score, number of matched peptides, and percentage of coverage are taken directly from the Mascot Daemon report. Theoretical MW and pI were either directly taken from the Mascot Daemon report or estimated based on the top protein sequence obtained by BLASTX. The proteins marked with an asterisk have not been reported in other cell wall proteomics work. Proteins that were also identified on the total soluble protein gel (Table III) are italicized. Organisms matched: Rice, *O. sativa*; Maize, *Z. mays*; Wheat, *Triticum aestivum*; Sorghum, *Sorghum bicolor*; Arabidopsis, *A. thaliana*.

Spot No.	Identification	Experimental		Theoretical		Accession No.	Mascot Score	No. of Peptides	Coverage %	SP	NSP	Organism Matched
		MW	pI	MW	pI							
1	Putative β -xylosidase	103	5.7	88	5.2	34894431	106	3	3	x		Rice
2	<i>Met synthase</i>	94	5.8	84	6.1	21213131	158	7	8		x	Maize
3	<i>Met synthase</i>	93	5.9	84	6.1	21213131	310	7	11		x	Maize
4	Putative Ser protease	93	7.5	74	6.4	22715488	66	2	30	x		Maize
5	β -D-Glucan exohydrolase	91	7.6	68	6.4	21206814	134	4	3	x		Maize
6	DnaK-type molecular chaperone*	85	5.1	22	9.5	4966786	61	4	12			Maize
8	α -L-Arabinofuranosidase*	68	5.4	82	5.7	32986991	114	3	2	x		Rice
10	Chain B of β -glucosidase	70	5.6	64	6.5	21212891	148	6	7		x	Maize
11	Chain B of β -glucosidase	71	5.8	64	6.5	21212891	434	15	16		x	Maize
12	Chain B of β -glucosidase	70	6.1	64	6.5	21212891	120	5	4		x	Maize
13	Chain B of β -glucosidase	69	5.9	64	6.5	21212891	221	11	8		x	Maize
14	Putative Leu aminopeptidase	65	5.4	62	8.1	21206625	366	13	16	x		Maize
17	Enolase 1 (2-phosphoglycerate dehydratase 1)	58	5.3	48	5.1	21207053	129	9	12			Maize
19	radc1: aspartyl protease family	52	8.1	45	6.9	32972250	109	4	4	x		Rice
20	Putative β -galactosidase	50	6.8	93	7.8	32977497	130	6	4	x		Rice
21	<i>Gln synthetase</i> *	45	5.2	39	5.3	286123	95	3	5			Maize
22	Reversibly glycosylated polypeptide*	45	6.5	42	6.0	4158231	85	3	5			Wheat
24	Aldolase	45	7.9	39	7.6	168419	145	6	10		x	Maize
25	Reversibly glycosylated polypeptide*	44	5.4	56	6.1	21215827	215	8	11			Maize
26	Reversibly glycosylated polypeptide*	44	5.9	56	6.1	21215827	214	9	16			Maize
27	<i>Adenosine kinase</i> *	41	4.8	58	6.1	21206958	100	5	9		x	Maize
28	Aldolase	45	7.5	39	7.6	168419	214	7	19		x	Maize
29	Putative α -galactosidase	42	6.4	46	7.9	21209074	134	4	9	x		Rice
30	<i>GAPC3</i>	42	7.7	37	6.9	34517179	71	2	7			Sorghum
31	Malate dehydrogenase	41	6.1	36	5.9	2286152	434	15	27			Maize
32	Cationic peroxidase isozyme 40K precursor	42	8.8	34	7.8	21212409	330	15	16	x		Maize
33	Putative malate dehydrogenase	40	6.7	34	8.6	21206939	228	7	12			Maize
34	Putative peroxidase P7X	38	8.8	34	6.7	15011985	85	4	7	x		Maize
35	Endoxyloglucan transferase	37	7.3	34	6.8	21207285	275	10	21	x		Maize
36	Peroxidase	36	8.8	33	8.7	34443238	63	2	12	x		Sorghum
37	Putative β -galactosidase	34	5.5	93	7.8	32977497	89	4	2	x		Rice
38	<i>Glyoxalase I</i>	33	6.1	32	5.6	21209394	135	4	10			Maize
39	Endo-1,3;1,4- β -D-glucanase*	33	7.2	33	7.1	21207797	175	7	19	x		Maize
40	Chitinase	32	8.4	32	6.8	37395885	90	3	12	x		Maize
41	Putative ascorbate peroxidase	29	5.5	27	5.4	21621062	60	3	7			Maize
43	L-Ascorbate peroxidase	29	6.0	27	5.3	11064967	64	4	16			Sorghum
44	Triosephosphate isomerase*	29	5.8	27	5.5	34508973	299	9	32		x	Sorghum
45	Superoxide dismutase [Cu-Zn]	29	6.0	15	5.6	134598	89	4	23		x	Maize
46	Osmotin	23	8.7	18	7.9	21207583	171	8	29	x		Maize
47	Peroxidase	43	3.8	36	4.6	21214399	120	3	7	x		Maize
48	Superoxide dismutase [Cu-Zn]	24	5.6	15	5.6	4753356	83	4	18		x	Maize
49	<i>Nucleoside diphosphate kinase I</i>	22	6.9	17	6.6	16918100	87	2	7		x	Maize
50	Putative receptor Ser/Thr kinase	19	5.5	15	6.5	12083304	141	7	32		x	Arabidopsis

of these spots are present on the 50- μ g CWP gel in Fig. 2A). Among the 31 additional proteins identified, five had signal peptides and 12 were predicted to be non-classical secretory proteins (Table IV). However, some cytosolic proteins were found on the 150- μ g gel, in-

cluding nuclear transport factor 2, mitochondrial chaperonin-60, actin depolymerization factor, and cytosolic glyceraldehyde-3-P dehydrogenase (GAPC3).

To examine the reproducibility of protein identification, we selected 17 protein spots (seven spots from

Table III. Identities of protein spots from the 2-DE gel of 50- μ g total soluble proteins

Spots are listed corresponding to the numbers on the gel image in Figure 2B, which were identified by MALDI-TOF MS analysis. PMFQ is an arbitrary score of 1 to 5, 5 being the best. N, NCBI nr; S, SwissPro; E, dbEST others; U, Unigenes of maize, rice, wheat, sorghum, and potato (*Solanum tuberosum*). N and S contain protein sequences, while E and U are nucleotide sequences. The E or U matches were BLASTX searched to obtain protein information. The listed protein information (theoretical MW and pI) is from the top BLASTX match. SP refers to the presence of a signal peptide sequence predicted by SignalP (version 3) with a Signal P threshold value of 0.900. NSP indicates nonclassical secretory proteins predicted by SecretomeP server (version 1) with an NN score greater than 0.600. Average Dppm \pm SD is the average of the differences (in ppm) between the submitted and matched masses and its SD. The number of matched peptides and percent coverage were taken directly from Protein-Prospector searching. Proteins that were also identified on the 50- μ g CWP gel (Table II) are italicized.

Spot No.	Identification	Experimental		Theoretical		Accession No.	Database	PMFQ	Peptide Coverage	Average Dppm \pm SD	Coverage %	SP	NSP	Organism Matched
		MW	pI	MW	pI									
t1	Lipoxygenase	106	7.1	98	6.7	8515850	U, E	3	17/60	5 \pm 7	23			Maize
t2	Elongation factor	105	7.1	94	6.2	21206885	U, E	4	25/49	4 \pm 5	37			Maize
t3	Elongation factor	104	7.0	94	6.2	21206885	U	4	22/60	5 \pm 6	35			Maize
t4	Lipoxygenase	81	7.5	98	6.7	8515850	U, E	4	22/32	5 \pm 7	28			Maize
t5	Aconitate hydratase	79	6.5	108	7.2	21207848	U	5	24/42	7 \pm 9	32			Maize
t6	Heat shock protein 82	79	5.3	80	5.0	P33126	S, N, U, E	4	20/50	7 \pm 9	26			Rice
t7	<i>Met synthase</i>	75	5.7	84	5.7	17017263	N, S, U, E	4	11/29	3 \pm 4	21	x		Maize
t8	Heat shock protein 70	68	5.0	70	5.6	21208061	U	5	11/60	4 \pm 6	17			Maize
t9	Vacuolar ATPase	64	5.6	68	5.4	21207832	U, N, S, E	5	24/42	5 \pm 7	29			Maize
t10	26S proteasome subunit4	62	6.1	50	5.9	3489202	E	4	12/60	7 \pm 10	27			Rice
t11	Protein disulfide isomerase	61	5.0	57	5.2	P52588	S, N, U, E	2	7/7	6 \pm 8	15	x		Maize
t12	Suc synthase	65	6.9	93	6.0	459895	N, S, U	4	20/60	9 \pm 8	36			Maize
t13	Hydroxymethyl transferase	59	8.3	51	7.5	21208416	U	3	10/24	3 \pm 4	23			Maize
t14	Chaperonin 60 mitochondrial precursor	51	5.5	61	5.7	Q43298	S, U	3	10/50	5 \pm 7	25			Maize
t15	α -Tubulin	45	6.1	49	4.9	135398	N, S, U	3	10/26	8 \pm 9	30			Maize
t16	Eukaryotic initiation factor 4A	43	5.7	46	5.6	21212601	U, N, S	4	10/32	11 \pm 11	27	x		Maize
t17	Actin	42	5.5	41	5.3	P30171	S, N, U	3	7/20	2 \pm 3	23			Potato
t18	Fru-bis-P aldolase	43	8.7	38	7.5	P08440	S, U, E	3	6/11	3 \pm 5	23			Maize
t19	UDP glucuronic acid decarboxylase	42	8.4	39	7.2	18447934	N, E	3	7/18	5 \pm 8	14			Rice
t21	α -1,4-Glucan protein synthase	40	6.9	41	5.8	P80607	S, N, U	2	4/9	5 \pm 8	16			Maize
t22	S-adenosyl-Met synthetase	39	6.2	43	5.3	P46611	S, N, U, E	3	14/24	9 \pm 9	34			Rice
t23	<i>GAPC3</i>	39	8.1	36	7.0	Q43247	S, U	3	5/11	4 \pm 6	19			Maize
t24	<i>Gln synthetase</i>	39	5.5	38	5.7	P38562	S, U	2	6/14	5 \pm 6	19			Maize
t26	Late-embryogenesis-abundant protein	38	4.9	35	5.1	21207903	U	2	11/22	5 \pm 6	28			Maize
t27	Adenosine kinase	38	5.2	36	5.2	4582787	N, U	3	12/38	8 \pm 9	52			Maize
t28	<i>GAPC3</i>	44	7.5	36	7.0	Q43247	S, U	3	5/15	2 \pm 4	19			Maize
t29	Fructokinase 1	38	6.4	34	4.9	31652274	N	3	8/30	4 \pm 5	35	x		Maize
t31	S-adenosyl-Met synthetase	37	6.1	43	5.3	21212903	U, N, S, E	3	7/23	3 \pm 4	15			Maize
t32	Fructokinase 1	36	4.9	34	4.9	31652274	N	4	8/21	4 \pm 6	35	x		Maize
t33	Fructokinase	35	5.8	35	5.6	21217113	U, E	2	11/24	4 \pm 5	32			Maize
t35	Putative YLP	33	8.0	26	7.4	21207460	U, E	4	19/60	3 \pm 5	40			Maize
t36	<i>Glyoxalase I</i>	32	8.8	32	5.7	21209394	U	4	12/50	6 \pm 7	41			Maize
t37	GF14-c protein	30	4.7	29	4.8	34515303	U, E	2	5/9	5 \pm 5	28			Sorghum
t40	Cyclophilin	26	9.3	18	8.9	P21569	S, N, U, E	3	5/15	1 \pm 2	23			Maize
t41	Gly-rich RNA-binding ABA-inducible protein	24	6.2	15	5.6	P10979	S	5	6/34	9 \pm 7	36			Maize
t42	<i>Nucleoside diphosphate kinase</i>	18	7.2	16	6.8	30970700	U, E	3	4/16	1 \pm 1	24			Sorghum

Table II and 10 spots from Table IV) that confidently matched spots on two gels of fraction 1 CWPs collected in independent experiments from specific regions within the root elongation zone (3–7 and 7–12 mm from the root apex). The region-specific gels (stained with SyproRuby) were created as part of an ongoing study of the spatial distribution of fraction 1 CWP composition within the elongation zone of well-watered and water-stressed roots (Sharp et al., 2004).

The selected protein spots were excised from each region-specific gel and analyzed by MS, resulting in a total of 32 identifications (Table V). Of the 17 original protein identifications (Tables II and IV), 14 were reproduced from both region-specific gels. The identities of two proteins, spots 66 and 80, were reproduced from one gel, but no identification was obtained from the other gel. If these two proteins are also considered as reproducibly identified, the success

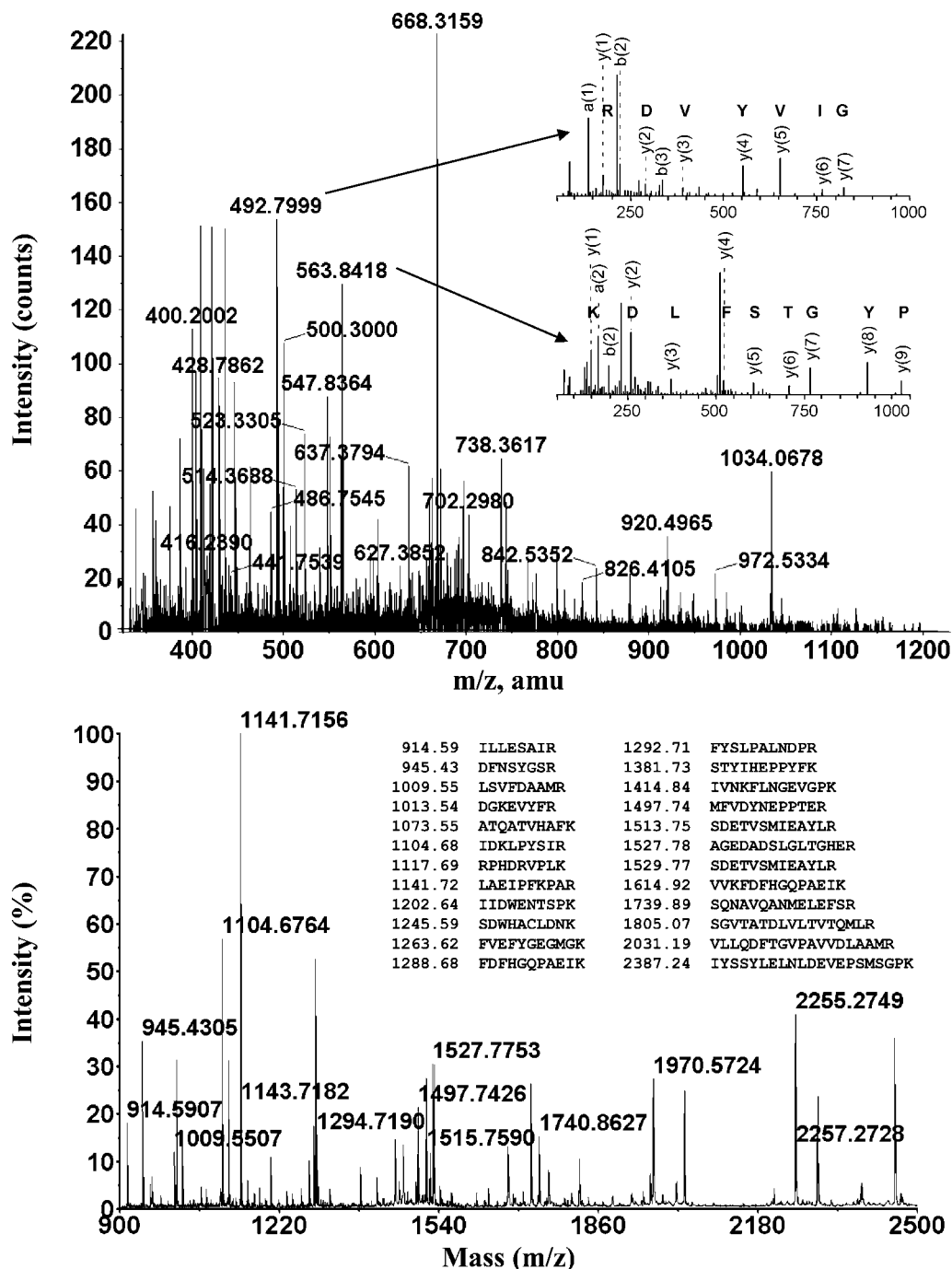


Figure 3. Mass spectra of two selected protein samples. Top, NanoESI-MS/MS precursor ion spectrum shows all the peptide ions at different charge states present in the tryptic digest. Two representative product ion spectra of m/z 492.7999 and m/z 563.8418 and their derived sequences are shown as insets. Database searching using the MS/MS data unambiguously identified the spot as chain B of β -glucosidase with a score of 434 and a sequence coverage of 16%. Bottom, MALDI-TOF MS of tryptic peptides eluted from spot t5 in Figure 2B. After baseline correction, noise removal, and peak deisotoping, 42 ions were submitted to Protein-Prospector. Twenty-four of the submitted ions were matched to theoretical tryptic peptides from aconitate hydratase (inset).

rate of protein reidentification was 94%. One protein spot, 54, resulted in a different protein identification from each of the three gels. Several factors may have contributed to the discrepancy in protein identification for spot 54. It should be noted that we used two dif-

ferent methods to introduce samples into the mass spectrometer for the protein identifications in Tables II and IV and the reidentifications in Table V. It is not uncommon for different protein identification results to be obtained from the same protein spot by direct

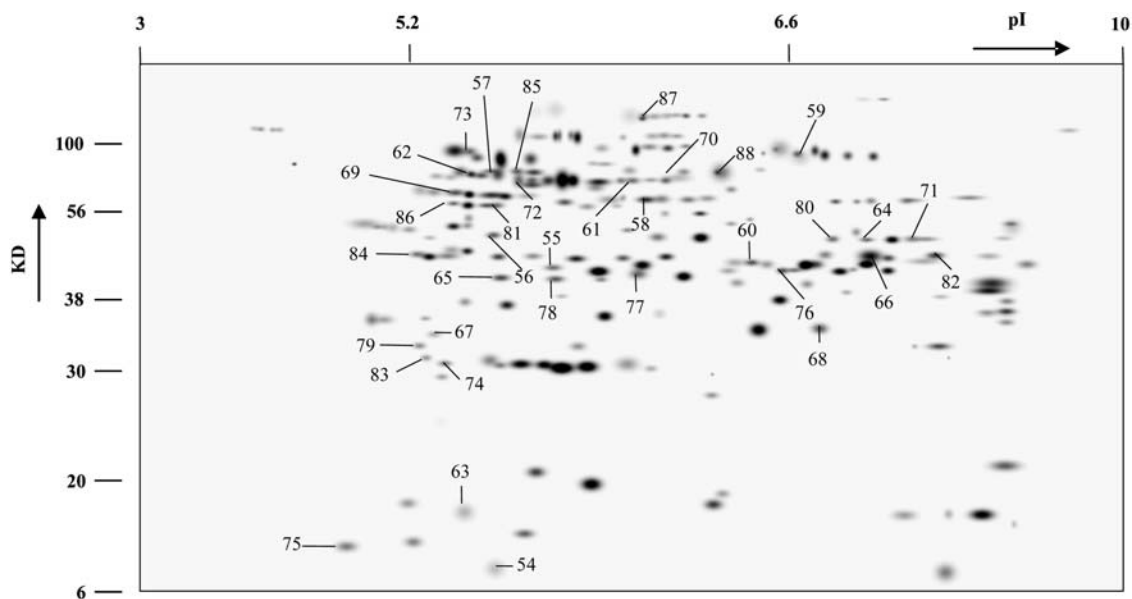


Figure 4. Image of 2-DE gel loaded with 150 μg of fraction 1 CWP from the root elongation zone. The gel was stained with Coomassie Blue and processed by PDQuest 7.2 (Bio-Rad). Thirty-five spots with reasonable intensity that were not identified on the 50- μg CWP gel (Fig. 2A) were labeled and picked for MS analysis.

infusion as compared to HPLC separation of peptides (Chen, 2005). This may reflect differential peptide ionization and selectivity of the different methods. In addition, a single protein spot on a 2-DE gel can contain more than one protein species, which may result in different protein identifications (Chen, 2005). There is also a slight possibility that the same protein spot on the different gels actually comprised different proteins that migrated to similar locations.

Functional Classification of Identified Fraction 1 CWPs and Total Soluble Proteins

To better understand the biological processes encompassed by the proteins identified using the 2-DE proteomics approach, fraction 1 CWPs and total soluble proteins were classified in functional categories (Fig. 5). It should be noted that these classifications are provisional because the biological role of many of the proteins identified has not been established experimentally (Tatusov et al., 1997). The largest proportion of the CWPs, 38%, was categorized in carbohydrate metabolism. In comparison, 24% of the total soluble proteins were in this category. For the CWPs, the second largest group (21%) was related to defense mechanisms, whereas only 2.8% of the total soluble proteins were in this category. A small number of the CWPs (7.0%) were classified as functionally unknown. The categories of total soluble proteins included translation, ribosomal structure and biogenesis, cytoskeleton, coenzyme transport and metabolism, lipid transport and metabolism, and RNA processing and modification. Proteins in these categories were not present in fraction 1 CWPs.

DISCUSSION

The Vacuum Infiltration-Centrifugation Method Enriches Fraction 1 CWPs from the Root Elongation Zone

One of the great challenges when working with the subproteome in cell walls is to isolate proteins free of contamination from membrane and cytosolic proteins. In this study, the vacuum infiltration-centrifugation technique was optimized to isolate the fraction 1 CWPs from the maize primary root elongation zone. Several lines of evidence indicate that this method can effectively enrich for CWPs. First, the infiltrate showed no detectable enzymatic activity of G6PDH, a cytosolic protein marker. Second, analyses of the gel images (Fig. 2) and protein identities (Tables II and III) showed that the protein profile of the CWP extract was clearly different from that of the total soluble protein extract. Only about 16% of the proteins identified from the 50- μg fraction 1 CWP gel overlapped with those from the total soluble protein gel. In addition, the differences in protein functional categories (Fig. 5), such as lacking translation and cytoskeleton groups in fraction 1 CWPs, further indicated the effectiveness of the extraction method. Third, about 40% of the proteins identified from fraction 1 CWPs have an N-terminal signal peptide, whereas only 3% of the proteins identified from the total soluble protein extract have signal peptides. Fourth, the majority of signal peptide-carrying proteins from the 50- μg fraction 1 CWP gel are related to cell wall structure, metabolism, and modification, including β -xylosidase, β -D-glucan exo-hydrolase, α -L-arabinofuranosidase, β -galactosidase, α -galactosidase, endoxyloglucan transferase, chitinase, endo-1,3;1,4- β -D-glucanase, and peroxidases (see Fry, 1988). Many of these proteins have also been isolated

Table IV. Identities of protein spots from the 2-DE gel of 150- μ g fraction 1 CWP

Spots are listed corresponding to the labels on the gel image in Figure 4, which were identified by electrospray ionization-MS/MS analysis. SP refers to the presence of a signal peptide sequence predicted by SignalP (version 3) with a Signal P threshold value > 0.900. NSP indicates nonclassical secretory proteins predicted by SecretomeP server (version 1) with an NN score > 0.600. The score, number of matched peptides, and percentage of coverage were taken directly from the Mascot Daemon report. Theoretical MW and pI were either directly taken from the Mascot Daemon report or estimated based on the top protein sequence obtained by BLASTX. Accession numbers were for either nucleotide sequences or proteins in GenBank.

Spot No.	Identification	Experimental		Theoretical		Accession No.	Mascot Score	No. of Peptides	Coverage	SP	NSP	Organism Matched
		MW	pI	MW	pI							
54	Nuclear transport factor 2	12	5.5	20	9.7	25195103	60	3	13		x	Wheat
55	Cytoplasmic malate dehydrogenase	43	5.8	36	5.8	2286153	136	9	19		x	Maize
56	Enolase	52	5.4	48	5.2	22273	146	5	13		x	Maize
57	β -Glucosidase	80	5.4	65	6.2	435313	42	5	8			Maize
58	UDP-Glc dehydrogenase	67	6.4	54	5.7	40317278	415	27	36		x	<i>Cinnamomum osmophloeum</i>
59	Exhydrolase	91	6.5	69	6.1	4731111	111	4	5	x		Maize
60	GAPC3	44	7.1	27	6.3	82694	82	6	23			Maize
61	β -Glucosidase	76	6.3	59	5.5	12084533	207	17	22			Maize
62	Mitochondrial chaperonin-60	80	5.2	61	5.7	22248	118	7	12			Maize
63	Actin depolymerizing factor	20	5.2	16	5.5	1419370	78	4	27		x	Maize
64	radc1	52	7.9	45	6.6	49532749	167	8	16	x		Rice
65	Fructokinase 2	40	5.3	36	5.3	31652276	358	16	46			Maize
66	Fru-bis-P aldolase	45	7.9	39	7.5	68196	252	11	31			Maize
67	Fructokinase 2	31	5.0	36	5.3	31652276	74	6	15			Maize
69	UDP-Glc pyrophosphorylase	69	5.1	25	5.8	33100756	100	6	17			Maize
70	Phe ammonia lyase	76	6.6	75	6.5	17467274	81	7	8			Maize
71	Nucleoid DNA-binding-like protein	52	8.2	47	9.2	50939761	218	8	14	x		Rice
72	β -Glucosidase	75	5.5	65	6.2	435313	109	8	10			Maize
73	OSJNBb0003B01.22	94	5.2	43	6.1	58531989	55	2	5	x		Rice
74	Triose-P isomerase	29	5.0	27	5.5	68426	159	7	24		x	Maize
75	Profilin 5	15	4.5	14	4.6	11493677	102	6	58		x	Maize
76	Cytosolic GAPC3	42	7.4	37	7.0	1184774	277	17	40			Maize
77	Cytoplasmic malate dehydrogenase	41	6.4	36	5.8	2286153	264	13	27		x	Maize
78	Malate dehydrogenase	40	5.8	36	7.6	19880701	133	4	11			Rice
79	6-Phosphogluconolactonase-like protein	30	4.9	35	7.7	50725145	126	5	15		x	Rice
80	radc1	51	7.4	45	6.6	49532749	164	7	16	x		Rice
81	Enolase	64	5.4	48	5.2	22273	104	7	23		x	Maize
82	Fru-bis-P aldolase	57	8.4	39	7.5	2213867	327	15	29			<i>Mesembryanthemum crystallinum</i>
84	Adenosine kinase	45	4.8	37	5.2	4582787	148	9	28		x	Maize
85	β -Glucosidase	82	5.5	65	6.2	435313	97	8	12			Maize
86	Enolase	64	5.1	48	5.2	22273	262	10	22		x	Maize

from cell walls using other approaches (Huber and Nevins, 1981; MacAdam et al., 1992a, 1992b; Nishitani and Tominaga, 1992; Kim et al., 2000), supporting their identification as true CWPs. None of these proteins appeared in the total soluble protein list.

In contrast to the major differences between the CWP and total soluble protein gels, highly similar profiles were observed on replicate CWP gels. Since the replicate samples were extracted from independent experiments, the results indicate a high reproducibility of the seedling culture system, the extraction protocol, and 2-DE. The reproducibility of CWP iden-

tification was also rigorously examined in this study. Seventeen protein spots were identified individually from three independent samples using MS analysis, and 94% of the proteins were reproducibly identified.

Cytosolic protein contamination was not obvious from the identities of the proteins from the 50- μ g CWP gel (Table II). However, some cytosolic contamination was evident from the additional 31 spots identified from the 150- μ g CWP gel (Table IV). Several proteins were annotated as cytosolic proteins, such as cytoplasmic malate dehydrogenase, nuclear transport factor 2, and mitochondrial chaperonin. It is possible that

Table V. Replicated identification of protein spots from 2-DE gels of fraction 1 CWP

Seventeen protein spots, corresponding to the labels on the gel images in Figures 2A and 4 and protein identifications in Tables II and IV (listed here as sample 1), were reidentified from two gels of fraction 1 CWP collected from specific regions in the elongation zone (3–7 and 7–12 mm from the root apex; listed as samples 2 and 3, respectively). The spots were excised and identified by liquid chromatography-MS/MS analysis.

Spot No.	Sample 1			Sample 2			Sample 3								
	Identification	Experiment		Identification	Experiment		Identification	Experiment		Mascot Score	No. of Peptides	Coverage			
		MW	pI		MW	pI		MW	pI						
1	Putative β -xylosidase	103	5.7	Putative β -xylosidase	99	5.6	142	3	3	Putative β -xylosidase	98	5.7	115	2	2
8	α -L-Arabinofuranosidase	68	5.4	α -L-Arabinofuranosidase	68	5.3	180	4	3	α -L-Arabinofuranosidase	69	5.4	195	4	3
27	Adenosine kinase	41	4.8	Adenosine kinase	38	5.0	189	5	14	Adenosine kinase	40	4.8	329	5	11
29	Putative α -galactosidase	42	6.4	Putative α -galactosidase	38	6.0	173	5	12	Putative α -galactosidase	39	6.1	160	4	7
39	Endo-1,3;1,4- β -D-glucanase	33	7.2	Endo-1,3;1,4- β -D-glucanase	29	6.7	289	4	13	Endo-1,3;1,4- β -D-glucanase	30	6.7	160	4	12
44	Triose-P isomerase	29	5.8	Triose-P isomerase	25	5.5	220	3	16	Triose-P isomerase	26	5.7	94	3	16
50	Putative receptor Ser/Thr kinase	19	5.5	Putative receptor Ser/Thr kinase	13	5.5	163	2	13	Putative receptor Ser/Thr kinase	13	5.5	118	3	16
54	Nuclear transport factor 2	12	5.5	Putative receptor Ser/Thr kinase	11	5.4	102	3	17	Glutaredoxin	11	5.3	63	2	7
59	Exhydrolase	91	7.4	Exhydrolase	72	6.8	330	6	5	Exhydrolase	74	6.8	263	5	4
64	radc1	52	7.9	radc1	42	7.5	134	4	4	radc1	45	7.6	165	4	12
66	Fru-bis-P aldolase	45	7.4	No ID	40	7.8	–	–	–	Fru-bis-P aldolase	41	7.6	152	3	8
70	Phe ammonia lyase	76	6.6	Phe ammonia lyase	75	6.2	361	8	9	Phe ammonia lyase	79	6.2	185	4	5
72	β -Glucosidase	75	5.5	β -D-Glucosidase	58	5.4	368	7	13	β -D-Glucosidase	59	5.5	368	8	14
77	Malate dehydrogenase	41	6.4	Malate dehydrogenase	37	6.0	317	8	17	Malate dehydrogenase	38	6.1	201	5	11
79	6-Phosphogluconolactonase	30	4.9	6-Phosphogluconolactonase	27	4.8	124	2	5	6-Phosphogluconolactonase	28	4.9	258	4	9
80	radc1	51	7.4	No ID	44	7.4	–	–	–	radc1	45	7.1	189	3	5
84	Adenosine kinase	45	4.8	Adenosine kinase	39	4.8	177	5	11	Adenosine kinase	40	4.7	227	5	13

cytosolic protein contamination occurred on the 50- μ g gel also, but in very low quantities that were not detected. However, when the gel was loaded with a larger amount of protein, the contamination became sufficient to be detected. It is also possible that the apparent cytosolic proteins could in fact be true CWPs for reasons discussed below.

Fraction 1 CWPs Include Proteins without a Traditional Signal Peptide Sequence

A high percentage of the identified CWPs from the 50- μ g gel (26/43; 60%), and an even higher percentage of the additional proteins identified from the 150- μ g gel (26/31; 84%), did not appear to contain an N-terminal signal peptide. This seems to be common based on other cell wall proteomics studies (Chivasa et al., 2002; Borderies et al., 2003; Canovas et al., 2004; Slabas et al., 2004; Watson et al., 2004). Increasing evidence suggests that proteins can be secreted into cell walls without a classic N-terminal signal peptide. For example, malate dehydrogenase, which is proposed to be involved in H₂O₂ production in cell walls (Gross, 1977; Fry, 1988), and β -glucosidase, both of which were identified in this study, were found in apoplastic fluid in barley (*Hordeum vulgare*) and oat (*Avena sativa*) primary leaves (Li et al., 1989). Enolase, which was also identified in this study, was detected in the cell walls of *Candida albicans*, *Arabidopsis*, and alfalfa (*Medicago sativa*) (Chivasa et al., 2002; Pitarch et al., 2002; Watson et al., 2004). Using immunolocal-

ization, enolase was shown to be secreted to the cell wall or extracellular space even though it lacked a signal peptide (Edwards et al., 1999). Glyoxalase 1 was identified in our study and was also present in a cell wall proteomics study of mature stems of alfalfa (Watson et al., 2004).

In mammalian systems, it has also been observed that many proteins that do not contain signal peptides can be secreted into the extracellular matrix. Bendtsen et al. (2004) have classified this group of proteins as nonclassical or leadless secretory proteins and have developed a program (SecretomeP 1.0b; <http://www.cbs.dtu.dk/services/SecretomeP-1.0>) based on a group of known extracellular localized proteins to predict this type of protein. Using this software with the proteins identified from the CWP gels that did not contain signal peptides revealed that 54% (14/26) from the 50- μ g gel and 46% (12/26) from the 150- μ g gel were predicted to be nonclassical secretory proteins. Interestingly, malate dehydrogenase, enolase, and β -glucosidase were included in this group of proteins, suggesting that this software could potentially be used for predicting nonclassical secretory proteins in plants. In contrast, only four of 35 proteins identified from the total soluble protein gel were predicted to be nonclassical secretory proteins.

Twelve proteins identified from the 50- μ g CWP gel did not have a signal peptide sequence nor were they predicted to be nonclassical secretory proteins. However, they could still be true CWPs because the protein identification process currently available has limitations

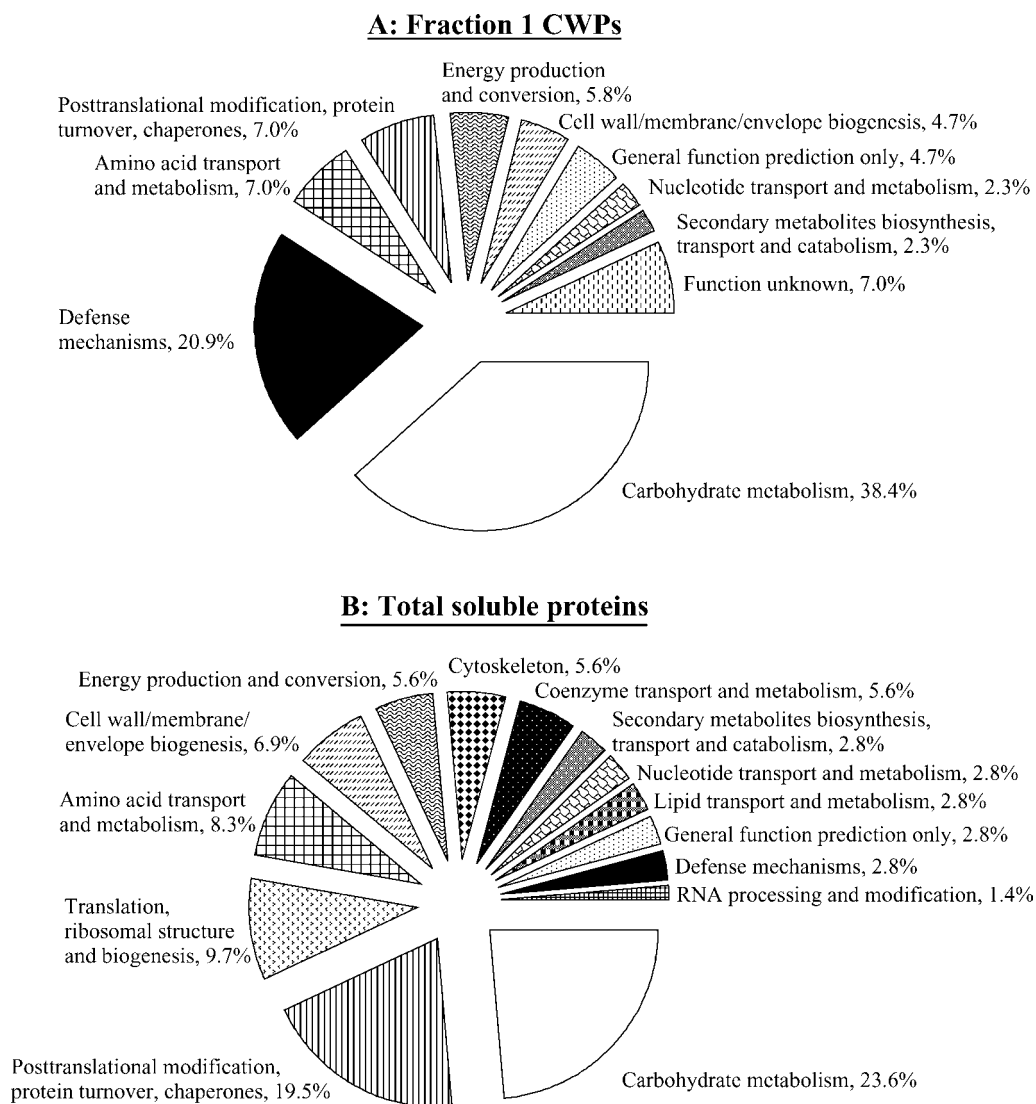


Figure 5. Comparison of the functional classifications of the 43 identified proteins from the 50- μ g fraction 1 CWP gel (A; Table II) and 36 of the most abundant total soluble proteins (B; Table III) as determined using the KOG classification.

for distinguishing family members. Most CWPs belong to multiprotein families, and proteins in the same family can have different cellular localizations. The 14-3-3 proteins were thought to be cytosolic or organelle-localized proteins involved in transcriptional regulation of ATP synthesis in plants (Voigt and Frank, 2003). Recently, it was found that some 14-3-3 proteins are constituents of the insoluble glycoprotein framework of *Chlamydomonas* cell walls (Voigt and Frank, 2003). In another example, a citrate synthase (At2g44350) was identified in a cell wall proteomics study, but was annotated as a mitochondria or peroxisome or glyoxysome protein (Slabas et al., 2004). By analyzing all the members in the citrate synthase family in *Arabidopsis*, Slabas et al. (2004) found one of the members

(At3g58750) not only contained an N-terminal signal peptide but also contained peptide sequence information for peroxisome targeting, indicating the proteins can be targeted to two different locations. Due to the limitation of current protein identification technology in distinguishing slight differences among members in the same protein family, it is possible that At2g44350 identified in the proteomics work was actually At3g58570 (Slabas et al., 2004). It is even more challenging to work with maize proteins because the maize genome information is incomplete. Accordingly, the CWP identifications in this study, based on limited peptide sequences, may have matched proteins in the database that represent other members in the protein families.

Fraction 1 CWPs from Maize Roots Include Proteins Associated with Type II Cell Walls

Maize plants have type II cell walls (Fry, 1988; Carpita and Gibeau, 1993) and are expected to have differences in composition and abundance of CWPs from type I cell walls. This study confirmed this notion by identifying nine proteins (indicated by asterisks in Table II) that were not reported in previous cell wall proteomics studies, which have focused only on type I walls. (Additional potentially novel CWPs have not been indicated in Table IV because of the indication of greater cytosolic contamination on the 150- μ g CWP gel.) These proteins include endo-1,3;1,4- β -D-glucanase and α -L-arabinofuranosidase, both of which were reproducibly identified (Table V). β -1,3;1,4-Mixed-linkage glucan is a unique polysaccharide for type II cell walls of grass species and is considered to be of major importance in cell wall metabolism or modification (Buckeridge et al., 2004). In maize coleoptiles or whole seedlings, it was found that mixed-linkage glucan was developmentally regulated and was associated with the cell elongation process (Carpita, 1996; Kim et al., 2000). Another major component in type II primary cell walls, but a minor fraction in type I cell walls, is glucuronoarabinoxylan (GAX), which can make up 30% of the wall mass (Fry, 1988). GAXs are considered as the counterpart of xyloglucan molecules in type I cell walls and the primary microfibril-tethering molecules in type II cell walls (Carpita, 1996; Buckeridge et al., 2004). α -L-Arabinofuranosidase is probably one of the major enzymes responsible for cleavage and modification of GAX (Fry, 1988). In contrast, no major pectin-related CWPs were identified in this study, while the pectin-related enzymes were always identified as one of the major constituents in cell wall proteomics work with type I cell walls. This finding is in line with the fact that pectin is a minor component in type II cell walls, whereas pectin can make up >30% of cell wall mass in type I cell walls (Fry, 1988; Carpita et al., 2001).

Involvement of Fraction 1 CWPs in Cell Elongation

Many of the CWPs identified in this study have been shown to be involved in the cell elongation process in maize or rice (*Oryza sativa*) coleoptiles. Both exo- and endoglucanase activities were found to be associated with cell walls of maize seedlings (Huber and Nevins, 1981; Kim et al., 2000). Exogenous application of an exo- β -D-glucanase (probably the β -D-glucan exohydrolase in this study) purified from cell walls of maize coleoptiles was capable of inducing cell elongation in the same tissue (Labrador and Nevins, 1989). Antibodies raised against exo- and endoglucanases (with high specificity to β -1,3;1,4-mixed-linkage glucan) from cell walls of maize seedlings inhibited auxin-induced cell elongation of maize and rice coleoptiles (Inouhe and Nevins, 1991; Thomas et al., 2000). An obvious question is whether similar proteins identified

in this study, as well as the putative CWPs that have not been identified in previous studies, are associated with cell elongation in maize roots. We are currently utilizing the methods developed here to study the changes in fraction 1 CWP composition in regions of the root elongation zone that exhibit maintenance or inhibition of cell elongation in response to water deficits (Sharp et al., 1988, 2004).

MATERIALS AND METHODS

Plant Materials

Maize (*Zea mays* L. cv FR697) seeds were surface sterilized in 0.3% NaOCl solution for 15 min, rinsed with distilled water, and imbibed for 24 h in aerated 1 mM CaSO₄. The seeds were germinated for 28 h in vermiculite (grade 3; Strong-Lite), well moistened with 1 mM CaSO₄ at 29°C and near-saturation humidity in the dark (Spollen et al., 2000). Seedlings with primary roots approximately 10 mm in length were then transplanted to a plastic container containing vermiculite well moistened with 1 mM CaSO₄ and grown under the same conditions. At 48 h after transplanting, the apical 12 mm were harvested (using a green safelight; Saab et al., 1990) for either CWP or total soluble protein extraction. The elongation zone constitutes the apical 12 mm in the primary root of well-watered seedlings of this cultivar (Sharp et al., 2004). In additional experiments, specific regions within the elongation zone (3–7 and 7–12 mm from the root apex) were harvested for extraction of CWPs. These samples were used to create region-specific gels that were utilized to test for reproducibility of CWP identification.

Extraction of Fraction 1 CWPs

Fraction 1 CWPs were extracted from the root elongation zone segments according to the methods described by Fry (1988) and MacAdam et al. (1992a, 1992b). Immediately after harvest, the segments were transferred into 20 mM ice-cold K₂HPO₄ solution (pH 6.0). The segments were then rinsed twice with 0.01 M MES buffer and oriented vertically with the root apex at the top in filter-free baskets of tared microfilterfuge tubes (approximately 33 segments per tube; Rainin Instrument Co.). The baskets with root segments were weighed and then placed in a scintillation vial where they were held in place by a stainless steel wire screen molded to fit inside the vial above the basket. Five milliliters of ice-cold, degassed 0.01 M MES buffer (pH 5.5) containing various concentrations of KCl plus protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 5 μ L of protease inhibitor cocktail; Sigma) were added to the vial, submerging the tissue. The whole assembly containing the root segments was vacuum infiltrated at -50 kPa for 15 min and for another 5 min without vacuum. Baskets were removed, drained, and excess buffer was blotted away from the segments through the perforated base of the baskets. Baskets with segments were then transferred to microfuge tubes and centrifuged for 15 min at 1,000g. All steps were conducted on ice or in a cold room at 4°C. Infiltration and centrifugation were then repeated twice. Apoplastic fluid from the three successive extractions was pooled (except in the initial optimization studies) in Centricon-10 Microconcentrators (Millipore), and the extract was desalted to less than 0.005 M KCl by adding 0.005 M MES buffer (pH 5.5, containing 1 μ L mL⁻¹ of protease inhibitor cocktail) and centrifuging for 80 min at 12,000g. The sample reservoirs were inverted inside new microfuge tubes and centrifuged at 12,000g for 20 min to collect the desalted apoplastic solution.

Extraction of Total Soluble Proteins

Total soluble proteins were extracted according to the method described by Tsugita et al. (1994). Immediately after harvest, the root elongation zone segments (approximately 50 segments/sample) were ground into fine powder in liquid N₂. Proteins were precipitated overnight at -70° C with 10% (w/v) TCA in acetone containing 0.07% 2-mercaptoethanol. The mixture was centrifuged at 35,000g at 4°C for 15 min, and the precipitate was washed three times with ice-cold acetone with 0.07% 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 2 mM EDTA. Pellets were dried by vacuum centrifugation.

Protein Quantification

The desalted CWP and total soluble proteins were quantified using the Bradford (1976) method with IgG (Bio-Rad Laboratories) as a standard.

G6PDH Assay

The activity of G6PDH (EC 1.1.1.49), a cytosolic protein marker (MacAdam et al., 1992b), was assayed in the fraction 1 CWP extracts as previously described (Li et al., 1989). Ten micrograms of protein were used for the G6PDH assay. The reaction mixture contained 0.1 M Tricine buffer (pH 8.0), 0.1 M $MgCl_2$, 10 mg mL^{-1} Glc-6-P, and 10 mg mL^{-1} NADP (Sigma).

Protein Separation by 2-DE

Prior to 2-DE, the CWP samples were precipitated overnight at $-70^{\circ}C$ with 10% (w/v) TCA, and the pellets were washed three times with ice-cold methanol and dried briefly by vacuum centrifugation. Total soluble protein (50 μg) or fraction 1 CWP (50 and 150 μg) samples were solubilized in an isoelectric focusing (IEF) buffer containing 5 M urea, 2 M thiourea, 2% CHAPS, 2% SB3-10, 20 mM dithiothreitol, 40 mM Tris, and 0.2% Bio-Lyte 3/10 ampholytes (Bio-Rad Laboratories), and were loaded onto Bio-Rad ReadStrip 11-cm immobilized pH gradient gel strips (pH range 3–10 NL). After the strips were rehydrated overnight, IEF was performed for approximately 10 h, reaching a total of 40 kVh at $20^{\circ}C$ on a flat-bed electrophoresis unit (Protean IEF cell; Bio-Rad). After electrophoresis in the first dimension, the immobilized pH gradient strips were equilibrated for 10 min in reducing buffer containing 6 M urea, 2% SDS, 0.375 M Tris-Cl (pH 8.8), 20% glycerol, and 2% dithiothreitol, followed by equilibration for 10 min in alkylation buffer containing 6 M urea, 2% SDS, 0.375 M Tris-Cl (pH 8.8), 20% glycerol, and 2.5% iodoacetamide. Second-dimension SDS-PAGE gels (8%–16% linear gradient, 8.7 \times 13.3 cm) were run on a Bio-Rad criterion cell at 60 V for 15 min and then at 180 V for 1 h. Gels were stained with either Bio-Rad BioSafe Coomassie Blue or SyproRuby (Molecular Probes) according to the manufacturer's recommendations. Additional gels were loaded with approximately 50 μg of CWP extracted from the 3- to 7- and 7- to 12-mm regions of the elongation zone, and stained with SyproRuby.

Image Analysis of 2-DE Gels

Coomassie Blue-stained gels were scanned with an Epson flat-bed scanner equipped with Adobe Photoshop 7.0. SyproRuby-stained gels were analyzed using a Typhoon laser scanner 9410 (GE Healthcare). Image analysis was carried out with Bio-Rad PDQuest version 7.2 software. After background subtraction and spot detection, spots were matched and normalized using the method of total density in gel image. Spots of interest were identified and excised using a Genetix GelPix protein spot excision system (Genetix USA).

Protein In-Gel Digestion and Peptide Clean-Up

Gel plugs were destained extensively with 50% (v/v) acetonitrile and 50 mM NH_4HCO_3 , followed by dehydration in acetonitrile for 5 min. Proteins were then digested at $37^{\circ}C$ in 25 μL of 50 mM NH_4HCO_3 containing 6 μg mL^{-1} trypsin (sequencing grade, modified; Promega) for 10 h. To extract the digested peptides from the gel matrix, 30 μL 1% formic acid/2% acetonitrile was added to the digest and incubated at $30^{\circ}C$ for 30 min on a shaking platform. The digested supernatant was transferred to a clean tube. The gel pieces were re-extracted with 25 μL of 60% acetonitrile for 15 min and the supernatant was combined with the first extraction. The pooled digest was lyophilized dry in a Speedvac. For whole elongation zone samples, the peptides were resuspended into 10 μL of 1% formic acid/2% acetonitrile, followed by solid-phase extraction using ZipTip microC18 (Millipore) columns according to the manufacturer's instructions, except the peptides were eluted into 4 μL of 60% acetonitrile in water with 0.1% formic acid. For the region-specific samples, peptides were resuspended in 8 μL of 0.1% formic acid/5% acetonitrile for nanoflow HPLC-tandem mass spectrometry (MS/MS).

MALDI-TOF and Electrospray Quadrupole TOF MS

Total soluble protein samples were analyzed using MALDI-TOF MS. Aliquots of protein digest (0.5 μL) were mixed with 0.5 μL of 5 mg mL^{-1} (w/v)

matrix-cyano-4-hydroxycinnamic acid (purified by recrystallization from ethanol) in 50% (v/v) acetonitrile and 0.1% (v/v) TCA for spotting onto a 96 \times 2 stainless steel target for MALDI-TOF MS (Voyager DE STR biochemistry workstation; Applied Biosystems). Standard peptide calibration mixtures (Applied Biosystems) were used as a close external lockmass for instrument and spectrum calibration. MALDI-TOF MS was run in reflector-positive mode for peptide mass fingerprinting.

An ABI QSTAR XL (Applied Biosystems) hybrid quadrupole TOF MS/MS system equipped with a nano-electrospray source (Protana XYZ manipulator) was used for protein identification analysis. For whole elongation zone samples, the peptides were directly infused into the mass spectrometer and the nano-electrospray was generated from a borosilicate nano-electrospray needle with an Au/Pd coating (2- μm tip; Proxeon Biosystems) usually at a voltage of 1,500 V. The instrument m/z response was calibrated daily with standards from the manufacturer. This calibration procedure provides a molecular mass measurement accuracy of <5 ppm for peptides. For nanoflow HPLC (Ultimate) analysis of the region-specific samples, 5 μL of protein digests were loaded onto a C18 precolumn (LC Packings) for desalting and concentrating. Peptides were then eluted from the precolumn and separated on a nanoflow analytical C18 column (PepMap 75- μm i.d., 3 μm , 100 A; LC Packings). The nano-electrospray was generated from a PicoTip needle (10- μm i.d.; New Objectives) at a voltage of 2,400 V. TOF MS spectra and product ion spectra were acquired using the information-dependent data acquisition feature in the Analyst QS software; mass ranges for TOF MS and MS/MS were 300 to 1,500 and 65 to 1,500, respectively. Every 1 s a TOF MS spectrum was accumulated followed by three product ion spectra, each for 3 s. The DP, DP2, and FP settings were 50, 10, and 200, respectively, and the collision energy was dependent on the m/z values of the ions.

Data Analysis and Database Searching

MALDI-TOF peptide mass fingerprints were used to gain an initial indication of the identity and homogeneity of the proteins. MALDI-TOF MS analyses provide a set of 15 to 50 abundant peptide masses from trypsin-digested protein samples. These masses were used via Protein-Prosector (prosector.ucsf.edu) to identify hits in several different maize-related protein and nucleic acid databases, including NCBItr, Swiss-Pro, dbESTothers, and Unigenes of maize, rice (*Oryza sativa*), wheat (*Triticum aestivum*), sorghum (*Sorghum bicolor*), and potato (*Solanum tuberosum*), with the maximum miscleavage of 1 and mass tolerance of 50 ppm. Cys carbamidomethylation was chosen as fixed modification, and acetylation of the N terminus, oxidation of Met, and pyroGlu formation of N-terminal Gln were considered as possible modifications. Matching was performed by the number of peptide matches and the coverage (typically greater than 20%), with consideration of Protein-Prosector scores, PMFQ (Watson et al., 2003), and the predicted MW and pI, which were compared with observed MW and pI values on the 2-DE gels. The peptide electrospray tandem mass spectra were searched against the above databases using the Mascot search engine (<http://www.matrixscience.com>) with a mass tolerance of 100 ppm and one allowed trypsin miscleavage. Search parameters allowed for the fixed Cys carbamidomethylation and the variable Met oxidation 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 MS/MS spectra.

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