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Proteomics of *Medicago sativa* cell walls

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Abstract

A method for the sequential extraction and profiling by two-dimensional gel electrophoresis (2-DE) of *Medicago sativa* (alfalfa) stem cell wall proteins is described. Protein extraction included freezing, grinding in a sodium acetate buffer, separation by filtration of cell walls from cytosolic contents, and extensive washing. Cell wall proteins were then extracted sequentially with a solution containing 200 mM $CaCl_2$ and 50 mM sodium acetate, followed by extraction with 3.0 M LiCl and 50 mM sodium acetate. Cell wall proteins from both the $CaCl_2$ and LiCl fractions were profiled by 2-DE. Approximately 150 protein spots were extracted from these two gels, digested with trypsin, and analyzed using nanoscale HPLC coupled to a hybrid quadrupole time-of-flight (Q-tof) tandem mass spectrometer (LC/MS/MS). More than 100 proteins were identified and used in conjunction with the 2-DE profiles to generate proteomic reference maps for cell walls of this important legume. Identified proteins include classical cell wall proteins as well as proteins traditionally considered as non-secreted. Two unique extracellular proteins were also identified.
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Keywords: *Medicago sativa*; Alfalfa; Cell wall proteins; Extraction method; Proteomics; Two-dimensional electrophoresis; 2-DE; Mass spectrometry; LC/ESI/MS/MS

1. Introduction

The plant cell wall is a dynamic structure composed of polysaccharides, phenolic compounds, and proteins. The cell wall is not only important for maintaining cell shape and rigidity, but also responds to environmental stress through release of putative signaling molecules (Baluška, 2003). In plants, there are two types of cell walls: the primary wall, formed at the cell plate during division with properties facilitating cell growth and elongation; and the secondary wall, produced at a later time of development with properties important for mechanical strength and defense.

Although plant cell walls consist primarily of carbohydrates and polyphenolics, proteins make up approximately 10% of the cell wall mass (Chivasa et al., 2002; Borderies et al., 2003). These wall proteins are involved in at least three categories of cell wall functions. Cell wall enzymes help regulate growth and development (Darley et al., 2001), structural proteins contribute to wall architecture (Cassab, 1998), and defense proteins aid in responses to biotic and abiotic stresses (Qin et al., 2003; Baluška, 2003).

To date, most proteomic studies of cell walls have been performed with microorganisms (Pitarch et al., 2002) or plant cell cultures (Borderies et al., 2003; Ndimba et al., 2003; Chivasa et al., 2002; Blee et al., 2001; Robertson et al., 1997). Cell cultures provide a convenient supply of cell wall material; however, they contain primarily undifferentiated cells and thus do not constitute a source of secondary wall proteins. A few culture types can contain both primary and secondary cell walls (zinnia, Norway spruce, transgenic tobacco) and of these, transgenic tobacco has been used for proteomic studies, but the identified proteins were limited to a few easily extractable proteins isolated from intact (live) cells (Blee et al., 2001). Few tissues from differentiated plants have been utilized for cell wall protein studies. Examples include whole seedlings of cucumber, pea, and soybean (McQueen-Mason et al., 1992; Melan and Cosgrove, 1988; Kleis-San Francisco and Tierney, 1990), tomato fruits (Rose et al., 2000), soybean seed coats (Cassab et al., 1985), and spruce xylem (McDougall, 2000). The cell walls of young

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plants consist only of primary wall material and fruits generally posses primary walls with little lignification. In fact, fruit ripening involves cell wall disassembly rather than wall synthesis (Qin et al., 2003). Although seed coats (Western et al., 2001) and spruce xylem do contain secondary walls, work on these tissues has predominantly consisted of isolation and characterization of single proteins or protein classes rather than obtaining an overall view of the protein population.

At least three problems are inherent in the isolation of cell wall proteins from mature, lignifying plant tissue. First, separating cell wall proteins from soluble or membrane-associated proteins is challenging. Isolation of subcellular fractions enhances the ability to identify low abundance proteins and yields knowledge of protein localization (Canovas et al., 2004); however, ensuring fraction purity is difficult. Second, cell wall proteins are known to be glycosylated, and some cell wall proteins are very basic (Borderies et al., 2003). Isoelectric focusing of proteins with these characteristics is often problematic. Third, during the extraction of proteins from the walls of mature plant tissues, other materials such as polysaccharides and polyphenolics are coextracted. High concentrations of these materials result in streaking and background staining on 2-DE gels, making analyses of the proteins separated by 2-DE difficult or impossible.

In this report, a 2-DE proteomic investigation of cell wall proteins isolated from mature stem tissue of alfalfa (*Medicago sativa* L) is described. Alfalfa is the world's primary forage legume, and the chemical and physical properties of its cell walls impact important agronomic traits such as forage digestibility and lodging. Furthermore, alfalfa is a target species for genetic modification of the lignin pathway to improve digestibility (Guo et al., 2001a,b). In this report, an extraction method is described that provides a population of cell wall proteins from alfalfa stems that appear to be free of contaminating proteins. These preparations exhibit only slight background staining in 2-D gels and, although not highly concentrated, the protein quantities were sufficient for identification by mass spectrometry. The identified proteins included both classical cell wall proteins and proteins that have traditionally been considered as non-secreted, but nevertheless have been reported to be wall-associated by other researchers (Chivasa et al., 2002; Kärkönen et al., 2002; Pitarch et al., 2002; Voigt and Frank, 2003). Because the number and function of plant cell wall proteins are still a topic of investigation, this work represents a significant extension of our knowledge of cell wall-related proteins. The identified proteins also provide a proteomics reference map and basis for future comparisons to the cell wall proteomes of immature stem tissue and stems from transgenic plants with modified lignin biosynthesis.

2. Results and discussion

2.1. Cell wall protein extraction

The extraction of ionically bound proteins from purified cell walls is commonly achieved using concentrated salt buffers, especially those containing $CaCl_2$ (Borderies et al., 2003; Robertson et al., 1997). Highly concentrated lithium chloride has also been reported for the extraction of glycoproteins (Voigt and Frank, 2003) and tightly bound proteins from cell walls (Melan and Cosgrove, 1988). In this report, an optimized sequential extraction utilizing both $CaCl_2$ and LiCl buffers was established for the extraction of cell wall proteins originating from mature stem tissue of *M. sativa.*

Purity is a significant concern when working with sub-proteomes. To address this concern, a rigorous purification regimen was employed. This began with stringent washing of cell walls using large volumes and numerous washes including a salt buffer wash, water washes, an organic wash, and a final buffer wash. To evaluate the effectiveness of the washing procedures, aliquots from the initial homogenization buffer and each sequential wash were analyzed by one-dimensional SDS–PAGE (Fig. 1). Fig. 1 demonstrates that the majority of cytosolic proteins were extracted in the initial homogenization buffer and that sequential washes were

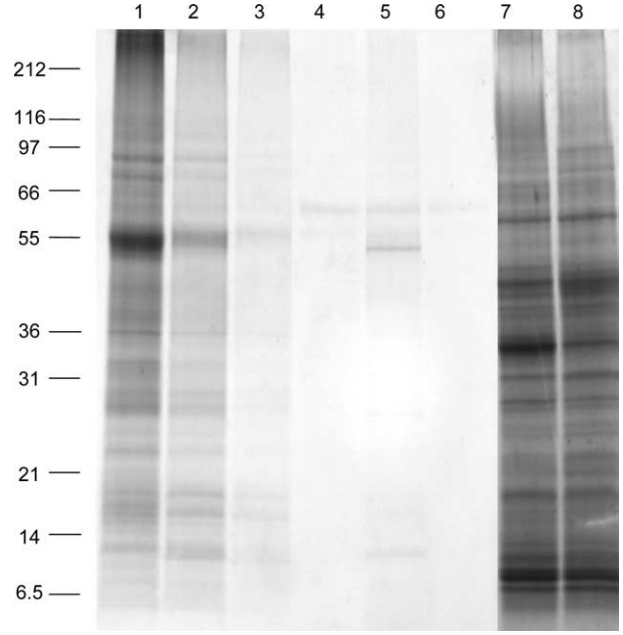

Fig. 1. One-dimensional SDS–PAGE analysis showing concentration and complexity of the proteins removed from cell walls by the various washes. Lane 1, crude extract in grinding buffer (20 μl); lane 2, 0.1 M NaCl wash (100 μl); lane 3, 1st dH_2O wash (200 μl); lane 4, acetone wash (500 μl); lane 5, 2nd H_2O wash (500 μl); lane 6, 10 mM NaAcet wash (500 μl); lane 7, 0.2 M $CaCl_2$ extract (5 μl/25 μg); and lane 8, 3.0 M LiCl extract (13 μl/25 μg). The numbers in parentheses are the volumes/amount of sample in each lane.

efficient in removing residual proteins. The purified and washed cell walls were then sequentially extracted with $CaCl_2$ (lane 7) and LiCl (lane 8) yielding a dramatic increase in protein compared to lane 6, supporting the effectiveness of $CaCl_2$ and LiCl for removal of *M. sativa* cell wall proteins from stem tissue.

The optimized method was used to extract cell wall proteins from alfalfa stem cell wall. These extracts were then profiled by 2-DE and silver stained to yield proteomic reference maps (Fig. 2). The observed protein populations extracted using $CaCl_2$ and LiCl appeared to be similar; however, there were lower concentrations of some proteins observed in the LiCl 2-DE gel. This was not surprising because of the sequential extraction protocol. There were, however, qualitative differences in the profiles, which consisted of a group of protein spots around 30 kDa from p*I* 6 to 8, several protein doublets at approximately 47–50 kDa, and a few higher molecular weight proteins.

2.2. Protein identifications

To improve the value of the proteomic reference maps, a large number of protein spots were excised, digested in-gel with trypsin, analyzed by LC/MS/MS, results queried, and proteins identified. Most of the proteins spots chosen for identification were excised from the $CaCl_2$ 2-DE gel because the majority of proteins appeared on both gels and because the concentrations of nearly all those were greater on the $CaCl_2$ gel. Selected protein spots that appeared more abundant in the LiCl 2-DE gel or were not readily apparent on the $CaCl_2$ gel were also excised, digested, analyzed, and identified from the LiCl gel. Several of the proteins identified from the LiCl 2-DE gel were the same as proteins identified from the $CaCl_2$ gel and served as landmarks for 2-DE gel alignments. The protein identifications and data documenting the analytical rigor of each of the identifications from both 2-DE gels are listed in Table 1. Although the protein concentrations loaded on the 2-DE gels were not high, and the gels were stained with silver rather than Coomassie, the digests yielded good quality MS data (Fig. 3; Table 1) for protein identification.

One hundred and thirty-nine protein digests were analyzed by LC/MS/MS and 97 of them (112 proteins because of multiple ids for some spots) were identified with high confidence. Although the goal of this project was not to generate a comprehensive listing of cell wall proteins, this report represents a significant advancement in the number of cell wall proteins characterized from *M. sativa*. All protein identifications reported had scores greater than or equal to two times the accepted significance threshold (determined at the 95% confidence level) calculated by Mascot. This criterion was chosen to provide additional confidence in the protein identifications. Most protein identifications were based on multiple peptide matches; however, a few protein identifications were based on a single peptide sequence (#35 both ids, #62nd id, #67 both ids, and #89), but their scores were still at least two times the significance threshold. Occasionally, two or more co-migrating proteins were identified in the same spot. Database queries often yielded multiple isozymes in the search results (#35, #76, #83, and #143), i.e., the first and second most confident identifications were to different isoforms of the same protein. It is probable that peptides which would allow differentiation between isoforms

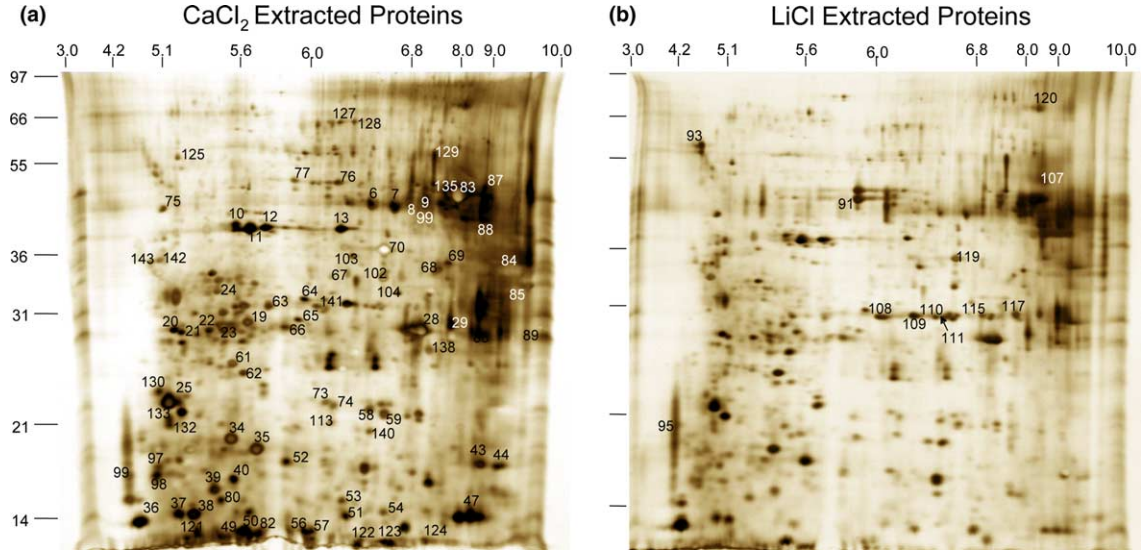

Fig. 2. 2-DE proteome reference maps of *M. sativa* cell wall proteins sequentially extracted using 200 mM $CaCl_2$ (a) and 3.0 M LiCl (b). Numbers indicate protein spots successfully identified by LC/MS/MS. The $CaCl_2$ gel contained 250 μg total protein and the LiCl gel 150 μg total protein. Molecular mass markers in kDa are indicated on the left of each gel and the p*I* calibration is noted at the top.

Table 1
Proteins identified in alfalfa stem cell walls

Spot #	Gel MW	Gel p*I*	Identification	SS	Accession #	Score	# Peptides	% Coverage	Theor MW	Theor p*I*
$CaCl_2$ gel										
6	48	6.4	Hypothetical protein precursor	x	T09642	91	4	8	40.7	7
7	48	6.5	Hypothetical protein precursor	x	T09642	90	3	12	40.7	7
8	48	6.9	GDSL-motif lipase/hydrolase protein	x	NP_196949	281	5	14	43.6	8.5
			Hypothetical protein precursor	x	T09642	156	4	13	40.7	7
9	48	8	GDSL-motif lipase/hydrolase protein	x	NP_196949	235	6	14	43.6	8.5
			Peroxidase 1B precursor	x	JC4780	212	4	14	38.2	7.5
10	43	5.4	Oxidoreductase, zinc-binding dehydrogenase family		NP_173786	74	2	13	41	8.4
11	42	5.5	Putative quinone oxidoreductase		AAL06644	1204	36	72	27.7	5.2
			Oxidoreductase, zinc-binding dehydrogenase family		NP_173786	437	11	30	27.5	5.4
12	42	5.6	Putative quinone oxidoreductase		AAL06644	1221	33	74	27.7	5.2
13	42	6.2	Malate dehydrogenase		T09286	961	29	53	35.8	8.6
19	30	5.5	Carboxymethylenebutenolidase related		NP_180811	84	2	12	25.9	5.5
20	30	5	Ribose-5-phosphate isomerase		AAL77589	444	14	45	29.9	5.5
21	30	5.1	Ribose-5-phosphate isomerase		AAL77589	492	18	45	29.9	5.5
22	30	5.3	In2-1 protein		AAG34872	276	6	23	25.7	5.8
23	30	5.3	In2-1 protein		AAG34872	333	6	23	27	5.6
24	33	5.3	Glyoxalase I, putative		NP_176896	382	12	27	38.8	6.6
25	24	5	Protease inhibitor	x	CAD29731	242	10	24	23	5.2
28	30	7.3	Expressed protein		NP_567141	89	2	12	30.4	9.4
29	29	7.8	Germin-like protein	x	CAC34417	125	5	14	21.6	7.8
34	20	5.5	Superoxide dismutase (Cu-Zn)		O65198	217	5	25	21.2	6.1
35	19	5.7	Superoxide dismutase (Cu-Zn)		O65198	83	1	11	21.2	6.1
			Superoxide dismutase (Cu-Zn)		Q02610	58	1	23	15.2	5.5
36	13	4.3	Plastocyanin		P16002	71	2	28	17	5
37	14	4.9	Profilin 1		P49231	149	5	33	14.1	4.9
38	14	5.1	Thioredoxin M-type 3		AAC49358	256	10	38	19.2	8.9
			Profilin 1		P49231	173	4	33	14.1	4.9
39	16	5.3	Cytochrome B6-F complex iron-sulfur subunit chloroplast precursor		P26291	360	9	26	23.9	7.6
40	17	5.4	Translational inhibitor protein		NP_188674	414	13	38	20	9
43	18	8	Oxygen-evolving complex 25.6 kD protein		NP_191093	278	6	19	25.6	9.4
44	18	9	Peroxiredoxin-related		NP_189235	293	5	22	23.1	9.5
47	13	7.9	Expressed protein		NP_566578	72	3	11	21	5.3
49	6	5.3	Rubisco small		AAL15646	182	2	23	10.9	6.6
50	7	5.4	Thioredoxin H-type		Q43636	388	13	41	12.8	5.8
51	14	6.2	Thioredoxin F-type		P29450	211	4	19	20	9.1
52	17	5.8	Expressed protein		NP_563841	140	4	15	19.4	5.2
53	15	6	Rubisco small		O65194	229	6	30	15	8
54	14	6.7	Rubisco small		O65194	227	4	24	18.4	8.8
56	7	5.4	Rubisco small		O65194	182	4	12	19.8	8.7
57	7	5.4	Rubisco small		O65194	209	7	30	15	8
58	21	6.6	Profucosidase	x	CAA09607	88	2	21	17	7
59	21	6.7	Profucosidase	x	CAA09607	98	2	21	17	7

61	27	5.4	Oxygen-evolving enhancer protein 2		P16059	433	10	48	28.1	7.7
62	26	5.5	Oxygen-evolving enhancer protein 2		P16059	67	2	10	28.1	7.7
			Glyoxylase I		CAA09177	61	1	4	20.9	5.6
63	32	5.7	Ascorbate peroxidase, putative		NP_192640	328	7	24	37.9	8.4
64	32	5.9	Malate dehydrogenase		T09286	291	7	13	35.8	8.6
65	31	5.8	Lectin 1 precursor	x	Q01806	150	4	19	30.5	5.8
66	30	5.7	20S proteasome alpha subunit B		NP_173096	411	11	32	25.6	5.5
67	34	6.4	Germin-like protein		AAB51752	65	1	5	23.3	6.3
			Germin-like protein	x	AAB51577	65	1	10	13.2	5.1
68	35	7.2	Glucan-endo-1,3-β-glucosidase	x	CAA10287	467	13	34	40.3	8.8
69	36	7.5	Glucan-endo-1,3-β-D-glucosidase	x	CAA10167	421	8	30	36.3	6. 2
			Peroxidase related	x	NP_200002	159	3	13	34.3	7.6
70	37	6.6	Endochitinase precursor	x	P36907	327	12	25	34.3	6.2
73	23	6.1	profucosidase	x	CAA09607	83	2	21	17	7
74	22	6.4	C2 domain-containing protein		NP_175292	110	2	13	21	8.4
			Profucosidase	x	CAA09607	88	2	21	17	7
75	46	4.8	Hypothetical protein precursor		T06363	236	11	21	43.8	5
76	52	6.1	Enolase (Mt TC)		Q42971	537	12	46	23.7	5
			Enolase (Gm TC)		Q42971	165	5	11	47.8	5.5
77	52	5.8	Putative nucellin protein	x	NP_567922	190	5	48	47.4	6.8
80	15	5.3	IgE-dependent histamine releasing factor homolog		S22489	70	2	7	14	5.2
82	7	5.7	Rubisco small precursor		O65194	175	5	27	18.4	8.8
83	50	7.9	Peroxidase 1C precursor	x	JC4781	626	25	23	38.3	6.2
			Peroxidase 1B precursor	x	JC4780	510	10	29	38.2	7.5
84	34	9.2	Peroxidase 2 precursor	x	JC4782	586	13	38	35.4	9.2
85	32	9.4	Expressed protein	x	NP_563851	89	3	9	43.3	8.4
86	29	8.3	Pectin acetylesterase	x	S68805	202	4	9	43.2	8.8
87	51	9.4	Probable peroxidase	x	T09165	233	4	14	34.5	9.1
88	44	8.3	Disease resistance protein family (LRR)	x	NP_188718	422	11	39	40	8.4
89	29	9.8	Rhicadhesin receptor precursor (germin-like protein)	x	Q9S8P4	71	1	6	22.5	8.9
97	18	4.4	Skp1		AAD34458	150	3	30	17.6	4.6
98	17	4.4	Glycine cleavage system H protein		P16048	133	3	21	17.8	5.1
99	17	4	Calmodulin		NP_189967	100	3	20	16.8	4.1
102	35	6.6	Ferredoxin-NADP reductase		P10933	201	5	15	40.3	8.4
			Cysteine proteinase XCP2	x	NP_564126	177	5	15	39.4	5.6
103	36	6.2	Ferredoxin-NADP reductase		P10933	372	11	22	40.3	8.4
104	33	6.7	NADH-cytochrome b5 reductase, putative		NP_568391	223	7	25	36.7	8.8
113	22	6.2	Malate dehydrogenase precursor		T09286	156	3	7	35.9	8.6
121	9	4.7	Cytochrome b5 domain-containing protein		NP_180066	311	7	50	11	4.9
122	6	6.5	Rubisco small		O65194	267	6	35	15	8
123	6	6.7	Macrophage migration inhibitory factor (MIF) family		NP_195785	230	7	28	12.1	6.6
			Rubisco small		O65194	113	3	14	14.6	6.5

(*continued on next page*)

Table 1 (*continued*)

Spot #	Gel MW	Gel p*I*	Identification	SS	Accession #	Score	# Peptides	% Coverage	Theor MW	Theor p*I*
124	6	7.4	Thioredoxin M-type 3		NP_179159	167	6	18	19.2	9.2
125	57	5.1	Glycosyl hydrolase family 17	x	NP_195174	166	4	7	37.8	5.7
127	66	6.2	NADP-dependent malic enzyme		P51615	124	5	8	65.3	6
128	66	6.4	NADP-dependent malic enzyme		P51615	83	3	7	65.3	6
129	58	7.5	Leucine-rich repeat protein family	x	NP_175397	163	3	8	54.4	8.1
130	24	4.5	Protease inhibitor	x	CAD29731	112	3	13	23	5.2
132	21	5	Protease inhibitor	x	CAD29731	79	3	13	23	5.2
133	22	5	Protease inhibitor	x	CAD29731	135	5	13	23	5.2
135	52	7.2	β-Galactosidase	x	CAC44501	82	3	12	21.1	8.4
138	28	7	NtPRp27	x	BAA81904	194	7	16	25.4	7
140	20	6.2	Acid phosphatase	x	T07086	143	6	13	29.9	6.6
141	32	6	Acid phosphatase	x	T07086	295	11	17	29.9	6.6
142	36	4.7	Glucan-endo-1,3-β-glucosidase	x	CAA10167	81	2	10	36.2	6.2
143	36	4.5	Glucan-endo-1,3-β-glucosidase	x	CAA10167	161	3	13	36.2	6.2
			Glucan-endo-1,3-β-glucosidase	x	T05957	153	4	25	22.6	4.8
LiCl gel										
91	47	5.7	Peroxidase pxdC precursor	x	T09665	125	3	5	37.9	5.8
93	59	4.3	Calreticulin precursor	x	P93508	398	8	15	48.4	4.4
95	21	4	Calmodulin		P04353	55	3	23	14.1	4.2
107	53	8.2	Leucine-rich repeat protein family	x	NP_175397	298	6	11	54.4	8.1
108	30	5.8	Acid phosphatase	x	T07086	287	11	17	29.9	6.6
109	30	6.3	Malate dehydrogenase		AAD56659	327	7	18	35.9	7.8
110	30	6.5	Acid phosphatase	x	T07086	154	6	13	29.9	6.6
111	30	6.6	Acid phosphatase	x	T07086	75	4	8	29.9	6.6
115	30	6.8	Acid phosphatase	x	T07086	92	3	10	29.9	6.6
117	30	7.4	Acid phosphatase	x	T07086	78	2	11	12.3	6.4
119	36	6.7	Endochitinase precursor	x	P36907	216	10	17	34.3	6.2
120	80	8.3	Subtilisin-like proteinase	x	T07171	108	3	5	82.7	8.3

Spot numbers correspond with the numbers on gel images (Fig. 2). Gel molecular weight was calculated using *HT Analyzer* and p*I* was estimated using information supplied by BioRad on the composition of their nonlinear gels. *SS* refers to the presence of a signal sequence directed for an extracellular location as predicted by SignalP. Identifications and accession numbers are from NCBI. The score, number of matched peptides, and percent coverage are taken directly from the Mascot Daemon report. Theoretical MW and p*I* are either taken from the Mascot Daemon report or calculated in Protein Calculator from the NCBI sequence.

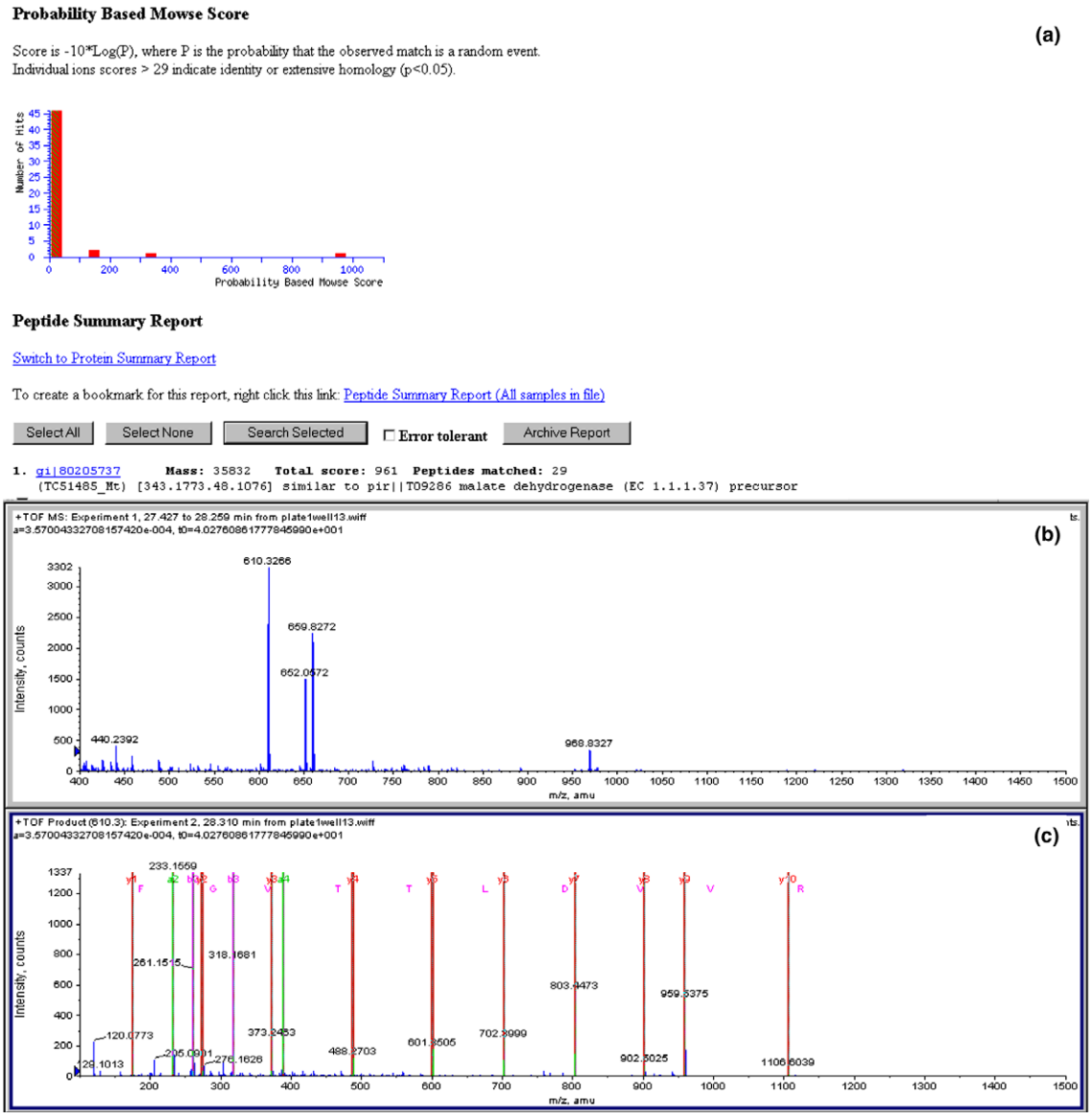

Fig. 3. LC/MS/MS data obtained from spot 13 on an ABI Qstar Pulsar hybrid quadrupole time-of-flight (Q-tof) mass spectrometer. (a) Database query results. All three significant identifications are to malate dehydrogenase; (b) Representative TOF-MS spectrum of eluted peptides; and (c) Representative product ion spectrum of *m/z* 610.3 and its sequence generated through de novo sequencing.

were not sequenced; however, the high similarity in the multiple isoforms lends validity to the identification. In addition, the same protein was often identified from multiple spots, suggesting that various post-translationally modified forms of that protein are present on the gel.

2.3. Identification and classification of classical and hypothetical cell wall proteins

Classical cell wall proteins contain a secretion signal sequence which is recognized by predictive software such as SignalP, and a known or predicted function in the extracellular matrix. A large number of classical cell wall proteins were identified in the alfalfa stem cell wall extracts that validate the reported purification and extraction method. Classical cell wall proteins identified include wall modifying proteins, proteins involved in defense, lignin biosynthetic proteins, possible extracellular signaling proteins, and proteins with unknown functions. More specifically, identified proteins involved in cell wall modification included carbohydrate modifying proteins such as profucosidase, glucosidases, a protein from the glycosyl hydrolase family, and pectin acetylesterases. Protein and lipid modifying proteins found in the walls of alfalfa stems include nucellin, an aspartyl protease like protein (#85 hypothetical, see below), a calreticulin precursor, and GDSL-motif lipase/

hydrolase. This protein is a proline-rich protein and thus could also function in the cell wall as a structural protein.

Several defense proteins were identified in this study. These include protease inhibitors, endochitinase, NtPRp27, a disease resistance leucine-rich repeat (LRR) protein, and a subtilisin-like proteinase. There were also several proteins containing a secretion signal which show sequence similarity to identified proteins, but which have no known function. These include another protein from the LRR protein family, germin-like proteins, and hypothetical proteins.

The tissue used in this study was from mature alfalfa stems and contained lignified walls (Guo et al., 2001a). Numerous class lll secretory peroxidases implicated in both defense and lignin biosynthesis (Hatfield and Vermerris, 2001; Anterola and Lewis, 2002) were identified (#9 2nd id, #69 2nd id, #83 both ids, #84, #87, and #91), however, no laccases or dirigent proteins were identified. It is possible that these proteins are not as abundant as peroxidases and thus were not visualized on the 2-DE and/or not excised for identification. Alternatively they could also be more difficult to extract from lignified tissue.

There have been several recent reports on the presence of phosphorylated proteins in the apoplast and cell walls of plants (Baluška, 2003). These proteins are proposed participants in extracellular signaling events. An extracellular acid phosphatase was identified in this report and could also be involved in signal mediation.

Six proteins were identified as hypothetical, suggesting they correspond to known proteins with no known function. Two of these are predicted by SignalP to be secreted. One of the two, #85, is predicted by PSI-Blast (http://www.ncbi.nlm.nih.gov/BLAST/producttable.shtml) to be similar to aspartyl proteases (*E*-value 7e − 39) that function in post-translational modification, protein turnover, or as chaperonins. In addition, two recent articles (Xia et al., 2004; Suzuki et al., 2004) describe an extracellular aspartic protease involved in resistance to *Pseudomonas syringae* in *Arabidopsis*. It is likely that this aspartic protease-like protein plays a role in signal transduction. The other protein, identified in spots #6, #7, and #8, is a unique protein (similar to DUF642, *E*-value 1e − 158) of unknown function. This family features a conserved region found in a number of uncharacterized plant proteins. Of the other four hypothetical proteins, two are predicted to be in the chloroplast (#28 and #75) by TargetP and two (#47 and #52) contain no signal peptide. Protein spot #28 is similar to a ribosome recycling factor protein (*E*-value 5e − 52) with a predicted function in translation, ribosomal structure, and biogenesis. Protein spot #75 shows similarity to a cyclophilin (peptidyl prolyl *cis–trans* isomerase with an *E*-value of 9e − 18) that functions in post-translational modification, protein turnover, or as a chaperonin. Protein spot #47 has no putative conserved domains and protein spot #52 is unique (similar to DUF538, *E*-value of 2e − 31). This family consists of several plant proteins of unknown function.

Certain classical cell wall proteins, often identified in other types of cell wall preparations (Blee et al., 2001; Borderies et al., 2003; Gao et al., 1999; Ndimba et al., 2003; Robertson et al., 1997), were not observed in this study. These include arabinogalactan proteins (AGPs), wall-associated kinases (WAKs), expansins, and xyloglucan transferases (XETs). It is possible that these proteins/classes of proteins were present in the $CaCl_2$ and LiCl protein extracts, but were not chosen for digestion or were of lower relative abundance and not visualized via 2-DE. Conversely, they could have been among the 30% of digested proteins for which we did not obtain confident identifications. The proteins reported in the previous studies were extracted either from living/intact cells or through less stringent procedures than the one employed here. In the current report, it is likely that these proteins were removed in the extensive washes. Extensins and other HRGPs are extensively glycosylated at the Hyp residue (Minorsky, 2002). This increases their solubility compared to other cell wall proteins, making it likely that these proteins were lost during the washes. Melan and Cosgrove (1988) also reported that 3.0 M LiCl extracts of homogenized and washed cell walls from pea epicotyls did not contain expansin activity. Further, AGPs and WAKs are often associated with the plasma membrane, either through an anchor sequence (AGPs) or by a transmembrane domain (WAKs) (Baluška, 2003), which allow easier extraction from the cell wall when compared to non-membrane-associated proteins.

SignalP (Nielsen et al., 1997, 1999) was used to predict the presence of a secretory signal peptide and in some cases TargetP (Emanuelsson et al., 2000) was used to predict intracellular location. As a result of these analyses, some of the proteins identified do not appear to contain a secretory signal peptide (47%). However, non-secreted or non-apoplastic proteins have also been reported in the cell walls of cell cultures of *Arabidopsis thaliana* (Chivasa et al., 2002) Norway spruce (Kärkönen et al., 2002), *Chlamydomonas reinhardtii* (Voigt and Frank, 2003), and the yeast and hyphal forms of *Candida albicans* (Pitarch et al., 2002).

Several of these non-classical cell wall proteins were identified in *M. sativa* cell walls (Table 1). In this respect, Kärkönen et al. (2002) found a wall-bound malate dehydrogenase; Chivasa et al. (2002) identified a cytochrome, peroxiredoxin, and enolase; and Pitarch et al. (2002) identified a cell wall enolase. The enolase in the cell wall of *C. albicans* (Pitarch et al., 2002) was so tightly bound that it had to be extracted by SDS/DTT or alternatively by NaOH, Quantazyme (a recombinant β-glycanase), and exochitinase in sequential extractions.

This is in agreement with the identification of the *C. albicans* enolase as a major glucan-associated cell wall protein (Angiolella et al., 1996, 2002). Using immunolocalization, enolase and peroxiredoxin were shown to be secreted to the cell wall or extracellular space even though they lack a signal peptide (Edwards et al., 1999; Robertson et al., 2000). There are also reports of wall-bound malate dehydrogenase (Gross, 1977; Li et al., 1989; Mäder and Schloss, 1979) which is thought to act to regenerate NAD(P)H needed by cell wall peroxidases for free radical generation associated with lignin polymerization. Though it is possible that the non-classical wall proteins found here are a result of contamination, the lack of discernible proteins in the washes (Fig. 1), and the corroborative evidence provided by other studies argues for their being true components of the wall.

There is a potential for certain cytosolic proteins to associate very tightly with the wall matrix such that most buffers do not disrupt the interaction; however, one report (Rose, 2003) indicates that detergents such as SDS could disrupt the interaction and allow resolubilization of the protein. The solubilization properties of the buffers could account for some non-classical proteins identified in other cell wall reports (Chivasa et al., 2002); however, it is not the case here because the 2-DE resolubilization buffer never came into contact with the cell walls.

It is possible that generally perceived cytosolic proteins may also be "moonlighting" proteins which perform more than one function in the cell (Pitarch et al., 2002; Copley, 2003). For example, 14-3-3 proteins are generally perceived as cytosolic or organelle-localized proteins involved in transcriptional control or regulation of ATP synthesis in plants. In *C. reinhardtii*, 14-3-3 proteins have reported cytoplasmic functions, but also have been documented as constituents of the insoluble glycoprotein framework of the cell wall and, in fact, are thought to be involved in the covalent cross-linking of Hyp-rich glycoproteins to the insoluble cell wall fraction (Voigt and Frank, 2003). All six *C. reinhardtii* 14-3-3 sequences listed in NCBI are predicted by SignalP to be non-secreted, including accession #P52908, the 14-3-3 protein from Voigt and Frank (2003). This may reflect incomplete knowledge of gene-splicing variants, signal peptides or alternative secretory pathways (Chivasa et al., 2002; Pitarch et al., 2002).

Although many other researchers have reported non-classical proteins localized to cell wall fractions, this is not sufficient to unambiguously verify that the *M. sativa* cell wall preparations are without contamination, as suggested by the presence of Rubisco small subunit. Even the lack of observable contaminants in the washes as determined by SDS–PAGE (Fig. 1) is not sufficient to guarantee the purity of the cell wall protein fraction. Further work such as immunolocalization or use of gene constructs expressing fluorescently labeled fusion proteins is needed to clarify this issue.

3. Conclusions

A method for the sequential extraction of cell wall proteins from mature stems of *M. sativa* has been described. It has been used to generate 2-DE proteome reference maps for this species. From these reference maps, a large number of proteins were excised, digested, analyzed by LC/MS/MS, and identified. Over 100 identified proteins are reported that further characterize the cell wall proteome of this important forage legume. Many of the identified proteins are verified cell wall proteins as evidenced by the literature and signal peptide sequences determined using the predictive software SignalP. Additional, non-traditional cell wall proteins were also identified. Although several of these may represent contamination, others are suggestive of localization of proteins traditionally supposed as cytosolic or non-cell wall proteins. The alfalfa cell wall proteomic reference maps provide initial information that will be expanded upon during future proteomic studies of plants modified in cell wall structure through targeted manipulation of the lignin pathway.

4. Experimental

4.1. General experimental procedures

Nitex membranes (Tetko Industries, Briarcliff, NY) were used for filtration. The protein samples were processed with BioRad's ReadyPrep 2-D Cleanup Kit. Samples were focused using a BioRad (Hercules, CA) Protean IEF Cell. Second dimension gels were run using the Hoefer SE 600 (Amersham, Piscataway, NJ) system and digitally imaged using a BioRad FluorS. Gel molecular weight was calculated using HT Analyzer software (Genomic Solutions, Ann Arbor, MI) and p*I* was estimated using information supplied by BioRad on the composition of their nonlinear gels. One dimensional SDS–PAGE was performed using precast 12.5% Criterion polyacrylamide gels (BioRad). Separation of protein digests was performed using a nano-HPLC system (LC Packings, San Francisco, CA) consisting of an autosampler (Famos), a precolumn switching device (Switchos), an HPLC pump system (Ultimate), a C18 precolumn (0.3 mm i.d. × 1.0 mm, 100 Å, PepMap C18 from LC Packings) and a nano-analytical C18 column (75 μm i.d. × 15 cm, 100 Å, PepMap C18, LC Packings). The nano-HPLC was coupled with an ABI QSTAR Pulsar (Applied Biosystems, Foster City, CA) hybrid quadrupole TOF MS equipped with a nano electrospray source (Protana XYZ manipulator) for LC/MS/MS

analysis. The nano electrospray was generated from a PicoTip needle (10 μm i.d., New Objectives, Woburn, MA) at a voltage of 2400 V. The instrument *m/z* response was calibrated with standards from the manufacturer.

4.2. Plant material

Stem tissue from 4th to 6th internodes (lignifying tissue) of branches with seven total internodes was collected from greenhouse grown alfalfa plants (cv Regen SY) and used as the source of cell wall proteins. The stems were frozen in liquid N_2 and stored at −80 °C until needed.

4.3. Isolation of cell wall proteins and protein quantification

Seven to eight grams of frozen and thawed tissue was ground with a mortar and pestle in ice-cold grinding buffer (50 mM Na acetate, pH 5.5, 50 mM NaCl, and 30 mM ascorbic acid) with 100 mg PVPP according to the method published at http://cellwall.genomicas.purdue.edu/techniques/index.html. The cell walls were isolated by filtering through nylon mesh (47 μm^2) membranes and washing sequentially with grinding buffer (100 ml total), 0.1 M NaCl (50 ml), dH_2O (100 ml), acetone (250 ml), dH_2O (100 ml), and 10 mM sodium acetate (50 ml) in 50 ml increments. Cell wall proteins were extracted sequentially, twice with 6–8 ml of 200 mM $CaCl_2$, 50 mM sodium acetate (incubated on ice for 30 min–1 h), followed by one extraction with 12–14 ml of 3.0 M LiCl, 50 mM sodium acetate (incubated on ice for 30 min–1 h). The $CaCl_2$ extracts were combined and the supernatants were centrifuged at 950*g* to remove any particulate matter. Proteins were concentrated and desalted (Amicon Ultra centrifugal filtration devices) and processed using the 2D Cleanup Kit. Samples were resuspended in 8 M urea, 4% chaps, 20 mM DTT, and 0.2% biolytes. Protein concentrations were determined using the Bradford method (Bradford, 1976) with BSA as the standard.

4.4. Two-dimensional electrophoresis and staining

Nonlinear, immobilized pH gradient (IPG) strips (11 cm, 3-10NL, BioRad) were rehydrated overnight and isoelectrically focused to 30,000 V h. After IEF and equilibration (Asirvatham et al., 2002), the strips were loaded onto second dimension gels of 12% polyacrylamide. The gels were fixed overnight and silver stained according to Blum et al. (1987). Three replicate protein preparations from separate biological samples were isolated and electrophoresed with similar results.

For the 1D PAGE analyses, samples from each step of the isolation procedure were precipitated with 10% TCA/acetone and resuspended in SDS sample buffer. Each gel sample was taken from the first 50 ml of the specific wash step. The $CaCl_2$ and LiCl extracted samples were processed using the 2D Cleanup kit and resuspended in SDS sample buffer.

4.5. Protein digestions and identification by LC/MS/MS analysis

Protein spots were excised and destained according to Asirvatham et al. (2002) and Sumner et al. (2002), and digested with trypsin according to Watson et al. (2003) with minor alterations. The concentration of trypsin was lowered to 8 ng/μl and the combined supernatants were concentrated to 10–15 μl rather than being completely dried. Separations of protein digests were performed using a nano HPLC system. Samples (5 μl) were loaded onto a C18 precolumn for desalting and concentrating at a flow rate of 50 μl/min in 5% acetonitrile containing 0.1% formic acid (solvent A). Peptides were then eluted from the precolumn and separated on a nano analytical C18 column at a flow rate of 200 nl/min using a linear separation gradient. The gradient was increased from 5% to 40% solvent B (95% acetontrile containing 0.08% formic acid) over 40 min, ramped to 95% B at 45 min, held at 95% B for 5 min, returned to 5% at 55 min, and held at 5% for 5 min to allow for column re-equilibration. The separated peptides were directly analyzed with an ABI QSTAR Pulsar hybrid quadrupole time-of-flight mass spectrometer (TOF-MS). TOF-MS and tandem TOF/MS/MS data were acquired on the QSTAR using an IDA (information dependent acquisition) feature in the Analyst QS software and the following parameters: selection of multiply charged precursor (charge state from 2 to 5) for fragmentation with a threshold at 5 cps (counts per second), precursor ion exclusion for 90 s and 6 amu, and CE (collision energy) dependent on the ions' *m/z* values.

4.6. Database queries and protein identifications

The acquired mass spectral data were searched against an in-house legume protein dataset using MASCOT search engine (Creasy and Cottrell, 2002; Perkins et al., 1999) with a mass tolerance of 150 ppm and one allowed trypsin miscleavage. Search parameters allowed for the variable oxidation of methionine and carbamidomethylation of cysteines. The legume protein dataset was generated at the Noble Foundation and contained annotated protein sequences. These sequences consisted of tentative consensus sequences or gene indices originally obtained from TIGR (http://www.tigr.org/tdb/tgi.shtml) and included: MtGI.0530002 (*Medicago truncatula*, 22,652 records), GmGI.052802 (soybean, 32,081 records), and LjGI.053102 (*Lotus japonica*, 7686 records). The dataset also included *M. truncatula*

chloroplast sequences (MtChI v.1, 156 records) and *Arabidopsis* mitochondrial proteins (AtMit v.1, 46 records) as these are underrepresented in the EST libraries (Asirvatham et al., in preparation). The inclusive legume protein dataset (62,651 records) was annotated using the custom software EST Analyzer (Liangjiang Wang, http://bioinfo.noble.org/). EST Analyzer is used to analyze EST or cDNA sequences for functional annotation, protein sequence derivation, and sequencing error detection and correction. For a given sequence query, EST Analyzer searches NCBI's NR protein dataset to identify a template for the query (the best hit of BLASTX search), then uses the template to annotate and translate the query sequence. Based on the alignment between the query sequence and template, frameshift errors are detected and corrected if possible. All protein sequences possible were annotated, given pseudo GI numbers, formatted similar to NCBInr to allow queries by MASCOT, and compiled as a legume specific protein dataset.

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References

Angiolella, L., Facchin, M., Stringaro, A., Maras, B., Simonetti, N., Cassone, A., 1996. Identification of a glucan-associated enolase as a main cell wall protein of *Candida albicans* and an indirect target of lipopeptide antimycotics. J. Infect. Dis. 173, 684–690.

Angiolella, L., Miccocci, M.M., D'Alessio, S., Girolamo, A., Maras, B., Cassone, A., 2002. Identification of major glucan-associated cell wall proteins of *Candida albicans* and their role in fluconazole resistance. Antimicrob. Agents Chemother. 46, 1688–1694.

Anterola, A.M., Lewis, N.G., 2002. Trends in lignin modification: a comprehensive analysis of the effects of genetic manipulations/mutations on lignification and vascular integrity. Phytochemistry 61, 221–294.

Asirvatham, V.S., Watson, B.S., Sumner, L.W., 2002. Analytical and biological variances associated with proteomic studies of *Medicago truncatula* by 2-DE. Proteomics 2, 960–968.

Baluška, F., Šamaj, J., Wojtaszek, P., Wolkmann, D., Menzel, D., 2003. Cytolskeleton–plasma membrane–cell wall continuum in plants. Emerging links revisited. Plant Physiol. 133, 482–491.

Blee, K.A., Wheatley, E.R., Bonham, V.A., Mitchell, G.P., Robertson, D., Slabas, A.R., Burrell, M.M., Wojtaszek, P., Bolwell, G.P., 2001. Proteomic analysis reveals a novel set of cell wall proteins in a transformed tobacco cell culture that synthesises secondary walls as determined by biochemical and morphological parameters. Planta 212, 404–415.

Blum, H., Beier, H., Gross, H.J., 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8, 93–99.

Borderies, G., Jamet, E., Lafitte, C., Rossignol, M., Januneau, A., Boudart, G., Monsarrat, B., Esquerré-Tugayé, M.-T., Boudet, A., Pont-Lezica, R., 2003. Proteomics of lossely bound cell wall proteins of *Arabidopsis thaliana* cell suspension cultures: a critical analysis. Electrophoresis 24, 3421–3432.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 292–297.

Canovas, F.M., Dumas-Gaudot, E., Recorbet, G., Jorrin, J., Mock, H.-P., Rossignol, M., 2004. Plant proteome analysis. Proteomics 4, 285–298.

Cassab, G.I., Nieto-Sotelo, J., Cooper, J.B., van Holst, G.-J., Varner, J.E., 1985. A developmentally regulated hydroxyproline-rich glycoprotein from the cell walls of soybean seed coats. Plant Physiol. 77, 532–535.

Cassab, G.I., 1998. Plant cell wall proteins. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 281–309.

Chivasa, S., Ndimba, B.K., Simon, W.J., Robertson, D., Yu, X.-L., Knox, J.P., Bolwell, P., Slabas, A.R., 2002. Proteomic analysis of the *Arabidopsis thaliana* cell wall. Electrophoresis 23, 1754–1765.

Copley, S.D., 2003. Enzymes with extra talents: moonlighting functions and catalytic propmiscuity. Curr. Opin. Chem. Biol. 7, 265–272.

Creasy, D.M., Cottrell, J.S., 2002. Error tolerant searching of uninterpreted tandem mass spectrometry data. Proteomics 2, 1426–1434.

Darley, C., Forrester, A., McQueen-Mason, S., 2001. The molecular basis of plant cell wall extension. Plant Mol. Biol. 47, 179–195.

Edwards, S.R., Braley, R., Chaffin, W.L., 1999. Enolase is present in the cell wall of *Saccharomyces cerevisiae*. FEMS Microbiol. Lett. 177, 211–216.

Emanuelsson, O., Nielsen, H., Brunak, S., von Heijne, G., 2000. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J. Mol. Biol. 300, 1005–1016.

Gao, M., Kieliszewski, M.J., Lamport, D.T.A., Showalter, A.M., 1999. Isolation, characterization and immunolocalization of a novel, modular tomato arabinogalactan-protein corresponding to the *LeAGP-1* gene. Plant J. 18, 43–55.

Gross, G.G., 1977. Cell wall-bound malate dehydrogenase from horseradish. Phytochemistry 16, 319–321.

Guo, D., Chen, F., Inoue, K., Blount, J., Dixon, R.A., 2001a. Downregulation of caffeic acid 3-*O*-methyltransferase and caffeoyl CoA 3-*O*-methyltransferase in transgenic alfalfa: impacts on lignin structure and implications for the biosynthesis of G and S lignin. Plant Cell 13, 73–88.

Guo, D., Chen, F., Wheeler, J., Winder, J., Selman, S., Peterson, M., Dixon, R.A., 2001b. Improvement of in-rumen digestibility of alfalfa forage by genetic manipulation of lignin *O*-methyltransferases. Transgenic Res. 10, 457–464.

Hatfield, R., Vermerris, W., 2001. Lignin formation in plants. The dilemma of linkage specificity. Plant Physiol. 126, 1351–1357.

Kärkönen, A., Koutaniemi, S., Mustonen, M., Syrjänen, K., Brunow, G., Kilpeläinen, I., Teeri, T.H., Simola, L.K., 2002. Lignification related enzymes in *Picea abies* suspension cultures. Physiol. Plant 114, 343–353.

Kleis-San Francisco, S.M., Tierney, M.L., 1990. Isolation and characterization of a proline-rich cell wall protein from soybean seedlings. Plant Physiol. 94, 1897–1902.

Li, Z.C., McClure, J.W., Hagerman, A.E., 1989. Soluble and bound apoplastic activity for peroxidase, β-D-glucosidease, malate dehydrogenase, and nonspecific arylesterase, in barley (*Hordeum vulgare* L.) and oat (*Avena sativa* L.) primary leaves. Plant Physiol. 90, 185–190.

Mäder, M., Schloss, P., 1979. Isolation of malate dehydrogenase from cell walls of *Nicotiana tobacum*. Plant Sci. Lett. 17, 75–80.

McDougall, G.J., 2000. A comparison of proteins from the developing xylem of compression and non-compression wood of branches of

Sitka spruce (*Picea sitchensis*) reveals a differentially expressed laccase. J. Exp. Bot. 51, 1395–1401.

McQueen-Mason, S., Durachko, D.M., Cosgrove, D.J., 1992. Two endogenous proteins that induce cell wall extension in plants. Plant Cell 4, 1425–1433.

Melan, M.A., Cosgrove, D.J., 1988. Evidence against the involvement of ionically bound cell wall proteins in pea epicotyl growth. Plant Physiol. 86, 469–474.

Minorsky, P.V., 2002. The wall becomes surmountable. Plant Physiol. 128, 345–353.

Ndimba, B.K., Chivasa, S., Hamilton, J.M., Simon, W.J., Slabas, A.R., 2003. Proteomic analysis of changes in the extracellular matrix of *Arabidopsis* cell suspension cultures induced by fungal elicitors. Proteomics 3, 1047–1059.

Nielsen, H., Engelbrecht, J., Brunak, S., von Heijne, G., 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their clevage sites. Prot. Eng. 10, 1–6.

Nielsen, H., Brunak, S., von Heijne, G., 1999. Machine learning approaches for the prediction of signal peptides and other protein sorting signals. Prot. Eng. 12, 3–9.

Perkins, D.N., Pappin, D.J., Creasy, D.M., Cottrell, J.S., 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20, 3551–3567.

Pitarch, A., Sánchez, M., Nombela, C., Gil, C., 2002. Sequential fractionation and two-dimensionsal analysis unravels the complexity of the dimorphic fungus *Candida albicans* cell wall proteome. Mol. Cell. Proteom. 1, 967–982.

Qin, Q., Bergmann, C.W., Rose, J.K.C., Saladie, M., Kolli, V.S.K., Albersheim, P., Darvill, A.G., York, W.S., 2003. Characterization of a tomato protein that inhibits a xyloglucan-specific endoglucanase. Plant J. 34, 327–338.

Robertson, D., Mitchell, G.P., Gilroy, J.S., Gerrish, C., Bolwell, G.P., Slabas, A.R., 1997. Differential extraction and protein sequencing reveals major differences in patterns of primary cell wall proteins from plants. J. Biol. Chem. 272, 15841–15848.

Robertson, L., Robertson, W.M., Sobczak, M., Helder, J., Tetaud, E., Ariyanayagam, M.R., Ferguson, M.A., Fairlamb, A., Jones, J.T., 2000. Cloning, expression and functional characterisation of a peroxiredoxin from the potato cyst nematode *Globodera rostochiensis*. Mol. Biochem. Parasitol. 111, 41–49.

Rose, J.K.C., Cosgrove, D.J., Albersheim, P., Darvill, A.G., Bennett, A.B., 2000. Detection of expansin proteins and activity during tomato fruit ontogeny. Plant Physiol. 123, 1583–1592.

Rose, J.K.C., 2003. In: The Plant Cell Wall, vol. 8. Blackwell Publishing Ltd., CRC Press, Oxford, UK.

Sumner, L.W., Wolf-Sumner, B., White, S.P., Asirvatham, V.S., 2002. Silver stain removal using H_2O_2 for enhanced peptide mass mapping by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom. 16, 160–168.

Suzuki, H., Xia, Y., Cameron, R., Shadle, G., Blount, J., Lamb, C.J., Dixon, R.A., 2004. Signals for local and systemic responses of plants to pathogen attack. J. Exp. Bot. 55, 169–179.

Voigt, J., Frank, R., 2003. 14-3-3 Proteins are constituents of the insoluble glycoprotein framework of the chlamydomonas cell wall. Plant Cell 15, 1399–1413.

Watson, B.S., Asirvatham, V.S., Wang, L., Sumner, L.W., 2003. Mapping the proteome of *Medicago truncatula*. Plant Physiol. 131, 1104–1123.

Western, T.L., Burns, J., Tan, W.L., Skinner, D.J., Martin-McCaffrey, L., Moffatt, B.A., Haughn, G.W., 2001. Isolation and characterization of mutants defective in seed coat mucilage secretory cell development in *Arabidopsis*. Plant Physiol. 127, 998–1011.

Xia, Y., Suzuki, H., Borewitz, J., Blount, J., Guo, Z., Patel, K., Dixon, R.A., Lamb, C., 2004. An extracellular aspartic protease mediates disease resistance in Arabidopsis. EMBO J. 23, 980–988.