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ORIGINAL ARTICLE

Tomomi Kaku · Satoshi Serada · Kei'ichi Baba
Fumio Tanaka · Takahisa Hayashi

Proteomic analysis of the G-layer in poplar tension wood

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Abstract Angiosperm trees bend their stems by forming tension wood at the upper side of leaning stems. Most tension wood has a cellulose-rich G-layer in the innermost surface of the fiber cell wall. Strong tensile stress is considered to occur in the G-layer. This study undertook to identify the proteins involved in G-layer formation and function through a proteomic analysis of G-layer-localized protein. G-layers of poplar were loosened by sonication and isolated as doughnut-shaped pieces of thinly sliced transverse sections. The proteins, once extracted with urea/detergent solution, were separated by two-dimensional polyacrylamide gel electrophoresis, and 110 spots were subjected to liquid chromatography tandem mass spectrometry (LC/MS/MS). A database search for these spots' mass spectrum patterns identified 72 proteins. In addition, all peptide digestion mixtures of G-layer proteins were separated by strong cation exchange chromatography and 39 proteins were identified using LC/MS/MS analysis. Proteins involved in wall formation, such as lignin biosynthesis-related protein, xyloglucan endotransglucosylase, and fasciclin-like arabinogalactan protein, were notably detected in the G-layer.

Key words G-layer · *Populus* · Proteomics · Tension wood

Introduction

Higher plants regulate their posture by bending their stems in response to environmental signals such as gravity, light, and mechanical stimuli. Woody plants bend not only their elongating apical stems by differential growth but also their secondary xylem by forming reaction wood on one side of their stems. Tension wood is one type of reaction wood that is formed on the upper sides of leaning stems of angiosperm trees. The other type of reaction wood is compression wood which is formed on the bottom sides of leaning stems of gymnosperm trees. In both types, the stems bend through mechanical action induced by abnormal growth stress, that is, either tension or compression. Tension wood bears tensile stress by forming a gelatinous layer known as the G-layer in the innermost surface of the cell wall, replacing the S2-layer. A typical G-layer consists mainly of highly crystalline cellulose, in which the microfibrils are oriented parallel to the axis of the fiber cell.[1] Chemical analysis indicates that G-layers contain small amounts of matrix components in addition to the cellulose. Recent work has revealed that, in poplar, xyloglucan is the most abundant noncellulosic component of the G-layer.[2] Several studies have reported the existence of other compounds in the G-layer, including pectin,[3] arabinogalactan,[4] β-1,4-galactan,[5] and lignin.[6] In spite of these discoveries, nothing is yet known about how the G-layer is produced and how it functions in tension wood.

Andersson-Gunnerås et al.[7] have published the results of a global analysis of gene transcripts and metabolites during tension wood formation in poplar. They showed that transcripts related to the major pathways involved in the biosynthesis of cell wall matrix carbohydrates and lignin were degraded during tension wood formation, and they identified several differentially expressed auxin-related and ethylene-related genes. Several unidentified proteins were found to be induced in the process of tension wood formation.[8] Beyond these observations, limited information is currently available on the formation and mechanical role of the G-layer.

Xyloglucan is incorporated into the G-layer by the activity of xyloglucan endotransglucosylase (XET).[2] The presence of XET protein in developing and mature G-layers was demonstrated by immunolabeling of tension wood with a polyclonal antibody against PttXET16A.[2] Interestingly, both XET protein and its activity persist in mature G-layers

T. Kaku · K. Baba · F. Tanaka · T. Hayashi (✉)
Research Institute for Sustainable Humanosphere, Kyoto University, Uji, Kyoto 611-0011, Japan
Tel. +81-774-38-3618; Fax +81-774-38-3618
e-mail: taka@rish.kyoto-u.ac.jp

S. Serada
National Institute of Biomedical Innovation, Ibaraki, Osaka 567-0085, Japan

for several years after cell death. In mature G-layers, a certain amount of protein is still detectable. We predicted that particular enzyme would still have a role for the maintenance of G-layers.

To determine the molecular basis of the G-layer, we performed a proteomic analysis of G-layer-localized protein in poplar mature xylem. We employed two proteomic approaches in this analysis: first, a two-dimensional gel electrophoresis coupled to liquid chromatography tandem mass spectrometry (LC/MS/MS); and second, a gel-free mass spectrometry technique that uses strong cation exchange chromatography coupled to LC/MS/MS. We expected that using two approaches would provide us with reliable information on which to base our theory of G-layer formation and function.

Materials and methods

Plant materials and isolation of G-layer

Field-grown poplars (*Populus alba*) were harvested in April 2006 in Fukuoka, Japan. Leaning stems and branches of about 5–10 cm in diameter were used as a source of tension wood. Isolation of the G-layers from the tension wood was performed as described by Norberg and Meier.[3] Twenty-micrometer-thick transverse sections were prepared from the tension wood with a sliding microtome. The sections were stored in 96% ethanol in order to loosen the G-layers from the S2-layers, and then they were treated with ultrasonic waves (Branson Ultrasonic Cleaner, Yamato, Tokyo, Japan) for 30 min on ice. The G-layers that were thus shaken out of the sections were isolated by filtering the sections through a 50-μm nylon mesh. Other small impurities were removed by filtration through a 10-μm nylon mesh.

Protein extraction

The obtained G-layers were rehydrated with several changes of water, and ground in 7 M urea containing 2 M thiourea, 4% (w/v) CHAPS buffer, and 10 mM dithiothreitol. The sample was centrifuged at 10 000 *g* for 20 min at 4°C, and the supernatant was retained. Proteins were precipitated using the 2-D Clean-Up Kit (GE Healthcare, Buckinghamshire, UK).

Sugar analysis

Hemicellulose of the isolated G-layer was extracted with 24% (w/w) KOH containing 0.02% (w/v) $NaBH_4$. The insoluble residue (cellulose) was solubilized with 72% (v/v) sulfuric acid. Total sugar content in each fraction was determined by the phenol–sulfuric acid method.[9] Methylation analysis was performed as described by Nishikubo et al.[2] Lignin content was determined by the Klason method.[10]

X-Ray diffraction

The G-layer preparation was subjected to X-ray diffraction measurement using a Rigaku UltraX-18HF with CuKα radiation at 30 kV and 100 mA. The degree of crystallinity of the G-layer was calculated from the peak for the (2 0 0) lattice plane according to the method by Segal et al.[11] The crystal size of cellulose microfibrils was calculated from the X-ray diffraction patterns according to the method of Scherrer.[12,13]

Two-dimensional gel electrophoresis

The proteins were solubilized in rehydration solution [7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.5% (v/v) IPG buffer pH 3-10 NL (GE Healthcare), 40 mM dithiothreitol]. The protein concentration was determined using a 2-D Quant Kit (GE Healthcare). One hundred micrograms of protein was directly loaded into an Immobiline DryStrip pH 3-10 NL, 7 cm (GE Healthcare), and isoelectric focusing was performed with a Multiphor II system (GE Healthcare) according to the manufacturer's protocol. The strips were shaken for 15 min in equilibration buffer [50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS)] containing 65 mM dithiothreitol, and for another 15 min in equilibration buffer containing 135 mM iodoacetamide. Then the strips were loaded on top of a polyacrylamide gel (90 × 70 × 1.0 mm) and subjected to SDS-polyacrylamide gel electrophoresis (PAGE).[14]

In-gel digestion and protein identification by LC/MS/MS

Proteins separated on 2D-PAGE were visualized with silver stain MS Kit (Wako, Osaka, Japan), and each band was cut into small pieces. The gel pieces were destained with 15 mM potassium ferricyanide in 50 mM sodium thiosulfate, and washed with 25 mM ammonium bicarbonate in 50% acetonitrile. They were dehydrated with acetonitrile and dried. The gel pieces were then rehydrated in 10 μl of 50 mM ammonium bicarbonate containing 0.1 μg of trypsin (Promega, Madison, WI, USA), and incubated at 37°C for 18 h. The resulting peptides were extracted with 50% acetonitrile in 5% trifluoroacetic acid, and dried. The digest was dissolved with 2% acetonitrile in 0.1% trifluoroacetic acid, separated with a column (20 × 15 cm) of C-18 RP (GL Sciences, Tokyo), and analyzed using a MAGIC 2002 HPLC system (Michrom Bioresources, Auburn, CA, USA) and an LCQ ion trap mass spectrometer (Thermo Electron, Waltham, MA, USA). Mass data were analyzed using the Mascot sequence database search program (Matrix Science, London, UK) against the National Center for Biotechnology Information (NCBI) nonredundant database with "Viridiplantae" as taxonomy. Homology identification was retained when the ion score was significant at $P < 0.05$. Where only one peptide was used in the identification of a specific protein, manual assignments of the spectra were performed.

Total peptides analysis by LC/MS/MS

The proteins (100 μg) that were extracted from G-layers were rehydrated in 10 μl of 50 mM ammonium bicarbonate containing 0.1 μg of trypsin (Promega), and incubated at 37°C for 18 h. The digest was dried and rehydrated with 10 mM ammonium formate (pH 3.8). The peptides were subjected to a column (1 × 1 cm) of Toyopearl SP-650M (Tosoh, Tokyo), and sequentially eluted with 0, 1, 10, 25, 50, and 100 mM ammonium sulfate. After the samples were treated with ZipTip C18 (Millipore, Milford, MA), LC/MS/MS analysis was performed as described above.

Results

Nature of isolated G-layers

Poplar G-layers were isolated as doughnut-shaped pieces from the thinly sliced transverse sections; they were loosened from their sections using sonication (Fig. 1). We examined a micrograph of isolated G-layers and confirmed that they remained intact after the treatment and there was little contamination of other cell wall layers (Fig. 1). Crude G-layers were treated with protein solubilization solution [7 M urea containing 2 M thiourea, 4% (w/v) CHAPS, and 40 mM dithiothreitol], and proteins were extracted. The protein-extracted G-layers were subjected to sugar composition analysis. Our data revealed that 10 mg dry weight of G-layer preparations consisted of 7.8 mg cellulose with 260 μg of proteins. The composition of the G-layers is shown in Table 1. Figure 2 shows the X-ray diffraction patterns of crude and protein-extracted G-layers. Four main peaks could be recognized at 14.8°, 16.4°, 22.7°, and 34.5°, corresponding to the (1 –1 0), (1 1 0), (2 0 0), and (0 0 4) lattice planes, respectively. The data indicates that these G-layer samples are the typical crystalline forms of cellulose I.[13] The crystallinity of the G-layers, estimated from the peak for the (2 0 0) lattice plane ($2\theta = 22.7°$), increased from 60% to 73% after the extraction of proteins. Apparent crystal size of cellulose microfibrils also increased from 3.55 nm to 3.64 nm after the extraction of proteins.

Protein identification using 2D-PAGE

Proteins extracted from G-layers were subjected to 2D-PAGE, in the course of which the protein preparation was subjected first to two isoelectric focusings (100 μg each) and then to two SDS-PAGEs, one with 7.5% polyacrylamide gel and the other with 12.5% polyacrylamide gel. After the proteins were stained with silver, a total of 110 spots were visible in the two kinds of 2D-PAGEs (Fig. 3). After in-gel digestion with trypsin, protein sequences were identified by an LC/MS/MS analysis coupled with a database search using the Mascot sequence database search program. The protein spots on each gel were numbered as shown in Fig. 3. Of these, 72 spots were selected to be summarized in Table 2. The differences of calculated molecular mass and pI from experimental mobility on 2D-PAGE suggest posttranslational modifications.

Table 1. Composition of G-layer from poplar tension wood

Component	Concentration (mg/10 mg dry weight)
Cellulose	7.8
Protein	0.26
Lignin	Not detected
Hemicellulose	1.9
Xylosyl terminal	0.022
Xylosyl 2- or 4-linked	0.056
Fucosyl terminal	0.0093
Glucosyl terminal	0.035
Glucosyl 4-linked	1.4
Glucosyl 4,6-linked	0.17
Galactosyl 2-linked	0.017
Galactosyl 3,6-linked	0.10
Mannosyl 4-linked	0.041

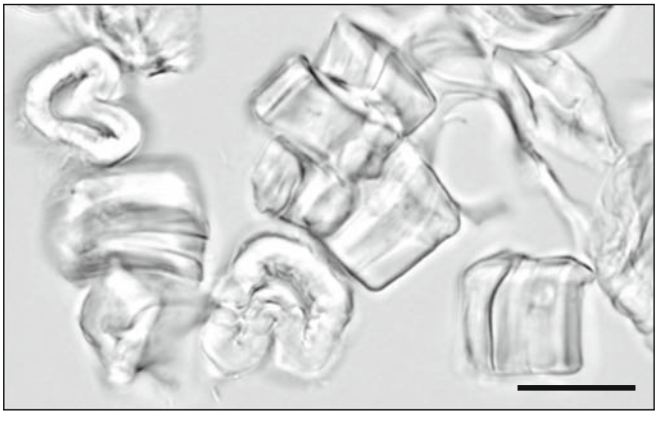

Fig. 1. Micrograph of the isolated G-layers obtained by ultrasonic treatment of transverse sections of tension wood. *Bar* 20 μm

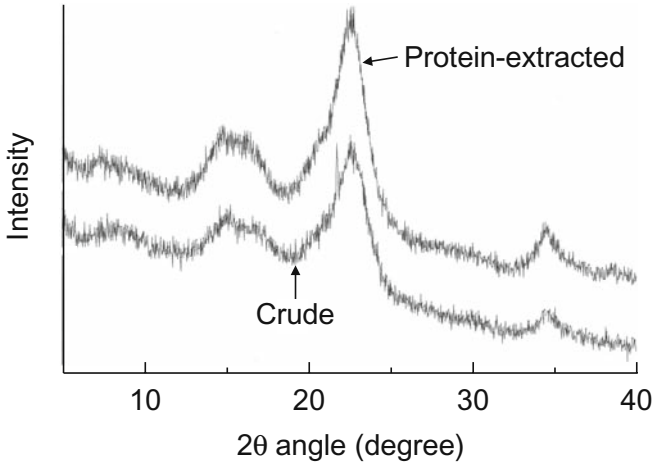

Fig. 2. X-Ray diffractograms of the G-layer. The crude isolated G-layer and the G-layer after extraction of proteins are shown

Table 2. Proteins identified from total spots from two-dimensional polyacrylamide gel electrophoresis

Spot no.	Accession no.	Protein name	Score	Mass	pI	Matched peptide no.	Sequence coverage (%)
7	gi|55168192	Putative aminotransferase [*Oryza sativa* (*japonica* cultivar-group)]	58	110 951	8.51	1	1
9	gi|50937821	Putative resistance complex protein I2C-1 [*Oryza sativa* (*japonica* cultivar-group)]	45	154 703	5.85	1	1
10	gi|9954728	Similar to tRNA-splicing endonuclease positive effector SEN1 [*Arabidopsis thaliana*]	47	242 277	8.14	1	1
11	gi|89257597	Hypothetical protein 27.t00104 [*Brassica oleracea*]	49	133 137	6.68	2	1
12	gi|51535846	Zinc finger protein-like [*Oryza sativa* (*japonica* cultivar-group)]	45	112 602	9.00	1	1
13	gi|1279654	Peroxidase [*Populus trichocarpa*]	104	37 081	4.74	2	8
17	gi|34908140	Putative HSP70 [*Oryza sativa* (*japonica* cultivar-group)]	173	71 306	5.10	6	12
	gi|8439545	Methionine synthase [*Solanum tuberosum*]	87	84 898	5.93	3	4
20	gi|3493039	Granule-bound starch synthase [*Sorghastrum nutans*]	57	27 876	5.76	1	5
	gi|68300810	RNA polymerase II second largest subunit [*Nicotiana sylvestris*]	49	123 992	9.07	1	1
	gi|7706850	ATP synthase beta subunit [*Androcymbium ciliolatum*]	48	50 326	5.08	1	3
21	gi|886471	Methionine synthase [*Catharanthus roseus*]	53	85 089	6.10	1	2
25	gi|51012459	Heat shock protein 70 [*Trypanosoma cruzi*]	55	71 876	5.15	1	2
26	gi|1279654	Peroxidase [*Populus trichocarpa*]	293	37 081	4.74	5	21
27	gi|2944417	Peroxidase FLXPER4 [*Linum usitatissimum*]	72	32 867	8.48	1	5
28	gi|60932273	UDP-glucuronic acid decarboxylase 3 [*Populus tomentosa*]	115	38 633	8.29	2	9
29	gi|20951	Lignin bispecific acid/5-hydroxyferulic acid methyltransferase [*Populus tremuloides*]	179	40 292	5.58	4	15
30	gi|7528266	Caffeic acid *O*-3-methyltransferase [*Populus tomentosa*]	179	39 662	5.24	4	15
	gi|4539543	Glyceraldehyde-3-phosphate dehydrogenase [*Nicotiana tabacum*]	131	36 816	7.70	4	9
	gi|60932273	UDP-glucuronic acid decarboxylase 3 [*Populus tomentosa*]	86	38 633	8.29	1	5
34	gi|56069874	Glyceraldehyde-3-phosphate dehydrogenase [*Musa acuminata*]	114	36 089	6.22	3	10
35	gi|56069874	Glyceraldehyde-3-phosphate dehydrogenase [*Musa acuminata*]	99	36 089	6.22	2	5
36	gi|20951	Lignin bispecific acid/5-hydroxyferulic acid methyltransferase [*Populus tremuloides*]	70	40 292	5.58	1	5
39	gi|56122822	Peroxidase [*Eucommia ulmoides*]	44	13 230	10.26	1	14
40	gi|1204054	*S*-Adenosylmethionine decarboxylase [*Brassica juncea*]	42	40 998	4.68	1	4
41	gi|34907582	Putative fructokinase I [*Oryza sativa* (*japonica* cultivar-group)]	77	34 869	5.07	1	5
42	gi|3114899	Phenylcoumaran benzylic ether reductase [*Populus balsamifera* subsp. *trichocarpa*]	129	33 986	5.66	4	11
	gi|31747338	Rubber elongation factor [*Hevea brasiliensis*]	57	14 713	5.04	1	12
43	gi|3114899	Phenylcoumaran benzylic ether reductase [*Populus balsamifera* subsp. *trichocarpa*]	73	33 986	5.66	2	6
44	gi|3114899	Phenylcoumaran benzylic ether reductase [*Populus balsamifera* subsp. *trichocarpa*]	356	33 986	5.66	11	33
	gi|108706728	Expressed protein [*Oryza sativa* (*japonica* cultivar-group)]	58	96 310	6.54	1	1
45	gi|3114899	Phenylcoumaran benzylic ether reductase [*Populus balsamifera* subsp. *trichocarpa*]	332	33 986	5.66	9	27
46	gi|3114899	Phenylcoumaran benzylic ether reductase [*Populus balsamifera* subsp. *trichocarpa*]	302	33 986	5.66	7	24
	gi|81239129	IAA-amino acid hydrolase 2 [*Brassica rapa*]	53	48 345	6.36	1	2
47	gi|3114899	Phenylcoumaran benzylic ether reductase [*Populus balsamifera* subsp. *trichocarpa*]	50	33 986	5.66	1	3
48	gi|3114899	Phenylcoumaran benzylic ether reductase [*Populus balsamifera* subsp. *trichocarpa*]	167	33 986	5.66	6	22
50	gi|3114899	Phenylcoumaran benzylic ether reductase [*Populus balsamifera* subsp. *trichocarpa*]	85	33 986	5.66	3	7

Table 2. *Continued*

Spot no.	Accession no.	Protein name	Score	Mass	pI	Matched peptide no.	Sequence coverage (%)
52	gi|1498338	Actin [Glycine max]	122	37 397	5.82	4	13
53	gi|124109181	XET16E [*Populus tremula*]	83	31 291	8.20	2	6
55	gi|46401612	Cysteine proteinase [*Dianthus caryophyllus*]	95	51 631	4.88	1	3
	gi|6752882	Nascent polypeptide associated complex alpha chain [*Pinus taeda*]	55	22 452	4.32	1	8
56	gi|8099063	14-3-3 protein [*Populus* × *canescens*]	142	29 421	4.68	4	12
	gi|3114899	Phenylcoumaran benzylic ether reductase [*Populus balsamifera* subsp. *trichocarpa*]	94	33 986	5.66	3	9
	gi|34907582	Putative fructokinase I [*Oryza sativa* (*japonica* cultivar-group)]	66	34 869	5.07	1	5
58	gi|4206687	Ribulose-1,5-bisphosphate carboxylase/oxygenase [*Rumohra adiantiformis*]	44	48 992	7.77	1	4
59	gi|56069874	Glyceraldehyde-3-phosphate dehydrogenase [*Musa acuminata*]	139	36 089	6.22	4	12
64	gi|12580867	60S ribosomal protein L13E [*Picea abies*]	54	23 815	10.95	1	5
66	gi|8515888	14-3-3 protein [*Populus* × *canescens*]	57	29 754	4.75	1	6
67	gi|8515888	14-3-3 protein [*Populus* × *canescens*]	111	29 754	4.75	3	12
	gi|34915072	Putative ribosomal protein S4 [*Oryza sativa* (*japonica* cultivar-group)]	100	28 658	10.09	3	8
71	gi|12381898	Eukaryotic initiation factor 4A [*Oryza sativa*]	72	47 320	5.51	1	4
75	gi|1498338	Actin [Glycine max]	58	37 397	5.82	1	5
78	gi|87247473	Putative 40S ribosomal protein S7 [*Populus* × *canadensis*]	43	18 975	9.95	1	5
79	gi|87247473	Putative 40S ribosomal protein S7 [*Populus* × *canadensis*]	55	18 975	9.95	1	7
83	gi|54291123	Hypothetical protein [*Oryza sativa* (*japonica* cultivar-group)]	44	18 717	10.11	1	8
84	gi|35187104	Kunitz trypsin inhibitor 3 [*Populus balsamifera* subsp. *trichocarpa* × *Populus deltoides*]	63	22 274	5.68	1	7
86	gi|54291123	Hypothetical protein [*Oryza sativa* (*japonica* cultivar-group)]	48	18 717	10.11	1	9
90	gi|13274148	Putative CuZn-superoxide dismutase [*Populus tremula* × *Populus tremuloides*]	138	21 593	6.23	2	15
91	gi|13274148	Putative CuZn-superoxide dismutase [*Populus tremula* × *Populus tremuloides*]	187	21 593	6.23	3	26
92	gi|13274150	Putative cytosolic CuZn-superoxide dismutase [*Populus tremula* × *Populus tremuloides*]	155	15 358	5.47	3	23
93	gi|18654	Unnamed protein product [Glycine max]	71	18 491	5.82	2	11
94	gi|18654	Unnamed protein product [Glycine max]	112	18 491	5.82	4	24
96	gi|95106179	Cytoplasmic Cu/Zn-superoxide dismutase [*Populus suaveolens*]	144	15 530	5.77	3	28
97	gi|95106179	Cytoplasmic Cu/Zn-superoxide dismutase [*Populus suaveolens*]	194	15 530	5.77	4	39
99	gi|563329	Histone 2B [*Asparagus officinalis*]	93	16 606	10.02	2	14
100	gi|563329	Histone 2B [*Asparagus officinalis*]	96	16 606	10.02	2	15
101	gi|16396	Nucleoside diphosphate kinase [*Arabidopsis thaliana*]	63	16 286	7.03	1	11
102	gi|16396	Nucleoside diphosphate kinase [*Arabidopsis thaliana*]	75	16 286	7.03	1	12
105	gi|71040669	Nucleoside diphosphate kinase I [*Arachis hypogaea*]	48	16 429	6.31	1	9
106	gi|19611	Histone H3 (AA 1-123) [*Medicago sativa*]	58	13 915	10.99	2	11
107	gi|71040669	Nucleoside diphosphate kinase I [*Arachis hypogaea*]	50	16 429	6.31	1	10
108	gi|71040669	Nucleoside diphosphate kinase I [*Arachis hypogaea*]	56	16 429	6.31	1	11

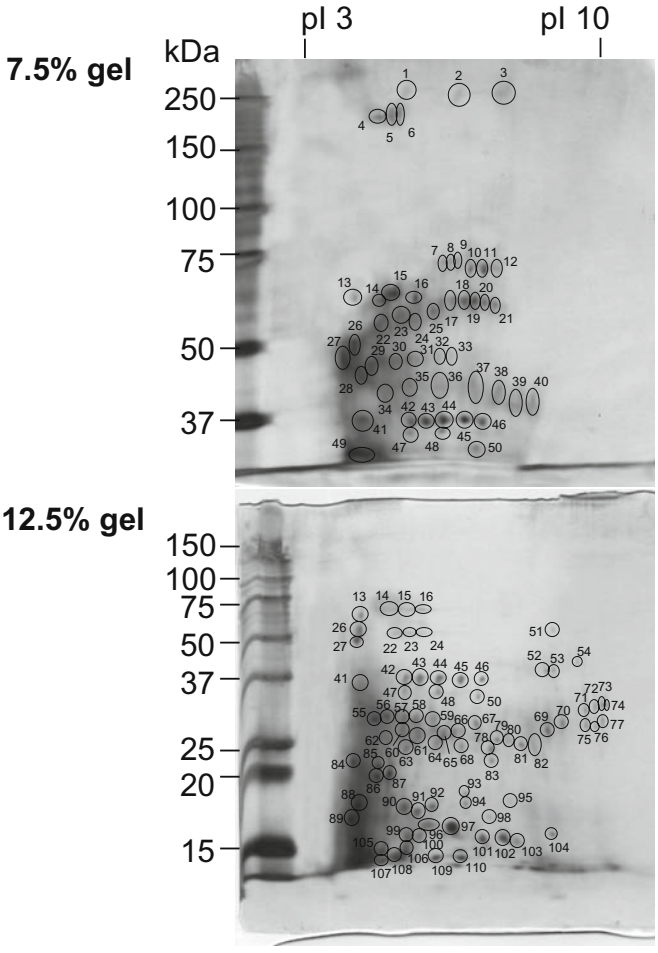

Fig. 3. Two-dimensional gel electrophoresis (2D-PAGE) of G-layer proteins. Isoelectric point (pI) gradient strips with pI 3–10 were loaded with 100 μg of extracted proteins. Proteins after the isoelectric focusing were separated by 7.5% (*upper*) or 12.5% (*lower*) polyacrylamide gels. Protein spots disclosed by silver staining were numbered and subjected to liquid chromatography tandem mass spectrometry

Protein identification from total peptides

We separated the trypsin-digested G-layer proteins by cation exchange chromatography by sequential elution with 0, 1, 10, 25, 50, and 100 mM ammonium sulfate. Each fraction was subjected to LC/MS/MS and analyzed by the Mascot sequence database search program. Sixty-three unique peptides corresponding to 39 proteins were identified. Table 3 shows a functional classification of the identified proteins.

Discussion

The proteins we identified using 2D-PAGE (Table 2) were classified according to function, and organized according to frequency in Fig. 4. Most abundant were lignin synthesis-related proteins, such as peroxidase,[15] lignin bispecific acid methyltransferase,[16] and phenylcoumaran benzylic ether reductase,[17] although the G-layers did not contain lignin

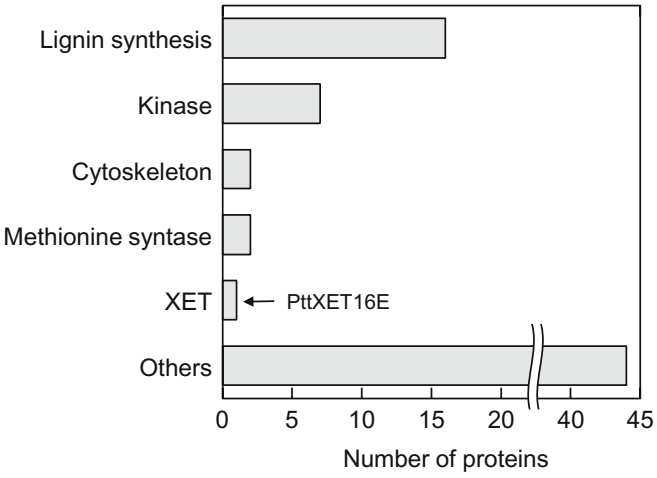

Fig. 4. Functional classification of proteins identified from 2D-PAGE. *XET* shows the one poplar xyloglucan endotransglucosylase (XET) isozyme, PttXET16E

itself (Table 1). Many putative functions of peroxidase have been suggested, and some peroxidases have been thought to be involved in lignin polymerization.[18] One of the identified peroxidases (gi number 1279654) has been reported to be present in differentiating xylem, supporting a lignin-specific function in secondary wall formation.[15] Although Andersson-Gunneråas et al.[7] has not sequenced this gene, they reported that some peroxidase genes had increased transcript levels in tension wood formation. They also reported that two caffeic acid *O*-methyltransferase gene transcripts (*COMT1* and *COMT2*), involved in lignification, were decreased during tension wood formation. It has been shown that phenylcoumaran benzylic ether reductase is the most abundant protein in the secondary xylem of poplar,[19] suggesting that it plays a general role in wood development. The transcripts of phenylcoumaran benzylic ether reductase genes were decreased in tension wood.[7] We also found one xyloglucan endotransglucosylase (XET) isozyme, PttXET16E, which might serve to tighten xyloglucan to give a tensile stress in the G-layer. The poplar cDNA microarray[7] revealed that the *PttXET16C* gene was highly expressed in developing tension wood compared with normal wood. Nishikubo et al.[2] established a XET gene-specific macroarray for 16 poplar-expressed XET genes, and reported that *PttXET16D*, *PttXET16G*, *PttXET16K*, and *PttXET16R* genes were up-regulated during tension wood formation. However, none of these gene products was detected in the G-layers isolated in the present study. XET is proposed to reinforce the xyloglucan network in the G-layer to form a further connection between wall-bound and secreted xyloglucans.

Proteins were also identified from the total trypsin-digested peptides of G-layer proteins. The observed proteins are classified as cytoskeleton proteins, lignin synthesis-related proteins, methionine synthesis-related proteins, cell wall-related proteins, or other proteins (Table 2). As discussed above, the lignin synthesis-related proteins were also identified through 2D-PAGE analysis. In

Table 3. Proteins identified from total peptides

	Accession no.	Protein name	Score	Mass	pI	Matched peptide no.	Sequence coverage (%)
Cytoskeleton	gi|5230841	Actin [*Malva pusilla*]	260	41 968	5.39	4	15
	gi|27819095	Alpha tubulin [*Populus tremuloides*]	243	50 262	4.95	4	17
	gi|29124983	Alpha-tubulin 1 [*Populus tremuloides*]	120	50 277	4.92	2	10
	gi|15242516	ACT7 [*Arabidopsis thaliana*]	112	41 937	5.31	2	8
	gi|20148289	Beta tubulin [*Arabidopsis thaliana*]	101	51 272	4.77	3	10
	gi|56181504	Putative actin 1 [*Chorispora bungeana*]	96	40 336	5.56	2	7
Lignin	gi|20951	Lignin bispecific acid/5-hydroxyferulic acid methyltransferase [*Populus tremuloides*]	197	40 292	5.58	3	14
	gi|7528266	Caffeic acid *O*-3-methyltransferase [*Populus tomentosa*]	97	39 662	5.20	2	10
	gi|3114899	Phenylcoumaran benzylic ether reductase [*Populus trichocarpa*]	65	33 986	5.66	1	6
	gi|1279654	Peroxidase [*Populus trichocarpa*]	55	37 081	4.74	1	7
Methionine	gi|1655578	*S*-Adenosyl-L-methionine synthetase 2 [*Catharanthus roseus*]	304	43 433	5.51	4	17
	gi|6716760	Methionine synthase [*Coffea arabica*]	222	24 659	5.69	3	18
	gi|55670137	Cobalamine independent methionine synthase [*Arabidopsis thaliana*]	120	84 283	6.02	1	2
	gi|30688090	5-Methyltetrahydropteroyltriglutamate-homocysteine *S*-methyltransferase [*Arabidopsis thaliana*]	105	90 993	8.17	2	2
	gi|166872	*S*-Adenosylmethionine synthetase [*Arabidopsis thaliana*]	100	43 573	5.50	2	8
	gi|4558554	*S*-Adenosylmethionine synthase 2 [*Arabidopsis thaliana*]	77	43 627	5.67	1	3
	gi|14532772	Methionine synthase [*Arabidopsis thaliana*]	61	84 900	6.09	1	2
Cell wall	gi|47717905	Fasciclin-like AGP 1 [*Populus alba* × *Populus tremula*]	44	27 546	7.01	1	3
	gi|47717907	Fasciclin-like AGP 2 [*Populus alba* × *Populus tremula*]	40	24 974	8.86	1	12
Others	gi|397482	Heat shock protein 70 cognate [*Arabidopsis thaliana*]	132	71 726	5.03	2	6
	gi|60932273	UDP-glucuronic acid decarboxylase 3 [Populus tomentosa]	109	38 633	8.29	1	4
	gi|95106179	Cu/Zn-superoxide dismutase [*Populus suaveolens*]	107	15 530	5.77	2	17
	gi|53139492	Glyceraldehyde-3-phosphate dehydrogenase [*Populus tremula*]	93	14 747	8.90	2	21
	gi|34907582	Putative fructokinase I [*Oryza sativa* (*japonica* cultivar-group)]	81	34 869	5.07	1	4
	gi|33945771	Alcohol dehydrogenase 1 [*Populus tremula*]	79	22 974	7.78	1	9
	gi|37790792	Sucrose synthase [*Populus tremuloides*]	78	93 149	6.02	1	3
	gi|4433627	Putative fructose-bisphosphate aldolase [*Dendrobium grex* Madame Thong-In]	63	19 268	9.21	1	14
	gi|20260174	Enolase (2-phospho-D-glycerate hydroylase) [*Arabidopsis thaliana*]	61	48 032	5.45	1	3
	gi|15218869	Isocitrate dehydrogenase (NADP+)/ oxidoreductase [*Arabidopsis thaliana*]	60	46 059	6.13	1	3
	gi|13924490	Glutamine synthetase [*Populus alba* × *Populus tremula*]	59	39 479	5.52	1	4
	gi|293887	Glyceraldehyde-3-phosphate dehydrogenase [*Zea mays*]	55	25 044	8.44	1	5
	gi|2551	Ubiquitin [*Coprinellus congregatus*]	51	8536	6.56	1	11
	gi|50911295	Hypothetical protein [*Oryza sativa* (*japonica* cultivar-group)]	50	7176	7.60	1	17
	gi|13274150	Putative CuZn-superoxide dismutase *Populus tremula* × *Populus tremuloides*]	49	15 358	5.47	1	6
	gi|16225	Calmodulin [*Arabidopsis thaliana*]	46	15 648	4.20	1	12
	gi|1087071	2-Phospho-D-glycerate hydrolase; enolase [*Mesembryanthemum crystallinum*]	43	48 661	5.62	1	4
	gi|90762150	Phosphomannomutase [Glycine max]	43	28 172	5.84	1	6
	gi|33411641	GID2 [*Oryza sativa* (*japonica* cultivar-group)]	42	23 265	6.63	1	6
	gi|556171	Triosephosphate isomerase [*Coptis japonica*]	41	27 244	5.54	1	5

addition, two fasciclin-like arabinogalactan proteins (AGP1 and AGP2) were detected in the total peptides analysis. Gene transcripts encoding fasciclin-like AGPs have been also reported to be highly expressed in tension wood,[4,7] indicating the importance of fasciclin-like AGP in the formation of tension wood. The poplar cDNA microarray[7] revealed that fasciclin-like *AGP1* gene was the most increased transcript during tension wood formation.

Plant hormones have long been implicated in tension wood formation. A large set of auxin-related and ethylene-related genes were reported to be differentially expressed in developing tension wood.[7] Our data did not reveal the existence of proteins related to hormonal signaling in the G-layer. Auxin and ethylene are considered to be important for induction of tension wood, but its signaling-related proteins are unlikely to remain in the G-layer.

Our proteome profiling of poplar G-layer is believed to provide new clues to highlight candidate proteins responsible for the G-layer mechanics. Previous studies using poplar expressed sequence tag (EST) libraries were based on comparisons of the two physiological tissues of wood. However, gene expressions do not link the properties of G-layers. This work highlights the G-layer-localizable protein set. Whether proteins localized in mature G-layers have enzymatic activities and functions is still unclear. Functional analysis for each of these identified proteins should extend our understanding of the role of the G-layer and the regulation of posture of woody plants.

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