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Proteomic Analysis to Identify Tightly-Bound Cell Wall Protein in Rice Calli

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Rice is a model plant widely used for basic and applied research programs. Plant cell wall proteins play key roles in a broad range of biological processes. However, presently, knowledge on the rice cell wall proteome is rudimentary in nature. In the present study, the tightly-bound cell wall proteome of rice callus cultured cells using sequential extraction protocols was developed using mass spectrometry and bioinformatics methods, leading to the identification of 1568 candidate proteins. Based on bioinformatics analyses, 389 classical rice cell wall proteins, possessing a signal peptide, and 334 putative non-classical cell wall proteins, lacking a signal peptide, were identified. By combining previously established rice cell wall protein databases with current data for the classical rice cell wall proteins, a comprehensive rice cell wall proteome, comprised of 496 proteins, was constructed. A comparative analysis of the rice and Arabidopsis cell wall proteomes revealed a high level of homology, suggesting a predominant conservation between monocot and eudicot cell wall proteins. This study importantly increased information on cell wall proteins, which serves for future functional analyses of these identified rice cell wall proteins.

INTRODUCTION

In higher plants, the cell wall, also known as the extracellular matrix, functions as a means of mechanical support as well as establishing an apoplastic matrix for signaling and defense. The initial cell wall is deposited during cytokinesis and is termed the primary wall; it is composed of polysaccharides, such as cellulose, hemicelluloses, and pectins (Keegstra, 2010; Pettolino et al., 2012; Tan et al., 2013; Yang et al., 2011). In the case where wall strengthening is required, this is achieved through the formation of secondary walls and, here, lignin is also incorporated into the polysaccharide matrix. These wall constituents are thought to play important roles in wall maintenance, metabolic and developmental regulation, as well as responses to environmental cues (Jamet et al., 2008; Rose and Lee, 2010). As a prerequisite to developing a comprehensive understanding of the functional importance of cell wall proteins, it will be necessary to compile cell wall proteomes for a number of model plant species. To this end, cell wall proteomics studies have been performed on alfalfa, Arabidopsis, chickpea, maize, rice, soybean, tobacco, tomato, and wheat (Bayer et al., 2006; Bhushan et al., 2006; Chen et al., 2009; Cho et al., 2009; Dahal et al., 2010; Komatsu et al., 2010; Kong et al., 2010; Millar et al., 2009; Watson et al., 2004; Zhu et al., 2007). Of these plant species, the most extensive database has been developed for Arabidopsis; here, some 500 proteins have been identified using a combination of 2D-gel/MudPIT and genome/bioinformatics tools (Bayer et al., 2006; Borderies et al., 2003; Chambon et al., 2005; Chivas et al., 2002; Ndimbra et al., 2003).

To date, rice cell wall proteomics studies have focused on identifying non-bound apoplastic and weakly-bound proteins, but not tightly-bound cell wall proteins. Other studies have investigated the induction of wall proteins in response to cold, dehydration, pathogen challenge and chemical stress (Chen et al., 2009; Cho et al., 2009; Cui et al., 2005; Ge et al., 2008; Jung et al., 2008; Kim et al., 2009; Pandey et al., 2010; Zhou et al., 2011). Previously, our group employed a rice callus culture system to identify non-redundant classical cell wall proteins containing conventional signal peptides. Here, 249 and 153 cell wall proteins were characterized as weakly-bound and secreted proteins, respectively (Chen et al., 2009; Cho et al., 2009). Given that the number of identified rice classical cell wall proteins was significantly below that observed for Arabidopsis, it would seem that this database is not yet comprehensive in nature.

Cell wall proteins have various affinities to the extracellular matrix, ranging from fully mobile proteins to those that are tightly integrated into the matrix, via covalent linkages; these latter proteins cannot be extracted by strong salt solutions (Jamet et al., 2008; Rose and Lee, 2010). Destructive methods, which require extensive grinding of the plant material, CaCl2 extraction and SDS/dithiothreitol extraction, have previously been employed to extract tightly-bound cell wall proteins (Feiz et al.,...
2006; Jamet et al., 2008; Rose and Lee, 2010). In the present study, an expanded rice cell wall proteome, based on callus cultured cells, was developed using mass spectrometry and bioinformatics methods. A combination of destructive and sequential extraction protocols was employed to purify tightly-bound wall proteins; this approach led to the identification of 1,999 candidate proteins. Based on bioinformatics analyses, 389 classical rice cell wall proteins, possessing a signal peptide, and putative 334 non-classical cell wall proteins, lacking a signal peptide, were identified. The classical rice callus cell wall proteins were compared to previously established rice callus cell wall protein databases, and a comprehensive rice callus cell wall proteome containing 496 proteins was constructed. A comparative analysis of the rice and Arabidopsis cell wall proteomes revealed a high level of homology, suggesting a predominant conservation between monocot and eudicot cell wall proteins.

**MATERIALS AND METHODS**

**Callus induction**

Rice callus cultures were established using the following procedure. Rice seeds (Oryza sativa L. cv. 'Dongjin') were first dehusked and washed in tap water to remove dust and other surface contaminants. Washed seeds were then surface-sterilized for 30 min in 2% NaOCl solution, rinsed extensively (3 times) with sterilized water and then inoculated on Nitsch's basal (NB) callus induction medium (N6 major salts, N6 minor salts, N6 vitamins, 1 g/L casamino acids, 30 g/L sucrose, 2 mg/L 2,4-D, 2 g/L Gelrite, pH 5.8), as previously described (Hiei et al., 1994). Callus formation was induced by culturing seeds at 30ºC in darkness for three weeks. Proliferating calli were sub-cultured on NB medium every two weeks.

**Isolation of cell walls from rice calli**

Aliquots (20 g) of cultured rice calli (Fig. 1A) were first frozen in liquid nitrogen and cells were then disrupted using several rounds of vortexing in a commercial blender that was pre-chilled. Disrupted rice calli were further homogenized using a mortar and pestle. Wall preparation buffer (WPB; 50 mM Tris, pH 8.0, 100 mM KCl, 10% v/v glycerol, 10 mM EDTA, 1 mM DTT and 1 mM phenylmethanesulfonylfluoride [PMSF]) was added to homogenized calli (4 ml/g of wall preparation) and the mixture was then centrifuged, at 400x g, for 5 min using a 5810 R centrifuge (Eppendorf AG, Germany). The supernatant was discarded and the pellet (Fig. 1B) was resuspended in 400 ml WPB, without PMSF. This suspension was then further homogenized using three rounds of French press treatment (13 MPa minimal outlet aperture pressure). Next, 200 ml of WPB was added, followed by sonication (1 min × 10 cycles). Aliquots of the suspended pellet (Fig. 1C) were equally distributed into four 250 ml tubes. Each tube was centrifuged at 428x g for 3 min, the pellet was then washed with 50 ml of WPB containing 0.1% triton X-100 and recentrifuged at 260x g for 3 min; this step was repeated twice, followed by centrifugation at 115x g for 3 min. After each centrifugation, the supernatant was removed. Finally, the pellet was washed five times with 50 ml of WPB without triton X-100 and then centrifuged at 115x g for 3 min, yielding a clear supernatant.

**Protein extraction from rice cell walls**

To extract proteins from purified rice callus cell walls, two volumes of 0.2 M CaCl_2 solution were added to the final cell wall pellet and the mixture was incubated for 2 h with stirring at 4ºC.
After centrifugation at 15000× g for 3 min, the supernatant was collected and extracted proteins were incubated with four volumes of cold acetone for 2 h at 4°C. The mixture was centrifuged at 15000× g for 15 min, and the resultant pellet was dried and resuspended in a minimal volume of sample buffer. The remaining pellet was further sequentially washed with 0.2 M CaCl₂ solution and TBS buffer. Residual proteins were extracted from the pellet by boiling for 5 min in two volumes of 2X sodium dodecyl sulfate (SDS) extraction buffer (62 mM Tris-HCl, pH 6.8, 2% SDS, 10% v/v glycerol, 0.005% bromophenol blue, 100 mM dithiothreitol).

**In-gel digestion and peptide sample preparation**

Extracted proteins were loaded onto a 12% polyacrylamide mini-gel (5 × 8 cm) for 1-D SDS-PAGE (100 V for 45 min in SDS-PAGE running buffer; 25 mM Tris, pH 8.3, 192 mM glycine, 0.1% SDS). Analytical and preparative gels were visualized with silver-staining (Yan et al., 2000) and Coomassie brilliant blue (CBB) staining buffer (50% methanol, 10% glacial acetic acid, 40% H₂O, 0.5 g/L CBB), respectively. Gels were washed thoroughly with destaining buffer (30% methanol, 10% glacial acetic acid in H₂O) and then cut into high and low molecular weight portions. These gel pieces were trypsin-digested and processed for LC-MS/MS analysis.

**MudPIT analysis**

MudPIT analysis was conducted as described previously (Cho et al., 2009). In brief, peptides were pressure-loaded onto a fused silica capillary column (100 µm i.d.) that contained 7 cm of 5-µm Aqua C18 material (Phenomenex, USA), 3 cm of 5 µm Partisphere strong cation exchanger (Whatman, USA), and 3 cm of 5-µm Aqua C18 reversed phase column material (Phenomenex). Peptides were eluted from the microcapillary column and electrosprayed into a LTQ linear ion trap mass spectrometer (ThermoElectron), with a 2.3-kV spray voltage and then cut into mass lists to yield high- and low molecular weight portions. The MS/MS spectra were reassigned with corresponding rice accession numbers, and maximum of two missed cleavage sites for trypsin digestion. Proteins that contained similar peptides and could not be differentiated, based on MS/MS analysis alone, were grouped to satisfy the principles of parsimony.

**Bioinformatics analyses**

SignalP 3.0 program including two different algorithms (Signal-NN and SignalP-HMM; [http://www.cbs.dtu.dk/services/SignalP](http://www.cbs.dtu.dk/services/SignalP)) was used to predict the presence and location of signal peptide cleavage sites in each identified protein sequence (Emanuelsson et al., 2007). The 0.43 value was employed as a default cutoff in both NN and HMM algorithms. If the score was higher than the selected cutoff, the comment would be ‘Yes’, otherwise the comment would be ‘No’. To predict transmembrane domains, in each identified protein sequences, the TMHMM v. 2.0 program was applied ([http://www.cbs.dtu.dk/services/TMHMM](http://www.cbs.dtu.dk/services/TMHMM); Möller et al., 2001). The cutoff value for the TMHMM program was 0.5, indicating the presence of predicted transmembrane helices.

The TargetP 1.1 was used to predict proteins localized to plastids and mitochondria with appropriate default parameters (Emanuelsson et al., 2007). Non-classical secreted proteins were predicted by the SecretomeP program and proteins with a neural network-score (NN-score) more than 0.5 were considered as non-classical secreted proteins (Bendtsen et al., 2004). Three different web-based programs, PredGPI prediction server ([http://gpcr2.biocomp.unibo.it/gpindex/index.htm](http://gpcr2.biocomp.unibo.it/gpindex/index.htm)), GPI-SOM ([http://gpi.unibe.ch/](http://gpi.unibe.ch/)) and Big-PI Predictor ([http://mendel.imp.ac.at/gpi/gpi_server.html](http://mendel.imp.ac.at/gpi/gpi_server.html)), were used to identify glycosylphosphatidylinositol (GPI) anchored signals (Eisenhaber et al., 2003; Fankhauser and Mäser, 2005; Pierleoni et al., 2008). In general, proteins with GPI-anchoring signals should possess a signal peptide for the secretory pathway; therefore, proteins having both N- and C-terminal signals were regarded as GPI-anchored proteins by the GPI-SOM program. The specificity cutoff for the PredGPI program should be more than 99% and the Big-PI program produced the quality of the site with P or S.

Conserved protein domains were identified by performing InterProScan ([http://www.ebi.ac.uk/Tools/InterProScan](http://www.ebi.ac.uk/Tools/InterProScan)) implemented in Blast2GO (Götz et al., 2008; Mulder and Apweiler, 2007). When a protein contained endoplasmic reticulum (ER) retention signals, like KDEL or HDEL sequences at the C-terminus, it was regarded as an ER protein (Denecke et al., 1997). To remove redundant rice proteins, all identified proteins were reassigned with corresponding rice accession numbers, using BLASTP ([http://blast.ncbi.nlm.nih.gov/Blast.cgi](http://blast.ncbi.nlm.nih.gov/Blast.cgi)) (Altschul et al., 1997). In order to find homologous Arabidopsis cell wall proteins, all rice proteins were blasted against the whole Arabidopsis proteome. For the BLASTP analysis, an E-value of 1E-6 was used as a threshold.

**Immunoblot analyses**

To perform immunoblot analyses of the extracted proteins, two different antibodies were used. One was anti-actin known to detect proteins localized in the cytoplasm and plastomes (PD). The second was anti-BiP, which detects the molecular chaperone, BiP, located in the ER lumen. Proteins were separated on 10% SDS-PAGE gels and electro-transferred onto PVDF membrane. After blocking with 7% skim milk, the membrane was incubated with rabbit polyclonal anti-actin (LabFrontier) or mouse monoclonal anti-BiP antibodies (Stressgen). Then horseradish peroxidase-conjugated donkey anti-rabbit, or goat anti-mouse, second-ary antibodies (Santa Cruz Biotechnology) were used to react with anti-actin and anti-BiP, respectively. The antibodies were detected by chemiluminescence with an ECL kit (Amersham Biosciences).

**Cloning, agroinfiltration and confocal imaging**

To validate cell wall localization of non-classical secreted proteins, two candidate proteins, LOC_Os02g37710.1 and LOC_Os01g71860.1, were selected. The respective full length CDS was amplified without its stop codon by PCR with gene specific primers.
profiles as compared to the cytosolic proteins (Fig. 1D). In both gels. These cell wall-extracted proteins displayed quite different as well as cytosolic proteins, were loaded onto 1-D SDS-PAGE in the low molecular weight regions below 20 kDa (Fig. 1D).

Note that the band intensity for cell wall proteins extracted with treatments, strong protein bands were detected predominantly in the cell wall pellet. The CaCl₂ and SDS extracted cell wall proteins, including 100 mM dithiothreitol, was used to remove residual tightly-bound cell wall proteins from the cell wall fractions are likely highly enriched with extracellular proteins, some 553 and 580 proteins were identified from the high and low molecular weight sections (Fig. 1D). Following in-gel trypsin digestion, proteins contained within the CaCl₂ and SDS extracted cell wall fractions are likely highly enriched with extracellular proteins. In this regard, actin detected in the SDS fraction may reflect protein derived from PD (Baluska et al., 2004). To prepare the isolated cell wall proteins for interrogation by mass spectrometry, samples were first separated on 1-D SDS-PAGE based on the scheme presented in Fig. 2. (A) CaCl₂-extracted proteins were separated and processed based on the scheme presented in Fig. 1. CaCl₂-H and CaCl₂-L; total number of proteins identified within the high (H) and low (L) molecular weight fractions. (B) SDS-extracted proteins were separated and processed based on the scheme presented in Fig. 1. SDS-H and SDS-L; total number of proteins identified within the high (H) and low (L) molecular weight fractions. (C) Comparison between rice proteomes extracted by CaCl₂ and SDS methods. A total of 1999 non-redundant rice callus proteins were identified using this sequential two-step extraction protocol.

Agrobacterium cells harboring each GFP fusion construct were grown to an OD600 of 1.0 in liquid yeast extract peptone (YPE) medium (1% peptone, 1% yeast extract, and 0.5% NaCl) supplemented with 50 µg/ml kanamycin, 50 µg/ml rifampicin and 25 µg/ml gentamicin. After inoculation at 28°C. Cells were pelleted, resuspended at OD600 = 1.0 in infiltration buffer [10 mM MgCl₂, 10 mM MES (pH 5.6), 100 µM acetoxymeron], incubated for 2.5 h at room temperature, and infiltrated into the abaxial side of 6-week-old Nicotiana benthamiana leaves using a 1-ml needleless syringe. Agrobacterium harboring the Tomato bushy stunt virus P19 silencing suppressor was also co-infiltrated in order to minimize the gene silencing effects on heterologous gene expression in tobacco.

After 36 h post-infiltration, green fluorescent signals were observed in leaves using an Olympus (Japan) confocal laser scanning microscope (model FV1000). Excitation and emission wavelengths for GFP were 488/ 510-540 nm. To perform plasmolysis experiments, cut leaf sections from N. benthamiana leaves transiently expressing GFP fusion constructs were vacuum-infiltrated in 0.8 M mannitol for 1 h. After a short incubation period, GFP signals were observed using a CLSM.

RESULTS

Extraction and identification of rice callus cell wall proteins

To extract tightly-bound cell wall proteins, two extraction buffers were sequentially employed. The highly purified cell wall fraction was first subjected to 0.2 M CaCl₂ extraction solution that has been well characterized as an excellent agent to remove cell wall proteins (Kim et al., 2013; Robertson et al., 1997). Next, an SDS extraction buffer, including 100 mM dithiothreitol, was used to remove residual tightly-bound cell wall proteins from the cell wall pellet. The CaCl₂ and SDS extracted cell wall proteins, as well as cytosolic proteins, were loaded onto 1-D SDS-PAGE gels. These cell wall-extracted proteins displayed quite different profiles as compared to the cytosolic proteins (Fig. 1D). In both cell wall protein fractions extracted by the CaCl₂ and SDS treatments, strong protein bands were detected predominantly in the low molecular weight regions below 20 kDa (Fig. 1D).

Note that the band intensity for cell wall proteins extracted with SDS was stronger than that for the CaCl₂ treatment. Furthermore, a number of high molecular weight bands were present in the SDS extracted cell wall proteins (Fig. 1D), indicating that SDS/dithiothreitol-based extraction is much effective compared to CaCl₂-based extraction.

To evaluate the purity of these cell wall-extracted proteins, western blot analysis was performed using antibodies specific for actin, a cytosolic/plasmodesmal marker, and BiP, an endo-

plasmic reticulum (ER) lumen-localized soluble protein. Strong BiP signals were detected in only the leaf (Ls) and callus (Cs) soluble fractions (Fig. 1E), but not in pellet fractions (Lp and Cp). Actin was detected strongly in the leaf (Ls), callus (Cs) soluble fractions and weakly in callus pellet (Cp) (Fig. 1F). Furthermore, actin was also detected in the SDS-extracted cell wall fraction (Fig. 1F). These results are consistent with the hypothesis that the proteins contained within the CaCl₂ and SDS extracted cell wall fractions are likely highly enriched with extracellular proteins. In this regard, actin detected in the SDS fraction may reflect protein derived from PD (Baluska et al., 2004). To prepare the isolated cell wall proteins for interrogation by mass spectrometry, samples were first separated on 1-D SDS-PAGE gels which were subsequently divided into high and low molecular weight sections (Fig. 1D). Following in-gel trypsin digestion, samples were analyzed by LC-MS/MS and protein identification achieved using a TurboSEQUEST program in conjunction with the rice protein database (Fig. 1G). Full details regarding peptide sequences are contained in Supplementary Tables S1-S4.

All identified rice proteins with GenBank identifier (GI) numbers were converted to the corresponding rice protein locus, based on the rice genome annotation (http://rice.plantbiology.msu.edu/) (Ouyang et al., 2007). After removal of redundant proteins, some 553 and 580 proteins were identified from the high and low molecular weight gel sections, respectively, based on the CaCl₂ extraction protocol (Fig. 2A). Statistics for SDS extracted proteins were 1216 and 668 proteins contained within the high and low molecular weight gel fractions, respectively (Fig. 2B). More proteins (1469 in total) were contained...
### Table 1. Representative rice classical cell wall proteins containing signal peptides predicted by SignalP 3.0

| Gi       | Function                          | Molecular mass | pl  | Sequence count | Sequence coverage |
|----------|-----------------------------------|----------------|-----|----------------|------------------|
| 21104619 | Thaumatin family                  | 26189          | 7.7 | 104            | 92%              |
| 62733218 | Glycosyl hydrolase                | 33947          | 9.3 | 64             | 79%              |
| 113683   | Alpha-amylose                     | 48708          | 5.6 | 62             | 73%              |
| 55168113 | Glycosyl hydrolase                | 32549          | 6.5 | 59             | 70%              |
| 50918709 | Periplasmic beta-glucosidase       | 67778          | 7.5 | 45             | 43%              |
| 34899554 | Glycosyl hydrolase family 31      | 102551         | 6.5 | 40             | 43%              |
| 50508897 | Glucan endo-1, 3-beta-glucosidase | 59363          | 5.4 | 33             | 64%              |
| 50940911 | Cysteine-rich repeat secretory protein 55 | 30046 | 7.9 | 33             | 67%              |
| 50905163 | Retrotransposon protein           | 70761          | 7.7 | 28             | 35%              |
| 51963488 | Polygalacturonase inhibitor        | 35465          | 7.4 | 28             | 76%              |
| 50940909 | Cysteine-rich repeat secretory protein 55 | 29629 | 7.9 | 26             | 62%              |
| 53792759 | Retrotransposon protein           | 56264          | 7.9 | 23             | 41%              |
| 113682   | Alpha-amylose                     | 47911          | 6.4 | 23             | 59%              |
| 51964350 | Dehydrogenase                     | 42702          | 7.8 | 21             | 55%              |
| 50940901 | Cysteine-rich repeat secretory protein 55 | 29695 | 7.9 | 21             | 57%              |
| 113766   | Alpha-amylose                     | 47756          | 5.3 | 20             | 59%              |
| 57900682 | Unknown protein                   | 44330          | 6.5 | 19             | 36%              |
| 50509692 | Beta-1,3-glucanase                | 53753          | 6.1 | 26             | 0.585 0.725      |
| 51964830 | Unknown protein                   | 67154          | 7.2 | 20             | 0.386 0.599      |
| 22830913 | Endo-1,3-beta-glucanase           | 67635          | 5.5 | 18             | 0.37 0.683       |
| 460989   | Beta tubulin                      | 43002          | 4.9 | 14             | 0.386 0.55       |
| 38345164 | Unknown protein                   | 71231          | 8.8 | 11             | 0.255 0.566      |
| 50912401 | Aldehyde dehydrogenase            | 45483          | 6.3 | 11             | 0.333 0.543      |
| 50938789 | Early nodulin 8 like protein      | 22215          | 8.2 | 9              | 0.47 0.582       |
| 3023713  | Enolase                           | 47987          | 5.6 | 8              | 0.215 0.53       |
| 38346903 | Unknown protein                   | 52786          | 8.3 | 8              | 0.176 0.503      |
| 50510001 | Prohibitin                        | 31955          | 9.8 | 8              | 0.343 0.607      |
| 51964530 | Unknown protein                   | 46830          | 8.2 | 8              | 0.208 0.702      |
| 730456   | 40S ribosomal protein S19         | 16387          | 10  | 7              | 0.507 0.703      |
| 34893994 | 40S ribosomal protein S5          | 22227          | 9.7 | 7              | 0.25 0.827       |
| 38344200 | Unknown protein                   | 55227          | 7.9 | 7              | 0.153 0.539      |
| 542153   | Translation initiation factor eIF-4A | 46932      | 5.5 | 6              | 0.232 0.573      |
| 37533060 | Unknown protein                   | 96740          | 5.8 | 6              | 0.085 0.589      |
| 42408023 | Phosphoribosyl pyrophosphate synthetase | 36149 | 6.8 | 6              | 0.265 0.823      |
| 50907169 | Unknown protein                   | 90986          | 6.5 | 6              | 0.129 0.587      |
| 51854281 | Unknown protein                   | 90130          | 5.5 | 6              | 0.117 0.525      |

*aGi (GenBank identifier) number indicates the protein accession number in NCBI.

### Table 2. Representative rice non-classical cell wall proteins that lack a signal peptide as predicted by SecretomeP 2.0

| Gi       | Function                        | Molecular mass | pl  | Sequence count | Sequence coverage | NN-scoreb |
|----------|---------------------------------|----------------|-----|----------------|------------------|-----------|
| 51963380 | Unknown protein                 | 67355          | 6.7 | 27             | 0.576 0.524      |
| 50509692 | Beta-1,3-glucanase              | 53753          | 6.1 | 26             | 0.585 0.725      |
| 51964830 | Unknown protein                 | 67154          | 7.2 | 20             | 0.386 0.599      |
| 22830913 | Endo-1,3-beta-glucanase         | 67635          | 5.5 | 18             | 0.37 0.683       |
| 460989   | Beta tubulin                    | 43002          | 4.9 | 14             | 0.386 0.55       |
| 38345164 | Unknown protein                 | 71231          | 8.8 | 11             | 0.255 0.566      |
| 50912401 | Aldehyde dehydrogenase          | 45483          | 6.3 | 11             | 0.333 0.543      |
| 50938789 | Early nodulin 8 like protein    | 22215          | 8.2 | 9              | 0.47 0.582       |
| 3023713  | Enolase                         | 47987          | 5.6 | 8              | 0.215 0.53       |
| 38346903 | Unknown protein                 | 52786          | 8.3 | 8              | 0.176 0.503      |
| 50510001 | Prohibitin                      | 31955          | 9.8 | 8              | 0.343 0.607      |
| 51964530 | Unknown protein                 | 46830          | 8.2 | 8              | 0.208 0.702      |
| 730456   | 40S ribosomal protein S19       | 16387          | 10  | 7              | 0.507 0.703      |
| 34893994 | 40S ribosomal protein S5        | 22227          | 9.7 | 7              | 0.25 0.827       |
| 38344200 | Unknown protein                 | 55227          | 7.9 | 7              | 0.153 0.539      |
| 542153   | Translation initiation factor eIF-4A | 46932      | 5.5 | 6              | 0.232 0.573      |
| 37533060 | Unknown protein                 | 96740          | 5.8 | 6              | 0.085 0.589      |
| 42408023 | Phosphoribosyl pyrophosphate synthetase | 36149 | 6.8 | 6              | 0.265 0.823      |
| 50907169 | Unknown protein                 | 90986          | 6.5 | 6              | 0.129 0.587      |
| 51854281 | Unknown protein                 | 90130          | 5.5 | 6              | 0.117 0.525      |

*aGi (GenBank identifier) number indicates the protein accession number in NCBI.
bCutoff value of the NN-score for non-classical cell wall proteins is greater than 0.5.
Table 3. GPI-anchored proteins isolated from the rice cell wall preparation identified by three prediction programs

| GIa  | Functionb | Rice locus | Evalue | Length | Molecular mass | pI | Omega-sitec |
|------|-----------|------------|--------|--------|----------------|----|-------------|
| 62733403 | Os3bglu8 - beta-glucosidase | LOC_Os03g49610.1 | 0 | 603 | 67245 | 7.4 | 573 |
| 55733786 | Glucan endo-1,3-beta-glucosidase-like protein 3 | LOC_Os05g43690.1 | 5.00E-52 | 228 | 21760 | 5.7 | 204 |
| 51964118 | Glycerophosphoryl diester phosphodiesterase family | LOC_Os02g37590.1 | 0 | 749 | 81084 | 6.1 | 723 |
| 51963864 | Glucan endo-1,3-beta-glucosidase | LOC_Os02g04670.1 | 0 | 489 | 53375 | 5.5 | 459 |
| 51243456 | LysM domain-containing GPI-anchored protein 1 | LOC_Os06g10660.1 | 0 | 409 | 40488 | 4.9 | 387 |
| 50943329 | Plastocyanin-like domain containing protein | LOC_Os08g17160.1 | 3.00E-69 | 193 | 19274 | 8.3 | 169 |
| 50941247 | Monocopper oxidase | LOC_Os08g05820.1 | 0 | 600 | 66229 | 6.8 | 576 |
| 50939031 | X8 domain containing protein | LOC_Os07g40940.1 | 7.00E-43 | 191 | 18902 | 5.7 | 167 |
| 50932835 | X8 domain containing protein | LOC_Os05g50490.3 | 1.00E-136 | 281 | 28222 | 6 | 255 |
| 50919115 | Glucan endo-1,3-beta-glucosidase | LOC_Os03g57880.3 | 0 | 491 | 52783 | 5 | 467 |
| 50918839 | X8 domain containing protein | LOC_Os03g54910.3 | 1.00E-68 | 175 | 17825 | 4.8 | 149 |
| 50912821 | Eukaryotic aspartyl protease domain containing protein | LOC_Os02g51540.1 | 0 | 520 | 56272 | 6 | 494 |
| 50907029 | Pectinesterase | LOC_Os02g18650.1 | 0 | 554 | 58764 | 9.5 | 536 |
| 38605955 | Fascilinin-like arabinogalactan protein | LOC_Os04g48490.1 | 1.00E-170 | 431 | 44536 | 6.7 | 410 |
| 37808880 | Retrotransposon protein | LOC_Os08g16810.1 | 8.00E-51 | 130 | 13441 | 10.4 | 102 |
| 37530136 | Thaumatin-like protein 1 | LOC_Os10g05600.1 | 1.00E-135 | 389 | 38581 | 4.8 | 362 |
| 34897712 | Monocopper oxidase | LOC_Os06g01490.1 | 0 | 593 | 65750 | 6.5 | 570 |

GI (Gene Identifier) number indicates the protein accession number in NCBI.

BPutative functions of 17 GPI-anchored proteins based on the annotation of the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/).

cOmega-site for GPI-anchored signal in protein sequences was predicted by the Big-PI program.

Table 4. A comparison between the classical cell wall proteins identified by four different cell wall proteome studies. Criterion for classical cell wall proteins is that they contain a signal peptide predicted by SignalP3.0

| Description | Weakly bound proteinsa | Purely secreted proteinst | Tightly bound proteins (CaCl2)c | Tightly bound proteins (SDS)d |
|-------------|-----------------------|--------------------------|-------------------------------|-------------------------------|
| No. of classical cell wall proteins | 205 | 153 | 251 | 312 |
| No. of proteins without a signal peptide | 1456 | 402 | 722 | 1157 |
| No. of total proteins | 1705 | 555 | 973 | 1469 |
| Ratio of classical cell wall proteins to total proteins | 14.6% | 27.6% | 25.8% | 21.2% |

aStudy on weakly-bound rice cell wall proteome (Chen et al., 2009)
bStudy on rice secretome (Cho et al., 2009)
cCurrent study on tightly-bound rice cell wall proteome extracted by CaCl2
dCurrent study on tightly-bound rice cell wall proteome extracted by SDS

within the SDS-extracted fraction when compared to the 971 proteins extracted by CaCl2 (Fig. 2C). The combined total of all rice proteins identified in this study was 1999; 530 and 1028 proteins were unique and 441 proteins were common for the CaCl2- and SDS-extracted fractions, respectively (Fig. 2C). Proteins predicted to contain a signal peptide

In general, protein targeting to the extracellular space requires an N-terminal signal peptide that mediates in the translocation of secreted proteins through the ER/Golgi pathway (Rabouille et al., 2012). The presence of a signal peptide in the N-terminus of identified cell wall proteins was investigated using the SignalP
Identification of proteins containing predicted transmembrane helices

Given the intimate association between the plant cell wall and the plasma membrane, it is technically difficult, if not impossible, to separate completely the proteins from these two cellular compartments. In the case of plasma membrane proteins, the plasma membrane contains both ER and plasma membrane components. Thus, it is likely that our cell wall proteome will include PD-localized proteins that are inserted within the matrix of the cell wall and contain both ER and plasma membrane components. Furthermore, PD are plant-specific intercellular organelles that are inserted within the matrix of the cell wall and contain both ER and plasma membrane components. Thus, it is likely that our cell wall proteome will include PD-localized proteins that are inserted within the matrix of the cell wall and contain both ER and plasma membrane components. However, in other cases, the plasma membrane components were identified as likely being targeting to plastids and mitochondria, plastids and mitochondria, and the ER v. 2.0 program was used to identify candidate proteins containing transmembrane helices (Möller et al., 2001). As expected, 243 cell wall proteins were predicted to contain one or more transmembrane domains (Supplementary Table S5). For example, 25% and 21% of the proteins within the CaCl₂ and SDS extracted fractions, respectively, were predicted to be membrane proteins (Fig. 3B).

The number of transmembrane helices for any given cell wall protein was highly variable, ranging from one to 20 domains. For instance, there were 170 CaCl₂ and 215 SDS extracted proteins that contained a single transmembrane domain (Fig. 3B). Here, some 27 predicted receptor-like kinase proteins were identified that contained a single transmembrane domain (Supplementary Table S6). In contrast, two proteins, callose synthase 1 (LOC_Os03g03610.3) and embryogenesis transcriptional protein (LOC_Os06g18880.1), have 15 and 20 predicted transmembrane domains, respectively (Supplementary Table S6).

Identification of classical cell wall proteins based on bioinformatics

A further analysis of the signal peptide-containing cell wall proteins (Fig. 3A) identified 458 non-redundant proteins, which were predicted as a signal peptide-containing cell wall protein by both the NN and HMM algorithms (Supplementary Table S6). Among them, 205 were classified as a signal peptide and these were included in the secreted protein category (Supplementary Table S6). Proteins contained within the ER possess classical retention signals, such as an HDEL or KDEL sequence located at the C-terminus. Interestingly, we identified only two DnaK family proteins (LOC_Os05g35400.1 and LOC_Os02g02400.1) and a hydrolase (LOC_Os01g37960.1) that possessed ER retention motifs (Supplementary Table S6). This finding supports the notion that our extracted rice proteins are specifically enriched for cell wall proteins. After exclusion of 66 plasma membrane and 3 ER proteins, based on the above criteria, we identified some 389 classical rice cell wall proteins predicted to be secreted into the extracellular matrix, via a default secretory pathway (Table 1 and Supplementary Table S7). In order to remove proteins targeted to organelles, such as plastids and mitochondria, the TargetP 1.1 program was employed to screen the remaining rice proteins (Emanuelsson et al., 2007). Based on this analysis, 205 and 226 proteins were identified as likely being targeting to plastids and mitochondria, respectively (Fig. 4). We next used the SecretomeP program to identify potential non-classical secreted proteins (Bendtsen et al., 2004). Although this program was initially designed for mammalian cells, we assumed that secreted proteins in eukaryotic cells might well have common properties. In any event, using this approach 334 putative non-conventional secreted proteins were identified (Table 2, Fig. 4 and Supplementary Table S8A). The cell wall localization of such proteins was ex-
contaminated proteins. Non-classical cell wall proteins were identified only 20 such proteins (Fig. 5).

GPI-anchored proteins, respectively, whereas the Big-PI program employed experimental data, predicted 62 and 2003). The GPI-SOM and Pred-GPI programs predicted 62 and 17 proteins predicted by the TargetP 1.1 program and considered as contaminated proteins. Non-classical cell wall proteins were predicted by the SecretomeP 2.0 program. Proteins containing an ER retention motif (KDEL or HEDL) at C-terminal region were identified as ER proteins.

Identification of GPI-anchored proteins

In plants, many proteins can be anchored to the extracellular surface of the plasma membrane following glycosylphosphatidylinositol (GPI) posttranslational modification. In general, GPI-anchored proteins have two highly conserved domains, composed of an N-terminal signal peptide and a C-terminal hydrophobic signal sequence, termed the omega site (Eisenhaber et al., 2003). This site is located between 20 and 30 residues upstream of the C-terminus. During posttranslational processing in the ER, the omega site in GPI-anchored proteins is cleaved and a glycolipid is then attached.

An earlier genome-wide analysis, using computational methods, identified some 198 and 180 GPI-anchored proteins in rice and Arabidopsis, respectively (Eisenhaber et al., 2003). To identify putative GPI-anchored proteins, we next used the Pre-GPI, Big-PI and GPI-SOM programs (Eisenhaber et al., 2003; Fankhauser and Mäser, 2005; Pierleoni et al., 2008). Among these programs, the Big-PI program employs experimental data, likely making it the more reliable method (Eisenhaber et al., 2003). The GPI-SOM and Pre-GPI programs predicted 62 and 73 GPI-anchored proteins, respectively, whereas the Big-PI identified only 20 such proteins (Fig. 5).

Distribution of molecular mass and isoelectric points for the rice cell wall proteome

The molecular mass distribution of the 1999 putative cell wall proteins was highly variable, ranging from 8 to 359 kDa, with the average being 60 kDa (Supplementary Table S6). Due to the unbiased characteristics of MudPIT technology, the molecular mass of these proteins exhibited a normal distribution (Supplementary Fig. S1A). The molecular mass distribution of the 389 cell wall and 243 membrane proteins was also examined; profiles similar to that of the total proteome were obtained (Supplementary Figs. S1B and S1C).

The isoelectric points (pI) of proteins in the rice cell wall proteome ranged from 4.3 to 11.9, with 7.5 being the average value (Supplementary Table S6). Here, only 49 and 34 proteins had values less than 5 and higher than 11, respectively (Supplementary Fig. S1D). The distribution patterns of the 1999 proteins and the 389 cell wall proteins were comparable, without any bias towards acidic or alkali proteins (Supplementary Figs. S1D and S1E). This result is similar to that of an earlier Arabidopsis cell wall proteome study (Bayer et al., 2006). Interestingly, as shown in Supplementary Fig. S1F, the 243 membrane proteins displayed a dissimilar pI pattern to that of the secreted proteins.

Functional characterization of the rice cell wall proteome

To gain insights into the role of the proteins contained within the
rice cell wall proteome, the Blast2GO program was used to assign putative functions, based on gene ontology (GO) (Götz et al., 2008; Jensen and Bork, 2010). Using this approach, some 1200 proteins were annotated in two GO categories, namely biological processes and molecular functions. Based on biological processes, many of these proteins were predicted to be involved in a wide range of metabolic pathways (890 proteins), from response to various stimuli (455 proteins) to developmental processes (413 proteins) and gene expression (263 proteins) (Supplementary Fig. S2A). These findings are consistent with previous studies which have established that cell wall proteins participate in numerous metabolic processes and responses to environmental cues (Jamet et al., 2008). Similarly, based on molecular function, many proteins were assigned to binding (969 proteins), catalytic (842 proteins), hydrolase (414 proteins), transferase (214 proteins), structural (108 proteins) and kinase (106 proteins) activities (Supplementary Fig. S2B).

To further analyze the functions likely associated with the 389 classical rice cell wall proteins, we next examined the conserved domains of each protein using the InterProScan database implemented in the Blast2GO program (Götz et al., 2008; Mulder and Apweiler, 2007). Proteins in the following families were found to be abundant: peroxidases (36), peptidase A1 (22) and glycoside hydrolases (36) (Fig. 6A). Protein families containing fewer members included the peptidase S10-serine carboxypeptidase (11), germin (8), phosphate-induced protein 1 (7), expansin (7) and alpha-amylase (7).

We next categorized these 389 conventional cell wall proteins into eight functional groups, according to the previous annotation of WallProtDB (http://www.polebio.scvs.ups-tlse.fr/WallProtDB/). Here, some 80% of the identified proteins could be assigned into functional groups: among them were proteins acting on cell wall polysaccharides (22%), proteases (14%), oxido-reductases (13%), but various miscellaneous proteins (15%) were also present (Fig. 6B).

Of the identified 334 non-classical rice cell wall proteins, a large fraction (approx. 150 proteins) was assigned as being of unknown function. To further analyze this group, they were examined for conserved domains using the InterProScan database: a total of 793 redundant domains were identified. Domains for armadillo-type fold, RNA recognition motif, nucleotide-binding, F-box domain, protein kinase-like domain, glycoside hydrolase, NAD(P)-binding domain, and tubulin were frequently found (Supplementary Table S8B).

Comparison of rice and Arabidopsis cell wall proteomes

To develop a comprehensive rice cell wall proteome database, we integrated data from our current and previous studies. For this purpose, we used only classical cell wall proteins that possess a signal peptide. As shown in Fig. 7, four independent rice cell wall proteomes have been established using rice callus cultured material. The first study used a non-destructive mannitol/CaCl₂ method to identify some 205 weakly-bound cell wall proteins (Chen et al., 2009). The second study focused on proteins secreted into the rice callus culture medium; here, 153 cell wall proteins were detected (Cho et al., 2009). The third and fourth studies used CaCl₂- and SDS-based protein extraction protocols; 251 and 312 tightly-bound cell wall proteins were extracted, respectively (current study).

Then, we established an expanded rice cell wall proteome database comprised of 496 classical secreted proteins (Supplementary Table S9). This rice database was next used to
analyze the level of conservation with the current Arabidopsis cell wall proteome, containing some 244 conventional secreted proteins (Bayer et al., 2006). To this end, the BLASTP program was used with an 1E-6 cutoff value; here, 166 and 56 cell wall proteins were identified that appeared to be specific for rice and Arabidopsis, respectively (Fig. 8, Supplementary Tables S10A and S10B). However, 330 rice (67%) and 188 Arabidopsis (77%) proteins were common to both proteomes, consistent with a high level of conservation between the cell wall proteomes of the monocots and eudicots (Fig. 8). Future functional analyses of these identified rice cell wall proteins will offer important insights into the roles they perform in wall chemistry, signaling and defense.

**DISCUSSION**

In the present study, a large scale proteomics experiment was performed to explore the nature of tightly-bound proteins present in the rice callus cell wall. To this end, we employed a combination of destructive methods, two protein extraction protocols and a MudPIT interrogation approach. Total 1999 proteins were identified by a combination of destructive methods (Fig. 2C). This total is relatively high as compared to 2-DGE-based proteomics, in which around 54-300 cell wall proteins have been identified (Jung et al., 2008; Kim et al., 2013; Zhou et al., 2011), indicating that this current study led to the identification of a significant number of additional plant cell wall-associated proteins.

In our current rice cell wall proteome, we identified 389 classical cell wall proteins possessing a conventional signal peptide, as well as 334 putative non-classical cell wall proteins that lacked such signal peptides. Approximately 80% of the classical cell wall proteins have functions related to cell wall polysaccharide chemistry, as well as to protease and oxido-reductase activities; however, some 40% of the identified non-classical cell wall proteins have unknown functions (Supplementary Tables S7 and S8A) and two candidate proteins LOC_Os02g 37710.1 and LOC_Os01g71860.1 have been experimentally validated for their secretion into extracellular space (Supplementary Fig. S3). This suggests that these non-classical proteins, which utilize unconventional export mechanisms, are likely to represent important components of the extracellular matrix. To advance plant cell wall biology, it will be important to unravel the molecular mechanisms that evolved to mediate unconventional protein secretion. In addition, it is important to note that a number of proteins identified in this study are homologous to known non-classical secreted proteins in other organisms, including bacteria, yeast, and mammals for which experimental evidence exists. Examples are annexin, enolase and elongation factor (Edwards et al., 1999; Marcilla et al., 2012; Marques et al., 1998). One such protein, elongation factor 1α that lacks a signal peptide was isolated from tobacco and has been shown to localize to the cell wall by immunogold localization (Zhu et al., 1994). In addition, GAPDH is another known non-classical cell wall protein in bacteria and yeast (Eichenbaum et al., 1996; Gozalbo et al., 1998). The GAPDH domain containing protein (LOC_Os04g40950.1) identified in our study was assigned as an unknown protein without predicted localization. Regardless of proteins that are secreted, via either the classical or non-conventional secretory pathway, it is necessary to stress that other proteins identified in the rice cell wall proteome could well function in unknown biological processes by interacting with various components of the cell wall. Molecular methods will be needed to probe the cell wall localization of these proteins (Groover et al., 2003).

In conclusion, we have analyzed the tightly-bound cell wall proteins in rice calli using a combination of destructive methods. This sensitive and relatively unbiased approach identified a range of proteins involved in metabolic processes and responses to environmental cues. A comparative analysis of the Arabidopsis cell wall proteome and comprehensive rice cell wall proteome generated by combining our current and previous studies (Chen et al., 2009; Cho et al., 2009), suggests a predominant conservation between monocot and eudicot cell wall proteins. This rice cell wall proteome dataset could increase information on cell wall proteins, thus it will act as a valuable resource for further functional analysis of cell wall proteins.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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**REFERENCES**

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389-3402.

Baluska, F., Hlavacka, A., Volkmann, D., and Menzel, D. (2004). Getting connected: actin-based cell-to-cell channels in plants and animals. Trends Cell Biol. 14, 404-408.

Bayer, E.M., Bottrell, A.R., Walsh, J., Vigouroux, M., Naldrett, M.J., Thomas, C.L., and Maule, A.J. (2006). Arabidopsis cell wall proteome defined using multidimensional protein identification technology. Proteomics 6, 301-311.

Bendtsen, J.D., Jensen, L.J., Blom, N., Von Heijne, G., and Brunak, S. (2004). Feature-based prediction of non-classical and leaderless protein secretion. Protein Eng. Des. Sel. 17, 349-356.

Bhushan, D., Pandey, A., Chattopadhyay, A., Choudhary, M.K.,...
Chakraborty, S., Datta, A., and Chakraborty, N. (2006). Extracellular matrix proteome of chickpea (Cicer arietinum L.) illustrates pathway abundance, novel protein functions and evolutionary perspect. J. Proteome Res. 5, 1711-1720.

Borderies, G., Jamet, E., Lafitte, C., Rossignol, M., Jaunaeu, A., Bouard, G., Monsarrat, B., Esquerré-Tugayé, M.T., Boudet, A., and Pont-Lezica, R. (2003). Proteomics of loosely bound cell wall proteins of Arabidopsis thaliana cell suspension cultures: a critical analysis. Electrophoresis 24, 3421-3432.

Brewis, I.A., Turner, A.J., and Hooper, N.M. (1994). Activation of the glycosyl-phosphatidylinositol-anchored membrane deipilipase upon release from pig kidney membranes by phospholipase C. Biochem. J. 303, 633-638.

Charmont, S., Jamet, E., Pont-Lezica, R., and Canut, H. (2005). Proteomic analysis of secreted proteins from Arabidopsis thaliana seedlings: improved recovery following removal of phenolic compounds. Phytochemistry 66, 453-461.

Chen, X.Y., Kim, S.T., Cho, W.K., Rim, Y., Kim, S., Kim, S.W., Kang, K.Y., Park, Z.Y., and Kim, J.Y. (2009). Proteomics of weakly bound cell wall proteins in rice calli. J. Plant Physiol. 166, 675-685.

Chivasara, S., Ndima, B.K., Simon, W.J., Robertson, D., Yu, X.L., Knox, J.P., Bolwell, P., and Slabas, A.R. (2002). Proteomic analysis of the Arabidopsis thaliana cell wall. Electrophoresis 23, 1754-1765.

Cho, W.K., Chen, X.Y., Chu, H., Rim, Y., Kim, S., Kim, S.T., Kim, S.W., Kang, K.Y., and Kim, J.Y. (2009). Proteomics of the secretome of rice calli. Physiol. Plant. 135, 331-341.

Cui, S., Huang, F., Wang, J., Ma, X., Cheng, Y., and Liu, J. (2003). A proteomic analysis of cold stress responses in rice seedlings. J. Proteome Res. 2, 10.

Curtis, M.D., and Grossniklaus, U. (2003). A gateway cloning vector set for high-throughput functional analysis of genes in plants. Plant Physiol. 133, 462-469.

Dahal, D., Pich, A., Braun, H.P., and Wydra, K. (2010). Analysis of cell wall proteins regulated in stem of susceptible and resistant tomato species after inoculation with Ralstonia solanacearum: a proteomic approach. Plant Mol. Biol. 73, 643-658.

Denecke, J., De Rycke, R., and Botterman, J. (1992). Plant and mammalian sorting signals for protein retention in the endoplasmic reticulum contain a conserved epitope. EMBO J. 11, 2345-2355.

Eisenhaber, B., Wildpaner, M., Schultz, C.J., Borner, G.H., Dupree, P., and Eisenhaber, F. (2003). Glycosylphosphatidylinositol lipid anchoring of plant proteins. Sensitive prediction from sequence-mammalian sorting signals for protein retention in the endoplasmic reticulum contain a conserved epitope. EMBO J. 11, 2345-2355.

Eisenhaber, F., Wildpaner, M., Schultz, C.J., Borner, G.H., Dupree, P., and Eisenhaber, F. (2003). Glycosylphosphatidylinositol lipid anchoring of plant proteins. Sensitive prediction from sequence-mammalian sorting signals for protein retention in the endoplasmic reticulum contain a conserved epitope. EMBO J. 11, 2345-2355.

Eisenhaber, F., Wildpaner, M., Schultz, C.J., Borner, G.H., Dupree, P., and Eisenhaber, F. (2003). Glycosylphosphatidylinositol lipid anchoring of plant proteins. Sensitive prediction from sequence-mammalian sorting signals for protein retention in the endoplasmic reticulum contain a conserved epitope. EMBO J. 11, 2345-2355.
Pierleoni, A., Martelli, P.L., and Casadio, R. (2008). PredGPI: a GPI-anchor predictor. BMC Bioinformatics 9, 382.
Rabouille, C., Malhotra, V., and Nickel, W. (2012). Diversity in unconventional protein secretion. J. Cell Sci. 125, 5251-5255.
Robertson, D., Mitchell, G.P., Gilroy, J.S., Gerrish, C., Bolwell, G.P., and 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.
Rose, J.K., and Lee, S.J. (2010). Straying off the highway: trafficking of secreted plant proteins and complexity in the plant cell wall proteome. Plant Physiol. 153, 433-436.
Tan, L., Eberhard, S., Pattathil, S., Warder, C., Glushka, J., Yuan, C., Hao, Z., Zhu, X., Avci, U., Miller, J.S., et al. (2013). An Arabidopsis cell wall proteoglycan consists of pectin and arabinoxylan covalently linked to an arabinogalactan protein. Plant Cell 25, 270-287.
Watson, B.S., Lei, Z., Dixon, R.A., and Sumner, L.W. (2004). Proteomics of Medicago sativa cell walls. Phytochemistry 65, 1709-1720.
Yan, J.X., Walt, R., Berkelman, T., Harry, R.A., Westbrook, J.A., Wheeler, C.H., and Dunn, M.J. (2000). A modified silver staining protocol for visualization of proteins compatible with matrix-assisted laser desorption/ionization and electrospray ionization-mass spectrometry. Electrophoresis 21, 3666-3672.
Yang, J.L., Zhu, X.F., Peng, Y.X., Zheng, C., Li, G.X., Liu, Y., Shi, Y.Z., and Zheng, S.J. (2011). Cell wall hemicellulose contributes significantly to aluminum adsorption and root growth in Arabidopsis. Plant Physiol. 155, 1885-1892.
Zhu, J.K., Damsz, B., Kononowicz, A.K., Bressan, R.A., and Hasegawa, P.M. (1994). A higher plant extracellular vitronectin-like adhesion protein is related to the translational elongation factor-1 alpha. Plant Cell 6, 393-404.
Zhu, J., Alvarez, S., Marsh, E.L., Lenoble, M.E., Cho, I.J., Sivaguru, M., Chen, S., Nguyen, H.T., Wu, Y., Schachtman, D.P., et al. (2007). Cell wall proteome in the maize primary root elongation zone. II. Region-specific changes in water soluble and lightly ionically bound proteins under water deficit. Plant Physiol. 145, 1533-1548.
Zhou, L., Bokhari, S.A., Dong, C.J., and Liu, J.Y. (2011). Comparative proteomics analysis of the root apoplasts of rice seedlings in response to hydrogen peroxide. PLoS One 6, e16723.