Cell wall proteomic of Brachypodium distachyon grains: A focus on cell wall remodeling proteins.

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Proteomic data are available at: http://www.polebio.lrsv.ups-tlse.fr/WallProtDBSeed/

Abstract

Cell walls play key roles during plant development. Following their deposition into the cell wall, polysaccharides are continually remodeled according to the growth stage and stress environment to accommodate cell growth and differentiation. To date, little is known concerning the enzymes involved in cell wall remodeling, especially in gramineous and particularly in the grain during development. Here, we investigated the cell wall proteome of the grain of *Brachypodium distachyon*. This plant is a suitable model for temperate cereal crops. Among the 606 proteins identified, 299 were predicted to be secreted. These proteins were distributed into 8 functional classes; the class of proteins that act on carbohydrates was the most highly represented. Among these proteins, numerous glycoside hydrolases were found. Expansins and peroxidases, which are assumed to be involved in cell wall polysaccharide remodeling, were also identified. Approximately half of the proteins identified in this study were newly discovered in grain and were not identified in the previous proteome analysis conducted using the culms and leaves of *B. distachyon*. Therefore, the data obtained from all organs of *B. distachyon* infers a global cell wall proteome consisting of 460 proteins. At present, this is the most extensive cell wall proteome of a monocot species.

Abbreviations: CAZy, carbohydrate-active enzyme; **CE**, carbohydrate esterase; CL, carbohydrate lyase; **CWP**, cell wall protein; **DAF**, day after flowering; **DUF**, domain of unknown function; **GH**, glycoside hydrolase; **LC-MS/MS**, liquid chromatography coupled to tandem mass spectrometry; **mRFP**, monomeric red fluorescent protein; **OR**, oxido-reductase; **PME**, pectin methylesterase; **XTH**, xyloglucan endotransglycosylase/hydrolase.

Keywords: *Brachypodium distachyon* / Grain / Plant cell wall / Polysaccharide / Proteome

1 Introduction

Plant cell walls are extracellular matrices that provide the skeletal framework for tissues and play essential roles in protection, cell-to-cell adhesion and growth regulation [1]. In cereal grains, cell walls impact grain filling and germination. In addition to the physiological roles of plant cell walls, they are actively studied for their use in the production of biofuel through the fermentation of the sugars that they contain [2]. As a source of dietary fibers, cell walls also have a major impact on the nutritional quality of cereal foods [3].

Cell walls are mostly composed of polysaccharides (pectins, cellulose and hemicelluloses); a smaller proportion consists in cell wall proteins (CWPs) such as structural and enzymatic proteins. In gramineous plants, cell walls contain a low amount of pectic polymers. Their hemicellulose composition consists in a low proportion of xyloglucans and a high content of arabinoxylans and (1-3)(1-4)-β-glucans that create the network structure between cellulose microfibrils [4-6]. With the exception of cellulose, oligosaccharide precursors are synthesized in the Golgi apparatus and then transported to the extracellular matrix. Inside the cell wall, these oligosaccharides likely are assembled into larger polysaccharides that can be re-arranged to respond to the physiological needs of the plant during their development and in response to external constraints [7].

Thus, a complex machinery is required to perform these adaptations. It encompasses the enzymes required for catalyzing the numerous modifications, including transglycosylation (‘cutting and pasting’ molecules), cross-linking, hydrolysis, transacylation, oxidation and acetylation. CWPs possibly involved in the assembly and remodeling of cell wall components have been described in different plant species [8]; however, the precise function of these proteins is often unknown. Today, the largest cell wall proteome available is that of the dicot model plant *Arabidopsis thaliana*; approximately 500 proteins with a predicted signal peptide

have been identified [9]. Monocot apoplastic or cell wall proteomes were also analyzed in *Saccharum officinalis* [10], in the tropical grass *Oryza sativa* [11-14], and in temperate grasses including *Zea mays* and *Triticum aestivum* [15, 16]. Among the proteins potentially involved in the reorganization of cell wall components, glycoside hydrolases (GHs) are thought to play a central role [17]. GHs belong to one of the largest protein families found in plants; many of these proteins are involved in cell wall remodeling [17]. Other proteins, such as class III peroxidases [18, 19] and expansins [20], have been shown to take part in the organization and remodeling of cell wall polysaccharides. Expansins are considered to be loosening agents that regulate cell wall enlargement in growing cells [21].

For a few years, *Brachypodium distachyon* has proved to be a suitable plant model for cereal crops such as wheat and barley; this is due to its small, diploid, and sequenced genome and the existence of an efficient transformation system [22, 23]. In addition, this monocot plant has a small size and a short lifecycle. It begins to produce grains after only a few months of growth. The grains of *B. distachyon* have been recently characterized [24-26], exhibiting the same major cell wall polysaccharides as those encountered in other cereals, but information on its cell wall proteome is still lacking. A proteomic analysis of the cell walls of culms and leaves of *B. distachyon* has been recently performed. Among the identified proteins, many of them were assumed to be involved in assembly and remodeling of the cell wall polysaccharides [27].

In this study, our goal was to identify the secreted grain proteins in the gramineous model plant *B. distachyon*. CWPs were extracted from purified cell walls. Protein identification was performed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Among the numerous CWPs identified, we have focused on those possibly involved in cell wall construction and remodeling. Functional data were obtained by checking the sub-cellular localization of some CWPs; the transcript level of genes encoding a subset of CWPs was also

96 analyzed. We compared the cell wall grain proteome with those of vegetative tissues of *B.*
97 *distachyon* that have been previously described. Altogether, these data lead to an enlargement
98 of the known *B. distachyon* cell wall proteome that further elucidate the mechanisms behind
99 the organization and remodeling of cell walls in gramineous plants.

100

101 **2. Materials and methods**

102 **2.1. Plant material**

103 *B. distachyon* line Bd21 was grown at temperatures of 24°C day/18°C night with a
104 photoperiod of 20 h light/4 h dark. Grains were harvested at 19 days after flowering (DAF)
105 and frozen in liquid nitrogen prior to storage at -20°C. *Nicotiana tabacum* plants were grown
106 in a growth chamber under a 16 h light/8 h dark photoperiod at temperatures of 24°C
107 day/18°C night for 5-6 weeks prior to *Agrobacterium tumefaciens* infiltration.

108 **2.2. Cell wall purification and extraction of CWPs**

109 Cell wall purification was performed as previously described [28]. The only protocol
110 modification was that the entire process was scaled-down to allow for the handling of only 0.5
111 g of fresh material. Proteins were extracted via 4 successive incubations with 0.2 M $CaCl_2$
112 (twice) and 2 M LiCl (twice) as described previously [29]. Two biological repeats were
113 performed.

114 **2.3. Identification of proteins by mass spectrometry and bioinformatics**

115 Proteins (50 µg) were separated by 1D-electrophoresis in 12% polyacrylamide gels. For MS
116 analyses, a short migration was performed in 2 cm of gel. After this separation, proteins were
117 stained with Coomassie Brilliant Blue G250 (Sigma, Saint Louis, MO) as previously

118 described [30]. Each gel lane was cut in 12 slices of approximatively 1.7 mm width. Briefly,
119 these gel pieces were washed three times in 50% (v/v) ACN/25 mM ammonium bicarbonate.
120 Proteins were then reduced using DTT and alkylated with iodoacetamide prior to tryptic
121 digestion as previously described [26].

122 Nanoscale LC-MS/MS analyses of the samples were performed using an Ultimate 3000
123 RSLC system (Thermo-Fisher Scientific, MA, USA) coupled with an LTQ-Orbitrap VELOS
124 mass spectrometer (Thermo-Fisher Scientific). Chromatographic separation was conducted on
125 a reverse-phase capillary column (Acclaim Pepmap C18 2 µm 100 Å, 75-µm i.d. x 15-cm in
126 length, Thermo-Fisher) at a flow rate of 300 $nL.min^{-1}$, as previously described [31]. MS data
127 acquisitions were performed using Xcalibur 2.1 software. Full MS scans were acquired at
128 high resolution (FWMH 30,000) using an Orbitrap analyzer (mass-to-charge ratio (m/z): 400
129 to 2000), while collision-induced dissociation (CID) spectra were recorded on the five most
130 intense ions in the linear LTQ traps.

131 The workflow corresponding to the MS data collection and protein identification is described
132 in Fig. 1 of the Supporting Information. LC-MS/MS spectrum files were processed via two
133 pipelines. Both pipelines were based on the X!tandem peptide search engine from the Global
134 Proteome Machine (GPM) [32]. The pipelines were as follows: (i) the X!tandemPipeline
135 available at http://pappso.inra.fr/bioinfo/xtandempipeline used X!tandem version 2008.02.01
136 (called TORNADO); and (ii) the Labkey Server platform (v. 13.1) available at
137 https://labkey.com integrated in the Trans Proteomic Pipeline (TPP v. 4.3) used tandem
138 pluggable scoring [33] on GPM X!tandem version 2007.07.01. Protein identification was
139 achieved by confronting MS data against the UniProt Knowledgebase restricted to *B.*
140 *distachyon* (http://www.uniprot.org/, October 2013) and a contaminant database including
141 human keratins and trypsin. Enzymatic cleavage was declared as a tryptic digestion with one
142 possible miscleavage event. The fixed modifications of cysteine residues by iodoacetamide

and the possible oxidation of methionines were considered. Precursor mass and fragment mass tolerance were set at 5 ppm and 0.5 Da, respectively. Results from the X!tandem analysis were validated by filtering the peptide e-values below 0.01 in X!tandemPipeline and p-values greater or equal to 0.995 in PeptideProphet [34], a component of TPP. In both processes, proteins were identified with at least two peptides according to the above specifications. For X!tandemPipeline, an additional protein e-value threshold was set to 10e-4.

The redundancy of the protein sequence data common to the two biological replicates and to the two pipelines was reduced by clustering; this was done for the later functional analyses of the identified proteins. The Cd-hit software clustered proteins sharing at least 90% of their sequence and produced a set of non-redundant (nr) representative sequences as its output [35].

Bioinformatic analysis of identified proteins was performed using the ProtAnnDB tool (www.polebio.lrsv.ups-tlse.fr/ProtAnnDB/) [36]. The sub-cellular localizations of proteins were predicted using different software: TargetP (http://www.cbs.dtu.dk/services/TargetP/), Predotar (https://urgi.versailles.inra.fr/predotar/predotar.html), SignalP (http://www.cbs.dtu.dk/services/SignalP/) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/). The protein functions were predicted using PROSITE (http://prosite.expasy.org/), Pfam (http://pfam.xfam.org/) and InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan5/) bioinformatic programs [36]. The proteomic data of the present work have been included in the WallProtDB database (www.polebio.lrsv.ups-tlse.fr/WallProtDB/). WallProtDB tools were used for cell wall proteome comparisons.

2.4. Immunolabeling

After the 1D-electrophoresis analysis, the separated CWPs (13 µg of proteins per sample) were electrotransferred to a nitrocellulose membrane (Invitrogen, CA, USA). The presence of CWPs was revealed using antibodies against *B. distachyon* GH1 and GH3, which have been previously described [27]. These antibodies were used at a 1: 20000 dilutions, and detection was performed using an alkaline phosphatase-conjugated goat anti-mouse antibody (1:2000 dilution). The enzymatic activity of the alkaline phosphatase was revealed with 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitroblue tetrazolium (NBT) (Kit Promega, Madison, WI, USA) according to the manufacturer's instructions.

Sample preparation for the immunofluorescence labeling of grain sections was conducted as described previously [24]. The antibodies were diluted in PBS containing 1% (w/v) BSA and 0.05% (w/v) Tween-20. Dilutions of 1:200 were used for the GH1 and GH3 antibodies. Alexa 546-conjugated secondary antibody (Molecular Probes, CA, USA) was diluted in PBS [1:100 (v/v)]. To remove (1–3) (1–4)-β-glucans, the sections were pre-treated with lichenase (40 U/mL, Megazyme International, Ireland) for 12 h at 38°C. The sections were then rinsed thoroughly with de-ionized water.

2.5. Sub-cellular localization of GH1 and GH3

Total RNA was extracted from 19 DAF grains, culms or leaves (all developmental stages were mixed) of *B. distachyon* using the RNA kit (Qiagen, Courtaboeuf, France). RNA samples were treated twice with the DNase set (Qiagen) and then purified using the RNeasy MinElute Cleanup kit (Qiagen) by following the manufacturer's instructions. Reverse transcription was carried out with 2 µg of total RNA, random hexamers, and the Transcriptor First Strand cDNA synthesis kit (Roche Applied Science, Mannheim, Germany).

The Gateway® cloning system was used to obtain different constructs of interest (Invitrogen, San Diego, CA). The cDNAs encoding GH1 (*Bradi1g10930*) and GH3 (*Bradi1g08570*) were

amplified by polymerase chain reaction (PCR) using specific primers (Supporting Information Table S1). A second PCR was performed with AttB1 and AttB2 primers (Supporting Information Table S1). Entry clones were obtained via a BP reaction in pDONR-207 (Invitrogen). The binary vector pH7RWG2 [37] was used to obtain the final constructs that encoded C-terminal mRFP fusions for each of the two CWPs.

Transient expression of the constructs in tobacco leaf epidermal cells was performed as previously described [38]. Transformed *A. tumefaciens* cultures were resuspended in infiltration buffer at OD_{600nm}=0.05. Fluorescence was examined with an inverted Nikon A1 confocal laser-scanning microscope at 2-3 days after infiltration.

Cell plasmolysis was performed using infiltrated leaf samples. Samples were placed in a solution containing 30% glycerol 5 min before imaging with the confocal microscope.

2.6. Semi-quantitative RT-PCR

RT-PCR using total RNA samples was carried out as described above; the primers used are described in Supporting Information Table 1. PCR reactions were performed using KOD polymerase (*Novagen* Inc, Madison, WI, USA) with appropriate buffer, forward and reverse primers (1 μL of 10 μM solutions), and in a total volume of 25 μL. PCR products were separated on 2% agarose gels by electrophoresis.

3. Results

3.1. Cell wall fractionation and protein extraction

For the proteomic analysis, *B. distachyon* grains were harvested at 19 DAF. This developmental stage corresponds approximately to the end of the cell differentiation, which occurs immediately before storage accumulation [25]. At 19 DAF, the mass of a single grain is estimated to be approximately 5 mg. The fractionation protocol was derived from Feiz *et al*. [28], with slight modifications. The protocol was adapted for the limited amount of available material, which was approximately 100 grains per replicate. Approximately 60 mg of lyophilized cell wall were obtained for each replicate. Approximately 150 µg of proteins were extracted from each sample using 0.2 M $CaCl_2$ and 2 M LiCl solutions. The resulting freeze-dried extracted proteins (50 µg) were separated by 1D-electrophoresis. The electrophoretic profiles of replicates were similar; large number of protein bands was observed between 130 and 20 kDa (Fig. 1).

To validate the fractionation, an immunoblot was performed using antibodies directed against two known CWPs that belong to the GH1 (*Bradi1g10930*) and GH3 (*Bradi1g08570*) families (Fig. 1). Both target proteins were detected at the expected molecular mass (MM) [27]; two specific bands were detected at 57 and 68 kDa for GH1, and a unique band at 80 kDa was detected for GH3. An immunoblot was performed using a total protein extract from *B. distachyon* grain at 19 DAF using the same antibodies, but no bands were observed (data not shown). This lack of detection may be due to the very low abundance of GH1 and GH3 in the total protein extract. This result confirms that the CWPs are enriched in the cell wall extract.

3.2. Protein identification by MS and bioinformatics

CWPs extracted from two independent biological replicates were analyzed by LC-MS/MS after 1D-electrophoresis. All gel slices were analyzed separately. Then, the results obtained

for the gel slices corresponding to the same extract were combined. Only proteins identified with at least two peptides present within the same extract were retained. The data were searched against the Uniprot databank using two separated pipelines, both of which were based on the same search engine (GPMX!Tandem) with two scoring algorithms. XtandemPipeline works with the X!Ttandem native score. TPP works with the k-score. The use of the XtandemPipeline and TPP pipelines resulted in the identification of 913 and 713 proteins, respectively; a common subset of 677 proteins was retained to strengthen the identification confidence. Their corresponding sequences were then clustered together according to sequence identity above a threshold of 90% to gather variants of identical proteins. The longest sequence within each of these clusters was reported as the representative sequence of the cluster. Altogether, a non-redundant dataset of 606 proteins was deduced from this data analysis and considered for further analyses.

3.3 CWPs identified in the grain cell wall proteome

Predictions of sub-cellular localization and of functional domains were performed using the ProtAnnDB tool [36]. Among the 606 proteins identified, between 48% and 52% were predicted to be secreted. This is consistent with previously reported cell wall proteomic analyses [9, 27] . In this work, depending on the prediction software used, between 10 to 13% of the proteins were predicted to be targeted to the chloroplast, 6 to 11% of the proteins were predicted to be targeted to the mitochondria, and the remaining 28 to 32% had no predicted signal peptide.

For further analyses, we considered proteins to be putative CWPs proteins having a signal peptide predicted by at least two different bioinformatic programs and lacking an ER retention signal. A total of 299 proteins met these criteria (Supporting Information Table 2). A list of

these proteins and the corresponding experimental data are available online in the WallProtDB database (http://www.polebio.lrsv.ups-tlse.fr/WallProtDB/).

CWPs were classified in 8 categories according to their predicted functions, which were predicted by PROSITE, Pfam and Interpro software [39]. Over a quarter of the proteins (27%) were predicted to act on carbohydrates, 12% were predicted to be oxido-reductases and 18% were proteins of yet unknown function. The other CWPs were predicted to be proteins related to lipid metabolism, proteins with interacting domains, proteases, signaling proteins or miscellaneous proteins (Fig. 2). No structural proteins, such as extensin, proline-rich protein, and glycine-rich protein, were identified.

Among the 299 CWPs identified in our analysis, 146 were newly found in the *B. distachyon* grain cell wall. The remaining proteins, approximately one half, were previously identified in the culm and leaf cell wall proteomes of *B. distachyon* [27].

3.4. Grain CWPs acting on cell wall polysaccharides and oxido-reductases

Eighty-one CWPs acting on cell wall polysaccharides were identified in the *B. distachyon* grain cell wall proteome. Compared to the culm and leaf cell wall proteomes of *B. distachyon*, a greater number of GHs and carbohydrate esterases (CEs) and fewer expansins and oxido-reductases were identified. No carbohydrate lyases (CLs) were found in the grain cell wall proteome, but only one in culm or leaf (Fig. 3A). This subset of proteins consisted mostly of GHs (69), which were sorted into 20 different families (Fig. 3B). GHs are among the largest protein families in plants; many of these proteins are involved in cell wall remodeling [17]. The GH families with the greatest number of members in our analysis were GH17, GH3, GH1 and GH28. The other well-represented GH families were GH35, GH51, GH16, GH18 and GH19; at least 3 members of each of these families were found. Among the 69 GHs identified, 32 were found only in the grain cell wall proteome and not in the previous

proteomes from *B. distachyon* culms and leaves [27]. GH13, GH51 and GH89 were found only in the grain cell wall proteome (Fig. 3 B).

Eight CEs were identified in the grain cell wall proteome. Half of these proteins were not previously found in culms and leaves. These proteins belong to the following two CE families: CE8, which includes enzymes with pectin methylesterase (PME) activity; and CE16, which includes proteins with acetylesterase activity. Five expansins were found in grains. Among them, only two were also found in vegetative organs (Bradi2g53580 and Bradi3g33150).

A total of 35 oxido-reductases (ORs) were identified in the grain cell wall proteome. Of these proteins, 18 were predicted to be peroxidases (51%), 8 were blue copper binding proteins (23%) and 4 were multicopper oxidases (11%). Although ORs were less represented in the grain than in culms and leaves (52 ORs), 18 novel ORs were identified in grains. Of these novel ORs, 11 were peroxidases (Fig. 3B; Supporting Information Table 2).

3.5. Expression pattern of genes corresponding to a subset of CWPs

For some of the CWPs identified in our grain proteomic analysis, we investigated the expression of the corresponding genes using total RNA extracted from grains at 19 DAF, culms and leaves of *B. distachyon*. Semi-quantitative RT-PCR experiments were conducted using primers specific for genes encoding four different CWPs identified in the proteomic analysis: a GH3 (*Bradi1g08570*), a GH18 (*Bradi4g09430*), a GH19 (*Bradi1g29880*) and a copper amine oxidase (*Bradi5g04070*). We used the gene encoding S-adenosylmethionine decarboxylase (SamDC) as a reference; this gene is suitable for normalizing gene expression data in *B. distachyon* [40]. As expected, all four genes were expressed in grain (Fig. 3C). This is consistent with the proteomic data. Regarding culms and leaves, there is also correlation between the presence of transcripts and the identification of the protein, except for the copper

amine oxidase gene since the protein has not been identified in the cell wall extracts of these organs [27]. Such discrepancies have already been observed in *A. thaliana* [41, 42].

3.6 Experimental validation of the cell wall localization of GH1 and GH3

For further experimental validation of our proteomic data, we used immunolabeling with specific polyclonal antibodies to detect two CWPs, GH1 (Bradi1g10930) and GH3 (Bradi1g08570), in *B. distachyon* grain sections [27]. As observed previously, immunoblotting with these two antibodies revealed bands of expected molecular masses with the grain CWP extract; in this way, their specificity was confirmed (Fig. 1).

Grain sections were first subjected to a lichenase pre-treatment to eliminate the large amount of β-glucan present in the cell walls of *B. distachyon* grains. It was shown that this pre-treatment is essential to increase labeling and reveal the antigenic sites [24].

Using anti-GH1 antibodies, a significant labeling occurred in the cell wall of the endosperm and external layers of grains (Fig. 4A). Incubation with the corresponding pre-immune serum revealed only weak background (Fig. 4B), confirming that the labeling observed with the antibodies was specific. A weak autofluorescence was noticed in the seed coat, the pericarp and at the sillon level, even when grain sections were not subjected to immunolabeling (data not shown). This made it difficult to detect a difference between autofluorescence and specific labeling in these tissues. In contrast, the fluorescence was doubtless specific in the endosperm and the nucellus epidermis.

Specific immunolabeling was also revealed with anti-GH3 antibodies at the cell wall level in the endosperm and the nucellus epidermis (Fig. 4 C, D). For the same reasons discussed above, it is difficult to differentiate autofluorescence from specific labeling in the other outer layers. However, fluorescent labeling with anti-GH3 or anti-GH1 antibodies appeared to be more intensive in the central endosperm than in the peripheral endosperm.

The immunolabeling experiments confirmed the presence of two CWPs (GH1, Bradi1g10930; GH3, Bradi1g08570) in the cell walls of *B. distachyon* grains and suggested their tissue localization. To go further, we generated C-terminal mRFP fusions of the two proteins under the control of the CaMV 35S promoter using an appropriate binary vector. The constructs contained the signal peptide of the corresponding GHs. The mRFP constructs were transiently expressed in *N. tabacum* leaves via *A. tumefaciens* infiltration. The sub-cellular localization of the fusion proteins was analyzed by confocal laser scanning microscopy. The fluorescence of GH1-mRFP and GH3-mRFP was detected at the periphery of the cells; this was consistent with localization to the cell wall (Fig. 5 A, C). A plasmolysis of the agro-infiltrated leaf pieces was performed to separate the plasma membranes from the cell walls. A calcofluor labeling of cells allowed for the demarcation of the cell wall. The results indicated that GH1 was present not only in the cell wall, but was also present in the apoplastic compartment (Fig. 5B). A similar result was obtained when the exogenous marker Sec-mRFP was used (Fig. 5F). The fluorescence of GH3-mRFP indicated localization of the fusion protein to the apoplast rather than to the cell wall (Fig. 5D).

4. Discussion

Although the seed proteome of several monocot species, including rice, wheat and barley, are well characterized, little is known concerning the cell wall proteome of monocot seeds. The cell wall of *B. distachyon* grains has been recently analyzed during all developmental stages, and the composition of its polysaccharides shares similarities with other cereal cell walls. This suggests that *B. distachyon* is a good model for the study of the cell wall of monocots. The proteomic analysis of the cell wall of *B. distachyon* grains performed in this work contributes to the knowledge of the cereal grain cell wall proteome, with a specific focus on the proteins involved in the assembly and remodeling of cell wall polysaccharides.

The protocol, initially developed to purify the cell walls of *A. thaliana* hypocotyls [28] and recently adapted for the culms and leaves of *B. distachyon* [27], was successfully optimized for the fractionation of cell walls from *B. distachyon* grains. The resulting cell wall fraction corresponded approximately to one third of the initial dry mass of the grain. The extraction of the proteins from these cell wall fractions allowed us to obtain a total protein amount sufficient for further work, with a yield of approximately 0.25% (m /m) of the lyophilized cell wall.

It is well documented that the use of different database search engines in proteomics may lead to some differences in protein identification [43]. A key component of search engines is the scoring method [33]. To generate meaningful results, the proteins were identified using two different pipelines. Those having a predicted peptide signal and no ER retention signal were considered to be CWPs. This list of CWPs corresponded to approximately one half of the total identified proteins; this reflects a good efficiency in cell wall fractionation and CWP extraction. Such a proportion of predicted secreted proteins in cell wall proteomes has been reported in the young leaves of *B. distachyon*; this proportion was greater in mature leaves and stems [27].

The CWPs identified in this work were distributed into 8 of the 9 previously defined functional classes [39]. As in the cell walls of the vegetative tissues of *B. distachyon*, no structural proteins were identified in the grain. This supports the idea that structural proteins are much less abundant in the grasses than in dicots, or that these proteins are more difficult to extract [44]. Among the CWPs identified in *B. distachyon* grains, the proteins acting on cell wall polysaccharides were the most highly represented (27%); a significant proportion of these proteins were GHs. These enzymes are involved in diverse processes in plants, including starch metabolism, defense, and cell-wall remodeling. In *B. distachyon*, 356 GH genes were identified, and the corresponding proteins were sorted into 34 GH families [45]. In this work, a total of 69 proteins in 20 GH families were identified. Of these proteins, 32 were not found in other organs analyzed thus far. The most highly represented families were the GH17, GH1 and GH3 protein families, with more than 6 proteins identified for each; this finding was consistent with the high number of genes belonging to each family, which is between 30 to 50 [45].

The GH17 family contains enzymes able to degrade mainly (1-3)- or (1-3)(1-4)-β-glucans. In *B. distachyon* grains, the presence of these enzymes can be correlated with a high abundance of (1-3)(1-4)-β-D-glucan in both the aleurone and endosperm cell walls [24]. The GH1 family members are well represented in both dicots and monocots. Enzymes from the GH1 family share a broad range of activities, such as β-D-glucosidase, β-mannosidase, β-galactosidase, β-xylosidase, β-D-fucosidase and exo-β-1,4-glucanase. The characterization of a GH1 from rice (Os4bglu12) revealed that the protein had high exoglucanase activity; this finding was consistent with a role for the enzyme in cell wall metabolism [46]. GH3 proteins were well represented in our study and in grass cell walls in general. These proteins exhibit broad substrate specificities and act on arabinoxylans and (1-3)(1-4)-β-D glucans, the most highly

represented hemicelluloses in grasses [17]. In both the GH1 and GH3 families, some identified proteins have already been found in the vegetative tissues of *B. distachyon* [27]. The immunolabeling of one GH1 (Bradi1g10930) and one GH3 (Bradi1g08570) revealed their presence mainly in the cell walls of the endosperm of *B. distachyon* grains. The sub-cellular localization of Bradi1g10930 (GH1) and Bradi1g08570 (GH3) confirmed their secretion into the apoplast. Only Bradi1g10930 was also localized to the cell wall; this finding suggests that GH1 possibly interacts with cell wall polysaccharides or cell wall anchored proteins.

The 32 GHs identified only in the grain belong to 14 GH families. Three of these families (GH13, 51 and 89) have never been found in other organs (either culms or leaves) in *B. distachyon*. GH13 is a very large family that includes mostly α-amylases, which are abundant in grain. The members identified here are predicted to contain an α-amylase catalytic domain; this suggests that these proteins can be contaminants coming from the intracellular compartment. On the contrary, the GH89 family is poorly represented in monocots. Only one or two GH89 genes have been identified in the rice and *B. distachyon* phylogenomic database of GHs [45, 47]. A unique activity, α-N-acetylglucosaminidase, has been described in this family. The GH51 family was better depicted, with four protein members identified. This family includes α-L-arabinofuranosidases and β-xylosidases. It was demonstrated that enzymes extracted from germinated barley grain belonging to the GH51 family modify the heteroxylan fine structure by removing arabinofuranosyl residues from cell wall arabinoxylans during growth and development in barley [48]. In the cell walls of wheat endosperm, the level of arabinoxylan branching decreases during the grain filling period; this phenomenon highly impacts the physico-chemical properties of grains [49, 50]. Further investigation into the grain-specific GH51 family of *B. distachyon* should be performed to

determine the precise role of the enzymes in arabinoxylan restructuring during grain development.

In addition to GH proteins, we identified CEs and expansins as proteins acting on cell wall polysaccharides. Only two CE families were found, CE8 and CE16, with a number of proteins slightly higher in the grain compared to the vegetative organs [27]. CE8 are PMEs that remove the methyl group from methyl-esterified galacturonic acid (GalA) residues within pectins in the apoplast [51]. This is a significant biological event in processes such as cell wall turnover, fruit ripening and pathogenesis [52]. In *A. thaliana* seeds, demethylesterification alters the physico-chemical properties of endosperm cell walls and contributes to the emergence of the radicle during germination [53]. The CE16 family includes acetylesterases that are active on pectins and xylans. The acetylation of arabinoxylans was detected in wheat during the early stages of development [54]. It was demonstrated that the modulation of pectin acetylation affects the remodeling and physiochemical properties of cell wall polysaccharides and plays a key role in cell extensibility.

Five expansins were found in the cell wall proteome of *B. distachyon* grains. The mechanism of action of these proteins consists in the disruption of hydrogen bonds between cellulose microfibrils and cross-linking glycans. Expansins induce the extension and loosening of plant cell walls and participate in plant cell growth, cell wall disassembly and cell separation [20]. In the wheat grain, they were also suggested as a potential factor in the final determination of grain size [55].

Among the 31 proteins with unknown function and only identified in grain, six belonging to the cupin superfamily were previously annotated as being 11S and 7S types of seed storage proteins [26], and could be considered as contaminants in a first instance. However, the cupin superfamily is extremely diverse, and may include proteins with other activities than seed

storage [56]. We therefore decided to keep cupin proteins in the CWP list. We also identified two DUF1680 proteins, which have been recently classified in the new GH127 family according to the CAZy database (Bradi1g36027 and Bradi3g07047) [57]. One DUF1680 protein was very recently characterized as a novel β-L-arabinofuranosidase which could play a role in the degradation of cell wall polysaccharides as well as hydroxyproline-rich glycoproteins [58]. Two DUF538 proteins were also found only in grain and not in vegetative organs of *B. distachyon* (Bradi1g25270 and Bradi4g21520). This protein family was recently nominated as the potential structural and functional homologue of the BPI (Bactericidal/Permeability Increasing) protein in plants [59].

In addition to the functional class of proteins that act on cell wall polysaccharides, enzymes belonging to the oxido-reductase superfamily may play an important role in cell wall assembly and remodeling. Eighteen class III peroxidases were identified in our analysis; 11 of these proteins were found only in the grain proteome. Class III peroxidases are involved in cell elongation, cell wall construction, and in the response to various abiotic stresses and biotic plant pathogens [18]. In rice shoot development, an increase in the activity of cell wall-bound peroxidases is correlated with diferulic acid content; this phenomenon suggests that wall-bound peroxidases are involved in the regulation of diferulic acid formation [60]. The high content of ferulic acid esterified to the arabinosyl residues of arabinoxylans in the *B. distachyon* cell wall could explain the large proportion of peroxidases found in the cell wall proteome of grains. Cross-linkages of arabinoxylans by diferulic acid (DFA) creates a rigid network within the cell wall architecture [61]. The level of wall-bound DFA is involved in determining the rigidity of the cell walls of gramineous plants [62]. Peroxidases may also play an important role in seed germination, as the up-regulation of peroxidase genes in barley and rice appears to be conserved [63].

Taken together, this work provides an overview of the proteins that are predicted to be secreted in the cell walls of *B. distachyon* grains harvested at 19 DAF. Combined with previous data obtained from culms and leaves, the global cell wall proteome of *B. distachyon* contains 460 proteins. Currently, this is the best-documented cell wall proteome from a monocot species. Although the precise functions of these proteins remain poorly understood, it is highly probable that many proteins are involved in the construction and remodeling of the cell wall. Further characterization of these CWPs should provide a better understanding of the mechanisms involved in cell wall organization.

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Legends to figures

Figure 1. A. 1D-electrophoretic pattern of the *B. distachyon* CWPs extracted from grains harvested at 19 DAF. Ten µg of CWPs were loaded on a 12% polyacrylamide gel and stained with Coomassie brilliant blue. **B.** Immunolabeling of GH1 (Bradi1g10930) and GH3 (Bradi1g08570) using antibodies specific to the respective proteins. **GH1**: theoretical molecular mass: 53.7 kDa; apparent molecular masses: 57 and 68 kDa. **GH3**: theoretical molecular mass: 67.5 kDa; apparent molecular mass: 80 kDa. Molecular mass (MM) markers are indicated in kDa.

Figure 2. Overview of the grain cell wall proteome: distribution of CWPs in functional classes according to predicted functions provided by Pfam and Interpro software programs.

Figure 3. Comparison of carbohydrate acting proteins and oxido-reductases, and the expression profile of some corresponding genes in the grain and vegetative organs of *B. distachyon* (culms and leaves). **A.** Distribution of CWPs into the oxido-reductase functional class and the different families of proteins acting on carbohydrates. GHs, glycosyl hydrolases; CEs, carbohydrate esterases; CLs, carbohydrate lyases; ORs, oxido-reductases **B.** Distribution of carbohydrates acting proteins, and oxido-reductases identified only or commonly in the cell wall proteomes of grains and of vegetative organs. Arrows indicate the GH families for which members were identified only in the grain proteome. Results are expressed as the numbers of identified proteins. **C.** Expression profile of genes corresponding to a subset of four CWPs identified in the proteomic analysis by semi-quantitative RT-PCR using total RNAs extracted from culms (C), leaves (L) or grain (G).The SamDC gene was used for the normalization of the PCR reactions.

Figure 4. Immunolocalization of Bradi1g10930.1 (GH1) and Bradi1g08570.1 (GH3) in *B. distachyon* grain harvested at 19 DAF. Serial cross-sections have been labeled with immune sera (**A**: anti-GH1; **C**: anti-GH3) or the corresponding PISs (**B**: PIS-GH1; **D**: PIS-GH3) used

as negative controls. For each cross section, higher magnification of an endosperm and external layers region are shown. Scale bars=50 µm.

Figure 5. Sub-cellular localization of Bradi1g10930.1 (GH1) and Bradi1g08570.1 (GH3) fused to a fluorescent protein in *N. tabacum* leaf epidermal cells. Confocal images showing mRFP (red) fusion proteins in leaf epidermal cells 2–3 days after agro-infiltration. Images of non-plasmolyzed cells expressing GH1-mRFP (**A**), GH3-mRFP (**B**), the apoplastic marker Sec-mRFP (**C**), and plasmolysis cells expressing GH1-mRFP (**B**), GH3-mRFP (**D**), the apoplastic marker Sec-mRFP (F) and staining with calcofluor (blue) and merged images. Arrows indicate the apoplastic compartment. Scale bars=10 µm.

Figure 1

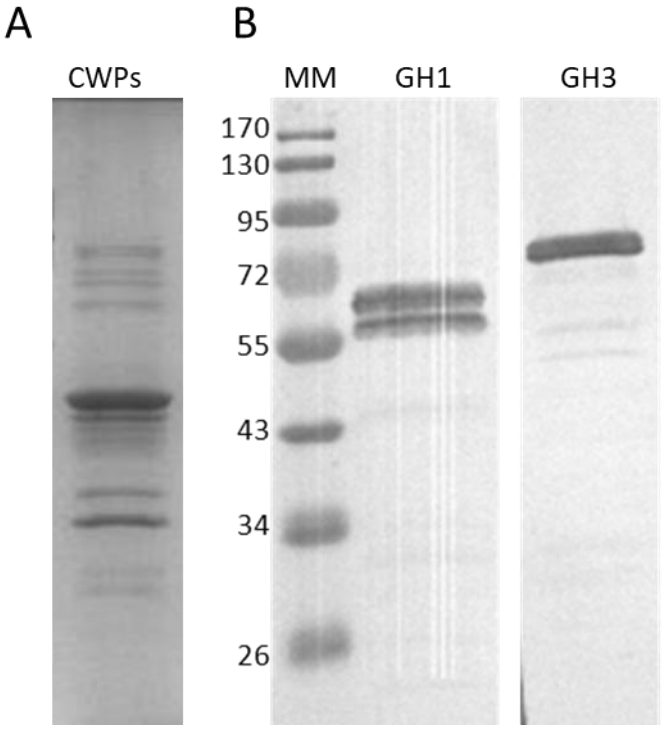
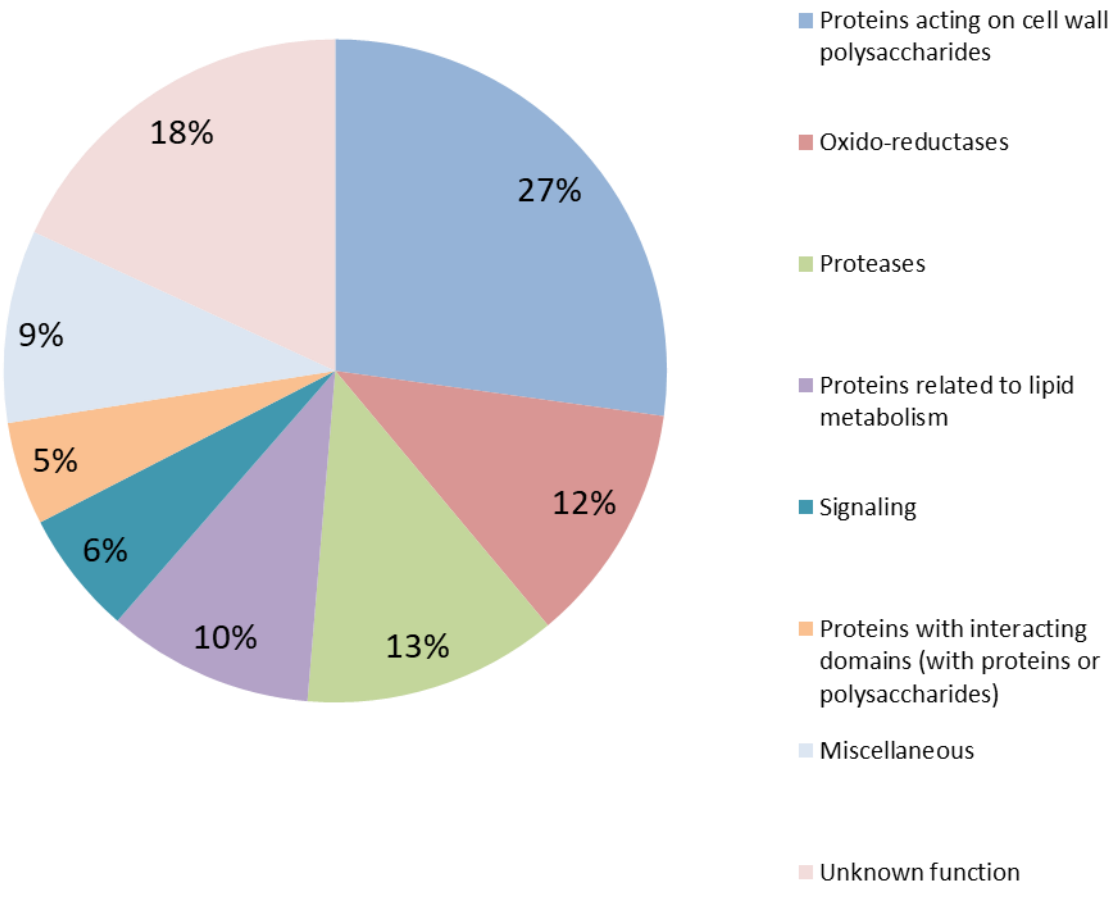


Figure 2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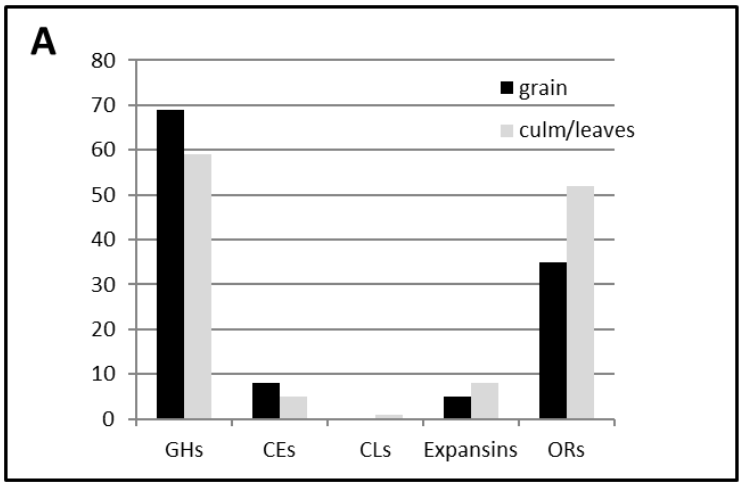
Signaling
Proteins with interacting domains (with proteins or polysaccharides)
Miscellaneous
Unknown function

Figure 3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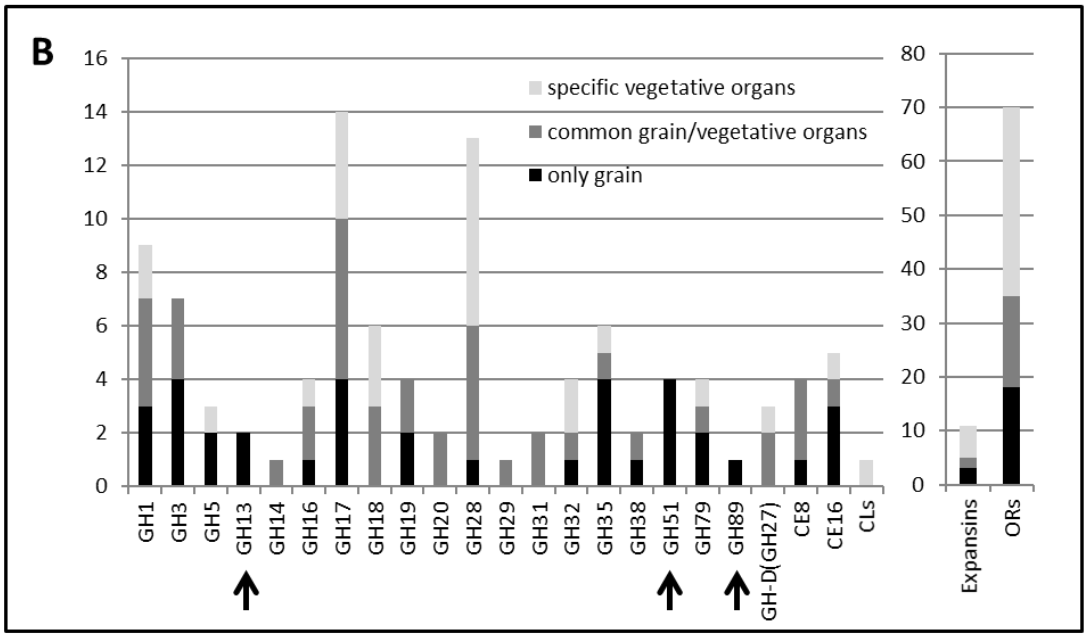
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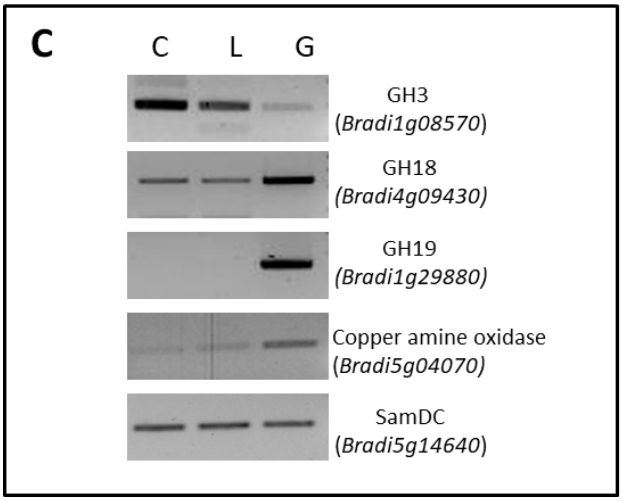
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Figure 4

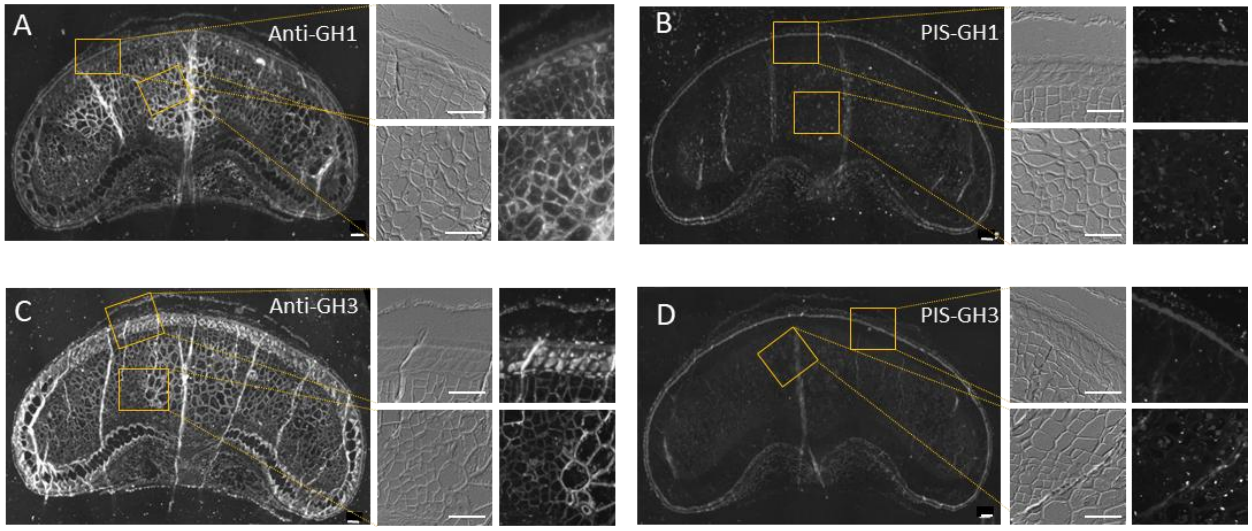

Figure 5