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**A sub-proteome of *Arabidopsis thaliana* mature stems trapped on Concanavalin A is enriched in cell wall glycoside hydrolases**

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**Abstract**

*N*-glycosylated proteins were isolated from *Arabidopsis thaliana* mature stems using affinity chromatography on Concanavalin A Sepharose, separated by 2D-electrophoresis and identified using nanoHPLC-MS/MS and MALDI-TOF MS. 102 glycoproteins were identified. 94% of these proteins were predicted by bioinformatics to be targeted to the secretory pathway and 87% of them were predicted to be localized in the cell wall or at the plasma membrane. 30% of these proteins belong to glycoside hydrolases (GHs) families with some of them possibly involved in the hydrolysis of cell wall polysaccharides. The second major class of identified proteins comprises aspartyl and serine proteases. Other proteins are predicted to be oxido-reductases, contain interacting domains, are potentially involved in signalling or have an unknown function. This is to our knowledge the first survey of plant cell wall *N*-glycosylated proteins.

**Key words:** *Arabidopsis thaliana*, cell wall, concanavalin A, glycoside hydrolase, glycosylation, plant stem.

## Introduction

Plant stems play different roles in growth and development, transport of nutrients and water and constitute a physical support for the plant. Major components of stem cell walls are polysaccharides (Carpita and Gibeault, 1993; Roberts, 1994). Polysaccharides represent up to 90% of plant cell walls and constitute three different kinds of polymers: cellulose, hemicelluloses and pectins. Plant cell wall polysaccharide composition and structure change during plant development and are different from one plant species to another (Cosgrove, 1997; Popper and Fry, 2003). Cell wall proteins (CWPs) contribute to wall architecture or are involved in the regulation of growth and development, or defence against biotic or abiotic stresses (Lee et al., 2004; Jamet et al., 2006). Cell wall modifying proteins such as glycoside hydrolases (GHs), esterases, transglycosylases, lyases and peroxidases are involved in the construction, remodelling or turnover of cell wall components (Heredia et al., 1995; Cosgrove, 1997; Fry, 2004; Stolle-Smits et al., 1999; Obel et al., 2002; Reiter, 2006).

The enzymes of GH and transglycosylase superfamilies are particularly important for the reorganization of cell wall polysaccharides after their deposition (Fry, 2004; Minic and Jouanin, 2006). They fall into several families whose distinction is based on amino acid sequence similarities (Henrissat, 1991; 1998). Exo-glycanases attack polysaccharides progressively from the non-reducing end or substituted side groups, thus releasing monosaccharides. Endo-glycanases attack polysaccharide backbones in an endo-fashion. They have a large impact on the molecular mass of polysaccharides. A third group of hydrolases can break some substituted non-carbohydrate groups linked to wall polysaccharides such as *O*-acetyl, *O*-methyl and *O*-feruloyl groups (Fry, 2004). Xyloglucan transglycosylase hydrolases (XTHs) can exhibit both endo-glycanase and transglycosylase activities (Fry, 2004).

Significant progress has been made in proteomic analysis of plant cell walls (Jamet et al., 2006). Interesting results were obtained using cell cultures, culture medium of seedlings, leaves, etiolated hypocotyls, protoplasts and roots (Chivasa et al., 2002; Borderies et al., 2003; Boudart et al., 2005; Charmont et al., 2005; Kwon et al., 2005; Jamet et al., 2006; Zhu et al., 2006). One cell wall proteome of mature stems was described in *Medicago sativa* L. (Watson et al., 2004). All these studies were based either on elution of cell wall proteins from living cells or on extraction of proteins from purified cell walls with salt solutions. However, since all CWPs are secreted proteins, they can be *N*-glycosylated with sugars such as D-glucose and D-mannose during their passage through endoplasmic reticulum and Golgi (Lerouge et al., 1998). It should be possible to trap them on Concanavalin A (Con A) which is a lectin extracted from *Canavalia ensiformis* L. able to bind molecules containing  $\alpha$ -D-mannopyranosyl,  $\beta$ -D-glucopyranosyl or sterically-related residues (Carlsson et al., 1998). Recently, the *N*-glycoproteomes of human urine and human bile were analysed using Con A Sepharose affinity chromatography followed by 2D-electrophoresis and mass spectrometry (Kristiansen et al., 2004; Wang et al., 2006). The majority of the proteins identified were predicted to be extracellular or membrane components. Con A affinity chromatography was also used for the characterisation of *N*-linked glycoproteins of *Ceanorhabditis elegans* (Kaji et al., 2003) and of GHs from various plant organs (Sheldon et al., 1998; Wilson and Altmann, 1998; Minic et al., 2004; Li and Kushad, 2005; Minic et al., 2006; Van Riet et al., 2006). In this work, we have developed a new proteomic approach starting from a crude protein extract and using Con A Sepharose affinity chromatography to identify soluble cell wall *N*-linked glycoproteins. This glycoproteome is significantly enriched for putative cell wall GHs compared to previous cell wall proteomes.

## Materials and methods

### *Plant material*

Wild-type *Arabidopsis thaliana*, Wassilewskija ecotype, was grown in the greenhouse at 20°C to 22°C with a 16 h-photoperiod at 150  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . Inflorescence stems of plants at mature stage (18–22 cm) were used for analysis.

### *Preparation of protein extracts from stems of A. thaliana*

Mature stems of *A. thaliana* measuring 18–22 cm in length at the late flowering stage were used for analysis. Approximately 10 g of stem tissues were suspended in 12 mL of ice-cold extraction buffer and grinded in a mortar with a pestle for 5 min. The extraction buffer consisted in 25 mM BisTris pH 7.0 (HCl), 200 mM  $\text{CaCl}_2$ , 10% (v/v) glycerol, 4  $\mu\text{M}$  Na-cacodylate, 1/200 (v/v) protease inhibitor cocktail (P-9599, Sigma Chemical, St Louis, MO, USA). The ground material was centrifuged twice at 4°C for 3 min at 10,000 g, and the supernatant was further centrifuged for 15 min at 17,000 g. The resulting supernatant was used for chromatographic analyses.

### *Con A Sepharose affinity chromatography*

A 1 x 6-cm column was filled with 3 mL of Con A Sepharose (Sigma Chemical, St Louis, MO, USA) and washed with 6 mL of 20 mM Tris pH 7.4 (HCl), 1 mM  $\text{CaCl}_2/\text{MgCl}_2/\text{MnCl}_2$  and 0.5 M NaCl buffer. The soluble protein extract (10 mL) was added and then washed with 15 mL of this buffer at a flow rate of 5  $\text{mL}\cdot\text{h}^{-1}$ . Proteins were eluted with 0.2 M methyl- $\alpha$ -glucopyranoside in the same buffer. The eluate was collected, concentrated by "Ultrafree-CL" (10 kDa) (Sigma Chemical, St Louis, MO, USA) to 300  $\mu\text{L}$  and dialysed against 7 M urea, 5 mM  $\text{K}_2\text{CO}_3$ , 0.125% SDS, 0.6% Triton X-100, 1 mM DTT, 2% carrier ampholytes 3–10 (GE Healthcare Europe GmbH, Orsay, France).

### *Glycoside hydrolase activities*

The reaction mixture contained 2 mM pNP-glycosides (Sigma Chemical, St Louis, MO, USA), 0.1 M acetate buffer (pH 5.0), 2 mM sodium azide, and 50  $\mu\text{L}$  of protein extract in a total volume of 0.5 mL. The reaction was carried out at 37°C for 5 to 60 min (depending on activity) and stopped by the addition of 0.5 mL 0.4 M sodium chloride. Controls were stopped at time 0. Concentration of the resulting pNP was determined spectrophotometrically at 405 nm by comparison to a calibration curve. Standard deviations values for 3 replicate assays were less than 5%.

### *2D-electrophoresis*

Isoelectric focusing (IEF) was performed using 24 cm-immobilized pH gradient (IPG) strips (GE Healthcare Europe GmbH, Orsay, France) with a linear pH gradient from 4 to 7 and 250  $\mu\text{g}$  of protein were applied on an IPG strip for in-gel rehydration in 7 M urea, 2 M thiourea, 2% CHAPS, 10 mM DTT, 2% IPG buffer pH 4–7 (Méchin et al., 2004). Focusing was achieved using a Protean IEF Cell (Bio-Rad, Hercules, CA, USA). An active rehydration was performed at 22°C during 12 h at 50 V prior to focusing. To improve sample entry, the voltage was increased step by step from 50 to 10,000 V (0.5 h at 200 V, 0.5 h at 500 V, 1 h at 1000 V then 10,000 V for a total of 94,000 V h). After IEF, IPG strips were successively incubated in 50 mM Tris pH 8.8 (HCl), 6 M urea, 30% glycerol, 2 % SDS, 1% DTT for 15 min, and in 50 mM Tris pH 8.8 (HCl), 6 M urea, 30% glycerol, 2 % SDS, 2.5% iodoacetamide for 15 min (Görg et al., 1987). Strips were further sealed on top of the 1 mm-thick second dimensional gel (24 x 24 cm) with the help of 1% low-melting agarose in SDS-electrophoresis buffer (25 mM Tris, 0.2 M glycine, 0.1% SDS). Continuous gels (11% T,

2.67% C gels with PDA as cross-linking agent) were used. Separation was carried out at 20 V for 1 h and subsequently at a maximum of 30 mA/gel, 120 V overnight, until the bromophenol blue front had reached the end of the gel.

#### *Protein staining*

Following 2D-electrophoresis, gels were stained with colloidal Coomassie blue G250 according to Mechin et al. (2004).

#### *Identification of proteins by mass spectrometry*

The individual protein spots obtained after 2D-electrophoresis were excised and in-gel digested with trypsin according to a standard protocol (Santoni et al., 2003). Tryptic peptides from each protein were analyzed by nanoHPLC-MS/MS or MALDI-TOF MS as previously described (Minic et al., 2004; Mechin et al., 2004). Proteins analysed by MALDI-TOF MS were identified via automated NCBI non redundant protein database (<http://www.ncbi.nlm.nih.gov/>) searching using the MASCOT programme ([http://www.matrixscience.com/search\\_form\\_select.html](http://www.matrixscience.com/search_form_select.html)). Only mowse scores exceeding threshold ( $p < 0.5$ ) were considered as positive results. Identification of proteins with nanoHPLC-MS/MS (ion trap) was performed with Biowoks<sup>TM</sup> (Thermo scientific, San Jose, USA). The main search parameters were methionine oxidation as differential modification and trypsin as enzyme. One miss cleavage was allowed. The *A. thaliana* protein database was downloaded from the mips website (<http://mips.gsf.de/projects/plants>). Identification was considered significant when the proteins were identified with at least 2 different tryptic peptides as first candidate, Xcorr > 1.7, 2.2 and 3.3 for respectively mono-, di- and tri-charged peptides and delta Cn > 0.1.

#### *Bioinformatics analyses*

Sub-cellular localization and length of signal peptides were predicted using PSORT (<http://psort.nibb.ac.jp/>) and TargetP (<http://www.cbs.dtu.dk/services/TargetP/>) (Nielsen et al., 1997; Emanuelsson et al., 2000). Prediction of transmembrane domains was done with Aramemnon (<http://aramemnon.botanik.uni-koeln.de/>) (Schwacke et al., 2003). Molecular masses and pI values were calculated using the aBi program ([http://www.up.univ-mrs.fr/~wabim/d\\_abim/compo-p.html](http://www.up.univ-mrs.fr/~wabim/d_abim/compo-p.html)). Homologies to other proteins were searched for using BLAST programs (<http://www.ncbi.nlm.nih.gov/BLAST/>) (Altschul et al., 1990). Identification of protein families and functional domains was performed using MyHits ([http://myhits.isb-sib.ch/cgi-bin/motif\\_scan](http://myhits.isb-sib.ch/cgi-bin/motif_scan)) and InterProScan (<http://www.ebi.ac.uk/InterProScan/>) (Quevillon et al., 2005). GHs and CEs were classified according to the CAZy database (<http://www.cazy.org/CAZY/>) (Coutinho et al., 1999). Peroxidases were named as in the PeroxiBase (<http://peroxidase.isb-sib.ch/index.php>) (Bakalovic et al., 2006).

#### *Protein measurements*

Protein concentration was determined by the method of Bradford (1996) using bovine serum albumin dissolved in extraction buffer as the standard.

## Results and discussion

### *Extraction of glycoside hydrolases from stem tissues of A. thaliana*

In a first attempt to study GHs from stem tissues of *A. thaliana* by using a proteomic approach it was necessary to establish a protocol for the extraction of these enzymes. Based on published experimental data on the purification of GHs from various plant organs (Sheldon et al., 1998; Wilson and Altmann, 1998; Minic et al., 2004; Li and Kushad, 2005; Van Riet et al., 2006), we developed a 2-step extraction procedure. Stems were ground in a buffer containing 200 mM CaCl<sub>2</sub>, followed by Con A Sepharose affinity chromatography. CaCl<sub>2</sub> was chosen as the most efficient salt for CWP extraction (Boudart et al., 2005). This protocol is different from those used in previous cell wall proteomic studies (Feiz et al., 2006): (i) the initial step is a grinding in a buffer containing 200 mM CaCl<sub>2</sub> to release CWPs instead of a low ionic strength buffer usually used to prevent CWP elution; (ii) there is no step of cell wall isolation to avoid losing CWPs weakly-bound to cell walls during the centrifugation steps required for cell wall isolation; (iii) the last step is an affinity chromatography to trap *N*-glycosylated proteins. Results show that the affinity chromatography step resulted in a significant increase in specific activities of several exo-GHs using artificial substrates compared to what was measured in the dialysed crude protein extract. These increases varied from 2.0 for  $\beta$ -D-xylosidase to 6.1 for  $\beta$ -D glucosidase (Table 1). On the basis of this observation, this protocol was adapted to analyze the *N*-glycoproteome of mature stems.

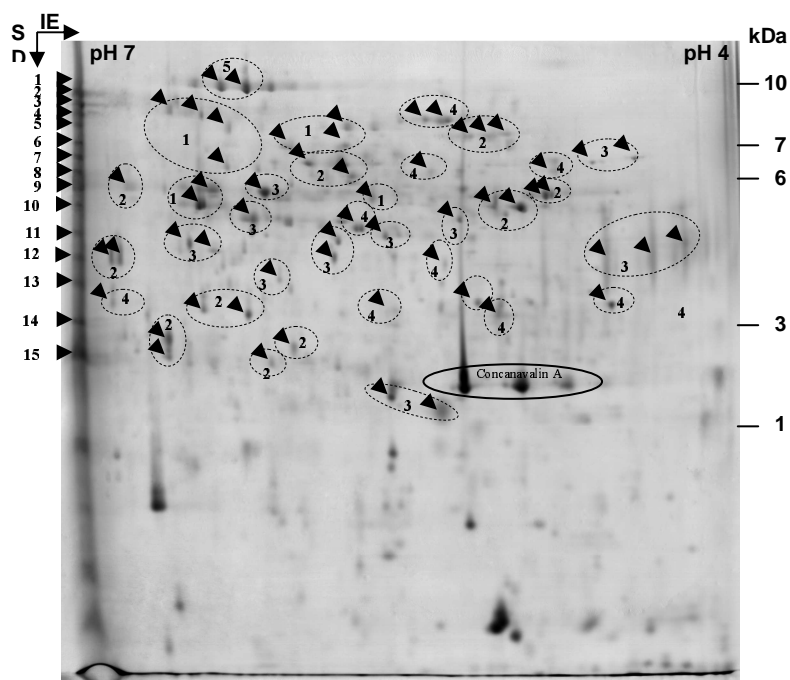
**Table 1.** *Specific activities of several glycoside hydrolases after Con A Sepharose affinity chromatography.*

All enzyme activities were measured *in vitro* at 37 °C, using 50  $\mu$ L of protein and pNP-glycosides as substrates.

Enzyme	Specific activities (nmol/min/mg protein)		Recovery (%)	Ratio of specific activities Con A Sepharose/ Crude protein extract
	Crude protein extract	After Con A Sepharose		
$\beta$ -D-xylosidase	32	89	43	2.8
$\alpha$ -L-arabinofuranosidase	103	442	66	4.3
$\beta$ -D-glucuronidase	10	22	34	2.0
$\beta$ -D-mannosidase	26	108	64	4.2
$\alpha$ -D-mannosidase	47	172	56	3.6
$\beta$ -D-glucosidase	82	497	93	6.1
$\alpha$ -D-galactosidase	85	468	84	5.5
$\beta$ -D-galactosidase	393	825	32	2.1
$\alpha$ -D-glucosidase	8	31	59	3.9

### *Proteomic analysis after enrichment of the soluble protein extract in glycoside hydrolases by lectin affinity chromatography*

The proteomic analysis was performed using protein extracts from 18-22 cm mature stems at the late flowering stage. About 10 g of stem tissues were used for the extraction of proteins. After grinding and centrifugation, the crude protein extract contained 2.5 mg of protein as determined by the Bradford method (1996). A fraction of this protein extract was subjected to Con A Sepharose affinity chromatography. Eluted proteins were concentrated and dialysed, resulting in a fraction of 400  $\mu$ L containing about 300  $\mu$ g of protein. A sample containing 250  $\mu$ g of protein was subjected to 2D-electrophoresis. Proteins were detected by colloidal Coomassie blue staining (Fig. 1). The number of resolved spots was about 200. Fifty-seven spots resolved by 2D-electrophoresis were analyzed using MALDI-TOF. Spots corresponding to proteins having molecular mass smaller than 20 kDa were not analyzed since they are not expected to contain GHs on the basis of calculations made from genes predicted to encode such proteins (Minic and Jouanin, 2006). The other proteins visible on the gel were also analyzed, but due to small quantity or mixture with other proteins their scores were not significant. Fifteen spots localized at the basic side of the gel were subjected to tryptic digestion and proteins were identified using nanoHPLC-MS/MS. Each of them was expected to contain more than one protein since previous studies showed that most CWP are basic (Jamet et al., 2006). A total number of 102 different proteins was identified (Table 2; Tables S1 and S2 at JXB online). Many of these proteins were present in several of the spots resolved by 2D-electrophoresis suggesting post-translational modifications such as glycosylations. On the basis on these results, those spots were collected into thirty-five groups as shown in Fig 1. Conversely, as expected, most of the 15 spots at the basic side of the gel contained more than one protein.



**Fig 1.** Analysis of *A. thaliana* proteins by 2D-electrophoresis. The 2D-gel was loaded with 250  $\mu$ g of the fraction obtained after Con A Sepharose affinity chromatography from stem tissues of *A. thaliana*. The gel was stained with colloidal Coomassie blue. Fifteen spots were picked for nanoHPLC-MS/MS (1 to 15) and fifty-seven for MALDI-TOF analyses. In the latter case, numbering refers to groups of spots containing the same protein. Arrows in the circles represent same identified proteins. Molecular mass markers are indicated on the right.

### *Bioinformatic prediction of sub-cellular localization and N-glycosylation of identified proteins*

PSORT, TargetP and Aramemnon programmes were used to predict the sub-cellular localization of the identified proteins. Seventy-seven out of the 102 identified proteins (77%) were predicted to be localized in the cell wall matrix, 13 at the plasma membrane, 6 into the endoplasmic reticulum, 2 in the cytoplasm and 3 in the chloroplast. Six proteins were predicted to be either targeted to vacuoles or to the cell wall. However, vacuolar targeting is not well-established in plants and the predictions are not yet very reliable (Hadlington and Denecke, 2000). Altogether, about 90% of the proteins have a predicted N-terminal signal peptide, which means that all of these proteins are targeted to the secretory pathway. Two proteins could not be assigned to any sub-cellular compartment due to discrepancies between predictions with PSORT and TargetP. Seven proteins were predicted to harbour a glycosyl phosphatidyl inositol (GPI) anchor. As expected, all of the identified proteins contained *N*-glycosylation sites as predicted by the MyHits programme (see Supplementary Table S1 at JXB).

These results show that the proposed protocol allowed the isolation of a protein fraction essentially composed of *N*-linked glycoproteins targeted to either the cell wall or to the plasma membrane. Despite the absence of a cell wall purification step, it should be noted that the proportion of proteins with a predicted intracellular localization was very low (12%). This protocol thus appears as an efficient alternative to previously described protocols used for *A. thaliana* cell wall proteomic analyses. Previous protocols include: (i) non-destructive methods such as analysis of culture media (Borderies et al., 2003; Charmont et al. 2005), washing of cells cultured in liquid medium with salt solutions (Borderies et al., 2003; Kwon et al. 2005) and vacuum infiltration of leaves (Boudart et al., 2005); (ii) destructive methods, *i.e.* cell wall purification, prior to CWP extraction with various buffers (Chivasa et al. 2002; Bayer et al., 2006; Feiz et al., 2006). The choice of a particular protocol will depend on the aim of the study and on the plant organ of interest.

### *Identification and functional classification of proteins*

Identification of protein families and functional domains were performed using several bioinformatic programmes. Proteins were classified according to the predicted functional classes of CWPs proposed by Jamet et al. (2006) (Tables 2, 3). Proteins belonging to seven functional classes were found according to the presence of functional domains predicted as described in the Material and methods: (i) proteins acting on polysaccharides include GH and esterases; (ii) oxido-reductases mainly include peroxidases and multicopper oxidases; (iii) proteins with interacting domains include proteins with lectin or LRR (leucine rich repeat) domains as well as enzyme inhibitors; (iv) proteins involved in signaling processes include fasciclin AGPs (arabinogalactan proteins); (v) proteases; (vi) proteins of yet unknown function; (vii) miscellaneous. However, this classification is provisional since the biological role of many of these proteins remains to be determined (Tatosov et al., 1997). For example, an enzyme of the GH 3 family (XYL3) shows amino acid homology with  $\beta$ -D-xylosidase. However, it was identified as an enzyme that efficiently hydrolyzed arabinosyl residues from arabinans, suggesting that it works as an  $\alpha$ -L-arabinofuranosidase (Minic et al., 2006).



**Table 2.** *Proteins identified through proteomic analysis of A. thaliana mature stems*

<b>Predicted or known functions<sup>a</sup></b>	<b>Accession</b>	<b>Spot number<sup>b</sup></b>
<b>Proteins acting on polysaccharides</b>		
GH family 1	At2g44450	7
GH family 1	At3g18080	13, 14, 15
GH family 1	At5g54570	47
GH family 1 (thioglucoside hydrolase 1) (TGG1)	At5g26000	41
GH family 3 (BXL1) (beta-xylosidase 1)	At5g49360	5, 9, 11, 13
GH family 3 (BXL2) (beta-xylosidase 2)	At1g02640	13, 15
GH family 3	At5g20950	5, 6, 8
GH family 16 (xyloglucan endotransferase) (EXGT-A1) (At-XTH4)	At2g06850	13, 14, 15
GH family 16 (xyloglucan endotransferase) (Meri5) (At-XTH24)	At4g30270	15
GH family 17	At3g07320	6, 7
GH family 17	At5g56590	11, 13
GH family 17	At4g31140	33
GH family 19 (chitinase), carbohydrate-binding module family 18 (chitin binding function)	At2g43570	31
GH family 20 (N-acetyl-beta-glucosaminidase)	At1g65590	7, 10
GH family 20 (N-acetyl-beta-glucosaminidase)	At3g55260	22
GH family 28 (polygalacturonase)	At1g80170	8, 9, 10

GH family 28 (polygalacturonase)	At3g07830	24
GH family 28 (polygalacturonase)	At3g16850	29
GH family 31 (alpha-glucosidase) (AtGLU1)	At5g11720	9, 13
GH family 31 (alpha-xylosidase) (XYL1)	At1g68560	17
GH family 32 (beta-fructofuranosidase) (ATBETAFRUCT4)	At1g12240	28
GH family 35 (beta-galactosidase) (BGAL1)	At3g13750	8
GH family 35 (beta-galactosidase) (BGAL9)	At2g32810	10
GH family 35 (beta-galactosidase) (BGAL10)	At5g63810	7, 13
GH family 38 (alpha-mannosidase)	At5g13980	13
GH family 38 (alpha-mannosidase)	At3g26720	42
GH family 51 (alpha-L-arabinofuranosidase)	At3g10740	27
GH family 79 (endo-beta-glucuronidase / heparanase)	At5g07830	10
GH family 79 (endo-beta-glucuronidase / heparanase )	At5g34940	7
CE family 8 (pectin methylesterase)	At3g43270	10
CE family 13 (pectin acylesterase)	At5g23870	9

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### **Oxido-reductases**

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homolog to SKU5 (SKS4) (multicopper oxidase domain)	At4g22010	6
homolog to SKU5 (SKS5) (multicopper oxidase domain)	At1g76160	7, 8
homolog to SKU5 (SKS6) (multicopper oxidase domain)	At1g41830	7, 8, 12

homolog to SKU5 (SKS7) (multicopper oxidase domain)	At1g21860	7, 12, 13
homolog to SKU5 (SKS17) (multicopper oxidase domain)	At5g66920	10
SKU5 (multicopper oxidase domain)	At4g12420	3, 6, 7, 8, 9
homolog to peroxidase (AtPrx22)	At2g38380	11
homolog to peroxidase (AtPrx28)	At3g03670	49
homolog to peroxidase (AtPrx33)	At3g49110	11, 13
homolog to peroxidase (AtPrx34)	At3g49120	23
homolog to <i>C. pepo</i> ascorbate oxidase (P37064)	At5g21100	11
homolog to berberine bridge enzyme (S)-reticulinn: oxygen oxido-reductase	At4g20830	13
homolog to germin (AtGER3)	At5g20630	32

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#### **Proteins with interacting domains**

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homolog to carrot EDGP and tomato XEGIP	At1g03230	9, 10
homolog to carrot EDGP and tomato XEGIP	At1g03220	39
expressed protein (LRR repeats)	At1g33590	26
expressed protein (LRR repeats)	At1g33600	8, 9
expressed protein (LRR repeats)	At2g42800	14
polygalacturonase inhibiting protein (PGIP2)	At5g06870	13, 15
homolog to lectin (legume lectin beta domain)	At1g53070	13
homolog to lectin (legume lectin beta domain)	At1g53080	13, 14, 15

homolog to lectin (legume lectin beta domain)	At3g16530	1, 13, 14, 15
homolog to lectin (curculin-like)	At1g78850	7, 8
homolog to lectin (curculin-like)	At1g78860	7, 8
homolog to lectin (curculin-like)	At5g18470	7, 8, 10

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### **Signalling**

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fasciclin-like arabinogalactan protein (AtFLA8)	At2g45470	1, 13, 14, 15
fasciclin-like arabinogalactan protein (AtFLA13)	At5g44130	30

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### **Proteases**

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homolog to aspartyl protease	At1g09750	11, 12, 13
homolog to aspartyl protease	At3g52500	8, 9
homolog to aspartyl protease	At5g07030	10, 11, 12, 13
homolog to aspartyl protease	At3g54400	21
homolog to serine protease	At1g30600	7
homolog to serine protease	At2g39850	50
homolog to serine protease	At4g21630	12, 13, 14, 15
homolog to serine protease	At4g21650	10, 13, 14, 15
homolog to serine protease	At5g67360	16
homolog to serine protease	At3g14067	18
xylem serine peptidase 1 (XSP1)	At4g00230	9

homolog to serine carboxypeptidase (SCPL11)	At2g22970	14, 15
homolog to serine carboxypeptidase (SCPL12)	At2g22920	15
homolog to serine carboxypeptidase (SCPL34)	At5g23210	12, 13
<b>Miscellaneous</b>		
homolog to anther specific proline-rich protein APG (lipase acylhydrolase domain)	At1g54000	13
homolog to anther specific proline-rich protein APG (lipase acylhydrolase domain)	At1g67830	12
homolog to purple acid phosphatase	At2g16430	7
homolog to purple acid phosphatase	At2g27190	13
homolog to purple acid phosphatase	At5g34850	19
homolog to <i>Chlamydomonas reinhardtii</i> apospory associated protein (aldose-1-epimerase domain)	At4g25900	13
homolog to glycerophosphoryl diester phosphodiesterase	At4g26690	35
<b>Unknown function</b>		
expressed protein	At1g21680	5, 6
expressed protein	At1g21670	2, 3
expressed protein	At3g14920	14, 15
expressed protein (DUF642)	At4g32460	44, 13
expressed protein (DUF642)	At5g11420	10, 11, 12
expressed protein (DUF642)	At5g25460	12
expressed protein (DUF1005)	At4g29310	13

expressed protein (DUF1184)	At4g18080	46
expressed protein (DUF1680)	At5g12950	5
expressed protein (cupin domains)	At3g22640	34
<b>Intracellular proteins</b>		
homolog to thioredoxin	At1g21750	45
GH family 1	At2g25630	7
homolog to serine protease	At2g05920	3, 9
homolog to serine carboxypeptidase	At4g36195	43
homolog to phosphorylase	At4g24350	12
homolog to gamma-glutamyltranspeptidase	At4g39640	12
homolog to aldo-keto reductase	At2g27680	37
homolog to peroxiredoxin	At3g52960	25
homolog to copper amine oxidase	At4g12990	21
expressed protein (PH domain)	At2g30880	36
expressed protein (RNA recognition motif)	At3g52980	40
expressed protein	At2g41950	48
expressed protein (CBS domains)	At3g48530	38

<sup>a</sup> Functions are predicted as described in Experimental

<sup>b</sup> Spot number refers to Fig. 1.

**Table 3.** *Predicted functional classes of proteins in the A. thaliana stem sub-proteome.* Functional classes have been defined according to Jamet et al. (2006). A list of all proteins identified in this study is provided in Table 2. The detailed bioinformatics functional analysis is given in Supplementary Table S1 at JXB online.

Functional classes	Number of proteins
<b>Proteins acting on polysaccharides</b>	<b>31</b>
Glycoside hydrolases	29
Esterases	2
<b>Oxido-reductases</b>	<b>13</b>
Peroxidases	4
Multicopper oxidases	6
Others	3
<b>Proteins with interacting domains</b>	<b>12</b>
Lectin domains	6
LRR domains	3
Others	3
<b>Signalling</b>	<b>2</b>
<b>Proteases</b>	<b>14</b>
Serine proteases	10
Aspartyl proteases	4
<b>Miscellaneous</b>	<b>7</b>
Homologs to phosphatase	3
Homologs to proline-rich protein (lipase acid hydrolase domain)	2
Others	2
<b>Unknown function</b>	<b>10</b>
<b>Intracellular proteins</b>	<b>13</b>
Total	102

Thirty-three proteins were expected to act on polysaccharides (Table 2). Furthermore, 30 proteins (29%) belong to the superfamily of GHs, 29 of which were predicted to be extracellular or plasma membrane-associated. The second largest group comprises 16 proteases, 14 of which were predicted to be localized in the extracellular matrix. Together GHs and proteases represent 47% of identified proteins. Among other proteins, oxido-reductases, proteins with interacting domains, miscellaneous proteins, proteins of unknown function, signalling and intracellular proteins were identified.

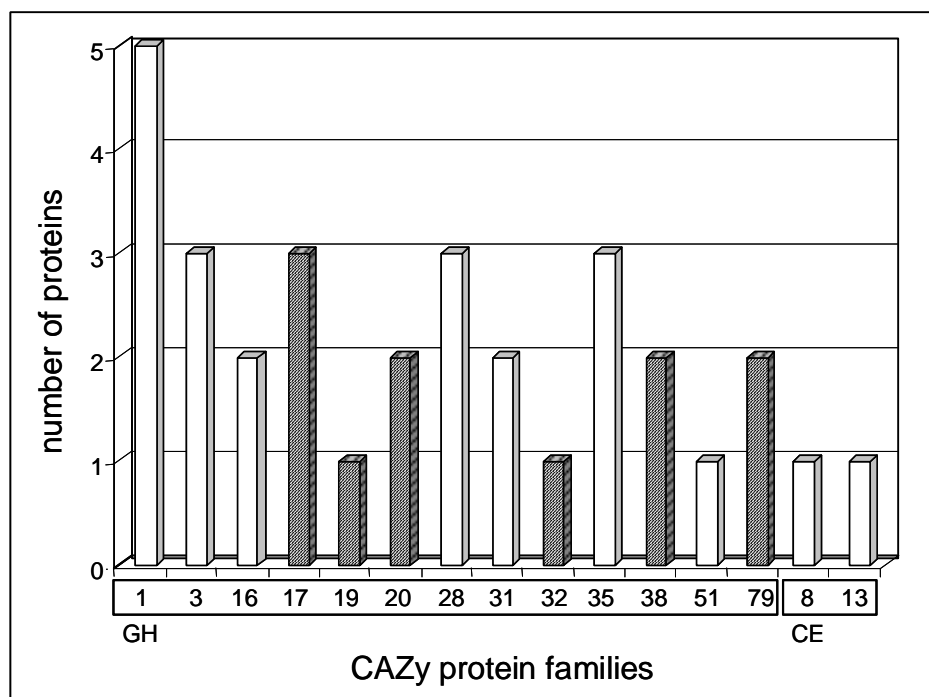
Proteins from the same functional classes as in previous cell wall proteomic studies were found, but 37 proteins were not identified before (Chivasa et al. 2002; Borderies et al. 2003; Borner et al., 2003; Schultz et al., 2004; Boudart et al., 2005; Charmont et al. 2005; Kwon et al. 2005; Bayer et al., 2006; Feiz et al. 2006). This stem N-glycoproteome thus appears to be very specific. Among the 90 proteins predicted to be at the plasma membrane or in the cell wall, only 5 also have been found in previously described proteomes: cell suspension cultures, rosette leaves and etiolated hypocotyls (Jamet et al., 2006). They encode a  $\beta$ -xylosidase that belongs to the GH 31 family (At1g68560), a multicopper oxidase (At1g76160), two lectins (At1g78850, At1g78860) and a protein homologous to the carrot extracellular dermal glycoprotein (EDGP) and to the tomato xyloglucan specific endoglucanase inhibitor protein (XEGIP) (At1g03220) (Qin et al., 2003). However, some protein families are missing. Since proteins with molecular masses lower than 20 kDa were not analyzed, it was not possible to identify homologs to protease or pectin methylesterase inhibitors, non-specific lipid transfer proteins, and blue copper binding proteins. Only one protein homolog to germins was identified. Although several expansins, which molecular masses are between 25 and 30 kDa, were previously identified in cell wall proteomes (Jamet et al., 2006), none was found in this study. This might be explained either by their low abundance or their low level of *N*-glycosylation. Finally, no structural protein could be identified either because of their strong binding to the extracellular matrix, or the absence of N-glycans.

#### *Possible roles of proteins identified in stem tissues of A. thaliana*

Proteins acting on polysaccharides constitute the major functional class. According to Coutinho et al. (1999), they belong to 12 GH families and to 2 carbohydrate esterase (CE) families (Fig. 2, Table 2). These enzyme families have diverse biological functions in defence, signalling, hydrolysis of starch, and cell wall modifications (Minic and Jouanin, 2006). A total of 18 GHs belonging to 7 different GH families were found. Three of them,  $\alpha$ -L-arabinofuranosidase (At3g10740),  $\alpha$ -L-arabinofuranosidase/ $\beta$ -D-xylosidase (At5g49360) and  $\beta$ -glucosidase AtGLU1 (At5g11720), were recently purified and characterized (Minic et al., 2004; Monroe et al., 1999). The XYL1  $\beta$ -xylosidase and two XTHs (Meri5/At4g30270, EXGT-A1/At2g06850) have been studied previously using both biochemical and genetic approaches (Akamatsu et al., 1999; Sampedro et al., 2001; Rose et al., 2002). A pectin methyl- and a pectin acyl-esterase were also identified. Previous studies have shown that pectin methylesterase activity is inversely correlated to the growth rate of expanding tissues, suggesting its possible involvement in wall rigidification (McQueen-Mason and Cosgrove, 1995).

Possible substrates *in muro* of the majority of these enzymes are xyloglucans and pectins. Most of them act as exo-enzymes whereas only enzymes belonging to GH families 16 and 28 act as endo-GHs on xyloglucans and homogalacturonans, respectively. Other cell wall GHs can hydrolyse  $\beta$ 1,4 glucan, arabinoxylan and xylan. These results suggest that xyloglucan and pectins, that are composed of homogalacturonans (HG), arabinans and galactans (RG-I), undergo structural changes in mature stem. However, some GH families can act on several natural polysaccharides showing broad substrate specificity. This low specificity has been reported in the case of several purified cell wall GHs (Leach et al, 1995; Kim and al., 2000; Sampedro et al., 2001; Steele et al., 2001; Rose et al., 2002; Lee et al., 2003; Minic et al, 2004; Minic et al., 2006). It has been hypothesized that it allows efficient modification of complex cell wall polysaccharides without requiring an extremely high number of enzymes (Minic et al, 2004; 2006).





**Fig. 2.** Distribution of families of glycoside hydrolases (GH) and carbohydrate esterases (CE) in the *A. thaliana* stem sub-proteome. Proteins are listed in Table 2. Proteins have been classified according to the CAZy nomenclature (Henrissat et al., 1998; <http://www.cazy.org/>). Glycoside hydrolase (GH) families from 1 to 79 are on the left whereas carbohydrate esterase (CE) families 8 and 13 are on the right. White bars correspond to families that might participate in cell wall modification and reorganization.

Many GH families described here could have other functions than cell wall modifications. Predicted extracellular GHs such as  $\beta$ -D-glucuronidase (GH 79),  $\alpha$ -D-mannosidase (GH 38) and acetyl-N-hexasaminase (chitinase-like enzymes, GH 19) could be involved in post-translational modifications of glycoproteins. Recently, an *A. thaliana*  $\beta$ -D-glucuronidase (AtGUS) was shown to hydrolyze glucuronic acids from carbohydrate chains of AGPs (Eudes, personal communication). Kinetic and structural analyses of Ginkgo  $\alpha$ -D-mannosidase acting on a pyridylamino derivative of oligo mannosides strongly suggested its involvement in the catabolism and turnover of *N*-linked glycoproteins (Woo et al., 2004). The pumpkin endo- $\beta$ -N-acetylglucosaminidase, partially purified from cotyledons, was highly active towards high-mannose type glycans (Kimura et al., 2002).

Among other GHs, one thioglucosyl hydrolase (GH 1), 3  $\beta$ -1,3-D-glucanase (GH 17) and 3 chitinase-like enzymes (GH 19 and 20) were identified. Thioglucosidases, also known as myrosinases, play diverse roles in cruciferous plant during growth, development and defence against microorganisms and insects (Rodman, 1991). Chitinase-like enzymes are able to degrade chitin in cell walls of fungal pathogens. However, the substrates and functions of most chitinase-like enzymes are not completely known. For example, a mutation in the chitinase-like gene classified in GH 19 family (AtCTL1/At1g05850) caused a cellulose deficiency as well as aberrant patterns of lignification with incomplete cell walls in the stem pith (Zhong et al., 2002; Rogers et al., 2005).

The second largest class of identified proteins comprises proteases. Seventeen putative proteinases including aspartyl and serine type proteases were found (Table 2). Fifteen of them were predicted to be secreted (Supplementary Table S1 at JXB online). The abundance of proteases in mature stem suggests that these enzymes may be actively involved in secondary

wall formation. Proteases may play various roles in plant development and during plant pathogen interactions through maturation of CWPs and generation of active peptides. It has been shown that the extracellular subtilisin-like serine protease SDD1 (STOMATAL DENSITY AND DISTRIBUTION 1) is involved in the regulation of stomatal density and distribution in *A. thaliana* (Berger and Altmann, 2000). ALE1 (ABNORMAL LEAF EPIDERMIS1) is also predicted to encode a subtilisin-like serine protease and is assumed to produce a peptide required for proper differentiation of epidermis (Tanaka et al., 2001). CDR1 (CONSTITUTIVE DISEASE RESISTANT 1) encodes a putative aspartic protease (Xia et al., 2004). Overexpression of CDR1 causes dwarfing and resistance to virulent *Pseudomonas syringae*. It was shown that CDR1 generates a small mobile signal (3-10 kDa) sensitive to heating and to protease. The substrates of these three proteases are yet unknown. On the contrary, it was shown that the CLE (CLV3/ESR-related) basic secreted proteins are processed at their C-terminus to generate 14 amino-acid peptides that carry a biological activity (Ito et al., 2006; Kondo et al. 2006). In those cases, the proteases have not yet been identified. Finally, several plant cell wall proteomic analyses show a large discrepancy between the observed and the expected molecular masses of proteins (Boudart et al., 2005; Kwon et al., 2005; Zhu et al., 2006). Proteases could be involved in processing and/or turnover of cell wall proteins.

Several oxido-reductases such as multicopper oxidase-like (6 proteins), peroxidases (4 proteins), germin-like protein (1 protein) and a homolog to berberine bridge enzyme were identified. In contrast to previously characterized cell wall proteomes, this study allowed the identification of numerous multicopper oxidase-like proteins. They catalyse full, four-electron reduction of dioxygen (O<sub>2</sub>) to water (H<sub>2</sub>O) using a variety of substrates (Solomon et al., 1997). They belong to a large gene family of 19 members in *A. thaliana* (Jacob and Roe, 2005). Only two members of the family have been previously studied, SKU5 (At4g12420) and SKS6 (At1g41830). It was shown that SKU5 is involved in the control of root growth (Sedbrook et al., 2002) and that SKS6 contributes to cotyledon vascular patterning during development (Jacob and Roe, 2005). Peroxidases are involved in many physiological and developmental processes that have been reviewed recently (Passardi et al., 2004). They can be involved in both cell elongation processes and in their arrest. In the latter case, they catalyze the formation of bridges across phenolic residues of lignins and between lignins and adjacent cell wall proteins or polysaccharides.

Three extracellular acid phosphatases were identified in this work. The presence of phosphorylated proteins and phosphatases in plant cell wall has been reported in several proteomes (Chivasa et al., 2002; Kwon et al., 2005; Jamet et al., 2006). However, no extracellular kinase has yet been found (Chivasa et al., 2005). Acid phosphatases may participate in extracellular signalling events or in regulation of cell wall proteins.

Some proteins contained interacting domains, such as LRRs. The polygalacturonase-inhibiting protein (PGIP2/At5g06870) plays a role in plant defence (Di Matteo et al., 2006). Two proteins identified as fasciclin-like AGPs (AtFLA8/At2g45470, AtFLA13/At5g45130) can participate in cell-to-cell adhesion in plant (Johnson et al., 2003; Groover and Robischon, 2006). Finally, 10 proteins of unknown function were found.

## Concluding remarks

This study demonstrates the effectiveness of the purification procedure to isolate cell wall glycoproteins. This includes novel GHs, multicopper oxidases and proteases. In contrast to these analyses, we did not identify homologs to protease or pectin methylesterase inhibitors, non-specific lipid transfer proteins, blue copper binding proteins, expansins and structural

proteins. The abundance of GHs suggests a great plasticity of polysaccharides in cell walls, even in well-differentiated tissues such as mature stems. Finally, the presence of phosphatases, proteases and GHs suggests a complex regulation of cell wall proteins involving various types of post-translational modifications such as de-phosphorylation and hydrolytic processing by proteases or glycosidases.

## Supplementary data

Supplementary data are available at *JEB* online. Table S1: Proteins identified in *Arabidopsis thaliana* mature stems. Table S2: LC-MS/MS data allowing identification of proteins in spots 1 to 15.

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