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Analysis of the xylem sap proteome of *Brassica oleracea* reveals a high content in secreted proteins

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Abbreviations: AGP, arabinogalactan protein; FLA, fasciclin AGP; GH, glycoside hydrolase; GRP, glycine-rich protein; LTP, lipid transfer protein; XTH, endotransglucosylase-hydrolase

Abstract

Xylem plays a major role in plant development, and is considered part of the apoplast. Here we studied the proteome of *Brassica oleracea* cv Bartolo and compared it to the plant cell wall proteome of another *Brassicaceae*, the model plant *Arabidopsis thaliana*. *B. oleracea* was chosen because it is technically difficult to harvest enough *A. thaliana* xylem sap for proteomic analysis. We studied the whole proteome and an *N*-glycoproteome obtained after Concanavalin A affinity chromatography. Altogether, 189 proteins were identified by LC-MS/MS using *Brassica* EST and cDNA sequences. A predicted signal peptide was found in 164 proteins suggesting that most proteins of the xylem sap are secreted. Eighty one proteins were identified in the *N*-glycoproteome, with 25 of them specific of this fraction, suggesting that they were concentrated during the chromatography step. All the protein families identified in this study were found in the cell wall proteomes. However proteases and oxidoreductases were more numerous in the xylem sap proteome, whereas enzyme inhibitors were rare. The origin of xylem sap proteins is discussed. All the experimental data including the MS/MS data were made available in the *WallProtDB* cell wall proteomic database.

1 Introduction

Xylem tissue is a major component of the vascular system of plants with a critical role in the transport of water, minerals and nutrients [1]. It is composed of tracheary elements, parenchyma cells, and fibers. During their differentiation, tracheary elements lose their nuclei and cellular content. A lignified secondary wall is formed. At the end of the process, cell death occurs, leaving a hollow tube which becomes a part of a vessel [2]. Xylem sap was shown to contain small molecular weight inorganic compounds and organic substances including hormones, amino acids, sugars, oligo- and polysaccharides, and proteins (for a review, see [3]). The question of the origin of xylem sap proteins was discussed and it was assumed that they could be breakdown products occurring during xylem formation or produced by parenchyma cells adjacent to xylem tissue [3, 4]. Until now, only a few xylem sap proteins were shown to be synthesized in roots [5, 6]. The current hypothesis is that proteins are secreted and/or synthesized by the stele cells and transported to the xylem vessels by the flow of water controlled by both transpiration in the leaves and pressure in the roots [3]. Then, the xylem sap is more and more considered as part of the apoplast [3].

Information on protein content of xylem sap is available for several plants such as *Brassica napus* [4, 7, 8], *B. oleracea* [7], *Cucumis sativus* [6, 7, 9], *Cucurbita maxima* [7], *Glycine max* [10, 11], *Malus domestica* [12], a poplar hybrid (*Populus trichocarpa* x *P. deltoides*) [13], *Prunus persica* [12], *Pyrus communis* [12], *Solanum lycopersicum* [14-16] and *Zea mays* [17]. Some of these studies were focused on some proteins, whereas others described xylem sap proteomes. However, because of the lack of genomic or EST sequences for all the plants studied, the identification of proteins by mass spectrometry (MS) and bioinformatics was mainly done using heterologous sequences. Several protein families were found such as glycosyl hydrolases (GHs) including xyloglucan endotransglucosylases-hydrolases (XTHs), peroxidases, proteases, lectins, pathogenesis-related proteins (PR-proteins), and cell wall structural proteins such as glycine-rich proteins (GRPs). It was also shown that the xylem sap proteome may change during plant-microbe interactions. Proteins homologous to basic glucanases (GH family 17, GH17) and PR4 were found in xylem sap of *B. napus* in response to infection by *Verticillium longisporum* and were assumed to contribute to defense [8]. Proteins homologous to cationic peroxidases and serine proteases were found to be induced in the soybean xylem sap in response to *Phytophthora sojae* elicitor and were related to programmed cell death [11]. After inoculation by the *Bradyrhizobium japonicum* symbiont, a XTH slightly accumulated in soybean xylem sap, but no function could be assigned to this

protein [11]. On the contrary, a major Cys-rich protein of the healthy tomato xylem sap was shown to disappear after infection by *Fusarium oxysporum* [14].

A description of the xylem sap proteome of the model plant *Arabidopsis thaliana* should help us to better characterize this important compartment of the plant and might allow the characterization of determinants limiting vascular pathogen infection or facilitating their growth. However, such an analysis is still missing, probably because efficient xylem sap harvesting in *A. thaliana* is technically difficult. Such an analysis should complete proteomic studies on this model plant [18-27]. In this study, we took advantage of the close genetic vicinity between *A. thaliana* and another *Brassicaceae*, *Brassica oleracea* to identify proteins of the xylem sap. *B. oleracea* has two main advantages: (i) the diploid *Brassica* species are descended from an hexaploid ancestor and the genome of *A. thaliana* is similar to each of their hypothetical diploid progenitors and (ii) it is one ($2n=18$, CC genome) of the two ancestors of the *B. napus* amphidiploid ($2n=38$, AACC genome) [28] for which a systematic program of EST sequencing has been developed (<http://compbio.dfci.harvard.edu/tgi/gi/bngi/GenInfo.html>). Two different proteomes of *B. oleracea* xylem sap were studied: a xylem sap proteome and the xylem sap *N*-glycoproteome because *N*-glycosylation was assumed to be a major post-translational modification (PTM) that occurs in secreted proteins. The identified proteins are homologous to previously described cell wall proteins, except that no structural protein was identified [29]. The origin of xylem sap proteins is discussed using *A. thaliana* root transcriptomic data available online.

2 Material and methods

2.1 Xylem sap harvesting

Xylem sap harvesting method from the cultivated plant Bartolo cabbage (*Brassica oleracea* var. *capitata*) was adapted from [30]. Harvesting was found to be optimal for 6-8 week-old plants. Briefly, stems were cut with a razor blade 2-3 cm above the cotyledons and below the first leaves. Before sampling from the remaining stem on the root side, the cut surface was washed with water to remove the content of cut cells and the phloem sap which exudes after cutting, and gently dried with a paper towel. Aliquots of xylem sap was collected in a tube using a micropipette (Supporting information Fig. 1) and stored at -20°C immediately after harvesting. All the xylem sap aliquots were pooled prior to further analysis. Before use, the samples were filtered using $0.45\ \mu\text{m}$ Millipore filters (Carrigtwohill, Ireland), to discard soil

particles, microbial cells or tissue remnants. After 8 h-sampling, we obtained from 0.3 to 0.7 mL of xylem sap from one plant. The experiment was performed twice.

2.2 Preparation of the protein samples for LC-MS/MS analysis

After harvesting the *B. oleracea* xylem sap, the sample was dialyzed against buffer 1 (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM MgCl₂/MnCl₂/CaCl₂) in a *Mega GeBAflex-tube* (MWCO 12-14 kDa, GeBA, Yavne, Israel). Half of this sample, the “xylem sap proteome”, was desalted using Econo-Pac 10 DG columns (Bio-Rad, Hercules, CA) and lyophilized. The second half of the sample used to get the “xylem sap *N*-glycoproteome” was directly separated by affinity chromatography on Concanavalin A (ConA) (Sigma, St Louis, MO). ConA lectin affinity chromatography is specific for Man residues and allows specific capture of *N*-glycoproteins [19]. Briefly, the resin was pre-washed with 20X volume of buffer 2 (20 mM Tris-HCl pH 7.4, 1 M NaCl, 3.3 mM MgCl₂/MnCl₂/CaCl₂) and equilibrated with 10X volume of buffer 1. The dialyzed xylem sap (10 mL) was mixed with the matrix (0.6 mL) in batch for 1 h at 4°C. After flow-through removal, the resin was washed three times with 1.5 mL of buffer 1. Proteins were eluted with 3X 1.5 mL of buffer 1 supplemented with 1 M methyl- α -D-glucopyranose (Sigma). The first and second eluted fractions were combined and, after desalting using Econo-Pac 10 DG columns (Bio-Rad) and lyophilization, employed to get the xylem sap *N*-glycoproteome.

2.3 Separation of proteins by SDS-PAGE

The two protein samples (xylem sap proteome and xylem sap *N*-glycoproteome) were suspended in 300 μ L and 100 μ L of UHQ water respectively. Fifty μ L of each sample were loaded on 10 x 12 x 0.15 cm SDS-polyacrylamide gels with a concentration of 12.50%/0.33% of acrylamide/bisacrylamide. Separation was performed as previously described [31]. The gel staining was carried out with Coomassie Brilliant Blue (CBB) [32], silver nitrate [32], or with the β -glucosyl Yariv reagent [33]. The rest of the samples were dried under vacuum prior to LC-MS/MS analysis.

2.4 LC-MS/MS analyses

Prior to analysis, proteins were briefly separated by SDS-PAGE to get three samples in order to increase the efficiency of tryptic digestion. In-gel digestion was performed as previously described [34]. Separation of tryptic peptides was performed by HPLC on a NanoLC-Ultra system (Eksigent, Dublin, CA). A 4 μ L sample was loaded at 7.5 μ L.min⁻¹ on a precolumn

cartridge (stationary phase: C18 PepMap 100, 5 μm ; column: 100 μm inner diameter, 1 cm in length; Dionex, Voisins le Bretonneux, France) and desalted with 0.1% HCOOH. After 3 min, the precolumn cartridge was connected to the separating PepMap C18 column (stationary phase: C18 PepMap 100, 3 μm ; column: 75 μm inner diameter, 150 mm in length; Dionex). Buffers were 0.1% HCOOH in water (A) and 0.1% HCOOH in ACN (B). The peptide separation was achieved with a linear gradient from 5 to 30% B for 28 min at 300 $\text{nL}\cdot\text{min}^{-1}$. One run took 45 min including the regeneration step at 95% B and the equilibration step at 95% A.

Eluted peptides were analyzed on-line with a LTQ XL ion trap (Thermo Electron, Thermo Fisher Scientific Inc, Courtaboeuf, France) using a nano electrospray interface as previously described with slight modifications detailed below [35]. Ionization (1.5 kV ionization potential) was performed with liquid junction and a noncoated capillary probe (10 μm inner diameter; New Objective). Peptide ions were analyzed using Xcalibur 2.07 (Thermo Fisher Scientific Inc) with the following data-dependent acquisition steps: (1) full MS scan (mass-to-charge ratio (m/z) 300 to 1400, centroid mode); and (2) MS/MS ($q_z = 0.25$, activation time = 30 ms, and collision energy = 35%; centroid mode). Step 2 was repeated for the three major ions detected in step 1. Dynamic exclusion was set to 30 s.

A database search was performed with X! Tandem (version 2010.01.01.4) (<http://www.thegpm.org/tandem/>) using parameters and protein identification specifications previously described [35]. Three databases were used: (i) a *Brassica napus* EST database (Compbio, http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=oilseed_rape); (ii) the Arabidopsis Information Resource (TAIR, <http://www.arabidopsis.org/>) database (32825 entries, version 8); and (iii) a contaminant database (trypsin, keratins). To take redundancy into account, proteins with at least one peptide in common were grouped. Within each group, proteins with at least one specific peptide relatively to other members of the group were reported as sub-groups.

2.5 Bioinformatics

Two databases were used to analyze the cell wall proteome of *B. oleracea*: *ProtAnnDB* (<http://www.polebio.scsv.ups-tlse.fr/ProtAnnDB/>) for the annotation of *A. thaliana* proteins [36]; and *WallProtDB* which collects *A. thaliana* cell wall proteomes (<http://www.polebio.scsv.ups-tlse.fr/WallProtDB/>) [37]. The *Brassica* proteins were annotated

as previously described for *A. thaliana* proteins [36]. Several available software were used to predict sub-cellular localization and functional domains of proteins: TargetP (<http://www.cbs.dtu.dk/services/TargetP/>) and SignalP (<http://www.cbs.dtu.dk/services/SignalP/>) for sub-cellular localization and InterProScan for prediction of functional domains (<http://www.ebi.ac.uk/Tools/InterProScan/>). The results were combined to improve the quality of the predictions and to propose a structural and a functional annotation. The PROSITE software was used to predict *N*-glycosylation sites (<http://www.expasy.org/prosite/>). The AREX database was used to look for the root pattern of expression of *A. thaliana* genes homologous to those of *B. oleracea* encoding xylem sap proteins (<http://www.arexdb.org/>) [38].

2.6 Implementation of MS/MS data in WallProtDB

This *WallProtDB* knowledgebase was developed with PHP5/AJAX/MySQL5. It presently contains CWPs (476 from *A. thaliana*, 263 from *Oryza sativa*) and ESTs (162 from *B. oleracea*) which were classified as described [29]. *B. oleracea* sequences are linked to their closest homologues in *A. thaliana* as inferred from BLASTX searches (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>, [39]). For *B. oleracea*, the proteomic data are linked to the MS data allowing protein identification. The spectra files are stored on a GPM server and usable with X! Tandem *via* a simple hyperlink. *WallProtDB* can be queried *via* an html form with various criteria including plant species, organ, and experimental conditions. User can refine their selection if necessary and export the result in a tab delimited text, or export the sequences of interest in the FASTA format.

3. Results

3.1 Harvesting of xylem sap and separation of proteins by SDS-PAGE

The xylem was collected during 8 h from cut stems of *B. oleracea* (Supporting information Fig. 1). To avoid contamination by phloem sap and intracellular proteins of cut cells, the cut surface was rinsed with water. It was not possible to quantify the amount of proteins with the Bradford reagent probably because of their low concentration [40]. However, CBB stained bands were clearly visible after separation of proteins of the total extract (xylem sap proteome) by SDS-PAGE (Fig. 1, lane A). After lectin affinity chromatography on ConA to separate *N*-glycoproteins (xylem sap *N*-glycoproteome), a distinct profile of proteins was obtained showing enrichment in certain proteins and depletion in others (Fig. 1, lane B). Again, it was not possible to get reliable quantification of proteins after ConA affinity

chromatography. In both cases, the profiles do not suggest any protein degradation. This was confirmed by a good distribution of the peptides allowing protein identification by LC-MS/MS all over the protein amino acid sequences (Supporting information Tables S1 and S2). A staining with silver nitrate showed additional bands of lower molecular masses (Fig.1, lanes C and D). The total extract was also submitted to β -glucosyl Yariv reagent staining to reveal arabinogalactan proteins (AGPs) which are poorly stained by CBB and silver nitrate because of their high degree of glycosylation. A smear was observed at the top of the gel showing the presence of AGPs in the sample (Fig. 1, lane E). Both the total extract and the *N*-glycoproteins retained on ConA were then analyzed by LC-MS/MS.

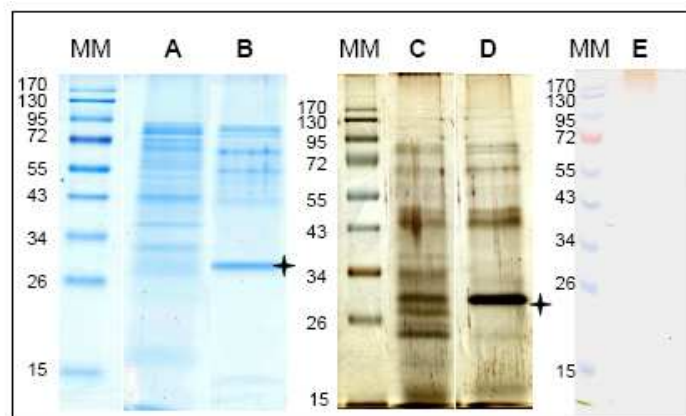


Figure 1. Separation of xylem sap proteins by SDS-PAGE.

Proteins from the xylem sap were separated by SDS-PAGE either directly after sampling and dialysis (lanes A and C, xylem sap proteome), or after an additional step of affinity chromatography on ConA (lanes B and D, xylem sap *N*-glycoproteome). The gel was stained with CBB (lanes A and B) or silver nitrate (lanes C and D). Bands shown by a star correspond to the ConA protein leaking from the column. The total extract was also stained by the β -glucosyl Yariv reagent (lane E). MM are molecular mass markers (kDa).

3.2 Identification by LC-MS/MS of proteins present in the xylem sap proteome and in the xylem sap *N*-glycoproteome trapped on ConA

Most of the previous studies on xylem sap relied on separation of proteins by 2D-electrophoresis prior to MS analysis. However, 2D-electrophoresis can be limiting in the case of secreted proteins which are mostly basic glycoproteins [41]. In our study, proteins were not separated before digestion and liquid chromatography tandem MS (LC-MS/MS). This technique also allowed working with limiting amount of proteins. *B. napus* cDNA and EST sequences were used for proteins identification. In all cases, it was possible to identify

proteins using *Brassica* EST or cDNA sequences. This was a great advantage since it allowed precise identification of the genes encoding the proteins especially in multigene families.

Altogether, 189 proteins were identified by LC-MS/MS with at least two peptides sequenced per protein (Table I, Supporting information Tables S1 and S2). One hundred and sixty four proteins were identified in the xylem sap proteome, whereas 81 proteins were identified in the xylem sap *N*-glycoproteome (Supporting information Table S3). Fifty six proteins were common to both proteomes. Twenty five proteins were only found in the xylem sap *N*-glycoproteome. This fraction was probably enriched in these proteins after selection by the ConA affinity chromatography. Identification of proteins in previously published *B. napus* and *B. oleracea* xylem sap proteomes were done by comparison to heterologous sequences, mainly from *A. thaliana* [4, 7, 8]. We performed a new TBLASTN analysis against *B. napus* ESTs with the peptide sequences and obtained the identification of 45 different proteins. Then it was possible to compare these results to our data. Twenty out of these 45 proteins were also found in our study (see Table I). On the contrary, 25 proteins present in one of these proteomes were not found in ours probably because of different culture conditions.

Table I. Proteins identified by LC-MS/MS in the xylem sap of *B. oleracea*

protein number ^a	<i>B. napus</i> EST, cDNA or protein accession number ^b	homologue in <i>A. thaliana</i>	predicted functional domain ^c	predicted sub-cellular localization ^d
Proteins acting on carbohydrates (48)				
71	TC38976	At3g18080	GH1 (β -glucosidase, β -mannosidase) (AtBGLU44 homologue)	chloroplast: 0.265 / SignalP-NN: 1-26, 0.679
125-1	TC20974/TC45939	At5g44640	GH1 (β -glucosidase) (AtBGLU3 homologue)	N-ter missing (secretory pathway: 1-22, 0.811)
125-2	TC50854	At2g44450	GH1 (β -glucosidase) (AtBGLU15 homologue)	N-ter missing (secretory pathway: 1-22, 0.853)
19-1	TC36871	At5g64570	GH3 (β -D-xylosidase) (AtXYL4 homologue)	N-ter missing (secretory pathway: 1-38, 0.773)
19-2	TC20792/CD814082	At5g64570	GH3 (β -D-xylosidase) (AtXYL4 homologue)	mitochondrion: 0.706 / SignalP-NN: 1-33, 0.797
40	TC20222/EE464798	At5g64570	GH3 (β -D-xylosidase) (AtXYL4 homologue)	chloroplast: 0.698 / SignalP-NN: 1-44, 0.644
83	TC32271	At5g10560	GH3 (β -D-xylosidase)	N-ter missing (secretory pathway: 1-18, 0.311)
102	TC49601	At4g33820	GH10	N-ter missing (secretory pathway: 1-23, 0.966)
23	TC32833	At4g15210	GH14	secretory pathway: 1-32, 0.977
52-1	TC52964	At4g25810	GH16 (xyloglucan:xyloglucosyltransferase) (AtXTH23 homologue)	N-ter missing (secretory pathway: 1-24, 0.988)
52-2	TC23545	At4g30270	GH16 (xyloglucan:xyloglucosyltransferase) (AtXTH24 homologue)	secretory pathway: 1-22, 0.971
3-1	TC38745 (<i>B. juncea</i>) [4, 8]	At3g57240	GH17 (β -1,3-glucanase)	secretory pathway: 1-34, 0.960
3-2	Q2HZ53 (<i>B. juncea</i>)	At3g57240	GH17 (β -1,3-glucanase)	N-ter missing (secretory pathway: 1-33, 0.980)
47-1	TC26578	At5g42720	GH17 (β -1,3-glucanase)	secretory pathway: 1-22, 0.911
47-2	TC24955	At4g34480	GH17 (β -1,3-glucanase)	secretory pathway: 1-22, 0.942
66	TC32141	At4g34480	GH17 (β -1,3-glucanase)	secretory pathway: 1-22, 0.931
95	TC30288	At5g55180	GH17 (β -1,3-glucanase)	secretory pathway: 1-22, 0.829
128	Q2VT22 (<i>B. rapa</i>)	At3g57260	GH17 (β -1,3-glucanase)	secretory pathway: 1-37, 0.713
159	TC49994	At3g55430	GH17 (β -1,3-glucanase)	N-ter missing (secretory pathway: 1-28, 0.973)
28	TC17043	At5g24090	GH18 (chitinase)	secretory pathway: 1-27, 0.985
88	TC54505	At4g19810	GH18 (chitinase)	N-ter missing (secretory pathway: 1-24, 0.944)
8	TC58115 [4]	At2g43590	GH19 (chitinase)	secretory pathway: 1-33, 0.938
110	TC15913	At3g12500	GH19 (chitinase)	secretory pathway: 1-20, 0.989
116	TC25879	At3g55260	GH20 (<i>N</i> -acetyl-hexosaminidase)	N-ter missing (secretory pathway: 1-20, 0.805)
56	TC37303	At3g56310	GH27	secretory pathway: 1-23, 0.689
144	EV101247	At5g08370	GH27	N-ter missing (secretory pathway: 1-25, 0.909)
26-1	TC25741/TC46151 [4]	At3g61490	GH28 (polygalacturonase)	N-ter missing (secretory pathway: 1-23, 0.765)

26-2	EV178995	At3g61490	GH28 (polygalacturonase)	N-ter missing (secretory pathway: 1-23, 0.765)
26-3	EV121625	At3g23500	GH28 (polygalacturonase)	N-ter missing (secretory pathway: 1-22, 0.948)
27	TC23229	At4g23500	GH28 (polygalacturonase)	secretory pathway: 1-25, 0.613
81-1	TC21367	At5g49215	GH28 (polygalacturonase)	N-ter missing (secretory pathway: 1-21, 0.825)
81-2	TC28593	At3g62110	GH28 (polygalacturonase)	N-ter missing (secretory pathway: 1-21, 0.946)
107	EE453748	At5g41870	GH28 (polygalacturonase)	N-ter missing (secretory pathway: 1-21, 0.989)
97	TC60884	At5g11720	GH31 (α -glucosidase) (AtGLU1 homologue)	N-ter missing (secretory pathway: 1-23, 0.976)
65	TC31486/TC29838	At2g28470	GH35 (β -D-galactosidase) (AtBGAL8 homologue)	secretory pathway: 1-23, 0.991
35	TC47393	At5g13980	GH38 (α -D-mannosidase)	secretory pathway: 1-23, 0.955
112	TC17346	At5g13980	GH38 (α -D-mannosidase)	N-ter missing (secretory pathway: 1-21, 0.953)
2-1	TC43276	At3g10740	GH51 (α -L-arabinofuranosidase/ β -D-xylosidase) (AtASD1 homologue)	N-ter missing (secretory pathway: 1-33, 0.962)
2-2	TC43393	At3g10740	GH51 (α -L-arabinofuranosidase/ β -D-xylosidase) (AtASD1 homologue)	N-ter missing (secretory pathway: 1-33, 0.962)
7-1	TC53916	At3g10740	GH51 (α -L-arabinofuranosidase/ β -D-xylosidase) (AtASD1 homologue)	N-ter missing (secretory pathway: 1-33, 0.962)
7-2	TC61736	At3g10740	GH51 (α -L-arabinofuranosidase/ β -D-xylosidase) (AtASD1 homologue)	secretory pathway: 1-33, 0.943
25	TC31356	At5g34940	GH79 (β -D-glucuronidase)	secretory pathway: 1-21, 0.988
122	TC41425	At5g09760	pectin methylesterase (CE8)	N-ter missing (SignalP-NN: 1-20, 0.803)
145	AY036606	At2g47550	pectin methylesterase (CE8)	secretory pathway: 1-23, 0.860
136	CD814593	At3g04910	pectin acylesterase (CE17)	N-ter missing (SignalP-NN: 1-44, 0.417)
91	TC16095	At5g45280	pectin acylesterase (CE23)	secretory pathway: 1-23, 0.989
51	TC16080	At4g17030	expansin-like B (AtEXLB1 homologue)	secretory pathway: 1-24, 0.988
138	DY023690	At5g02260	alpha-expansin (AtEXPA9)	N-ter missing (secretory pathway: 1-21, 0.949)

Oxido-reductases (39)

58	TC47947	At1g05260	peroxidase (AtPrx03 homologue)	secretory pathway: 1-24, 0.970
99	TC33540	At1g71695	peroxidase (AtPrx12 homologue)	secretory pathway: 1-31, 0.856
64	TC25610	At2g22420	peroxidase (AtPrx17 homologue)	secretory pathway: 1-20, 0.762
43	TC36205 [4, 8]	At2g37130	peroxidase (AtPrx21 homologue)	secretory pathway: 1-29, 0.981
20-1	TC30566	At4g08770	peroxidase (AtPrx37 homologue)	secretory pathway: 1-29, 0.514
20-2	TC27727	At3g32980	peroxidase (AtPrx32 homologue)	chloroplast: 1-54, 0.309/SignalP-NN: 1-30, 0.684
20-3	TC25612	At3g49120	peroxidase (AtPrx34 homologue)	secretory pathway: 1-32, 0.820
20-4	NP1447930	At3g49120	peroxidase (AtPrx34 homologue)	secretory pathway: 1-31, 0.525
77	TC18276	At4g11290	peroxidase (AtPrx39 homologue)	secretory pathway: 1-23, 0.962
49-1	TC58934 [4]	At4g33420	peroxidase (AtPrx47 homologue)	secretory pathway: 1-30, 0.772
49-2	TC61099 [4]	At4g33420	peroxidase (AtPrx47 homologue)	secretory pathway: 1-25, 0.920
13-1	TC35223 [7]	At5g05340	peroxidase (AtPrx52 homologue)	secretory pathway: 1-21, 0.796

13-2	TC45523 [4]	At5g05340	peroxidase (AtPrx52 homologue)	N-term missing (secretory pathway: 1-20, 0.916)
13-3	P00434 (<i>B. rapa</i>)	At5g05340	peroxidase (AtPrx52 homologue)	N-term missing (secretory pathway: 1-20, 0.916)
11-1	TC55805 [4]	At5g19890	peroxidase (AtPrx59 homologue)	secretory pathway: 1-25, 0.739
11-2	EE424445 [7]	At5g39580	peroxidase (AtPrx62 homologue)	secretory pathway: 1-22, 0.962
11-3	TC16466	At5g64120	peroxidase (AtPrx71 homologue)	secretory pathway: 1-23, 0.719
59	TC48654	At4g12420	multicopper oxidase (AtSKU5 homologue)	N-ter missing (secretory pathway: 1-20, 0.989)
111	TC16200	At4g12420	multicopper oxidase (AtSKU5 homologue)	secretory pathway: 1-20, 0.990
22-1	TC16404	At1g41830	multicopper oxidase (AtSKS6 homologue)	secretory pathway: 1-24, 0.970
22-2	TC38876	At1g76160	multicopper oxidase (AtSKS5 homologue)	secretory pathway: 1-23, 0.991
22-3	TC37557	At1g76160	multicopper oxidase (AtSKS5 homologue)	N-ter missing (secretory pathway: 1-23, 0.984)
22-4	TC19179	At1g76160	multicopper oxidase (AtSKS5 homologue)	secretory pathway: 1-23, 0.989
60-1	TC39082	At1g72230	uclacyanin (AtUCC8 homologue)	secretory pathway: 1-25, 0.972
60-2	TC20750	At1g72230	uclacyanin (AtUCC8 homologue)	secretory pathway: 1-24, 0.985
117	TC26850	At2g02850	plantacyanin (AtPNC homologue)	secretory pathway: 1-33, 0.825
38	TC29181	At2g25060	plastocyanin (AtEN7 homologue)	secretory pathway: 1-23, 0.988
39-1	TC32771	At4g27520	plastocyanin (AtEN12 homologue)	secretory pathway: 1-27, 0.975
39-2	TC47186	At4g27520	plastocyanin (AtEN12 homologue)	secretory pathway: 1-28, 0.967
63-1	TC21582	At4g31840	plastocyanin (AtEN13 homologue)	secretory pathway: 1-22, 0.989
63-2	TC17946	At4g31840	plastocyanin (AtEN13 homologue)	secretory pathway: 1-21, 0.986
63-3	TC23721	At4g31840	plastocyanin (AtEN13 homologue)	secretory pathway: 1-25, 0.991
130	TC21566	At5g15350	plastocyanin (AtEN22 homologue)	secretory pathway: 1-26, 0.970
153	TC25008	At5g15350	plastocyanin (AtEN22 homologue)	secretory pathway: 1-24, 0.981
5	TC18996	At4g20840	berberine-bridge oxido-reductase	secretory pathway: 1-26, 0.872
15-1	TC17262	At1g30760	berberine-bridge oxido-reductase	secretory pathway: 1-29, 0.979
15-2	TC38473	At2g34790	berberine-bridge oxido-reductase	N-ter missing (secretory pathway: 1-26, 0.989)
142	ES903641	At4g20840	berberine-bridge oxido-reductase	secretory pathway: 1-23, 0.803
143	EV087794	At5g44390	berberine-bridge oxido-reductase	N-ter missing (secretory pathway: 1-30, 0.825)

Proteases (28)

1-1	TC18683/TC28749/ TC22282 [4]	At5g67360	Ser protease (ATSBT1.7, ARA12 homologue)	secretory pathway: 1-29, 0.977
1-2	TC19435	At5g67360	Ser protease (ATSBT1.7, ARA12 homologue)	N-ter missing (secretory pathway: 1-24, 0.973)
12/33	ES901600/TC56831/ TC46249 [4]	At1g20160	Ser protease (ATSBT5.2 homologue)	secretory pathway: 1-29, 0.991
29-1	TC27270	At4g36195	Ser carboxypeptidase	secretory pathway: 1-21, 0.704
29-2	TC25039	At4g36190	Ser carboxypeptidase	secretory pathway: 1-25, 0.595
75	TC27534	At4g12910	Ser carboxypeptidase (AtSCPL20 homologue)	secretory pathway: 1-23, 0.988
30-1	TC34104	At4g30810	Ser carboxypeptidase (AtSCPL29 homologue)	secretory pathway: 1-27, 0.992
30-2	TC47262	At4g30810	Ser carboxypeptidase (AtSCPL29 homologue)	secretory pathway: 1-25, 0.984

69	EV124833 [7]	At1g11080	Ser carboxypeptidase (AtSCPL31 homologue)	N-ter missing (secretory pathway: 1-30, 0.902)
74	EV124923	At1g11080	Ser carboxypeptidase (AtSCPL31 homologue)	N-ter missing (secretory pathway: 1-30, 0.902)
14	TC45864	At3g45010	Ser carboxypeptidase (AtSCPL48 homologue)	secretory pathway: 1-25, 0.603
62	TC23586/EV048732	At2g27920	Ser carboxypeptidase (AtSCPL51 homologue)	secretory pathway: 1-20 0.810
18-1	TC21318	At5g10770	Asp protease	N-ter missing (secretory pathway: 1-25, 0.276)
18-2	Q8LK82	At5g10770	Asp protease	N-ter missing (secretory pathway: 1-25, 0.276)
18-3	ES904551	At5g10770	Asp protease	N-ter missing (secretory pathway: 1-25, 0.276)
18-4	EE486131	At5g10770	Asp protease	N-ter missing (secretory pathway: 1-25, 0.276)
44	EV196557/TC46757	At5g10770	Asp protease	N-ter missing (secretory pathway: 1-25, 0.276)
93-1	TC21632	At3g18490	Asp protease	N-ter missing (SignalP-NN: 1-24, 0.895)
93-2	TC23324	At3g18490	Asp protease	chloroplast: 0.342 / SignalP-NN: 1-24, 0.833
93-3	TC33329	At3g18490	Asp protease	secretory pathway: 1-26, 0.456
100	TC17031	At5g07030	Asp protease	secretory pathway: 1-19, 0.805
115	TC23722	At3g54400	Asp protease	secretory pathway: 1-19, 0.294
120	TC33693	At1g79720	Asp protease	secretory pathway: 1-28, 0.967
96-1	B1Q3A2	At1g47128	Cys protease (RESPONSIVE TO DEHYDRATION, RD21 homologue)	N-ter missing (secretory pathway: 1-21, 0.993)
96-2	TC59035	At5g43060	Cys protease	secretory pathway: 1-20, 0.987
104	TC53234	At5g60360	Cys protease (ALEURAIN LIKE PROTEASE, AALP homologue)	secretory pathway: 1-22, 0.976
119	TC33308 [4]	At4g35350	Cys protease (XYLEM CYSTEINE PEPTIDASE 1, XCP1 homologue)	secretory pathway: 1-29, 0.986
134	TC26010	At4g01610	Cys protease	secretory pathway: 1-29, 0.979

Lipid metabolism (8)

21	TC36034 [7]	At3g53980	lipid transfer protein	secretory pathway: 1-23, 0.936
132	TC19947	At2g44300	lipid transfer protein	secretory pathway: 1-23, 0.937
57	TC45634	At5G05960	lipid transfer protein	secretory pathway: 1-25, 0.720
98	O82582 (<i>B. oleracea</i>)	At2g38540	lipid transfer protein (LTP1)	secretory pathway: 1-25, 0.970
68	Q9S9F9	At2g38530	lipid transfer protein (LTP2)	N-ter missing (secretory pathway: 1-23, 0.967)
73	EG019134	At5g59310	lipid transfer protein (LTP4)	N-ter missing (secretory pathway: 1-23, 0.976)
139	DY024942	At1g09390	lipase acylhydrolase (GDSL family)	secretory pathway: 1-27, 0.983
160	TC50035	At4g26690	glycerophosphoryl diester phosphodiesterase (MORPHOGENESIS OF ROOT 5, MHR5 homologue) (GPI-anchored protein)	N-ter missing (SignalP-NN : 1-27, 0.761)

Signaling (8)

16-1	TC35372	At5g55730	FLA (AtFLA1 homologue)	secretory pathway: 1-26, 0.799
16-2	TC31355	At5g55730	FLA (AtFLA1 homologue)	secretory pathway: 1-26, 0.799

46	TC39357	At4g12730	FLA (AtFLA2 homologue)	secretory pathway: 1-23, 0.817
37	TC21253	At2g04780	FLA (AtFLA7 homologue)	secretory pathway: 1-23, 0.882
24-1	TC21238	At2g45470	FLA (AtFLA8 homologue)	N-ter missing (secretory pathway: 1-25, 0.936)
24-2	TC30763	At3g60900	FLA (AtFLA10 homologue)	N-ter missing (secretory pathway: 1-25, 0.869)
61-1	TC32307	At1g03870	FLA (AtFLA9 homologue)	secretory pathway: 1-25, 0.912
61-2	TC23584	At1g03870	FLA (AtFLA9 homologue)	secretory pathway: 1-25, 0.912
45	TC26664	At5g03170	FLA (AtFLA11 homologue)	secretory pathway: 1-24, 0.815

Interacting domains (8)

54	ES901495	At1g17860	protease inhibitor	N-ter missing (secretory pathway: 1-19, 0.910)
163	B2KSD1 (<i>B. oleracea</i>)	At1g73260	protease inhibitor I3 (Kunitz legume)	N-ter missing (secretory pathway: 1-28, 0.985)
113	TC20389 [4]	At1g47960	pectinesterase inhibitor (PMEI)	secretory pathway: 1-20, 0.974
17	TC40783	At1g03220	xylanase inhibitor I (EDGP)	secretory pathway: 1-23, 0.917
53	TC32327 [4]	At1g78850	lectin (curculin-like, mannose binding)	secretory pathway: 1-22, 0.990
67-105	TC53636 [4]	At1g78830	lectin (curculin-like, mannose binding)	secretory pathway: 1-22, 0.962
123	TC41889	At1g26450	unknown function (X8 domain)	secretory pathway: 1-19, 0.809
114	TC22255	At1g21880	unknown function (LysM domain)	secretory pathway: 1-27, 0.775

Miscellaneous (14)

36	TC59434	At4g23690	dirigent protein	secretory pathway: 1-29, 0.994
146	TC16085	At1g13900	purple acid phosphatase (AtPAP2 homologue)	N-ter missing (secretory pathway: 1-19, 0.983)
42	TC32617	At3g07130	purple acid phosphatase (AtPAP15 homologue)	N-ter missing (secretory pathway: 1-19, 0.859)
72	TC62786	At3g07130	purple acid phosphatase (AtPAP15 homologue)	N-ter missing (secretory pathway: 1-19, 0.859)
10-1	TC21902	At5g34850	purple acid phosphatase (AtPAP26 homologue)	secretory pathway: 1-27, 0.991
10-2	TC50714	At5g34850	purple acid phosphatase (AtPAP26 homologue)	secretory pathway: 1-22, 0.772
55	TC25081	At3g19320	thaumatin	secretory pathway: 1-26, 0.974
82	TC28594	At2g14610	cysteine-rich secretory protein (SCP) (PR1 homologue)	secretory pathway: 1-26, 0.798
86	TC38503 [4]	At4g34180	cyclase	secretory pathway: 1-23, 0.922
106	TC55340	At3g62020	germin (GLP10 homologue)	N-ter missing (secretory pathway: 1-21, 0.970)
126	TC54095 [4]	At4g11650	osmotin (AtOSM34 homologue)	secretory pathway: 1-26, 0.991
41-1	TC22018	At2g02990	ribonuclease T2 (AtRNS1 homologue)	secretory pathway: 1-26, 0.995
41-2	TC22953	At1g26820	ribonuclease T2 (AtRNS3 homologue)	secretory pathway: 1-19, 0.983
154	TC33600	At1g78680	homologous to gamma-glutamyl hydrolase	secretory pathway: 1-24, 0.672

Unknown function (10)

4	TC27548	At5g48540	unknown function (DUF26)	secretory pathway: 1-20, 0.930
32	EV115188	At3g22060	unknown function (DUF26)	secretory pathway: 1-27, 0.880

48	TC55540	At1g07390	unknown function (DUF568) (AIR12, AUXIN-INDUCED IN ROOT CULTURES 12) (GPI-anchored protein)	N-ter missing (SignalP-NN: 1-46, 0.260)
6-1	TC30155	At5g11420	unknown function (DUF642)	secretory pathway: 1-22, 0.978
6-2	TC41195	At5g11420	unknown function (DUF642)	N-ter missing (secretory pathway: 1-22, 0.989)
6-3	TC28652	At5g25460	unknown function (DUF642)	N-ter missing (secretory pathway: 1-19, 0.979)
50/109	TC57337/TC59437/ EV164447	At5g12950	unknown function (DUF1680)	N-ter missing (secretory pathway: 1-24, 0.952)
103	TC50570	At2g15220	unknown function (basic secretory protein)	secretory pathway: 1-22, 0.986
147	TC16416	At1g76020	unknown function (thioredoxin fold)	secretory pathway : 1-19, 0.759
150	TC18289	At3g06035	unknown function	secretory pathway: 1-26, 0.987

Intracellular (25)

9-1	TC23761	At5g17920	methionine-synthase	mitochondrion: 0.414
9-2	TC20982	At5g17920	methionine-synthase	mitochondrion: 0.212
31	Y09437	At1g52030	MYROSINASE-BINDING PROTEIN 2 (MBP2 homologue)	other: 0.860
34	TC16377	At4g33090	peptidase M1	mitochondrion: 0.408
70	TC16864	At3g52930	fructose-bisphosphate aldolase	other: 0.530
76	TC17072	At5g13420	trans-aldolase	chloroplast: 0.990
78-1	TC18919	At5g57330	aldose 1-epimerase	other: 0.850
78-2	TC26676	At5g57330	aldose 1-epimerase	other: 0.836
79-1	TC17406	At1g53240	malate dehydrogenase	mitochondrion: 0.700
79-2	TC18964	At1g53240	malate dehydrogenase	mitochondrion: 0.789
80	TC20514	At1g54040	unknown function (kelch-type beta-propeller)	other: 0.827
84	Q14U56 (<i>B. oleracea</i>)	At1g09010	glycoside hydrolase family 2 (GH2)	other : 0.713
85-1	TC46191	At5g20620	ubiquitin (UBQ4)	N-ter missing (other: 0.847)
85-2	EE426154	At1g31340	ubiquitin (RUB1)	other: 0.833
92	TC18282	At5g60390	translation elongation factor	other: 0.855
94	TC28096	At2g01140	fructose bisphosphate aldolase	chloroplast : 0.828
101	TC49144	At3g26380	unknown function (aldolase-type TIM barrel)	N-ter missing (other: 0.355)
118	TC31117	At5g11670	NADP-malic enzyme 2 (AtNADP-ME2 homologue)	N-ter missing (other: 0.963)
124	TC42226	At4g03920	nucleoside diphosphate kinase	N-ter missing (other: 0.383)
133	TC22085	At1g11840	glyoxalase (AtGLX1)	other: 0.807
137	DW999965	At2g19760	homologous to profilin	other: 0.805
148	TC17883	At3g55440	triose-phosphate isomerase	other: 0.745
149	TC18121	At1g09310	unknown function (DUF538)	other: 0.917
152	TC20640	At3g04120	glyceraldehyde-3-phosphate dehydrogenase	N-ter missing (other: 0.619)
156	TC37476	ArthCp029	ATPase, alpha/beta subunit	other: 0.760

- a.** Protein numbers refer to Supporting information Tables S1, S2, and S3. Protein numbers in bold refer to proteins identified in the xylem sap *N*-glycoproteome.
- b.** Nucleotide or amino acid sequences can be found either in the CompBio (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=oilseed_rape) or the NCBI (<http://www.ncbi.nlm.nih.gov/>) databases. Sequences are from *B. napus* otherwise stated. Numbers between brackets refer to previously published xylem sap proteomes.
- c.** Functional domains were found as described in Material and methods. GH were annotated according to CAZy (<http://www.cazy.org/>) and [59].
- d.** Prediction of sub-cellular localization was done with TargetP (<http://www.cbs.dtu.dk/services/TargetP/>) and SignalP (<http://www.cbs.dtu.dk/services/SignalP/>). When both predictions are consistent, only the TargetP result is shown (size of the predicted signal peptide, score). When it is not the case, both predictions are shown. When the *B. oleracea* protein sequence is not complete at its N-terminus (N-ter), the prediction for the closest homologue in *A. thaliana* is shown.

3.3 General features of proteins identified in the *B. oleracea* xylem sap

All the *Brassica* protein sequences were analyzed with bioinformatics software to predict their sub-cellular localization and the presence of functional domains. The same work was done for *A. thaliana* proteins homologous to *Brassica* sequences. The proteins could then be classified in (i) intracellular when they were devoid of signal peptide or of any signal targeting them to an intracellular compartment, and (ii) secreted proteins (Table I, Supporting information Table S3). Twenty five proteins (13%) were predicted to be intracellular whereas 164 proteins (87%) were predicted to be secreted. The latter proteins could be distributed in eight of the nine functional classes previously defined for *A. thaliana* cell wall proteins [29]: proteins acting on carbohydrates (29.2%), oxido-reductases (23.8%), proteases (17.1%), proteins related to lipid metabolism (4.9%), proteins involved in signaling (5.5%), proteins with domains interacting with carbohydrates or proteins (4.9%), miscellaneous proteins having diverse functions (8.5%), and proteins with yet unknown function (6.1%). No structural protein was identified in these analyses. Among the 48 proteins acting on carbohydrates, two glycoside hydrolase (GH) families were well represented: eight proteins belonged to GH17 (β -1,3-glucosidases), and seven to GH28 (polygalacturonases). Nearly half of the oxido-reductases were peroxidases (17), the others being proteins homologous to plastocyanins (11), multicopper oxidases (6), and berberine bridge enzymes (5). Proteases were mainly predicted as Ser proteases (12) and Asp proteases (11). Most of the proteins included in the lipid metabolism functional class were lipid transfer proteins (LTPs). Finally, all the proteins included in the signaling functional class were fasciclin AGPs (FLAs). Finding such proteins was expected because of the positive Yariv staining observed after analysis of xylem sap proteins by SDS-PAGE (Fig. 1).

All the proteins of the xylem sap proteome were also analyzed for the presence of putative *N*-glycosylation sites. Proteins homologous to LTPs, ribonuclease T2, and osmotin had no predicted *N*-glycosylation sites and were only found in the xylem sap proteome. All the other proteins had predicted *N*-glycosylation sites. However, 70 proteins predicted to be secreted and having *N*-glycosylation sites were only found in the xylem sap proteome. One cannot exclude the possibility that they were poorly or not *N*-glycosylated consistent with the fact that they were lost during the affinity chromatography step. Interestingly, although 17 out of 25 proteins predicted to be intracellular had predicted *N*-glycosylation sites, none was retained on the ConA affinity column. It suggested that the *N*-glycosylation sites of these proteins were not occupied.

LC-MS/MS data allow getting some information on the abundance of proteins relying on spectral counting. The rationale is that the number of spectra collected for a protein in a MS/MS run is correlated to the abundance of the protein (Supporting information Tables S4). Among the proteins predicted to be intracellular, two proteins homologous to methionine synthase (proteins 9-1, 9-2) were identified with 35 and 24 spectra respectively, a protein homologous to jacalin (protein 31) with 11 spectra, and a protein homologous to peptidase M1 (protein 34) with 10 spectra. Ten out of the remaining 18 proteins predicted to be intracellular were identified with only 2 spectra which meant that they were probably present at a low level. In two independent experiments, the most abundant proteins predicted to be secreted were the following: two Ser proteases (proteins 1-1 and 12 identified with 74 and 25 spectra respectively in the reported experiment); an Asp protease (protein 18-1; 20 spectra); two α -arabinofuranosidases (proteins 2-1 and 7-1; 43 and 29 spectra respectively); a protein homologous to peroxidase AtPrx59 (protein 11-1; 23 spectra); three berberine-bridge oxidoreductases (proteins 5, 15-1 and 15-2; 36, 23 and 20 spectra respectively); a protein homologous to AtFLA1 (protein 16-1; 20 spectra); a protein homologous to purple acid phosphatase AtPAP26 (protein 10; 24 spectra); a protein of yet unknown function with a DUF26 domain (protein 4; 32 spectra). Altogether, these results show that the proteins predicted to be intracellular are mostly minor components of the xylem sap proteome.

3.4 Extension and screening of *WallProtDB* with the *B. oleracea* xylem sap proteome

As mentioned above, *WallProtDB* was built up to collect plant cell wall proteomic data [37]. It was initially devoted to *A. thaliana* and *O. sativa* because (i) their genomes are fully sequenced thus allowing the precise identification of proteins by MS and bioinformatics, and (ii) many proteomic studies were published on different organs. *WallProtDB* displays information about experimental conditions, and cell wall proteomes previously analyzed. Each protein accession number in *WallProtDB* has been linked to the *ProtAnnDB* database including bioinformatics predictions about subcellular localization, functional domains, and gene networking [36]. Literature dealing with plant cell wall proteomics has been included.

Two novelties were introduced to describe the *B. oleracea* xylem sap proteome and to allow comparisons with the *A. thaliana* cell wall proteomes characterized in different organs (Supporting information Fig. 2). First, considering the closeness of both genomes, each *B. oleracea* EST is linked to the closest *A. thaliana* gene as determined by BLASTX analysis. In many cases, two or more *B. oleracea* proteins corresponded to a single *A. thaliana* gene were

found thanks to the presence of proteotypic peptides. This is consistent with the evolution of the *Brassica* genomes [42]. Second, LC-MS/MS peptide sequencing data allowing identification of *B. oleracea* proteins encoded by ESTs are provided using the X! Tandem software. The position of sequenced peptides on the translated EST or cDNA sequence can be visualized as well as MS/MS spectra and data (Supporting information Fig. 3). All this information provides a comprehensive view of the identification process using MS data.

A search was performed in *WallProtDB*, a cell wall proteomic database, to look for the *A. thaliana* proteins homologous to the *B. oleracea* xylem sap proteins. Eighty six proteins were already identified in at least one cell wall proteome. For example, 30 proteins were found in the leaf apoplastic proteome [18], 23 in the stem cell wall *N*-glycoproteome [19], 28 and 27 in the 5- and 11-day-old etiolated cell wall proteomes respectively [20]. Seventy eight proteins were new proteins never found in previously characterized cell wall proteomes.

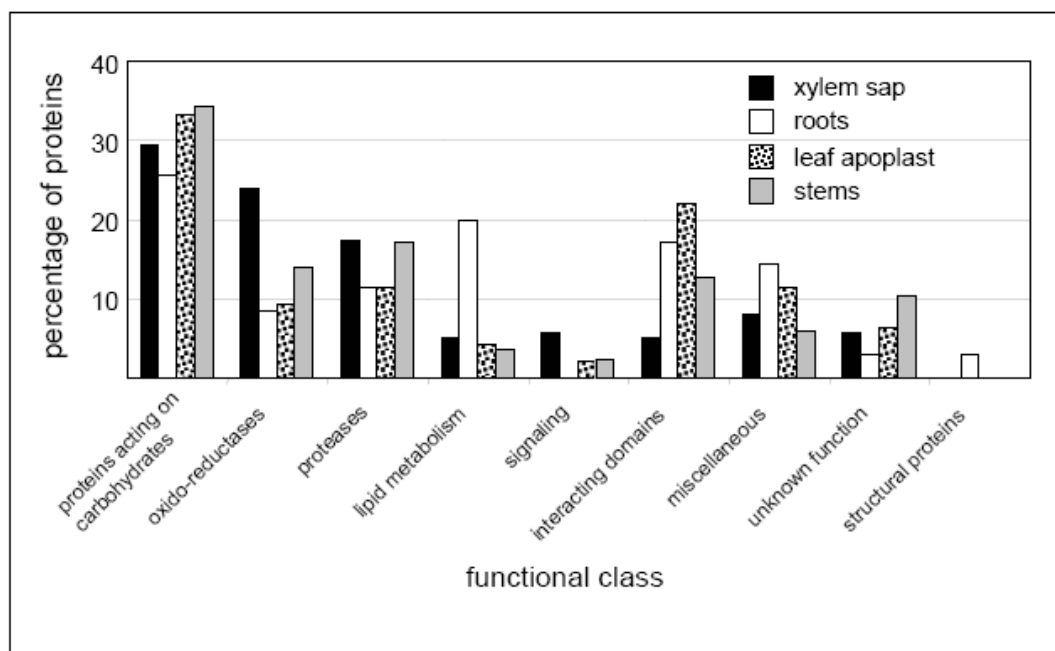


Figure 2. Comparison between the *B. oleracea* xylem sap proteome and previously characterized *A. thaliana* cell wall proteomes.

A. thaliana cell wall proteomes were found in the *WallProtDB* database (<http://www.polebio.scsv.ups-tlse.fr/WallProtDB/>). As in *WallProtDB*, *B. oleracea* proteins were annotated with regard to predicted functional domains and distributed in functional classes accordingly [29]. The distribution of the xylem sap proteins (xylem sap proteome, black bars) in these functional classes was compared to that of proteins previously identified in *A. thaliana* cell wall proteomes: roots (white bars) [43], rosette leaves (punctuated bars) [18], and stems (grey bars) [19]. Only proteins predicted to be secreted are considered in all cases, *i.e.* proteins having a predicted signal peptide and no known targeting signal in any cell compartment.

Using *WallProtDB*, the whole xylem sap proteome was compared to *A. thaliana* cell wall proteomes of roots [43], rosette leaves [18], and stems [19]. In all cases only proteins predicted to be secreted were considered. Fig. 2 highlights several differences. There were two times more oxido-reductases in the xylem sap proteome compared to the other cell wall proteomes. The proportion of proteases was higher in the xylem sap and the stem proteomes than in the rosette leaves and root proteomes. On the contrary, there were fewer proteins with interacting domains in the xylem sap than in the other proteomes, with only two proteins homologous to lectins, two to protease inhibitors, one to xylanase inhibitors, and one to pectin methylesterase inhibitors. Structural proteins were missing as in the rosette leaves and stem proteomes.

3.5 Expression of *A. thaliana* genes homologous to genes encoding proteins identified in the xylem sap of *B. oleracea* in roots

The presence of proteins in the xylem sap raises the question of the origin of these proteins. To better understand it, we looked at the pattern of expression of *A. thaliana* genes homologous to *B. oleracea* genes encoding xylem sap proteins in the AREX database which collects *A. thaliana* genes patterns of expression based on transcriptomics data [38]. The root is characterized by different developmental zones and tissues. We focused our attention on the root tip where xylem vessels are formed and where root absorption occurs. From outside to inside, there are different cell layers, namely epidermis, cortical cells, endodermis, pericycle and the stele comprising the xylem and the phloem vessels. Expression data could be found for 90% of the *A. thaliana* genes corresponding to proteins identified in the xylem sap and all those genes were found to be transcribed even at low level in root tips. Most relevant patterns were the following (Supporting information Fig. 4): expression in all root tissues except in stele parenchyma cells (pattern 1: 17% of the genes including one third of the genes encoding proteins predicted to be intracellular); expression mostly in epidermis including or not root hairs, or cortical cells (pattern 2: 10%); expression mostly in cortical cells, and eventually in endodermis, pericycle or stele (pattern 3: 16%); expression in stele parenchyma stele including or not vessels (pattern 4: 37%); expression mostly in phloem cells (pattern 5: 8%); expression mostly in xylem cells (pattern 6: 12%). Very few genes had transcripts neither in the stele, the pericycle or the endodermis.

4. Discussion

Previous studies showed the presence of proteins in the xylem sap of different species [4, 6, 8-17]. However, with the exception of the xylem sap proteome of an hybrid poplar [13], identification of proteins was done against heterologous sequences because of the lack of genomic or EST sequences for all the plants studied. The most complete xylem sap proteomes are those of *B. napus* [5], an hybrid poplar [13] and *Z. mays* [17] with 69, 97, and 154 proteins identified respectively. However, a detailed examination of the results indicates that several of the identified proteins show homology to different parts of the same protein and/or are identified with identical peptides. Such proteins can be present in different spots of 2D-gels or in different bands of 1D-gels, thus indicating the presence of isoforms of the same gene product as a consequence of PTMs or resulting from protein degradation. As a consequence, the number of proteins in each proteome is certainly lower. This was discussed in the case of the *Z. mays* xylem sap proteome, thus leading to the conclusion that only 59 different proteins were identified instead of 154 [17]. To our knowledge, our proteomic study provides the characterization of the largest xylem sap proteome with 189 different proteins identified. When compared to previous *B. napus* xylem sap proteomes [4, 7, 8], only 20 proteins out of the 189 identified in this study were already found. In addition to data obtained by MS analysis of the *B. oleracea* xylem sap, this study provides information on the xylem sap *N*-glycoproteome. About half of the proteins (81 proteins) predicted to be secreted identified in the *B. oleracea* xylem sap were retained on the ConA column, showing that they are *N*-glycosylated and confirming that *N*-glycosylation is a major PTM of extracellular proteins. The high proportion of secreted *N*-glycosylated proteins was expected because they pass through the endoplasmic reticulum where *N*-glycosylation occurs [44]. As expected, the thirteen proteins predicted to be secreted and devoid of *N*-glycosylation sites were only found in the xylem sap proteome. For the remaining 70 proteins not retained on ConA, it is assumed that their *N*-glycans were removed in the xylem sap. Indeed all the GH families possibly involved in *N*-glycan degradation were found, namely β -D-xylosidases (GH3), N-acetylhexosaminidases (GH19), β -D-galactosidases (GH35), and α -D-mannosidases (GH38) [45]. Besides, ConA affinity chromatography was assumed to enrich the protein mixture in glycoproteins present in low amount, thus allowing to increase the coverage of the xylem sap proteome.

The proportion of proteins predicted to be intracellular (13%) is rather low in this study. All except three of the proteins predicted to be intracellular have predicted *N*-glycosylation sites.

However, none of them was retained on the ConA column, suggesting that their *N*-glycosylation sites are not occupied. All these proteins are present at a low level apart from a protein homologous to methionine synthase. Sixty six percent of the proteins identified in hybrid poplar xylem proteome are devoid of predicted signal peptide [13]. The current hypothesis to explain the presence of intracellular proteins in xylem sap is that such proteins originate from differentiating xylem cells, and that they are released in xylem sap after cell death [13]. In the case of perennial plants such as poplar, secondary wall formation and xylem growth are more intensive than in annual plants. This would explain why there are more types of intracellular proteins in the poplar xylem sap. On the contrary, the *Z. mays* xylem proteome only contained proteins predicted to be secreted [17].

A major difficulty encountered in xylem sap proteome analysis relies in the harvesting step. The plants have to be decapitated and the harvesting can last for several hours. With regard to the harvesting duration, a detailed study performed in *G. max* showed that the 1D-electrophoresis pattern of xylem sap proteins was constant over a 28 h-period of harvesting [10]. It suggests that major xylem sap proteins are stable xylem sap constituents and are continuously produced. With regard to the stress, it is probable that not only the cells located at the stem cut surface, but also the underground part of the plants, undergo a stress which causes changes in xylem sap composition. Many protein families identified in xylem sap proteomes could be induced by this stress, *e.g.* oxido-reductases, pathogenesis-related (PR)-proteins, proteases and ribonucleases ([7, 12, 13, 17], this study). However, there is no way to avoid this stress and all the xylem sap proteomes are obtained in similar conditions, thus allowing comparison of results.

Xylem sap proteomes appear to be very different from phloem sap proteomes. The phloem is the part of the vascular system which delivers sugars and amino acids to plant organs and carries informational molecules such as proteins, mRNAs and hormones (for a review, see [46]). Phloem protein 2 (PP2) was described as a major protein of phloem sap having RNA-binding properties or lectin activity [47]. Other proteins were identified with putative roles in defense reactions, gibberellin biosynthesis and transport [48]. The most complete phloem proteomic study identified more than 1000 proteins such as RNA-binding proteins (82 proteins) and proteins involved in protein synthesis (100 proteins) and turnover (116 proteins) [49]. Other proteins could play roles in vesicle trafficking, membrane dynamics, stress response, and redox regulation. Only one of the 45 most abundant proteins, a peroxidase

homologous to At5g07630, was predicted to be secreted. Altogether, the xylem and phloem sap proteomes thus appear to be very different, as expected from their different physiological roles.

All the proteomic data were included in *WallProtDB*, a database dedicated to cell wall proteomics. *WallProtDB* is a tool complementary to existing databases since it allows direct comparison between cell wall proteomes of various organs of *A. thaliana* and *O. sativa*. Only data from plants with genomes completely sequenced or large collection of ESTs are included since unequivocal identification of proteins by peptide mass fingerprinting or peptide sequencing can be done. Other proteomic databases such as the Plant Proteome Database (PPDB, <http://ppdb.tc.cornell.edu/>) and the Atproteome database (<http://fgcz-atproteome.unizh.ch/>) are built in a different way. For each gene, the latter databases give information on the conditions in which the proteins were identified as well as MS data when available. Being devoted to cell wall proteomes only, *WallProtDB* resembles AT_CHLORO (http://www.grenoble.prabi.fr/at_chloro/) which describes the chloroplast proteome. In this new version, MS data are also included for the *B. oleracea* xylem sap proteome. The next step would be to crosslink all the plant proteomic databases to get all the information at the same place as was done in the Human Proteomic reference database (<http://www.hprd.org/>). It would give the plant community a great tool to better understand protein structure and gene regulation.

An interesting outcome of the new version of *WallProtDB* is the comparison between the *B. oleracea* xylem sap proteome and previously characterized cell wall proteomes of *A. thaliana*. Indeed, the fact that xylem sap is considered as part of the apoplast and the closeness of the two species allowed this comparison. Three features distinguish the *B. oleracea* proteome from previously characterized cell wall proteomes [29]: there are more oxido-reductases, more proteases, and less enzyme inhibitors. As discussed above, because of the technical constraints to collect xylem sap, we cannot exclude that some of the proteins related to stress response may change during the harvesting period. Among oxido-reductases, peroxidases represent one tenth of the predicted secreted proteins identified. Peroxidases were previously described as important proteins in xylem sap proteomes. They were associated either to lignin biosynthesis in xylem vessels undergoing differentiation or to plant defense [4, 7, 12, 13]. Proteins homologous to multicopper oxidases were also found to be numerous in *A. thaliana* stems at late flowering stage when lignification occurs [19]. SKU5 (SKEWED 5) was shown

to be involved in root growth and SKS6 (SKU5-SIMILAR 6) to contribute to cotyledon vascular patterning [50, 51]. Blue copper binding proteins were abundant in *A. thaliana* cell suspension cultures. Although their exact role in cell walls is not known, they have been associated to redox processes as electron transfer proteins with small molecular weight compounds [52]. A great proportion of proteases is the second feature of the *B. oleracea* xylem sap proteome. It is the first time that so many proteases are identified. Different specificities could be predicted such as Ser proteases (subtilases), Ser carboxypeptidases, Cys proteases, and Asp proteases. Proteases are assumed to play roles in maturation of enzymes, signaling, protein turnover, and defense against pathogens [53]. It was previously shown that maturation of enzymes occur in the cell wall [54]. Over-expression of CDR1 (CONSTITUTIVE DISEASE RESISTANT 1) encoding an *A. thaliana* Asp protease causes dwarfism and resistance to virulent *Pseudomonas syringae* [55]. Finally, in all previously characterized plant extracellular proteomes, enzymes and the corresponding inhibitors are present, probably allowing fine regulation of enzymatic activities [20]. In the *B. oleracea* xylem sap, there are only a few enzyme inhibitors (two protease inhibitors, a pectin methyl esterase inhibitor, and a xylanase inhibitor). It suggests that the enzymes are fully active. Some of them may play roles in defense reactions against pathogens invading the xylem vessels.

Three additional protein functional classes deserve comments. The main one comprises proteins acting on carbohydrates. Forty two proteins having GH domains were identified. Several of them could play roles in hydrolysis of PTMs of *N*-glycoproteins as discussed above. Others are assumed to contribute to defense reactions, such as β -1,3-glucanases (GH17, 6 proteins) and chitinases/lysozymes (GH18-19, 5 proteins) [45]. More puzzling are the roles of polygalacturonases and α -L-arabinofuranosidases/ β -D-xylosidases. Polygalacturonases (GH28, 7 proteins) are assumed to play roles in the organization of pectins and in their modification in response to pathogen attack [45]. However, the contribution of pectins to secondary walls is very low. α -L-arabinofuranosidases/ β -D-xylosidases (GH51, 4 proteins) are major proteins in xylem sap as estimated from MS data. Such proteins were also identified in the poplar xylem sap [13]. Their preferred substrates in cell wall are assumed to be arabinoxylan, and arabinan as inferred from *in vitro* tests [56]. The second functional class to be mentioned is that of proteins possibly involved in signaling. It is the first time the importance of FLAs in xylem sap can be stressed. Indeed, seven proteins homologous to *A. thaliana* FLAs (AtFLA1, 2, 7-10) were identified. Their roles in cell walls

are not yet understood, but they were found to accumulate at the inner side of the G-layer of the xylem of poplar tension wood. They were assumed to have a specific function in the building of this cell wall layer [57]. Finally no structural protein could be identified in the *B. oleracea* xylem sap although a glycine-rich protein (GRP) was previously found in the *B. napus* xylem sap proteome [4]. Such proteins were shown to be present in cucumber xylem sap and to accumulate in the walls of cucumber root metaxylem cells [6]. A bean GRP was shown to be synthesized by living protoxylem cells and xylem parenchyma cells, and to be transported from xylem parenchyma cells to the protoxylem wall after cell death [58].

Several authors have already discussed the origin of xylem sap proteins [3, 10, 12]. In the root, the endodermis constitutes a barrier preventing the movement of organic substances and even water from the epidermis and cortical cells to the stele through the apoplast. It is assumed that proteins present in the xylem sap are synthesized in the stele cells. They would then be delivered apoplastically to the xylem sap thus considered as part of the apoplast. In this study, we looked at the pattern of expression of the *A. thaliana* genes homologous to the *B. oleracea* xylem sap proteins using the AREX database. All the genes were found to be expressed in the root tip, and only a few of them were expressed neither in the stele, the pericycle, or the endodermis. It suggests that the proteins were synthesized in the root tip, and then loaded into the xylem sap. These data are consistent with previous experimental data. A cucumber GRP was shown to be synthesized in the vascular tissues of the root and assumed to be transported over long distance *via* the xylem sap to vessels and sclerenchyma of aboveground organs [6]. Xylem sap proteins as diverse as a cucumber lectin [5], a cucumber peroxidase [12], and a tomato Cys-rich protein with structural similarity to LTPs [14] were found in roots and the genes encoding the lectin and the Cys-rich protein were shown to be transcribed in roots. Altogether, these data strengthen the hypothesis of the production of xylem sap proteins in the root stele, and their further loading into the xylem sap. The water flow would then ensure their long distance transport to aboveground organs. The composition of the xylem sap proteome suggests roles in xylem differentiation and in plant defense. However, additional investigations are required to better understand the function of xylem sap proteins.

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Supporting information

Supporting information Table S1. Identification by LC-MS/MS of proteins present in the *B. oleracea* xylem sap (xylem sap proteome).

Supporting information Table S2. Identification by LC-MS/MS of proteins of *B. oleracea* retained by ConA affinity chromatography (xylem sap *N*-glycoproteome).

Supporting information Table S3. Description of the xylem sap proteome and of the xylem sap *N*-glycoproteome of *B. oleracea*.

Supporting information Table S4. Spectral counting data for the most abundant proteins identified in the *B. oleracea* xylem sap proteome and for the proteins predicted to be intracellular.

Supporting information Fig. 1. Xylem sap sampling from *B. oleracea* cut stems.

Supporting information Fig. 2. Content of *WallProtDB*.

Supporting information Fig. 3. LC-MS/MS data as shown in *WallProtDB*.

Supporting information Fig. 4. Root pattern of expression of *A. thaliana* genes encoding proteins homologous to *B. oleracea* xylem sap proteins.