

Proteomic analysis of tomato (*Solanum lycopersicum*) secretome

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Abstract In fleshy fruits, fruit texture features are mainly related to chemical and mechanical properties of the cell wall. The description of tomato fruit cell wall proteome is a first step in the process of linking tomato genetic variability to fruit texture phenotypes. In this study, the proteome of 3 ripe tomato fruit lines with contrasted texture traits were studied. Weakly bound and soluble proteins were extracted from cell wall of the three cultivars using both destructive and non-destructive methods, respectively. Wall proteins were separated on 1D-PAGE, bands were excised and identified by LC-MS/MS. The software SignalP which searches for the leader peptide was used to discriminate between protein with or without signal peptide. In combine, seventy-five different cell wall proteins were recorded for both weakly bound and soluble cell wall fractions. The major identified functions included several proteins acting on polysaccharides, proteins involved in “lipid metabolism”, proteins having interacting domain, “oxido-reductases” and “proteases” whose putative roles in ripe fruit cell wall is discussed. Several proteins with no obvious

signal peptide, however, with accumulating supportive evidences to be bona fide wall proteins, were also identified. Some variations in protein repertoires were observed among the lines, demonstrating the possibility to characterize cell wall protein genetic variability by such in muro proteome analyses.

Keywords Secretome · Tomato · Cell wall protein · Proteome · Pericarp

Introduction

The extracellular matrix of plant cells is a dynamic compartment composed of a complex mixture of carbohydrates, proteins, lignin, water, metabolic and inorganic compounds. It is involved in a variety of key functions related to plant structure development and functioning. During normal growth, it has a primary role in cell nutrition, because cells import ions and metabolites from the apoplast and in plant development, by determining the capacity and the rate of cell division and expansion. During adverse conditions, such as biotic or abiotic stress, the cell wall also plays a major role in plant adaptation and protection.

The wall carbohydrates account for about 90 % of the total dry cell wall weight. Cellulose microfibrils, hemicelluloses and pectins constitute a complex network embedded in the apoplastic fluid. Three types of cell wall proteins (CWPs) are classically distinguished on the basis of their chemical interactions with cell wall components, from strongly to weakly and loosely bound CWP (Jamet et al. 2008). Their masses represent less than 10 % of the cell wall mass, however they are numerous (more than 5,000 genes identified in *Arabidopsis thaliana*) and show a great variety of functions from structural role to enzymatic

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activities participating in cell wall biogenesis and remodeling (Carpita et al. 2001; Farrokhi et al. 2006; Rose et al. 2004). Their chemical diversity, their frequent low amount within the cell wall and their interaction with cell wall components pose some methodological problems for exhaustive studies. The use of several complementary protocols aimed for protein extraction and characterization are required to specifically target and describe this sub-cellular compartment (Borderies et al. 2003; Boudart et al. 2005; Feiz et al. 2006; Lee et al. 2004). Furthermore, the choice of separation methods must integrate the intrinsic properties of CWPs which are mainly basic glycoproteins and are weakly resolved by classical two-dimensional electrophoresis (Jamet et al. 2006). In addition to experimental difficulties, inventory of CWPs needs the use of appropriate bioinformatic tools for predicting secreted proteins (San Clemente et al. 2009). However some proteins do not have canonical signal peptides suggesting that, beside the classic ER-Golgi secretion route, unknown mechanisms of protein secretion remain to be characterized (Agrawal et al. 2010; Rose and Lee 2010; Slabas et al. 2004).

Studies aiming at deciphering the cell wall protein encoding gene network at transcriptomic level are not so numerous, they rely either on in silico whole sequenced genome bioinformatic analysis or on microarray data with special attention to genes exhibiting at N-terminus peptides targeting the protein to the secretion pathway. Many discrepancies are noticed when transcriptomic and proteomic data are compared suggesting post-transcriptional mechanisms of regulation of expression of genes encoding cell wall proteins (Minic et al. 2009).

In their review Schroder and his colleagues emphasized that under in vivo conditions it is difficult to mimic the actual scenario which occurs inside the cell wall micro-environment (Schroder et al. 2009). This justifies such descriptive approaches and the necessity to validate their activities in planta.

Until now, the best well characterized cell wall proteome is that of Arabidopsis plant (Albenne et al. 2009; Bayer et al. 2006; Borderies et al. 2003; Chivasa et al. 2002; Jamet et al. 2006, 2008), but consistent data are also available, among others, on rice (Chen et al. 2009; Cho et al. 2009), maize (Zhu et al. 2006, 2007), medicago (Watson et al. 2004) and tobacco (Blee et al. 2001; Millar et al. 2009). The main part of these data was obtained from stems, roots, leaves or calli analysis.

To our knowledge, until now only two papers devoted to cell wall proteome were published on grape skin and seeds besides another one on tomato skin (Negri et al. 2008; Yeats et al. 2010). In addition, an unpublished analysis of tomato fruit cell wall based on the use of a yeast secretion trap screen wall is also reported (Vicente et al. 2007).

Nevertheless, CWPs are of primary importance first, during fruit development in relation to cell division, expansion and final fruit size, secondly during fruit ripening with cell wall loosening phenomena which determines, in major part, fruit texture that is a main criteria perceived by consumers during consumption. However, a great amount of data was obtained on tomato cell wall proteins by in depth works dealing with specific functions such as expansins, glycosylhydrolases, extensins, etc. and was reviewed in Harker et al. (1997), Vicente et al. (2007) and Waldron et al. (2003).

The tomato cell wall proteome analysis presented here, is based on the combined use of destructive and non-destructive methods for isolation of weakly bound and solubly wall proteins, respectively. 1-SDS-PAGE was undertaken for protein separation followed by subsequent mass spectrometry for protein identification. This study was undertaken with the objective to isolate the easily accessible cell wall proteins fractions from three texture contrasted tomato pericarp, using two adapted protocols to isolate the weakly bound (WB-CWP) and soluble wall proteins (S-CWP), respectively. This texture variability would allow constructing a comprehensive proteomic map for tomato. On the other hand, we also aimed to assess the genetic variability at the pericarp cell wall level by comparing 3 tomato genotypes already known for their contrasted texture characteristics (Chaib et al. 2007).

Materials and methods

Plant material

Three tomato lines were compared: Cervil, a cherry tomato line (*Solanum lycopersicum* var. *cerasiforme*) and two large-fruited accessions Levovil and VilB (*S. lycopersicum* Mill). Five plants per line were grown in a greenhouse under standard conditions. Fruits were harvested on several trusses at red ripe stage (7 days after breaker stage). Pericarps with epidermis were separated and immediately frozen with liquid nitrogen before grounding for total and WB-CWP extractions. For S-CWP fresh pericarp pieces were used.

Protein extraction methods

Total protein extraction

Five hundred milligrams of pericarp powder material was directly extracted in 1.2 mL of Laemmli sample buffer (Laemmli 1970) for 15 min at room temperature. After 15 min centrifugation at 5,500g the protein content of the supernatant was assayed using the Biorad RC-DC protein

assay with bovine serum albumin (BSA) as a standard according to the manufacturer instructions.

Soluble cell wall proteins extraction method

The method was adapted from Zhu et al. (2006) with some modifications. Unless otherwise stated all steps were carried out at 4 °C. Red ripe tomato pericarps were cut with a cleaned razor into small pieces and immediately immersed in ice-cold 20 mM K_2HPO_4 pH 6.0 buffer dried on filter paper before weighing. They were then rinsed twice with degassed ice-cold 3 mL/g 10 mM MES buffer, pH 5.5 and immersed in flasks containing the same solution. Rinsing buffer was discarded and tissue pieces were vacuum infiltrated at 60 kPa for 30 s for removal of gas trapped in veins. Ice-cold degassed 100 mM KCl, 10 mM MES, pH 5.5 buffer supplemented with 5 μ L of protease inhibitor cocktail (Sigma) was added on the basis of 5 mL per gram of plant material then soaked pericarp segments were subjected to 60 kPa vacuum for 3 min with gentle intermittent shaking. Vacuum was released; pericarp pieces were allowed to stand in the infiltration buffer for 3 min. Infiltration buffer was then removed and excess buffer was blotted away from tomato segments. Pericarp pieces were then transferred into 15 mL Falcon tube internally lined with mesh into a U-shape and previously holed at their bottom part. The whole unit was inserted into a 50 mL clean Falcon tube and centrifuged for 10 min at 1,000 rpm. Infiltration juice collected at the bottom of the 50 mL Falcon tube from various vacuum infiltration experiments was collected, concentrated with Macrosep centrifugal device (15 mL capacity/10 kDa membrane cutoff) followed by desalting by washing using three volume of 50 mM Na-Acetate buffer, pH 6 with 1 μ L/mL protease inhibitor cocktail. Protein content was quantified with Bio-Rad Bradford assay protein using BSA as the standard.

Weakly bound cell wall protein extraction method

This was carried out as reported by Watson et al. (2004) with some modifications. In brief, in a cold mortar and pestle tomato pericarp was ground under liquid nitrogen till coarse particles were obtained. 3 mL/g of grinding buffer (50 mM Na-Ac, pH 5.2, 50 mM NaCl, 30 mM ascorbic acid) was added to powdered pericarp and grinding was further continued until a viscous slurry was obtained. Ground tissue was poured onto nylon mesh filter in the Buchner funnel (pore size 70–100 μ m). Suction vacuum was applied to drain buffer off. Evenly distributed debris was washed thrice with grinding buffer (5 mL/g) each. Debris was washed with 7 mL/g cold buffer I (100 mM NaCl). Buffer was poured over the tissue debris and removed by vacuum filtration followed by twice wash with

7 mL/g cold doubled distilled water. Finally the tissues were fully dehydrated with five times wash with 7 mL/g cold acetone (total of 35 mL). The obtained wall fragments were allowed to completely dry. Finally the wall fragments were washed with 15 mL cold wash buffer II (10 mM Na-Ac, pH 5.2) and extracted with ice cold 2 mL/g extraction buffer (200 mM $CaCl_2$, 50 mM Na-Ac pH 5.2) for 45 min with shaking (250 rpm). Wall protein extract obtained upon gentle centrifugation at 1,000 rpm for 15 min was filtered by bacterial filter (2 μ m). Clear protein solution was concentrated and desalted exactly as described for soluble wall protein. Protein was quantified using Bio-Rad protein estimation kit and preserved at –80 °C till further use.

Purity assessment

This was carried out by following the intracellular marker glucose-6 phosphate as reported (Li et al. 1989) dehydrogenase activity as follows: in 1 mL reaction volumes. Glucose-6 phosphate dehydrogenase (G6P-D, EC 1.1.1.49) in a reaction mixture containing 25 μ M tricine, pH 8, 25 μ M glucose-6-phosphate, 0.1 M $MgCl_2$, 25 μ M NADP, and enzyme, following the reduction of $NADP^+$ at 340 nm.

Protein electrophoresis

Cell wall proteins were separated by SDS-PAGE according to Laemmli (1970). 40 μ g protein samples were loaded on 13 % acrylamide SDS-PAGE gels. Gels were stained with Coomassie colloidal as per Scheler et al.'s (1998) procedures. Briefly, proteins were first fixed ca 1 h in 50 % ethanol, 2 % phosphoric acid. Gels were then washed 1 h in 2 % phosphoric acid. Gels were submitted to a sensitization step for 20 min in 17 % ethanol, 15 % ammonium sulfate, and 2 % phosphoric acid, and 0.1 % Coomassie colloidal was then added to this buffer. After 3 days of staining, gels were washed 10 min in deionized water, 10 min in 20 % ethanol, and finally, 10 min in deionized water.

Trypsin digestion and protein identification by mass spectrometry

Stained bands were excised from gels and digested with trypsin as follows. Gel bands were washed with 100 μ L of 25 mM NH_4HCO_3 , followed by 100 μ L of 50 % acetonitrile in 25 mM NH_4HCO_3 . Proteins were then reduced by incubation with 10 mM DTT (1 h, 57 °C), and alkylated with 100 μ L of 55 mM iodoacetamide (45 min at room temperature). Gel bands were further washed as described above and digested overnight at 37 °C by the addition of 10–10 μ L of Trypsin (12.5 ng/ μ L in 25 mM NH_4HCO_3 ;

modified trypsin purchased from Promega, Madison, WI, USA). The resulting peptides mixture was stored at -20°C until analysis.

Mass spectrometry analyses were conducted by the platform “Biopolymers-Structural Biology” located at the INRA Center of Angers-Nantes (http://www.angers-nantes.inra.fr/plateformes_et_plateaux_techniques/plateforme_bibs).

Nanoscale capillary liquid chromatography–tandem mass spectrometry (LC–MS/MS) analyses of the digested proteins were performed using a Switchos-Ultimate capillary LC system (Dionex, Amsterdam, The Netherlands), coupled with a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-TOF Global, Waters, Manchester, UK). Chromatographic separation was conducted on a reverse-phase capillary column (Pepmap C18, 75- μm i.d., 15-cm length, Dionex) at a flow rate of 200 nL min^{-1} . The gradient consisted of a linear increase from 2 to 50 % of acetonitrile in 50 min, followed by a rapid increase to 60 % of acetonitrile within 10 min. Mass data acquisitions were processed with Masslynx software (Waters) using the so-called “data dependent acquisition” mode: MS data were recorded for 1 s on the mass-to-charge (m/z) range of 400–1,500, after which the most intense ions were selected and fragmented in the collision cell (MS/MS measurements) for 8 s; MS/MS data were recorded on the m/z range of 50–1,500.

Databank searches

Raw data were processed with Protein Lynx Global Server software (version 2.1, Waters). Protein identification was performed by confronting the collected LC–MS/MS data against the tomato SGN databank version 3. Databank searches were performed using the Mascot server 2.2 program (Matrix Science).

Mass tolerance was set at 120 ppm for parent ions (MS mode) and 0.3 Da for fragment ions (MS/MS mode); one missed cut per peptide was allowed, and the oxidation of methionines was set as a variable modification.

Validation of protein identifications

The peptide sequences, unigenes accession numbers, description, scores and sequence coverage are provided in Supplementary Table 1. Each identification was manually inspected and validated. In general, proteins were identified with a minimum of two MS/MS spectra matching the databank sequence with individual MASCOT ion scores above the significance threshold ($p < 0.05$). Protein identification generated by single MS/MS spectra were considered valid only when the MS/MS spectra displayed a wide series of intense fragments which could be assigned

to major predicted fragments (i.e. b or y ions) of the proposed peptide sequence (Supplementary Table 2). These single hits were carefully inspected to validate protein identification. Database protein searches identified 185 proteins from single peptides.

Results were validated and exported in an analytical report using the OVNip software developed by INRA Nantes (<http://www.appli.nantes.inra.fr:8180/OVNip/>) (Tessier et al. 2010).

Bioinformatic analysis

The nucleotide sequence of the each unigene was blasted against Uniprot database.

The presence and location of signal peptide cleavage sites were predicted by the SignalP 3.0 software (<http://www.cbs.dtu.dk/services/SignalP/>) (Emanuelsson et al. 2007). The default threshold was 0.43 for both SIGNAL-NN and SIGNALP-HMM.

Results and discussion

Fruit softening is a major ripening process that gives final fruit texture. Modifications of the cell wall architecture, is supposed to occur mainly by cell wall disassembly and reorganization of polysaccharidic polymers during loss of firmness. This deconstruction process is being extensively studied and the role of some enzymes whose activities may be mechanistically involved in these wall changes are yet to be fully characterized in tomato (for review see Brummell and Harpster 2001; Vicente et al. 2007). However, the fruit cell wall compartment is highly complex and biosynthesis of cell wall polymers is known to occur even in ripening tomato fruit (Greve and Labavitch 1991). Until now, no global overview of fruit cell wall proteome is available, even though a recent description of tomato cuticle proteome was provided (Yeats et al. 2010).

In order to get the first description of tomato fruit cell wall proteome we compared its expression in 3 tomato lines elsewhere known for their contrasted firmness phenotype. Weakly bound (WB-CWP) and soluble proteins (S-CWP) were obtained from tomato pericarp which is predominantly a parenchyma system with large cells surrounded by epidermis layer and irrigated by xylem vessels.

Two different protocols were adapted for extraction of weakly bound-cell wall proteins (WB-CWP) and soluble-cell wall proteins (S-CWP) of three tomato genotypes. The first method involved cellular destruction followed by consecutive buffer/salt/solvent washings to get rid of cytosolic contaminant to finally obtain cell wall fragments which were then extracted with salt added buffer. The second method was milder and aimed at obtaining soluble

Table 1 Pericarp total, weakly bound (WB) and soluble (S) CWP content and extraction yield of the 3 tomato varieties

	Cervil	Levovil	VilB
Total protein content ($\mu\text{g/g}$ FM)	5,251.6 (± 229.4)	1,630 (± 80.90)	2,290 (± 264.6)
WB-CWP ($\mu\text{g/g}$ FM)	27.27 (± 12.52)	13.83 (± 2.81)	15.72 (± 3.30)
S-CWP ($\mu\text{g/g}$ FM)	4.08 (± 0.31)	2.1 (± 0.27)	2.98 (± 0.52)
Total pericarp protein content (% DM)	4.80	2.60	5.70
WB-CWP extraction yield (% total proteins)	0.52	0.85	0.69
S-CWP extraction yield (% total proteins)	0.08	0.12	0.13

The extraction process was repeated 3 times for each sample

wall proteins by implementation of vacuum infiltration-centrifugation. Both methods were based on use of none dehydrated pericarp material (either frozen or fresh tissues). Table 1 summarizes the pericarp protein contents and yields obtained for each extraction method and each genotype.

Cell wall protein yield

Tomato pericarp total protein content represented less than 6 % of pericarp dry matter. WB-CWP extraction yield varied from 0.5 % to approximately 1 % of total proteins, which is quite low but still 6 to 7 fold more than S-CWP extraction yield. S-CWP represented about 0.1 % of total proteins which is very low in term of protein quantity and explains why more than 50 g of fresh pericarp for WB-CWP and about 500 g for S-CWP were necessary to obtain sufficient protein quantity to perform repeats of extractions and electrophoresis. Very few data are available in literature concerning CWP extraction yields. Haslam and his colleagues extracted S-CWP from *Arabidopsis* leaves in which they obtained about tenfold higher yield as compared to our results with tomato fruit pericarp (Haslam et al. 2003). In the same way, our WB-CWP yield was also lower by 2–3 fold than that obtained by Feiz and his coworkers using calcium chloride as salt, from *arabidopsis* stems (Feiz et al. 2006). It is tempting to correlate these lower yields to the higher total protein content reported by these authors in *arabidopsis* leaves and stems. However, comparing the yields among tomato varieties investigated in our study no such correlation could be noticed, Cervil and VilB showed twofold higher protein content as compared to Levovil but this difference had no obvious consequence on the WB-CWP and S-CWP extraction yields.

Assay for intracellular contamination

To properly evaluate both the efficiency of extraction methods and the effect of genotype on CWP content, it was important to assess the purity extend of the obtained protein fractions. We followed the cytosolic protein marker

glucose-6-phosphate dehydrogenase (G6PDH) (Li et al. 1989). Total activity from whole cell extracts was lower than 770 nmol/min/g FW and only about 0.1 % of this activity remained in S-CWP extracts, indicating the high purity of our preparation. On the other hand, no G6PDH activity could be detected in WB extracts. Nevertheless, as will be shown and discussed later, some proteins with no clear cell wall assignment were still detected in our extracts by mass spectrometry analysis. Similar results were reported by Feiz et al. (2006) and highlights the advantages of mass spectrometry and bioinformatics tools (San Clemente et al. 2009) over the enzymatic assays in order to properly evaluate CWP extraction methods.

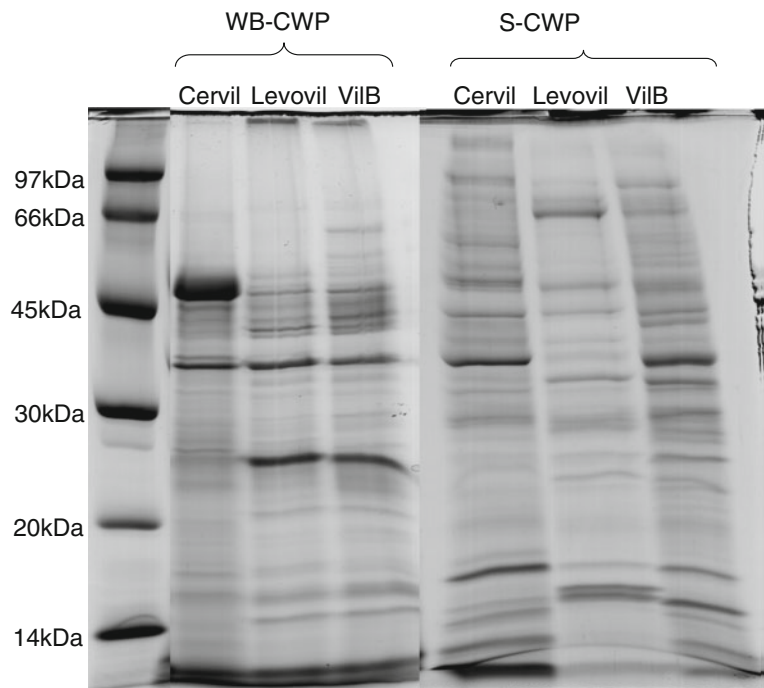
Cell wall protein separation by SDS-PAGE

Taking into account the fact that two-dimensional electrophoresis is not well suited for CWPs separation; we performed simple SDS polyacrylamide gel electrophoresis (Fig. 1). Obvious differences in the electrophoretic patterns were obtained between the soluble and WB extracts from the 3 lines, as well as between the genotypes. The distribution of the S-CWP on the gel almost spanned the entire molecular marker ranges, from high mass proteins (higher than 97 kDa) to protein masses as low as less 14 kDa or less. In contrast, WB-CWP bands were clearly focused below 66 kDa, with little or no bands above 66 kDa. In total, 77 clearly resolved bands were observed on the S-CWP gels and 62 bands on the WB-CWP gels. Levovil and VilB WB-CWP patterns showed similar bands spatial distribution whereas Cervil pattern was markedly distinct among all. The pattern similarity between VilB and Levovil was not noticed in S-CWP protein pattern, in contrary, in this case, VilB looked more of like Cervil than Levovil.

Protein identification by LC–MS–MS

One hundred and thirty-nine bands, reproducibly detected were excised, subjected to trypsin digestion and peptide mixtures were analyzed by LC–MS–MS (Supplementary Figure 1). Nine bands did not bring any identification.

Fig. 1 SDS-PAGE analysis of tomato fruit cell wall protein extract. 40 µg of weakly bounded and soluble cell wall protein extracts (WB-CWP and S-CWP) of 3 tomato lines (Cervil, Levovil and VilB) were loaded on a 13 % acrylamide gel and stained with colloidal blue. The exact position of the bands which were excised and submitted to LC–MS/MS is indicated on Supplementary Fig. 1



The remaining 130 bands gave 433 positive hits against SGN Tomato Unigenes database representing 140 distinct unigenes entries. Among the protein hits, 241 were obtained by WB-CWP extraction procedure and 192 by S-CWP extraction procedure. More than two-third (329) of the identified unigenes aligned with *Solanaceae* protein sequences in Uniprot database. The complete lists of peptides and proteins identifications are available [Supplementary Table 1 (peptides) and Table 2 (proteins)]. From one up to 14 valid MS/MS spectra could be obtained for a given protein identification. The majority of the proteins (288) were identified with two or more MS/MS spectra matching the database sequence. For the 185 protein identifications generated by single MS/MS spectra, careful inspection was performed to validate protein identification (see “Materials and methods” and Supplementary Figure 1).

Redundancy in protein identifications was often observed within several bands of the same gel lane, some unigenes being present in a very high number of bands. For example, pectin methylesterase could be identified 41 times either with UG312757 accession number (35 cases) or with UG318232 accessions number (6 cases). It was detected in all genotypes and in both cell wall protein extract types, although more in WB-CWP fraction. The seven more frequent unigenes were confirmed cell wall proteins (Biotic cell death protein, hevein like protein, osmotin-like protein, LRR disease resistance protein, polygalacturonase, peroxidase and pectin methylesterase). On the other hand, 60 unigenes could be found in only one

band and thus appeared to be putatively genotype and fraction specific.

Unlike Arabidopsis, no database is available for easy prediction of sub-cellular localization and functional domains (San Clemente et al. 2009) in case of tomato. Therefore, for each unigene or corresponding protein sequence in Uniprot or NCBI database, the Signal P software was used to check the existence of a N-terminal leader peptide. When a signal peptide was predicted, the protein was considered as classical cell wall protein (cCWP). Other proteins with no software detectable signal that would destine them to cell wall (denoted NS).

In total, 297 identifications could be assigned to cell wall using SignalP predictive tool, 289 were predicted as cCWP and remaining 145 identifications were assumed to be either contaminants or cell wall proteins with no obvious secretory signal (NS, Supplementary Table 2). The total number of identifications was 170 for VilB, 157 for Cervil and 106 for Levovil. As several identifications were similar between genotypes and cell wall compartments, it turned out that the identified CWPs corresponded to 75 different SGN accessions themselves corresponding to 68 Uniprot accessions (Table 2; Supplementary Table 3).

For each method, the percentage of cell wall proteins (cCWP) and proteins with no clear to cell wall targeting signal (NS) for the three genotypes is represented in Fig. 2. The number NS proteins varied according to the genotype, the lowest level of NS proteins was obtained for Levovil pericarp while Cervil extraction reported the highest quantity of NS. Whatever the genotype, the WB method

Table 2 List weakly bound and soluble CWP identified by LC-MS/MS

Unigene identifier	E value ^a	Uniprot accession	Protein function	Location	Extract	Species	Functional classes	pI	M _r
SGN-U312757	0	P14280	Pectinesterase 1 precursor	ccwp	WB + S	<i>Solanum lycopersicum</i>	Acting on polysaccharides	6.33	60,065.61
SGN-U318232	0	B9SAU4	Pectinesterase	ccwp	WB	<i>Ricinus communis</i>	Acting on polysaccharides	9.06	62,006.58
SGN-U312703	0	P05117	Polygalacturonase-2 precursor	ccwp	WB	<i>Solanum lycopersicum</i>	Acting on polysaccharides	6.4	50,051.54
SGN-U312701	3e–45	P05117	Polygalacturonase-2 precursor	ccwp	WB	<i>Solanum lycopersicum</i>	Acting on polysaccharides	6.4	50,051.54
SGN-U313742	0	Q8L5J1	Mannan endo-1,4- β -mannosidase 4 precursor (GH5)	ccwp	WB	<i>Solanum lycopersicum</i>	Acting on polysaccharides	8.92	45,338.67
SGN-U320073	0	Q9SP05	α -Galactosidase (GH27)	ccwp	S	<i>Solanum lycopersicum</i>	Acting on polysaccharides	5.3	44,942.23
SGN-U324817	1e–36	Q9LYL2	α -Galactosidase, putative (GH27)	ccwp	WB	<i>Arabidopsis thaliana</i>	Acting on polysaccharides	4.73	48,019.14
SGN-U320267	0	Q9SP05	α -Galactosidase (GH27)	ccwp	WB	<i>Lycopersicon esculentum</i>	Acting on polysaccharides	5.3	44,942.23
SGN-U342004	0	Q43778	Glucan endo-1,3- β -D-glucosidase precursor (GH17)	ccwp	WB + S	<i>Solanum lycopersicum</i>	Acting on polysaccharides	5.38	37,866.76
SGN-U312944	0	Q01413	Glucan endo-1,3- β -glucosidase B precursor (GH17)	ccwp	WB	<i>Solanum lycopersicum</i>	Acting on polysaccharides	7.84	39,719.15
SGN-U313265	1e–149	Q05539	Acidic 26 kDa endochitinase precursor (GH19)	ccwp	S	<i>Solanum lycopersicum</i>	Acting on polysaccharides	5.93	27,631.98
SGN-U313266	1e–145	Q05540	Acidic 27 kDa endochitinase precursor (GH19)	ccwp	S	<i>Solanum lycopersicum</i>	Acting on polysaccharides	4.68	26,583.55
SGN-U312562	0	Q05538	Basic 30 kDa endochitinase precursor (GH19)	ccwp	WB + S	<i>Solanum lycopersicum</i>	Acting on polysaccharides	6.19	34,345.43
SGN-U312558	1e–180	O81145	Class I chitinase (GH19)	ccwp	WB + S	<i>Solanum tuberosum</i>	Acting on polysaccharides	5.66	35,331.41
SGN-U322252	1e–173	Q43576	Chitinase, class V (GH18)	ccwp	WB + S	<i>Nicotiana tabacum</i>	Acting on polysaccharides	8.94	41,779.98
SGN-U322253	1e–173	Q43576	Chitinase, class V (GH18)	ccwp	S	<i>Nicotiana tabacum</i>	Acting on polysaccharides	8.94	41,779.98
SGN-U315168	1e–179	Q40144	Xyloglucan endotransglucosylase/hydrolase 1 (GH16)	ccwp	WB	<i>Solanum lycopersicum</i>	Acting on polysaccharides	8.8	34,010.64
SGN-U314145	6e–97	B9RIY8	β -Glucosidase, putative (GH3)	ccwp	S	<i>Ricinus communis</i>	Acting on polysaccharides	6.22	83,603.07
SGN-U317583	0	Q944F3	Arabinosidase ARA-1	ccwp	S	<i>Solanum lycopersicum</i>	Acting on polysaccharides	5.3	74,231.51
SGN-U329313	0	Q76MS5	LEXYL1 (GH3)	ccwp	S	<i>Solanum lycopersicum</i>	Acting on polysaccharides	4.73	48,019.14
SGN-U336993	2e–75	Q9LEC9	α -Glucosidase (GH31)	ccwp	S	<i>Solanum tuberosum</i>	Acting on polysaccharides	6.31	10,4687.5
SGN-U314735	0	Q2WGN6	Glycerophosphodiesterase-like protein	ccwp	S	<i>Nicotiana tabacum</i>	Acting on polysaccharides	5.34	82,005.24
SGN-U314384	2e–60	Q4AIN1	Non-specific lipid-transfer protein	ccwp	WB + S	<i>Solanum lycopersicum</i>	Lipid metabolism	8.74	11,910.1
SGN-U324217	2e–27	Q93YX9	Non-specific lipid-transfer protein	ccwp	WB + S	<i>Davidia involucrata</i>	Lipid metabolism	9.18	12,057.24
SGN-U315630	3e–52	Q8H6L4	Non-specific lipid-transfer protein 2 precursor	ccwp	WB	<i>Solanum lycopersicum</i>	Lipid metabolism	9.24	11,510.81
SGN-U315060	7e–62	P93224	Non-specific lipid-transfer protein 2 precursor	ccwp	WB	<i>Solanum lycopersicum</i>	Lipid metabolism	8.04	11,484.5
SGN-U315061	7e–62	P93224	Non-specific lipid-transfer protein 2 precursor	ccwp	WB + S	<i>Solanum lycopersicum</i>	Lipid metabolism	8.04	11,484.5
SGN-U314281	3e–23	B9GNC0	Predicted protein lipid transfer protein	ccwp	S	<i>Populus trichocarpa</i>	Lipid metabolism	8.68	9,926.79
SGN-U316556	3e–27	Q93YX9	Non-specific lipid-transfer protein	ccwp	S	<i>Davidia involucrata</i>	Lipid metabolism	9.18	12,057.24

Table 2 continued

Unigene identifier	E value ^a	Uniprot accession	Protein function	Location	Extract	Species	Functional classes	pI	M _r
SGN-U312310	0	Q946Z2	Putative acyl-CoA synthetase	ccwp	S	<i>Capsicum annuum</i>	Lipid metabolism	6.55	56,341.64
SGN-U315693	0	P15003	Suberization-associated anionic peroxidase 1 precursor	ccwp	WB + S	<i>Solanum lycopersicum</i>	Oxido-reductase	4.91	38,749.69
SGN-U318102	1e-114	O65358	Germin-like protein	ccwp	WB	<i>Solanum tuberosum</i>	Oxido-reductase	8.79	23,234.89
SGN-U317475	8 e-74	A5AXM4	Putative uncharacterized protein	ccwp	WB	<i>Vitis vinifera</i>	Oxido-reductase	4.95	28,860.53
SGN-U316807	1e-176	Q94IQ1	Peroxidase	ccwp	WB	<i>Nicotiana tabacum</i>	Oxido-reductase	5.99	39,062.92
SGN-U313409	1e-96	B7UCP4	Peroxidase 4	ccwp	WB	<i>Litchi chinensis</i>	Oxido-reductase	5.53	39,313.62
SGN-U314196	1e-155	Q9XIV9	Peroxidase	ccwp	WB	<i>Nicotiana tabacum</i>	Oxido-reductase	9.4	34,369.85
SGN-U322187	1e-131	A9PBM7	Aspartyl protease	ccwp	WB + S	<i>Populus trichocarpa</i>	Protease	9.5	45,612.45
SGN-U313997	0	Q40143	Cysteine proteinase 3 precursor	ccwp	S	<i>Solanum lycopersicum</i>	Protease	8.59	38,944.52
SGN-U317724	0	A9XG40	Subtilisin-like protease	ccwp	S	<i>Nicotiana tabacum</i>	Protease	6.55	81,187.6
SGN-U313775	0	O04678	P69B protein (protease)	ccwp	S	<i>Solanum lycopersicum</i>	Protease	6.26	78,930.52
SGN-U312452	4e-65	Q5W111	Putative ML domain protein	ccwp	WB + S	<i>Nicotiana glauca</i>	Having an interacting domain	6.55	20,152.33
SGN-U317036	1e-150	B9GM86	Predicted protein (leucine rich repeats)	ccwp	WB	<i>Populus trichocarpa</i>	Having an interacting domain	8.78	40,337.56
SGN-U312623	1e-126	Q9LEG1	Cathepsin D inhibitor precursor	ccwp	WB	<i>Solanum lycopersicum</i>	Having an interacting domain	8.84	24,188.08
SGN-U313725	0	Q43143	Pectinesterase/pectinesterase inhibitor U1 precursor	ccwp	WB	<i>Solanum lycopersicum</i>	Having an interacting domain	8.97	64,103.07
SGN-U314637	9 e-63	B9R9Q7	21 kDa protein, putative (pectinesterase inhibitor)	ccwp	WB	<i>Ricinus communis</i>	Having an interacting domain	9.41	21361.61
SGN-U317539	3e-90	O82001	Tomato invertase inhibitor precursor	ccwp	WB	<i>Solanum lycopersicum</i>	Having an interacting domain	8.3	18,764.84
SGN-U320854	1e-81	Q9SWC6	Hypersensitive response assisting protein	ccwp	WB	<i>Capsicum annuum</i>	Having an interacting domain	8.74	29,936.3
SGN-U314071	0	Q8GT67	Xyloglucan-specific fungal endoglucanase inhibitor	ccwp	WB + S	<i>Solanum lycopersicum</i>	Having an interacting domain	7.88	46,637.03
SGN-U313878	7e-26	B9RH71	Ribonuclease L2, putative	ccwp	WB	<i>Ricinus communis</i>	Having an interacting domain	9.21	28,985.7
SGN-U317739	7e-93	Q850R9	Biotic cell death-associated protein	ccwp	S	<i>Nicotiana glutinosa</i>	Having an interacting domain	8.85	25,157.92
SGN-U328496	1e-14	Q6TPK3	Cysteine protease inhibitor, putative	ccwp	S	<i>Actinidia delictiosa</i>	Having an interacting domain	10.1	12,898.04
SGN-U346984	2e-38	Q9T0K5	Extensin-like protein	ccwp	WB	<i>Arabidopsis thaliana</i>	Structural protein	6.49	82,246.46
SGN-U316008	4e-82	P32045	Pathogenesis-related protein P2 precursor	ccwp	WB + S	<i>Solanum lycopersicum</i>	Defense response	8.53	16,026.12
SGN-U312653	1e-152	Q5XUH6	Osmotin-like protein	ccwp	WB + S	<i>Solanum phureja</i>	Defense response	6.67	26,669.92
SGN-U324419	1e-46	Q948Z4	Snakin-1 precursor	ccwp	WB	<i>Solanum tuberosum</i>	Defense response	9.06	9,663.59
SGN-U314266	4e-40	B1N681	Defensin protein	ccwp	WB	<i>Solanum pimpinellifolium</i>	Defense response	9.14	8,799.35
SGN-U314481	2e-54	Q40128	Gamma-thionin-like protein/acidic protein precursor	ccwp	WB	<i>Solanum lycopersicum</i>	Defense response	6.57	11914.18
SGN-U318558	1e-154	Q41350	Osmotin-like protein precursor	ccwp	WB	<i>Solanum lycopersicum</i>	Defense response	8.15	27,265.05
SGN-U313533	6e-30	A9PGW3	Rapid alkalization factor (RALF) family protein	ccwp	WB	<i>Populus trichocarpa</i>	Defense response	8.85	14,148
SGN-U312654	1e-153	P12670	Protein NP24 precursor	ccwp	WB	<i>Solanum lycopersicum</i>	Defense response	8.28	26,646.21
SGN-U312655	1e-81	P12670	Protein NP24 precursor	ccwp	WB	<i>Solanum lycopersicum</i>	Defense response	8.28	26,646.21

Table 2 continued

Unigene identifier	E value ^a	Uniprot accession	Protein function	Location	Extract	Species	Functional classes	pI	M _r
SGN-U312367	1e-107	B2LW68	PR-1 protein	ccwp	S	<i>Solanum lycopersicum</i>	Defense response	8.48	20,123.47
SGN-U314796	1e-93	Q04108	Pathogenesis-related leaf protein 4 precursor	ccwp	S	<i>Solanum lycopersicum</i>	Defense response	7.57	17,438.58
SGN-U315430	3e-38	B9SY08	Putative uncharacterized protein (DUF538)	ccwp	WB + S	<i>Ricinus communis</i>	Unknown	7.76	18,166.17
SGN-U315886	1e-165	O24329	Protein of unknown function (DUF642)	ccwp	WB	<i>Vitis vinifera</i>	Unknown	7.57	40,005.99
SGN-U317723	1e-168	A7Q261	Protein of unknown function (DUF642)	ccwp	WB	<i>Vitis vinifera</i>	Unknown	8.72	39,861.88
SGN-U315665	1e-140	A7PWF2	Protein of unknown function (DUF642)	ccwp	WB	<i>Vitis vinifera</i>	Unknown	9.06	39,615.63
SGN-U318898	1e-69	A5BXB9	Uncharacterized protein (SOUL-heme domain)	ccwp	WB	<i>Vitis vinifera</i>	Unknown	4.6	25,179.26
SGN-U318899	2e-76	A5BXB9	Uncharacterized protein (SOUL-heme domain)	ccwp	WB	<i>Vitis vinifera</i>	Unknown	4.6	25,179.26

The table gives the distribution of each protein per tomato line and cell wall fraction type

WB weakly bound cell wall proteins, S soluble wall protein

^a E values for blast of SGN unigenes sequence against Uniprot corresponding protein

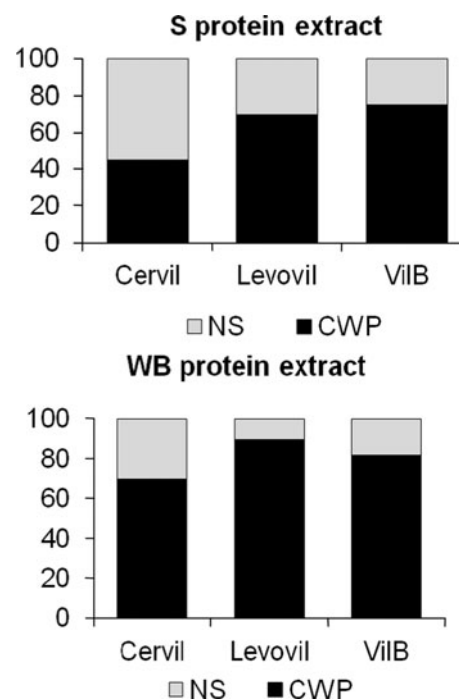


Fig. 2 Efficiency of both CWP extraction methods according to the tomato line. The graph shows the percentages of CWP and of probable contaminant not secreted proteins (NS) that were obtained in WB and S extracts per tomato line. The presence and location of signal peptide cleavages sites were predicted by the SignalP 3.0 software

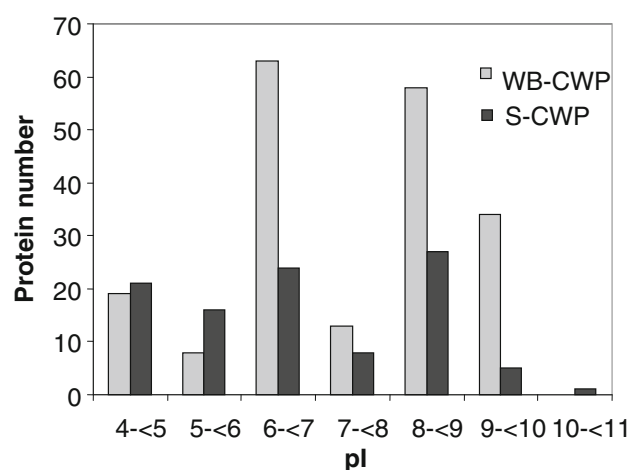


Fig. 3 Distribution of soluble and weakly bound cell wall protein pIs. The pI of the Uniprot proteins corresponding to the identified tomato SGN database unigenes were computed using the “Compute pI/MW” tool on the ExPASy website

gave markedly lesser NS proteins as compared to the vacuum infiltration method. Excluding NS proteins, we compared the pI distribution of CWPs for both methods (Fig. 3). As expected, the pIs were homogeneously

distributed between 4 and 9 for vacuum infiltrated proteins (S-CWP). Whereas WB-CWPs were dominantly of basic pI, these results are in accordance with previous observation for arabidopsis CWP (Jamet et al. 2006).

Moonlighting proteins or experimental artifact?

The classical secretion of proteins across biological membranes in the most of the cases so far known is mediated by translocation machinery adapted to recognize a specific N-terminal sequence called leader or signal peptide, however, a non-classical protein release independent of such common secretory pathway has also been reported for an increasing number of proteins lacking the N-terminal signal sequence. However, could still be successfully translocated to cell wall.

Our work, to our knowledge, is the first cell wall proteomic study of fruit pericarp. Two previous studies dealing with fruit wall proteins were already documented; the first was of weakly bound cell wall protein of grape skin and seeds which exhibited 26 % of total protein with signal peptide whereas analysis of tomato fruit cuticle resulted in 39 % proteins with a secretion signal (Yeats et al. 2010). In literature, several proteins, which have been found to display a function in the cytoplasm, have also been shown to actively participate in biological processes in the extracellular environment (Schaumburg et al. 2004). Recently Wang and his colleagues reported the existence of an unconventional secretion system composed of spherical double membrane structure (named EXPO) close to exosomes, that brings an innovative view towards plant secretion mechanisms (Wang et al. 2010). Evidences are now accumulating in support of occurrence of such translocation in plant system (Hawes et al. 2011). In the current investigation, as well as in many other studies (Chivasa et al. 2002; Watson et al. 2004), proteins such as histones, thioredoxin, superoxide dismutase, fructose-bisphosphate aldolase, glyceraldehyde 3-phosphate dehydrogenase, ribosomal proteins etc have been identified among cell wall proteins (Supplementary Table 2). These findings may rule out or at least weaken the allegation that argues for contamination possibility or experimental artifacts. Histone is found in eukaryotic cell nuclei that package and order the DNA into structural units called nucleosomes. Histones were proved to act extracellularly by providing antimicrobial activities in some specific cells of a diverse range of organisms from shrimps, plant to humans (Hawes et al. 2011; Hiroaki 2008), a role for extracellular histone H1 during skeletal muscle regeneration has also been reported (Henriquez et al. 2002). Thioredoxin is a small redox protein, known to be present in all organisms. In plants, it is mainly found in chloroplast, though it may also be present in the cytosol. It performs a spectrum of critical

functions, ranging from photosynthesis to growth, flowering, the development and germination of seeds. It is documented to be associated with plasma membrane playing a role in cell-to-cell communications (Meng et al. 2010). Glyceraldehyde 3-phosphate dehydrogenase mostly is a cytoplasmic protein; however, recent evidences indicated the involvement of the enzyme with the yeast cell wall activities (Barbosa et al. 2006; Gozalbo et al. 1998). Fructose bisphosphate aldolase is a glycolytic enzyme but it was repeatedly isolated as a cell wall protein (Kelly and Kavanagh 2010; Pitarch et al. 2002; Zhu et al. 2007). While working with *Bacillus subtilis* secretome Tjalsma et al. have reported as high as third of *Bacillus subtilis* cell wall proteins as ribosomal proteins (Tjalsma et al. 2008). Heat shock proteins (HSPs) are cytosolic group of proteins the expression of which is increased when the cells are exposed to various biological stresses, including heat, high pressures, toxic compounds etc. By immunolocalization heat shock protein was found to be associated with cell wall (Cunningham and Spreadbury 1998; Lopez-Ribot et al. 1996; Vasques et al. 2010). The repetitive occurrence of these proteins in cell wall isolates though of their apparent lack of a leader peptide, may shedlight on the possibilities that these proteins are either targeted to cell wall via a signal that yet to be revealed or may lead an unknown secretory pathway. Though it may be early to affiliate these proteins to cell wall as non-classical residents, their report by many cell wall researchers, may enhance their possibility of being genuine cell wall members.

Functional assignments

Comparison of proteins identified between S-CWP and WB-CWP fractions showed that some functions were strictly related to biochemical properties of these physiological roles. For example, Leman4, polygalacturonase, a probable XTH, predicted leucine rich and aspartyl protease were only present in WB extract, while β -glucosidase, arabinosidase and xylosidase were specifically detected in soluble fraction. On the contrary, some proteins, probably corresponding to more abundant ones (pectinesterase, suberization associated peroxidase, pathogenesis-related protein P2 and one uncharacterized protein) were identified in both fractions. In a few cases, such as galactosidases, distinct isoforms corresponding to the same function were specifically detected in for each fraction suggesting possibility of different roles in the cell wall.

The distribution of proteins into functional classes was established on the basis of their biochemical or biological functions according to Jamet et al. (2006). Of the non-redundant list of identified proteins, 21 were classified as “protein acting on polysaccharides” class, 11 in “protein

having interacting domain” class, 9 in “lipid metabolism” class, 7 in “oxido-reductase” class, 4 in “protease” class, 3 in “structural protein” class, 8 in “unknown function” class. At last, 12 proteins were clustered in an additional “defense response” class (Table 2). The putative roles of the most important functions in case of ripe fruit organ are discussed below.

Fruit ripening is accompanied by major cell wall modifications leading to cell wall loosening. The pectin-rich middle lamella dissolves and a reduction in the molecular weight of cell-wall polysaccharides through hydrolytic enzyme action is observed. Among the “Proteins acting on polysaccharides” class, two major functions involved in pectin depolymerization process were found. Pectinesterase, the most frequently identified function of this class, plays a role in cell wall metabolism during fruit growth and development prior to ripening. It is thought to be responsible for the demethylation of galacturonyl residues in pectin and may be required for preparing cell walls for softening by polygalacturonase during fruit ripening. One form was found in all extracts (SGN-U312757) and corresponded to tomato PME1 whose activity was previously correlated with fruit softening (Phan et al. 2007). Another isoform (SGN-U318232) was only detected in WB-CWP.

Polygalacturonase (PG)-2 precursor, is one of the major lytic enzymes involved in texture modification of the fruit, it hydrolyses (1 → 4)- α -D-galactosiduronic linkages in pectate and other galacturonans. The protein is thought to be associated with other proteins to form PG1 and PGx, this association leading to modulation of the enzyme in vivo (Tomassen et al. 2007).

We detected three enzymes having activities directed towards xyloglucan and glucomannan that could be linked to hemicellulose modification. First, a precursor of a xyloglucan endotransglucosylase/hydrolase 1 (XHT, UG315168) was found in ViLB and Cervil WB-CWP extracts. This enzyme is supposed to cleave and religate xyloglucan polymers and thus to catalyze xyloglucan endohydrolysis (XEH) and/or endotransglycosylation (XET). Some XHTs have been shown to depolymerize hemicellulose in vitro, although they did not make the wall more extensible (Saladie et al. 2006). The second enzyme: LeMan4 endomannase was detected in the WB-CWP for all cultivars extracts. This enzyme is known to be tightly associated to the wall and to have, besides its endomannase activity, a mannan transglycosylase activity (MTH) (Schroder et al. 2009). Thus may play a role in cell wall breakdown that leads to fruit softening as many non-ripening mutants were noticed to lack activity (Bourgault and Bewley 2002). Thirdly, an arabinosidase was present in S-CWP extract in the Levovil extract. This function is negatively regulated by ethylene which may control its transcript decline during ripening (Itai et al. 2003). In the present investigation, the presence of this protein in a ripe

tomato raises the question of its role in cell wall modification. Recent unpublished results show that the structure of xyloglucans and glucomannans is modified all along the Levovil fruit development and even during ripening suggesting remodeling of glucomannans and xyloglucans (Adeline Moïse, personal communication).

In S-CWP fraction, different exoglycosydases were identified, some of them being detected in only one genotype. For example, an acidic α -glucosidase was identified in ViLB S-CWP extract. α -Glucosidase occurs widely in plants but their function is still elusive due of the lack of corresponding substrate in the apoplast (Monroe et al. 1999). β -Xylosidase 1 was only detected in ViLB. In tomato fruit, its mRNA expression was related to late stages of ripening probably playing a role in breakdown of xylans (Itai et al. 2003). It is widely assumed that β -galactosidase plays an important role in tomato ripening by chopping galactosyl residues from cell wall polymers (Brummell and Harpster 2001). In this work, we did not identify β -galactosidase in our proteome map, though of the fact that when purified wall protein was tested against β -galactosidase's pseudosubstrate *p*-nitrophenyl β -galactopyranoside, strong activity for the enzyme was detected (not shown). β -galactosidase is of N-linked glycosylation enzyme (Smith et al. 1998) and it is usually challenging to identify glycoproteins with mass spectrometry as they would usually escape proteomic analyses (Jamet et al. 2008; Yin et al. 2008), secondly since the enzyme was initially detected in wall extract it might have not been chosen for digestion due to lower relative abundance and consequently not visualized in the gel. The missing of β -galactosidase in several proteome publications is a recurrent phenomenon, which may indicate the difficulty encountered during extracting of this enzyme in cell wall proteome studies (Chen et al. 2009; Negri et al. 2008; Soares et al. 2007). These authors did not justify for the absence of β -galactosidase in their proteome maps, however similar reasons, discussed in this paper, cannot be excluded. Other important classical cell wall proteins which were not identified in this investigation are expansins family proteins; the cell wall loosening proteins. Tomato expansins are highly glycosylated basic structural proteins known to associate tightly with cell wall matrix, and these proteins usually form insoluble network makes their extraction difficult (Brady et al. 1996; Jamet et al. 2008). Moreover, Weston and his colleagues, who worked with medicago sativa cell wall proteins, did not identify any of expansin family proteins in their study (Watson et al. 2004), and since we used a protocol similar to the one used by this group our result becomes justifiable.

Though we did not detect any β -galactosidase, 3 unigenes corresponding to α -galactosidase were identified. This enzyme is capable of hydrolysing α -1,6 linked

α -galactoside residues and its activity or quantity was shown to increase substantially during ripening in tomato fruits (Faurobert et al. 2007; Jagadeesh et al. 2004) and papaya (Soh et al. 2006).

Four enzymes were classified as “proteases”, including two serine proteases (P69B and subtilisin-like protease), one cysteine protease and one aspartic protease. It is noteworthy to mention that P69B was only detected in Levovil and aspartic protease was only present in WB fraction. Cell wall proteases may be involved in the processing of cell wall proteins and in their turnover. Occurrence of a proteolytic activity in cell wall proteins was proposed to explain the often observed discrepancies between observed and expected molecular masses of cell wall proteins (Jamet et al. 2008). The expression of cysteine protease 3 (Q40143) is already known to be up-regulated during Cervil fruit development and maturation (Faurobert et al. 2007). Moreover, the formation of peptides hormones from precursor polypeptides may be due to subtilases activity (Jamet et al. 2008). As a matter of fact, several peptides with hormonal effect such as snakins and defensins were also found in our proteome list (see below).

In the category under “Proteins having an interacting domain” we detected some proteins with inhibitory activity of importance during fruit ripening. Among them, we identified a tomato cell wall invertase inhibitor precursor in Cervil WB fraction. Recently, Jin and his research fellows demonstrated the role of this protein in tight post-translational regulation of cell wall invertase in planta and suggested that it regulates leaf senescence and fruit development in tomato (Jin et al. 2009). Two different unigenes annotated as pectinesterase inhibitors were also identified, which could play a role in regulating fruit cell wall loosening. Recently, structure similarities of complexes between cell wall invertase and pectin methyl-esterase (PME) with their corresponding inhibitors were described (Hothorn et al. 2010). In addition to that, a protein with a large lipid binding cavity (ML domain) was detected; it is likely to be involved in lipopolysaccharides signaling and lipid metabolism (Inohara and Nunez 2002). This protein was previously detected as a component of the surface proteome of tomato fruit, and probably involved in cuticle biogenesis (Yeats et al. 2010), this protein may originate from the epidermis layer that was present at the surface of tomato pericarp of the 3 varieties.

Under the category of proteins involved in lipid metabolism, several non-specific lipid transfer proteins (LTPs) were found in both extracts. These small extracellular basic proteins are major allergens in plant-derived food products. Among many other roles assigned for these proteins, some LTPs were established as cell wall loosening proteins. Their hydrophobic cavity may interact with hydrophobic molecules of the cellulose/xyloglucan network of the cell wall

and could disrupt hydrogen bonds between cellulose and hemicellulose (Nieuwland et al. 2005; Yeats and Rose 2008). Interestingly, they seem to play a functional role in the pectin-degrading protein complex by stimulating the activity and the stability of polygalacturonase 2 and thus being involved in fruit softening (Tomassen et al. 2007). Lastly, the glycerophosphodiesterase-like protein might be involved in glycerophosphodiester degradation related to fruit senescence (van der Rest et al. 2002).

As previously shown in case of total proteins from tomato pericarp (Faurobert et al. 2007), several CWPs were related to defense response. Thaumatin-like NP24, osmotin, defensin, gamma thionin-like and snakins are all small molecular weight proteins, involved in defense against pathogen attacks but also known to be up regulated during fruit ripening (Choi et al. 2008; Kim and Triplett 2004; Pressey 1997; Segura et al. 1999; Stotz et al. 2009). Their peculiar role in fruit physiology is still unknown but many of them have allergenic properties that have to be taken into account for fruit breeding purposes.

Another protein related to defense but also to fruit development processes was RALF protein (rapid alkalisation factor). This 5-kDa polypeptide is ubiquitous in plants and known to cause alkalisation of the cell wall pH which may play a role in activation of processing peptidases and in induction of MAP kinase activity (Germain et al. 2005; Pearce et al. 2001).

Within the “oxido-reductase” class it is worth to mention the finding of 3 WB and 2 soluble suberization associated peroxidase and phospholipid hydroperoxide glutathione peroxidase. Peroxidases affect the mechanical properties of the cell wall; they can cause reduction of cell wall extensibility by formation of diferuloyl bridges between pectin residues or isodityrosine bridges between hydroxyproline-rich proteins like extensin (Andrews et al. 2002; Fry 1986).

As a matter of fact, one extensin-like protein was found in Levovil WB extract. Because of their covalent binding to cell wall matrix such structural proteins are rarely identified by proteomic studies. They are supposed to participate in cell wall assembly (Cannon et al. 2008). Our results also pointed out the presence of two profilins. Such proteins were previously detected by Zhu et al. (2006) as non classical CWPs, in maize root cell wall, they are small proteins involved in actin dynamic and supposed to play a role in cell wall synthesis. Again, they were reported as tomato fruit allergenic compounds.

Finally, we also identified 8 proteins of “unknown function” in tomato cell wall extracts.

Genotype-related variation of CWPs among genotypes

One of our aims was to evaluate the possibility for genetic variability assessment at the pericarp cell wall level by

comparing 3 tomato genotypes already known for their contrasted fruit texture characteristics (Chaib et al. 2007). Up to now, and probably due to the experimental difficulty to assess cell wall proteome, only two studies have compared CWP from different species or from different genotypes of a given species (Dahal et al. 2010; Haslam et al. 2003). However, genotype comparison is of main interest first to extensively describe a species cell wall proteome, but also to identify which proteins networks may correlate with cell wall properties among distinct phenotypes.

Our study revealed some repeatable qualitative differences between genotypes in terms of protein band patterns on gels and in terms of protein identification by mass spectrometry. As no quantitative analysis was performed, genotype differences were exclusively considered in terms of the presence or absence of a given protein (or one of its different isoforms) between genotypes. In case of WB extract, the SDS-PAGE protein patterns of Levovil and VilB fitted well with their relative genetic proximity as compared to Cervil which is a more distinct genotype belonging to the cerasiforme type (Ranc et al. 2008). Surprisingly, when moving to S-CWP, Levovil pattern was repeatedly observed to be clearly distinct from that of VilB which, in this case looked closer to Cervil's one.

Although not all bands were picked from each genotype, the main and prominent bands were analyzed for each genotype and they were shown to contain either “common” or “genotype specific” proteins. However, one must keep in mind that the absence of a protein may reveal genotype specificity but may also be attributed to the analytical difficulty to detect low abundance or highly glycosylated proteins, especially when present in complex mixtures.

Twenty-two proteins were common to the 3 tomato genotypes and 8 of them were detected in both cell wall fractions (a putative ML domain protein, the pectinesterase 1 precursor, a putative uncharacterized protein, two non-specific lipid-transfer proteins, the suberization-associated anionic peroxidase 1 precursor, a pathogenesis-related protein and an osmotin-like protein). Twelve WB-CWPs were present in the 3 genotypes (snakin-1 precursor, Polygalacturonase-2 precursor, LeMan4, defensin protein, flower-specific gamma-thionin-like protein, non-specific lipid-transfer protein 2 precursor, 2 Proteins of unknown function, germin-like protein, pectinesterase, aspartyl protease) and only 2 S-CWPs were common to the 3 genotypes (the cysteine proteinase 3 precursor, an α -galactosidase, putative).

Functions detected in only one genotype and thus, suspected to be genotype specific were for example a α -galactosidase isoform or tomato invertase inhibitor precursor, in case of Cervil line, while β -glucosidase, arabinosidase, extensin-like and RALF protein were expressed

only in Levovil. Finally, xylosidase 1 and α -glucosidase, were only found in VilB pericarp. These proteins may therefore represent candidate proteins for fruit phenotypic differences between the lines.

Further analyses would be necessary to conclude on their physiological relevance in relation to differences observed between the 3 lines especially regarding fruit sugar content and texture attributes (Chaib et al. 2007; Page et al. 2010; Ranc et al. 2008).

Conclusion

Gaining genetic control of cell wall composition and dynamic modifications is a key goal for the improvement of fresh fruits. The extensive knowledge of cell wall proteins network and the evaluation of their natural genetic diversity are prerequisites to further improvement. A first overview of tomato pericarp cell wall proteome has been established using two different extraction procedures of fractions of proteins: weakly bound and soluble cell wall proteins for 3 different tomato lines. A set of 75 main proteins related to fruit ripening was described with some variations according to the genotype.

Our results added to unceasing progress in proteomics methods, argue in favor of further in muro fruit candidate protein fishing by exhaustive proteomic approach. Comparison and quantification of cell wall protein networks from relate genotypes such as mutants or isogenic lines should provide important clues for understanding how a fruit develops and ripens. However, the ability of a protein to moonlight, or to have more than one function, can complicate the interpretation and application of the results from these proteomics studies.

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Supplementary- Figure1.

