DEVELOPMENT OF A METHOD FOR THE EXTRACTION AND ANALYSIS OF GRAPE SKIN PROTEINS STRONGLY BOUND TO CELL WALLS

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Abstract

Aim : To better understand the protein composition of grape skin cell walls, we have developed a method to analyse the strongly bound cell wall proteins.

Methods and results : The protocol was developed with grape skins at full maturity. The critical steps of this protocol were : (i) the elimination of cellular aggregates, (ii) the elimination of soluble proteins, and (iii) the localization of the identified proteins within the cell wall. To verify whether these three conditions were met, the decrease in the quantity of cellular aggregates was followed by optical microscopy, the removal of soluble proteins was measured by chemical assay, and the presence of proteins located in cell walls was demonstrated by extensive bioinformatic analysis. The process made it possible to obtain a four-fold reduction in the amount of cellular aggregates, a reduction in the concentration of soluble proteins below the method detection limit, and a high proportion of proteins predicted to be secreted (79 %).

Conclusion : The protocol described in this paper constitutes the first method to analyse proteins strongly bound to cell walls in grape skins. However, this method excludes the identification of labile proteins or proteins weakly bound to the cell wall.

Significance and impact of the study : This protocol can be used for studying the role that strongly bound cell wall proteins play in development and defense processes in grape skins.

Key words : cell walls, proteins, extraction, grape skin, localization prediction, microscopy

Résumé

Objectif : Pour mieux comprendre la complexité des parois cellulaires, nous avons mis au point une méthode d'analyse des protéines fortement liées aux parois cellulaires de la pellicule du raisin.

Méthodes et résultats : Le protocole a été développé avec des pellicules de raisin au stade maturité. Les étapes critiques de ce protocole étaient : (i) l'élimination des agrégats cellulaires, (ii) l'élimination des protéines solubles et (iii) la localisation des protéines identifiées dans la paroi cellulaire. Pour vérifier si ces conditions ont été remplies, la diminution de la quantité d'agrégats cellulaires a été suivie par microscopie optique, l'élimination des protéines libres a été vérifiée par dosage chimique et la présence de protéines localisées dans les parois cellulaires a été démontrée par analyse bioinformatique. Le processus a permis de diviser par quatre la quantité d'agrégats cellulaires, de réduire la concentration en protéines solubles en dessous du seuil de détection de la méthode et d'obtenir une proportion élevée de protéines avec une prédiction de sécrétion (79 %).

Conclusion : Le protocole décrit dans ce papier constitue la première méthode d'analyse des protéines fortement liées aux parois cellulaires des pellicules du raisin. Cependant, les caractéristiques du tissu étudié ne permettent pas l'identification de protéines labiles ou faiblement liées aux parois cellulaires.

Signification et impact de l'étude : Le protocole peut être utilisé pour étudier le rôle que les protéines fortement liées aux parois cellulaires jouent dans les processus de développement et de défense des pellicules du raisin.

Mots clés : parois cellulaire, protéines, extraction, pellicule de raisin, prédiction de localisation, microscopie

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INTRODUCTION

Fruit ripening is accompanied by many physiological changes that lead to tissue softening. This softening is related to the degradation of cell walls and results in an increased susceptibility to pathogens. The plant cell wall, while primarily composed of polysaccharides and proteins, is a highly dynamic network whose composition and structure is affected by environmental conditions (Cassab, 1998). Cell wall proteins (CWP) can be distinguished on the basis of their interactions with networks formed by polysaccharides and other components present in cell walls (Jamet *et al*. 2006). For instance, labile proteins have few or no interactions with cell wall polysaccharides and thus move freely in the intercellular space. There are also proteins that are weakly bound to the matrix by van der Waals interactions, hydrogen bonds, and hydrophobic or ionic interactions. Finally, a third group of cell wall proteins are strongly bound to cell wall components. Labile proteins can be lost during cell wall preparation or can be extracted from tissues by non-destructive protocols. In the same way, weakly bound CWP can be extracted from living cells using non-destructive techniques such as salts or chelating agents (Borderies *et al*., 2003 ; Boudart *et al*., 2005).

Cell wall proteomes in plant species such as *Arabidopsis thaliana* (L.) Heynh., *Medicago sativa* L., *Zea mays* L., and *Oryza sativa* L. have been extensively described, in spite of specific difficulties encountered when dealing with cell walls (Chivasa *et al*., 2002 ; Watson *et al*., 2004). These difficulties include the loss of cell wall proteins during the isolation procedure and the existence of polysaccharide networks that may retain contaminants such as intracellular proteins (Jamet *et al*., 2008a). Grape skin is a complex matrix that contains high amounts of sugar, amino acids, aroma compounds and phenolic compounds that can interact with proteins and interfere in their identification if they are not eliminated. It is generally for this reason that previous studies focusing on grape skin cell walls have used harsh techniques in order to obtain a purified cell wall (Nunan *et al*., 1997). In an attempt to better understand the complexity of the cell wall of grapes, we have developed a new method to identify CWP strongly bound to cell walls. The efficacy of this method was evaluated by optical microscopy, soluble protein quantification (difference between total proteins and proteins strongly bound to cell walls), and identification of these strongly bound proteins through bioinformatic analysis.

MATERIAL AND METHODS

1. Plant material

Grapes (*Vitis vinifera* L. cv. Cabernet-Sauvignon) were sampled from a vineyard consisting of 34 rows of 60 vines located in the *Appellation d'Origine Contrôlée* Pessac-Léognan (Bordeaux, France) during the 2009 growing season. Grape clusters were collected at full maturity, corresponding to 116 days after anthesis. Random samples (30 berries per vine) were selected from 30 clusters, immediately placed in liquid nitrogen and stored at -80°C. The berries were separated from the stems and the skins were removed with a scalpel and ground in a mortar with a pestle under liquid nitrogen prior to analysis. Part of the powdered skin was lyophilised and retained for microscopic analysis.

2. Isolation of the cell walls from grape skins

Three g of powdered grape skin (in triplicate) and 9 mL of extraction buffer (EB) (0.5 M Tris–HCl pH 7.5, 10 mM EDTA, 1 mM PMSF, 2 % (v/v) β-mercaptoethanol, 0.1 M KCl, 0.7 M sucrose, 1 % (w/v) polyvinylpolypyrrolidone (PVPP) were stirred in a Teflon tube (25 mL) at 4 °C for 1 h. An equal volume of phenol-Tris–HCl 10.6 M pH 7.5 was added and the mixture was shaken at 4 °C for 1 h and then centrifuged at 9,000 *g* for 30 min at 4 °C. The phenol phase was eliminated and 3 mL of EB + 3 mL of phenol-Tris–HCl were added to the pellet, stirred for 15 min, and centrifuged. The phenol phase was eliminated and 20 mL of EB + 0.5 % (v/v) Triton X-100 was added to the pellet and the mixture was sonicated (3×1 min), shaken for 15 min at 4 °C, and then centrifuged at 18,000 *g* for 30 min at 4 °C. The upper phase was recovered, constituting ***wash 1***. Twenty mL of Tris–HCl 0.5 M pH 7.5 was added to the pellet and the mixture was sonicated (3×1 min) and centrifuged (30 min, 1,000 *g*, 4 °C). This procedure was repeated five times with the upper phases, constituting ***washes 2 to 6***. Finally, the pellet was washed two times with 20 mL of ultrapure water, sonicated (3×1 min), and centrifuged (30 min, 6,000 *g*, 4 °C for the first wash and 30 min, 18,000 *g*, 4 °C for the second wash). The upper phases were recovered, constituting ***washes 7 and 8***. The purified pellet was lyophilised overnight and consisted primarily of cell walls.

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a) Analysis of cellular aggregate by optical microscopy

Five mg of lyophilised grape skin or aggregatecell wall were suspended in 0.5 mL of calcofluor 0.1 % (w/v) (Meadows 1984) and homogenized for 10 min at room temperature in the dark, before centrifugation (5 min, 1,000 *g*, 4 °C). The supernatant was removed and the pellet washed with 0.5 mL of phosphate-buffered saline (PBS) (NaCl 8 g/L, KCl 0.2 g/L, Na_2HPO_4 1.44 g/L, KH_2PO_4 0.24 g/L) prior to centrifugation (5 min, 1000 *g*, 4 °C). Finally, the pellet was suspended in 40 μL of ultrapure water and mounted between a slide and cover slip for observation. The slides were observed under a light microscope (Olympus BX 51) equipped with a U-RFL-T Olympus mercury vapour lamp. The fluorescence signal of the calcofluor staining was detected with a DAPI filter set (excitation at 365 nm, emission at 400 nm). Images were acquired using Olympus digital camera system Camedia Master and DP-Soft 3.2 software. GNU Image Manipulation Program (GIMP) software was used in order to determine the amount of residual cellular aggregates present in the cell wall. Image resolution was 200 PPP or 680×512 pixels for a total of 348,160 pixels/image. Images obtained with visible light were overlapped with those obtained using fluorescence before pixel subtraction. This allowed the determination of the number of pixels representing cellular aggregates. The average number of pixels (and the standard deviation) corresponding to cellular aggregates was calculated using six images of initial grape skin compared to six images of lyophilised cell walls.

b. Verification of the elimination of soluble proteins by chemical assay

Protein concentrations were determined in six replicates with ovalbumin as standard (Ramagli *et al*. 1985). One mL of *washes 1* to *8* (diluted with ultrapure water, 1/40) and 250 μl of Bradford reagent (BioRad) were mixed in an Eppendorf tube, vortexed, allowed to react for 5 min, and read for absorbance at 595 nm using a UV/Vis spectrophotometer (Thermo Scientific Evolution 100).

3. Cell wall protein extraction

100 mg of lyophilised cell walls was mixed with 1.5 mL of EB (1 M Tris–HCl pH 6.8, 2 % (w/v) SDS, 0.1 M DTT) in an Eppendorf tube and heated to 100°C for 10 min (Feiz *et al*. 2006). After cooling, the mixture was sonicated for 2 min and centrifuged (20 min, 20,000 *g*, 20 °C). The pellet was discarded; PVPP 1 % (w/v) was added to the upper SDS phase and then centrifuged (20 min, 20,000 *g*, 20 °C). Protein precipitation from the SDS phase was accomplished by adding 1/5 volume of acetone/TCA 20 % (v/v) for 1 h at -20 °C. After complete precipitation, the mixture was centrifuged (20 min, 20,000 *g*, 4 °C) and the pellet was washed three times with 80 % acetone (v/v) and twice with pure acetone. The resulting pellet was dried under nitrogen.

a. Cell wall proteins rehydration, tryptic digestion and MS analysis

One mg of proteinsCell wall proteins rehydration (in duplicate) was resuspended in 100 μl of solubilisation buffer (7 M urea, 2 M thiourea, 25 mM DTT, 4 % CHAPS), submitted to ultrasounds for 5 min, and centrifuged (10 min, 10,000 *g*, 4 °C). Then 10 μl of supernatant was added to 90 μl of digestion buffer (40 mM Ambic, 10 % ACN, 0.1 mM HCl) previously diluted 1/10, 10 μl of 0.5 M triethylammonium bicarbonate (TEAB) pH 8.5 and 10 μl of trypsin in 0.1 μg/μl HCl 1 mM before overnight incubation at 37°C. The peptide mixture was analysed by on-line capillary HPLC (LC Packings, Amsterdam, The Netherlands) coupled to a nanospray LTQ-Orbitrap XL mass spectrometer. Ten μl of peptide digests were loaded onto a 300 μm × 5 mm C18 PepMap™ trap column (LC Packings, Amsterdam, The Netherlands) at a flow rate of 30 μL/min. The peptides were eluted from the trap column onto an analytical 75 μm × 15 cm C18 PepMap™ column (LC Packings) with a 5-40 % linear gradient of solvent B in 35 min (solvent A was 0.1 % formic acid in 5 % ACN and solvent B was 0.1 % formic acid in 80 % ACN). The flow rate was set at 200 nL/min. The mass spectrometer was operated in positive ion mode with a 2.2 kV needle voltage and a 28 V capillary voltage. Data were acquired in a data-dependent mode alternating a MS scan survey over the range *m*/*z* 300-1700 and 10 MS/MS scans in an exclusion dynamic mode. MS/MS spectra were acquired using a 2-*m*/*z*-unit ion isolation window, a 35 % relative collision energy, and a 0.5 min dynamic exclusion duration.

b. Verification of the presence of proteins classified as « secretory pathway signaling peptides » by bioinformatic analysis

Data were searched by SEQUEST through the Bioworks 3.3.1 interface (ThermoFinnigan, San Jose, CA) against a subset of the UniProt database (version 15.15, downloaded in March 2010) restricted to *Vitis vinifera* species (50943 entries).

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DTA files were generated for MS/MS spectra that reached a minimal intensity (100) and had a sufficient number of ions (5). Spectra from precursor ions with molecular weights higher than 4500 Da or lower than 500 Da were rejected. Search parameters were as follows: mass accuracy of the peptide precursor and peptide fragments was set to 10 ppm and 0.5 Da, respectively. Only b- and y-ions were considered for mass calculation. Oxidation of methionine (+16) and carbamidomethylation of cysteine (+57) was considered as differential modifications. Two missed trypsin cleavages were allowed. Only peptides whose cross-correlation score (Xcorr) was over 1.5 (single charge), 2.0 (double charge), 2.5 (triple charge) and 3.0 (four charges and more) were retained. In all cases, ΔCn was above 0.1 and peptide *p*-value was lower than 1.10×10^{-3}. All protein identifications were based on a minimum of two peptide assignments. The presence or absence of the signal peptide and subcellular location was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/), TargetP (http://www.cbs.dtu.dk/services/TargetP/), PSORT (http://psort.hgc.jp/), Predotar (http://urgi.versailles.inra.fr/predotar/predotar.html) and ARAMEMNON (http://aramemnon.uni-koeln.de/).

RESULTS AND DISCUSSION

The development of a method for analysing cell wall proteins requires taking into account several factors that can influence the quality of the final analysis (Figure 1). Two major factors include the large quantity of cellular aggregates not containing walls and the free proteins that must be removed from the skin prior to analysis. To verify whether these two conditions were met, the removal of « impurities » was followed by optical microscopy and the presence of soluble proteins by Bradford assay. Finally, the presence of proteins that do not possess a signaling peptide involved in the secretory pathways (non-canonical) was determined through identification by extensive bioinformatic analysis of the mass spectrometry data (Charmont *et al.* 2005, Jamet 2004). It was then possible to evaluate the quality of the cell wall preparations by calculating the proportion of predicted secreted proteins to intracellular ones.

Non-destructive methods described for cell suspension cultures or model plants, such as *Arabidopsis thaliana* (L.) Heynh, were not applicable in our case. The main reason is due to the fact that the grape skin is a rigid and complex system with high quantity of sugars, amino acids, aroma compounds, and a particularly high concentration of phenolic compounds, requiring a cell wall purification step prior to protein extraction (Barnavon *et al.,* 2001; Nunan *et al.*, 1997). As a result, the modification of the ionic strength induced by the use of salts, as reported in the extraction procedure of labile and weakly bound proteins, is not possible (Jamet *et al.,* 2008b).

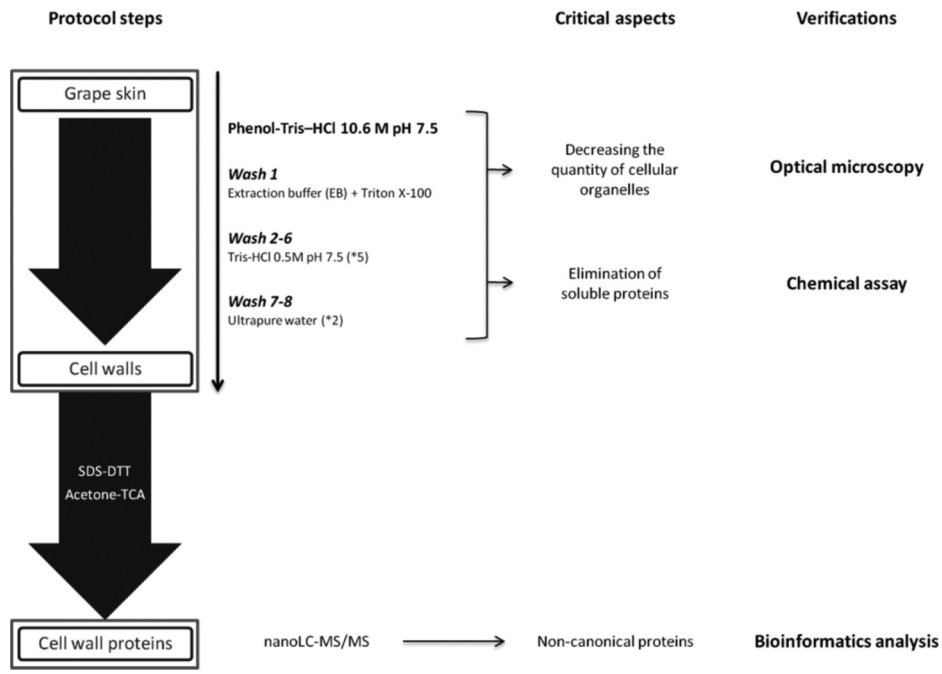


Figure 1 - General scheme of the protocol steps with critical points and verification of the grape skin cell wall protein analysis.

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1- Verification of the decrease in cellular aggregates not containing walls

The removal of cell wall-free cellular aggregates was monitored in the grape skin and extracted cell walls by optical microscopy. The images (Figure 2) show the difference between unpurified grape skin powder (A) and purified cell wall powder (B). Examination of the particle size and density of the two samples under visible light indicates the procedure provided good cell disruption. Ultrasound was used to ensure complete cell disintegration (Lepeschkin and Goldman, 1952). In addition, the use of calcofluor as a fluorescent marker for glycans allowed the specific staining of cellulose walls. Therefore, it was possible to estimate the amount of cellular aggregates in the samples by overlapping images obtained by fluorescence with images obtained with visible light (A_3 and B_3). The amount of cellular aggregates in the grape skin powder (A) was 43.3 % (± 2.9%), whereas in the cell wall powder (B) it was only 11.4 % (± 3.3%). The purification process developed in this study made it possible to decrease by four-fold the number of contaminants that could potentially distort the subsequent analysis. These cell wall-free aggregates, such as peroxisomes, mitochondria, chloroplasts or nucleus, contain proteins that may contaminate the cell wall fractions and thus change the final protein profile. Elimination of cellular aggregates was assured by a combination of successive washing steps with Tris–HCl. Differential low speed centrifugation was used to remove the less dense aggregates that remained suspended, while the cell wall fractions accumulated at the bottom of the centrifugation tube (Laurent *et al.* 1999).

2 - Verification of the elimination of soluble proteins

The number of consecutive washing steps was optimized by quantifying soluble proteins in the different supernatants (***wash 1 to 8***) (Figure 3). The first wash was performed with EB and Triton X-100, the Tris–HCl buffer (pH 7.5) was used for the following five washes, and purified water was used for the last two washing steps. The average concentration of soluble proteins at the beginning of the purification protocol was 4.36 (± 1.43) mg/g of fresh skin, whereas it was below the method detection limit at the end of the protocol. The first part of the developed protocol is based on a methodology of Saravanan and Rose (2004), which allows the removal of the majority of the soluble proteins and the proteins loosely and weakly bound to cell wall in grape skins. Phenol was used as denaturing agent, allowing the elimination of the interactions between these proteins and other cellular elements (Carpentier *et al.*, 2005). Following this, different washing steps were carried out on the cell wall-enriched pellets. The washing step using Triton X-100 allowed the solubilisation of plasma membrane (Schnaitman, 1971).

3 - Verification of the presence of proteins that possess signaling peptides believed to be involved in secretory pathways

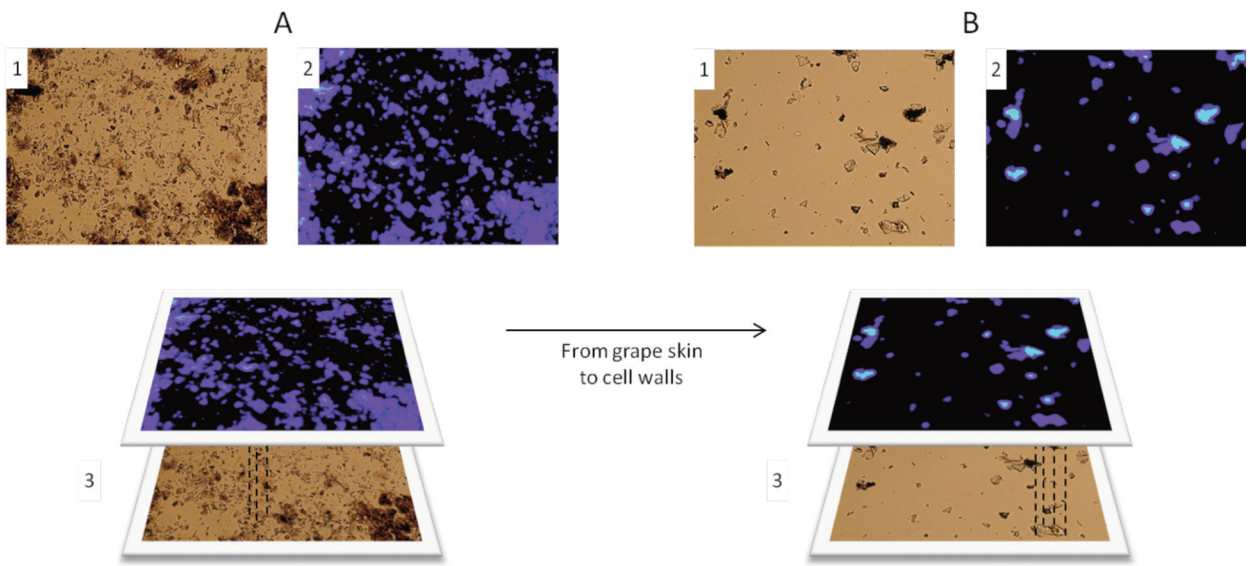


Figure 2 - Assessment of cellular aggregates "from grape skin to cell walls" by optical microscopy. A- Grape skin powder; B- Grape skin cell wall; A1 and B1- Visible light; A2 and B2- Epifluorescence; A3 and B3- Overlapping of visible light and epifluorescence.

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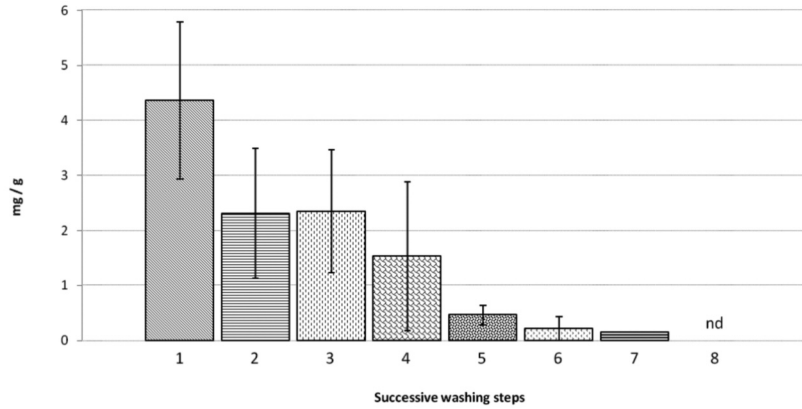


Figure 3 - Evolution of free proteins "from grape skin to cell walls" expressed in mg proteins/g of fresh skin. nd, not detected. Data are means ± standard deviation of six replicates.

Table 1 - Identified proteins from grape skin cell wall extract and corresponding predicted localization.

UniProt Id [a]	Protein matched [b]	Th M_r (kDa) / pI [c]	Total peptide [d]	Coverage % [e]	Localization prediction [f]	Secretory pathways location score [g]
A5BB66	uncharacterized protein	42.9 / 5	12	30.05	Se	33.8
F6HAU0	uncharacterized protein	60 / 4.4	9	20.29	Se	10.3
F6HZ64	Pectinesterase	61.6 / 8.9	9	19.49	Se	30.7
A5B7E6	uncharacterized protein	50.1 / 5.8	2	5.19	Se	26.5
D7T860	uncharacterized protein	82.3 / 5.4	12	20.48	Se	11.5
A5B702	uncharacterized protein	21.8 / 9.4	3	7.25	Se	28.7
A5BTU9	uncharacterized protein	20.1 / 7.8	2	8.95	Se	32.4
Q9LNU5	T20H2.2 protein	58.6 / 9.7	3	7.78	Se	33.9
A7PW81	Polygalacturonase-inhibiting protein	36.9 / 8.8	3	9.31	Se	38.8
A5BQP3	uncharacterized protein	27.2 / 4.9	2	11.07	Se	33.0
F6HRK8	uncharacterized protein	13 / 6.6	2	21.92	Se	32.1
F6HQE0	uncharacterized protein	54.3 / 6.6	2	5.04	Se	23.9
Q9M4H8	Putative ripening-related protein	21.1 / 9.2	2	11.62	Se	31.8
O04708	uncharacterized protein	24 / 5.1	2	9.00	Se	38.4
O24530	Class IV endochitinase	27.2 / 5.4	2	27.22	Se	40.7
P93622	Polyphenol oxidase	67.4 / 6.4	4	7.91	Ch	nd
A5AML5	Thioredoxin	12.8 / 5.2	2	20.18	-	0
D7T5Q6	uncharacterized protein	32.6 / 4.2	2	11.76	-	4.5
A5B8K3	uncharacterized protein	11.8 / 5.2	2	34.21	-	0

[a] Accession no. of the matched protein as reported in the UniProt database. [b] Protein matched after searching against an in-house database from UniProt database. [c] Theoretical molecular weight expressed in kDa and theoretical isoelectric point. [d] Number of unique peptides matching the protein sequence. [e] Percentage of the protein sequence covered by matching peptides. [f] Localization prediction (Se: secretion; Ch: chloroplast). [g] Individual predictions for each protein combined to a built-in consensus prediction using the Bayes' theorem. A consensus score is based on the ratio of the posterior probabilities of both hypotheses, i.e., that a protein is targeted and that it is not targeted given positive prediction results. The corresponding likelihoods of the hypotheses were estimated using a training set of plant proteins with known subcellular location.

To ensure the effectiveness of the procedure, all the proteins extracted from cell walls were identified to determine whether they predicted to secretory pathway and, if not, whether they contained peptide signals for other cell compartment. The proteins revealed in this work are listed in Table 1. Nineteen proteins were found in mature grape skin cell walls. This classification takes "non-canonical" CWP into account. To our knowledge, none of the studies concerning plants such as *Medicago sativa* L. (Watson *et al.,* 2004), *Zea mays* L. (Zhu *et al.,* 2006), *Cicer arietinum* L. (Bhushan *et al.,* 2006), *Oryza sativa* L. (Chen *et al.,* 2009) and *Arabidopsis thaliana* (L.) Heynh (Chivasa *et al.,* 2002; Jamet *et al.,* 2008) were able to obtain a fraction containing 100 % cell wall proteins. These non-canonical proteins do not contain a predicted signal peptide, an element needed for targeting to

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the secretory pathway, which renders difficult the interpretation of their presence in cell walls. Therefore, the existence of an unknown export mechanism should not be excluded, although it may be possible that contamination with mitochondria, chloroplast or nucleus proteins exists. Among the 19 proteins found, 15 were predicted to be involved in the secretory pathway, 1 was predicted to be chloroplast targeted, and 3 had no prediction. This shows that the majority of the proteins found are exported to an extracellular compartment. The proportion of proteins predicted to be secretory in the mature berry cell walls was 79 %. This corresponds well with other studies such as that by Feiz *et al.* (2006), which presented a score of 73 % of proteins involved in the secretory pathway in *Arabidopsis thaliana* (L.) Heynh.

CONCLUSION

The method developed involved cell wall purification and CWP extraction. The protocol eliminated, in part, cellular aggregates and soluble proteins and allowed the sequencing and identification of 19 CWP at berry maturity. The presence of a signal peptide and their predicted localization within the cell wall showed that the majority of the proteins found are correlated to a secretory pathway.

Each step of the purification process, taken individually, has been previously described in the literature. Nevertheless, the procedure described in the present work has confirmed the effectiveness of this multi-step purification process, which allows the extraction of cell wall proteins from complex material like grape skin. To the best of our knowledge, this procedure, as a whole, has not been previously reported. The overall procedure, thus validated, can be applied to better understand the mechanisms behind grape maturation and defense.

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Authors' contributions: GP carried out cell wall preparations, protein extractions, and bioinformatic analyses; DL carried out protein separations; JWD, AML, and SC contributed to LC/MS-MS analyses; LG, MB, and BD conceived data evaluation, contributed to the writing of the manuscript, and participated in discussions all along the study; and PLT performed literature screening, designed research, and coordinated writing of the manuscript. All authors read and approved the final manuscript.

REFERENCES

Barnavon L., Doco T., Terrier N., Ageorges A., Romieu C. and Pellerin P., 2001. Involvement of pectin methyl-esterase during the ripening of grape berries: partial cDNA isolation, transcript expression and changes in the degree of methyl-esterification of cell wall pectins. *Phytochemistry*, **58**, 693-701.

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

Borderies G., Jamet E., Lafitte C., Rossignol M., Jauneau A., Boudart G., Monsarrat B., Esquerre-Tugaye M. T., Boudet A. and Pont-Lezica R., 2003. Proteomics of loosely bound cell wall proteins of *Arabidopsis thaliana* cell suspension cultures: A critical analysis. *Electrophoresis*, **24**, 3421-3432.

Boudart G., Jamet E., Rossignol M., Lafitte C., Borderies G., Jauneau A., Esquerre-Tugaye M. T. and Pont-Lezica R., 2005. Cell wall proteins in apoplastic fluids of *Arabidopsis thaliana* rosettes: Identification by mass spectrometry and bioinformatics. *Proteomics*, **5**, 212-221.

Carpentier S. C., Witters E., Laukens K., Deckers P., Swennen R. and Panis B., 2005. Preparation of protein extracts from recalcitrant plant tissues: An evaluation of different methods for two-dimensional gel electrophoresis analysis. *Proteomics*, **5**, 2497-2507.

Cassab G. I., 1998. Plant cell wall proteins. Annual Rev. *Plant Physiol. Plant Mol. Biol.*, **49**, 281-309.

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

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

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

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Feiz L., Irshad M., Pont-Lezica R. F., Canut H. and Jamet E., 2006. Evaluation of cell wall preparations for proteomics: A new procedure for purifying cell walls from *Arabidopsis* hypocotyls. *Plant Methods*, **2**, 10.

Jamet E., 2004. Bioinformatics as a critical prerequisite to transcriptome and proteome studies. *J. Exp. Botany*, **55**, 1977-1979.

Jamet E., Canut H., Boudart G. and Pont-Lezica R. F., 2006. Cell wall proteins: A new insight through proteomics. *Trends in Plant Science*, **11**, 33-39.

Jamet E., Albenne C., Boudart G., Irshad M., Canut H. and Pont-Lezica R., 2008a. Recent advances in plant cell wall proteomics. *Proteomics*, **8**, 893-908.

Jamet E., Boudart G., Borderies G., Charmont S., Lafitte C., Rossignol M., Canut H. and Pont-Lezica R., 2008b. Isolation of plant cell wall proteins. *Methods in Molecular Biology*, **425**, 187-201.

Laurent P., Voiblet C., Tagu D., de Carvalho D., Nehls U., De Bellis R., Balestrini R., Bauw G., Bonfante P. and Martin F., 1999. A novel class of ectomycorrhiza-regulated cell wall polypeptides in *Pisolithus tinctorius*. *Molecular Plant-Microbe Interaction*, **12**, 862-871.

Lepeschkin W. W. and Goldman D. E., 1952. Effects of ultrasound on cell structure. *J. Cellular Comparative Physiology*, **40**, 383-397.

Meadows M. G., 1984. A batch assay using Calcofluor fluorescence to characterize cell wall regeneration in plant protoplasts. *Analytical Biochem*., **141**, 38-42.

Nunan K. J., Sims I. M., Bacic A., Robinson S. P. and Fincher G. B., 1997. Isolation and characterization of cell walls from the mesocarp of mature grape berries (*Vitis vinifera*). *Planta*, **203**, 93-100.

Ramagli L. S. and Rodriguez L. V., 1985. Quantitation of microgram amounts of protein in two-dimensional polyacrylamide gel electrophoresis sample buffer. *Electrophoresis*, **6**, 559-563.

Saravanan R. S. and Rose J. K. C., 2004. A critical evaluation of sample extraction techniques for enhanced proteomic analysis of recalcitrant plant tissues. Proteomics, **4**, 2522-2532.

Schnaitman C. A., 1971. Effect of ethylenediaminetetraacetic acid, Triton X-100, and lysozyme on the morphology and chemical composition of isolate cell walls of *Escherichia coli*. *J. Bacteriology*, **108**, 553-563.

Watson B. S., Lei Z., Dixon R. A. and Sumner L. W., 2004. Proteomics of *Medicago sativa* cell walls. *Phytochemistry*, **65**, 1709-1720.

Zhu J., Chen S., Alvarez S., Asirvatham V. S., Schachtman D. P., Wu Y. and Sharp R. E., 2006. Cell wall proteome in the maize primary root elongation zone. I. Extraction and identification of water-soluble and lightly ionically bound proteins. *Plant Physiology*, **140**, 311-325.

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