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RAPID COMMUNICATION

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Preparative laser capture microdissection and single-pot cell wall material preparation: a novel method for tissue-specific analysis

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Abstract In adaptation to their function the walls of plant cell display tissue-specific variations of composition according to their developmental stage, cell type and stress of various origin. It is therefore important to obtain a precise analytical data describing the cell wall composition with respect to these different factors. In the present work, laser capture microdissection (LCM) was used for isolating different tissues from the stem of *Urtica dioica* L. at a semi-preparative scale. The technique was associated for the first time to a one-pot sequential cell wall preparation and hydrolysis for the carbohydrate analysis of each cell type. The results demonstrate that the combination of LCM and micro-analytical methods can provide individual cell type composition and should improve our knowledge of the biochemical diversity of cell walls in plants. This approach will be of potential interest for the understanding of the effects of stress or genetic engineering on the composition of the cell walls.

Keywords Cell wall microanalysis · Laser capture microdissection · Polysaccharide hydrolysis · *Urtica*

Abbreviations CWM: cell wall material · GC-MS: gas chromatography–mass spectroscopy · LCM: laser capture microdissection

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Introduction

One difficulty in describing plant cell wall is that plants are made of a multiplicity and diversity of tissues that have specific structural and physiological functions. According to the plant origin and tissue (Mellerowicz et al. 2001), their developmental stage and in response to the environmental factors, the cells develop walls with different compositional and mechanical characteristics. Consequently, besides cellulose, which is present in every plant cell wall, cells synthesize polysaccharide constituents that vary in types, primary structures and relative abundance. It is clear that functional characteristics of cell walls depend on the proportion and macromolecular arrangement of their structural polymers. Depending on their function in the plant, some cells deposit in their walls lignin and suberin, which bring resistance and hydrophobicity to the corresponding tissues. Other cells such as epidermal cells have walls impregnated with waxes (Varner and Lin 1989). Deciphering the processes that accompany cell wall biogenesis and maturation requires precise quantitative compositional analysis. Cell-specific wall compositional analysis is also important for evaluating the impact of genes related to cell wall formation in genetically engineered plants (Mellerowicz et al. 2001; Somerville et al. 2004). However, compositional analysis of cell wall material is generally obscured by the fact that analyses are performed on ground tissues in which cells of different types are mixed, with the consequence that the data so obtained reflect only the averaged values of each compound, regardless of the cell type. For this reason the polymer composition of plants is often expressed according to the species or plant organ, such as wheat straw and various wood species (Timell 1982), but rarely describes the analysis of individual cell types. Hardell and Westermark (1981) and Whiting and Goring (1982) analysed the quantitative distribution of cellulose and hemicelluloses through the carbohydrate composition of the secondary walls of the whole wood of hard-

woods and softwoods. Earlier, Meier (1961) followed the carbohydrate distribution across the middle lamellae and the primary and secondary walls of woods after separation with a micromanipulator. The results were later confirmed by Takabe et al. (1983) using the same methods and also demonstrated variation within the different layers of the secondary wall of tracheids and showed that cell walls differ markedly according to their cell type (Meier 1958). However, the approach by dissection using a micromanipulator followed by chemical analysis is long and tedious and only a few attempts to improve and diversify the method have been successful (Westermark 1985; Parker et al. 2005). More recently the introduction of Fourier-transform infrared and Fourier-transform Raman microscopy has allowed relatively precise in situ analysis of different categories of polysaccharides and phenolic constituents of primary cell walls (McCann et al. 1992; Séné et al. 1994). Despite this progress, a more precise quantitative description of the ultrastructural organisation of each polymer in the wood cell wall and a better understanding of their respective function in the wall assembly are still missing. Specific labelling in transmission electron microscopy provides evidence about the non-uniform distribution of wall polymers, including glucuronoxylans (Awano et al. 1998, 2002), and lignins (Joseleau and Ruel 1997).

To obtain cell specific analytical data, we developed the laser capture microdissection (LCM) technique on a preparative micro-scale and coupled it to a single-pot cell wall material preparation for cell wall carbohydrate analysis. Up to now, the new microdissection technique by LCM has been principally applied to the animal tissues. Its utilisation in plant tissues is more recent and has been almost exclusively devoted to cell-specific gene expression profiling, in association with RT-PCR technique (Asano et al. 2002; Kerk et al. 2003; Nakazano et al. 2003; Casson et al. 2005). To our knowledge this is the first time that LCM technique is used to establish the quantitative composition of plant tissues. We describe the use of this combination of techniques for the carbohydrate analysis of the polysaccharides from the walls of lignified and unlignified parenchyma cells, and of xylem fibres of a perennial dicotyledon.

Materials and methods

Plant material and tissue

Stems from *Urtica dioica* L. were collected in October in Grenoble. Segments 5–10 cm in length were cut and fixed in the mixture of 0.2% glutaraldehyde and 2% formaldehyde in 0.05 M phosphate buffer (pH 7.0–7.2). In the laboratory, stem segments were split longitudinally into four pieces and each piece was trimmed to a final length of 3–5 mm and 1.5 mm in thickness. Finally, the tissues were vacuum infiltrated with a fixative and stored at 4°C overnight. For paraffin embedding, the tissues were washed in distilled water (three changes, 5 min each) and gradually dehydrated in increasing concentrations of ethanol (30, 50, 70, 80, 96, 100%, 15 min at each step). After two more changes in 100% ethanol, the tissues were transferred to paraffin through mixtures of toluene/ethanol 1:2, 1:1 and 2:1 (v/v), washing the tissues in each mixture for 30 min. After two more changes in pure toluene for 30 min each, the tissues were transferred to an equal volume of paraffin/toluene, and placed in an oven at 60°C for 24 h. Two more changes of pure, melted paraffin were performed every 4 h, then, the tissues were cast into moulds using a freshly melted paraffin (Rozin 1999).

Serial thin sections of the embedded material (4 µm) were collected on membrane-coated slides (Palm Microlaser technology, Bernried, Germany) and dried 2 h in an oven at 56°C. Just prior to the dissection, the slides were dewaxed in toluene (2 × 5 min) and air dried. For laser microdissection, the P.A.L.M. Laser Microbeam System (Bernried, Germany), was employed. This system consists of a low heat UV (337 nm nitrogen) laser and an inverted microscope (IX70, Olympus). Microdissection, were performed at 20 × (UPLanFL AN 0.5 Olympus) or 40 × (LD AchroPlan AN 0.6 Zeiss) magnification. The tissues were laser catapulted and collected in a 0.5 ml microtube containing a drop of distilled water.

Preparation of cell wall material from tissues

The preparation of cell wall material for carbohydrate analysis were performed on an estimated amount of about 70–100 µg calculated from the number of cells isolated from the 20 µm thick sections. The tissues were transferred from the microscope slides to conical centrifuge tubes with ethanol. Lipids were extracted with the chloroform–methanol mixture (1:1, v/v), then the tissues were soaked in ethanol–toluene mixture (1:1, v/v, 50°C) to extract the pigments. At each extraction step, the solvents were carefully removed with a glass Pasteur pipette whose tip had been tapered by drawing out the flame of a burner. The extraction was repeated at 50°C. This was followed by two successive extractions of cytoplasmic soluble sugars with hot water (95°C, 30 min). The resulting material, considered as crude cell wall material (CWM), was then suspended in ethanol and dried before being submitted to acid hydrolyses.

Total polysaccharide sulphuric hydrolysis

Quantitative monosaccharide analysis of the cell walls including total hydrolysis of cellulose was performed in a conical centrifuged tube with 72% sulphuric acid at 30°C (Saeman et al. 1954; Chambat et al. 1997) for 1 h. Water was then added to give a final acid concentration of 2 M. A known amount of the internal standard *m*-inositol was added at the same time. The hydrolysis was performed at 100°C for 4 h. The mixture of sugars was neutralised with barium carbonate and deionised. The monosaccharides were reduced with sodium borohydride and acetylated in a mixture of acetic anhydride–pyridine (1:1, v/v) to give

the corresponding alditol acetate derivatives (Joseleau et al. 1992). After evaporation to dryness the resulting mixture was dissolved in minimum amount of chloroform and injected in the gas chromatograph (Agilent 6850 Series GC System equipped with a SP-2380 macrobore column (25 × 0.53 mm, Agilent Technologies, Palo Alto, CA, USA). Whenever needed peaks were analysed in GC-MS (gas chromtograph Delsi Di 700) fitted with capillary column SP-2380 (Supelco) with mass spectrometer Nermag R10.10C (France).

Results and discussion

Our current interest is in the understanding of the plant cell wall biogenesis and its macromolecular assembly, as well as deciphering the tissue-specific impact of genes from cell wall polymer biosynthesis, it has become necessary to achieve cell specific wall polymer analysis. To this end, isolation of various cell types, in a pure state and in sufficient amounts was important. The xylem fibres and two kinds of parenchyma cells from *U. dioica* L. from the Urticacea family having different functions in the stem were studied since they do not have the same cell wall organisation, one being lignified and the other being unlignified. It was anticipated that these different cell types should differ in their cell wall carbohydrates, and be good models for the development of the preparative LCM-microanalytical method.

Selection of tissue preparation method

Fixation and embedding is needed to preserve the tissues before LCM sectioning and cell harvest. In the present case these steps required the evaluation of several preparation methods for the embedding procedures best suited for laser dissection. It also required an easy removal of the cross-linking fixative and embedding medium during the subsequent cell wall material preparation that involved sequential extractions of the cell contents, the efficacy of which is highly dependent on the chemical nature of the preparation (Kerk et al. 2003).

Embedding in the LR-White resin (as described in Joseleau and Ruel 1997) turned out not to be appropriate for LCM since it cracked under the laser beam, proving impossible to clearly separate different cell types. Polystyrene embedding was tried next, but showed the same disadvantage as LR-White resin. Polyethylene glycol (PEG, MW 1500) provided good and easy embedding, but it required several attempts before it could be catapulted, probably because PEG tends to retain too much water. The best results, in our hands, were obtained with paraffin embedding.

Preparative laser capture microdissection

In view of the downstream preparation of cell wall material from the different cell types separated and harvested by LCM, and to have enough material in hand for the subsequent quantitative sugar analysis, it was important to collect sufficient amounts of each kind of cell. On the basis of 10 μm thick sections, and taking into account the respective areas of a section occupied by the xylem fibres and lignified and unlignified parenchyma cells, as estimated in light microscopy, the number of sections to be subjected to LCM was calculated that should provide an amount of cell wall material (dry weight) from each cell type estimated to about 70–100 μg. This amount corresponded to the minimum quantity required for significant quantitative cell wall carbohydrate analysis. A major difficulty encountered during LCM sectioning was the particular resistance of the lignified middle lamellae that unit cells together through their primary walls. It was difficult to completely cut the middle lamellae, especially those of the lignified cells, to loosen the clumps of cells. Whereas only one passage of the laser beam around the unlignified parenchyma was enough, several (two to three) passages of the laser beam were sometimes necessary to clearly cut the delineated area of the lignified parenchyma and fibres to be released. In the case of more resistant middle lamellae, the robot LPC was used in the catapulting mode in several spots on the laser tracing to totally free the area of interest for harvesting it (Fig. 1). This mode uses a setting of higher laser energy that is also implemented for collecting the detached cells.

Single-pot preparation of cell wall materials from harvested cells

Cell wall preparation (Chambat et al. 1981) involved the sequential removal of most of the cytoplasmic components, especially cytoplasmic sugars. It consisted of ethanol extraction, then removal of pigments and lipids with a mixture of ethanol and toluene (1:1, v/v), followed by an extraction with a mixture of methanol–chloroform (2:1, v/v). Cytoplasmic sugars and starch that would interfere in cellulose estimation through the release of glucose after total hydrolysis of the cell walls were removed by two consecutive hot water extractions. Finally, the remaining clean cell wall material was treated with ethanol and dried in the oven at 45°C. To perform all the sequential extraction steps on such a minimal amount of harvested cell materials, the crucial difficulty was to achieve an efficient extraction without losing the remaining cell walls. A single conical centrifuge tube was used for that purpose since it allowed concentration of the cell material at the bottom of the tube. A difficulty was to ensure that the material was still present in the tube between each extraction step since it was not visible to the naked eye. This was achieved in examining the preparation under a binocular lens at each step. The extracting solvents were removed by carefully aspirating the supernatants with a tapered glass pipette. It is worth mentioning that the needle of a medical syringe was too wide and caused significant loss of cell wall material.

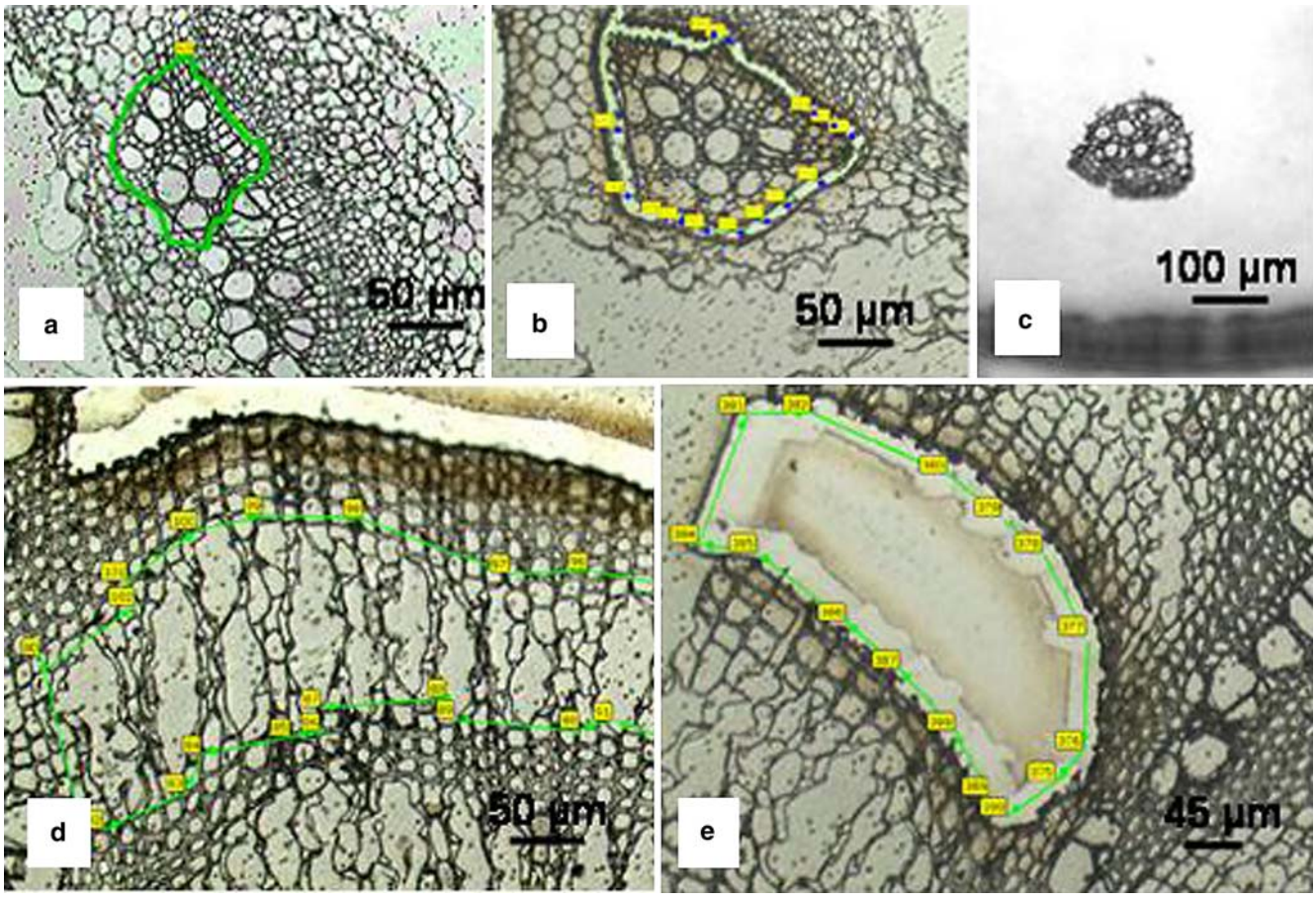

Fig. 1 Laser capture microdissection of different tissues from *Urtica dioica* stem. Isolation of vascular bundles (**a**–**c**) and separation of unlignified from lignified parenchyma (**d**, **e**). Cells are encircled (*green line*, **a**) on the computer screen to delineate the cells of interest. The *cutting line* and the *spots* of laser shots of higher intensity (*yellow rectangles*) are visible around a vascular bundle (**b**). **c** Vascular bundle collected into the cap of an Eppendorf tube after the capture step. Unlignified parenchyma cells before (**d**) and after (**e**) cutting and removal leaving lignified-enriched parenchyma cells in the section (**e**)

Carbohydrate analysis of the cell wall materials

Total hydrolysis of the cell wall carbohydrates was carried out according to the method of Saeman (1954) using 72% sulphuric acid and in the presence of an internal standard. Here again, the hydrolytic step was performed in the centrifuge glass tube used for the cell wall preparation to avoid any transfer of the material. Only at the end of the hydrolysis, and because at this stage all the polysaccharide constituents of the cell walls were converted into soluble monosaccharides, was the solution transferred into a flask and the reduction and acetylation steps converting the free reducing sugars into alditol acetates completed.

In all the chromatogram tracings resulting from the hydrolyses, extraneous peaks were observed in addition to the expected cell wall carbohydrates. In order to evaluate the possible contribution of the embedding medium to the extraneous peaks observed in the chromatogram and interfering with the peak of arabinitol pentacetate, mass spectroscopy of the peaks was carried out by GC-MS. Sections containing only paraffin without plant tissues were submitted to the complete sequence of hydrolysis, reduction and acetylation and the products injected in the gas chromatograph. A multiplicity of peaks were observed, some of them with retention times close to those of sugar derivatives. Indeed the strong peak appearing as a shoulder on the peak of the arabinose derivative was characterised as a degradation product originating from paraffin, and was therefore eliminated from the quantitative integration of arabinose. The results of the analyses are given in Table 1.

In summary, the combination of selective microdissection using LCM on a semi-preparative scale with the adaptation of cell wall preparation and hydrolysis in a

Table 1 Total cell wall carbohydrate analysis from different cell types isolated by LCM technique

Cell type	Ara	Xyl	Man	Gal	Glc
Unlignified parenchyma	13 ± 3	50 ± 5	t	t	37 ± 4
Lignified parenchyma	11 ± 3	46 ± 5	t	t	43 ± 4
Xylem fibres	5.5 ± 1	39 ± 4	1.5 ± 1	2.7 ± 1	51 ± 4

Sugars are expressed in relative molar proportions. Values represent the average of three replicates (± SE)
t trace amount

single pot on micro-quantities was efficient to carry out comparative cell wall carbohydrate analysis. This approach, implemented for the first time on plant tissues is a valuable tool for the investigation of cell-specific analyses in plants. The approach could be generalised for quantitative analysis of all types of cell and cell wall components other than carbohydrates. We are currently using the method of cryosectioning (Inada and Wildermuth 2005) to investigate the modification of the carbohydrate status of plants genetically transformed on the biosynthetic pathways of the cell wall polymers (Ruel et al. 2001).

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