Biochemical and immunohistochemical analysis of pectic polysaccharides in the cell walls of Arabidopsis mutant QUASIMODO 1 suspension-cultured cells: implications for cell adhesion

Edouard Leboeuf¹,², Fabienne Guillon¹, Séverine Thoiron² and Marc Lahaye¹*

¹ INRA-Biopolymères, Interactions, Assemblages, BP 71627, F-44316 Nantes Cedex 3, France
² Université de Nantes, Groupe de Physiologie et Pathologie Végétales, Faculté des Sciences et Techniques, 2 rue de la Houssinière, BP 92208, F-44322, Nantes Cedex 3, France

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Abstract

Mutation in the Arabidopsis thaliana QUASIMODO 1 gene (QUA1), which encodes a putative glycosyltransferase, reduces cell wall pectin content and cell adhesion. Suspension-cultured calli were generated from roots of wild-type (wt) and qua1-1 A. thaliana plants. The altered cell adhesion phenotype of the qua1-1 plant was also found with its suspension-cultured calli. Cell walls of both wt and qua1-1 calli were analysed by chemical, enzymatic and immunohistochemical techniques in order to assess the role of pectic polysaccharides in the mutant phenotype. Compared with the wt, qua1-1 calli cell walls contained more arabinose (23.6 versus 21.6 mol%), rhamnose (3.1 versus 2.7 mol%), and fucose (1.4 versus 1.2 mol%) and less uronic acid (24.2 versus 27.6 mol%), and they were less methyl-esterified (DM: 22.9% versus 30.3%). When sequential pectin extraction of calli cell walls was performed, qua1-1 water-soluble and chelator-soluble extracts contained more arabinose and less uronic acid than wt. Water-soluble pectins were less methyl-esterified in qua1-1 than in wt. Chelator-soluble pectins were more acetyl-esterified in qua1-1. Differences in the cell wall chemistry of wt and mutant calli were supported by a reduction in JIM7 labelling (methyl-esterified homogalacturonan) of the whole wall in small cells and particularly by a reduced labelling with 2F4 (calcium-associated homogalacturonan) in the middle lamella at tricellular junctions of large qua1-1 cells. Differences in the oligosaccharide profile obtained after endopolygalacturonase degradation of alkali extracts from qua1-1 and wt calli indicated variations in the structure of covalently bonded homogalacturonan. About 29% more extracellular polymers rich in pectins were recovered from the calli culture medium of qua1-1 compared with wt. These results show that perturbation of QUASIMODO 1-1 gene expression in calli resulted in alterations of homogalacturonan content and cell wall location. The consequences of these structural variations are discussed with regard to plant cell adhesion.

Key words: Arabidopsis thaliana, cell adhesion, cell culture, cell wall, pectin.

Introduction

Higher plant cells are surrounded by a wall, which provides mechanical support and mediates cell adhesion (Knox, 1992; Jarvis et al., 2003). In growing plant cells, walls are primarily composed of cellulose, hemicelluloses, pectins, and small amounts of structural proteins (Carpita and Gibeaut, 1993). Pectin-rich cell wall interfaces (middle lamella and cellular junctions) are thought to play a key role in cell–cell adhesion. Typically, pectins consist of a complex family of acidic polysaccharides built from several structural domains (Vincken et al., 2003). These structural domains are built on more or less methyl- and acetyl-esterified galacturonan, referred to as homogalacturonan, and alternating rhamnose and galacturonic acid residues, obtained after endopolygalacturonase degradation of alkali extracts from qua1-1 and wt calli indicated variations in the structure of covalently bonded homogalacturonan. About 29% more extracellular polymers rich in pectins were recovered from the calli culture medium of qua1-1 compared with wt. These results show that perturbation of QUASIMODO 1-1 gene expression in calli resulted in alterations of homogalacturonan content and cell wall location. The consequences of these structural variations are discussed with regard to plant cell adhesion.
referred to as rhamnogalacturonan I. Homogalacturonan can be further substituted by apiose, xylose or by complex side chains, and is then referred to as apio-1, xylo-1, or rhamnogalacturonan II, respectively. Rhamnogalacturonan I can be branched on rhamnose residues with galactan, arabinan, or arabinogalactan chains. According to Voragen et al. (1995), these domains would be organized linearly along the pectic polysaccharides by as-yet undefined distribution patterns. A more recent model of pectin structure proposed that a rhamnogalacturonan I would constitute the backbone on which would be branched all other pectic domains (Vincen et al., 2003). Regardless of the pectic domain distribution, unesterified homogalacturonan cross-linked by calcium in the middle lamella is thought to be responsible for cell adhesion (Jarvis et al., 2003). Evidence to support this hypothesis is: (i) unesterified homogalacturonan forms in vitro three-dimensional networks through calcium-ion-mediated junction zones (Jarvis, 1984), (ii) treatment of some plant tissues by pectolytic enzymes and calcium-chelating agents leads to networks through calcium ion-mediated junction zones, and (iii) low methyl-esterified homogalacturonan and calcium were co-localized in the middle lamella and tricellular junctions at critical zones where cell adhesion needs to be reinforced to withstand stress induced by cell turgor pressure (reviewed by Jarvis et al., 2003).

The cell wall characterization of mutants showing defects in cell adhesion support the role of pectins in cell–cell attachment (Iwai et al., 2001, 2002; Orfila et al., 2001, 2002; Atkinson et al., 2002; Oomen et al., 2002). However, it is difficult to determine the exact role of the different pectic structural domains on cell adhesion because most of these mutants are pleiotropic. Two allelic mutants, known as QUASIMODO 1 (qua1-1 and qua1-2), displaying a dwarf and a reduced cell adhesion phenotype, were recently isolated (Bouton et al., 2002). Mutant seedlings, as well as rosette leaves, cell walls showed a 25% reduction in galacturonic acid content compared with the wild type (wt), indicating a reduced pectin content, whereas neutral sugars remained unchanged. Moreover, immersion of roots in solutions of JIM5 and JIM7 monoclonal antibodies that recognize homogalacturonan epitopes revealed reduced labelling in the mutants compared with wt. Both mutants carry a T-DNA insertion in a gene (QUA1) that encodes a putative membrane-bound glycosyltransferase of family 8. The mutant phenotype is consistent with the central role of pectins in cell adhesion and particularly that of homogalacturonan. To characterize the cell wall modifications in the mutant QUASIMODO 1 further, suspension-cultured qua1-1 calli were generated. This material was produced to reduce the heterogeneity of the whole plant cell wall by having only undifferentiated cells. The present study reports on the biochemical characterization of different pectic extracts and the in situ immunocytochemical analyses of homogalacturonan epitopes in wt and mutant calli. The results are discussed with regard to the role of pectic polysaccharide in mediating cell adhesion.

Materials and methods

Plant material and initiation of calli suspension cultures

Wild-type and qua1-1 Arabidopsis plants (Wassilewskija ecotype) were grown under sterile conditions on solid (Difco Bacto Agar 0.8%) 0.5× MS nutrient solution (Murashige and Skoog, 1962) containing 0.5% glucose at pH 5.8. Plants were cultivated for 2 weeks at 21 °C with a day/night regime of 12 h of light (50 μE m−2 s−1).

To induce callus formation, pieces of roots of wt and qua1-1 Arabidopsis plants were plated on callus-inducing medium (CIM), which consisted of solid (Bacto Difco Agar 0.8%) Gamborg B5 nutrient solution (Gamborg et al., 1968) at pH 5.8 containing 2% glucose, 0.05% MES, 0.05 mg ml−1 kinetin, and 0.5 mg ml−1 2,4-dichlorophenoxy acetic acid. After 4 weeks, small calli formed from roots and were transferred on the same fresh medium. One month later, well-developed calli were subcultured once more for 4 weeks. Calli suspension cultures were initiated from this material and maintained as described by Axelos et al. (1992), but the Jouanneau and Pêaud-Lenoël medium was replaced by CIM liquid medium. The calli suspension culture was subcultured every 2 weeks. All replicates of analyses were made from subculture cycles of calli suspension displaying a similar growth, stable in time (observed after at least 8 subcultures from the initial culture initiation).

Determination of cell growth parameters

Fresh weight was measured after the harvesting of calli by filtration on a filter paper (Macherey-Nagel, Baekkerroo Labo, Jacou, France) under mild pressure. The dry weight of harvested calli was determined after drying for 24 h at 60 °C.

The number of single cells in the medium was estimated by determining cell density on 10 fields on a Malassez cell counter and is expressed on the fresh weight basis of suspension-cultured calli.

Preparation of alcohol-insoluble material (AIM) and extracellular polymers (ECP)

Eight-day-old calli were harvested by filtration through a 10 μm stainless-steel mesh. The alcohol-insoluble material was prepared from about 10 g of calli, according to the method used by Bouton et al. (2002).

The cell-free filtrates were freeze-dried and redissolved in deionized water (1 g l−1). Extracellular polymers were precipitated by adding ethanol to reach 80% final concentration. The precipitate was collected by centrifugation (10 000 g, 10 min), dissolved in deionized water, dialysed against deionized water, and freeze-dried.

Extraction of the alcohol-insoluble material (AIM)

AIM was extracted sequentially with deionized water (three times, 150 ml g−1) at 25 °C for 1 h with agitation, 1% potassium oxalate (three times, 150 ml g−1) at 25 °C for 1 h with agitation followed by deionized water (twice and combined with the oxalate extract), 0.05 N NaOH (three times, 100 ml g−1) at 4 °C for 30 min with agitation followed by deionized water (twice and combined with the dilute alkali extract), and 4 N NaOH containing 0.3% sodium borohydride (twice, 100 ml g−1) at 20 °C for 30 min with agitation. The last residues were washed with deionized water until the washes reached pH 5.5. After each extraction step, residues were dehydrated by 95% ethanol and acetone prior to drying at 40 °C in vacuo over P2O5. Soluble polymers in the extracts were recovered after centrifugation (9000 g, 15 min) and filtration (5–15 μm porosity) of
suspensions. The alkaline extracts were acidified by concentrated HCl to pH 4.8 for the 0.05 N NaOH extract and 6.0 for the 4 N NaOH extract. All extracts were concentrated, dialysed (except the water extract), and freeze-dried. These extracts are referred to in the text as the water-soluble (WS), chelator-soluble (ChS), dilute alkali-soluble (DAS) and the concentrated alkali soluble (CAS) fractions.

Chemical analyses

Identification and quantification of neutral sugars by gas–liquid chromatography (GC) were performed after sulphuric acid degradation (Hoebler et al., 1989). Insoluble samples were dispersed in 13 M sulphuric acid for 30 min at 25 °C, prior to dilution to 1 M and hydrolysis (2 h, 100 °C). Soluble samples were directly dispersed in 1 M sulphuric acid solution and hydrolysed (2 h, 100 °C). For calibration, standard monosaccharide solutions and inositol (the internal standard) were used. The starch content in AIM was determined by GC (Englyst and Cummings, 1988). Uronic acids were quantified by colorimetry using the automated m-phenylphenol-sulphuric acid method (Thibault, 1979). The nitrogen content in AIM was performed using the Kjeldahl method (Miller and Miller, 1948). The protein amount was deduced using a conversion factor of 6.25. The degrees of methyl- and acetyl-esterification of pectins were determined by HPLC following saponification (Levigne et al., 2002).

Detection of arabinoxylan-proteins in ECP

ECP were suspended at room temperature in 0.15 M NaCl solution (10 mg ml⁻¹) for 1 h with shaking. The preparations were assayed for arabinoxylan-proteins by the β-glycosyl-Yariv reagent (Bio-supplies, Parkville, Victoria, Australia) using the single radial gel diffusion method (van Holst and Clark, 1985).

Enzymatic degradations of pectin-rich fractions and ECP, and HPAEC analysis of released oligomers

WS, ChS, DAS, and CAS fractions were degraded by an endo-polygalacturonase from Aspergillus niger (Megazyme, Bray, Ireland), ECP and Tamarind xyloglucan (purified in the laboratory) were degraded by a glucanase from Trichoderma longibrachiatum (Megazyme, Bray, Ireland). Extracted fractions, ECP, and Tamarind xyloglucans were suspended (10 mg dry weight 1.5 ml) and then thoroughly washed with deionized water. Control sections were treated in parallel, but with the omission of the primary antibody.

Immunolabelling

Sections were labelled with JIM7 (Van den Bosch et al., 1989; Knox et al., 1990) and 2F4 antibodies (Liners et al., 1989) as described by Guillemin et al. (2005). For electron microscopy observations, the average gold cluster was increased by silver enhancement as recommended by the manufacturer (Aurion R-GENT SE-EM, Wageningen, NL). The grids were finally post-stained with 4% aqueous uranyl acetate for 15 min.

In some cases, sections were submitted to enzymatic or chemical treatments before immunolabelling, for the in situ de-esterification of pectins. Enzymatic de-methylesterification was carried out by incubating the sections with 50 μl of pectin-methylesterase (from orange peel, 105 U ml⁻¹ Sigma-Aldrich) at 0.2 mg ml⁻¹ in TRIS-HCl pH 7.5 for 1 h at 30 °C in a wet chamber. Then they were washed six times with deionized water for 30 min at room temperature. For chemical de-esterification, the sections were incubated with 50 μl of NaOH 0.05 N for 30 min at 4 °C and then thoroughly washed with deionized water. Control sections were treated in parallel, but with the omission of the primary antibody.

For light microscopy, sections were examined with a LEICA DMRD microscope equipped for epifluorescence. Images were acquired using the objective ×20/0.60 and a CCD camera (ATX 200L Photometrics).

Immunolabelled sections were examined with a JEOL 100 S electron transmission microscope with an accelerating voltage of 80 kV. Each immunolabelling was observed on about ten sections from two different sample blocks.

Statistical analysis

Mean values obtained for cell growth, single cell counts, cell wall material yield and composition, exopolysaccharide yields, and sugar compositions were compared between wt and quai-1 using a Student
t-test. When not specified, significant differences mentioned in the results were observed at the 95% confidence level.

To compare immunofluorescence means, a Student t-test was performed on values obtained from image analysis of JIM 7 labelling of wt and qua1-1 calli. For 2F4, analysis of variance and the F-test in the ANOVA table were applied to evaluate the influence of the factors (genotype and treatments). The statistic analysis was performed with Statgraphics Plus 3.0 (Sigma Plus, Fr).

Results

Qua1-1 calli suspension cultures are more turgid and show a cell adhesion defect

Qua1-1 and wt calli were generated from plant roots. Northern blot analysis (data not shown) showed that QUA1 gene was expressed in wt calli suspension culture. The longer RNA detected in the mutant plant (Bouton et al., 2002) was also produced in the qua1-1 calli.

Qua1-1 calli grew significantly faster than wt on the fresh weight basis, while no differences were observed on the dry weight basis (Fig. 1A). This indicated that fresh qua1-1 calli contained more water than wt. Compared with wt, qua1-1 calli were more fragmented into spherical clumps (Fig. 1B) and released a higher number of single cells into the culture medium (Table 1).

Cell wall polysaccharides composition from qua1-1 calli differs from wt

Cell walls were prepared as alcohol-insoluble material (AIM) from actively growing 8-d-old calli. Due to the higher water content of qua1-1 calli, more AIM was recovered from fresh wt (6.5±0.4%) than qua1-1 (4.8±0.2%). As previously observed for cell cultures (Leboeuf et al., 2004), in both types of callus the AIM was low in polysaccharide (30% dry matter), rich in protein (60% dry matter; Table 2) and contained about 5% starch. Qua1-1 cell walls contained significantly higher proportions of starch.

Table 1. Number of single cells of wild type (wt) and qua1-1 mutant in the culture medium

<table>
<thead>
<tr>
<th>Samples</th>
<th>Days in culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td>wt</td>
<td>0.20±0.06 a</td>
</tr>
<tr>
<td>qua1-1</td>
<td>0.18±0.06 a</td>
</tr>
</tbody>
</table>

Fig. 1. (A) Cell growth of wild-type (WT) and qua1-1 mutant calli expressed as fresh and dry weight per volume of culture. Bars indicate standard deviation (n=4 cultures). (B) Sections of wild-type and qua1-1 mutant calli grown for 8 d in suspension, stained by Toluidine Blue. General organization of the calli.
of arabinose, rhamnose, and fucose, and a lower proportion of uronic acid. The degree of methyl esterification of pectins was significantly lower in qua1-1 (by about 24%) compared with wt. The overall content of acetic acid substituting pectins and hemicelluloses in AIM of mutant and wt calli was similar (Table 2).

**Cell walls of qua1-1 calli are poorer in chelator-soluble pectins than wt**

To identify cell wall polymers affected by the qua1-1 mutation, sequentially extracted water-soluble (WS), chelator-soluble (ChS), dilute alkali-soluble, and concentrated alkali-soluble (CAS) polysaccharides from AIM were chemically characterized. Similar yields were obtained between qua1-1 and wt extracts (WS: about 10%; DAS: about 18%, CAS: about 27% w/w) except for the ChS fraction, which was lower for the mutant (6.9 ± 0.4%) compared with wt (7.8 ± 0.1%). Recovery of materials at each extraction step was high for WS, ChS, and DAS extracts (from 97–99%), but indicated losses in protein and/or sugar during concentrated alkali extraction (50.4% and 67.9% recovery for wt and qua1-1, respectively).

Sugars in WS, ChS, and DAS fractions were principally uronic acid, galactose, and arabinose, with minor amounts of rhamnose. Because the origin of glucose was not able to be distinguished between cell wall and starch, it was analysed separately. Qua1-1 WS and ChS fractions were poorer in uronic acid than wt and this defect appeared to be compensated for mainly by arabinose (Table 3). The proportion of the other sugars in the different extracts was similar between wt and qua1-1.

Although WS, DAS, and, in particular, ChS fractions, were rich in homogalacturonan, as indicated by the molar ratio of rhamnose to uronic acid ranging from 0.12 to 0.05 (Table 3), these ratios also indicated some enrichment of qua1-1 WS and ChS fractions in rhamnogalacturonan I compared with wt. The opposite was observed for the DAS fraction. Further extraction with strong alkali released additional pectic material along with hemicelluloses as revealed by the high xylose content (about 25 mol%). Based on the rhamnose to uronic acid ratio of 0.2, the CAS fraction was richer in rhamnogalacturonan I than the other extracts. The cumulative recovery of uronic acid in qua1-1 fractions was similar to wt and amounted to 36.1 ± 4.3% and 37.4 ± 4.1% of the initial uronic acid content in AIM, respectively.

The degree of methyl- (DM) and acetyl-esterification (DA) of pectic extracts are reported in Table 3. The DM of WS pectins was higher in wt (56%) than in qua1-1 (48%), while the DA of WS pectins was similarly high in wt and qua1-1 (about 35%). The DM and DA of ChS pectins were lower than those of WS pectins. The DM of ChS pectins was similar in wt and qua1-1, but the DA was higher in qua1-1 (17%) than in wt (11%).

**Table 2. Chemical composition of the alcohol-insoluble material (AIM) from 8-d-old wild-type (wt) and qua1-1 mutant calli**

<table>
<thead>
<tr>
<th>Samples</th>
<th>AIM composition</th>
<th>Sugar composition (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methanol (DM)a</td>
<td>Rha, Fuc, Ara, Xyl, Man, Gal, Glc, UA, uronic acids.</td>
</tr>
</tbody>
</table>

Measurements are ± standard deviation, n=4 cultures, different lower case and upper case letters refer to significant differences at the 95% and 99% confidence levels, respectively.)

a Mol of methanol for 100 mols of uronic acids.

b Dry weight %.

c Rha, rhamnose; Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; UA, uronic acids.
The final residue, whose glucose accounted for about 23% (w/w), still contained uronic acid (about 14%), arabinose (about 6%), galactose (about 4%), and rhamnose (about 2%) without significant differences between the two types of calli.

**The fine structure of dilute alkali-soluble pectins from *qua1-1* differs from *wt***

The proportion of unsubstituted homogalacturonan in extracts was assessed from the amount of endopolygalacturonase-generated galacturonic acid and oligogalacturonans (Fig. 2). Larger amounts of uronic acid were released from ChS and DAS (about 80 and 30%, respectively) than from WS and CAS fractions for both types of calli (Fig. 2). These results agreed with the higher esterification degree of WS pectins and the higher proportion of rhamnogalacturonan I in CAS pectins. There were no differences in the proportions of DP1-3 released from *wt* and *qua1-1* WS and ChS extracts on the basis of total uronic acid content of the fractions. Only minor differences were observed for

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**Table 3. Sugar composition and degree of methyl- (DM) and acetyl-esterification (DA) of pectins in WS, ChS, DAS and CAS fractions expressed as molar percentage (± standard deviation between duplicate extracts)**

<table>
<thead>
<tr>
<th></th>
<th>WS wt</th>
<th>ChS wt</th>
<th>ChS <em>qua1-1</em></th>
<th>DAS wt</th>
<th>DAS <em>qua1-1</em></th>
<th>CAS wt</th>
<th>CAS <em>qua1-1</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total carbohydrate (weight % of fractions)</td>
<td>28.6±1.6</td>
<td>28.3±1.9</td>
<td>18.1±2.2</td>
<td>16.5±3.2</td>
<td>8.1±0.7</td>
<td>8.9±2.0</td>
<td>10.4±1.2</td>
</tr>
<tr>
<td>Sugar (mol%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rha</td>
<td>3.1±0.2</td>
<td>3.5±0.2</td>
<td>3.2±0.3</td>
<td>4.6±0.5</td>
<td>3.2±0.2</td>
<td>2.8±0.3</td>
<td>4.1±0.6</td>
</tr>
<tr>
<td>Fuc</td>
<td>1.0±0.3</td>
<td>1.3±0.3</td>
<td>0.2±0.2</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.7±0.2</td>
<td>3.0±0.6</td>
</tr>
<tr>
<td>Ara</td>
<td>25.6±0.8</td>
<td>27.3±1.1</td>
<td>15.4±0.3</td>
<td>19.8±0.4</td>
<td>33.2±0.6</td>
<td>34.2±0.3</td>
<td>18.4±1.8</td>
</tr>
<tr>
<td>Xyl</td>
<td>6.6±1.4</td>
<td>6.3±1.1</td>
<td>4.4±0.2</td>
<td>3.6±0.0</td>
<td>6.0±0.2</td>
<td>6.0±0.5</td>
<td>24.5±3.0</td>
</tr>
<tr>
<td>Man</td>
<td>1.6±0.3</td>
<td>1.7±0.3</td>
<td>1.5±0.4</td>
<td>0.0±0.0</td>
<td>4.5±0.6</td>
<td>3.1±0.4</td>
<td>6.6±0.9</td>
</tr>
<tr>
<td>Gal</td>
<td>25.7±3.0</td>
<td>27.1±3.2</td>
<td>9.5±1.2</td>
<td>10.9±0.1</td>
<td>25.7±2.6</td>
<td>24.7±0.5</td>
<td>22.4±1.6</td>
</tr>
<tr>
<td>UA</td>
<td>36.4±0.6</td>
<td>32.8±0.8</td>
<td>65.9±1.5</td>
<td>61.0±1.0</td>
<td>27.5±1.0</td>
<td>28.4±0.5</td>
<td>21.0±0.6</td>
</tr>
<tr>
<td>Glucose (weight % of fractions)</td>
<td>9.9±5.9</td>
<td>5.2±1.5</td>
<td>1.0±0.2</td>
<td>0.7±0.1</td>
<td>1.3±0.8</td>
<td>1.1±0.4</td>
<td>16.5±6.7</td>
</tr>
<tr>
<td>Rha/UA</td>
<td>0.09</td>
<td>0.11</td>
<td>0.05</td>
<td>0.08</td>
<td>0.12</td>
<td>0.10</td>
<td>0.20</td>
</tr>
<tr>
<td>DM</td>
<td>56.5±3.0</td>
<td>48.4±2.9</td>
<td>27.2±9.7</td>
<td>23.7±6.0</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>DA</td>
<td>34.0±0.4</td>
<td>36.4±4.5</td>
<td>11.4±1.1</td>
<td>16.5±1.7</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

* Rha, rhamnose; Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; UA, uronic acids.

b Molar ratio.

*nd, not detected.

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Fig. 2. Content of galacturonic acid (DP1) and oligogalacturonans of degree of polymerization (DP) 2 to 6 recovered after endopolygalacturonase degradation of water-soluble (WS), chelator-soluble (ChS), dilute and concentrated alkali-soluble (CAS) fractions from wild-type (*wt*) and mutant (*qua*) calli AIM. The data are expressed as weight percentages of total uronic acid in the fractions. Bars indicate differences between duplicate extracts.
higher DP oligos from ChS. Thus, the overall distribution of substituent groups on homogalacturonan in WS and ChS fractions did not markedly differ between wt and quai-1. Compared with wt, the cumulative recovery of galacturonic acid released as monomers and oligomers was significantly lower (23.5%) on the dry weight basis of the quai-1 ChS fraction. Thus, ChS from quai-1 contained less homogalacturonan than wt. In the case of the dilute alkaline extract, different proportions of oligogalacturonides were released from quai-1 and wt. On the basis of the uronic acid content of the fraction, 25% less uronic acid were released from quai-1 as galacturonic acid and oligogalacturonans than wt. Thus, DAS soluble pectins from quai-1 contained less homogalacturonan blocks and/or more alkali-stable substituents than wt.

**Qua1-1 calli contain less calcium dimerized homogalacturonan at the tricellular junction zones of ‘mature’ cells than wt**

Monoclonal antibodies JIM7 and 2F4 were used to compare the abundance and the *in situ* distribution of homogalacturonan in sections of resin embedded 8-d-old wt and quai-1 calli. JIM7 antibody recognizes methyl-esterified residues with adjacent or flanking unesterified GalA (Clausen et al., 2003), while 2F4 antibody recognizes homogalacturonan associated with calcium (Liners et al., 1989). Sections were also treated with plant pectin-methyl-esterases (PME) or alkali partially to de-methyl-esterify or totally to de-esterify pectins.

The JIM7 epitope was abundant in the cell wall (Fig. 3). At the light microscopy level, the mean immunofluorescent labelling of wt calli (1104 ± 83) was significantly higher than that of quai-1 calli (858 ± 63). This result agreed with the higher methyl-esterification of wt pectins compared with quai-1 (Tables 2, 3) and with the lower homogalacturonan content in the cell wall of the mutant. Close examination by electron microscopy revealed that labelling was not equally distributed between ‘young’ (small and densely packed cells) and ‘mature’ cells (large vacuolated cells; Fig. 3). In particular, walls of ‘young’ cells exhibited a stronger labelling compared with ‘mature’ cells. Labelling was particularly abundant at filled cell corners for both wt and quai-1 calli. After treatment of sections with plant PME or alkali, labelling disappeared (data not shown).

2F4 labelling was observed at tri-cellular junctions of both wt and quai-1 calli sections, particularly in ‘mature’ cells (Fig. 4). At the light microscopy level, the mean fluorescence was low and did not significantly differ between wt and quai-1 calli (768 ± 49 and 762 ± 73, respectively). After plant PME and alkali treatments, sections of both wt and quai-1 calli showed an increased labelling intensity. No significant effect was observed for the type of de-esterification treatment on the 2F4 labelling intensity. At the electron microscope level, 2F4 labelling was detected in ‘mature’ cells, essentially localized in the middle lamella expanding at the tri-cellular junctions. It was reduced in quai-1 sections (Fig. 4). NaOH removal of esters on pectins led to the wall-wide 2F4 labelling of the two types of calli. Labelling increased in the middle lamella towards cell corners. However, ‘mature’ cell walls were more densely labelled in wt than in quai-1 calli (Fig. 4). Although polysaccharide losses were possible during
sample preparation for electron microscopy, it is unlikely that they affected calcium dimerized pectins. The extraction of such homogalacturonans would have required chelator agents and such compounds were not used in the preparation of samples. These observations showed that the lower homogalacturonan content in qua1-1 calli cell wall particu-
larly affected tricellular junction zones of ‘mature’ cells. More polysaccharide is released in the culture medium by qua1-1 calli than wt and contains more pectin

The dry weight of extracellular polymers (ECP) recovered in 8-d-old calli culture medium expressed as a percentage of AIM dry weight was significantly lower in wt (7.4±1.2%) than in qua1-1 (10.4±0.9%). Sugars, including glucose, accounted for 40.5±4.3% in wt ECP and 48.7±7.1% in qua1-1 ECP (w:w). The major sugars were glucose, xylose, galactose, and arabinose, while uronic acid, rhamnose, mannose, and fucose represented the minor sugars (Table 4). Small differences were observed between wt and qua1-1 sugar composition. In particular, the proportions of uronic acid and rhamnose were significantly higher in qua1-1 than in wt. The presence of uronic acid, rhamnose, galactose, and arabinose, and the rhamnose to uronic acid ratio of 0.1, suggested the release of pectins with homogalacturonan

Table 4. Sugar composition of extracellular polysaccharides (ECP) from wild-type (wt) and qua1-1 mutant calli grown for 8 d in suspension

Measurements as ± standard deviation; n=3 cultures; different lower case and upper case letters refer to significant differences at the 95% and 99% confidence levels, respectively.

<table>
<thead>
<tr>
<th>ECP sugars composition (mol%)</th>
<th>Rha</th>
<th>Fuc</th>
<th>Ara</th>
<th>Xyl</th>
<th>Man</th>
<th>Gal</th>
<th>Glc</th>
<th>UA</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>0.6±0.1A</td>
<td>0.9±0.1 a</td>
<td>22.6±0.5 a</td>
<td>14.9±0.5 a</td>
<td>4.2±0.3 a</td>
<td>27.0±0.6 a</td>
<td>24.0±0.6 a</td>
<td>5.8±0.3 a</td>
</tr>
<tr>
<td>qua1-1</td>
<td>1.0±0.1B</td>
<td>0.9±0.1 a</td>
<td>19.8±1.0 b</td>
<td>16.5±0.9 a</td>
<td>3.2±0.5 a</td>
<td>24.8±1.2 b</td>
<td>25.5±0.4 b</td>
<td>8.2±1.2 b</td>
</tr>
</tbody>
</table>

* Rha, rhamnose; Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; UA, uronic acids.

Fig. 4. Electron microscopy of ‘mature’ wild-type (wt) and mutant (qua1-1) suspension cultured cells labelled with 2F4.
and rhamnogalacturonan I from wt and qua1-1 calli. The degree of methyl-esterification of pectins was higher in wt (36.0±5.3%) compared with qua1-1 (23.5±1.7%). The weight percentage of acetic acid esters substituting pectins and hemicelluloses relative to the weight of ECP was similar in wt (0.6±0.1%) and qua1-1 (0.7±0.1%). The presence of AGPs and xyloglucans in ECP was revealed by the coloured reaction with the β-glucosyl-Yariv reagent and the release of typical xyloglucan oligosaccharides upon endoglucanase hydrolysis (data not shown). These polymers accounted for the presence of glucose, xylose, galactose, arabinose, and fucose in ECP.

Discussion

Calcium cross-linked homogalacturonan in the middle lamella is widely considered to play a key role in cell–cell adhesion (Fry, 1986; Knox, 1992; Jarvis et al., 2003). The QUASIMODO 1 Arabidopsis mutant, defective in a glycosyltransferase-like gene which reduces cell adhesion and homogalacturonan content (Bouton et al., 2002), appears to be a good biological model to further our understanding of the role of homogalacturonan in cell adhesion. For that purpose, wt and qua1-1 calli suspension cultures were generated. Such undifferentiated cells provide a more homogeneous material than whole plants.

The cell adhesion defect and the lower wall uronic acid content of QUASIMODO 1 plants compared with wt (Bouton et al., 2002) were also observed with qua1-1 calli (Tables 1, 2; Fig. 1). However, the decrease in homogalacturonan in calli was about half that observed for the plant (12% versus 25% reduction), suggesting that the function of the QUA1 gene may be more important in differentiated cells. To determine which pectic polysaccharides were affected by the mutation, the cell wall of wt and qua1-1 calli was sequentially extracted according to their putative wall interactions and each fraction was chemically characterized.

The yield of loosely interacting polymers (water-soluble pectins, WS) and covalently-linked polymers (dilute and concentrated alkali-soluble pectins, DAS and CAS fractions) did not markedly differ between the wt and mutant calli and slightly differed for the calcium-interacting polymers (chelator-soluble, ChS). However, the composition of the fractions clearly differed. Qua1-1 WS and ChS pectic extracts contained less uronic acid originating particularly from homogalacturonan, and showed differences in the esterification levels. The high DM and DA of WS pectins can explain their loose self-association or interactions with other wall polymers. Pectin interactions through calcium bridges require that the DM be sufficiently low (<40%) with at least nine consecutive un-methyl esterified galacturonic acids (Liners et al., 1989). Furthermore, a degree of acetyl-esterification of galacturonic acid above 15% hinders pectin gel formation (Ralet et al., 2003) and has been related to callus friability (Liners et al., 1994). The higher degree of acetyl esterification of qua1-1 ChS pectins (17%) compared with wt (11%) is of interest because this pool of pectins is assumed to be located in the middle lamella (Jarvis et al., 2003) and to be involved in cell adhesion. The reduction in ChS pectin quantity and their high substitution by acetyl esters may contribute to the adhesion defect of the mutant cells.

Endopolygalacturonase degradations revealed small structural disparities between qua1-1 and wt alkaline soluble pectins (Fig. 2). This population of pectins is involved in cell–cell attachment by mechanisms that are not clear (Jarvis et al., 2003) and among which galacturonoyl ester links with hydroxyl groups of other chains were proposed (Brown and Fry, 1993; Hou and Chang, 1996). The structural variations observed between qua1-1 and wt alkali-soluble pectins may reflect distinct homogalacturonan and rhamnogalacturonan distributions and/or the presence of alkali-stable substitutions such as xylose on homogalacturonan (Willats et al., 2004). These structures affect the in vitro interconnecting ability of homogalacturonan and thus may contribute to the defective cell adhesion phenotype of qua1-1.

The immunolabelling results supported the biochemical data and indicated a decrease of the methyl ester content of pectin during cell ageing. Stronger JIM7 labelling was observed in the wall of ‘young’ cells compared with ‘mature’ cells. Likewise, 2F4 labelling was stronger in cell corners of ‘mature’ cells than in ‘young’ cells. This chemical heterogeneity is most likely related to the different growth status of cells within calli. Similar results were reported by Liner and Van Cutsem (1992) in walls of micro-colonies of suspension-cultured carrot cells. The overall lower labelling of qua1-1 cell walls by JIM7 resulted from the combination of a reduced homogalacturonan content and a lower methyl-esterification of pectins in the mutant cell wall. The lower 2F4 labelling observed by electron microscopy in the middle lamella at tricellular junctions of qua1-1 mature cells compared with wt (Fig. 4) clearly showed that there is less low methyl-esterified homogalacturonan associated with calcium in the mutant. The low labelling in qua1-1 observed after alkali treatment of the sections (Fig. 4) further indicated that the initial low 2F4 labelling in qua1-1 did not originate from differently esterified homogalacturonan. This result also showed that the middle lamella of qua1-1 was not particularly rich in acetyl-esterified pectins. The ChS pectins generally assumed to represent middle lamella pectins are more likely to originate from the whole wall. These minor structural alterations that were only clearly observed at the electron microscopy scale occur in critical areas where intercellular adhesion strength is required to withstand cell turgor pressure and to maintain cell cohesion (Jarvis, 1998). The decrease in calcium-associated homogalacturonan appears to be an important factor for cell adhesion, but seems to
concern mainly ‘mature’ cells (Sobry et al., 2005). Thus, 
QUA1 may participate in the turnover of homogalacturonan 
during cell ageing or cell differentiation in plants.

The small enrichment of qua1-1 cell wall in neutral 
sugars and the low degree of methyl-esterification of 
pectins are other consequences of the QUA1 mutation. 
These chemical modifications can result from alterations in 
the physiology of the mutant, with its peculiar higher 
uptake of water compared with wt and/or from a compensating 
mechanism to supplement the decrease in homogalacturonan 
content with other polymers contributing to cell 
adhesion. The higher arabinose content in qua1-1 may 
originate from an increase in the synthesis of arabinose 
containing polymers and/or a reduction in their metabolism. 
Recently, low content, low branching or misplaced pectic 
arabinan in the wall were related to low cell adhesion in cell 
cultures (Iwai et al., 2001; Leboeuf et al. 2004) and in fruits 
(Orfila et al., 2001; Brumme1 et al., 2004; Peña and 
Carpita, 2004). Arabinogalactan proteins identified in the 
extracellular polymers of wt and qua1-1 calli cultures and 
probably contributing to the high protein content of MIA, 
are known to be major proteins synthesized by cells in 
culture (Showalter, 2001) and Arabidopsis cells in particu-
lar (Darjania et al., 2002; Leboeuf et al., 2004). Among 
the many putative functions of these proteins, roles in cell 
adhesion have been proposed (Johnson et al., 2003). It 
therefore appears that down-regulation of the QUA1 gene 
may also have led to modifications of other pectic domains 
(arabinan side chains of RGI) and wall polymers (AGP). 
Whereas the reduction of cell adhesion in qua1-1 can be 
understood by the low content in calcium dimerized homo-
galacturonan, the mechanisms involving arabinose rich 
wall polymers (rhamnogalacturonan I and AGPs) remain 
to be elucidated.

Alteration in QUA1 also led to an increase in the amount 
of ECP released in the culture medium of qua1-1 compared 
with wt. Such an increase could result from higher cell wall 
polymer biosynthesis or degradation rates due to the 
peculiar growth of qua1-1. The latter possibility would be 
supported by the lower methyl-esterification of pectins in 
qua1-1 ECP compared with wt since newly synthesized 
pectins are generally highly methyl-esterified (Carpita and 
Gibeaut, 1993). Another alternative is the modification of 
wall polymer interactions or cross-links. Such modification 
would affect wall porosity and allow an easier diffusion of 
polymers, such as AGP, xyloglucans, and pectins through 
the wall to the culture medium. This would also increase the 
sloughing off of cell wall surface polymers in the culture 
medium. The higher amount of pectins released by qua1-1 
calli may correspond in part to non-cross-linked homoga-
lacturonans that have diffused through the wall and were 
sloughed off from the cell wall surface. Their release into 
the culture medium may have contributed to lower the 
content of homogalacturonan in AIM. This interpretation 
assumes that the affected homogalacturonan domain is not 
integrated within linear pectin chains (Voragen et al., 1995) 
or ramified on rhamnogalacturonan I (Vincken et al., 2003) 
depending on the model of pectin structure considered. As 
a consequence, the capacity of these pectins to form strong 
interactions with other wall polymers, particularly at the 
cell wall surface, would be affected and would directly 
impact cell adhesion.

Conclusion

This work confirms that suspension-cultured cells are good 
models to study plant cell adhesion (Leboeuf et al., 2004) 
and facilitate the characterization of cell wall mutants 
(Pauly et al., 2001). As in the plant, down-regulation of 
the gene QUASIMODO-1 decreases cell adhesion and 
homogalacturonan content in qua1-1 calli although the 
chemical modification was reduced. This work showed that 
the reduction in homogalacturonan in the cell walls of 
qua1-1 calli particularly affect the tricellular junction 
zones, which are critical areas for cell adhesion. Such 
defects can largely contribute to the high fragmentability of 
the mutant calli and by extension, of the plant. Other subtle 
modifications in the chemistry and physico-chemistry of 
the mutant wall were also noticed, probably as compensatory 
mechanisms for the homogalacturonan deficiency. In particu-
lar, those affecting arabinose-containing polymers need 
to be investigated further with regard to cell adhesion. 
Although the biochemical function of the QUA1 protein 
remains to be defined, the appearance of the cell wall 
homogalacturonan defect in ‘mature’ cells suggests that 
the putative galacturonosyl-transferase may be develop-
mentally regulated. The higher content of pectin released 
in the culture medium of qua1-1 calli compared with wt 
also suggests a homogalacturonan synthease function 
linking homogalacturonan domain to other pectin struc-
tures. Expression of QUA1 has been shown to be up-
regulated in suspension-cultured cells that are deficient 
in cellulose and enriched in pectin (Manfield et al., 
2004). Other kinetics studies of QUA1 gene expression 
in relation to the physiological status of cells in culture 
/division, enlargement and senescence states) and the cell 
adhesion defect will help in elucidating the role of this gene 
in cell wall construction.

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