Down-regulation of an Auxin Response Factor in the tomato induces modification of fine pectin structure and tissue architecture

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Abstract

It has previously been shown that down-regulation of an auxin response factor gene (DR12) results in pleiotropic phenotypes including enhanced fruit firmness in antisense transgenic tomato (AS-DR12). To uncover the nature of the ripening-associated modifications affecting fruit texture, comparative analyses were performed of pectin composition and structure in cell wall pericarp tissue of wild-type and AS-DR12 fruit at mature green (MG) and red-ripe (RR) stages. Throughout ripening, pectin showed a decrease in methyl esterification and in the content of galactan side chains in both genotypes. At mature green stage, pectin content in methyl ester groups was slightly higher in AS-DR12 fruit than in wild type, but this ratio was reversed at the red-ripe stage. The amount of water- and oxalate-soluble pectins increased at the red-ripe stage in the wild type, but decreased in AS-DR12. The distribution of methyl ester groups on the homogalaturonan backbone differed between the two genotypes. There was no evidence of more calcium cross-linked homogalacturan involved in cell-to-cell adhesion in AS-DR12 compared with wild-type fruit. Furthermore, the outer pericarp contains higher proportion of small cells in AS-DR12 fruit than in wild type and higher occurrence of (1→5) α-L-arabianan epitope at the RR stage. It is concluded that the increased firmness of transgenic fruit does not result from a major impairment of ripening-related pectin metabolism, but rather involves differences in pectin fine structure associated with changes in tissue architecture.

Key words: Auxin, cell wall, firmness, fruit, pectin, pericarp, tomato.

Introduction

Fruit texture is among the key quality traits determining consumer’s preferences, shelf life and disease resistance (Knee, 2002; Redgwell and Fisher, 2002). Fruit texture is dependent on the assembly of cell wall polymers (Jackman and Stanley, 1995; Rose and Bennet, 1999) and cell turgor pressure (Lin and Pitt, 1986; Shackel et al., 1991; Konstankiewicz and Zudnek, 2001). The highly hydrophilic and viscoelastic cell wall of fleshy fruits is made of pectin, cellulose, and hemicelluloses. The cellular interfaces (middle lamella and cell corners) are rich in pectins cross-linked by calcium (Seymour and Gross, 1996; Redgwell and Fisher, 2002) and play important roles in cell adhesion (Jarvis et al., 2003). Pectins are complex polysaccharides (Willats et al., 2001a) that are generally described to contain different structural domains: contiguous (1→4)-α-L-linked GalA known as homogalacturonic acid (HG), (1→4)-α-L-linked GalA interrupted by the insertion of (1→2) linked α-L-Rha (type I rhamnogalacturan, RG1), and a substituted galacturan (type II rhamnogalacturan, RGII) which exhibits a very complex structure with diverse sugars and linkages. HG contains 100–200 GalA residues (Bonnin et al., 2002) that can be methyl-esterified at position 6, acetylated at position 2 and/or 3 (Quéméner et al., 2003) or substituted by xylose (Le Goff et al., 2001), apiose or short xylose side chains on O-2 and/or
Materials and methods

Material

Tomato fruits: Lycopersicon esculentum Mill. cv. Kermer fruits mature green and red ripe were collected 30 d and 50 d (20 d after the turning stage) after anthesis. They are referred to as MG wild-type and AS-DR12. Per sections, several mosaic images with and without bundles were considered. In total, 40 mosaic images were computed.

Preparation of the alcohol-insoluble material (AIM): Frozen fruits (10–12) were boiled for 30 min in 3 vols of 96% ethanol. After cooling, the pericarp of wild-type and AS-DR12 fruits at the two maturation stages was collected and washed with 70% ethanol until no sugar was detected in the washes (Dubois et al., 1956). The material was depigmented by repeated acetone washings, dried at 40 °C in vacuum over P2O5 and milled to pass through a 2 mm sieve.

Monoclonal antibodies: The antibodies used in this study were the rat monoclonal antibodies JIM7, LM5, and LM6 and the mouse monoclonal antibody 2F4. JIM7 recognizes partially methyl-esterified HG epitope: methyl-esterified residues with adjacent or flanking unesterified GalA alternating methyl-esterified GalA residues (Knox et al., 1989; Clausen et al., 2001). LM5 recognizes epitopes in (1→4)-linked β-D-galactan present in rhamnogalacturonan I (Jones et al., 1997; Willats et al., 2000) and LM6 recognizes epitopes in (1→5)-linked α-L-arabinan present in rhamnogalacturonan I (Willats et al., 1998). 2F4 recognizes an epitope on calcium dimerized homogalacturonan chains of at least nine contiguous galacturonic acids (Liners et al., 1989, 1992).

Pectins: Citrus pectin with a degree of methyl esterification of 41 and a random distribution of the methyl groups was a gift from Dr Bonnin. It is refereed as P41. Polygalacturonic acid (PGA) was from Sigma Aldrich Chemicals (St Quentin Fallavier, France).

Cell morphology

Preparation of samples: Hand-cut sectioning of MG pericarp was carried out in order to preserve cell morphology, to minimize sample preparation, and to observe the whole cross-section of the pericarp. Samples were about 1 cm large and 1 mm thick and were stained using acridine orange (0.02% in 0.1 M phosphate buffer, pH 7) in order to make cells walls fluorescent.

Image acquisition: Images were acquired using a confocal laser scanning microscope (Zeiss, LSM 410). The excitation wavelength was 488 nm and the light emitted over 515 nm was collected using a long pass filter. The ×10 lens was used and under these conditions, the 512×512 pixel image was 1277.5 μm large. As such an area was not sufficient to observe the whole thickness of the pericarp tissue on the same view, several adjacent images were acquired from the external to the internal cuticle. Adjacent images were assembled to form a mosaic image as described by Guillemín et al. (2004). The pericarp mosaic images were considered for image analysis.

Pericarp sections of 3 or 4 fruits were examined for both wild-type and AS-DR12. Per sections, several mosaic images with and without bundles were considered. In total, 40 mosaic images were computed.

Image analysis of pericarp sections: The objective was to extract information on cell morphology from 2D mosaic images of pericarp from MG wild-type and AS-DR12 fruits.

Extraction of morphological features: the uneven background illumination was first corrected. This was done by performing morphological top-hat filtering using a square of size 15×15 pixels as the structuring element (Soille, 2003). Then, a binary version of the image was created by applying a single threshold, the value of which was visually determined for each mosaic image from the grey level histogram. Objects with an area lower than 200 μm2 pixels were eliminated as well as those with an area larger than 30 000 pixels (badly segmented objects). The cell label images were computed. Morphological features were extracted for each cell. The following Matlab morphological parameters were used: length and width along the major axis; area of the convex hull; elongation factor calculated as the width-to-length ratio; and cell orientation. The convex hull of a cell is defined as the smallest convex polygon that can contain the cell. Convex hulls were considered in order to
smooth the edges of the cells. The cell position within the pericarp was assessed as the distance in pixels from the cuticle. The result of feature extraction was a data table containing the measured variables for each cell.

Cell clustering: clustering procedures were performed to classify cells into groups on the basis of their individual morphology, orientation, and position in the pericarp. No prior information on the groups was necessary. The procedure, implemented as described by Guillén et al. (2004), combined a hierarchical clustering and k-means clustering steps (Lebart et al., 1995; Filzmoser et al., 1999). Divisive hierarchical clustering was applied to partition the cell population into an increasing number of groups. The k-means procedure was applied at each divisive step to adjust the frontiers between the groups. The procedure splits the cell population into an increasing number of groups in a completely data driven way and, in particular, does not depend on random starting groups. At each step, the group to be divided and the initial centres of the new groups are chosen from principal component analyses (Guillén et al., 2004). Labelling the cells according to their membership groups made it possible to visualize and interpret the groups in the mosaic images during clustering. The operator chooses the final number of clusters by visual examination of these label images. The whole procedure was developed within the Matlab environment (The MATHWORKS, USA, http://www.mathworks.com).

Height mosaic images corresponding to cross-sections of pericarp with and without bundles acquired for two tomatoes of each genotype were chosen as calibration images. The centres of the groups assessed from the calibration images were used to assign the cells of the other mosaic images.

Comparison of the two genotypes: the relative area proportion of each group was computed for each image and variance analysis was applied to compare the two genotypes. The statistic analysis was performed with Statgraphics Plus 3.0 (Sigma Plus, Fr).

Chemical analyses

Extractions of water- and oxalate-soluble pectins from AIM: AIM from wild type and AS-DR12 pericarp at MG and RR stages was sequentially extracted with distilled water and chelating agent.

AIM (4 g) were agitated in 600 ml of de-ionized water at 25 °C for 1 h. Residues were recovered by centrifugation (9000 g, 15 min) and extracted twice more. Pooled supernatants were passed through a sintered glass filter G4 (5–15 μm porosity), concentrated and freeze-dried. The final insoluble residues were dehydrated by 96% ethanol and acetone washes prior to drying at 40 °C in vacuum over P2O5.

The water extraction residues were then extracted by 1% potassium oxalate in a solid:liquid ratio of 1 g:150 ml at 25 °C for 1 h with agitation. The extracts were recovered as above and residues re-extracted similarly twice more. Final residues were washed with water and the washes were combined with the pooled oxalate extracts. The residues were then dehydrated and dried as above. Extracts were concentrated, dialysed extensively against de-ionized water, and freeze-dried.

Sugars analysis: Neutral sugars composition was determined by gas liquid chromatography as described by Høeble et al. (1989). AIM and extract residues were dispersed in 72% sulphuric acid for 30 min at 25 °C prior dilution to 1 M and hydrolysis (2 h, 100 °C). Extracts were directly hydrolysed in 1 M sulphuric acid at 100 °C for 2 h. The sugars were reduced by sodium borohydride, acetylated, and their alditol acetates analysed by gas-liquid chromatography.

Uronic acids were quantified by colorimetry using the automated m-phenyl-phenol-sulphuric acid method without tetraborate (Thibault, 1979) either on acid hydrolysates produced for neutral sugar analysis of AIM and oxalate-insoluble material or directly on solutions of de-esterified extracts.

Degree of methyl- and acetyl-esterification were determined by HPLC following saponification according to Lévine et al. (2002). They are expressed as mol of acetic acid or methanol per 100 mol of uronic acid.

Proteins in insoluble materials were quantified as N×6.25 following the Kjeldahl method.

Immunochemistry: competitive ELISA: Microplates (Maxisorp, Nunc) were coated (100 μl well−1) overnight at 4 °C with P41 (250 μg ml−1; JIM7) in 20 mM phosphate buffer (pH 7.4) for JIM7, with PGA (100 μg ml−1; 2F4) in Ca/Na TRIS buffer (20 mM TRIS–HCl, pH 8.2; CaCl2 0.5 mM; NaCl 150 mM) for 2F4. After brief washing with buffer, plates were blocked at room temperature for 1 h with 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for JIM7 and for 2 h with 2% skimmed milk (200 μl) in Ca/Na TRIS buffer for 2F4. Following brief washing, competitor solutions were applied (100 μl well−1). The competitor solution consisted of 200 μl of the hybridoma solutions diluted 100 times in PBS for JIM7, and 25 times in Ca/Na TRIS buffer for 2F4 and pre-incubated with 200 μl of pectic material from wild-type and AS-DR12 pericarp at different concentrations in the same solution for 1 h at 37 °C. The pectic samples were omitted in the blanks.

After 1 h, the plates were washed extensively with corresponding buffer using an Immunowash (NUNC) and second antibodies (anti-rat horseradish peroxidase conjugate for JIM7 and anti-mouse horseradish peroxidase for 2F4, Sigma diluted 1/1000 in PBS for JIM7 and in Ca/Na TRIS buffer for 2F4) applied (100 μl well−1) and incubated for 1 h at 37 °C. After a second cycle of washing, the binding of the antibodies was revealed by a chromogen-substrate solution made of O-phenylenediamine (10 mg in 25 ml of 0.05 M citrate buffer, pH 6) and H2O2 (final concentration 0.03%). After 30 min, the reaction was stopped with 2 M H2SO4, (100 μl well−1) and the absorbance of the solution was measured with a Biotek-Els800 microplate reader (Universal Microplate reader, INC) at 490 nm.

Concentration of competitors (expressed as equivalent anhydro-galacturonic acid) resulting in 50% inhibition (IC50) of antibody binding were determined by plotting competitor concentrations against absorbance. Values from the blank with no competitor were taken as 0% inhibition of antibody binding.

Statistical analysis: All extractions were performed in triplicate. Sugar contents and esterification patterns were determined for each replicate. Variance analyses were applied to evaluate the effect of genotype, ripening stage, and their interaction. The analysis was performed with Statgraphics Plus 3.0 (Sigma Plus, Fr).

Microscopy

Immunoocytochemistry: resin embedded samples: Small pieces of fresh pericarp were taken from the equatorial region of tomato fruits. They were put into 3% agar and fixed 4 h by 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2. Fixed samples were then rinsed in buffer and de-ionized water prior dehydration by washes in 30, 50, 70, 85, 95, and 100% ethanol. Samples were impregnated in ethanol:LR White (LRW, London Resin Company Limited, Reading UK), v/v: 80/20, 60/40, 40/60, 20, 80, and pure LR White. Samples were then embedded in gelatin capsules. The resin was polymerized in the presence of a catalyst for 2 d at 5 °C. Semi-thin sections of 1 μm were cut on an ultramicrotome (MICROM MT-7000) equipped with a diamond knife and mounted on glass microscope slides for cytochemical staining or multi-well
slides for bright field observation or immunostaining. For transmission electron microscopy (TEM), ultra-thin sections (80–100 nm) were collected on nickel grids.

**Immunolabelling**: Sections were incubated in a blocking solution of 3% (v/v) BSA in 20 mM PBS, pH 7.2 for JIM7, LM5, and LM6, or of 5% skimmed milk in a Ca/Na TRIS buffer (20 mM TRIS–HCl, pH 8.2; CaCl2 0.5 mM; NaCl 150 mM) for 2F4 for 30 min at room temperature. Sections were then incubated in a solution containing the monoclonal antibodies (JIM7, JIM5, LM5, LM6: goat-anti-rat IgG; 2F4: goat-anti-mouse IgG,) linked to ALEXA Fluor 546 (Molecular Probe, Oregon, USA) diluted 1:100 in buffer (JIM7, LM5, LM6: 20 mM PBS supplemented with 3% BSA, 2F4: Ca/Na TRIS buffer supplemented with 1% skimmed milk) for 1 h at room temperature. The sections were washed extensively with the supplemented buffer and then incubated in a solution containing the secondary antibody (JIM7, JIM5, LM5, LM6: goat-anti-rat IgG; 2F4: goat-anti-mouse IgG,) linked to a microscope equipped with epifluorescence (LEICA DMRD).

For TEM, sections were treated following the same procedure as that of resin embedded semi-thin sections, using as secondary Abs, gold conjugates (goat anti-rat-IgG or goat anti-mouse-IgG conjugated with 1 nm colloidal gold complexes, Aurion, NL), diluted 1:20 (v/v) in the respective buffer used for diluting the primary Abs. Labelling was intensified with the silver enhancement kits (Aurion, NL), diluted 1:5 in Ca/Na TRIS buffer supplemented with 1% low-fat dried milk) for 1 h at room temperature at obscurity. The sections were then incubated in a solution containing the secondary antibody (JIM7, JIM5, LM5, LM6 diluted 1:10 in 20 mM phosphate buffered saline supplemented with 3% BSA, 2F4 diluted 1:5 in Ca/Na TRIS buffer supplemented with 1% low-fat dried milk) for 1 h at room temperature. The sections were washed extensively with the supplemented buffer and then incubated in a solution containing the secondary antibody (JIM7, JIM5, LM5, LM6: goat-anti-rat IgG; 2F4: goat-anti-mouse IgG,) linked to ALEXA Fluor 546 (Molecular Probe, Oregon, USA) diluted 1:100 in buffer (JIM7, LM5, LM6: 20 mM PBS supplemented with 3% BSA, 2F4: Ca/Na TRIS buffer supplemented with 1% skimmed milk) for 1 h at room temperature at obscurity. The sections were washed extensively with buffer and examined with a microscope equipped with epifluorescence (LEICA DMRD).

In some cases, enzymatic treatments were used before immunolabelling, for on slide de-methyl-esterification of pectins: the sections were treated for 60 min at 30 °C with 0.05 M NaOH (pH 12.6) for 30 min at 4 °C. Sections were rinsed thoroughly with deionized water.

Incubation of sections with the second antibody only was done as the control. All the observations were done on embedded samples prepared from two different fruits and on each sample. Sample treatments and staining were repeated twice.

**Results**

**Histological description of pericarp tissues in wild-type and AS-DR12 fruit**

In order to get a better insight into the histological structure of the fruit pericarp in wild-type and AS-DR12 lines, a quantitative description of cell size and shape was performed. Images of adjacent area were acquired by confocal microscopy and assembled as mosaic images to reconstruct the whole pericarp thickness. The mosaic images reveals that pericarp tissue of transgenic fruit is thicker (9.41 ± 0.25 mm) than that of wild type (8.21 ± 0.25 mm). The clustering procedure retains six groups based on cell morphology and cell position (Fig. 1) and for each of them the average feature values corresponding to the centre are assessed (Table 1). The red group corresponds to small isodiametric cells just under the cuticle. Cyan cells, located at mid-pericarp are large, elongated, and orientated. Green cells are small to medium-sized, orientated, and occur mainly in images containing bundles. Yellow cells correspond to medium-sized round cells located close to the cuticle. The relative proportions of each group in the mosaic images of the pericarp tissue were computed (Table 2). For both wild type and AS-DR12, variance analysis highlights differences between cell groups in mosaic images with and without bundles. Bundle-containing images have more medium-sized and orientated cells in the mesocarp (green group) while large and elongated cells (cyan group) are more numerous in images with only parenchymatous tissue.

At the mature green stage, the differences between wild type and AS-DR12 are small. The yellow cells close to the cuticle and the green cells in the bundle regions are significantly more abundant in transgenic fruit than in wild type. By contrast, the cyan group is more represented in wild-type fruit (Table 2).

Beside differences in the pericarp histology, further examination of the epidermis region reveals a difference in cuticle thickness between the two genotypes. The cuticle thickness increases similarly in both genotypes from mature green to red ripe fruits (data not shown), but AS-DR12 displays a thinner cuticle at red ripe stage compared to wild type (Fig. 2).

**Compositional analysis of pericarp cell wall**

Sugar and methyl-ester contents of cell wall materials from wild-type and AS-DR12 fruit were assessed at mature green and red ripe stages. Table 3 indicates that the sugar content decreases during ripening while the amount of protein increases in both genotypes. Sugar compositions of wild-type and AS-DR12 cell wall are similar at the same ripening stage. Ripening is associated with a net decrease in galactose content and pectin methyl-esterification in both genotypes. However, the degree of methylation is slightly higher in AS-DR12 fruit at mature green stage compared with the wild type, but becomes lower at red ripe stage. These data indicate that transgenic fruit undergo a more intensive de-methylation process throughout ripening.

**Extraction yield and characterization of pectins**

Processes responsible for ripening-related textural changes are still not fully understood, although they are thought to involve pectin modification, in particular methyl de-esterification of homogalacturonans and an increase in water-soluble pectin. To investigate whether the metabolism of homogalacturonans is altered during ripening in AS-DR12, the alcohol-insoluble material (AIM) of
wild-type and transgenic fruit were sequentially treated with water and chelating agents to isolate readily soluble pectins and calcium cross-linked pectins. These extracts were then characterized in terms of sugar composition and reactivity with JIM7 and 2F4 antibodies.

The yield and chemical composition of pectins are shown in Tables 4 and 5. In wild-type fruit, taking into account the fractions yield and sugar content, the cumulative amounts of sugar extracted by water and oxalate increase during ripening. It represents 11.3% and 13.8% of total sugars and 42.2% and 55.2% of total uronic acid in the cell wall from mature green and red ripe fruit, respectively. By contrast, this cumulative amount of

<table>
<thead>
<tr>
<th>Group</th>
<th>Length (μm)</th>
<th>Width (μm)</th>
<th>Area of convex hull (μm²)</th>
<th>Elongation</th>
<th>Orientation</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red</td>
<td>122</td>
<td>75</td>
<td>9139</td>
<td>0.62</td>
<td>93</td>
<td>0.88</td>
</tr>
<tr>
<td>Green</td>
<td>115</td>
<td>70</td>
<td>7919</td>
<td>0.61</td>
<td>17</td>
<td>0.38</td>
</tr>
<tr>
<td>Yellow</td>
<td>120</td>
<td>95</td>
<td>11094</td>
<td>0.81</td>
<td>19</td>
<td>0.82</td>
</tr>
<tr>
<td>Magenta</td>
<td>287</td>
<td>132</td>
<td>33917</td>
<td>0.47</td>
<td>2</td>
<td>0.37</td>
</tr>
<tr>
<td>Cyan</td>
<td>459</td>
<td>245</td>
<td>93509</td>
<td>0.55</td>
<td>8</td>
<td>0.38</td>
</tr>
<tr>
<td>White</td>
<td>252</td>
<td>197</td>
<td>42708</td>
<td>0.78</td>
<td>34</td>
<td>0.73</td>
</tr>
</tbody>
</table>

Table 2. The percentage of the cross-sectional area occupied by each cell group in the pericarp images

<table>
<thead>
<tr>
<th>Group</th>
<th>Red</th>
<th>Green</th>
<th>Yellow</th>
<th>Magenta</th>
<th>Cyan</th>
<th>White</th>
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</thead>
<tbody>
<tr>
<td>Wild type parenchyma</td>
<td>8.3</td>
<td>4.2</td>
<td>4.1</td>
<td>16.9</td>
<td>37.3</td>
<td>29.4</td>
</tr>
<tr>
<td>SD</td>
<td>2.5</td>
<td>2.9</td>
<td>3.0</td>
<td>5.9</td>
<td>14.4</td>
<td>9.7</td>
</tr>
<tr>
<td>Wild type bundle</td>
<td>9.4</td>
<td>7.3</td>
<td>5.3</td>
<td>21.4</td>
<td>27.0</td>
<td>29.6</td>
</tr>
<tr>
<td>SD</td>
<td>2.2</td>
<td>3.2</td>
<td>2.0</td>
<td>7.7</td>
<td>8.2</td>
<td>8.8</td>
</tr>
<tr>
<td>AS-DR12 parenchyma</td>
<td>7.9</td>
<td>8.0</td>
<td>10.7</td>
<td>17.4</td>
<td>25.1</td>
<td>30.9</td>
</tr>
<tr>
<td>SD</td>
<td>4.7</td>
<td>4.0</td>
<td>5.2</td>
<td>7.1</td>
<td>15.3</td>
<td>7.9</td>
</tr>
<tr>
<td>AS-DR12 bundle</td>
<td>14.4</td>
<td>15.3</td>
<td>9.0</td>
<td>26.0</td>
<td>11.7</td>
<td>24.0</td>
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<tr>
<td>SD</td>
<td>10.9</td>
<td>4.6</td>
<td>5.4</td>
<td>7.3</td>
<td>8.5</td>
<td>11.0</td>
</tr>
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</table>
total sugars extracted from AS-DR12 fruit decreases during ripening. It represents 12.6% and 9.9% of the total carbohydrate and 49.1% and 41.2% of the total uronic acid in the cell wall from mature green and red ripe fruit, respectively. Tables 4 and 5 indicate that the amount of pectin and uronic acid-extracted material from transgenic fruit are lower than wild type. For both genotypes, uronic acid is by far the major sugar in all extracts (Tables 4, 5).

Sugar composition of water extracts
At the mature green stage, neutral sugars account for about 40% of the total sugars present in water extracts.
either wild-type or AS-DR12 fruit (Table 4). Glucose, mainly originating from contaminating starch, and to a lesser extent galactose are the major neutral sugars. Water extracts of red ripe fruits contain less glucose as a result of starch degradation during ripening and a higher proportion of uronic acid compared to mature green fruits. At the red ripe stage, water extracts of transgenic fruit have a greater proportion of neutral sugars (about 26% of total sugars present) than the corresponding fractions from wild type (about 16% of total sugar present). Galactose and xylose are the major neutral sugars. At the mature green stage, the degree of pectin methylation in water extracts is similarly high in wild-type and transgenic fruits (Table 4), but decreases during ripening for both genotypes, with AS-DR12 fruit displaying the lowest decrease. By contrast, the degree of acetyl-esterification of pectins in water extracts increases during ripening for both genotypes, with AS-DR12 showing the more substantial increase (Table 4).

**Sugar composition of oxalate extracts**

For both genotypes, uronic acid in oxalate extracts accounts for over 75% of total sugars at the mature green stage and over 90% at the red ripe stage (Table 5). The degree of methylation of pectin at the mature green stage is less than 40% and decreases similarly in both genotypes during ripening. The degree of acetylation of the pectin in these oxalate extracts from either wild type or AS-DR12 is low at both mature green and red ripe stages (Table 5).

**Characterization of pectins by immunochemistry methods**

Competitive ELISA methods were used to characterize the fine structure of homogalacturonan domains in the water and oxalate extracts. The JIM7 antibody reveals the presence of methyl-esterified pectins while the 2F4 monoclonal antibody reveals unesterified homogalacturonan blocks. Comparing the IC50 of the pectic extracts gives a good indication of the extent of their recognition by the antibodies. The higher is the IC50, the less is the recognition of the pectic extracts by the antibodies. JIM7 epitope is abundant in water extracts of both wild-type and AS-DR12 fruits at the mature green stage and its occurrence slightly decreases during ripening (Fig. 3A). However, water extracts from wild type inhibit more JIM7 binding to the model pectin P41 than those from AS-DR12 at both the mature green and red ripe stages. Accordingly, competitive ELISA with 2F4 indicates that, at the mature green stage, water extracts of AS-DR12 fruits contain more de-esterified homogalacturonan blocks than wild type (Fig. 3B). Interestingly, while wild-type water extract displays dramatic change in 2F4 reactivity during ripening, AS-DR12 shows only reduced changes. At the mature green stage, oxalate extracts of wild type contain a higher amount of JIM7 epitope than those of AS-DR12 at red ripe stage (Fig. 4A). Its occurrence decreases in oxalate extracts from both wild type and AS-DR12 during ripening. However, oxalate extracts from wild type inhibit more JIM7 binding to P41 than those of AS-DR12 at red ripe stage (Fig. 4B). All together, the competitive ELISA tests does not reveal differences in 2F4 reactivity towards oxalate extracts between the two genotypes at any stage of fruit ripening (Fig. 4B).

**Characterization of the oxalate-insoluble material**

The sugar composition of the oxalate-insoluble material was analysed. The total amount of residual sugars after water and oxalate extractions is higher in AS-DR12 than in wild type at both mature green and red ripe stages (Table 6). This is in agreement with the extraction yield of water and oxalate-soluble material. These residues contain uronic acid and glucose as the main sugar components. The degree of methyl-esterification of residual pectins is

### Table 5. Yield from AIM and sugar composition of oxalate extracts

Degrees of methyl- (DM) and acetyl-esterification (DA) of pectins in oxalate extracts. Variance analysis: significance for variety, stage effect, and interaction (ns=not significant, * <5%, ** <1%, *** <0.1%).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Yield</th>
<th>Total sugars</th>
<th>Rha</th>
<th>Ara</th>
<th>Xyl</th>
<th>Man</th>
<th>Gal</th>
<th>Glc</th>
<th>UA</th>
<th>DM</th>
<th>DA</th>
</tr>
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<tbody>
<tr>
<td>MG wild type</td>
<td>10.6</td>
<td>44.8</td>
<td>0.3</td>
<td>0.3</td>
<td>0.1</td>
<td>0.2</td>
<td>1.1</td>
<td>7.3</td>
<td>35.5</td>
<td>35.0</td>
<td>5.1</td>
</tr>
<tr>
<td>SD</td>
<td>1.9</td>
<td>1.7</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
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* a % of dry AIR.
* b Amount of total sugars (% dry wild type of the extracts).
* c Rha, rhamnose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; UA, uronic acid.
similar in wild-type and AS-DR12 fruit at the mature green stage, but 1.5 times higher in wild-type fruit at the red ripe stage.

**Cytochemistry and microstructure**

Cell wall dissociation was investigated by light and electron microscopy observation of pericarp sections and immunolabelling with anti-pectic antibodies. Methyl-esterified pectins and blocks of de-esterified galacturonic acid residues cross-linked by calcium were localized by JIM7 and 2F4 labelling, respectively. All cell walls remained unlabelled when the primary mAbs were omitted from control sections in both LM and TEM (not shown). As shown in Fig. 5A–D, at the mature green stage strong JIM7-labelling of pericarp tissue from wild-type and AS-DR12 fruit is observed by light and electron microscopy indicating that the overall abundance of methylated pectins is similar in both genotypes. The epitope is equally distributed in all parenchyma cell walls which appear labelled across their width and entire length including filled corners (Fig. 5C, D). Ripening induces a marked cellular dissociation and JIM7 labelling remains intense in both wild-type and AS-DR12 fruits (Fig. 5E–H). At the red ripe stage, the epitope is distributed throughout the walls apart from the disintegrated and dispersed material in the middle lamella region (Fig. 5H). The walls of cells under the epidermis are more intensely labelled (Fig. 5E–G). For a given stage, no difference in the occurrence of the 2F4 epitope can be detected between wild type and AS-DR12 and hence, only pictures of AS-DR12 are shown to illustrate the changes that take place during ripening (Fig. 6A–D). At the mature green stage, 2F4 epitopes are detected in the region of cell walls lining intercellular spaces and at tricellular junctions (Fig. 6A). Immunogold electron microscopy reveals the presence of 2F4 epitopes throughout the middle lamella at mature green stage (Fig. 6B), while it is spread over the cell walls at the red ripe stage (Fig. 6D). In contrast to JIM7, the occurrence of the 2F4 epitope is not uniform within the mesocarp of red ripe fruit with a faint labelling in the inner subepidermal layer while cell walls in the mesocarp exhibited higher labelling intensity with variation observed from cell to cell (Fig. 6C). Pectin methyl esterase and NaOH treatments, both resulted in an increase of the labelling in all sections observed under light and electron microscopy in both DR12 and WT at any of the ripening stages considered (result not shown).

LM5 and LM6 antibodies were used to locate galactan and arabinan side chains of rhamnogalacturonan 1 domains in pectins, respectively. At the mature green stage, galactan side-chains of pectin are detected by LM5 monoclonal antibody labelling in all cell walls except in the epidermis of both wild type and AS-DR12 (Fig. 7A). In all sections observed, LM5 epitopes are always present
at the plasmalemma–cell wall interface and at the lining of intercellular spaces, while it is absent in the areas of expanded middle lamella at intercellular space and pit fields (Fig. 7B–D). Compared with mature green, the labelling dramatically decreases at the red ripe stage for both wild-type and transgenic fruit (Fig. 7E, F). In mature green pericarp tissues, the α-(1,5)-arabinan epitope revealed by LM6 antibodies is distributed in all cell walls including those of subepidermal cells in both wild type and AS-DR12 (Fig. 8A, C). The overall cell wall labelling is far more intense in mature green than in ripe pericarp tissue. In this latter stage, the subepidermal cells display higher reactivity with LM6 antibodies in both genotypes (Fig. 8B, D). Cell walls of red ripe pericarp tissues of wild type and AS-DR12 fruit are weakly labelled and when labelled, they appear irregularly labelled (Fig. 8B, D). In the inner pericarp tissue, the LM6 epitope appears more abundant in AS-DR12 than in wild type (Fig. 8B, D). At the mature green stage, electron microscopy observations indicate that LM6 epitopes are present throughout the primary walls but are nearly absent at the cell corners and expanded lamella (Fig. 8E, F). At the ripe stage, the epitope is more scarce (Fig. 8H) and always absent from disintegrated fibrillar material (Fig. 8I). The high LM6 labelling of walls of superepidermal cells seen by light microscopy was confirmed at the ultrastructural level (Fig. 8G). Immunogold label was restricted to wall regions close to plasmalemma (Fig. 8G).

**Discussion**

**Histological traits**

Down-regulation of the tomato DR12 gene encoding an Auxin Response Factor was reported to result in enhanced firmness of ripe fruit associated with unusual cell division in the pericarp tissue (Jones et al., 2002). A number of studies investigated the role of cell wall enzymes and proteins in fruit softening using transgenic lines under- or over-expressing the corresponding genes. Changes in tissue structure have been studied using genotypes displaying contrasted fruit firmness (Chaib et al., 2007; Devaux et al., 2007). In this study, in order to minimize the genetic background effect, isogenic tomatoes lines were used differing in the expression of a single gene encoding a transcriptional regulator known to mediate auxin-responses (Guilfoyle et al., 1998; Ulmasov et al., 1999). To understand the effect of the AS-DR12 mutation further, the histological changes in fruit pericarp tissues and associated modifications of cell wall structure and composition were explored. Our data indicate that pericarp of transgenic fruit is thicker than that of wild type and that the increased thickness is not due to larger cells but rather to a higher proportion of small cells in the subepidermal area compared to wild type fruit. It was shown that the sub-epidermal part of the pericarp tissue is the main area where cell division occurs (Cheniclet et al., 2005) and this area corresponds to a transition zone where cell size and shape change gradually from epidermal type to parenchymal type. Since cell size is related to the level of cell endoreplication (Cheniclet et al., 2005), it can be speculated that down-regulation of DR12 by promoting cell division with cytokinesis, alters the cell cycle leading to cells of lower ploidy compared to wild type.

**Changes in pectin metabolism**

Since it was shown that ripening-associated softening of tomato fruit involves cell wall disassembly by affecting pectin structure (Huysamer et al., 1997; Redgwell et al., 1997; Brummell and Harpster, 2001; Orfila et al., 2001), the impact of the DR12 mutation on pectin components of the cell wall was investigated in transgenic fruit. Our data indicate that the overall chemical composition of the cell wall was similar in wild type and transgenic pericarp tissues. During ripening, the combined action of pectin methyl esterases and endo-polygalacturonases is thought to depolymerize pectins, which in turn become increasingly soluble in water and chelator solutions (Koch et al., 2002). A number of studies investigated the role of pectin components and associated modifications of cell wall structure and composition were explored. Our data indicate that pectin of transgenic fruit is thicker than that of wild type and that the increased thickness is not due to larger cells but rather to a higher proportion of small cells in the subepidermal area compared to wild type fruit. It was shown that the sub-epidermal part of the pericarp tissue is the main area where cell division occurs (Cheniclet et al., 2005) and this area corresponds to a transition zone where cell size and shape change gradually from epidermal type to parenchymal type. Since cell size is related to the level of cell endoreplication (Cheniclet et al., 2005), it can be speculated that down-regulation of DR12 by promoting cell division with cytokinesis, alters the cell cycle leading to cells of lower ploidy compared to wild type.

**Table 6. Yield (% of dry AIM) and sugar composition (% dry matter) of the oxalate insoluble material**

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* Degree of methyl-esterification of residual pectins.
Nevins, 1989; Huber and O'Donoghue, 1993; Wakabayashi et al., 2003). It is noteworthy that such an increase in pectin solubility was not observed in red ripe AS-DR12 fruit, indicating that pectin cleavage in transgenic fruit may occur at a lesser extent than in wild type and/or that cleaved pectins may be retained in the wall by covalent linkages (Brown and Fry, 1993; Qi et al., 1995; Popper and Fry, 2005) or may get entangled in other wall polymers (Hwang and Kokini, 1992). As reported previously for such pectin extracts (Selvendran, 1985), wild type and AS-DR12 water-soluble pectins are highly methyl-esterified and rich in neutral sugars, while chelator-soluble pectins contain high amount of uronic acid and are weakly methyl-esterified. Extracts from AS-DR12 fruits have lower uronic acid content than wild type and differ in the distribution of methyl esters. The different methyl ester distribution of AS-DR12 soluble pectins compared to wild type and the lower amount of pectins extracted with water and oxalate from red-ripe transgenic fruit clearly indicate that down-regulation of DR12 affects pectin synthesis and/or metabolism.

These data indicate that the distribution of methyl esters along the backbone of water- and oxalate-soluble pectins differs in wild-type and AS-DR12 fruits. Oxalate-soluble pectins was of interest because this pool of pectins is assumed to be located in middle lamella and to be involved in cell adhesion (Fry, 1988; Jarvis, 2003). There was no evidence of more calcium cross-linked

Fig. 5. Immunolabelling of pectic homogalacturonic by the monoclonal antibody JIM7 of radial sections of resin-embedded pericarp from wild-type and AS-DR12 fruits at the MG and RR stage. (A, B, E, F) Immunofluorescent micrographs; (C, D, G, H) immunogold electron micrographs. Uranyl acetate counterstaining. (A) MG wild-type fruit; (B, C, D) MG AS-DR12 fruit; (E) RR wild-type fruit; (F, G, H) RR AS-DR12 fruit. At the RR stage, labelling was still intense. The epitope occurred throughout the primary wall but was absent from the disintegrated material in the middle lamella region. Arrowheads in (E) and (F) showed the intense immunofluorescent labelling of cell walls beneath the cuticle.
homogalacturan involved in cell-to-cell adhesion in AS-DR12 compared to wild-type fruit. The results also suggest that the kinetic of demethyl- esterification of pectins during ripening may differ between wild-type and AS-DR12 fruits. HG and particularly low methyl-esterified HG cross-linked by calcium have been proposed to play key roles on fruit mechanical properties (Jarvis et al., 2003). The lower degree of methyl esterification of pectins in the oxalate residues in AS-DR12 may result in more calcium-binding sites for pectin cross-linking which may therefore contribute to the increased firmness of red-ripe AS-DR12 fruit. Other factors, such as the distribution of unesterified homogalacturonan blocks, the presence of other covalent linkages and/or a higher level of wall polymer entanglements have also to be considered.

Modifications of HG and RGI in primary walls and middle lamella investigated by the immunolabelling approach indicate that pericarp tissue at the mature-green stage is composed of highly cohesive cells in both wild type and AS-DR12. Blocks of un-esterified (2F4 epitope) HG were confined to the middle lamella, at cell junctions, and lining of intercellular spaces in the mesocarp tissue as reported (Bush and McCann, 1999; Willats et al., 1999, 2001b; Orfila et al., 2001). Densely packed cells beneath the cuticle contained no acidic pectin in their walls. These data show that cell-to-cell adhesion in these layers is not due to the presence of long stretches of de-esterified pectins susceptible to cross-linking by calcium ions. Intermediate forms of soft acidic gels involving H-bonding and hydrophobic interactions described in vitro (Tibbits et al., 1998; Gilsenan et al., 2000) could play an important role in cellular adhesion in planta.

At the ripening stage, the breakdown of the middle lamella leads to cell separation in both AS-DR12 and wild-type fruit. Although the degree of pectin esterification decreases during ripening, the pectic motifs recognized by JIM7 remain at a similar level in red-ripe and MG pericarp tissue for both AS-DR12 and wild-type tissue as revealed by the density of labelling. Furthermore, the similarity of 2F4 immunolabelling density observed after pectin de-esterification in AS-DR12 and wild-type pericarp tissue indicates that pectins in both genotypes are equally substituted by other groups, such as acetyl esters.

Galactan and arabinan side chains

The enhanced firmness of the ripe fruit cannot be explained by differences in RGI side chains metabolism. However, heterogeneous distributions of both methyl-esterified HG and RGI galactan and arabinan side chains are observed in the walls of ripe fruit and particularly those from AS-DR12. Such heterogeneity may reflect the high regulation of endogenous galactosidases and arabinofuranosidases in AS-DR12. This higher disparity may also reflect the blotchy ripening behaviour of the transgenic fruit (Jones et al., 2002) and may lead to different local enzyme kinetics, which could create spatially limited modifications in cell wall assembly with consequences on firmness at the macroscopic scale. RGI has often been associated with fruit texture, but its roles remain unknown. It is branched by galactan and arabinan side chains, the distribution of which varies in cell walls (Jones et al., 1997; Orfila and Knox, 2000; Orfila et al., 2001). The content of these side chains decreases during ripening of tomato (Seymour et al., 1990; Orfila and Knox, 2000; Orfila et al., 2001; Rondeau-Mouro et al., 2003). RGI galactan has been associated with firmness and mechanical strength of tissues (McCartney et al., 2000; McCartney and Knox, 2002) and with tomato mealiness (Devaux et al., 2005), but direct relationships involving RGI galactan in mechanical and physicochemical properties of cell walls wait to be demonstrated (Redgwell et al., 1997; Smith et al., 2002). RGI arabinan side chains when deficient or modified have been associated with loosely adhering cells (Iwai et al., 2001; Nara et al., 2001; Orfila et al., 2002; Leboeuf et al., 2005). In apple, loss of branched arabinans occurred before loss of texture (Pena Fig. 6. Immunolabelling of pectic homogalacturonic by the monoclonal antibody 2F4 of radial sections of resin-embedded pericarp from the AS-DR12 fruits at the MG and RR stages. (A, C) Immunofluorescent micrographs; (B, D) immunogold electron micrographs. Uranyl acetyl counterstaining. (A, B) MG AS-DR12 fruit; (C, D) RR AS-DR12 fruit. At the MG stage, labelling was more intense at the cell corners and at the middle lamella. At the RR stage, the 2F4 epitope was not equally distributed in all mesocarp cell walls. At the ultrastructural level, the labelling was irregularly distributed throughout the wall.
Several putative functions of RGI and side chains have been proposed with regard to wall mechanics and fruit texture. Pectic matrix may play a role in transmitting mechanical stresses to cellulose microfibrils (Ulvskov et al., 2005) and, in fact, RGI-arabinan side chains are able to complex with cellulose (Zykwinska et al., 2005, 2006). RGI side chains may control the microenvironment of walls and particularly water activity and matrix flow properties (Ulvskov et al., 2005). In doing so, they were proposed to modulate access of hydrolases to wall components and thus, prevent or retard cell wall disassembly (Smith et al., 2002; Sorensen et al., 2000).

The implication of RGI to wall structuring was particularly revealed with a mutant of potato tubers in which the RGI backbone was fragmented (Oomen et al., 2002). The transformant had aberrant cell wall architecture. In the case of AS-DR12, ripening induced loss of RGI galactan and arabinan side chains to similar extents as in wild-type fruit.

Although wild-type and AS-DR12 fruits samples are collected at the same stage based on their post-anthesis age, the above-observed variations related to extracted pectin chemistry and in situ HG and RGI structures support possible differences in fruit ripening kinetics that may affect wall disassembly. These data also point to differences in cell wall composition and ripening-associated changes between the skin (cuticles, epidermis, and variable number of hypodermal cell layers) and the mesocarp of both...
wild-type and AS-DR12 fruits. Pectin galactan is absent from the skin at the mature green stage. At the red ripe stage, the cells are still adhering with homogalacturonans and cell walls are reacting to both JIM7 and 2F4. The thickness of the pericarp tissue of AS-DR12 can largely contribute to firmness of the fruit, but the modification of the cuticle region in the mutant can also be of importance. Cuticle structure and organization were shown to affect the mechanical properties of tomato pericarp tissue (Allende et al., 2004; Matas et al., 2004) and more generally, the sub-epidermal region of turgid organs is thought to be important for the mechanical properties as it contains the stress of turgid parenchymatous cells (Hejnowicz and Sievers, 1995).

**Conclusion**

AS-DR12 tomato was obtained by altering a response gene to auxin, a hormone with known multiple effects and signalling pathways. Differences in developmental and biochemical characteristics were observed. At this point, it is not possible to decide whether auxin plays a direct or
indirect role in these differences. However, these data highlight several cell wall features at different scales that can contribute to the enhanced firmness of red-ripe AS-DR12 fruit. At the molecular scale, differences in methyl-esterification of HG and lower methyl esterification of residual pectins refractory to extractions with water and oxalate solution can result in a tighter cell wall polymer assembly withstanding better mechanical stresses. At the microscopic scale, histological differences with a higher amount of small cells in the outer sub-epidermal area and modifications of the cuticle region attest for different pericarp tissue architecture in transgenic fruit. At the macroscopic scale, AS-DR12 pericarp tissue is thicker compared to wild type. Thus, the pleiotropic phenotype described for AS-DR12 can be extended to cell wall-related defects at different scales including histology, all of which have, potentially, a contribution to texture. All these features are likely to take place during fruit construction rather than during fruit ripening only. The example of AS-DR12 attests for the importance in considering different scales to address the mechanisms underlying changes in fruit texture. Indeed, while extensive researches addressed the contribution of cell wall disassembly in the softening process of tomato fruit, little attention has been paid to microscopical and macroscopical approaches. Although anatomical features of plant tissue are often mentioned as contributors to texture (Waldron, 2004), only a few studies have really reported on the influence of mechanical properties.

Acknowledgements

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