In muro fragmentation of the rhamnogalacturonan I backbone in potato (*Solanum tuberosum* L.) results in a reduction and altered location of the galactan and arabinan side-chains and abnormal periderm development

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Summary

Rhamnogalacturonan (RG) I is a branched pectic polysaccharide in plant cell walls. Rhamnogalacturonan lyase (*e*RGL) from *Aspergillus aculeatus* is able to cleave the RG I backbone at specific sites. Transgenic potato (*Solanum tuberosum* L.) plants were made by the introduction of the gene encoding *e*RGL, under the control of the granule-bound starch synthase promoter. The *e*RGL protein was successfully expressed and translated into an active form, demonstrated by *e*RGL activity in the tuber extracts. The transgenic plants produced tubers with clear morphological alterations, including radial swelling of the periderm cells and development of intercellular spaces in the cortex. Sugar compositional analysis of the isolated cell walls showed a large reduction in galactosyl and arabinosyl residues in transgenic tubers. Immunocytochemical studies using the LM5 (galactan) and LM6 (arabinan) antibodies also showed a large reduction in galactan and arabinan side-chains of RG I. Most of the remaining LM5 epitopes were located in the expanded middle lamella at cell corners of *e*RGL tubers, which is in contrast to their normal location in the primary wall of wild type tubers. These data suggest that RG I has an important role in anchoring galactans and arabinans at particular regions in the wall and in normal development of the periderm.

Keywords: pectin, fungal *e*RGL, *Solanum tuberosum*, transgenic plant, cell wall modification, immunolabelling.

Introduction

The primary cell wall is composed of several polysaccharides (90%) and some structural proteins (10%) (McNeil *et al.*, 1984). Of these polysaccharides, cellulose and cross-linking glycans form a load-bearing network that is embedded in a matrix of pectic polysaccharides (Carpita and Gibeaut, 1993). The pectic molecules comprise mainly homogalacturonan (HGA) and rhamnogalacturonan I (RG I). HGA is composed of unbranched α-1,4-linked galacturonic acid (GalA) residues. RG I has a backbone composed of repeating α-(1,2)-L-rhamnose(Rha)-α-1,4-D-GalA

disaccharide units. Side-chains, mainly consisting of arabinan and/or galactan, may be attached to the RG I backbone at the C-4 position of the Rha residues (Carpita and Gibeaut, 1993; O'Neill *et al.*, 1990; Schols and Voragen, 1994). The GalA residues in the HGA and RG I backbone may be methyl-esterified and/or O-acetylated (Carpita and Gibeaut, 1993). The functions of the individual pectic polysaccharides are still unclear. They have been implicated in regulating wall porosity, cell separation, cell expansion, organogenesis, textural changes during fruit

ripening and as a source of oligosaccharins which can act as signalling molecules (Aldington and Fry, 1993; Darvill *et al.*, 1992; McCann and Roberts, 1994). The presence and composition of the pectic polysaccharides has been shown to depend not only on the plant tissue, and the position in the cell wall, but also on the developmental stage of the plant (tissue) (reviewed by Willats *et al.*, 2001). These observations support the idea of specific functions for the different pectic polysaccharides in plant cell walls.

Pectin can be modified *in planta* by mutagenesis (Reiter *et al.*, 1997), but this is difficult to achieve in potato because it is a tetraploid crop. A more direct approach is to modify pectin *in planta* by the apoplastic expression of genes encoding glycanase activities (Vincken *et al.*, 2000). Whilst many potential pectin-modifying enzymes are annotated in the *Arabidopsis* database, very few of these have been authenticated by demonstration of a corresponding biochemical activity. However, recombinant glycanases from fungi have well-characterized activities on cell-wall substrates. The introduction of an endo-galactanase from *Aspergillus aculeatus* in potato has been shown to result in the specific removal of part of the galactan sidechains attached to the RG I (Sørensen *et al.*, 2000). Tubers expressing the endo-galactanase developed normally. In this paper, we report the consequences of fragmenting the RG I backbone rather than the attached side-chains.

We have generated potato plants over-expressing *Aspergillus* RG I lyase (*e*RGL: Kofod *et al.*, 1994; Mutter *et al.*, 1996; Mutter *et al.*, 1998), driving expression with the granule bound starch synthase promoter, which is highly active in potato tubers (Visser *et al.*, 1991). All transformants were analysed for RNA expression and the presence and activity of the introduced enzyme in tuber extracts. Furthermore, the effect on cell wall composition was analysed by determining the monosaccharide composition, immunogold labelling and with Fourier Transform Infrared (FTIR) Microspectroscopy. Our results provide evidence that the side-chains of RG I become reduced in abundance and abnormally localised to the middle lamella at cell corners of transgenic tubers. Further, a phenotype of abnormal periderm development is observed, perhaps a consequence of abnormal cell division and cell swelling. This approach provides useful tools with which to evaluate the functions of pectin *in planta*.

Results

*e*RGL transgenic plants make tubers with altered morphology

A construct was made to introduce the *e*RGL from *A. aculeatus* into potato tubers via *Agrobacterium tumefaciens*-mediated plant transformation. The granule bound
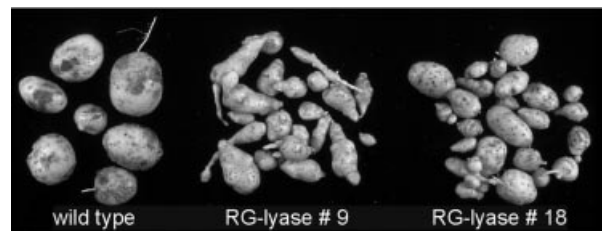

Figure 1. A selection of tubers expressing the *e*RGL and a wild type control.
The two transformant clones, #9 and #18, show a high and low RNA expression of the *e*RGL, respectively.

starch synthase promoter was used to obtain a high expression in potato tubers. Twenty-one individual transformants were obtained and transferred to the greenhouse for tuber production. Compared to wild type (WT), the transformants showed characteristic differences. The transformants grew slower and their tubers were slower to produce a new plant after planting in soil. Crossing experiments demonstrated that the *e*RGL transgenic plants, in contrast to WT plants, produce only a limited number of flowers and have a low male fertility.

The *e*RGL plants produced tubers with a clearly altered morphology (Figure 1). These tubers were smaller compared to WT, had deeper 'eyes' and the tuber surface had a wrinkled appearance. This phenotype was observed in plants with a high (#9) *e*RGL expression, but also in plants with a (relatively) low (#18) expression of the gene. Compared to the low *e*RGL expressers, the high expressers showed a more severe tuber phenotype. The average yield (grams of tuber material per plant) of the *e*RGL transformants was comparable to wild type due to a higher number of smaller tubers produced by each plant.

*e*RGL transformants express the transgenic RNA

DNA was isolated from the leaves of transformed plants and used for Southern analysis. In all plants, which were used for further analysis, the transgene could be detected. Furthermore, the number of T-DNA insertions was shown to vary from 1 to 4 (data not shown). Tubers were harvested and used for RNA isolation followed by Northern blot analysis (Figure 2a,b). A partial cDNA probe, corresponding to the introduced gene, was used with a 28S ribosomal RNA probe as a control (Figure 2b). In all transformants, RNA expression of the transgene could be detected in the potato tubers and a number of high and low expressers were identified (Figure 2a).

*Transgenic enzyme activity: e*RGL *activity correlates to its level of RNA expression*

Transgenic and wild type tuber extracts were made and subsequently measured for *e*RGL activity. Tuber extracts
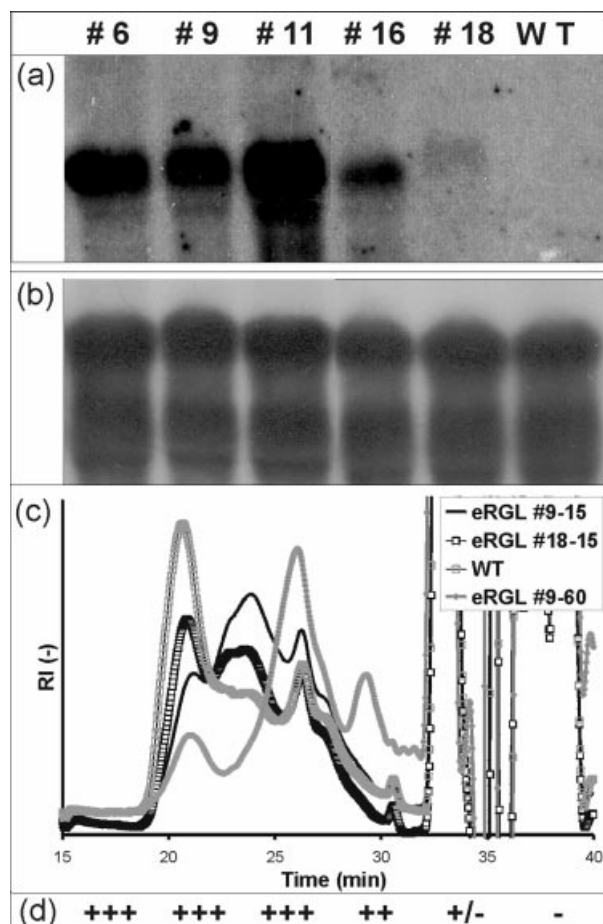
Figure 2. Northern blot analysis and *e*RGL enzyme activity determination in WT and *e*RGL transformants.
(a,b) For all transformants total tuber RNA (40 µg per lane) was separated by gel electrophoresis, transferred onto a nylon membrane and probed using the *e*RGL (a)and ribosomal RNA (b) ^{32}P-labelled cDNAs.
(c) HPSEC elution patterns of the degradation of a saponified apple RG preparation by different potato tuber extracts. *Blocked Grey line* is representative for incubations with WT extracts and inactivated *e*RGL extracts. *Black line e*RGL #9, 15 min incubation. *Circled Grey line e*RGL #9, 60 min incubation. *Blocked black line e*RGL #18, 15 min incubation. The peaks from retention time 32 min onwards result from salts and other impurities present in the crude protein extracts.
(d) Based on the results as shown for *e*RGL transformants #9 and #18 in panel c, the relative enzyme activity of these and other transformants is expressed as + + + for the highest activity and – for no detectable activity.

Table 1. Glycosyl residue composition (mol%) of the cell wall material isolated from tubers of wild type and rhamno-galacturonan lyase expressing potato plants. Values represent the average of a triplicate analysis ± the variance

	WT	eRGL #9
Rha	1.4 ± 0.11	0.9 ± 0.25
Ara	8.6 ± 0.10	5.2 ± 0.11
Xyl	2.4 ± 0.13	4.4 ± 0.07
Man	1.8 ± 0.04	2.5 ± 0.09
Gal	21.4 ± 1.83	4.9 ± 0.05
Glc	41.8 ± 0.26	45.1 ± 0.22
uronic acid	22.6 ± 2.12	37.0 ± 0.28

showed varying enzyme activity levels among the different transformants; the MHR-S populations were clearly further degraded upon treatment with the *e*RGL #9 extract than with the *e*RGL #18 extract. Furthermore, the level of *e*RGL enzyme activity for these transformants is clearly correlated to the RNA expression levels determined by Northern blot analysis (Figure 2a). Transformants #9 and #18 were subsequently marked as high and low expressers, respectively (Figure 2d). The data above are in line with a rapid screening of WT and transgenic tuber cell walls by FTIR microspectroscopy (data not shown), which demonstrated that complex changes had occurred in the transgenic walls.

Monosaccharide analysis shows gross changes in the composition of e*RGL* cell walls

Cell wall material (CWM) was isolated from WT and *e*RGL expressing tubers (transformant #9). Comparison of sugar composition from the *e*RGL transformants with WT showed some clear changes in the different cell wall components (Table 1). The *e*RGL #9 transformant has a markedly lower galactose (4.9 and 21.4 mol%, for *e*RGL #9 and WT plants, respectively) and arabinose content (5.2 and 8.6 mol%, for *e*RGL #9 and WT plants, respectively). These decreases in galactose and arabinose are in line with the decrease in rhamnose (0.9 and 1.4 mol%, for *e*RGL #9 and WT plants, respectively), one of the backbone residues of RG I. Finally, this transformant shows an increase in the uronic acid content (37.0 and 22.6, for *e*RGL #9 and WT plants, respectively). The sugar composition of *e*RGL #18 CWM showed similar, but less pronounced changes as that of *e*RGL #9 when compared to WT CWM (data not shown).

WT and eRGL CWM have different extraction characteristics

The isolated CWM from WT and *e*RGL #9 was sequentially extracted with a series of solvents to determine: (i) if the

were added to saponified (saponification removes acetyl groups which inhibit the enzyme activity) RG from apple (MHR-S; Schols *et al.* 1990b), and after 15 min and 1 h incubations, degradation of the MHR-S was analysed by high performance size exclusion chromatography (HPSEC). Extracts from WT tubers had no effect on the HPSEC elution pattern, but the *e*RGL transformants were able to degrade the polymeric population of apple RG-S into oligomers, as seen by a shift in the molecular weight distribution (Figure 2c). After 1-h incubations, all extracts from the different *e*RGL transformants gave the same degradation pattern. However, the 15 min incubations

Table 2. Glycosyl residue composition (mol%) of various extracts from cell wall material isolated from tubers of wild type and rhamnogalacturonan lyase expressing potato plants. The values represent the average of a duplicate analysis. All extracts have been corrected for the presence of starch as detailed in the section experimental procedures

	Rha	Ara	Xyl	Man	Gal	Glc	uronic acid
WT-ChSS	3.7	13.7	2.1	3.2	52	4.2	21.0
WT-DASS	2.9	11.6	2.9	10.2	43.5	5.8	18.9
WT-1 M ASS	0	8.8	19.3	3.3	27.5	38.5	2.2
WT-6 M ASS	4.6	16.8	4.1	2.6	50	8.2	13.8
WT-residue	0.5	1.8	1.8	1.4	1.5	84.7	8.4
*e*RGL #9-ChSS	1.8	5.7	1.8	2.2	9.8	3.6	75.2
*e*RGL #9-DASS	4.3	12.8	5.0	5.0	14.2	9.9	58.9
*e*RGL #9–1 M ASS	0	12.4	23.7	5.7	6.7	47.4	3.6
*e*RGL #9–6 M ASS	3.1	14.6	13	7.8	15.1	26	20.8
*e*RGL #9-residue	0	2.4	0.7	1.2	3.9	87.1	4.5

various polymers from WT and *e*RGL #9 CWM have different extraction characteristics, and (ii) to obtain fractions that are enriched in RG I with side chains. Table 2 shows that the extraction efficiency was good because there were only small quantities of sugars left in the final residue apart from Glc, which is celllulose. As expected, the chelator soluble solid (ChSS) and dilute alkali soluble solid (DASS) extracts contain mainly pectic polysaccharides, whereas the 1M alkali soluble solid (ASS) extract was rich in xyloglucan and some (arabino)galactan. Typically, the 6 M ASS fractions are rich in pectic polysaccharides (besides xyloglucan). The pectin-containing fractions (ChSS, DASS, 6 M ASS) of *e*RGL #9 tuber CWM are distinguished from those of WT tuber CWM by their much lower galactosyl and much higher uronosyl content. This is in accordance with the data in Table 1.

Figure 3(a) indicates the proportion of the various polysaccharides extracted from the total WT and *e*RGL #9 CWM. The most important conclusion from these data is that the RG I-rich 6 M ASS fraction from both WT and *e*RGL #9 potato tuber CWM (52 and 34%, respectively) forms an important part of the cell wall. The diagrams indicate that polymers are easier extracted from the transgenic CWM than from WT, because more material is recovered with the relatively mild extractants in the case of *e*RGL #9.

RG I is degraded in the transgenic e*RGL* #9 tubers

In order to investigate if the *e*RGL had fully degraded its substrate in the transgenic tuber walls, isolated CWM of WT and *e*RGL #9 tubers were treated *in vitro* with a fungal *e*RGL and the resulting digest analysed for diagnostic RG I oligomers by HPAEC. Release of oligosaccharides was not observed in this experiment, probably because the fungal *e*RGL could not access its substrate in the isolated CWMs. Therefore, the 6 M ASS extracts, which are large fractions, rich in RG I, were treated with the fungal *e*RGL. Figure 3(b)
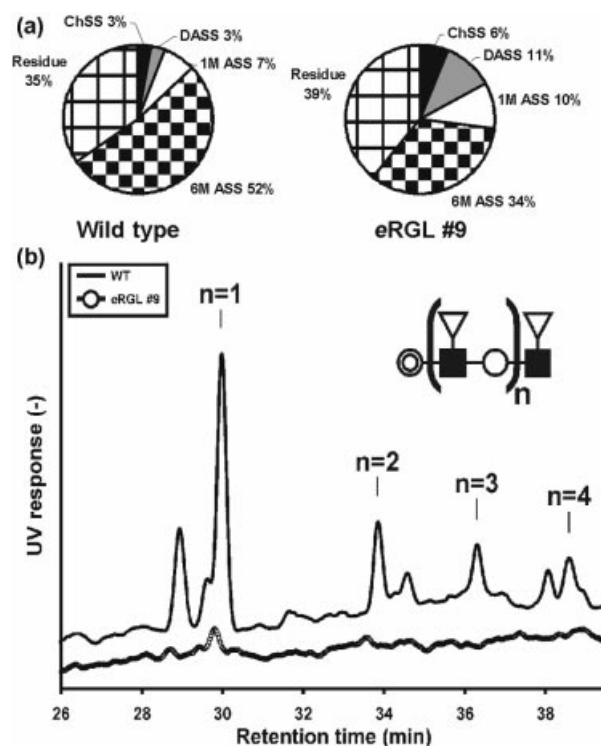

Figure 3. (a) Pie charts showing the amount (% w/w) of polymers, sequentially extracted from cell wall material of wild type and transgenic potato tubers using different solvents.
(b) HPAEC elution patterns of 6 M ASS extracts from wild type and transgenic potato tuber cell wall material, which was treated with a fungal *e*RGL. Diagnostic oligosaccharides are indicated by $n = 1$–4, and were identified by comparison of their retention times to those of a digest with known composition (Mutter *et al.*, 1998). The structure of the repeated oligomeric moiety is shown; Squares indicate Rha residues; circles, GalA residues; concentric circles, unsaturated Δ4,5-GalA residues; triangles, Gal residues. The control digests (6 M ASS material incubated with inactivated enzyme) have a similar appearance as the lower line. For clarity, these have been omitted from the figure.

clearly shows that the fungal *e*RGL releases the typical, galactosylated RG oligosaccharides only from the WT-6 M ASS material (Mutter *et al.*, 1998). In contrast, only traces

of oligosaccharides were released from the transgenic 6 M ASS fraction, indicating that the eRGL substrate was depleted *in muro* from the transgenic tuber walls during development. Incubation of the extracts with inactivated fungal eRGL yielded a chromatogram comparable to that of eRGL #9. These results demonstrate that the majority of the RG I backbones were degraded in the transgenic potato tubers.

eRGL expressing tubers show a developmental phenotype

Comparison of toluidine-blue stained sections of WT and eRGL-expressing tubers, showed differences in the periderm and cortex (Figure 4). The normal appearance of the WT periderm is that of ordered layers of cells (generated by periclinal divisions) which become flattened and suberized as they mature (Figure 4a). The underlying cortex is arranged into roughly concentric rows of cells containing few starch granules and intercellular spaces (Figure 4a). In contrast, the periderm of the high eRGL-expressing tubers (#9) is disorganized (Figure 4b): there is a complete absence of ordered cell layers, due to the random division planes and expansion of the periderm cells. The periderm of the low expressing transformants (#18, data not shown) is more similar to WT, except that the cells in the peripheral layers expand isodiametrically. Compared to WT tubers, both high and low eRGL-expressers show marked increases in the number and size of cortical intercellular spaces (Figure 4b), but the perimedullary and vascular tissues exhibit a WT appearance.

Galactan and arabinan epitopes are reduced in abundance and appear at altered locations in eRGL tuber walls

The mAb that recognizes Sycamore RG I, CCRC-M2 (Puhlmann *et al.*, 1994), does not cross-react with potato (Bush and McCann, 1999). However the mAbs LM5 and LM6 recognize (1→4)-β-D-galactan and (1→5)-α-L-arabinan, respectively, which are components of RG I side-chains and hence are indirect markers of the RG I backbone. We used reflectance confocal laser scanning microscopy

(CLSM) and transmission electron microscopy to image immunogold-labelled sections of potato tuber from WT and eRGL transformants #9 and #18. The distribution of the
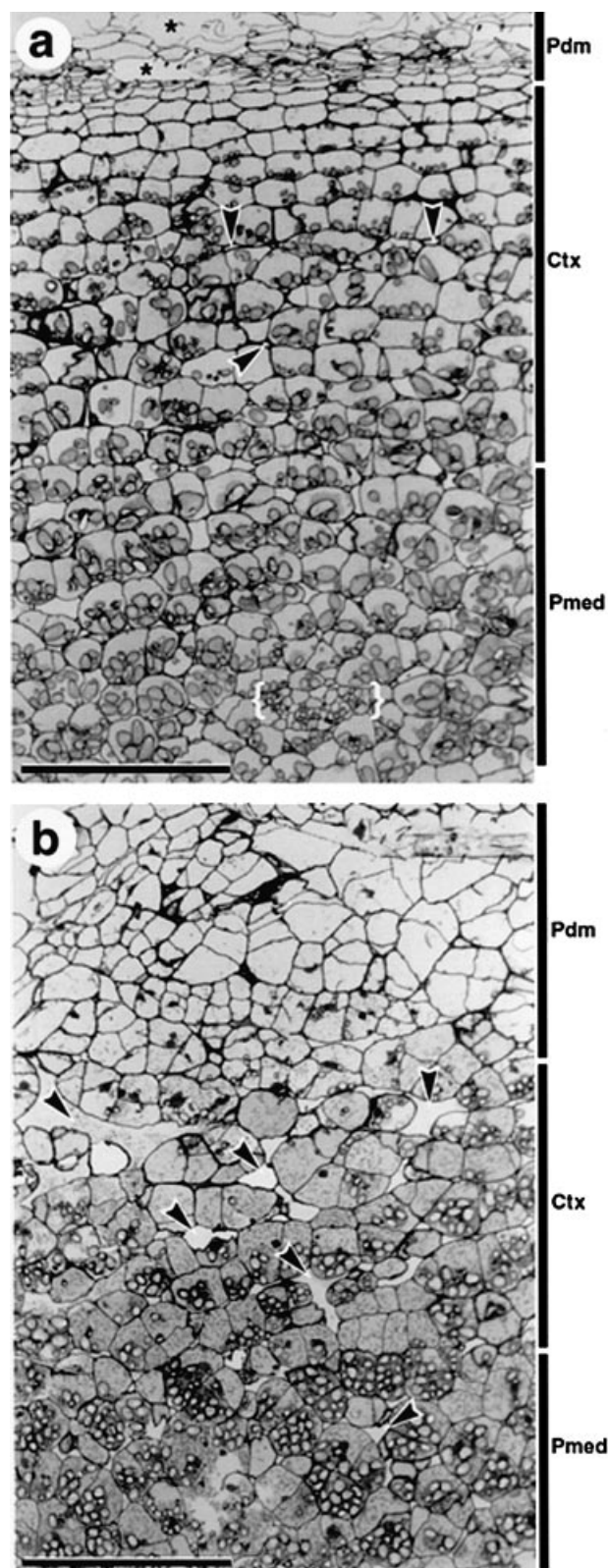

Figure 4. Micrographs of toluidine-blue-stained 0.5 μm-thick resin sections of wild type (a) and high eRGL (#9) expressing (b) tubers.
The wild type periderm (Pdm) is composed of regularly arranged flattened cells, but has poor sectioning qualities (asterisks indicate tears in the section); in contrast the transgenic periderm consists of randomly-arranged swollen cells. Large intercellular spaces (arrowheads), which are absent in the wild type tuber, are a characteristic feature of the transgenic cortex (Ctx). Scale bar represents 0.5 mm; Pmed, perimedullary tissue.
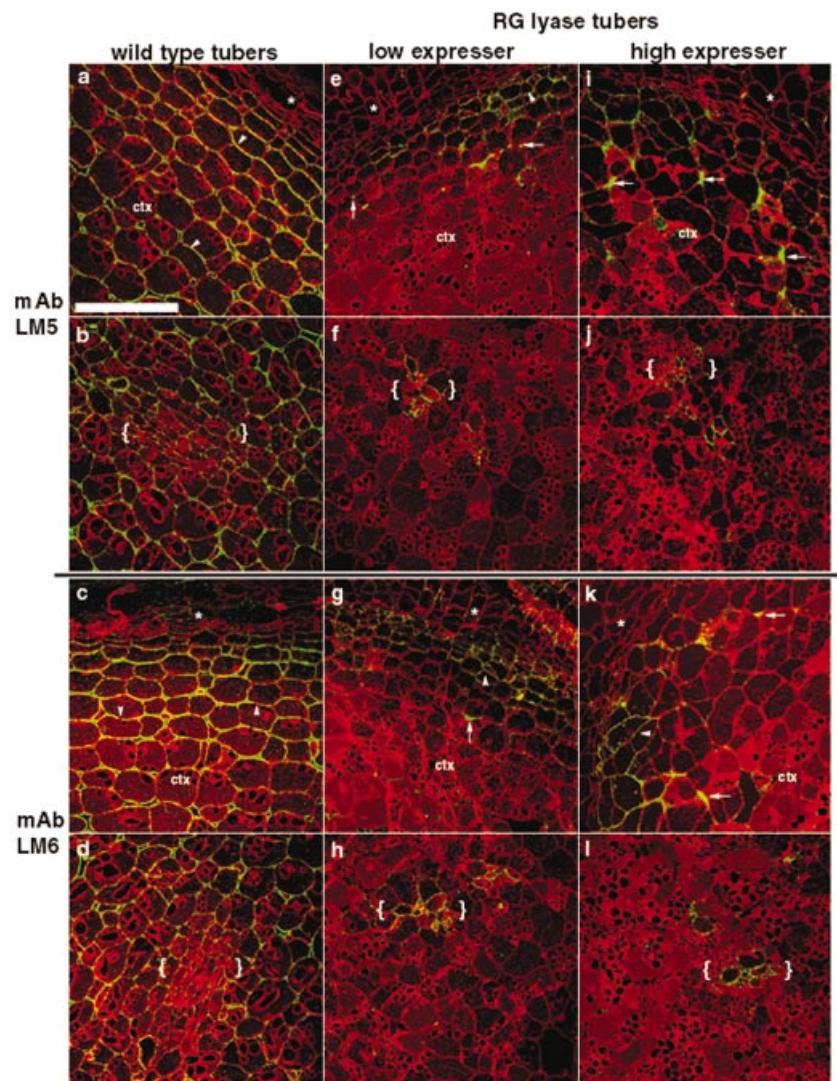
RG lyase tubers

wild type tubers | low expresser | high expresser

mAb LM5

mAb LM6

Figure 5. Confocal scanning laser microscope images of wild type and *e*RGL expressing (#9 high expresser, #18 low expresser) tubers immunogold-labelled with LM5 (upper panel) and LM6 (lower panel) that recognize 1,4-galactan and 1,5-arabinan epitopes, respectively.

Images a, c, e, g, i and k show periderm (asterisk) and cortical (ctx) tissues, whilst b, d, f, h, j and l show vascular tissue (bracketed) from the perimedullary region of the tuber. Wild type cell walls (arrowheads) of starch parenchymal cells contain abundant galactan (a, b) and arabinan (c, d) epitopes, the vascular walls are less enriched in these components (brackets in b and d). These epitopes are barely detectable in most starch parenchymal cell walls from the transgenic tubers (e-l), notable exceptions are some cells from the outer cortex (arrowhead in e, g and k) and the vascular walls (f, h, j and l), of which the latter ones show labelling intensities similar to the WT situation. LM5 and LM6 strongly label a few cell corners in the transgenic cortical tissues (arrows in e, g, i and k). Scale bar is 200 μm and applies to all images.

LM5 and LM6 epitopes is well-documented in WT tubers (Bush and McCann, 1999; Bush *et al.*, 2001). Essentially, the WT periderm shows gradients of both epitopes (most abundant towards the cortex), the parenchymal cell walls throughout the tuber label strongly with both mAbs, whilst the vascular walls show relatively lower abundancies of both epitopes (Figure 5). The low and high expressing *e*RGL tubers gave similar labelling results to each other with both mAbs: the periderm walls labelled similarly to WT (compare Figure 5a,c with Figure e,g,i,k). However, in low *e*RGL-expressers, galactan and arabinan epitopes were detected in the walls of the outer cortical cells, but deeper into the cortex, they were restricted to just a few cell corners (Figure 5e,g); in high *e*RGL-expressing tubers, they only occurred at a few cortical cell corners (Figure 5i,k). In the perimedullary tissue that comprises the bulk of the tuber, both epitopes were only detectable in

the walls of the vascular tissue where the labelling intensities were unaffected and comparable to WT levels (compare Figure 5b,d with Figure f,h,j,l).

In the electron microscope, it is clear that in WT tubers, the galactan epitope is restricted to the primary wall of parenchymal cells and absent from the middle lamella, whilst the arabinan epitope occurs throughout the walls, but both epitopes are absent from the expanded middle lamella at corners (Figure 6a,b).

In all *e*RGL transformants, both galactan and arabinan epitopes are greatly reduced throughout parenchymal walls, except in vascular cell walls (Figure 6c). The labelling of cell corners with mAb LM5 seen by CLSM is due to an altered location of the epitopes from their normal WT location in the primary wall to the expanded middle lamella or lining of intercellular spaces at cell corners (Figure 6d,e). The tuber cells showed characteris-

Figure 6. Transmission electron micrographs of cell walls from wild type (a-b) and low *e*RGL (#18) expressing (c-f) tubers immunolabelled with LM5 (a, c-e) and LM6 (b and f).

In wild type starch parenchymal walls galactan (LM5) is restricted to the primary wall (a, arrowhead), whilst arabinan (LM6) is only absent from the middle lamella (asterisk) where it expands at corners (b). Starch parenchymal cells (sp.) in transgenic tubers have considerably reduced amounts of galactan (c, d and e) and arabinan (f) in their walls (arrowhead), but vascular parenchymal cells (vp in c) retain relatively high amounts of both galactan (c) and arabinan (not shown) epitopes. Epitopes are excluded from the primary wall and localised in the expanded middle lamella (asterisk in d) and lining of intercellular spaces (e) at some cortical corners. Starch parenchymal cells contain abundant biosynthetic organelles. Scale bars a, b and e represent 2 μm, d and f 4 μm and c 10 μm; st, sieve tube.
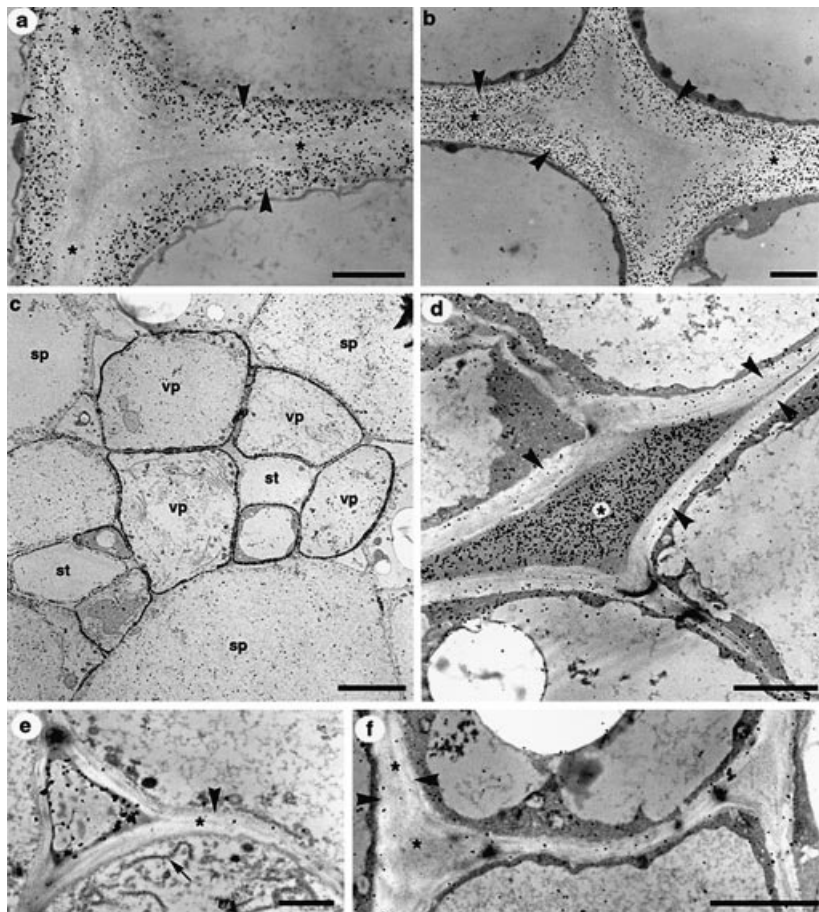
tic features that were absent from WT cells: these were the increased abundance of biosynthetic organelles (Figure 6e) and the presence of a diffuse cytoplasmic label.

Discussion

This study reports the introduction into potatoes of an *A. aculeatus* gene that encodes a rhamnogalacturonan lyase (*e*RGL). The aerial portions of *e*RGL transformed plants appear normal, but the plants produce tubers with an altered morphology and pollen with a low fertility. The altered tuber morphology is a consequence of changes in the periderm and cortex tissues of the transgenic tubers, the result of random cell divisions and abnormal expansion in these tissues. It appears that RG I is necessary for normal periderm development, perhaps by constraining the expansion of periderm cells during development, but the exact underlying reason is not yet clear.

The isolation of CWM, followed by sugar compositional analysis showed a change in several of the constituent pectic monosaccharides (Table 1). These changes involve a reduction of both galactose and arabinose, the major constituents of RG I side-chains. Furthermore, the relative proportion of homogalacturonan was increased.

Consistent with these data, immunocytochemical studies (Figure 5 and Figure 6) show a reduction in the abundance of galactan and arabinan epitopes (side-chains of RG I) and an altered location of the residual galactan epitopes in parenchymal cell walls. Considering that the *e*RGL can only degrade the RG I backbone (Kofod *et al.*, 1994), the subsequent altered location of the galactan and arabinan can only be explained if they are not cross-linked to other wall polymers.

The GBSS promoter is activated during the process of stolon initiation followed by the tuber formation (Visser *et al.*, 1991). During this process of tuber development *e*RGL is released into the apoplast of the stolons. Once the *e*RGL is released into the wall, it may fragment the RG I present in the wall or newly synthesized and secreted RG I molecules before they are integrated into the wall. *e*RGL cleaves the RG I backbone (at alternating regions of α-L-Rha-(1,4)-α-D-GalA bearing single unit Gal side-chains attached to Rha; (Kofod *et al.*, 1994; Mutter *et al.*, 1998)) to give Rha-GalA fragments of DP 4–10 with Rha at the reducing end, and possibly also larger fragments. The loss

and altered location of the LM6 epitope and the decreased arabinose content is less profound compared with the decrease in galactan. The monosaccharide compositional data confirm these observations. This suggests a larger decrease of RG I with galactan side-chains compared to RG I with arabinan side-chains. A possible explanation is the higher affinity of the *e*RGL for the galactose-containing RG I which is in line with the kinetic studies of Mutter *et al.* (1998).

In WT plants, the LM5 epitope is found in the primary cell wall, the LM6 epitope occurs throughout walls, but both are excluded from the expanded middle lamella at cell corners. The loss and altered location of the majority of the LM5 and LM6 epitopes suggests that the RG I has been degraded into smaller fragments which are subsequently re-distributed into the middle lamella and cell corners. This suggests that the RG I backbone is important for anchoring these side-chains within the primary wall. The galactan and arabinan side-chains are thus not likely to be cross-linked to other immobile wall molecules. This is in line with ^{13}C NMR spectroscopic studies of onion pectin which show that galactan side-chains are highly mobile but glycosidically linked to RG I (Foster *et al.*, 1996; Ha *et al.*, 1996). The mechanism by which the fragments are subsequently re-distributed is as yet unknown, but the process of phase separation proposed by MacDougall *et al.* (1997) to be involved in cell wall assembly is a possible mechanism.

There is little effect on the distribution of LM5 and LM6 epitopes in the outer cortical walls and vascular tissue of the *e*RGL expressing tubers. Both of these tissues are characterized by a reduction in the number of intracellular starch granules, so presumably the GBSS promoter is relatively inactive here (compared to the perimedullary parenchymal cells) and hence it would be expected that the *e*RGL activity would also be reduced. It is also possible that the turnover of the RG I in these walls is greater than for other cells, or that the RG I in these cells has a higher degree of acetylation, resulting in a less efficient degradation by the eRGL (Kauppinen *et al.*, 1995; Kofod *et al.*, 1994; Schols *et al.*, 1990a). Further, the vascular walls of WT tubers contain more LM6 arabinan than LM5 galactan epitopes (Bush and McCann, 1999). The reduced affinity of *e*RGL for the arabinan-containing RG I (Mutter *et al.*, 1998), could be another explanation for the relatively low reduction of LM5 and LM6 epitopes in these tissues.

Our results prove that the *e*RGL has indeed been active and able to modify the composition of the tuber cell wall, which resulted in several modifications, including a decrease in RG I, and a relative increase in homogalacturonan. The histological appearance and tuber morphology may be a result of the re-location and removal of the RG I backbone from the cell wall.

The degradation of the RG I backbone correlates to a decrease of both galactan and arabinan that are normally attached to this backbone. Different studies (as reviewed by Willats *et al.*, 2001) have shown that different forms of RG I (bearing either galactan or arabinan) may occur in a developmental fashion at specific locations in the plant suggesting that they have a specific function. The deposition and degradation of galactan has been correlated with the firmness of several plant tissues (Jones *et al.*, 1997; McCartney *et al.*, 2000; Redgwell *et al.*, 1997). However, Sørensen *et al.* (2000) showed that in transgenic tubers expressing an endo-1,4-β-galactanase in the apoplast, the galactose content of the wall is reduced to 30% of the wild type, but this had no effect upon the histological or morphological structure of tubers. One explanation for this is that the removal of galactan by the endo-galactanase was not co-ordinated with other developmental processes in the plant, and that therefore the impact on the tuber's morphology and histology was less pronounced.

The specific removal of RG I arabinan side-chains by the expression of an apoplast-directed endo-1,5-α-arabinanase (Skjøt *et al.*, 2002) resulted in transgenic potato plants with severe phenotypic changes: the plants did not produce side shoots, flowers or stolons, and were unable to produce tubers (Skjøt *et al.*, 2002). When the same endo-arabinanase was targeted to the Golgi complex, the potato plants and tubers showed no detrimental phenotype, but tuber cell walls contained significantly reduced levels of arabinose and LM6 epitopes (Skjøt *et al.*, 2002). Furthermore, the *Arabidopsis mur4* mutant (Burget and Reiter, 1999), showing a reduction of L-arabinose to 50% of WT levels, does not show any phenotypic changes. Although arabinan side-chains are developmentally and spatially regulated structures (Bush *et al.*, 2001; Orfila and Knox, 2000; Orfila *et al.*, 2001; Willats *et al.*, 1999; 2001) they have yet to be linked to a physiological function.

Even though RG I galactan and arabinan side-chains have an, as yet enigmatic, role to play *in planta*, the backbone must also play an important role in the integrity and function of the wall, since its degradation by *e*RGL in potato tubers results in morphological changes. This function may be to secure side-chains and possibly other polysaccharides to specific locations in the wall. Our experiments confirm that the approach of introducing fungal cell wall degrading enzymes *in muro*, can be very useful for attributing a biological significance to a particular wall polysaccharide.

Experimental procedures

Materials

The cDNA vector pYES2/*e*RGL, containing the *e*RGL from *A. aculeatus* (Kofod *et al.*, 1994), was a gift from S. Kauppinen (Novo Nordisk A/S, Bagsværd, Denmark). The α-amylase and

pullulanase to de-starch cell walls were obtained from Boehringer (Alkmaar, The Netherlands) and Megazyme (Bray, Ireland), respectively.

Vector construction and transformation of potato plants

The granule bound starch synthase promoter was amplified by PCR from the vector pPGB121s (Visser *et al.*, 1991) with primers 5′GATTACGCCAAGCTTTAACG3′ and 5′GGTTTTGTCGACGAA-ATCAGAAATAATTGGAGG3′ introducing a *Hind*III site 5′-and a *Sal*I site in the 3′ end of the PCR product. Subsequently the fragment was cloned into the pBI121 vector (Datla *et al.*, 1992) as a *Hind*III/*Sal*I fragment. This pPGB121s-new vector was digested with the restriction enzyme *Sal*I, blunt-ended with Klenow enzyme, and after heat inactivation of the two enzymes further digested with the restriction enzyme *Xba*I. The vector pYES2/ *e*RGL was digested with the restriction enzyme *Bam*HI, blunt ended with Klenow enzyme, and after heat-inactivation of the two enzymes further digested with the restriction enzyme *Xba*I. The treated vector and the cDNA insert were purified by agarose gel electrophoresis and ligated creating the DNA construct pPGB121s-new-*e*RGL.

In vitro shoots of the *Solanum tuberosum* cultivar Karnico were used for *Agrobacterium tumefaciens* mediated transformation (Visser *et al.*, 1989). After regeneration of *in vitro* shoots on selective kanamycin medium, the shoots were transferred to the greenhouse to generate mature plants.

Northern analysis

RNA was extracted from 1 tuber of each transgenic line as described by Kuipers *et al.* (1994). RNA gel blotting and hybrid-isation was performed using 40 μg of tuber RNA per sample, as described by Sambrook *et al.* (1989). The membranes were hybridized with a ^{32}P-ATP labelled 1-kb *Kpn*I-*Xba*I fragment of pYES2/*e*RGL. A 2.3-kb *Eco*RI fragment of a potato 28S ribosomal RNA gene was used as a control (Landsmann and Uhrig, 1985).

Activity measurements

Frozen potato tissue was ground to a powder under liquid N_2 using a mortar and pestle. Approximately 1 g of the ground tissue was then homogenized using an Ultra-Turrax TP 18–10 (14 000 rotations min^{-1}, Ika Werk, Staufen, Germany) in 2 ml 0.25 M sodium phosphate buffer (pH 6.5, 4°C) containing 0.4 M NaCl. After 1 h at 4°C (with periodic shaking), the suspension was centrifuged (10 min, 2000 *g*) and the supernatant was used as a crude enzyme extract (Vincken *et al.*, 1997).

A portion (25 μl) of this supernatant was added to 225 μl (or 5 and 245 μl in case of high activity) 0.25% (w/v) solution of saponified rhamnogalacturonan from apple (modified hairy regions, MHR (Schols *et al.*, 1990b) in 0.1 M sodium phosphate buffer pH 5.0. The use of apple RG fractions in this experiment is supported by its well described degradation patterns and its easy extractability (in contrast to potato RG). After a 15-min, or overnight, incubation at 40°C in a head over tail-mixer, the enzymes were inactivated by heating for 5 min at 100°C. Fragmentation of the saponified apple RG was determined using HPSEC (BioGel TSK 40XL, 30XL, and 20XL columns in series) (Mutter *et al.*, 1996; Schols *et al.*, 1990a; Vincken *et al.*, 1997).

Isolation of cell wall material from potato tubers

For each isolation of cell wall material (CWM), 300 g (FW) of frozen potato tuber cubes was ground to a fine powder under liquid N_2. The tissue was homogenised in a mixed-cation buffer (10 mM NaOAc, 3 mM KCl, 2 mM MgCl$_2$, 1 mM CaCl$_2$, pH 6.5), containing Triton 100 (2 mg ml^{-1}), using an Ultra-turrax homo-genizer. The residue was washed with mixed-cation buffer, resuspended in 50% acetone and extracted by saturation with phenol. This residue (washed with mixed-cation buffer) was cryomilled, added to mixed-cation buffer and heated for 20 min at 70°C. Starch was removed by an overnight incubation with α-amylase and pullulanase. When starch removal was complete the cell walls were filtered on a grade 3 sintered glass funnel and sequentially dried in 50, 75, 90 and 100% acetone. (The method is described in more detail as method 1 A in Jardine *et al.*, 2002 for publication).

Sequential extraction of CWM of wildtype (WT) potato and transformant eRGL #9

Potato CWM (1.5 g) was sequentially extracted with 0.5 M imidazole-HCl, pH 7 (3 times 150 ml) at room temperature for 2 h (Chelating Agent Soluble Solids, ChSS), and washed with distilled water (2 times 200 ml); the water and ChSS extracts were combined. After this, the forth-coming residue was extracted with 0.05 M NaOH (3 times 150 ml) at 4°C for 2 h (Dilute Alkali Soluble Solids, DASS). Subsequently, 1 M KOH containing 0.02 M NaBH$_4$ (3 times 150 ml) was used as an extractant at room temperature for 2 h (1 M Alkali Soluble Solids, 1 M ASS). Finally, the residue was extracted with 6 M KOH containing 0.02 M NaBH$_4$ (3 times 150 ml) at room temperature for 2 h (6 M Alkali Soluble Solids, 6 M ASS), and washed with distilled water (3 times 200 ml); the water and 6 M ASS extracts were combined. After each extraction step, solubilised polymers were separated from the insoluble residue by filtration over a grade 3 sintered glass funnel. When changing extractants, the funnel was rinsed in the opposite direction with the new extractant applying low air pressure. The filtrate was collected and added to the next extraction step. All extracts were acidified to pH 5.2 (if necessary) by glacial acetic acid, dialysed against distilled water, concentrated by vacuum evaporation, dialysed again, and finally freeze-dried. The 6 M ASS fraction was dialysed against tap water before dialysing against distilled water. The final residue was suspended in water, acidified to pH 5.2, dialysed and freeze-dried.

Degradation of the 6 M ASS fraction of WT and eRGL #9 potato CWM

The 6 M ASS fraction of both WT and *e*RGL #9 (10 mg) was suspended in 5 ml of a 0.05-M NaOAc buffer pH 5 containing 0.01% NaN$_3$ at 80°C. Subsequently, 490 μl of this substrate solution was incubated head-over-tail at 40°C with 10 μl (0.08 mg protein/ml) of the commercially available *e*RGL (Batch PPJ 4471; Novo Nordisk, Bagsvoerd, Denmark). After 16 h the incubation mixtures were heated at 100°C for 15 min to inactivate the enzyme, and then centrifuged (10 min, 20 000 *g*). The deg-radation products in the supernatant were analysed by High-Performance Anion-Exchange Chromatography (HPAEC) at pH 12 as described by Daas *et al.* (1998). Samples were separated on a CarboPac PA-1 column preceded by a CarboPac PA-100 guard column. A 40-min linear gradient of 0.1–0.5 M NaOAc in 0.1 N NaOH at a flow rate of 1 ml min^{-1} was used, followed by a 5-min

gradient to 1 M NaOAc. After 5 min of elution with 1 M NaOAc in 0.1 N NaOH, the column was equilibrated with 0.1 M NaOAc in 0.1 N NaOH for 15 min. The compounds were detected with a PAD-detector (Electrochemical detector ED40, Dionex, Bavel, The Netherlands) in series with a UV-detector (Spectrasystem UV1000) set at a wavelength of 235 nm. A 0.25% w/v solution of saponified apple MHR was digested with fungal eRGL under the same conditions as the 6 M ASS fractions for peak identification. To ensure that the observed degradation was not caused by the incubation conditions, enzyme blanks containing 490 μl of 6 M ASS fraction and 10 μl of the inactivated enzyme were analysed.

Immunogold labelling and microscopy

Small pieces of cortex and perimedullary tissue cut from freshly harvested wild type and eRGL transformed tubers were fixed in 2.5% (v/v) glutaraldehyde-0.05 M sodium cacodylate containing 0.05% (v/v) NP-40 (Sigma, Poole, UK) and then processed for low temperature L.R. White resin embedding, as described previously (Bush and McCann, 1999). Tissue sections (0.5 μm and ultra-thin) were labelled with mAbs LM5 (recognizes (1→4)-β-D-galactan (Jones et al., 1997)) and LM6 (recognizes (1(r)5)-α-L-arabinan (Willats et al., 1998)), followed by silver enhancement of 5 nm gold conjugates (Bush and McCann, 1999). Gold-labelled and silver-enhanced resin sections were examined by confocal laser scanning microscopy or by transmission electron microscopy as described previously (Bush and McCann, 1999).

Analytical procedures

Residual starch was determined using the Boehringer (Mannheim) enzymatic package. Neutral sugar composition was determined by gas chromatography according to Englyst and Cummings (1984), using inositol as an internal standard. The samples (for CWM and the residue) were pretreated with 72% (w/w) H_2SO_4 (1 h, 30°C) followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100°C and the constituent sugars were analysed as their alditol acetates. Sugar composition analysis of the various extracts was performed according to Englyst and Cummings (1984) using 2 M TFA (for 3 h at 121°C) instead of H_2SO_4 for polysaccharide hydrolysis. It should be noted that during the extraction procedure some residual starch in the CWM is also extracted, notably in the 1 M ASS and DASS fractions. The values for ChSS, DASS, 1 M ASS and 6 M ASS have been corrected for the presence of starch. For this, it was assumed that (i) all cellulose is present in the residue (ii) the residue contains no starch (iii) all xylosyl residues are derived from xyloglucan (only small amounts of xylan are present in potato (Ryden and Selvendran, 1990)) and (iv) the Glc : Xyl ratio in potato xyloglucan is 2 (Vincken et al., 1996). With this, the amount of glucose derived from starch can be estimated for the various extracts, which was subsequently subtracted from the total amount of glucose in these fractions.

Uronic acid content was determined by the automated colorimetric *m*-hydroxydiphenyl assay (Blumenkrantz and Asboe-Hansen, 1973; Thibault, 1979) using an auto-analyser (Skalar Analytical BV, Breda, The Netherlands). Corrections were made for interference by neutral sugars present in the sample.

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