Pectin engineering: Modification of potato pectin by *in vivo* expression of an endo-1,4-β-D-galactanase

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Potato tuber pectin is rich in galactan (oligomer of β-1,4-linked galactosyl residues). We have expressed a fungal endo-galactanase cDNA in potato under control of the granule bound starch synthase promoter to obtain expression of the enzyme in tubers during growth. The transgenic plants displayed no altered phenotype compared with the wild type. Fungal endo-galactanase activity was quantified in the transgenic tubers, and its expression was verified by Western blot analysis. The effect of the endo-galactanase activity on potato tuber pectin was studied by Fourier transform infrared microspectroscopy, immuno-gold labeling, and sugar analysis. All analyses revealed alterations in pectin composition. Monosaccharide composition of total cell walls and isolated rhamnogalacturonan I fragments showed a reduction in galactosyl content to 30% in the transformants compared with the wild type. Increased solubility of pectin from transgenic cell walls by endo-polygalacturonase/pectin methylesterase digestion points to other changes in wall architecture.

Pectic polysaccharides comprise between 30 and 50% of the cell walls of dicotyledonous plants (1). The pectic matrix of plant cell walls is a complex mixture of homogalacturonan (HGA), rhamnogalacturonan I (RGI), and rhamnoglacturonan II polymers (2). HGA is an unbranched chain of α-1,4-linked galacturonic acid (GalA) residues that can be differentially methyl-esterified and/or acetylated. RGI is a branched heteropolymer of alternating α-1,2-linked rhamnose and α-1,4-linked GalA residues (3) that carries neutral side-chains of predominantly 1,4-β-D-galactose and/or 1,5-α-L-arabinose residues attached to the rhamnose residues of the RGI backbone. RGI side-chains may be decorated with arabinosyl residues (arabinogalactan I) or other sugars, including fucose, xylose, and mannose. The highly conserved rhamnogalacturonan II molecule has a HGA backbone with side-chains containing the richest diversity of sugars and linkages known and can dimerize through a borate di-ester cross-link (4). Although the composition of these molecules is well characterized, their assembly into higher-order structures and their function in wall architecture is unclear.

Diverse roles have been proposed for pectic polymers (5–9), including the regulation of cell-cell adhesion, cell expansion, wall mechanical properties, mediation of wall porosity, a source of signaling molecules (oligosaccharins), and involvement in cell differentiation and organogenesis. To address the function of pectins in these processes, we need to manipulate the quantities of different types of wall polysaccharides and examine the downstream consequences. One strategy is the random generation of cell wall mutants with altered monosaccharide composition (10). Only one of these, the *Arabidopsis mur8*, is deficient in rhamnose and is likely to be a mutant in which pectin composition is specifically altered (10). An alternative strategy is to directly manipulate pectins in transgenic plants that overexpress cell-wall degrading enzymes. As many fungal enzymes have evolved to degrade specific parts of the pectic network, this strategy offers a wide range of potential cell-wall phenotypes.

We have chosen potato (*Solanum tuberosum* L.) as a model system for these studies because it is easy to transform and provides large quantities of transformed tissue and hence cell walls for characterization. In addition, during the process of starch isolation, the potato starch industry generates large volumes of waste pulp that is relatively rich in pectins (11). Potato pectin is less suitable for many food industrial uses (e.g., as a gelling agent) than pectins from other sources in part because of its high content of neutral galactans. A remodeling of pectin structure by transgenic approaches may allow improvement of potato pectin quality.

In this paper, we report the generation of potato transformants expressing a 38-kDa endo-galactanase (EC 3.2.1.89) isolated from *Aspergillus aculeatus* (12). The endo-galactanase degrades unsubstituted 1,4-β-D-galactan to galactose and galactobiose at optimal conditions of pH 4.0 and 50–55°C (13, 14). The coding region of the endo-galactanase contains a signal sequence for secretion. Because signal sequences among eukaryotes are very similar (15), we expect the fungal signal sequence to function in a plant cell, resulting in secretion of the enzyme to the apoplast. We have determined endo-galactanase activity in tubers of transgenic potato plants and studied the effect of the introduced gene on tuber pectic composition as determined by monosaccharide composition, Fourier transform infrared microspectroscopy, and immuno-gold labeling with a galactan-specific antibody. This is a direct modification of the monosaccharide profile of a plant cell-wall polysaccharide by transgenic experiment.

Materials and Methods

Reagents and Enzymes. Unless otherwise stated, chemicals and reagents were purchased from Sigma. The porcine pancreatic α-amylase was purchased from Merck, and the pullulanase from *Bacillus acidopullulyticus* and endo-polygalacturonase (EPG) from *Aspergillus niger* were purchased from Megazyme International. The purified recombinant pectin methylesterase (PME) from *A. aculeatus* was a gift from K. Schnorr (Novo Nordisk A/S, Bagsværd, Denmark).

Transformation and Regeneration of Potato Plants. The granule bound starch synthase promoter was amplified by PCR from the plasmid pPGB121s (16) by using oligonucleotide primers 5′-GATTACGCC*AAGCTT*TAACG (*Hin*dIII site italicized) and 5′-GGTTT*TCTAGA*GTCGACGAAATCAGAAATAATT-GGAGG (*Xba*I site italicized) and was inserted as a *Hin*dIII-*Xba*I fragment into pBI121 (17), generating pPGB121-new. The coding region of β-glucuronidase was removed by digestion with *Sma*I and *Sac*I followed by Klenow DNA polymerase and religation, resulting in pPGB121s-new. The *Hin*dIII site upstream of the promoter

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Abbreviations: EPG, endo-polygalacturonase; FTIR, Fourier transform infrared microspectroscopy; GalA, galacturonic acid; HGA, homogalacturonic acid; PCA, principal component analysis; PME, pectin methylesterase; RGI, rhamnogalacturonan I; UA, uronic acid.

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region was deleted by digestion with *Hin*dIII, fill in of the 5′-overhangs with *Taq* DNA polymerase and religation to generate pPGB121s-B. A polylinker with restriction sites for *Hin*dIII, *Xba*I, and *Xho*I was produced by annealing of the two synthetic oligonucleotides 5′-*TCGA*CAAGCTTTCTAGAGCCTCGAGG (*Sal*I overhang italicized) 5′- *GATC*CCTCGAGGCTCTAGAAAGCTTG (*Bam*HI overhang italicized) and was cloned into *Sal*I/*Bam*HI-cleaved pPGB121s-B, generating the plasmid pGED. The 1.3-kb cDNA encoding an *A. aculeatus* endo-galactanase was excised from the vector pC1G1 (a gift from S. Kauppinen, Novo-Nordisk A/S, Bagsværd, Denmark) by digestion with *Hin*dIII and *Xba*I and was cloned into pGED, giving rise to the plasmid pGED-GAL. This plasmid was introduced into *Agrobacterium tumefaciens* strain LBA 4404 by electroporation. Transformation of potato leaf discs followed a two-media regeneration procedure essentially as described by Edwards *et al.* (18) by using kanamycin selection. Both transformed and wild-type plants were regenerated and transferred to the greenhouse. Tubers were harvested and used as seed potatoes to generate the potato plants and tubers to be analyzed. These plants were grown for 16 weeks in the greenhouse before harvest.

Preparation of Tuber Extracts. Freshly harvested tubers were cut into cubes, were frozen in liquid N_2, and were comminuted in an electric grinder to a fine powder. The powder was extracted with 3 ml of low-salt extraction buffer [25 mM NaOAc (pH 4.0) containing 5 mM EDTA, 0.5 mM phenylmethylsulphonylfluoride, 1 μM Pepstatin, and 10 μM E64] per gram of tissue. The sample was incubated for 10 min on ice, and insoluble material was sedimented by centrifugation (10 min at 12,000 $\times$ g_{max}). The supernatant was stored at -20°C.

Galactanase Activity Assays. Tuber extracts of wild-type and transformants ($T_{11.1}$ and $T_{13.1}$) were assayed for endo-galactanase activity by using the *p*-hydroxybenzoic acid hydrazine assay (19) with potato galactan (P-GALPOT from Megazyme) at a concentration of 0.1% in 0.1 M NaOAc (pH 4.0) as substrate at 40°C.

SDS/PAGE and Western Blot Analysis. Tuber extracts were subjected to SDS/PAGE and Western blot analysis according to standard procedures. The blot was probed with a rabbit antibody raised against purified *A. aculeatus* endo-galactanase.

Fourier Transform Infrared Microspectroscopy. Vibratome sections (60 μm) from 20 individual freshly harvested wild-type and transgenic ($T_{11.1}$, $T_{11.2}$, and $T_{13.1}$) tubers were mounted on barium fluoride windows and were air-dried. The barium fluoride window was supported on the stage of a UMA500 microscope accessory of a Bio-Rad FTS175c FTIR spectrometer equipped with a liquid nitrogen-cooled mercury cadmium telluride detector. Areas of 100 μm^2 of the cortex and perimedullary regions were selected and spectra obtained. Sixty-four interferograms were collected in transmission mode with 8 cm^{-1} resolution and were co-added to improve the signal-to-noise ratio for each sample. The spectra were baseline-corrected and area-normalized. Exploratory principal component analysis (PCA) of area-normalized spectra in the region 1,800–800 cm^{-1} were carried out by using WIN-DISCRIM software (E.K. Kemsley, Institute of Food Research, Norwich, U.K.).

Isolation of Cell Wall Material from Potato Tubers. Cell wall material was isolated by using the procedure of C. Doeswijk-Voragen (Wageningen Agricultural University, The Netherlands). Thirty grams of comminuted tuber tissue was resuspended in 150 ml of ice-cold extraction buffer B [1% sodium deoxycholate (Calbiochem), 20 mM Hepes (pH 7.5), and 5 mM $Na_2S_2O_5$ (Merck)] and was homogenized with an ultra-turrax (20,000 rpm) for 10 $\times$ 60 sec. The suspension was centrifuged (15,000 $\times$ g_{max}) in a Sorvall SLA-1500 rotor at 4°C for 15 min, the supernatant was filtered through a nylon mesh (pore size 30 μm), and the resulting residue on the filter was added back to the pellet. The pellet was resuspended in 100 ml of ice-cold buffer C (0.5% sodium deoxycholate/20 mM Hepes, pH 7.5/3 mM $Na_2S_2O_5$,) and was stirred at 4°C for 30 min. The centrifugations and subsequent washes in buffer C were repeated three times. The final wash was done overnight. The pellet was resuspended and centrifuged three times in 150 ml of ice-cold Milli-Q water before extraction with 150 ml PAW (phenol:acetic acid:water, 2:1:1 by volume) for 3 h at room temperature followed by centrifugation (15,000 $\times$ g_{max}) for 20 min at 24°C. The pellet was washed three times with 150 ml of water before resuspension in 150 ml of 25 mM NaOAc (pH 4.0); it was then heated for 20 min at 80°C to gelatinize the starch and was subsequently cooled on ice. The pH was adjusted to 5.0 with 1 M NaOH before addition of 6 mg of α-amylase and 60 μl of pullulanase (1,000 units/ml). The mixture was incubated for 3 h at 37°C, the enzymes were inactivated by stirring in a boiling water-bath for 10 min, and the mixture was cooled and centrifuged at 15,000 $\times$ g_{max} for 15 min. The pellet was washed on a nylon filter (30-μm pore size) with 1 liter of water, then was resuspended in $\approx$50 of ml water and was finally lyophilized. The enzyme treatment including gelatinization and subsequent washing was repeated. Starch content of the lyophilized cell wall material was quantified by using test 207748 from Boehringer Mannheim. The supernatant of the primary extraction was tested for endo-galactanase activity by incubation for 1 h at 4°C (conditions identical to extraction procedure) using the assay described above. No endo-galactanase activity could be detected (data not shown).

Isolation of Pectic Polysaccharides. Following the procedure of O'Neill *et al.* (20), de-starched cell wall material (10 mg) was suspended in 2 ml of 50 mM ammonium formate (pH 4.5) containing 0.05% sodium azide. EPG (1 unit) and PME (1 unit) were added, and the suspension was incubated for 16 h at 40°C. The suspension was then filtered through a double layer of nylon (30-μm pore size) to separate the EPG/PME extracts from the remaining wall material, which was suspended in 1 ml of ice-cold 50 mM sodium carbonate containing 10 mM sodium borohydride. This suspension was incubated for 1 h at 4°C, followed by a 4-h incubation at room temperature. Insoluble material was recovered by filtration through a double layer of nylon. The pH of the filtrate was adjusted to pH 5, EPG (1 unit) was added, and the mixture was incubated for 4 h at 40°C to partially depolymerize solubilized pectic material. This digested soluble wall material was termed the carbonate extract. Both the EPG/PME and carbonate extracts were subjected to size exclusion chromatography using a Superose 12 column (1 $\times$ 30 cm) (Amersham Pharmacia). The column was equilibrated in 50 mM ammonium formate (pH 5.0) before the extracts were applied to the column and eluted isocratically with the same buffer at a flow rate of 0.4 ml/min. The eluent was monitored by using refractive-index detection (Model 131, Gilson), and the uronic acid (UA) content of the collected fractions (0.8 ml) was determined by using the *m*-hydroxybiphenyl assay (21). Selected fractions were combined (see *Results*) and freeze-dried, and their monosaccharide composition was determined. The molecular mass of the eluted components was estimated by comparing their retention times to those of dextran standards (Fluka) and GalA.

Monosaccharide Composition Analysis. Seaman hydrolysis, as described in ref. 22, was used to hydrolyze crude or insoluble cell wall residues for monosaccharide composition analysis whereas solubilized wall fractions were hydrolyzed to monosaccharides by using 2 M aqueous trifluoroacetic acid for 1 h at 121°C. Monosaccharide mixtures (5–15 μg) were analyzed by high-performance anion exchange chromatography on a Carbo-Pac PA10 column (Dionex, Sunnyvale, CA) and were eluted isocratically using water as an eluent, according to the manufacturer's instructions. The UA content, expected in plant cell walls

Table 1. Endo-galactanase activity and cell wall yield

	Galactanase activity*	Cell wall yield, mg/g fresh weight	Starch, mass %	Extraction yield†	
				EPG/PME‡	Na_2CO_3‡
Wild type	1.6	13.1 ± 2.0	14.6 ± 5	71.9 ± 2.5	14.0 ± 1.4
$T_{11.1}$	104	8.6 ± 2.1	11.9 ± 6	115.5 ± 20.7	11.5 ± 0.9
$T_{13.1}$	214	9.5 ± 2.5	9.1 ± 5	142.2 ± 14.3	20.3 ± 6.5

Data (± SD) is the average of three independent experiments.
*Galactanase activity is given as μmol of galactose eq./min/g fresh weight.
†Determined by the *m*-hydroxybiphenyl assay given as μg of UA-eq./mg dry walls.
‡Extraction specifics are described in *Materials and Methods.*

to be mainly GalA, of each monosaccharide mixture was determined by the *m*-hydroxybiphenyl assay (21).

Immunogold Labeling of Potato Tubers. Low-temperature resin-embedded sections of glutaraldehyde-fixed tubers from the wild type and $T_{13.1}$ were immunogold-labeled with monoclonal antibody LM5 [which recognizes 1,4-β-galactan (23)] and were silver-enhanced and analyzed by reflection laser scanning confocal microscopy and electron microscopy as described (24).

Results

Transformation and Regeneration of Potato Plants. Fourteen individual transgenic potato plants have been generated. The transformation frequency with the granule bound starch synthase promoter-endo-galactanase cassette was low (mean 8% of four transformation experiments) compared with control transformations with the vector only (88%). In galactanase transformants, the time of shoot appearance was 6–8 weeks whereas that of the control was 2 weeks. Despite this delay, the transformants grew and displayed a phenotype indistinguishable from wild-type plants. The phenotype and yield of tubers on harvest was similar for wild-type and transformed plants (data not shown).

Galactanase Activity in Tuber Extracts. Endogenous endo-galactanase activity was almost undetectable in tuber extracts of wild-type potato plants whereas for $T_{11.1}$ and $T_{13.1}$ endo-galactanase activity was higher (Table 1). Endo-galactanase activity in the transgenic plants was quantitatively extracted in low salt buffer, indicating that the enzyme is not tightly bound to the cell wall. Extracts from transgenic and wild-type plants were also analyzed by Western blotting. All extracts having endo-galactanase activity contained a protein with a molecular mass of 38 kDa similar to that of isolated recombinant endo-galactanase (12, 25), which was recognized by a polyclonal antibody specific for the endo-galactanase (data not shown).

FTIR Spectroscopy. FTIR spectroscopy and exploratory PCA has been established as a rapid screen for altered cell wall phenotypes (26). Comparison of averaged spectra showed little apparent differences between wild-type and the endo-galactanase-expressing transformants (Fig. 1*A*). Therefore, exploratory PCA was used. We screened 20 tubers from the wild type and three selected endo-galactanase transformants and found that sections of potato tuber tissue could be discriminated for two of the transformants. For example, $T_{13.1}$ could be discriminated from the wild type with a success of 67/68 by using 5 PC scores (Fig. 1*B*). PC loadings 3 and 5 for this transformant showed characteristic features of pectin (Fig. 1*C*), an ester peak at 1,745 cm^{-1}, and features similar to those of polygalacturonic acid at 1,250, 1,150, 1,105, and 1,018 cm^{-1} (27). Interestingly, discrimination was clearest from the comparisons of cortical tissue, where galactan is more abundant in the wild type, than in the perimedullary tissue.

Isolation of Cell Wall Material and Pectic Polysaccharides. Based on the FTIR screening, cell walls were prepared in triplicate from selected transformants ($T_{11.1}$ and $T_{13.1}$) and wild type. The yield of cell wall material was lower in the transformants than in the wild type (Table 1). Monosaccharide composition of the wall preparations revealed a dramatic reduction to ≈30% in galactose content in both transformants compared with the wild type (Table 2). Galactosyl residues are present in different wall polysaccharides (hemicellulose, arabinogalactan proteins, and RGI), but only RGI

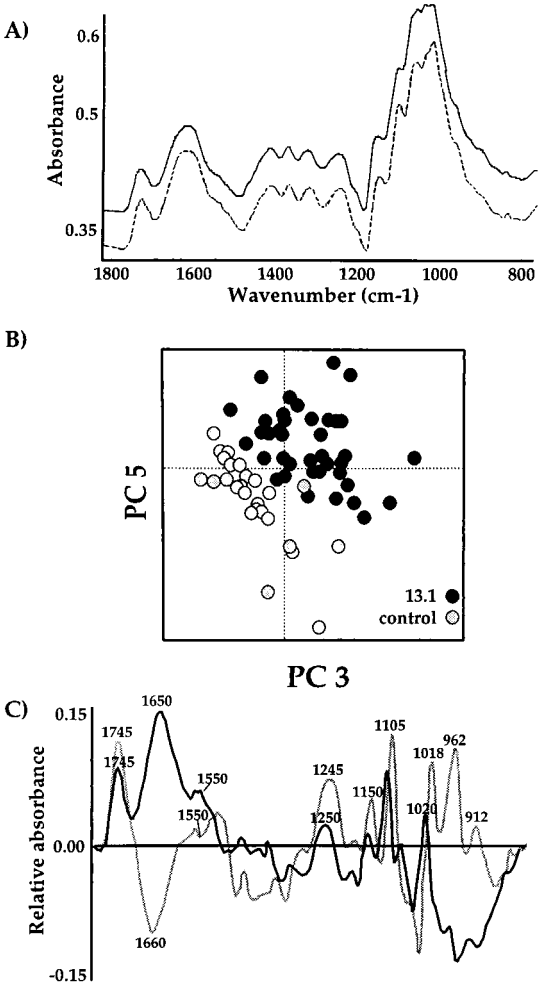

Fig. 1. (*A*) Averaged FTIR spectra of potato tuber cortex from 20 wild-type tubers (solid line) and 20 $T_{13.1}$ tubers (dashed line). (*B*) Exploratory PC analysis discriminates $T_{13.1}$ from the wild type, using the third and fifth PC scores. (*C*) The loadings for PC3 (black line) and PC5 (gray line) show features characteristic of pectins at 1,745, 1,250, 1,150, 1,105, and 1,018 cm^{-1}.

Table 2. Sugar compositions (mol%) of material obtained from transgenic and wild type potato tubers

	Fucose	Arabinose	Rhamnose	Galactose	Glucose	Xylose	Mannose	UA
Walls before extraction								
Wild type	0.3 ± 0.3	6.2 ± 2.7	1.4 ± 1.2	9.4 ± 1.5	53.0 ± 7.0	2.6 ± 0.2	0.4 ± 0.4	26.7 ± 1.7
$T_{11.1}$	0.3 ± 0.1	6.8 ± 2.8	1.4 ± 1.2	3.3 ± 1.1	61.0 ± 5.6	3.0 ± 2.3	0.6 ± 0.5	23.5 ± 8.0
$T_{13.1}$	0.3 ± 0.1	4.8 ± 1.2	0.8 ± 0.4	2.6 ± 0.4	63.5 ± 5.9	1.3 ± 0.3	0.8 ± 0.4	25.9 ± 6.6
EPG/PME extract								
A								
Wild type	0.2 ± 0.2	15.8 ± 1.2	5.7 ± 2.0	64.6 ± 4.1	0.3 ± 0.2	0.4 ± 0.3	nd	13.0 ± 2.5
$T_{11.1}$	nd	30.3 ± 9.9	10.4 ± 8.4	20.5 ± 2.5	0.4 ± 0.4	2.4 ± 0.8	nd	36.0 ± 2.2
$T_{13.1}$	nd	25.1 ± 6.9	7.0 ± 4.5	15.8 ± 3.6	0.2 ± 0.3	1.4 ± 1.5	nd	50.5 ± 12.3
B*								
$T_{11.1}$	nd	32.3 ± 1.1	7.8 ± 3.1	20.3 ± 0.5	0.3 ± 0.4	0.7 ± 0.6	nd	38.6 ± 4.3
$T_{13.1}$	nd	20.8 ± 7.8	5.5 ± 2.2	13.3 ± 3.9	0.4 ± 0.6	0.4 ± 0.8	nd	59.6 ± 11.8
C								
Wild type	nd	2.2 ± 0.7	2.5 ± 0.5	3.8 ± 1.5	24.8 ± 24.9	0.1 ± 0.2	nd	66.4 ± 22.8
$T_{11.1}$	nd	2.0 ± 0.6	2.5 ± 2.2	1.4 ± 0.6	28.9 ± 25.4	0.3 ± 0.3	nd	64.9 ± 24.3
$T_{13.1}$	nd	4.2 ± 2.8	1.1 ± 0.3	1.5 ± 1.0	9.5 ± 3.3	1.5 ± 2.6	0.4 ± 0.7	81.9 ± 10.3
Na_2CO_3 extract								
Wild type	nd	23.0 ± 7.9	7.1 ± 3.3	52.0 ± 10.9	5.2 ± 5.9	2.8 ± 2.8	nd	9.9 ± 3.7
$T_{11.1}$	nd	38.9 ± 13.7	13.6 ± 6.9	18.9 ± 3.3	5.5 ± 5.0	3.7 ± 1.3	1.5 ± 2.6	17.9 ± 8.3
$T_{13.1}$	nd	34.2 ± 14.9	9.7 ± 6.6	18.3 ± 4.6	3.4 ± 4.8	2.3 ± 2.1	nd	32.1 ± 28.3
Walls after extraction								
Wild type	nd	4.2 ± 0.5	nd	2.4 ± 1.1	46.0 ± 5.9	15.6 ± 5.4	1.5 ± 1.9	30.2 ± 0.9
$T_{11.1}$	nd	3.6 ± 0.2	nd	2.4 ± 1.5	73.3 ± 7.6	7.8 ± 5.9	5.8 ± 9.2	7.0 ± 2.9
$T_{13.1}$	nd	3.4 ± 1.1	nd	1.8 ± 0.5	74.2 ± 7.6	10.5 ± 6.4	0.3 ± 0.1	9.7 ± 1.0

Data (± SD) is the average of three independent experiments. nd, not detectable.
*Fraction B was not present in EPG/PME extracts from wild-type plants (see Fig. 2).

is known to contain β-1,4-linked galactan, so our subsequent analysis of cell wall material focused on this polymer.

RGI is specifically extracted from walls by treatment with a combination of fungal EPG and PME (20). This EPG/PME treatment released nearly twice as much UA from the cell walls of transformed tubers compared with the wild type (Table 1). The EPG/PME-solubilized pectin was analyzed by size exclusion chromatography to separate the digest fragments according to molecular size. The RGI extracted by EPG/PME from wild-type cell walls contained two major fractions, as indicated by UA content (data not shown) and refractive index detection (Fig. 2): these were termed fraction A (molecular mass >500 kDa) and fraction C (molecular mass 0.2–8 kDa). EPG/PME extracts from $T_{11.1}$ and $T_{13.1}$ have a different profile from the wild type (Fig. 2), containing less of fraction A, substantially more of fraction C, and, in addition, fragments of ≈120 kDa (fraction B), not present in wild-type extracts. By sugar analysis (Table 2), fraction A of the wild-type tuber contained high proportions of UA, rhamnose, arabinose, and galactose with virtually no other monosaccharides, indicative of high molecular weight HGA and RGI polymers. However, although fraction A from wild-type tubers contained 64 mol% galactosyl residues, transgenic tubers contained only 15–20 mol%, suggesting a major reduction in the amount of galactan. Furthermore, the UA content is significantly higher when taking into account the rhamnose:UA:galactose:arabinose ratios (wild-type, 1.0:2.3:11.3:2.8; $T_{11.1}$ 1.0:3.6:2.0:3.0; $T_{13.1}$ 1.0:7.1:2.2:3.6). Interestingly, fucosyl residues could not be detected in the transgenic fraction A but were present, albeit in a very low percentage, in wild-type fraction A. Fucosyl residues may be substituents of the galactan side-chains of potato RGI, as has been shown for sycamore RGI (28). The monosaccharide composition of fractions A and B from transgenic tubers was similar (Table 2). Fraction C from wild-type and transformant tuber cell walls contained mainly HGA fragments, but some small molecular weight RGI fragments were also detected. The RGI fragments derived from the transgenic tubers, like those of fractions A and B, also had a lower galactosyl content (1.4–1.5 mol%) compared with those of wild-type tubers (3.8 mol%). However, the relatively large amount of glucosyl residues may represent a contamination of the wall material with oligosaccharides released by the α-amylase treatment during cell wall preparation.

Enzymatic treatment of the walls with EPG/PME does not completely depectinate the walls (29). Therefore, additional sequential extractions were performed by using sodium carbonate at 4°C and room temperatures (Table 1). De-esterification with carbonate solubilized very little pectin and, unlike the EPG/PME extraction, the yield from the wild-type and transgenic cell wall preparations was similar. Size exclusion chromatography of the carbonate extracts resulted in two UA containing fractions (data not shown), one in the void volume (molecular mass >500 kDa) and one with a retention time equivalent to GalA, indicating the presence of monosaccharides or small oligosaccharides. The latter fraction could not be analyzed for its monosaccharide composition because of the high salt concentration present in the sample. Monosaccharide analysis of the extracts containing the large polymers (Table 2) showed high proportions of arabinose, galactose, UA, and rhamnose, indicative of RGI. However, significant amounts of glucosyl, and xylosyl residues were probably derived from xyloglucan solubilized by the carbonate extractions. The carbonate extracts of the transgenic cell walls also showed reduced galactosyl content relative to the wild type (18–19% versus 52%). Again, the arabinosyl content of the RGI present in carbonate extract is constant whereas the UA content is only higher in the cell wall preparations of $T_{13.1}$ but not in $T_{11.1}$.

Pectins may remain in the wall after the enzymatic and carbonate buffer extractions. The monosaccharide compositions of cell walls before and after the sequential treatments with EPG/PME and carbonate were compared to quantify the efficiency at which pectins were solubilized (Table 2). No rhamnose could be detected in the walls after pectin extraction, suggesting that the RGI present in the unextracted walls was completely removed by the sequential extractions. However, the extracted walls of wild-type and trans-

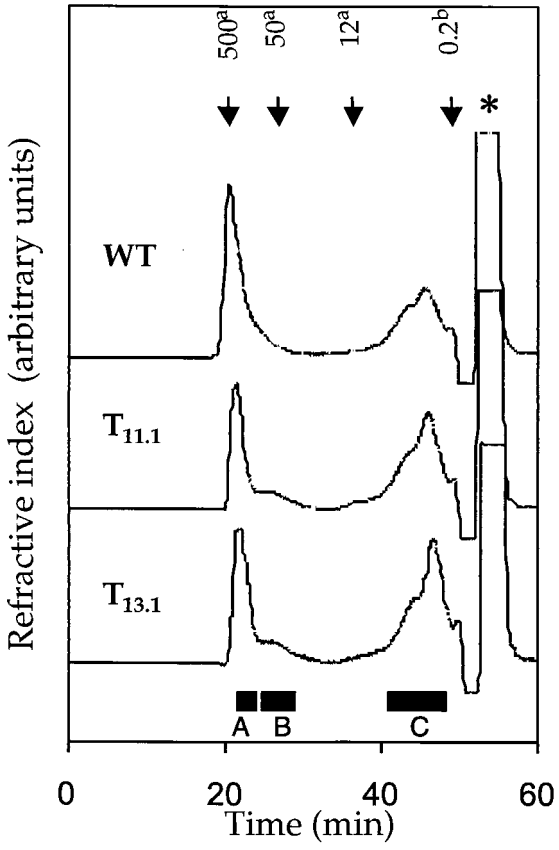

Fig. 2. Superose 12 profiles of EPG/PME extracts solubilized from potato tubers. The eluent was monitored by using a refractive index detector. Bars (A, B, C) indicate fractions that were pooled. The asterisk indicates a large peak attributable to the presence of sample buffer salts that is devoid of pectic material. Arrows, elution time of molecular mass markers (kDa). [a], dextrans; [b], galacturonic acid.

formed tubers still contained galactose in similar quantities. This galactose most likely originates from other wall components than RGI, such as xyloglucan, which are known to contain β-1,2-linked galactosyl residues (31), and cannot be hydrolyzed by the endo-galactanase. Xyloglucans are only extracted by concentrated alkali or xyloglucanase treatment (31) and would therefore be expected to be present in walls treated by the procedure used in this study. Interestingly, the UA content is more than 3-fold higher in the remaining walls of the wild type than in the transformants, indicating an increased extractability of pectin from the transformants.

Immunogold Labeling. To determine whether, specifically, 1,4-β-D-galactan has been hydrolyzed by the endo-galactanase, we used monoclonal antibody LM5, which recognizes tetramers of 1,4-β-D-galactan to immunogold-label wild-type and $T_{13.1}$ mature tuber tissue (Fig. 3). Perimedullary parenchymal cell walls of the wild type labeled strongly, indicating a high abundance of the epitope recognized by LM5 (Fig. 3 *A* and *C*) whereas similar walls in transformed tubers (Fig. 3*B*) were only weakly stained at some cell corners. Electron microscopy showed that the immunoreactive material at the corners in transformed tissue was associated with the wall close to the plasma membrane (Fig. 3*D*).

Discussion

Potato plants expressing a fungal (*A. aculeatus*) endo-galactanase under the control of the tuber-specific granule bound starch synthase promoter have been generated. Apart from a low transformation efficiency, the obtained plants displayed no altered phenotype compared with wild-type plants. The lower frequency may indicate that the promoter is active during *in vitro* culture and that a high level of endo-galactanase activity at this very early stage of development may be lethal to the transformed cells.

During the isolation and extraction of cell walls, several precautions were taken to avoid enzymatic degradation of the pectic material by the introduced endo-galactanase as well as endogenous pectinases. Buffers containing sodium deoxycholate were used to denature and solubilize proteins, which were also diluted by several subsequent ice-cold extractions in buffers with a pH unfavorable for optimal activity of plant pectinases (32–34) and in particular the introduced endo-galactanase (13, 14). The endo-galactanase activity was also undetectable in the cell wall extraction buffer during preparation of cell walls.

Two of the transformants, $T_{11.1}$ and $T_{13.1}$, could be clearly distinguished from the wild type by FTIR and were therefore selected for further analyses. All RGI polymers isolated from transgenic walls had a significantly reduced galactose content showing that the secreted enzyme was active in the walls and hydrolyzed most galactan side-chains of RGI. Higher expression of endo-galactanase in $T_{13.1}$ compared with $T_{11.1}$ did not result in any further reduction of galactan content of RGI. The residual galactose content of RGI may be attributable to several factors: potato RGI might contain traces of β-1,3- and/or β-1,6-linked galactosyl residues, as those residues have been found in potato walls (35). Galactans can be substituted with arabinosyl substituents (then termed arabinogalactan I) inhibiting hydrolysis of the galactan side-chains (12, 13). However, the similar arabinan content of both wild-type and transgenic cell walls and the extracted RGI molecules indicates that the hydrolyzed galactan contains few, if any, arabinosyl residues. Antibody labeling experiments indicated a near quantitative removal of the LM5-epitope in the transformants. The LM5 antibody requires tetrameric and the galactanase trimeric unsubstituted β-1,4-galactan for binding. It is thus reasonable to infer that transgenic RGI might contain β-1,4 linked galactosyl residues of such low degree of polymerization such that the LM5 antibody is unable to bind and the galactanase is unable to hydrolyze them any further. However, the LM5 labeling at cell corners in perimedullary parenchymal walls suggest that some of the galactan chains may be inaccessible to the enzyme for steric or wall architectural reasons. From the electron micrographs, it is evident that this residual labeling is confined to the part of the wall closest to the plasmamembrane, which is considered to be the most recently deposited wall material. Therefore, newly synthesized RGI might be deposited at a higher rate than hydrolysis can occur.

Further changes apart from the reduced galactose content were detected. The transgenic modified RGI polymers extracted with EPG/PME had a higher UA content, suggesting that they contain a larger proportion of HGA. Fraction B from size exclusion chromatography of the extracted transgenic polymers (of the EPG/PME extract) may result from the removal of galactosyl residues from RGI side-chains, thereby reducing the molecular weight of the polymer(s). Other characteristics of the transgenic cell walls were a larger yield of total pectic material as well as a larger proportion of HGA released by EPG/PME extraction. These data indicate that the pectic material in the transgenic walls is more accessible to EPG and PME compared with wild-type walls. An intriguing hypothesis is that the reduced galactan content of RGI results in a more porous wall architecture. The modified solubility of pectic polymers in the transformants points either to a particular role of the galactans in wall architecture or to other structural changes in the wall compensating for the reduced galactan content. The lack of altered

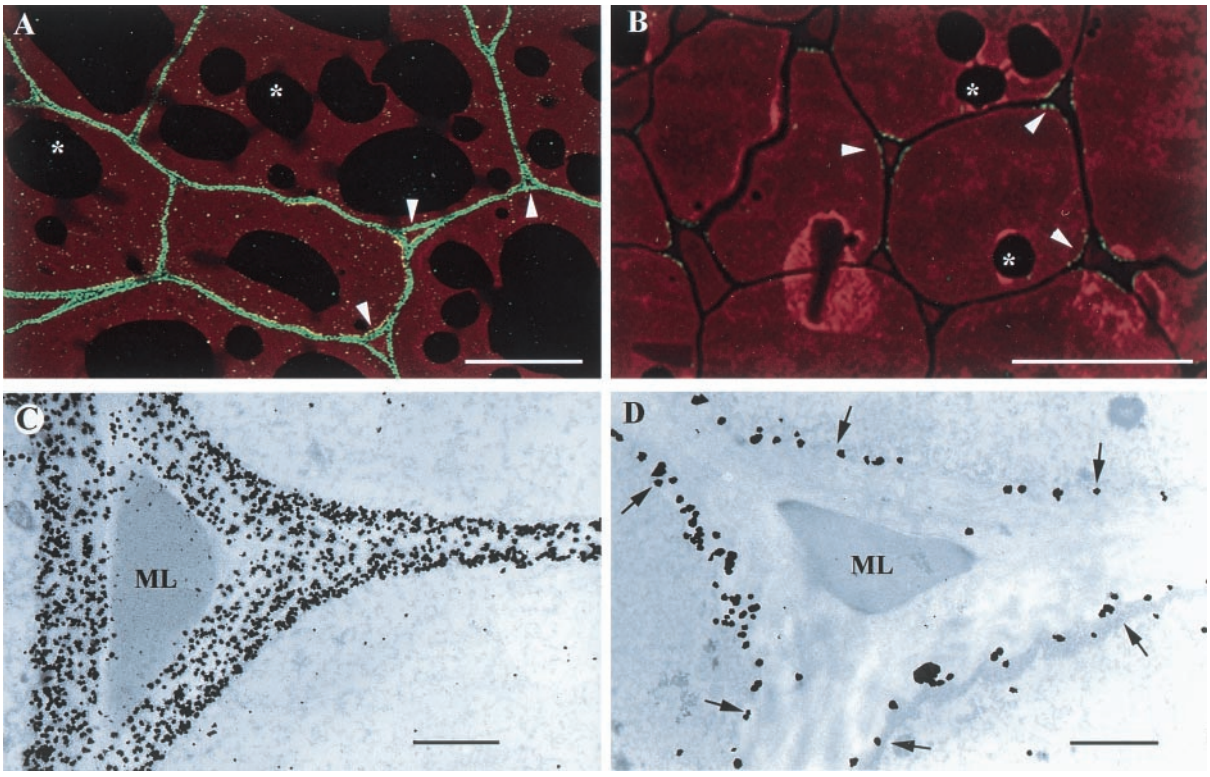

Fig. 3. Sections of wild-type (*A* and *C*) and endo-galactanase-expressing ($T_{13.1}$) (*B* and *D*) potato tubers gold-labeled with monoclonal antibody LM5, silver enhanced and viewed by reflection confocal scanning microscopy (*A* and *B*) and transmission electron microscopy (*C* and *D*). The walls of wild-type parenchymal cells are strongly labeled (green in *A*, black particles in *C*) whereas in $T_{13.1}$ tubers, the labeling density is greatly reduced and localized only to some cell corners (arrowheads in *B*) close to the plasma membrane (arrows in *D*). Asterisks represent spaces once occupied by starch granules. ML indicates the expanded middle lamella of these filled corners. [Bars = 100 μm (*A* and *B*) and 2 μm (*C* and *D*).]

phenotype in the transformed tubers suggests that homeostatic mechanisms operate to compensate for the loss of galactan and produce a functional wall architecture without detriment to the plant under the growth conditions used.

In conclusion, the targeted approach of generating plants with modified cell walls by expressing polysaccharide hydrolases in the plant complements other mutant approaches. Both expected wall structural changes, removal of galactan in the present case, and unforeseen changes, e.g., increased solubility of pectins or increased UA content, were observed and provide us with experimental tools for assigning particular physiological and structural roles to these wall components.

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