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In situ, Chemical and Macromolecular Study of the Composition of *Arabidopsis thaliana* Seed Coat Mucilage

Audrey Macquet [1], Marie-Christine Ralet [2], Jocelyne Kronenberger [1], Annie Marion-Poll [1] and Helen M. North [1,*]

[1] *Laboratoire de Biologie des Semences, UMR 204, INRA, INAPG, Institut Jean-Pierre Bourgin, INRA, F-78026 Versailles Cedex, France*
[2] *INRA, UR1268 Biopolymères Interactions Assemblages, F-44000 Nantes, France*

A comprehensive analysis was carried out of the composition of seed coat mucilage from *Arabidopsis thaliana* using the Columbia-0 accession. Pectinaceous mucilage is released from myxospermous seeds upon imbibition, and in *Arabidopsis* consists of a water-soluble, outer layer and an adherent, inner layer. Analysis of monosaccharide composition in conjunction with digestion with pectolytic enzymes conclusively demonstrated that the principal pectic domain of both layers was rhamnogalacturonan I, and that in the outer layer this was unbranched. The macromolecular characteristics of the water-soluble mucilage indicated that the rhamnogalacturonan molecules in the outer layer were in a slightly expanded random-coil conformation. The inner, adherent layer remained attached to the seed, even after extraction with acid and alkali, suggesting that its integrity was maintained by covalent bonds. Confocal microscopy and monosaccharide composition analyses showed that the inner layer can be separated into two domains. The internal domain contained cellulose microfibrils, which could form a matrix with RGI and bind it to the seed. In effect, in the *mum5-1* mutant where most of the inner and outer mucilage layers were water soluble, cellulose remained attached to the seed coat. Immunolabeling with anti-pectin antibodies indicated the presence of galactan and arabinan in the inner layer, with the latter only present in the non-cellulose-containing external domain. In addition, JIM5 and JIM7 antibodies labeled different domains of the inner layer, suggesting the presence of stretches of homogalacturonan with different levels of methyl esterification.

Keywords: *Arabidopsis thaliana* — Cellulose — Pectin — Rhamnogalacturonan I — Seed mucilage.

Abbreviations: BSA, bovine serum albumin; GLC, gas–liquid chromatography; HG, homogalacturonan; HP-SEC, high-performance size-exclusion chromatography; *mum, mucilage modified*; MALLS, multiple angle laser light scattering; PBS, phosphate-buffered saline; RGI, rhamnogalacturonan I; RGII, rhamnogalacturonan II.

Introduction

The angiosperm seed coat or testa is a multifunctional organ that surrounds the plant embryo to protect it from mechanical damage and pathogen attack. It also acts as a barrier for the regulation of water uptake upon imbibition and the control of germination (Haughn and Chaudhury 2005). The maternally derived seed coat consists of multiple specialized cell layers and, in some species, including members of the *Brassicaceae*, *Solanaceae*, *Linaceae* and *Plantaginaceae*, the epidermal cells accumulate a large quantity of mucilage that is liberated upon imbibition to form a gel-like capsule around the seed; this specialization is known as myxospermy. In *Arabidopsis thaliana*, the differentiation of the outer cell layer involves the secretion of mucilage between the primary cell wall and the plasma membrane, with the concomitant formation of a column-shaped cytoplasm at the centre of the cell (Beeckman et al. 2000, Western et al. 2000, Windsor et al. 2000). This cytoplasmic column is later surrounded by a thickened secondary cell wall (Western et al. 2000, Windsor et al. 2000) that forms a volcano-shaped structure known as the columella. On seed imbibition the outer tangential cell wall of the epidermal cell is ruptured and mucilage released (Western et al. 2000). As extruded mucilage is strongly stained with the dye ruthenium red (Western et al. 2000, Penfield et al. 2001, Western et al. 2001, Willats et al. 2001, Usadel et al. 2004. Western et al. 2004), which stains acidic polymers and mainly pectic polysaccharides (Sterling 1970), this suggested that the major component was pectin.

Pectin is an extremely complex polysaccharide that can be viewed as a multiblock co-biopolymer (Ralet et al. 2002). The simplest, and the most abundant, of these blocks is homogalacturonan (HG), an unbranched polymer of (1→4)-α-D-Gal*p*A (galacturonic acid) residues that are partly methyl esterified and sometimes partly acetyl esterified. A second major block, rhamnogalacturonan I (RGI), is mainly composed of a repeating disaccharide unit $[\rightarrow 2)$-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→$]_n$ (Rha, rhamnose)

*Corresponding author: E-mail, helen.north@versailles.inra.fr; Fax, +33-1-30-83-31-11.

(RG backbone) decorated primarily with arabinan and (arabino)-galactan side chains. A minor block, rhamnogalacturonan II (RGII), is a highly complex molecule made of a short HG backbone with four conserved side chains consisting of 12 different monosaccharides including Rha. The presence of large amounts of pectic polysaccharides in *Arabidopsis* mucilage, suggested by ruthenium red staining, was confirmed by sugar analysis of the extracted mucilage which indicated an abundance of Rha and GalA (Goto 1985, Western et al. 2000, Penfield et al. 2001, Western et al. 2001, Usadel et al. 2004, Western et al. 2004). It remains unclear, however, which pectic domains are present and if other cell wall-related polysaccharides (cellulose, hemicelluloses) are also part of the mucilage. Significant amounts of neutral sugars, other than Rha, mainly galactose (Gal) and glucose (Glc), but also fucose (Fuc), arabinose (Ara), xylose (Xyl) and mannose (Man), were detected [34–48% (w/w) of the total neutral and acidic sugars in mucilage (Western et al. 2000, Penfield et al. 2001, Usadel et al. 2004)]. In addition, ruthenium red staining revealed two distinct layers of mucilage: an outer diffuse layer and an inner intensively stained one (Western et al. 2000, Western et al. 2001, Fig. 1A, B). Results of immersion immunofluorescence analyses, using antibodies which recognize particular pectin structures, and epifluorescence microscopy suggested that there is a lower degree of methyl esterification in the inner layer that would allow calcium-based cross-linking, thus making it more dense (Willats et al. 2001). Furthermore, an antibody that recognizes both RGI and HG strongly labeled mucilage, but no labeling was detected with probes for arabinan and type I galactan chains (Willats et al. 2001, Western et al. 2004). It was also reported that seed mucilage is extensively labeled with Calcofluor, a fluorescent probe for β-glycans, indicating that cellulose is a component of mucilage (Windsor et al. 2000, Willats et al. 2001). In addition, cellulose-directed non-catalytic carbohydrate-binding modules have been demonstrated to recognize components in seed mucilage (Blake et al. 2006). Sugar analyses related to cellulose content have yielded variable results, with Glc contents ranging from <1 to 15% (w/w) of the total sugars in mucilage (Goto 1985, Western et al. 2000, Penfield et al. 2001, Western et al. 2001, Usadel et al. 2004, Western et al. 2004); this variability could be due to natural variation between the different *Arabidopsis* accessions used in these studies.

Studies aimed at identifying elements involved in the differentiation of the seed coat to produce mucilage have employed molecular genetic approaches, and a number of mutants have been identified (Koornneef 1981, Koornneef et al. 1982, Jofuku et al. 1994, Léon-Kloosterziel et al. 1994, Penfield et al. 2001, Western et al. 2001, Johnson et al. 2002). The majority of those identified to date, *ttg1*, *ttg2*, *ap2*, *gl2*, *ats*, *myb61*, *tt8* and *egl3*, are defective in proteins involved in transcription regulation (Jofuku et al. 1994, Rerie et al. 1994, Walker et al. 1999, Nesi et al. 2000, Penfield et al. 2001, Johnson et al. 2002, Zhang et al. 2003, McAbee et al. 2006) and have pleiotropic phenotypes. Nonetheless, some mutants have been identified that appear to be more directly affected in mucilage synthesis or composition; notably, the *mucilage modified* (*mum*) mutants (Western et al. 2001). In effect, the mutation in the *mum4* mutant was shown to affect a putative UDP-Rha synthase (Usadel et al. 2004, Western et al. 2004), and *mum2* and *mum5* mutations have been reported to alter the activities of a putative β-galactosidase or pectin methylesterase, respectively (Western et al. 2006). The latter is of particular interest as although the mutant liberated mucilage, its ruthenium red staining properties were altered and it appeared to be affected in mucilage composition (Western et al. 2001).

The present work was undertaken to clarify the structure of *Arabidopsis* seed mucilage using both chemical analyses and in situ labeling. Previous data concerning *Arabidopsis* mucilage have been obtained using a variety of accessions (Sendai, Wassilewskija, Landsberg *erecta*, Col-2, Col-0 and C24), and no complete analysis has been carried out using one in particular. In this study, the *Arabidopsis* accession Col-0 has been analysed in detail for an ensemble of characteristics; this accession was chosen as a reference accession as its genome has been sequenced (Arabidopsis Genome Initiative, 2000). A detailed study of the macromolecular parameters of extracted polymers from the outer mucilage layer has been carried out; molar mass, radius of gyration, polydispersity and intrinsic viscosity, and digestion with polysaccharide-degrading enzymes made possible chemical analysis of the inner layer. Furthermore, in situ labeling showed that the inner layer of mucilage was not homogeneous, but contains two domains with different polysaccharide compositions. The major component of seed mucilage in both inner and outer mucilage layers was unsubstituted RGI.

Results

Comparison of seed mucilage extracted by ammonium oxalate or water

Ruthenium red staining of *Arabidopsis* seed mucilage has indicated that two structurally different domains exist, an outer layer that is easily washed off seeds, or removed by shaking, and an inner strongly staining layer (Western et al. 2000, Fig. 1A, B, C). The physical and staining properties of these suggested that they were structurally different and these differences could be indicative of specific physiological roles for each. In order to obtain information about their structural particularities, we set out to determine the

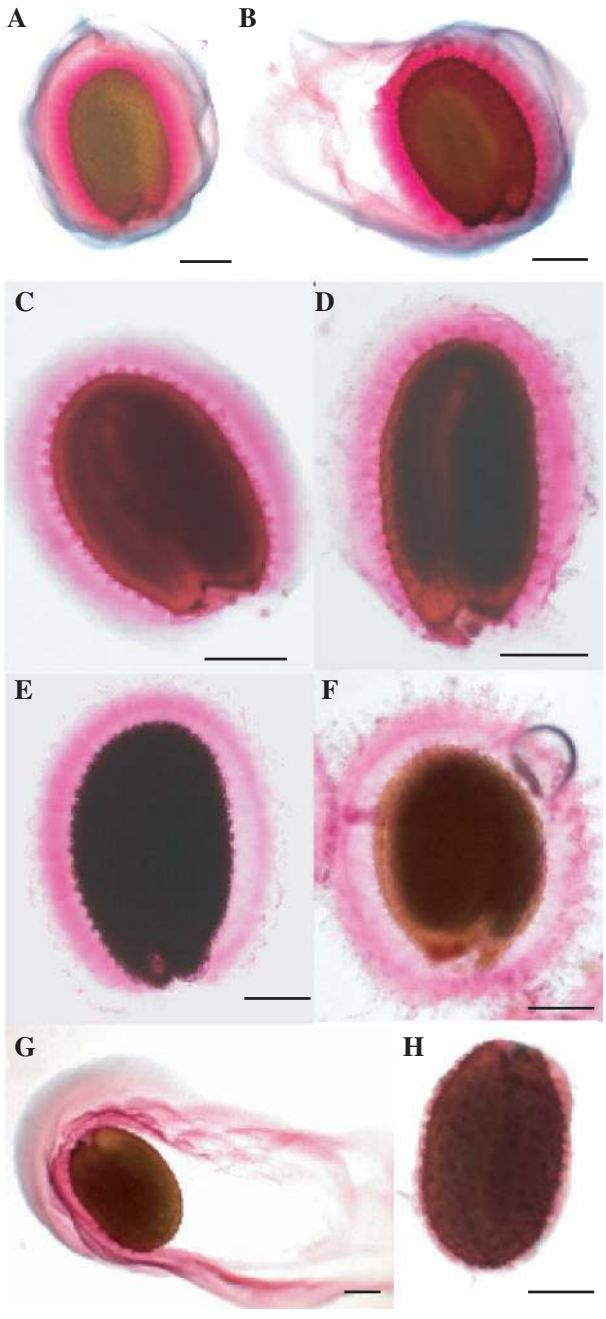

Fig. 1 Visualization of mucilage liberated on imbibition of mature *Arabidopsis* seeds by ruthenium red staining. (A–F) Wild-type seeds stained without agitation (A and B), with agitation equivalent to that used in immunolabeling experiments (C), following treatment with 0.2% (w/v) ammonium oxalate overnight at 40°C with vigorous agitation (D), after treatment with 0.05 N HCl for 30 min at 85°C with vigorous agitation (E) and after incubation in 0.1 N NaOH for 2 h at 80°C with vigorous agitation (F). (G) *mum5-1* seeds stained without agitation. (H) *mum5-1* seeds after agitation equivalent to that used in immunolabeling experiments. Seeds were from the Col-0 (A–F) or Col-2 accessions (G and H). Scale bars: 150 μm.

composition of each individually. To analyze the composition of the outer mucilage layer, extractions were carried out, therefore, using water and vigorous vortexing. Interestingly, the results obtained (Table 1) were very similar to those obtained in previous analyses of *Arabidopsis* seed mucilage composition using ammonium oxalate, a chelating agent, which extracts Ca^{2+}-cross-linked pectins (Goto 1985, Western et al. 2000, Western et al. 2001, Usadel et al. 2004, Western et al. 2004). Furthermore, the amount of freeze-dried mucilage recovered by water extraction was similar, 20.0 ± 0.5 mg of dialyzed freeze-dried material g^{-1} of seed, to values for ammonium oxalate-extracted mucilage calculated from data of Usadel et al. (2004) and Deng et al. (2006). Ammonium oxalate extractions were carried out, therefore, and analyzed in our experimental conditions, and these gave similar results to water-extracted mucilage (Table 1). In addition, ruthenium red staining of seeds after both extraction techniques yielded equivalent staining even after vigorous shaking (compare Fig. 1C and D), with most of the inner layer of mucilage remaining attached to the seeds. Analysis of mucilage composition carried out using water and ammonium oxalate extracts would therefore appear to be equivalent and only representative of the outer layer of mucilage.

Analysis of the water-soluble and ammonium oxalate mucilage extracts showed that the outer layer of mucilage was mainly composed of sugars (18.5 and 17.6 mg g^{-1} of intact seeds for water and oxalate extracts, respectively) with only trace amounts of proteins (Table 1). GalA and Rha represented >90% (w/w) of the sugars detected, and no cellulosic Glc was identified in freeze-dried water-extracted mucilage. In addition, mucilage appeared neither methylated nor acetylated. Analysis of mucilage extracted from two other accessions, Col-2 and Bay-0, found that the distribution of the different sugars between accessions was very similar, with the molar ratio of the most abundant sugars, Rha and GalA, being stable around 1. Nonetheless, the total amount of sugars isolated from each accession differed, being significantly higher in Col-0. This indicates that the amount of seed mucilage in the external layer is subject to natural variation.

The inner layer of seed mucilage is resistant to extraction by dilute acid and alkali

The retention of compact mucilage close to the seed coat has been proposed to be due to macromolecular entanglement or the association of the mucilage with the columella and outer cell wall remnants (Western et al. 2000). To try to obtain an extract containing the polysaccharides from both the outer and inner mucilage layer, more aggressive extraction protocols using hot dilute acid or alkali were tested. Hot dilute acid extracts pectins by hydrolysis of some glycosidic linkages and is used for

Table 1 Composition (mg of dialysed material g^{-1} of intact seeds) of Col-0, Col-2, Bay-0 and *mum5-1* water-extracted mucilage and ammonium oxalate-, acid-extracted- and alkali-extracted- Col-0 mucilage

	Water-extracted mucilage				Oxalate-extracted mucilage	HCl-extracted mucilage	NaOH-extracted mucilage
	Col-0	Bay-0	Col-2	*mum5-1*	Col-0	Col-0	Col-0
GalA	9.1 (0.55)	6.5 (0.83)	7.6 (0.70)	14.2 (0.55)	8.6 (0.01)	14.7 (0.59)	11.1 (0.36)
Rha	8.1 (0.37)	5.2 (0.69)	6.0 (0.53)	11.1 (0.37)	7.6 (0.17)	7.3 (0.31)	7.7 (0.13)
Fuc	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ara	0.1 (0.03)	0.1 (0.02)	0.1 (0.01)	0.1 (0.01)	0.1 (0.02)	3.2 (0.75)	1.2 (0.10)
Xyl	0.6 (0.17)	0.4 (0.05)	0.4 (0.02)	0.3 (0.04)	0.9 (0.07)	0.8 (0.12)	n.d.
Man	0.1 (0.09)	0.2 (0.02)	0.1 (0.08)	0.1 (0.01)	n.d.	n.d.	n.d.
Gal	0.2 (0.03)	0.2 (0.01)	0.2 (0.01)	0.2 (0.02)	0.2 (0.01)	0.8 (0.09)	2.7 (0.10)
Non-cellulosic Glc	0.35 (0.08)	0.3 (0.05)	0.1 (0.18)	0.3 (0.02)	0.2 (0.32)	2.4 (0.22)	n.d.
Cellulosic Glc	n.d.[a]	–	–	–	–	–	–
GalA/Rha (mol/mol)	0.93 (0.09)	1.04 (0.01)	1.05 (0.01)	1.06 (0.06)	0.94 (0.02)	1.67 (0.14)	1.20 (0.02)
Total sugars	18.5 (0.94)	12.9 (1.67)	14.3 (1.45)	28.3 (0.28)	17.6 (0.60)	29.2 (1.01)	22.6 (0.49)
Proteins	<0.2	<0.2	<0.2	0.2 (0.02)	<0.2	–	–
Methanol	n.d.	–	–	–	–	–	–
Acetic acid	n.d.	–	–	–	–	–	–

Values in parentheses are SDs. All experiments were performed in duplicate except for Col-0 water extractions, which were repeated four times. Values were obtained from at least four individual measurements.
[a] Performed on freeze-dried material.
n.d.: not detected; –: not determined.

industrial pectin extraction. Dilute alkali extracts pectins by de-esterification and β-elimination. Neutralization was carried out after acid extraction, as ruthenium red staining requires alkaline conditions (Sterling 1970). Ruthenium red staining of seeds after extraction in either 0.1 N NaOH for 2 h at 80°C or 0.05 N HCl for 30 min at 85°C followed by neutralization at room temperature showed that mucilage was still present around seeds (Fig. 1E, F). No clear reduction in the amount of mucilage attached to the seed seemed to occur with either treatment, indicating that the pectin in the mucilage is extremely aggregated and/or attached covalently, either directly or indirectly, to the columella or the outer seed coat cell wall.

Analysis of the composition of mucilage extracted with hot dilute acid or hot dilute alkali indicated that total sugar levels were higher compared with water or ammonium oxalate extracts (Table 1). Notably, GalA, Ara and Gal were increased (Table 1) and the GalA/Rha molar ratios were significantly higher than those obtained with water or ammonium oxalate. Furthermore, soluble mucilage from acid extracts released reducing ends on digestion with endo-arabinase or endo-galactanase, 98 and 270 μg g^{-1} of seeds, respectively, whereas no release was detected from water-soluble mucilage. This demonstrated that increased Ara and Gal in acid extracts was at least partly from α-(1→5)-linked arabinans and β-(1→4)-linked galactans, and not due to leaching of soluble sugars, and that additional pectins were extracted with the outer layer of mucilage by acid extraction. These could be derived from the inner mucilage layer, but could also originate from pectins present in the primary cell wall of the outer seed coat layer or the embryo.

Macromolecular characterization of mucilage

High-performance size-exclusion chromatography (HP-SEC) coupled with refractive index, multiple angle laser light scattering (MALLS) and viscosimetry detection was employed to determine the macromolecular parameters of seed mucilage. Extracts were obtained from seeds using the HP-SEC elution buffer in order to avoid the appearance of a negative refractive index peak at the total volume of the column. Ruthenium red staining of seeds extracted with this buffer was identical to that of seeds shaken vigorously in ammonium oxalate or water (data not shown). Elution profiles found two polymeric populations of polysaccharides: one eluting at the column void volume and one eluting at an elution volume of 13 ml (Fig. 2). No A_{280} signal was detected in these two populations, in agreement with the presence of only trace amounts of proteins in the water-extracted mucilage. Added together, the two polymeric populations accounted for 17.8 ± 0.5 mg of freeze-dried mucilage g^{-1} of whole seeds treated, in good agreement with values calculated by sugar analysis. Standard SEC was also performed in order to have sufficient material in fractions for analysis of sugar composition.

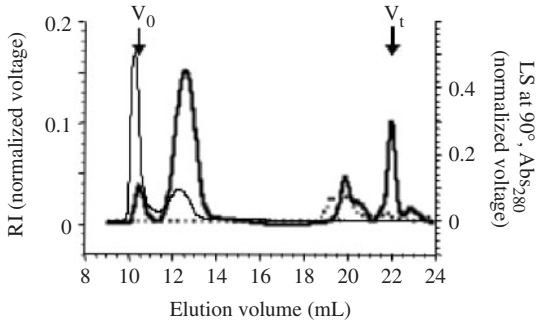

Fig. 2 Analysis of the macromolecular properties of Col-0 water-extracted mucilage by high-performance size-exclusion chromatography combined with multiple angle laser light scattering. Bold line, refractive index signal (RI); thin line, light scattering signal at 90° (LS at 90°); dotted line, absorbance at 280 nm (Abs_{280}); V_0, column void volume; V_t, column total volume.

Again two populations were observed, one eluting at the void volume and the second at K_{av} 0.2 (data not shown). Both populations contained Rha and GalA exclusively in a molar ratio close to 1 (data not shown). The extracts separated by HP-SEC were not dialyzed prior to analysis and some material eluted just before and at the total volume of the column (Fig. 2). These peaks were absent from dialyzed extracts analyzed by standard SEC (data not shown) and so do not correspond to complex macromolecules. The HP-SEC populations of very low molar mass probably correspond, therefore, to small sugars, ions or pigments. The macromolecular characteristics of the two polymeric populations of Col-0 mucilage are given in Table 2. The first population, representing around 9% of the extracted mucilage, eluted at the void volume, and was of very high average molar mass (close to 50,000 kDa), but exhibited moderate radius of gyration and intrinsic viscosity values. This reveals the presence of entangled, collapsed or aggregated macromolecules, which could represent pectin coming from the inner layer of compact mucilage directly around the seed, in agreement with the molecular entanglement proposed by Western et al. (2000). The second population was of high average molar mass (around 600 kDa) with an average radius of gyration of around 60 nm and an intrinsic viscosity of around 700 ml g^{-1}. This population was particularly homogeneous with respect to molar mass as revealed by the polydispersity index value close to 1. Fig. 3 shows the dependence of size upon mass for the second mucilage population, which fitted a linear relationship ($r^2 > 0.97$) of the logarithmic form of:

$$\mathrm{Rg} = k\ \mathrm{M}\nu$$

with Rg the radius of gyration and M the molar mass at each point of the chromatogram. A hydrodynamic parameter ν of 0.77 was calculated, which is characteristic of a slightly expanded random coil conformation (Burchard 1994).

Table 2 Macromolecular characteristics of Col-0 water-extracted mucilage

	Polymeric population 1	Polymeric population 2
Elution volume (ml)	10.3 (V_0)	13.0
Yield (%)	9.2 (1.5)	90.8 (1.5)
$<M_n>$ (kDa)	48 500 (2 300)	636 (40)
I ($<M_w>/<M_n>$)	–	1.04 (0.01)
$<Rg_n>$ (nm)	211 (6)	60 (3)
$<[\eta]_n>$ (ml/g)	671 (5)	714 (3)
ν	–	0.77 (0.06)

Values in parentheses are SDs of four independently extracted samples.
V_0, column void volume; $<M_n>$, number-average molar mass; $<M_w>$, weight-average molar mass I, polydispersity index; $<Rg_n>$, number average radius of gyration; $<[\eta]_n>$, number-average intrinsic viscosity; ν, hydrodynamic parameter.

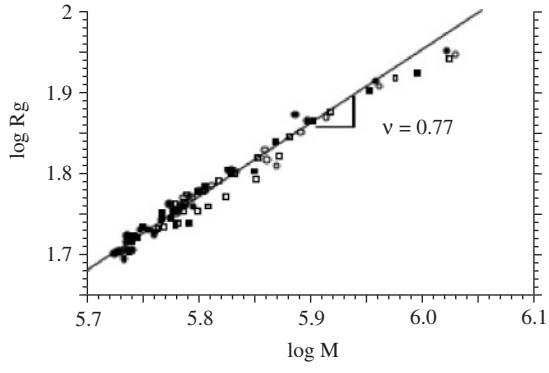

Fig. 3 Determination of the hydrodynamic parameter of pectin macromolecules present in Col-0 water-extracted mucilage from Log radius of gyration (Rg) as a function of log molar mass (M). The four different symbols represent four independent extractions.

Results obtained from HP-SEC-MALLS analysis of mucilage from Col-2 and Bay-0 accessions were similar to those of Col-0 extracts with two populations of macromolecules: the first polymeric population, representing around 10% of the total, of very high molar mass (>40,000 kDa), and the second centered on 600 kDa (data not shown).

Immunolabeling of the inner layer of seed mucilage with anti-pectin antibodies

As the inner layer of seed mucilage could not be extracted efficiently by the different protocols tested, its composition was examined in situ using anti-pectin antibodies. Whole-mount immersion immunofluorescence has previously been employed to examine the composition

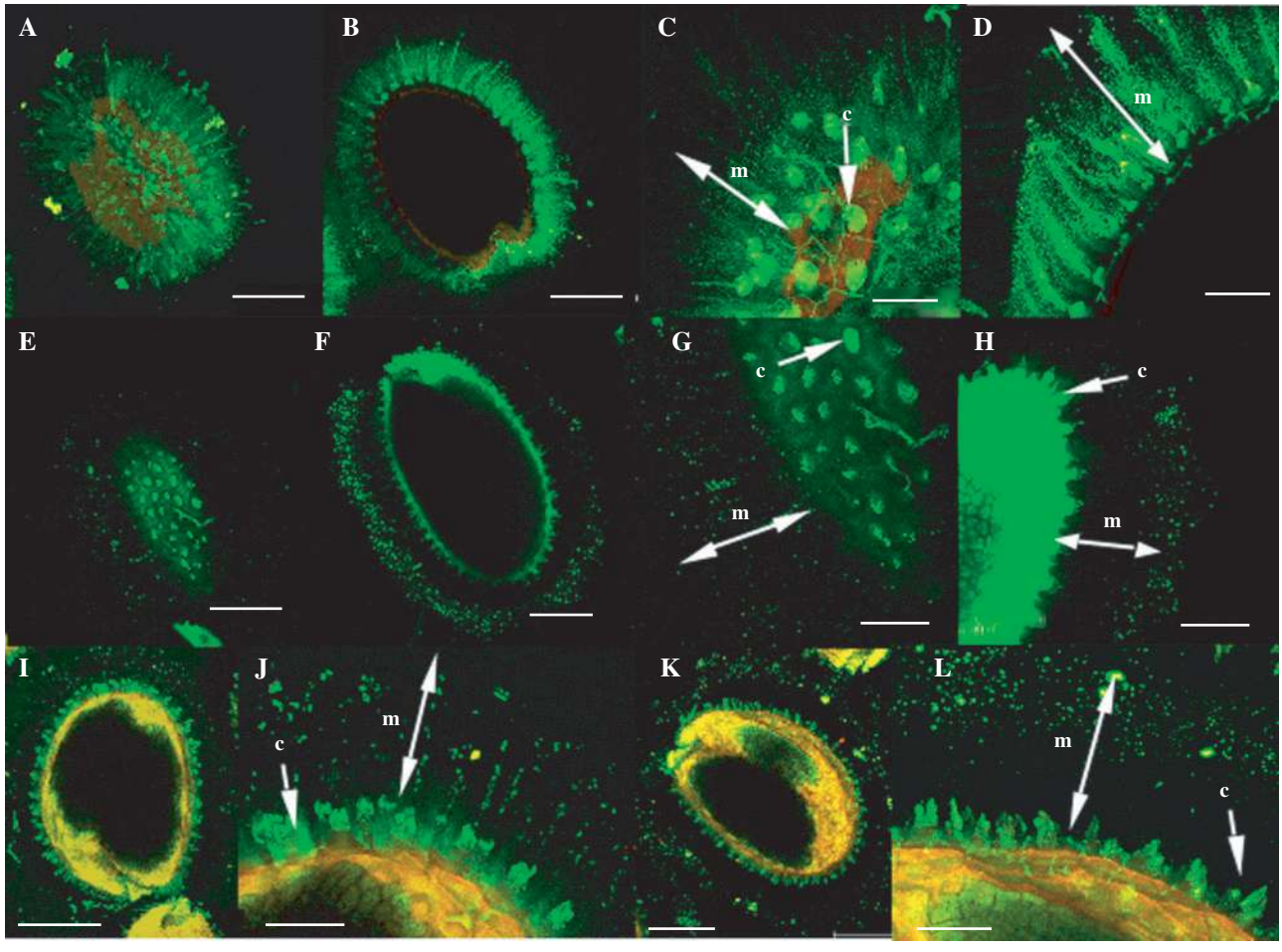

Fig. 4 Antipectin antibody labeling of the inner layer of *Arabidopsis* seed mucilage observed as immunofluorescence. Whole, imbibed seeds were labeled with (A–D) JIM5, (E–H) JIM7, (I and J) LM5 and (K and L) LM6 antibodies. Confocal microscopy was used to take optical sections either at the seed surface (A, C, E and G) or passing through the seed (B, D, F, H, I, J, K and L); m, mucilage; c, columella. Seeds were from the Col-0 accession. Scale bars: 150 μm (A, B, E, F, I and K), 60 μm (G and H), 50 μm (C and D) and 45 μm (J and L).

of seed mucilage in C24, Landsberg *erecta*, Col-2 and Col-0 accessions (Willats et al. 2001, Moffatt et al. 2002, Western et al. 2004) using epifluorescence microscopy. Here we used confocal microscopy, which has the advantage of enabling optical sections to be obtained from intact samples, and when applied to the analysis of immunofluorescence images increases their resolution, thus allowing more detailed information to be obtained concerning the localization of different pectin epitopes. Furthermore, analysis of the spectral quality of the signal obtained allows specific labeling to be distinguished from background or autofluorescence. Immunolabeling was carried out with four monoclonal antibodies that recognize different pectin epitopes. JIM5 and JIM7 both bind HG and recognize specific methyl esterification patterns that are restricted to moderate methyl esterification for the former and moderate to high methyl esterification for the latter, whereas LM5 and LM6 recognize (1→4)-β-galactan and (1→5)-α-arabinan, respectively (Jones et al. 1997, Willats et al. 1998, Willats et al. 2000). All four antibodies showed strong labeling of the columella; this appeared to be strongest at the top and associated with cell wall debris that remained attached to the columella (Fig. 4C, G, J, L). The JIM5 antibody showed strong labeling of the inner mucilage layer (Fig. 4A, B), which was not evenly distributed, but was punctate and showed regions that were more dense, as if sprayed out from the top of the columella (Fig. 4D). Labeling with JIM7 was very weak (Fig. 4E, F) and appeared restricted to the outer edge of the inner mucilage layer (Fig. 4G, H). In contrast to previous studies employing epifluorescence microscopy (Willats et al. 2000), using confocal microscopy mucilage labeling was detected with both LM5 and LM6. LM5 labeling resembled that of JIM5, but was much weaker (Fig. 4I, J), whereas LM6 was similar to JIM7

labeling, being clearly on the perimeter of the inner mucilage layer (Fig. 4K, L). Together the results suggest that HG is present in the inner mucilage layer as two distinct populations; the largest one, present in a halo close to the seed coat, is moderately methyl esterified, and the second, at the outer edge, is highly methyl esterified. Furthermore, arabinan was found in the same area as the highly methyl-esterified HG.

Cellulose is present in an internal domain of the inner mucilage layer

Labeling with Calcofluor, a fluorescent probe for β-glycans, had previously indicated that *Arabidopsis* seed mucilage contained cellulose in addition to pectin (Windsor et al. 2000, Willats et al. 2001). The increased resolution possible using confocal microscopy enabled the Calcofluor labeling to be observed in more detail and showed that the inner mucilage layer clearly contained β-glycans (Fig. 5A, C) which, like the JIM5 labeling, appeared to be more intense in rays associated with the tops of the columella (Fig. 5B, D). Calcofluor stains cellulose, callose and other non-substituted or weakly substituted β-glycans. To determine whether the β-glycans observed corresponded to the β (1→3)-glycans found in callose, staining was carried out using aniline blue; only the columella were stained (Fig. 5E). In contrast, staining with Congo Red, another β-glycan stain, also clearly marked the seed mucilage layer (Fig. 5F), indicating that the inner layer of seed mucilage did indeed contain cellulose. To determine whether the observed staining of cellulose microfibrils overlapped with both JIM5 and JIM7 labeling, double labeling of seed mucilage was carried out with JIM5 or JIM7 and Calcofluor. The double labeling showed that JIM5 labeling was localized to an area similar to Calcofluor staining (Fig. 6A–D), whereas the JIM7 labeling was clearly excluded from the mucilage labeled with Calcofluor (Fig. 6E–H).

The JIM7 labeling observed in a previous study suggested that highly methylated HG was present throughout the inner layer of seed mucilage (Willats et al. 2001). In contrast, our results (Figs. 4E–H, 6E–H), and those of Moffatt et al. (2002) indicated that JIM7 labeling was restricted to an outer domain of the inner mucilage layer. The protocol for immersion fluorescence labeling used by Willats et al. (2001) included fixation prior to labeling. To determine whether the more restricted labeling observed with Col-0 was due to the lack of fixation, we carried out double labeling of seed mucilage with JIM5 or JIM7 and Calcofluor after fixation with paraformaldehyde for 1 h. Although Calcofluor staining was identical to that in non-fixed tissue, the labeling patterns observed for JIM5 and JIM7 both showed strong labeling localized at the periphery of the mucilage

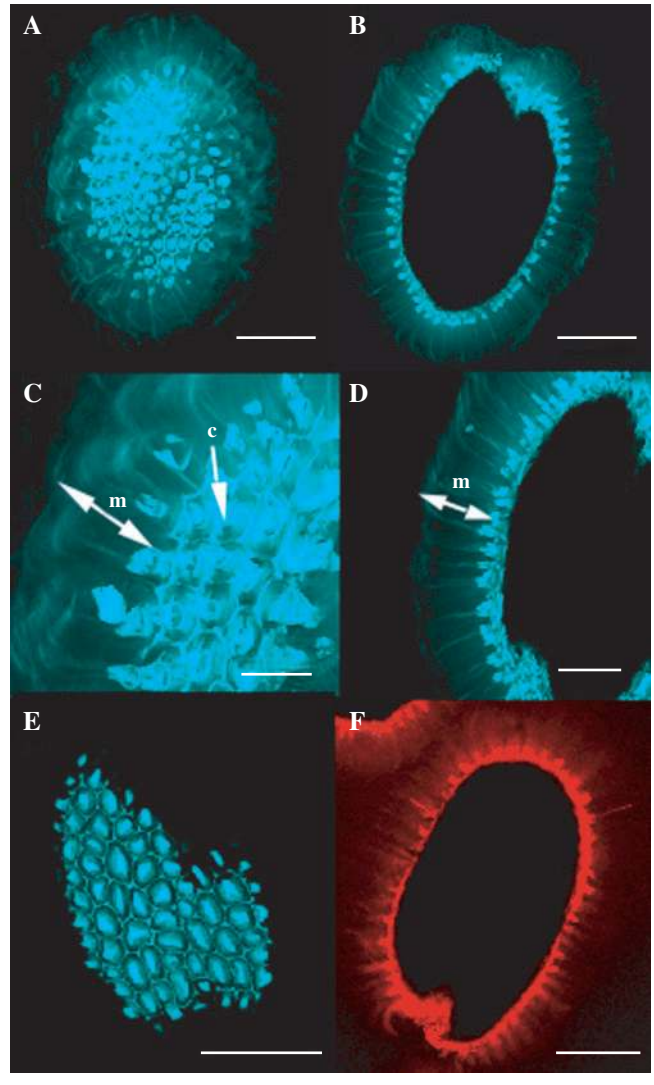

Fig. 5 Staining of β-glycans in the inner layer of *Arabidopsis* seed mucilage with Calcofluor (A–D), aniline blue (E) and Congo Red (F). Confocal microscopy was used to take optical sections of whole, imbibed Col-0 seeds either at the seed surface (A, C and E) or passing through the seed (B, D and E). m, mucilage; c, columella. Scale bars: 150 μm (A, B, E and F), 45 μm (C) and 90 μm (D).

in two accessions, Col-0 and Bay-0 (Fig. 6I, J, and data not shown). The localization of the JIM5 labeling pattern was, therefore, significantly altered, indicating that the paraformaldehyde had changed the pectic epitopes present in the mucilage. In addition, when seeds were immunolabeled after overnight fixation in paraformaldehyde all the labeling of the seed mucilage was lost with either JIM5 or JIM7 antibodies (data not shown). Mucilage fixation with paraformaldehyde would seem, therefore, inappropriate for use with antibodies against certain epitopes.

Determination of mucilage composition by enzymatic digestion

Two major pectolytic enzymes (endo-polygalacturonase and rhamnogalacturonan hydrolase) were tested for their ability to degrade water-extracted mucilage. Endo-polygalacturonase II, active on polygalacturonic acid, appeared unable to degrade seed mucilage (<5 mg of reducing GalA g^{-1} of freeze-dried mucilage), precluding the presence of stretches of (1→4)-linked GalA residues in the mucilage. In contrast, rhamnogalacturonan hydrolase was able to degrade seed mucilage extensively (390 mg of reducing Rha g^{-1} of freeze-dried mucilage). The water-soluble outer layer of *Arabidopsis* seed mucilage appeared, therefore, to be largely composed of unbranched RGI.

Degradation of the inner mucilage layer was also analyzed by digestion with enzymes after removal of the outer soluble layer of mucilage (Fig. 7). Digestion was carried out with a rhamnogalacturonan hydrolase to determine whether RGI was present in both the inner and outer mucilage layers. Ruthenium red and Calcofluor staining showed that the inner mucilage layer was significantly reduced, with patches of pectin and filaments remaining (Fig. 8B); these filaments appeared to correspond to cellulose microfibrils (Fig. 8G). This altered appearance correlated with the large amounts of Rha and GalA released into digest extracts (Fig. 7) in a molar ratio of 1.18 ± 0.27. As immunolabeling and Calcofluor staining results suggested the presence of HG and cellulose, polygalacturonase

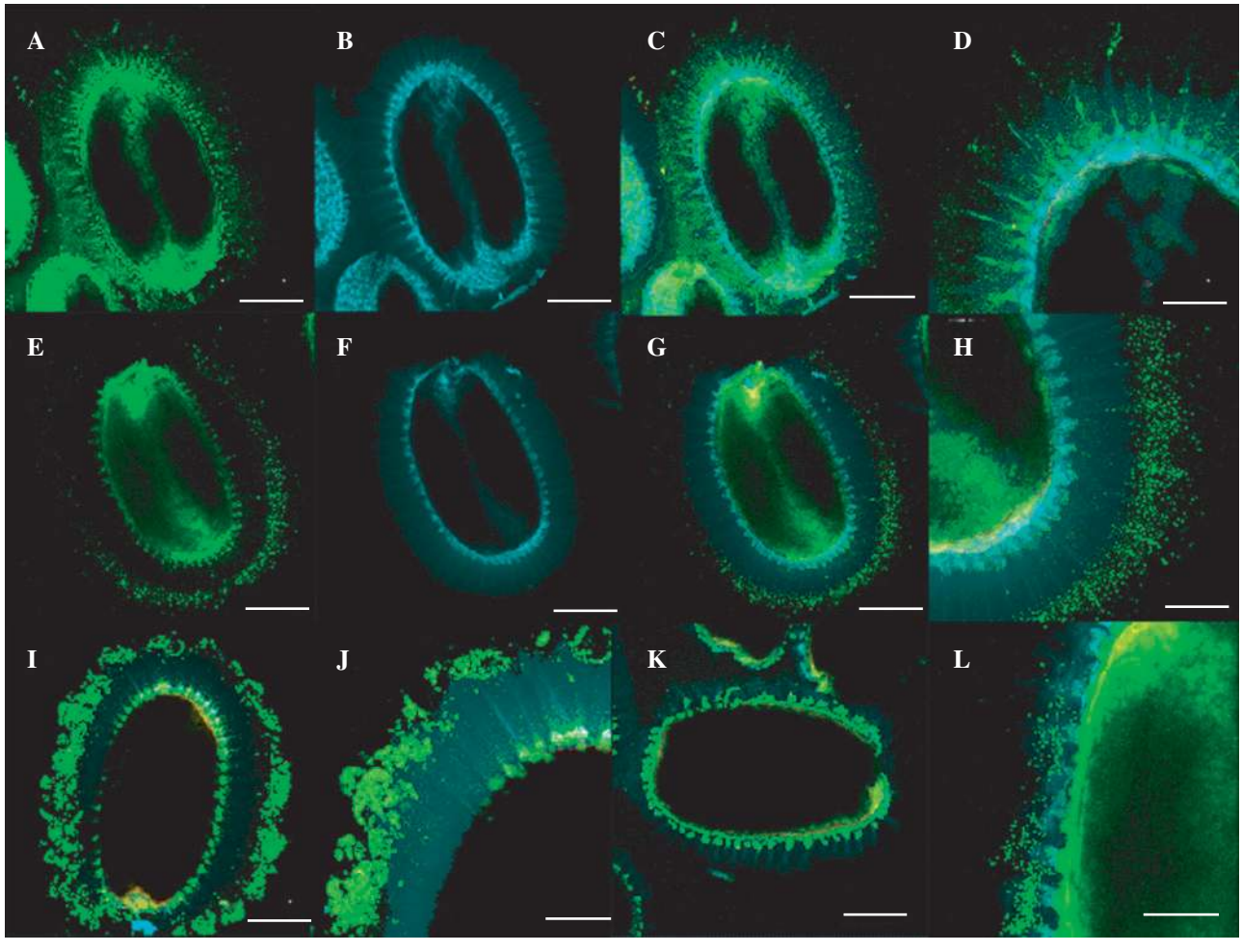

Fig. 6 Double labeling of methyl-esterified pectin epitopes and β-glycans in the inner layer of seed mucilage. (A) JIM5 labeling; (B and F) Calcofluor staining; (C) composite image of A and B; (D) detail of JIM5 and Calcofluor double labeling; (E) JIM7 labeling; (G) composite images of E and F; (H) detail of JIM7 and Calcofluor double labeling; (I) composite image of JIM5 and Calcofluor double labeling after fixation in paraformaldehyde for 1 h; (J) detail of composite image in I; (K) composite image of JIM5- and Calcofluor-labeled *mum5-1*; (L) composite image of JIM7- and Calcofluor-labeled *mum5-1*. Confocal microscopy was used to take optical sections through imbibed, whole, mature seeds. Seeds were from the Col-0 (A–J) or Col-2 accessions. Scale bars: 150 μm (A, B, C, E, F, G, I and K), 70 μm, (J) and 65 μm (D, H and L).

and cellulase treatments were carried out. Ruthenium red and Calcofluor staining were very similar to those of untreated samples after both enzyme treatments (Fig. 8C, D, H, I), although the polygalacturonase-treated mucilage seemed slightly less compact (Fig. 8A compared with C and F with H).

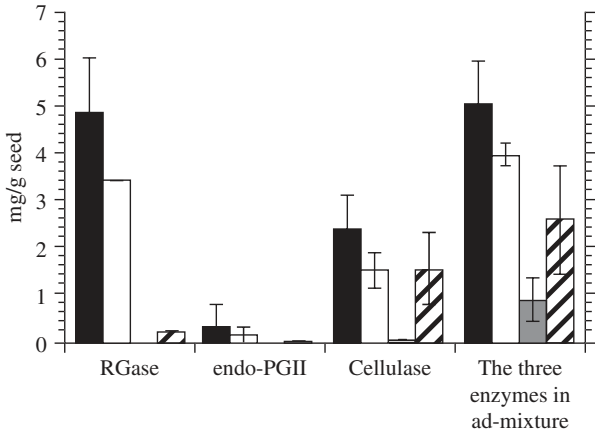

Fig. 7 Sugar analysis of extracts recovered after enzymatic hydrolysis of the inner seed mucilage. Values were calculated after deduction of the amount of the different individual sugars released by buffer alone. Only those sugars whose levels were significant are presented: GalA, black bars; Rha, white bars; Gal, gray bars; Glc, hatched bars. Enzymes used were rhamnogalacturonan hydrolase, RGase; endo-polygalacturonase II, endo-PGII; and cellulase. All experiments were performed in duplicate. Error bars represent the SD ($n = 2$).

In agreement, sugar analysis of the digest extracts detected smaller amounts of released sugars (Fig. 7). The cellulase used for digestion also contains traces of pectolytic enzymes (Panouillé et al. 2006), which probably explains the increase in GalA and Rha (molar ratio 1.30 ± 0.09) in addition to Glc. Digestion with all three enzymes virtually eliminated the inner mucilage layer and most of the filaments, with Calcofluor labeling significantly reduced (Fig. 8E, J). The sugars released on this treatment (Fig. 7) were, therefore, probably the most representative of the composition of the inner layer of mucilage, although some may originate from the seed coat cell walls and the embryo. The predominant sugars were GalA and Rha (molar ratio of 1.05 ± 0.13), and significant amounts of Glc were also present, in agreement with the digestion of cellulose microfibrils. Digestion with the cellulase alone was limited in comparison with digestion with cellulase, rhamnogalacturonan hydrolase and polygalacturonase in ad-mixture, which might be due to the difficulty for the enzyme to penetrate into the dense mass of pectin. Significant amounts of Gal were also released (Fig. 7) which appeared to be derived from the inner mucilage layer, but could come from cell wall components of the seed coat or the embryo.

Composition of mum5-1 mucilage

The *mum5-1* mutant has an altered mucilage composition, which may reflect different pectin branching and cross-linking properties (Western et al. 2001). In effect,

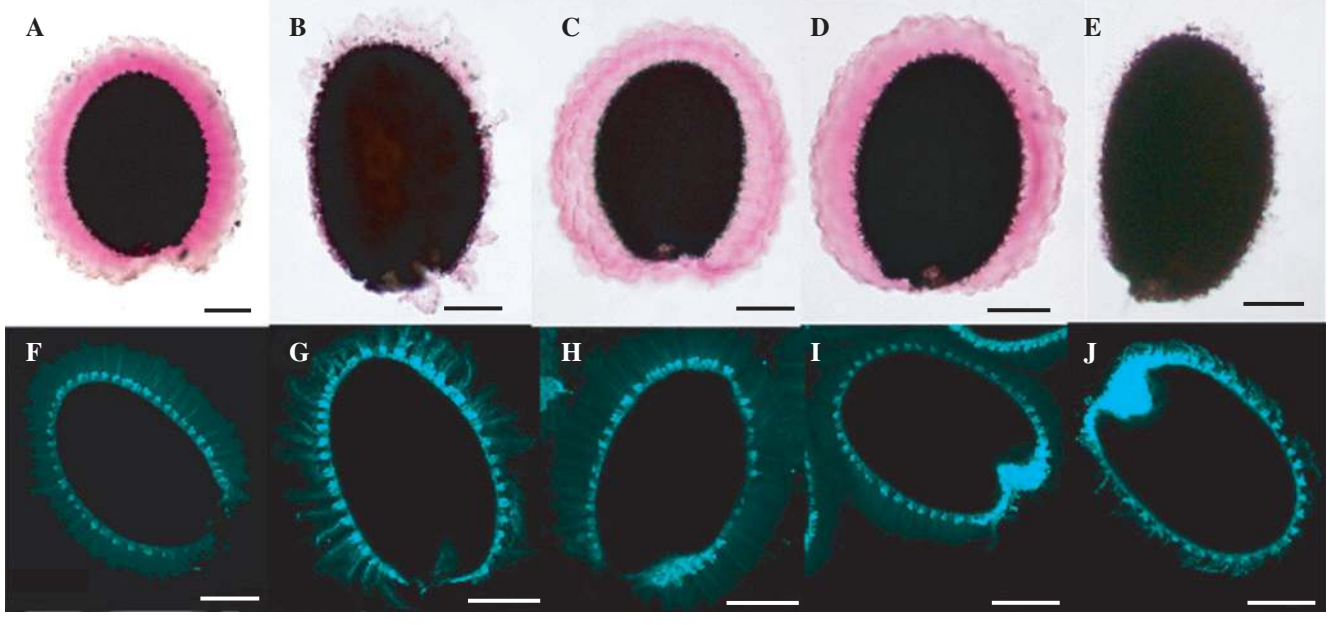

Fig. 8 Histochemical staining of the inner seed mucilage layer after digestion with pectolytic enzymes. (A and F) Undigested control, (B and G) rhamnogalacturonan hydrolase treatment, (C and H) polygalacturonase treatment, (D and I) cellulase treatment, (E and J) digestion with all three enzymes. (A– E) Bright field images of ruthenium red staining. (F–J) Confocal microscopy images of Calcofluor staining with optical sections taken through whole, mature seeds. Seeds were from the Col-0 accession. Scale bars in A–J = 150 μm.

ruthenium red staining of *mum5-1* seed mucilage is lost on shaking (Western et al. 2001; Fig. 1G, H). To investigate whether water-extracted mucilage from *mum5-1* contained inner mucilage layer components, monosaccharide composition and macromolecular characteristics were determined (Table 1 and Fig. 9). A clear increase was observed in the amount of total sugars present in *mum5-1* water-soluble mucilage compared with that of wild-type Col-2. In particular, the amount of GalA and Rha increased, and their molar ratio remained close to 1. This indicated that inner mucilage layer components were released in addition to those of the outer layer. The macromolecular structure of the mutant mucilage showed major differences from the wild type, although two polymeric populations were found in *mum5-1* mucilage; the high molar mass population (50,000 kDa) was absent and a new 500 kDa population was observed (Fig. 9). The *mum5-1* mutation would thus appear to alter the structure and amount of the water-soluble mucilage layer, but not its composition.

JIM5 and JIM7 labeling was carried out to determine whether the distribution of highly methyl-esterified and moderately methyl-esterified HG populations was affected in the mutant. No JIM5 labeling of mucilage was observed (Fig. 6K) whereas a small amount of JIM7 labeling was seen within the cellulose domain of the inner mucilage (Fig. 6L). In addition, the appearance of the inner layer of mucilage was altered, with the ring of Calcofluor-stained mucilage reduced in size (Fig. 6K, L) and showing spaces between the rays originating from the columella. This confirmed that the increased levels of sugars in water-soluble mucilage were because the majority of those normally present in the inner, adherent mucilage layer were now water-soluble in the *mum5-1* mutant.

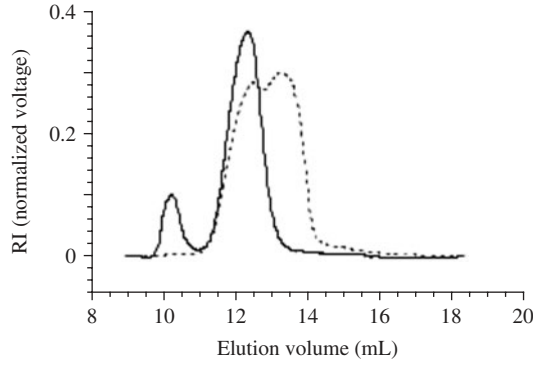

Fig. 9 Quantification of the different macromolecular populations present in water-extracted mucilage from the wild type and *mum5-1*. Mucilage extracts were separated using high-performance size-exclusion chromatography on a system comprising a Shodex OH SB-G pre-column followed by two columns in series (Shodex OH-Pack SB-804 HQ and OH-Pack SB-805 HQ). Refractive index signal: wild-type Col-2, solid line; *mum5-1*, dotted line.

Discussion

The pectinaceous capsule of mucilage that surrounds the *Arabidopsis* seed on imbibition is composed of two layers (Fig. 1A; Western et al. 2000). The outer diffuse layer is easily removed by agitation, whereas the inner layer remains attached to the outer seed coat (Fig. 1C). Analysis of the composition of seed mucilage has been carried out previously using ammonium oxalate extracts (Goto 1985, Western et al. 2000, Western et al. 2001, Usadel et al. 2004, Western et al. 2004). Ruthenium red staining results were identical with this technique and water extraction (Fig. 1D, and data not shown), and as most of the inner mucilage layer remained attached to the seed coat it did not present an advantage over a simple water extraction. In effect, analyses of monosaccharide composition were equivalent with both techniques (Table 1). The monosaccharide composition of the external layer of seed mucilage has been analysed in several different accessions and found to be mainly composed of Rha and GalA (Table 1; Goto 1985, Western et al. 2000, Penfield et al. 2001, Usadel et al. 2004). Although the sugar composition between different accessions was very similar, the quantity varied (Table 1). This natural variation in the amount of the outer layer of mucilage may reflect different mucilage requirements with regard to the environmental conditions in which the accessions are found. The role of seed mucilage remains an enigma. It has been suggested that it is important for seed dispersal, adherence to soil particles or the control of germination (Young and Evans 1973, Gutterman and Shem-Tov 1996). Such functions imply that the mucilage be tightly fixed to the outer seed coat. This is true of the inner layer of seed mucilage, but the facility with which the outer layer of mucilage diffuses from around the seed would suggest that it plays a different role. Pectins are targets for degradation by bacterial and fungal enzymes (Willats et al. 2001) and it is possible that release of soluble pectins into the environment around the seed conditions the rhizosphere in preparation for seedling germination. The correlation of outer seed mucilage amount with the ecosystem in which *Arabidopsis* accessions are found may provide a useful tool for determining the role of the outer layer of mucilage.

The outer layer of water-soluble mucilage consists of unbranched, non-esterified RGI

Sugar composition analyses (Table 1) showed that the most abundant monosaccharides in water-soluble mucilage were GalA and Rha in a molar ratio of approximately 1, indicating that this mucilage was mostly composed of RGI as suggested by previous analyses (Goto 1985,

Penfield et al. 2001). These studies also proposed that Rha was the major monosaccharide, whereas our analyses found that GalA was consistently the most abundant sugar (Table 1); however, GalA measurements were not presented in the former study and the latter analyzed whole seed monosaccharide composition rather than water-extracted mucilage. The extensive digestion of water-soluble mucilage with rhamnogalacturonan hydrolase confirmed that it is indeed mainly RGI.

Analysis of the physical properties of the water-extracted mucilage identified two populations with different molar masses (Fig. 2 and Table 2); the majority of the mucilage had a molar mass of around 600 kDa. This population probably corresponds to those identified by Goto (1985) using the Sendai accession, estimated to have a molar mass of 2,000 kDa, or by Usadel et al. (2004) with Col-0, estimated at 5,200 kDa. The size differences presumably reflect differences in resolution between the techniques used; SEC with dextran size markers or field flow fractionation, respectively. The hydrodynamic parameter calculated for the 600 kDa population indicated that it was present in a slightly expanded random coil conformation. The identification of a second minor population with a very large molar mass (50,000 kDa) suggested either that the macromolecular structure of water-soluble mucilage is not uniform or that some of the inner mucilage was in fact extracted in water, perhaps as a result of the vigorous vortexing. The physical properties of the 50,000 kDa population indicated the presence of aggregated macromolecules (Table 2). As this was replaced by a new 500 kDa population in the *mum5-1* mutant (Fig. 9), it would appear that pectin synthesis itself is not affected in the mutant, but that it is defective in the process of aggregation or cross-linking which forms the high molecular weight population.

Methyl esters of (1→4)-α-D-GalA were not detected in linkage analysis of soluble polysaccharides from whole seed extracts (Penfield et al. 2001), and our results also indicated that water-soluble mucilage was not methylated or acetylated (Table 1). This is in accord with the digestion of the water-soluble mucilage with rhamnogalacturonan hydrolase, which cannot digest methyl-esterified RGI. Furthermore, enzymatic hydrolysis with endo-polygalacturonase did not indicate the presence of HG in the water-soluble mucilage and the GalA/Rha molar ratio was close to 1. Nonetheless, the JIM7 antibody has previously been shown to bind strongly in immuno-dot assays to water-soluble mucilage from the C24 accession (Willats et al. 2001), indicating the presence of highly methyl-esterified HG. This suggests either that different accessions exhibit different levels of esterification or that other epitopes recognized by the JIM7 antibody are present in the outer water-soluble mucilage layer.

The inner mucilage layer, although structurally different, is mainly RGI

The inner layer of seed mucilage appeared to be strongly attached to seeds, as it could not be removed with either hot acid or alkali (Table 1 and Fig. 1E, F). Analysis of the composition of the inner mucilage layer was possible after treatment with various pectolytic enzymes. These showed that like the outer mucilage layer, the major components of the mucilage were GalA and Rha in a molar ratio of 1.05 ± 0.13 and the most extensive mucilage digestion was observed with rhamnogalacturonan hydrolase (Fig. 8B, G), indicating that the major pectin was RGI. As 38% of the sugars in seed mucilage are Rha, it is not surprising that a mutation in a putative NDP-L-rhamnose synthase (*RHM2*/*MUM4*) caused a strong reduction in the amount of mucilage accumulated (Usadel et al. 2004, Western et al. 2004).

The GalA/Rha ratio was slightly higher for the inner seed mucilage layer compared with that observed for the outer layer, which could be due to the presence of small stretches of GalA. In agreement with previously published results (Willats et al. 2001, Moffatt et al. 2002), immunolabeling with antibodies against HG indicated that both highly methyl-esterified and moderately methyl-esterified HG were present in the mucilage. Furthermore, our results showed that they were localized to different domains of the inner mucilage (Figs. 4A–H, Fig 6A–H). Nonetheless non-methylated HG must represent a very minor component of the inner layer of mucilage, as digestion with polygalacturonase had little effect on the integrity of the mucilage capsule (Fig. 8C, H) and only small amounts of GalA were released (Fig. 7).

Other monosaccharides were also detected as minor components of the inner layer of mucilage; notably Glc and Gal (Fig. 7). Immunolabeling results confirmed the presence of Gal within the inner mucilage layer (Fig. 4I, J) and also indicated that some Ara was present and showed a domain-specific localization at the periphery of the inner layer where highly methyl-esterified HG was found (Fig. 4K, L). Previous experiments using LM5 and LM6 antibodies against mucilage from the C24 accession did not detect Gal and Ara. It is probable that the improved resolution obtained using confocal microscopy allowed the relatively low levels of labeling to be detected. Indeed, Gal represented only 6.6% of the monosaccharides present in the inner mucilage layer, and only traces of Ara were identified. Alternatively, the difference in LM5 and LM6 labeling might be due to differences in the composition of the inner mucilage between the C24 and Col-0 accessions, in contrast to the outer water-soluble layer whose composition was similar in the different accessions tested.

Cellulose is present in an internal domain of the inner mucilage layer

Comparison of Calcofluor, Congo Red and aniline blue staining of the seed coat and mucilage confirmed that the inner layer of seed mucilage contained cellulose (Fig. 5; Windsor et al. 2000, Willats et al. 2001) as callose was only present in the columella (Fig. 5E). No cellulosic Glc was detected in extracts of the outer layer of water-soluble mucilage (Table 1), and double labeling of mucilage with JIM7 and Calcofluor indicated that cellulose was not present throughout the inner mucilage (Fig. 6F–H), but was limited to an inner domain. In accordance, digestion of the inner seed coat layer with cellulase released Glc (Fig. 7). Furthermore, significantly more Glc was released when cellulase was used in conjunction with rhamnogalacturonan hydrolase and polygalacturonase, indicating that their activity rendered more cellulose accessible to digestion; this would be in agreement with the localization of the cellulose in an inner domain. Although it cannot be excluded that the cellulase digested cell wall components of the seed coat, Glc derived from digestion of cellulose within the mucilage can be estimated as between 12 and 19% of inner mucilage layer sugars. This cellulose may be important for maintaining the inner mucilage layer attached to the seed coat as the microfibrils appeared to radiate out from the surface of the seed coat, and these could serve as a scaffold in which large RGI polymers are entangled or cross-linked. Seed mucilages from other plant species have also been found to contain cellulose (Azuma and Sakamoto 2003), and in quince seeds it was observed as helicoidal arrays within unreleased mucilage (Willison and Abeysekera 1988).

The temperature-sensitive *radial swelling 3* (*rsw3*) mutant shows cellulose-deficient phenotypes and is also affected in mucilage secretion (Burn et al. 2002). The *RSW3* gene encodes the α-subunit of a glucosidase II, and it was postulated either that mucilage secretion was affected due to defective processing of pectin biosynthetic enzymes by the Golgi or that mucilage was retained because of structural changes resulting from cellulose deficiency (Burn et al. 2002). As cellulose is present within the mucilage itself, it is also possible that the reduced levels of cellulose affect mucilage composition such that either accumulation or hydrophilic properties are reduced and mucilage is not released from the epidermal cells of the seed coat. Images of the surface of dry *rsw3* seeds revealed a flattened aspect with no distinguishable columella compared with seed produced under non-restrictive conditions (Burn et al. 2002), indicating that reduced amounts of mucilage are synthesized (Western et al. 2006). Further analyses of mucilage and the outer seed coat layer in the mutant are required to determine the exact effect of the *RSW3* gene.

The role of de-esterification for inner mucilage layer structure

Ruthenium red staining of the *mum5-1* mutant without agitation has shown that two layers of mucilage are present, but that staining of the inner layer was lost on shaking (Western et al. 2001). Observation of mucilage using India ink indicated it was nevertheless present, but was now colorless (Western et al. 2001). In contrast, our ruthenium red staining indicated that only a small amount of the inner mucilage layer remained attached to the seed on shaking (Fig. 1H compared with G). Calcofluor staining and analysis of the sugar composition of water-soluble mucilage showed that a large part of the pectin present in the inner layer of mucilage had in fact become water soluble (Table 1 and Fig. 6K, L). Nonetheless, significant amounts of the cellulose present in the internal domain of the inner mucilage layer apparently remained attached to the seed (Fig. 6K, L).

The *mum5-1* mutant is thought to be defective in a pectin methylesterase (M. Facette and C. Somerville personal communication, cited in Western, 2006) and in accordance JIM7 and JIM5 labeling of the inner mucilage layer was altered, with JIM7 epitopes being present in the inner domain of the inner mucilage layer, whereas all JIM5 labeling was lost (Fig. 6K, L). In plant cell walls it has been hypothesized that HG is synthesized in a highly esterified form and is de-esterified in muro (Schols and Voragen 2002). The altered distribution of methyl-esterified epitopes in the *mum5-1* mutant would imply that HG regions in the inner mucilage layer are synthesized already methyl esterified and that subsequently de-esterification occurs in an internal domain of the inner mucilage layer.

De-esterified HG can form ionic cross-links with Ca^{2+}, which could play a role in maintaining the inner layer of mucilage attached to the seed. Polygalacturonase digestion of the inner mucilage layer did not, however, significantly affect its appearance, although its volume appeared to have increased slightly (Fig. 8C, H). Furthermore, Calcofluor staining of mucilage from wild-type seeds after shaking in the Ca^{2+} chelator EDTA also indicated an increased mucilage volume (data not shown), compared with that of seeds shaken in water. This is in agreement with immuno-labeling results obtained after CDTA treatment and the absence of binding to C24 seed mucilage with the antibody PAM1, which recognizes long stretches of de-esterified blocks of HG (Willats et al. 2001). Together, these results imply that although some Ca^{2+}–HG cross-links may be present, these are not responsible for the structural resistance of the inner mucilage. As most of the pectic components of the inner mucilage layer were water soluble in the *mum5-1* mutant, which is potentially defective in a pectin methylesterase, this means that de-esterification could be important for mucilage synthesis during seed

development for structuring the pectin present in the inner mucilage layer. Alternatively, de-esterified HG or RG could form cross-links with other molecules or polymers.

What retains the inner mucilage layer attached to the seed?

The resistance of the inner mucilage layer to hot dilute acid or alkali treatments (Fig. 1E, F; Table 1) suggested that it was covalently attached to the outer seed coat. The question arises, therefore, as to what would be the nature of these covalent links. As the major pectic component of the inner mucilage layer, RGI, was water soluble in the *mum5-1* mutant, de-esterification might be implicated in the structuring of the inner mucilage layer. Furthermore, cellulose found in the internal domain of the inner mucilage layer was still attached to the seed coat in the *mum5-1* mutant (Fig. 6K, L). Indeed the aspect of the Calcofluor staining resembled that of wild-type seeds digested with rhamnogalacturonan hydrolase (Fig. 8G) although the amount of cellulose appeared to be reduced. It is possible that de-esterification is important for the generation of covalent bonds between RGI and cellulose. Nonetheless, cellulose was not present throughout the inner mucilage and such links could not be the only ones that retain the inner mucilage layer. The high molar mass population present in water-soluble mucilage extracts (Table 2) might represent some inner mucilage detached by the vigorous mixing used. Indeed, although the majority of the mucilage appeared intact on ruthenium red staining, some of the most external mucilage appeared to be removed by this mixing (Fig. 1D and data not shown). Such non-cellulose-linked RGI might, therefore, be less resistant to the mechanical forces applied in this extraction. Analyses of the macromolecular parameters of this 50,000 kDa molar mass size population suggested aggregated or entangled macromolecules, and these could be maintained by inter-RGI cross-links via branching. Further information is required concerning the behaviour of RGI in different physico-chemical environments in order to determine what type of interactions and molecular structures can be formed.

In conclusion, our results show that the major pectin in both water-soluble and adherent seed mucilage is RGI. In addition, the adherent inner mucilage layer can itself be separated into two domains, with the internal domain containing cellulose (Fig. 10). This cellulose appeared to be present as microfibrils that might form a matrix involved in maintaining the inner mucilage layer attached to the seed coat. Anti-pectin antibodies indicated further structural differences between the two domains involving minor sugar components, Ara and Gal, and the level of methyl-esterification (Fig. 10). Understanding the structure of pectic polysaccharides is often hampered, as naturally occurring pectins are often mixtures of polysaccharide types. The availability of an easily extractable and relatively pure unbranched RG resource will be of considerable use for the advancement of our understanding of pectin structure. Indeed *Arabidopsis* seed mucilage has recently been used as an RG substrate for the development of a method for selective methyl esterification and cleavage of GalA residues (Deng et al. 2006).

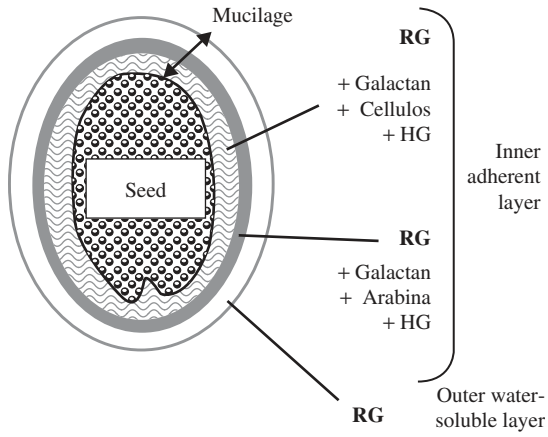

Fig. 10 Diagramatic representation of the structure of *Arabidopsis* seed mucilage based on the results obtained in this study. The major component of mucilage is a rhamnogalacturonan (RG) backbone shown in bold type. The mucilage is composed of an inner adherent layer and an outer water-soluble layer. The latter is probably in a slightly expanded random coil conformation and has a molar mass of around 600 kDa. The inner mucilage layer has a very high molar mass (50,000 kDa) and is probably composed of aggregated polysaccharides. It can be separated into two structural domains; an inner cellulose-containing domain, which also contains a small amount of galactan, and an outer domain, which contains both galactan and arabinan as minor constituents. Both inner and outer domains appear to contain small stretches of homogalacturonan (HG) with differing degrees of methyl esterification.

Materials and Methods

Plant material and growth conditions

Wild-type seeds used in this study were from *Arabidopsis* (*Arabidopsis thaliana*) accessions Col-0, Col-2 and Bay-0. Seeds for the mutant *mum5-1* (accession Col-2) were obtained from the Nottingham *Arabidopsis* Stock Center (http://nasc.nott.ac.uk). Seeds were harvested from plants grown in greenhouse conditions (22–28°C) with a minimum photoperiod of 13 h assured when required by supplementary white light. Seed lots used in individual experiments were harvested from plants grown simultaneously. Plants were grown in compost (Tref Substrates, Rotterdam, The Netherlands) and watered with Plan-Prod nutritive solution (Fertil, Boulogne-Billancourt, France) 2–3 times a week.

Cytochemical probes

Seed mucilage was stained with 200 $\mu g\,ml^{-1}$ ruthenium red or 25 $\mu g\,ml^{-1}$ Calcofluor (Sigma-Aldrich, St Quentin Fallavier, France) essentially as described by Willats et al. (2001).

Staining with 1% (w/v) Congo red or aniline blue was carried out for 10 or 30 min, respectively, at room temperature before rinsing twice with water and observation. Examination of ruthenium red staining was carried out with a light microscope (Axioplan2; Zeiss, Jena, Germany). Calcofluor, Congo Red and aniline blue staining were observed with an inverted Leica TCS-SP2-AOBS spectral confocal laser microscope (Leica Microsystems, Manheim, Germany) equipped with a 405 nm laser diode for excitation.

Immunolabeling procedures

Four primary monoclonal antibodies were used for immunolabeling; JIM5, JIM7, LM5, and LM6 (Plant Probes, Leeds, UK) which bind low-ester HG, high-ester HG, (1→4)-β-D-galactan or (1→5)-α-D-arabinan respectively (Knox et al. 1990, Jones et al. 1997, Willats et al. 2001). Intact mature seeds were imbibed in water without shaking for 30 min at room temperature before immunostaining. For fixed mature seed samples, incubation was carried out directly in 4% (w/v) paraformaldehyde for 1 h or overnight as described by Willats et al. (2001). Epitope demasking was carried out prior to incubation in JIM5, LM5 and LM6 antibodies in a protocol adapted from Leon and Sormunen (1998) by heating samples in 8 mM sodium citrate, 2 mM citric acid in a microwave oven until boiling then washing in phosphate-buffered saline (PBS). Samples were then incubated for 2 h at 37°C in primary antibody diluted 10-fold in PBS containing 1% (w/v) bovine serum albumin (BSA), washed with PBS (three changes, 3 min per wash) then incubated for 2 h at 37°C in secondary antibody, goat anti-rat-IgG antibody conjugated to Alexa Fluor 488 (Molecular Probes, Invitrogen, Cergy Pontoise, France), diluted 1 : 500 in PBS, 1% (w/v) BSA. After washing in PBS (three changes, 3 min per wash), the samples were mounted in anti-fade agent (Citifluor, Oxford Instruments, Orsay, France). For double labeling, seeds were first labeled with the appropriate antibody as described above, and after rinsing once in PBS stained with Calcofluor as above.

Confocal microscopy

Observations were carried out using an inverted Leica TCS-SP2-AOBS spectral confocal laser microscope (Leica Microsystems, Mannheim, Germany), using either an PL APO 20 × 0.70 NA or 63 × 1.20 NA water-immersion objective. Fluorescence labeling from Alexa Fluor and Congo Red were observed with a 488 or 514 nm light wavelength, respectively, generated by an argon laser, whereas Calcofluor and aniline blue were excited with a 405 nm UV diode. Emission bands of 498–567, 554–681 and 418–474 nm were used for fluorescent dye, Congo red and Calcofluor or aniline blue detection, respectively. Each image presented consists of the maximum projection of 20–60 optical sections. Each section is the average of three scans conducted at the resolution of 512 × 512 pixels.

The noise level generated by autofluorescence of tissues and the specificity of the labeling were systematically examined using two spectral bands. A first photomultiplier collected the light emitted by the secondary antibody between 498 and 567 nm, represented by green in the images. The second photomultiplier collected the light emitted between 605 and 683 nm. This spectral band corresponded to autofluorescence (represented by red or yellow in the images). Noise level was determined by superposing the two channels. To confirm labeling specificity, wavelength scans were obtained in the optical section of each image where maximum labeling was observed. A stack of 20 individual images was recorded, which corresponded to the signal detected at a specific wavelength between 495 and 605 nm. At least 10 regions within the image were selected as polygonal sections and the wavelength at which the maximal emission signal was obtained confirmed to correspond to that of the probe used.

Mucilage extraction

Extruded mucilage was extracted from approximately 300 mg of intact seeds by mixing head-over-tail overnight in either distilled water (3 ml) at 4°C for 16 h or 0.2% (w/v) ammonium oxalate at 40°C for 16 h. The resulting suspension was then vortexed vigorously prior to centrifugation (8,000×*g*, 3 min). Supernatants were recovered and seeds were rinsed twice with 1 ml of water or 0.2% (w/v) ammonium oxalate. Supernatants and washes were pooled and filtered through a disposable glass microfiber filter (13 mm diameter, 2.7 µm pore size) (Whatman Inc., Sanford, ME, USA). Extracts were then dialyzed (molecular weight cut-off 6,000–8,000) three times against 500 ml of water. A large-scale extraction was also carried out on Col-0 seeds using approximately 2 g of seeds and 20 ml of distilled water. The extracted mucilage was recovered as described above. The washes were pooled with the extract before dialysis and freeze-drying.

Mucilage was also extracted from Col-0 samples by lateral shaking in 0.1 N NaOH (300 mg seed per 11 ml) at 80°C for 2 h or 0.05 N HCl (200 mg per 5 ml) for 30 min at 85°C followed by addition of 5 ml of 0.3 N NaOH and incubation at room temperature for 10 min. Suspensions were filtered on a G1 sintered glass, and seeds were rinsed twice with 1 ml of water. Extracts were then dialyzed three times against 500 ml of water prior to analysis.

For HP-SEC, mucilage was extracted from samples of 50 or 100 mg of intact seeds by mixing head-over-tail overnight in HP-SEC elution buffer [50 mM sodium nitrate, 0.2% (w/v) sodium azide] (1 ml) at 4°C. Suspensions were then vortexed vigorously prior to centrifugation (8,000×*g*, 2 min). An aliquot of extract was filtered sequentially through a glass microfiber filter (13 mm diameter, 2.7 µm pore size) and a PVDF filter (13 mm diameter, 0.45 µm pore size) (Whatman Inc., Sanford, ME, USA) prior to analysis.

Enzyme assays

Rhamnogalacturonan hydrolase (Swiss-Prot Q00018) was provided by Novozymes (Copenhagen, Denmark). It was originally cloned from *Aspergillus aculeatus* and expressed in *Aspergillus oryzae*. Endo-polygalacturonase II (Swiss-Prot P26214) was purified from a crude preparation of a cloned *Aspergillus niger* (Sakamoto et al., 2003) (Sigma-Aldrich, St Quentin Fallavier, France). Endo 1,5-α-L-arabinanase and endo-1,4-β-D-galactanase were purified from *A. niger* as previously described (Bonnin et al. 2002). Enzymatic activities towards different polysaccharide substrates present in water-soluble mucilage were calculated from the increase in reducing ends (Nelson 1944), using Rha, GalA, Ara or Gal for standard curves. Assays were carried out in microtiter plates as described by Sturgeon (1990). Reaction mixtures for rhamnogalacturonan hydrolase and endo-polygalacturonase II contained 0.9 ml of substrate (1 mg of freeze-dried mucilage ml^{-1} in 50 mM sodium acetate buffer, pH 4.5) and 0.1 ml of appropriately diluted enzyme (i.e. 0.1 nkat ml^{-1} of reaction mixture). Reaction mixtures for endo-arabinanase and endo-galactanase consisted of 0.4 ml of substrate (4 mg of freeze-dried mucilage ml^{-1} in 50 mM sodium acetate buffer, pH 4.5) and 1 µl of arabinase or galactanase (i.e. 0.8 nkat ml^{-1} of reaction mixture). Total reducing sugars were measured after 0 and 24 h of digestion at 40°C.

Prior to digestion of the inner mucilage layer, the outer mucilage layer was washed from 200 mg of seeds by lateral shaking

twice in 5 ml of water for 5 min followed by shaking in 20 ml of 50 mM sodium acetate buffer, pH 4.5 (three changes). Cellulase (0.9 nkat) (Maxazyme®, DSM, Seclin, France), rhamnogalacturonan hydrolase (0.1 nkat) and endo-polygalacturonase II (0.7 nkat) were added to the imbibed seeds in 3 ml of sodium acetate buffer and the reactions incubated at 40°C for 16 h. A control reaction without enzyme was also carried out. After vortexing, and centrifugation (3,000 *g*, 30 s), the supernatant was removed for analysis. Duplicate reactions were carried out for each condition.

Size-exclusion chromatography

HP-SEC was performed at room temperature on a system comprising a Shodex OH SB-G pre-column followed by two columns in series (Shodex OH-Pack SB-804 HQ and OH-Pack SB-805 HQ, Shodex, Showa Denko KK, Miniato, Japan). Elution was carried out with 50 mM sodium nitrate buffer containing 0.02% (w/v) sodium azide at a constant flow rate of 42 ml h^{-1}. Measurements were obtained using four detectors; a refractometer (RI) (ERC 7517A), a UV detector operating at 280 nm, a differential viscosimeter (T-50A, Viscotek, Houston, TX, USA) and a MALLS (Mini Dawn, Wyatt, Santa Barbara, CA, USA) operating at three angles (41°, 90° and 138°). Samples were injected automatically through a 50 µl loop. Data for molar mass determinations were analyzed using Astra software (Wyatt, Santa Barbara, CA, USA) taking d*n*/d*c* as 0.146. Data for viscosimetry determinations were analyzed using TriSEC software (Version 3.0, Viscotek). No data smoothing was applied.

Standard SEC was performed on a Sephacryl S-500 column (85 × 5 cm) equilibrated with 0.1 M sodium acetate buffer, pH 4. Water-soluble dialyzed and freeze-dried mucilage were solubilized by mixing in elution buffer (15 mg per 8 ml) overnight. The solution was loaded onto the column and eluted by upward elution at 60 ml h^{-1}. Fractions (5 ml) were collected and analyzed for their galacturonic and neutral sugar contents by colorimetry using the automated *m*-hydroxybiphenyl and orcinol methods, respectively (Thibault 1979, Tollier and Robin 1979). Fractions were also assayed for individual neutral sugars by gas–liquid chromatography (GLC; Blakeney et al. 1983).

Analytical

The uronic acid content was determined by the automated *m*-hydroxybiphenyl method (Thibault 1979). Individual neutral sugars were analyzed as their alditol acetate derivatives (Blakeney et al. 1983) by GLC after hydrolysis of mucilage extracts with 2 M trifluoroacetic acid at 121°C for 2.5 h. The quantification of cellulosic Glc was carried out on freeze-dried mucilage after pre-hydrolysis in 72% (v/v) H_2SO_4 (30 min, 25°C) followed by hydrolysis with 1 M H_2SO_4 (2 or 6 h, 100°C) (Seaman et al. 1954). Methanol and acetic acid released by alkaline de-esterification of mucilage were quantified by HPLC (Levigne et al. 2002). Protein concentrations were determined by the Bradford method (Bradford 1976) using BSA (Sigma-Aldrich, St Quentin Fallavier, France) for the standard curve.

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References

Arabidopsis Genome Initiative, (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* 408: 796–815.

Azuma, J.-I. and Sakamoto, M. (2003) Cellulosic hydrocolloid system present in seed of plants. *Trends Glycosci. Glycotechnol*. 15: 1–14.

Beeckman, T., De Rycke, R., Viane, R. and Inzé, D. (2000) Histological study of seed coat development in *Arabidopsis thaliana*. *J. Plant Res*. 113: 139–148.

Blake, A.W., McCartney, L., Flint, J.E., Bolam, D.N., Boraston, A.B., Gilbert, H.J. and Knox, J.P. (2006) Understanding the biological rationale for the diversity of cellulose-directed carbohydrate-binding molecules in prokaryotic enzymes. *J. Biol. Chem*. 281: 29321–29329.

Blakeney, A.B., Harris, P.J., Henry, R.J. and Stone, B.A. (1983) A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydr. Res*. 113: 291–299.

Bonnin, E., Dolo, E., Le Goff, A. and Thibault, J.-F. (2002) Characterization of pectin subunits released by an optimised combination of enzymes. *Carbohydr. Res*. 337: 1687–1696.

Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem*. 72: 248–254.

Burchard, W. (1994) Light scattering techniques. *In* Physical Techniques for the Study of Food Biopolymers. Edited by Ross-Murphy, S.B. pp. 152–213. Blackie Academic and Professional, London.

Burn, J.E., Hurley, U.A., Birch, R.J., Arioli, T., Cork, A. and Williamson, R.E. (2002) The cellulose-deficient *Arabidopsis* mutant *rsw3* is defective in a gene encoding a putative glucosidase II, an enzyme processing N-glycans during ER quality control. *Plant J*. 32: 949–960.

Deng, C., O'Neill, M.A. and York, W.S. (2006) Selective chemical depolymerization of rhamnogalacturonans. *Carbohydr. Res*. 341: 474–484.

Goto, N. (1985) A mucilage polysaccharide secreted from testa of *Arabidopsis thaliana*. *Arabidopsis Inf. Serv*. 22: 143–145.

Gutterman, Y. and Shem-Tov, S. (1996) Structure and function of the mucilaginous seed coats of *Plantago coronopus* inhabiting the Negev Desert of Israel. *Isr. J. Plant Sci*. 44: 125–133.

Haughn, G. and Chaudhury, A. (2005) Genetic analysis of seed coat development in Arabidopsis. *Trends Plant. Sci*. 10: 472–477.

Jofuku, K.D., du Boer, B.G.W., Van Montagu, M. and Okamro, J.K. (1994) Control of Arabidopsis flower and seed development by the homeotic gene *APETALA2*. *Plant Cell* 6: 1211–1225.

Johnson, C.S., Kolevski, B. and Smyth, D.R. (2002) *TRANSPARENT TESTA GLABRA2*, a trichome and seed coat development gene of Arabidopsis, encodes a WRKY transcription factor. *Plant Cell* 14: 1359–1375.

Jones, L., Seymour, G.B. and Knox, J.P. (1997) Localization of pectic galactan in tomato cell walls using a monoclonal antibody specific to (1→4)-β-D-galactan. *Plant Physiol*. 113: 1405–1412.

Knox, J.P., Linstead, P.J., King, J., Cooper, C. and Roberts, K. (1990) Pectin esterification is spatially regulated both within cell walls and between developing tissues or root apices. *Planta* 181: 512–521.

Koornneef, M. (1981) The complex syndrome of the *ttg* mutants. *Arabidopsis Inf. Serv*. 18: 45–51.

Koornneef, M., Dellaert, L.W.M. and Vanderveen, J.H. (1982) EMS-induced and radiation-induced mutation frequencies at individual loci in *Arabidopsis thaliana* (L) heynh. *Mutat. Res*. 93: 109–123.

Leon, A.S.-Y. and Sormunen, R.T. (1998) Microwave procedures for electron microscopy and resin-embedded sections. *Micron* 29: 397–409.

Léon-Kloosterziel, K.M., Keijer, C.J. and Koornneef, M. (1994) A seed shape mutant of Arabidopsis that is affected in integument development. *Plant Cell* 6: 385–392.

Levigne, S., Thomas, M., Ralet, M.-C., Quemener, B. and Thibault, J.-F. (2002) Determination of the degrees of methylation and acetylation of pectins using a C18 column and internal standards. *Food Hydrocoll*. 16: 547–550.

McAbee, J.M., Hill, T.A., Skinner, D.J., Izhaki, A., Hauser, B.A., Meister, R.J., Reddy, G.V., Meyerowitz, E.M., Bowman, J.L. and Gasser, C.S. (2006) *ABERRANT TESTA SHAPE* encodes a KANADI family member, linking polarity determination to separation and growth of Arabidopsis ovule integuments. *Plant J*. 46: 522–531.

Moffatt, B.A., Stevens, Y.Y., Allen, M.S., Snider, J.D., Pereira, L.A., Todorova, M.I., Summers, P.S., Weretilnyk, E.A., Martin-McCaffrey, L. and Wagner, C. (2002) Adenosine kinase deficiency is associated with developmental abnormalities and reduced transmethylation. *Plant Physiol*. 128: 812–821.

Nelson, N. (1944) A photometric adaptation of the Somogyi method for determination of glucose. *J. Biol. Chem*. 153: 375–380.

Nesi, N., Debeaujon, I., Jond, C., Pelletier, G., Caboche, M. and Lepiniec, L. (2000) The *TT8* gene encodes a basic helix–loop–helix domain protein required for the expression of *DFR* and *BAN* genes in Arabidopsis siliques. *Plant Cell* 12: 1863–1878.

Panouillé, M., Bonnin, E. and Thibault, J.-F. (2006) Cellulases and proteases preparations can extract pectins from various plant by-products. *J Agric. Food Chem*. 54: 8926–8935.

Penfield, S., Meissner, R.C., Shoue, D.A., Carpita, N.C. and Bevan, M.W. (2001) *MYB61* is required for mucilage deposition and extrusion in the *Arabidopsis* seed coat. *Plant Cell*. 13: 2777–2791.

Ralet, M.-C., Bonnin, E. and Thibault, J.-F. (2002) Pectins. *In* Biopolymers Vol 6: Polysaccharides II—Polysaccharides from Eukaryotes. Edited by De Baets, S., Vandamme, E.J. and Steinbüchel, A. 6 pp. 345–380. Wiley-VCH Verlag, Weinheim.

Rerie, W.G., Feldmann, K.A. and Marks, M.D. (1994) The *GLABRA2* gene encodes a homeodomain protein required for normal trichome development in Arabidopsis. *Genes Dev*. 8: 1388–1399.

Sakamoto, T., Bonnin, E. and Thibault, J.-F. (2003) A new approach for studying interaction of the polygalacturonase-inhibiting proteins with pectins. *Biochim. Biophys. Acta* 1621: 280–284.

Schols, H.A. and Voragen, A.G.J. (1996) Complex pectins: structure elucidation using enzymes. *In* Pectins and Pectinases. Edited by Visser, J. and Voragen, A.G.J. pp. 3–19. Elsevier Science, Amsterdam.

Seaman, J.F., Moore, W.E., Mitchell, R.L. and Millet, R.A. (1954) Techniques for the determination of pulp constituents by quantitative paper chromatography. *Tappi* 37: 336–343.

Sterling, C. (1970) Crystal-structure of ruthenium red and stereochemistry of its pectin stain. *Amer. J. Bot*. 57: 172–175.

Sturgeon, R.J. (1990) Monosaccharides. *In* Methods in Plant Biochemistry Vol. 2. Edited by Dey, P.M. and Harborne, J.B. pp. 1–37. Academic Press, London.

Thibault, J.-F. (1979) Automatisation du dosage des substances pectiques par la méthode au métahydroxydiphényle. *Lebensmittel Wiss. Technol*. 12: 247–251.

Usadel, B., Kuschinsky, A.M., Rosso, M.G., Eckermann, N. and Pauly, M. (2004) *RHM2* is involved in mucilage pectin synthesis and is required for the development of the seed coat in *Arabidopsis*. *Plant Physiol*. 134: 286–295.

Walker, A.R., Davison, P.A., Bolognesi-Winfield, A.C., James, C.M., Srinivasan, N., Blundell, T.L., Esch, J.J., Marks, M.D. and Gray, J.C. (1999) The *TRANSPARENT TESTA GLABRA1* locus, which regulates trichome differentiation and anthocyanin biosynthesis in Arabidopsis, encodes a WD40 repeat protein. *Plant Cell* 11: 1337–1349.

Western, T.L. (2006) Changing spaces: the *Arabidopsis* mucilage secretory cells as a novel system to dissect cell wall production in differentiating cells. *Can. J. Bot*. 84: 622–630.

Western, T.L., Burn, J., Tan, W.L., Skinner, D.J., Martin-McCaffrey, L., Moffatt, B.A. and Haughn, G.W. (2001) Isolation and characterization of mutants defective in seed coat mucilage secretory cell development in *Arabidopsis*. *Plant Physiol*. 127: 998–1011.

Western, T.L., Skinner, D.J. and Haughn, G.W. (2000) Differentiation of mucilage secretory cells of the *Arabidopsis* seed coat. *Plant Physiol*. 122: 345–56.

Western, T.L., Young, D.S., Dean, G.H., Tan, W.L., Samuels, A.L. and Haughn, G.W. (2004) *mucilage-modified4* encodes a putative pectin biosynthetic enzyme developmentally regulated by *APETALA2*, *TRANSPARENT TESTA GLABRA1*, and *GLABRA2* in the *Arabidopsis* seed coat. *Plant Physiol*. 134: 296–306.

Willats, W.G.T., Limberg, G., Buchholt, H.C., van Alebeek, G.-J., Benen, J., Christensen, T.M., Visser, J., Voragen, A., Mikkelsen, J.D. and Knox, J.P. (2000) Analysis of pectic epitopes recognised by hybridoma and phage display monoclonal antibodies using defined oligosaccharides, polysaccharides and enzymatic degradation. *Carbohydr. Res*. 327: 309–320.

Willats, W.G.T., Marcus, S.E. and Knox, J.P. (1998) Generation of a monoclonal antibody specific to (1→5)-α-l-arabinan. *Carbohydr. Res*. 308: 149–152.

Willats, W.G.T., McCartney, L. and Knox, J.P. (2001) *In-situ* analysis of pectic polysaccharides in seed mucilage and at the root surface of *Arabidopsis thaliana*. *Planta* 213: 37–44.

Willison, J.H.M. and Abeysekera, R.M. (1988) Helicoidal arrays of cellulose in quince seed epidermis: evidence for cell wall self-assembly in the plant cell periplasm, *In* Cellulose and Wood: Chemistry and Technology. Edited by Schuerch, C. pp. 765–781. Wiley, New York.

Windsor, J.B., Symonds, V.V., Mendenhall, J. and Lloyd, A.M. (2000) *Arabidopsis* seed coat development: morphological differentiation of the outer integument. *Plant J*. 22: 483–93.

Young, J.A. and Evans, R.A. (1973) Mucilaginous seed coats. *Weed Sci*. 21: 52–54.

Zhang, F., Gonzalez, A., Zhao, M., Payne, C.T. and Lloyd, A. (2003) A network of redundant bHLH proteins functions in a TTG1-dependent pathways of Arabidopsis. *Development* 130: 4859–4869.

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