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Altered pectin composition in primary cell walls of *korrigan*, a dwarf mutant of *Arabidopsis* deficient in a membrane-bound endo-1,4-β-glucanase

Isabelle His[1], Azeddine Driouich[1], Frédéric Nicol[2], Alain Jauneau[3], Herman Höfte[2]

[1]UMR CNRS 6037, Centre de Microscopie Electronique, IFRMP 23, Université de Rouen, 76821 Mont-Saint-Aignan Cedex, France
[2]Laboratoire de Biologie Cellulaire, Institut National de Recherche Agronomique, Route de Saint-Cyr, 78026 Versailles Cedex, France
[3]Laboratoire Signaux et Messages Cellulaires chez les Végétaux, IFR 40 Pôle de Biotechnologie Végétale, 24 Chemin de Borde Rouge, B.P. 17 Auzeville, 31326 Castanet-Tolosan, France

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Abstract. *Korrigan* (*kor*) is a dwarf mutant of *Arabidopsis thaliana* (L.) Heynh. that is deficient in a membrane-bound endo-1,4-β-glucanase. The effect of the mutation on the pectin network has been studied in *kor* by microscopical techniques associated with various probes specific for different classes of pectic polysaccharides. The localisation of native crystalline cellulose was also examined using the cellobiohydrolase I-gold probe. The investigations were focused on the external cell walls of the epidermis, a cell layer that, in a number of plant species, has been shown to be growth limiting. Anionic sites associated with pectic polymers were quantified using the cationic gold probe. Homogalacturonans were quantified using polyclonal anti-polygalacturonic acid/rhamnogalacturonan I antibodies recognising polygalacturonic acid, and monoclonal JIM7 and JIM5 antibodies recognising homogalacturonans with a high or low degree of methyl-esterification, respectively. Rhamnogalacturonans were quantified with two monoclonal antibodies, LM5, recognising β-1,4 galactan side chains of rhamnogalacturonan I, and CCRCM2. Our results show a marked increase in homogalacturonan epitopes and a decrease in rhamnogalacturonan epitopes in *kor* compared to the wild type. A substantial decrease in cellobiohydrolase I-gold labelling was also observed in the mutant cell walls. These findings demonstrate that a deficiency in an endo-1,4-β-glucanase, which is in principle not directly implicated in pectin metabolism, can induce important changes in pectin composition in the primary cell wall. The changes indicate the existence of feedback mechanisms controlling the synthesis and/or deposition of pectic polysaccharides in primary cell walls.

Key words: *Arabidopsis* (mutant, pectin) – Cellulose – Cell wall – *kor* mutant (*Arabidopsis*) – Pectin (immunocytochemistry) – Polysaccharide

Introduction

The plant cell wall is a complex and dynamic structure which has many crucial functions in plant development and survival (Roberts 1990; Albersheim et al. 1994; McCann and Roberts 1994). Primary cell walls consist of cellulose microfibrils embedded in a heterogeneous matrix of polysaccharides (hemicelluloses and pectins) and proteins. According to the model of McCann and Roberts (1991), the polysaccharides in a typical dicotyledonous primary cell wall are organised into two networks, a cellulose/hemicellulose network and a pectin network. Pectins are principally composed of the three polysaccharides, homogalacturonans, rhamnogalacturonan I (RG I), and rhamnogalacturonan II (RG II) (O'Neill et al. 1990). Cellulose microfibrils are synthesised by cellulose-synthesising complexes embedded in the plasma membrane (Delmer and Amor 1995), whereas other polysaccharides of the cell wall matrix are assembled in the Golgi apparatus and transported in secretory vesicles to the cell surface (Driouich et al. 1993). The structure, function and biosynthesis of various wall polysaccharides are not fully understood. An important but unsolved question is how the synthesis and the assembly of the different polysaccharide classes are co-ordinated in the expanding primary wall of growing cells. A number of reports illustrate the high plasticity of cell wall polysaccharide biosynthesis in response to varying environmental factors. For instance, changes in cell wall composition have been reported in plants growing under polyethyleneglycol (PEG)-induced osmotic stress. Cell walls of tobacco cell cultures grown in the presence of PEG showed a decrease in the percentage of cellulose and an increase in the hemicellulosic fractions, whereas the pectic fractions remained

Abbreviations: CBH-I = cellobiohydrolase I; CMC = carboxymethylcellulose; Egase = endo-1,4-β-glucanase; *kor* = *korrigan* mutant of *Arabidopsis*; PATAg = periodic acid-thiocarbohydrazide-silver proteinate; PGA = polygalacturonic acid; RG I = rhamnogalacturonan I

Correspondence to: A. Driouich;
E-mail: adriouic@crihan.fr; Fax: +33-2-35146535

unchanged (Iraki et al. 1989). In *Cicer arietinum* epicotyls (Muñoz et al. 1993), the PEG-induced inhibition of elongation was correlated with a decrease in hemicellulose and cellulose contents. In contrast, variations in pectin composition, as observed during the different cell growth stages in an elongating epicotyl, were not detected. Recently, Burton et al. (2000) showed that a decrease in cellulose content caused by a virus-induced silencing of a cellulose synthase gene in tobacco was compensated by an increase in homogalacturonans. McCann et al. (1993) reported differences in pectin composition between growing tobacco cells that were either non-adapted or adapted to high NaCl concentrations. Throughout the culture period, pectic polymers of non-adapted cells showed an increase in methyl-esterification during elongation followed by a decrease during the stationary phase. In contrast, the degree of pectin esterification remained unchanged in adapted cells. Several studies have reported that the synthesis and/or deposition of the cell wall components are under control of calcium. For instance, in Norway spruce, high concentrations of calcium in the soil stimulate the deposition of non-cellulosic polysaccharides, especially pectic polymers (Eklund and Eliasson 1990). Calcium also affects the frequency and distribution of anionic sites within the wall of parenchyma cells of apple fruit (Roy et al. 1994). His et al. (1997) observed an increase in acidic pectins, as well as inter-pectin calcium bridges within the hypocotyl cell walls of flax seedlings grown in the presence of calcium. Interestingly, these changes could not be correlated with changes in hypocotyl length.

The first indications for the existence of feedback mechanisms regulating the polysaccharide composition of cell walls came from the study of tomato and tobacco cells adapted to the inhibitor of cellulose synthesis 2,6-dichlorobenzonitrile (DCB). The dramatic reduction of cellulose in these cells was associated with important changes in the composition of non-cellulosic polysaccharides (Shedletsky et al. 1990). The synthesis of xyloglucan was not inhibited, but most of it was secreted in a soluble form into the culture medium. The walls of these cells showed a significant enrichment in pectins, including homogalacturonan and rhamnogalacturonan-like polymers. The integrity of the wall appeared to be maintained by an increase in Ca^{2+}-bridged pectates. These findings point to the existence of an independent pectic network in the primary wall, the composition of which is altered in the absence of a cellulose/xyloglucan network. In contrast, walls from DCB-adapted monocot cells did not show increased pectin contents, but normal to elevated amounts of other non-cellulosic material (Shedletsky et al. 1992), confirming the fundamental differences between dicot and monocot cell walls, including the way they compensate for the reduction of their cellulose content. It is not known, however, whether the observed changes in the DCB-adapted cells reflect the plasticity of the machinery involved in cell wall construction, or whether any genetic changes are responsible for these adaptations.

In this report we took advantage of a recently identified dwarf mutant of *Arabidopsis*, *kor*, to determine how the pectin network is implicated in the plant elongation phenomenon. Plants carrying the recessive nuclear mutation *kor* show a cell elongation defect in all cell types except for tip-growing cells (Nicol et al. 1998). This growth defect is caused by a T-DNA insertion abolishing the expression of a gene encoding a membrane-bound endo-1,4-β-glucanase (EGase). Although the precise role of the enzyme remains to be determined, all the evidence suggests a function for KOR in the assembly and/or metabolism of the cellulose/hemicellulose network in growing cells (Nicol et al. 1998).

To investigate the interrelation between the synthesis and assembly of the cellulose/hemicellulose network and the pectic network in the primary wall, and knowing the primary defect of the mutant, we compared the composition and distribution of pectic polysaccharides in cell walls of wild-type and mutant hypocotyls. We focused our investigations on the epidermal cell wall known to control the rate of organ elongation in stems and coleoptiles (Kutschera 1992). Using immuno-affinity methods and microscopical techniques, we observed marked modifications in the pectic network. In particular, we show an increase in homogalacturonan epitopes and a decrease in rhamnogalacturonan epitopes in *kor* compared to the wild type. Our results suggest that a mutation eliminating an enzyme that is not normally directly implicated in pectin metabolism can cause important changes in the composition of pectins within the wall.

Materials and methods

Plant culture conditions

Seedlings of *Arabidopsis thaliana* (L.) Heynh., ecotype Wassilewskija (seeds provided by K. Feldman, University of Arizona, Tucson, Ariz., USA), and the mutant *kor* were grown in vitro, in the dark for 7 d as previously described (Nicol et al. 1998).

Fixation and embedding

Seven-day-old seedlings were fixed for 90 min in 4% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). After post-fixation in 1% osmium tetroxide for 1 h, the samples were dehydrated in a graded aqueous ethanol series and embedded in LR White resin as previously described (His et al. 1997; Nicol et al. 1998). Some *Arabidopsis* samples were submitted to a subtractive treatment with hot water (100 °C) for 2 h to solubilise pectins as described in His et al. (1997).

Calcofluor staining of cellulose

Light microscopy was carried out on a Axioskop microscope (Zeiss) equipped with epifluorescence optics. Calcofluor staining was done as described by Mori and Bellani (1996). Briefly, sections (0.5 μm in thickness) mounted on glass slides were incubated with the fluorescent probe (1 mg/ml) for 30 min in the dark. After several washes, the sections were observed with a Zeiss filter set (excitation filter 350–410, barrier filter 470).

Immunolabelling procedures

The antibodies used in this study were: (i) the monoclonal antibodies JIM5 and JIM7, specific for pectins with a low and

high degree of methyl-esterification, respectively (Knox et al. 1990); (ii) the monoclonal antibody LM5, specific for β-1,4 galactans (Jones et al. 1997); and (iii) the polyclonal antibodies anti-polygalacturonic acid/rhamnogalacturonan I (PGA/RG I), recognising non-esterified pectins (Moore and Staehelin 1988).

All incubations were carried out overnight at 4 °C, or 4 h at room temperature. Thin sections (90 nm) mounted on gold grids were incubated in a blocking solution of 3% (v/v) non-fat dried milk in TTBS [0.05 M Tris-HCl (pH 7.5), 0.1% Tween 20 (w/v), 0.15 M NaCl) for 30 min. They were incubated on droplets of undiluted JIM5, JIM7, or LM5, or diluted (diluted 1:10 in TTBS) anti-PGA/RG 1 antibodies. After washing with TTBS, the grids were transferred to (i) a droplet of goat anti-rat IgG conjugated to 10-nm colloidal gold diluted 1 to 50 in TTBS (for JIM5, JIM7 and LM5-labelled sections) or to (ii) a goat anti-rabbit IgG diluted 1 to 50 (for anti-PGA/RG 1-labelled sections) for 1 h at room temperature. The sections were rinsed thoroughly with TTBS followed by distilled water. After immunolabelling, the sections were stained with 2% (w/v) uranyl acetate in 50% (v/v) ethanol for 30 min or submitted to periodic acid-thiocarbohydrazide-silver proteinate (PATAg) staining as previously described (His et al. 1997; Jauneau et al. 1997). These sections were examined at 80 kV on a Zeiss EM 109 electron microscope. All micrographs illustrate representative labelling patterns. Controls were performed either by omission of primary antibodies or by pre-incubation of the probe for 1 h with PGA (1 mg/ml) for JIM5, anti-PGA/RG 1 and the cationic gold probe, or with methylated pectin from citrus (Sigma) for JIM7.

Enzyme-gold affinity labelling of cellulose

Ultra-thin sections were incubated for 10 min with citrate-phosphate buffer (CPB; 0.05 M, pH 4.8) at room temperature, followed by labelling with the cellobiohydrolase I (CBH I)-gold complex at a concentration of 1/100 in CPB for 1 h at room temperature. The sections were thoroughly rinsed with CPB and water, stained with PATAg and observed with a Zeiss electron microscope (EM 109). Control sections were incubated in the presence of 1 mg/ml of CMC as described in Andème-Onzighi et al. (2000). Pre-incubation of the CBH I probe with tamarind xyloglucan (1 mg/ml) did not change the labeling pattern (data not shown).

Detection of anionic sites with the cationic gold probe

The labelling with the cationic gold probe was performed on ultra-thin sections (90 nm) from LR White-embedded samples collected on gold grids and treated with 3% acetic acid for 15 min at room temperature (Roy et al. 1994). The sections were incubated on a droplet of 10-nm cationic poly-L-lysine–colloidal gold complex (Biocell) diluted 1 to 100 (v/v) in 3% acetic acid for 1 h at room temperature. They were rinsed in 3% acetic acid followed by distilled water, and stained with uranyl acetate (see above) before observations.

Quantification of gold labelling density

The number of gold particles was determined for 10 electron micrographs (from 3 independent experiments) and correlated to the surface of the different wall areas (His et al. 1997). The density of gold labelling was expressed as the average number of gold particles per μm^2.

Polysaccharide detection using PATAg

The PATAg staining for polysaccharide detection was performed as previously described (Roland and Vian 1991).

Results

Histological features of mutant cell walls

In *Arabidopsis* seedlings grown in the dark under our conditions, hypocotyl elongation takes place between days 2 and 7, whereby cell elongation follows an acropetal gradient (Gendreau et al. 1997). Hypocotyls of seedlings homozygous for the recessive nuclear mutation *kor* have the same growth kinetics but are only 25% of the length of those of the wild type at the end of the growth period (Fig. 1A; Nicol et al. 1998). We studied the cell wall in fully elongated cells in the middle region of 7-d-old dark-grown hypocotyls. No remarkable differences in the radial organisation of the hypocotyl could be noticed on transverse sections (Fig. 1B–D), confirming previous observations (Nicol et al. 1998). Both hypocotyl types were composed of an epidermal layer, two layers of cortical parenchyma cells, one layer of endodermis and the stele. However, in the mutant, all cells, including those of the central cylinder, showed increased radial expansion (Nicol et al. 1998). External epidermal walls, as well as cortical walls were thicker in *kor* than in the wild type (for quantification, see Nicol et al. 1998).

Staining with Calcofluor, a specific dye for fibrillar β-glucans (e.g. cellulose) of plant cell walls (Haigler and Benziman 1982; Mori and Bellani 1996), revealed a more heterogeneous staining of epidermal cell walls than in the wild type, which may reflect irregularities in the deposition of cellulose, not observed in the wild type (compare Fig. 1C and D). Besides the epidermis, parenchyma cells showed frequent separation of two adjacent walls (Fig. 1D, arrowheads), indicating abnormal cell adhesion. These observations confirm an essential role for the membrane-bound endo-1,4-β-glucanase encoded by *KOR* in normal cell elongation and the correct assembly of cellulose in primary cell walls.

Two parts within the epidermal outer wall could be discriminated in the wild type and mutant by their reactivity to PATAg staining (Fig. 2A, B). The inner layer of the wall, near the plasmalemma membrane, was more reactive to the PATAg than the outer layer of the wall near the cuticle. Within these two areas, polysaccharide organisation seemed to be quite different. The inner layer of the epidermal wall had a fine fibrillar structure, whereas it was rather granular in the outer layer of the wall beneath the cuticle (Fig. 2A). In contrast to the *kor* mutant, in the wild type, the reactivity to PATAg staining of a specific wall layer (e.g. inner or outer) in the epidermal cells was homogeneous. In the inner layer of epidermal cell walls of *kor*, some areas showed a strong reactivity to PATAg, while others were only very weakly reactive (Fig. 2B).

One remarkable feature of the *kor* mutant is that cell shape is highly irregular not only in dark-grown hypocotyls but also in all other tissues investigated (Nicol et al. 1998). In the same tissue small cells could be found alternating with fully expanded cells. As shown below, this variability in cell shape was paralleled by cell-to-cell differences in wall structure and composition, as revealed by the large standard deviations observed for

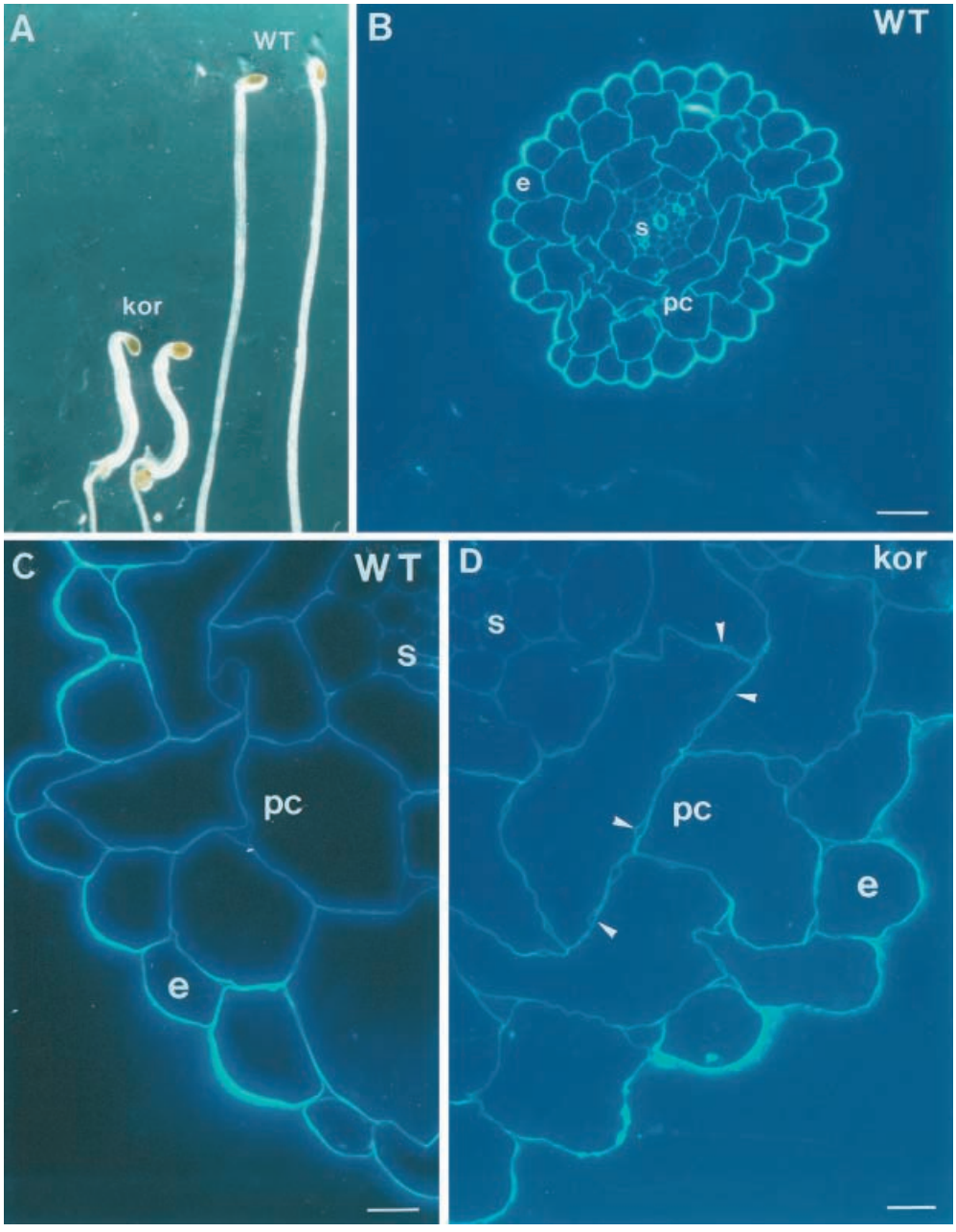

Fig. 1A–D. Phenotype and cell wall features of the *korrigan* (*kor*) mutant of *Arabidopsis thaliana*. **A** Seven-day-old dark-grown wild-type (*right*) and *kor* mutant (*left*) seedlings. **B** Calcofluor-stained, transverse section through the hypocotyl of a 7-d-old dark-grown wild-type seedling, showing the spatial organisation of different cell types. **C, D** Higher magnifications of similar Calcofluor-stained sections from the wild type (**C**) and *kor* (**D**). Note the radial enlargement of the epidermal and cortical cells in *kor* as compared to the wild type. In *kor*, important wall separations are seen between cortical cells (*arrowheads*). *e* Epidermal cells; *pc* parenchyma cells; *s* stele. Bars = 55 μm (**B**) and 22 μm (**C, D**)

the quantification of the pectic epitopes revealed by cationic gold and four antibodies in the mutant.

Distribution of anionic sites in the epidermal cell wall

The cationic gold probe was used to detect negatively charged groups, which are known to be essentially associated with pectins in the wall (Roy et al. 1994; Fig. 3A, B). As mentioned above, in parallel with the large cell-to-cell variation in the *kor* phenotype, large differences from one wall to another were obtained for the quantification of the density of gold particles in kor (not shown). Even so, the average labelling density (±SD) was higher in *kor* than in the wild type (658 ± 289 and 428 ± 83 gold particles per μm^2, respectively). The anionic sites were preferentially associated with the outer layer of the epidermal walls, with a homogeneous distribution in the wild type (Fig. 3A), whereas in the *kor* mutant, gold particles formed aggregates (Fig. 3B).

Distribution of pectic epitopes in the cell wall

In both the wild type and *kor*, labelling with anti-PGA/RG 1 polyclonal antibodies directed against polygalacturonic acid (Moore and Staehelin 1988) was observed all over the thickness of the external epidermal wall, with a slightly higher labelling in the outer layer of the wall (Fig. 4A, B). In *kor*, the labelling density was 3-fold higher than in the wild type (294 ± 64 vs. 101 ± 31, respectively; Fig. 5). Also, note the higher standard deviations for *kor*.

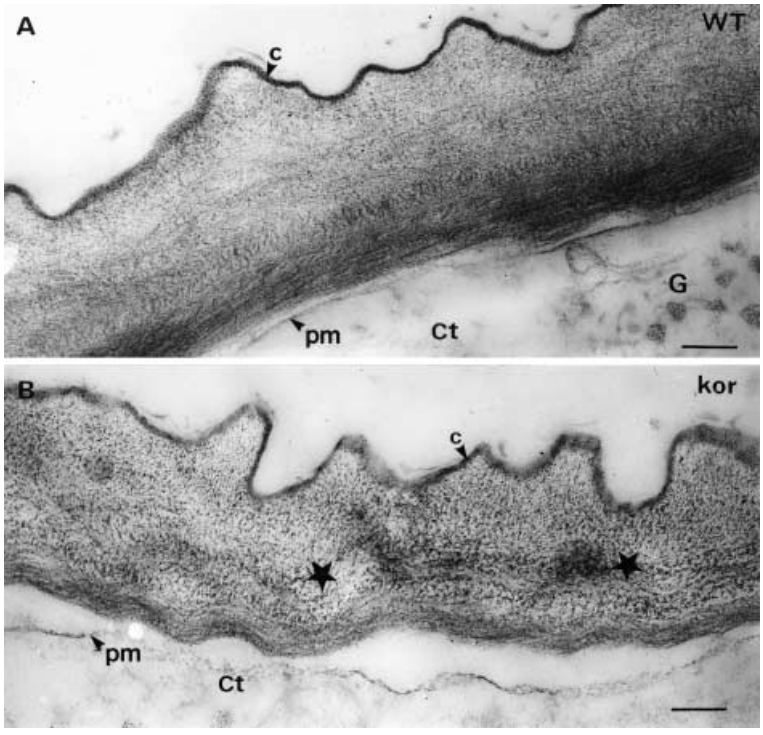

Fig. 2. Staining of polysaccharides, using the PATAg test, in epidermal cell walls of wild-type (**A**) and *kor* (**B**) hypocotyls of *Arabidopsis*. The inner layer near the plasmalemma of the wall is more reactive to the PATAg than the outer layer for both hypocotyls. Note the heterogeneous staining in *kor* walls (★). *c* Cuticle; *Ct* cytoplasm; *G* Golgi stack; *pm* plasma membrane. Bars = 0.20 μm

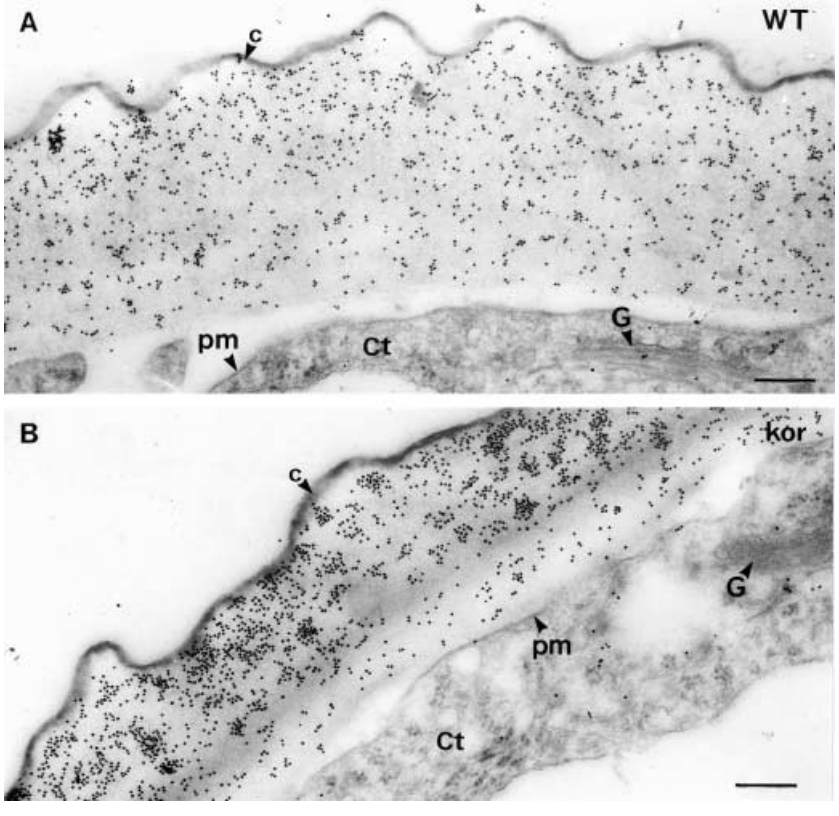

Fig. 3. Distribution of anionic sites within epidermal cell walls of wild-type (**A**) and *kor* (**B**) *Arabidopsis* plants as determined by cationic gold labelling. Note the homogeneous and abundant labelling of the epidermal wall of the wild type (**A**). Labelling is also abundant and even denser in the wall of *kor* (**B**). *c* Cuticle; *Ct* cytoplasm; *G* Golgi stack; *pm* plasma membrane
Bars = 0.20 μm

To further investigate the distribution of pectins, we used JIM5 and JIM7, monoclonal antibodies specific for homogalacturonans with a low and high degree of methyl-esterification, respectively (Knox et al. 1990). The distribution of JIM5-recognised epitopes within the epidermal cell wall allowed us to distinguish between *kor* mutant and wild-type walls. In the wild-type walls, JIM5 labelling was distributed all over the entire wall, except in the innermost layer of the wall close to the plasmalemma (Fig. 4C). In *kor*, the distribution of

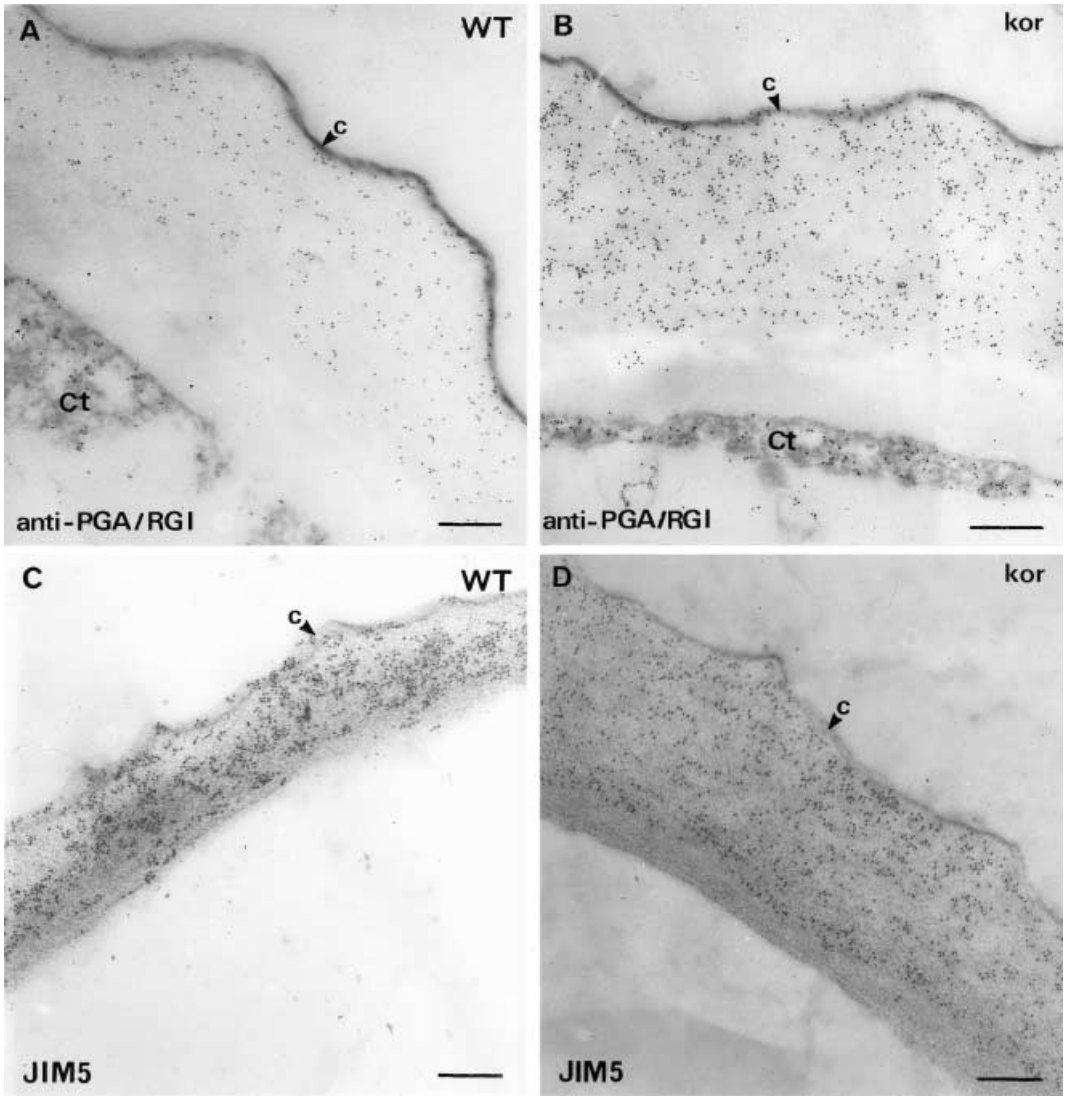

Fig. 4. Immunogold labelling of epidermal cell walls of wild-type (**A, C**) and *kor* (**B, D**) *Arabidopsis* plants with antibodies specific for pectin epitopes (anti-PGA/RG 1: **A, B**; JIM5: **C, D**). Note the increase in the labelling density, with both antibodies, in *kor* cell walls as compared to the wild type (see Fig. 5, for quantification). *c* Cuticle; *Ct* cytoplasm. Bars = 0.25 μm

gold particles was quite similar to that of the wild type, although labelling was denser (Fig. 4D). As shown in the histogram (Fig. 5), the labelling density for JIM5 was significantly higher in *kor* than in wild-type walls (496 ± 133 vs. 259 ± 64, respectively). The distribution of highly methylated pectins (JIM7-recognised epitopes) was homogeneous in both wild-type and *kor* epidermal cell walls (Fig. 6A, B), and there were no differences in labelling densities between the inner and outer wall layers. The labelling density for JIM7 was higher in *kor* than in wild-type walls (127 ± 71 vs. 59 ± 21; Fig. 5). Thus, the wild type and *kor* had a relatively homogeneous distribution of label all over the wall, both for JIM5 and JIM7, but a higher labelling density for JIM5 than for JIM7 (Fig. 5). The ratios of JIM7 to JIM5 labelling were unaltered between mutant and wild type, suggesting the absence of significant differences in pectin methylesterification.

To visualise the pectic polysaccharide RG 1, wild-type and *kor* plants were probed with the monoclonal antibody LM5 specific for 1,4-β-galactans associated with the side chains (Jones et al. 1997). LM5 immunolabelled the epidermal cell wall of the wild type abundantly. The gold particles appeared to be generally distributed throughout the cell wall, although the labelling was denser in the innermost region of the wall (Fig. 6C). In contrast, in *kor*, LM5 labelling, although not very dense, was associated with the part of the wall immediately adjacent to the plasma membrane (Fig. 6D). Quantification analysis indicated that the density of the labelling was significantly lower in the mutant (190 ± 22) than in the wild type (309 ± 85; Fig. 5). Similar labelling was also found with CCRCM2, another monoclonal antibody specific for RG 1 (Puhlmann et al. 1994; data not shown).

To investigate the effect of polysaccharide solubilisation on the distribution of pectins, we used an on-block

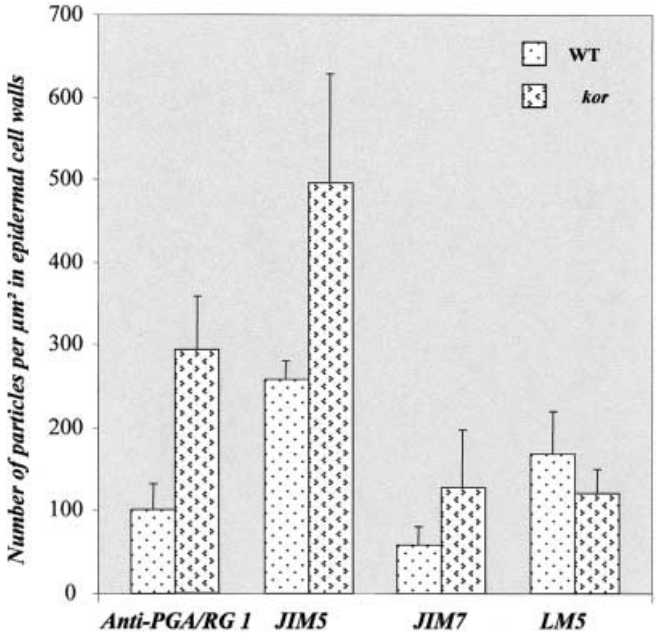

Fig. 5. Histogram illustrating the density of anti-PGA/RG 1, JIM5, JIM7 and LM5 immunogold labelling in epidermal cell walls of wild-type (*WT*) and *kor* epidermal cell wall of *A. thaliana*. For each antibody, the mean density of gold particles was calculated from 10 micrographs, and expressed as the number of particles per μm^2 (mean ± SD) in epidermal cell walls

subtractive treatment with hot water (Reis and Roland 1974) in conjunction with JIM5 labelling. This extraction induced a marked swelling of the wall, with, in most cases, a loosening of the cuticle. As illustrated in Fig. 7, the sensitivity of epidermal walls to this treatment was different in wild-type and *kor* plants. Hot-water treatment resulted in a swelling of epidermal walls with a slight loosening of the wall network in the wild type (Fig. 7A). In contrast, in *kor*, the same treatment caused not only a swelling of the walls but also an important separation of wall material in the innermost half of the wall (Fig. 7B, C). Labelling with JIM5 antibody was homogeneous all over the epidermal wall except in the inner part, which was devoid of labelling (Fig. 7B). Loosened fibrillar material (presumably cellulose microfibrils) could be seen in this area of the mutant wall (Fig. 7C). Almost no labelling with JIM5 was observed in hot-water-extracted wall of the wild type (Fig. 7A).

Distribution of cellulose in the cell wall

We also examined the localisation of cellulose using the CBH I-gold probe. Labelling was quite abundant and uniformly distributed throughout the entire epidermal walls of the wild type (Fig. 8A). In *kor*, binding of the cellulose probe to the epidermal cell walls was always low compared to the wild type (Fig. 8B). Subtractive treatment of pectins with hot water caused an increase in CBH I-gold labelling of epidermal cell walls of both *kor* and the wild type (Fig. 8C, D). Again, hot-water-induced loosening of wall material was much more pronounced in *kor* than in the wild type and most of the CBH I-gold labelling was associated with the loosened fibrillar material in *kor* walls (Fig. 8D). These data indicate that in *kor* walls, cellulose microfibrils are embedded in a network composed essentially of pectic polysaccharides, which may play a crucial role in their stability, and that this network is essential for the cohesion and stability of the mutant walls.

Discussion

Using microscopical techniques, we observed important changes in the presence and distribution of pectic epitopes in the primary wall of an *Arabidopsis* dwarf mutant deficient in a membrane-bound EGase. Endo-1,4-β-glucanases hydrolyse β-1,4-linkages behind unsubstituted glucose residues, present in cellulose and hemicellulose in plants. Although the exact in-vivo substrate of KOR remains unknown, enzymes of this family undoubtedly act on the cellulose/hemicellulose network in plant cells and not on pectins. We therefore reasoned that an altered pectin composition in this mutant should reflect changes in the synthesis, secretion and/or processing of pectins induced by modifications in the cellulose/hemicellulose network.

We used antibodies to visualise the distribution pattern of specific epitopes carried by pectin molecules. Immunological detection techniques have obvious disadvantages, especially when the exact nature of the epitopes is not known. However, the antibodies used in this study have been previously shown to reliably detect various pectins in plant tissues (VandenBosch et al. 1989; Knox et al. 1990; Lynch and Staehelin 1992; Jones et al. 1997).

The results demonstrate that the composition of the pectic network is affected in *kor*. The different probes used indicate that the epidermal cell wall of *kor* hypocotyls is characterised by an increased amount of homogalacturonans and a reduced amount of β-1,4-galactans.

In contrast to the wild type, in *kor* a large cell-to-cell variation in labelling density was observed for the cationic gold probe and anti-pectins. This variation parallels the variation in cell shape observed in the *kor* hypocotyl. The molecular basis for this variability is not understood, but may reflect stochastic events associated with an incomplete reduction of KOR activity in the mutant.

An enrichment for homogalacturonans in *kor* walls is indicated by the labelling density obtained with cationic gold and three antibodies, JIM5, JIM7 and anti-PGA/RG 1. The cationic gold probe labels the COO^- groups of pectins (Roy et al. 1994; His et al. 1997; Jauneau et al. 1998) and the antibodies label specific epitopes of galacturonic acid residues (Knox et al. 1990; Moore and Staehelin 1988). Thus, although the two types of probe bind differently, they are both reliable markers for acidic pectins. The increased average labelling density together with the large standard deviations suggest an increase in COO^- groups, at least in a subset of cells of the mutant

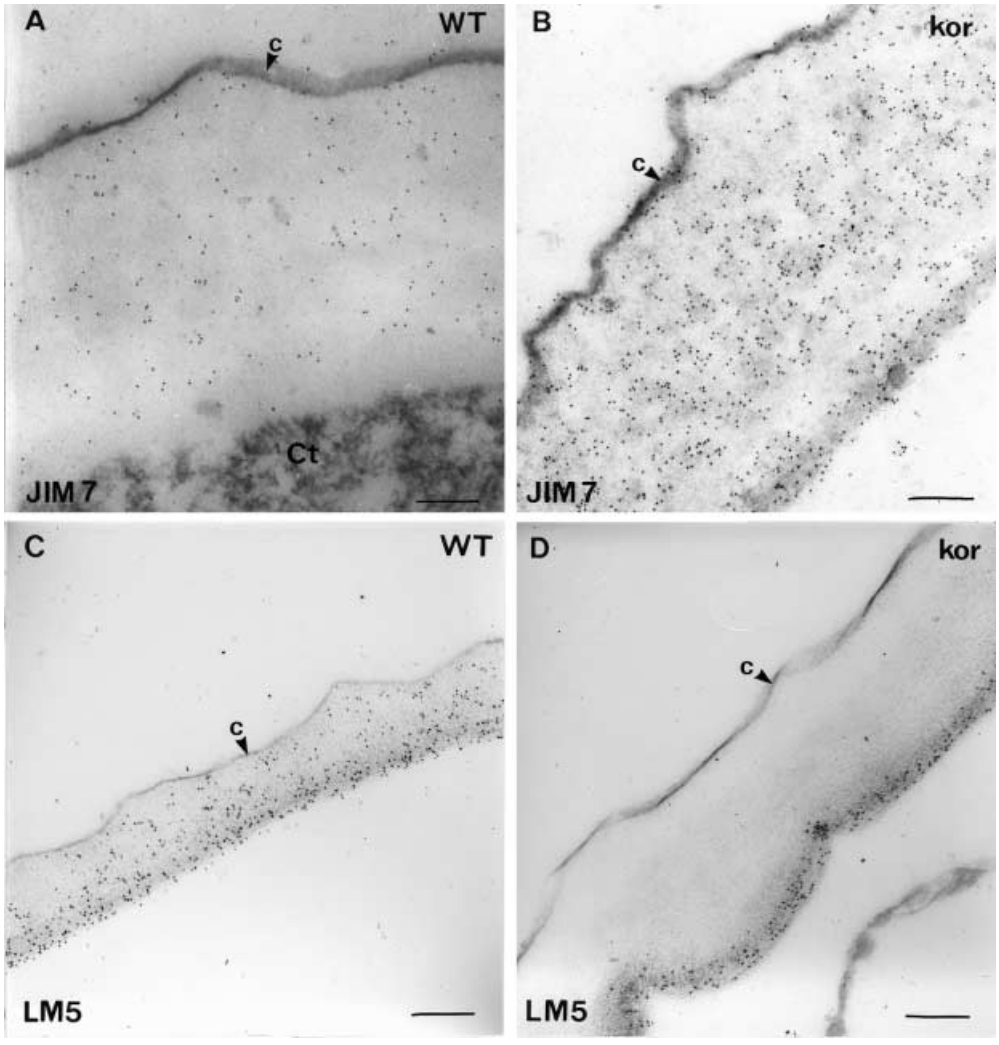

Fig. 6. Immunogold labelling of epidermal cell walls of wild-type (**A, C**) and *kor* (**B, D**) *Arabidopsis* plants with the monoclonal antibodies JIM7 (**A, B**) and LM5 (**C, D**). Note the increase in the JIM7 labelling density in *kor* cell walls (**A**) compared to the wild type (**B**). The LM5 labelling is homogeneous within epidermal cell walls of the wild-type samples (**C**), whereas it is exclusively associated with the innermost layer of the *kor* epidermal cell walls (**D**). *c* Cuticle; *Ct* cytoplasm. Bars = 0.25 μm

hypocotyl. Similar observations were made for JIM7, PGA/RG 1 and JIM5 whereby the most dramatic difference between *kor* and the wild type was observed with the latter antibody. The JIM5/JIM7-labelling ratio was significantly higher than 1 for both mutant and wild-type walls, suggesting that the majority of the pectins are acidic, especially in the outer half of the epidermal wall. Interestingly, although the average labelling density for both antibodies was higher in the mutant, the JIM5/JIM7 ratio remained comparable in the mutant and wild type, suggesting that the cell wall defect in the mutant did not induce a change in pectin methyl-esterification.

Another striking observation is that extraction of pectins with hot water induced a dramatic disorganisation of the innermost layer of the wall, more in *kor* than in the wild type (Fig. 7). This reflects a profound alteration not only of the composition of pectins (e.g. homogalacturonans) but also of their cross-linking within the wall of the *kor* mutant. In addition, it indicates that in *kor*, the structural integrity of that region of the wall, which is very rich in cellulose/hemicellulose polysaccharides, is much more dependant on pectins in *kor* than in the wild type.

The monoclonal antibody LM5 recognises short neutral chains of β-1,4-galactan associated with lateral chains of RG 1 (Jones et al. 1997; Vicré et al. 1998), which define the "hairy" zones of pectin molecules (O'Neill et al. 1990). It cannot be excluded, however, that some galactans are free within the wall. LM5 labelling was much stronger in the wild type than in *kor* walls, and was mostly found in the inner half of the wall. However, whereas LM5 labelling disappeared from the wild-type wall, after subtractive treatments (i.e. pectin removal), it remained associated with the innermost layer of the wall, which exhibits a marked dissociation in *kor* (data not shown). Together these results indicate modifications in the synthesis, transport or processing of RG 1-containing polysaccharides in the mutant, whereby fewer β-1,4-galactan side chains are incorporated in the wall, or that RG 1 processing is altered in mutant walls, reducing the "hairiness" of the polymers.

What could be the cause of the observed changes in the abundance and distribution of pectin epitopes in the epidermal cell wall of the *kor* mutant? First, pectin composition may be different in growing walls and walls of cells that have completed their elongation. This is most unlikely to be the cause of the observed differences

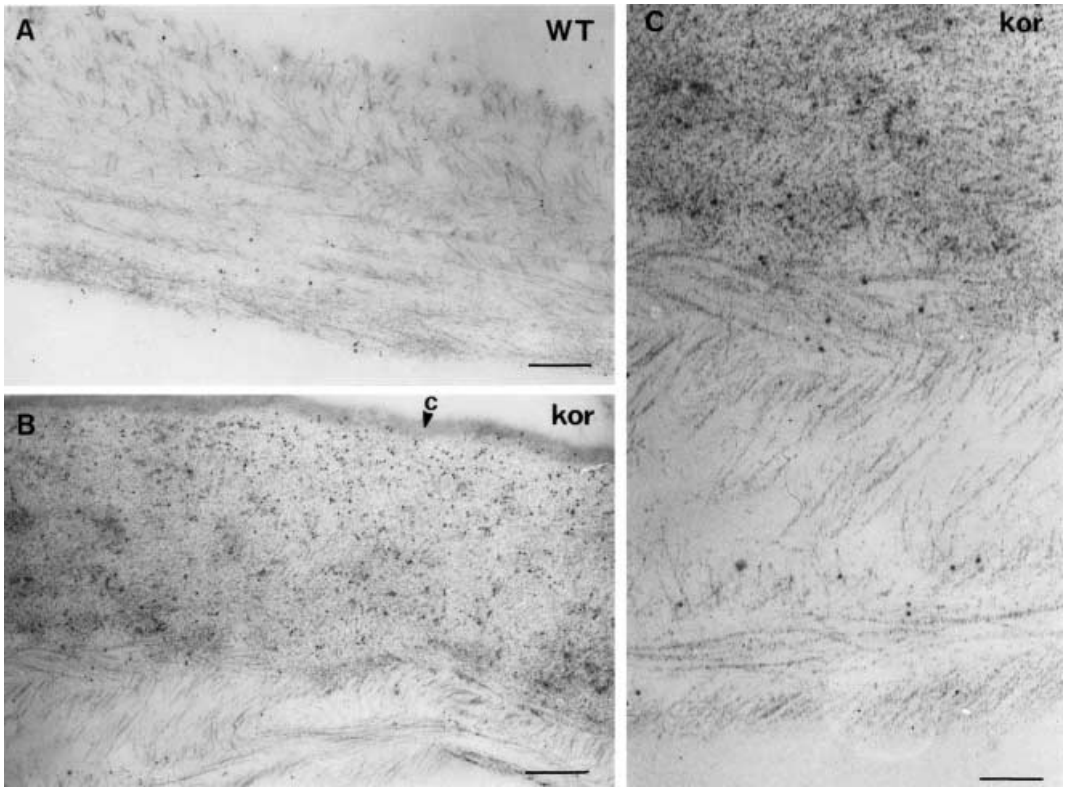

Fig. 7A–C. Immunogold labelling of epidermal cell walls of wild-type and *kor Arabidopsis* plants with the monoclonal antibody JIM5 after subtractive treatment with hot water. Note that labelling with the JIM5 antibody disappears in hot-water-extracted walls of the wild type (**A**) but not in those of *kor* (**B**). Note also the important hot-water-induced disorganisation of wall material in the inner layer of the wall, especially in *kor* (**B**). **C** Higher magnification of **B**, showing the disorganised wall zone in *kor*. *c* Cuticle; *Ct* cytoplasm. Bars = 0.25 μm (**A**), 0.35 μm (**B**) and 0.15 μm (**C**)

between the mutant and the wild type since observations were done on sections obtained from fully expanded cells in the middle of the hypocotyl. Second, the alterations in the cellulose/hemicellulose network were caused by the absence of KOR in the mutant wall may in turn induce changes in pectin composition and organisation within the wall. The KOR enzyme has been shown to hydrolyse the artificial substrate carboxymethylcellulose (CMC) in vitro (Nicol et al. 1998); however, the exact in-vivo substrate remains to be determined. Staining of mutant cell walls with Calcofluor and gold-labelled CBH I, both of which specifically visualise cellulose, showed important perturbations in the deposition of crystalline cellulose, indicating an essential role for KOR in the normal deposition of the cellulose/hemicellulose network in the expanding cell wall. The labelling obtained with the CBH I-gold probe in epidermal cell walls of *kor* was much lower than that in the wild-type walls. This suggests that *kor* walls may contain either much less cellulose or cellulose with a lower level of crystallinity that is not recognised by the CBH I-gold probe (i.e. is not accessible). Primary cell walls of plants are known to be composed of $I\alpha$ cellulose with a high degree of crystallinity and microfibrils that are parallel to the elongation axis (Kataoka and Kondo 1999). These authors have shown that under stress conditions, the degree of cellulose cristallinity increases. If the *kor* mutation were considered to be a stress by *Arabidopsis* seedlings, we would have observed an increase in the CBH I-gold labelling in epidermal cell walls of *kor*. The absence of such a rise in *kor* walls may reflect either a reduction in the synthesis of cellulose, in the size of microfibrils, or its incorrect assembly within the walls. Preliminary data obtained by transform infrared microspectroscopy indicated a decrease in cellulose content in *kor* (data not shown). In addition, in *kor* epidermal cell walls, loosening of the cellulose/hemicellulose domain was much more pronounced after pectin solubilisation than in the wild type, suggesting a role for the pectic network in the stabilisation of the cellulose/hemicellulose network. Thus, stresses induced by a reduction in the tensile strength of this network may therefore be compensated by changes in the pectin network, and the modified pectin network may in turn cause alterations in cellulose deposition and assembly, as recently reported (Chanliaud and Gidley 1999). Our observations can be interpreted in the following way. In *kor,* the presence of an increased amount of acidic homogalacturonans in the epidermal wall of the mutant may indicate increased cross-linking of these components, which would promote rigidity and strength. In addition, the wall of the mutant contains reduced amounts of galactan-side-chain-containing RG I regions. These branched "hairy" blocks of pectins may prevent the formation of cross-links between pectins (Hwang and Kokini 1991), and are thought to contribute

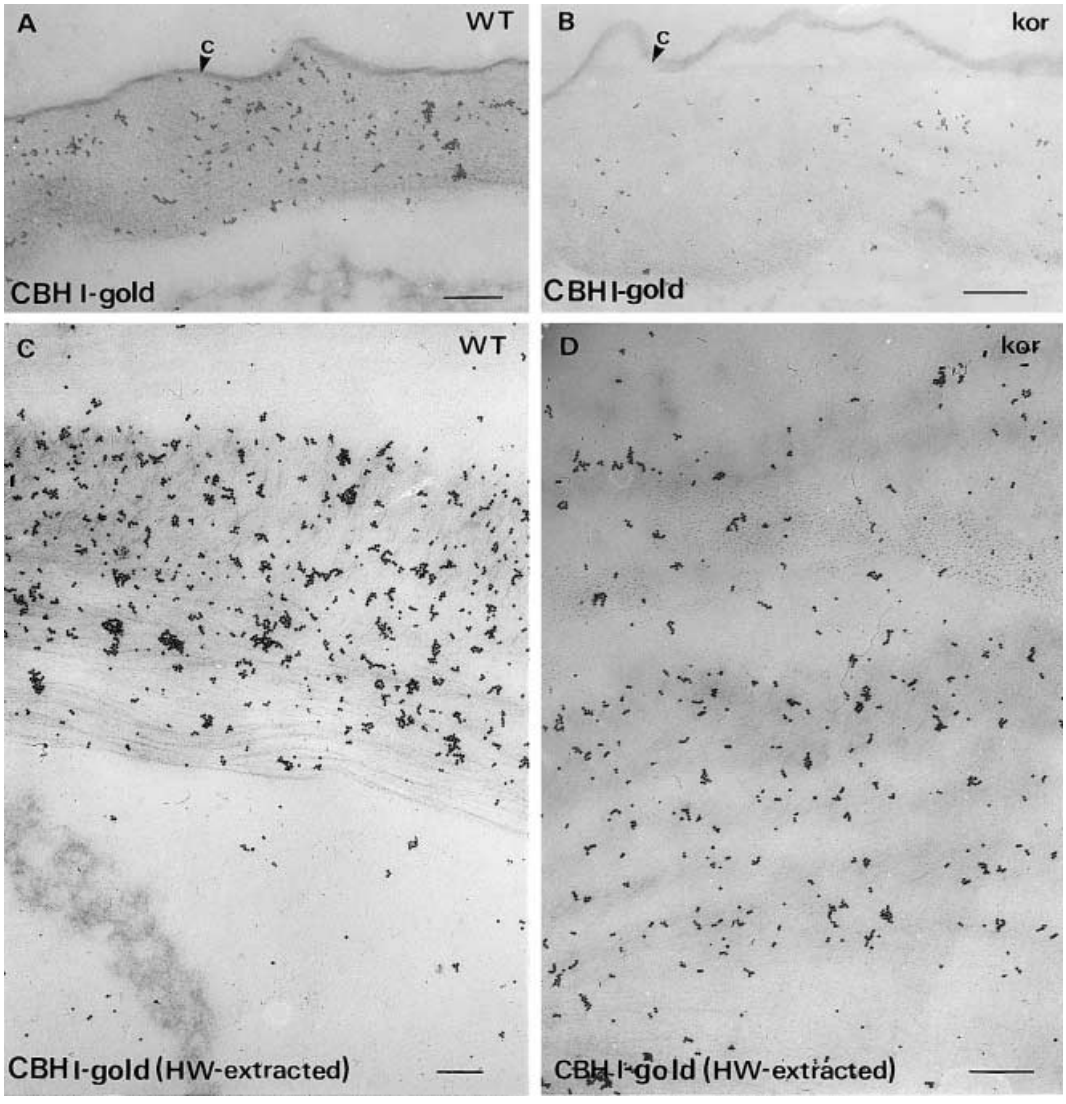

Fig. 8. Subtractive treatments and CBH I-gold labelling (specific for cellulose) of epidermal cell walls of wild-type (**A, C**) and *kor* (**B, D**) *Arabidopsis* plants. **A, B** Non-extracted samples. **C, D** Samples subjected to hot-water (*HW*) treatment to solubilise pectins. Labelling is higher in epidermal cell walls of the wild type than in *kor* (compare **A** and **B**). In the extracted samples, an increase in the labelling density is observed in both wild-type and *kor* plants (**C, D**). *c* Cuticle. Bars = 0.30 μm (**A, B**) and 0.25 μm (**C, D**)

to the extensibility of the wall (Ha et al. 1996). Together, these findings may reflect an adaptation of the cell to compensate for a reduced strength of the cellulose/hemicellulose network by increasing the strength of the pectin network. This would suggest the existence of feedback control mechanisms that translate changes in wall strength into changes in the synthesis, secretion and/or processing of wall polysaccharides. Such mechanisms may be responsible to a large extent for the plasticity of the plant cell wall and the extreme adaptability of plant growth to environmental variations.

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