If Homogalacturonan Were a Side Chain of Rhamnogalacturonan I. Implications for Cell Wall Architecture[1]

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Pectin, an important cell wall component of dicotyledonous plants, is probably the most complex macromolecule in nature. Here, we critically summarize the large amount of data on pectin structure. An alternative model for the macromolecular structure of pectin is put forward, together with ideas on how pectins are integrated into the plant cell wall.

CONSTITUENT POLYMERS, PRIMARY STRUCTURE

Pectin is composed of as many as 17 different monosaccharides (Fig. 1; for review, see Ridley et al., 2001; Voragen et al., 2003). These monosaccharides are organized in a number of distinct polysaccharides, the structures of which are schematically shown in Figure 1. Together, these polymers form the pectin network (Visser and Voragen, 1996; Ridley et al., 2001; Voragen et al., 2003).

The first structural elements of pectin to be discussed have a backbone of 1,4-linked α-D-Gal*p*A residues and are referred to as galacturonans. Three types of galacturonan can be distinguished in the plant cell wall: the unsubstituted homogalacturonan (HG), rhamnogalacturonan II (RG-II; see below), and xylogalacturonan (XGA). The GalA residues of HG can be methyl-esterified at *C*-6 and carry acetyl groups on *O*-2 and *O*-3. XGA is a branched galacturonan with β-D-Xylp-(1→3) side chains (Schols et al., 1995; Visser and Voragen, 1996). The degree of xylosylation can vary between 25% (watermelon [*Citrullis vulgaris*]) and 75% (apple [*Malus domestica*]; Schols et al., 1995; Visser and Voragen, 1996). The GalA residues of XGA can be methyl-esterified as in HG (Schols et al., 1995). The methyl-esterification of HG, in particular, has drawn the attention of many research groups, because it determines to a large extent the industrial applicability of pectin. Not only the amount of methyl-esterification is important, but also the distribution of methyl groups on the HG backbone. Blocks of more than 10 unesterified GalA residues generally yield pectin molecules, which are sensitive to Ca^{2+}-cross-linking (Daas et al., 2001).

HGs can contain clusters of four different (heterooligomeric) side chains with very peculiar sugar residues (such as Api, AceA, Dha, and Kdo). These side chains, together with the approximately nine galacturonyl residues to which they are connected, are referred to as RG-II (O'Neill et al., 2001; Ridley et al., 2001). The name RG-II is somewhat misleading, because it suggests that this structure contains a rhamnogalacturonan backbone just like rhamnogalacturonan I (RG-I). However, the Rha residues are much less abundant in RG-II than in RG-I, and they are present in the side chains of RG-II instead of in the backbone. Although the structure of RG-II is highly conserved in plants, new structural details are continuously being added to this complex element (Ridley et al., 2001). The distribution of the four side chains in RG-II, as well as the distribution of RG-II in HG, still remain to be established. It is not known whether XGA can harbor RG-II elements.

Another constituent polysaccharide of pectin, RG-I, is composed of a repeating disaccharide unit [→2)-α-L-Rha*p*-(1→ 4)-α-D-Gal*p*A-(1→]$_n$, where *n* can be larger than 100 (McNeil et al., 1980; Visser and Voragen, 1996). The galacturonyl residues can carry acetyl groups on *O*-2 and *O*-3. The rhamnosyl residues can be substituted at *O*-4 with neutral sugars (McNeil et al., 1980; Lau et al., 1987). The proportion of branched Rha residues generally varies from approximately 20% to approximately 80% depending on the source of the polysaccharide (Visser and Voragen, 1996), although essentially unbranched RG-I molecules have also been reported in the literature (Penfield et al., 2001). The side chains can be single unit [β-D-

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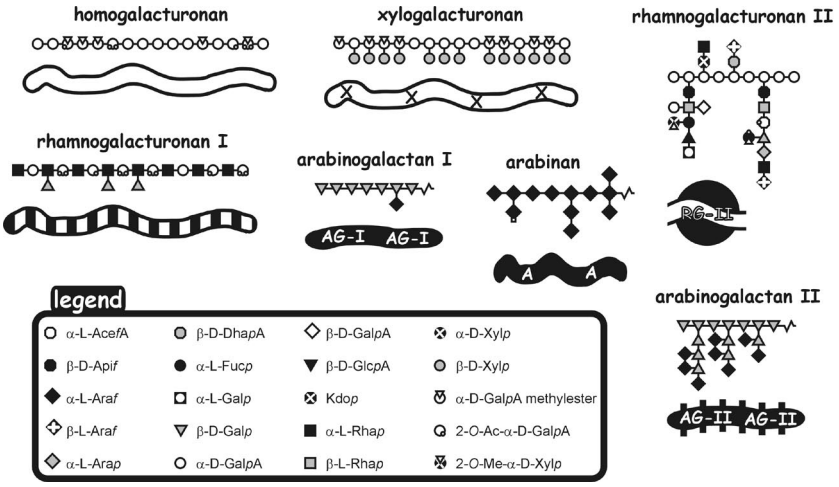

Figure 1. Schematic representative structures of the constituent polysaccharides of pectin. The symbols for the various monosaccharide building units are explained in the accompanying legend. The predominant linkage types are indicated in the text. Underneath the diagrams with the monosaccharide building units, the symbol for the polymeric structure of that polysaccharide is indicated. These symbols will be used in the rest of the paper.

Gal*p*-(1→4)] but also polymeric, such as arabinogalactan I (AG-I) and arabinan (50 glycosyl residues or more). AG-I is composed of a 1,4-linked β-D-Gal*p* backbone; α-L-Ara*f* residues can be attached to the *O*-3 of the galactosyl residues (Ridley et al., 2001). The arabinans consist of a 1,5-linked α-L-Ara*f* backbone, which can be substituted with α-L-Ara*f*-(1→2)-, α-L-Ara*f*-(1→3)-, and/or α-L-Ara*f*-(1→3)-α-L-Ara*f*-(1→3)- side chains (Ridley et al., 2001). Complexes of RG-I, AG-I, and arabinan are often referred to as pectic hairy regions (HR), in which AG-I and arabinan are the "hairs." The abundance of HR and the amount/nature of the hairs can differ considerably from species to species.

Arabinogalactan II (AG-II), is mainly associated with proteins (arabinogalactan proteins or AGPs), and it is still unclear whether this polysaccharide is part of the pectin complex. Pectin and AG-II often seem to co-extract and are subsequently difficult to separate from each other, suggesting that they can be covalently linked. AG-II is composed of 1,3-linked β-D-Gal*p* backbone, containing short side chains of α-L-Ara*f*-(1→6)-[β-D-Gal*p*-(1→6)]$_n$ (n = 1, 2, or 3; Ridley et al., 2001). The galactosyl residues of the side chains can be substituted with α-L-Ara*f*-(1→3) residues. The major moiety of AGPs (>90%) consists of polysaccharides. The protein moiety is rich in Pro/Hyp, Ala, Ser, and Thr (Gaspar et al., 2001, and refs. therein). In Arabidopsis, mature AGPs are between 10 and 369 amino acids long. At the C terminus of these proteins, a glycosylphosphatidylinositol anchor signal sequence is present. This signal can be replaced by a glycosylphosphatidylinositol anchor, which allows the protein to be inserted in the cell membrane.

MODELS FOR THE MACROMOLECULAR STRUCTURE OF PECTIN

Although the fine structure of the various pectic constituents is largely known, it is still unclear how these structural elements are combined into a macromolecular structure. It is likely that these constituents are covalently linked to each other (Ridley et al., 2001; Voragen et al., 2003). For a long time, pectin was either depicted as is shown in Figure 2A, in which HG and RG-I form one continuous backbone (Visser and Voragen, 1996), or the connection between HG and RG-I was left undiscussed. The most important feature of the model in Figure 2A is that the "pectic backbone" is an extended chain comprising RG-I (including the neutral hairs [not shown]), HG (or XGA [not shown]), and (isolated) Rha residues interspersing the HG regions. This model was consistent with the observed length periodicity of HG (approximately 70–100 GalA residues; Thibault et al., 1993), endo-polygalacturonase-resistant rhamnogalacturonan regions with a GalA to Rha ratio of >1, and the typical RG-I structure (McNeil et al., 1980; Visser and Voragen, 1996). Recently, we have summarized evidence for an alternative macromolecular structure of pectin (Fig. 2, B–D) that is more consistent with the most recent results in pectin research. The main difference between the alternative models and that in Figure 2A is that the HG constituents are depicted as side chains of RG-I. The most important motivations for proposing this alternative structure are briefly described below (for a detailed discussion, see Voragen et al., 2003). (a) It seems unlikely that the biosynthetic machinery inserts rhamnosyl residues in the nascent pectic backbone in an irregular pattern. (b) The length periodicity of HG is explained in a different way. Upon treatment of pectin with dilute acid, the α-L-Rha*p*-(1→4)-α-D-Gal*p*A linkages in rhamnogalacturonans are (selectively) split. It seems likely that similarly sized HG fragments would be obtained with both models. (c) A thorough study in which pectin was exhaustively treated with endo-polygalacturonase, followed by a careful fractionation of the resulting fragments, has not provided evidence for the existence of isolated Rha residues interspersing two HG structural elements (Zhan et al., 1998). (d) Studies in which pectin preparations were treated with exoPG or endo-XGA

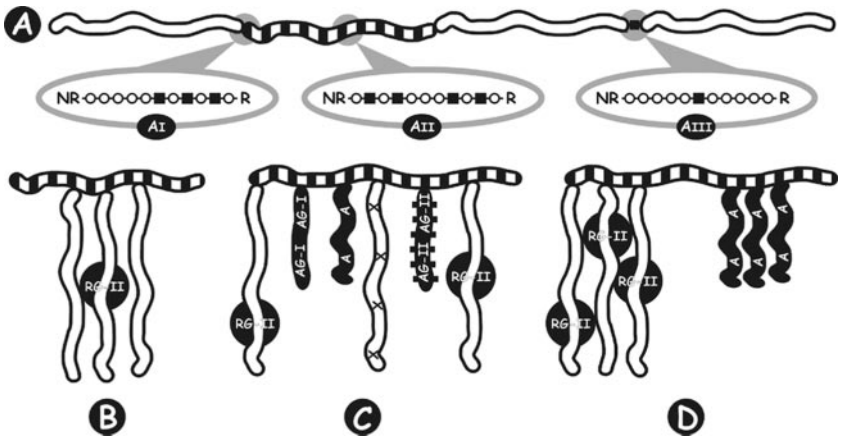

Figure 2. Schematic representation of macromolecular structures of pectin, which are in accordance with the observed length periodicity of HG. A, In this model, the pectic backbone is an extended chain with HG and RG-I regions (adapted from Visser and Voragen, 1996). HG regions can be interspersed with rhamnosyl residues. The "smooth" regions are mainly composed of HG. The "hairy" regions consist of RG-I, XGA, arabinan, and AG-I. For simplicity, XGA, arabinan, and AG-I are not shown. AI, AII, and AIII illustrate the transition of HG to RG-I, rhamnogalacturonan regions with a GalA to Rha ratio of >1, and a Rha residue interspersing two HG constituents, respectively. R is reducing end; NR is nonreducing end. B through D, Models for pectin in which RG-I is decorated with neutral (AG-I, arabinan, and possibly AG-II) and HG/XGA side chains (adapted from Voragen et al., 2003). It is unknown how HG and XGA are attached to RG-I. It is possible that unique pectin molecules exist, in which only one type of side chain is present (B), or in which various side chains are distributed at random in RG-I polymer (C), or in which the side chains are arranged in a cluster-like fashion (D). The abundance of the various polysaccharide units and their length is arbitrary, as is the position of RG-II in the HG polymer. For simplicity, all hairs are drawn to one side of the RG-I backbone. The exact orientation of hairs with respect to each other remains to be established. It is very possible that the hairs point out in various directions, away from the RG-I backbone. For symbols, see Figure 1.

hydrolase suggest that XGA is not an integral part of the pectic backbone (J.-P. Vincken, G. Beldman, and H.A. Schols, unpublished data). Because XGA and HG have the same backbone structure, it is not unreasonable to hypothesize that this holds also for HG. Recently, Oechslin et al. (2003) have also suggested that XGA might be a side chain of RG-I, based on a detailed structural investigation of pectic substances that were strongly associated with cellulose. (5) Atomic force microscopy (AFM) of citrus pectin indicated that HG branches might be connected to a pectic backbone (Round et al., 2001). Although the interpretation of the micrographs is rather complex, we think that the AFM results could indicate that HG side chains are attached to an RG-I backbone.

AFM will be a powerful tool to elucidate the macromolecular structure of pectin further, in particular when this technique is combined with selective (enzymic) degradation procedures. Selective degradation of pectin (enzymes and/or dilute acid) combined with chromatographic separation of the products is required to elucidate the exact nature of the point of attachment of HG to RG-I. Other challenges will be to establish how the various hairs, including HG, are distributed in the RG-I backbone. In principle, an unprecedented number of "hair styles" is possible. RG-I may carry only one kind of side chain (Fig. 2B). Other possibilities are a random distribution of hairs (Fig. 2C), cluster-like distributions of hairs of the same kind (Fig. 2D), and cluster-like distributions of different kind of hairs (not shown). It should be mentioned that it is unlikely that all hairs point into one direction. It is probably more realistic to draw the hairs in various directions, perpendicular to the RG-I backbone. However, in Figure 2 (and Fig. 4) this has not been done, because it would make the picture(s) too complicated. The fact that HRs can be partially degraded by treatment with endo-rhamnogalacturonan hydrolase or endo-rhamnogalacturonan lyase (RGL) lends some support to the existence of cluster-like distributions. The HG, arabinan, and AG-I (and AG-II) hairs are very different in nature (charge and conformation). In principle, different micro-environments may be created within the wall by combining different kind of hairs. Possibly, this plays an important role in regulating wall properties at various developmental time points and in different tissues.

CROSS-LINKS BETWEEN PECTIC (MACRO) MOLECULES RESULT IN THE FORMATION OF NETWORKS

In the previous section we addressed the large structural diversity of pectin macromolecules. In the plant cell wall, these macromolecules assemble in much larger networks. Three different kinds of cross-links for interconnecting pectic molecules will be discussed below.

Ca^{2+} Gels

HGs can adopt different conformations in solution of which the right-handed 2- (2_1) and 3-fold (3_1) helices seem to be most favorable in terms of minimal energy (Braccini et al., 1999). Two unesterified HG chains can engage in a complex (sometimes referred to as egg boxes; Fig. 3A), in which the carboxyl groups of two GalA residues form a negatively charged pocket that can accommodate a Ca^{2+} cation (Braccini et al., 1999; Willats et al., 2001a). At least 10 contiguous unesterified galacturonyl residues are required to build a stable cross-link between chains (Liners et al., 1992). In this way, Ca^{2+}-pectate gels can be made.

Borate-Diol Esters

Two molecules of RG-II can complex with boron, forming a borate-diol ester (Fig. 3B; Ishii et al., 1999). Only the apiofuranosyl residues of the 2-*O*-methyl-D-Xyl-containing side chains in each of the subunits of the dimer participate in the cross-linking (Ishii et al., 1999). Because RG-II is an integral part of HG (Ishii and Matsunaga, 2001), borate-diol esters can cross-link two HG chains. Certain cations (Ca^{2+}, Pb^{2+}, Sr^{2+}, and La^{3+}) promote dimer formation in vitro in a concentration- and pH-dependent manner (Ishii et al., 1999). The widespread occurrence of RG-II in the plant kingdom and its structural conservation indicate a distinct role in wall integrity for this constituent of pectin.

Uronyl Esters

Some time ago it was suggested that HG could be cross-linked to other components by uronyl esters (Fig. 3C; Brown and Fry, 1993). Up to approximately 2% of the GalA residues could be cross-linked in this way. Recently, these observations have been revisited (Gelineo-Albersheim et al., 2001). It was suggested that (particular) pectin methylesterase(s) (PME) can catalyze a transesterification reaction, using methyl-esterified HG as a donor substrate and other HG molecules as an acceptor substrate. Because HG is mainly deposited in the cell wall in a methyl-esterified form, it is evident that these molecules hold an enormous potential for cross-linking. Interestingly, in Arabidopsis, about 60 PME genes have been found that await further characterization (Henrissat et al., 2001). It is possible that PMEs specialized in catalyzing the formation of uronyl esters can be found among these. Much more work is needed to substantiate the abundance, formation, and role of this cross-link further.

DISTRIBUTION OF PECTIC EPITOPES IN THE WALL

Over the years, a number of monoclonal antibodies (mAbs) have been generated that recognize different pectic structures in the wall. The epitopes of these mAbs have recently been reviewed (Willats et al., 2001a). Microscopic analysis of walls labeled with various mAbs have shown that pectin greatly contributes to the heterogeneity of the plant cell wall. This was elegantly demonstrated by the work of Willats et al. (2001b), in which three antibodies (PAM1, JIM5, and LM7) recognizing different patterns of methoxyl esterification were used. They showed that the wall around one cell can contain discrete microdomains, each accumulating a particular type of HG. Pectic polysaccharides are also de-

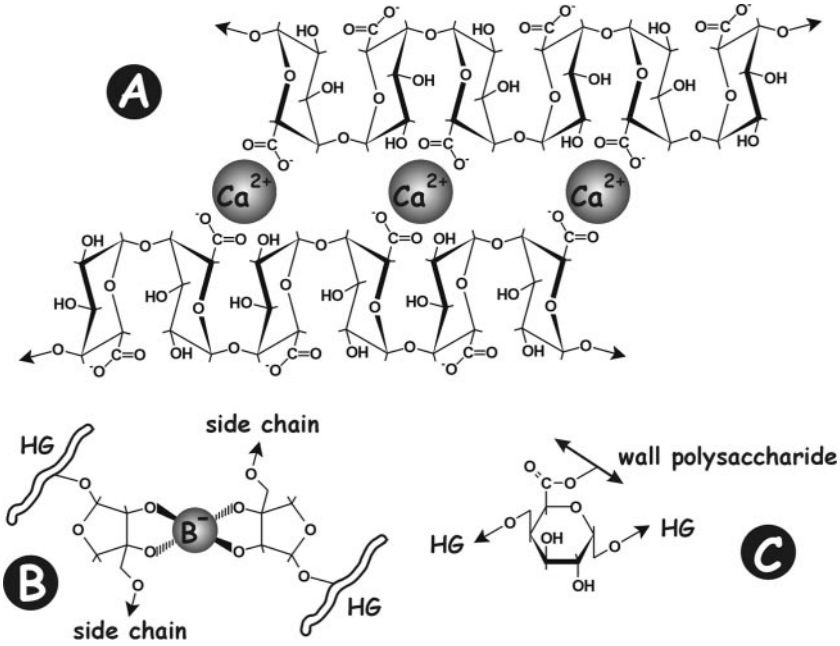

Figure 3. Pectin molecules can be cross-linked in different ways. A, Interaction through insertion of Ca^{2+} ions between the unesterified carboxyl groups of the galacturonosyl residues of two HG chains. More than nine contiguous calcium bridges are required to generate a stable connection (Liners et al., 1992). B, HG molecules can also be cross-linked by borate-diol esters, which can be formed between the apiofuranosyl residues of the 2-*O*-methyl-D-Xyl-containing side chains of RG-II. C, It has been suggested that uronyl esters can be formed as a result of a transesterification reaction, in which a methyl-esterified GalA residue is the donor substrate. In principle, any wall polysaccharide can serve as an acceptor substrate.

posited in a tissue-specific manner. For example, the distribution of pectic epitopes in the periderm of potato (*Solanum tuberosum*) tubers differs from that of the parenchymatous cells (for review, see Voragen et al., 2003, and refs. therein). Last but not least, different pectic epitopes are deposited at different stages of development. For instance, galactan epitopes appear later in the process of tuberization in potato than those of arabinan.

The observations above suggest that the wall acquires a set of pectic molecules fitted to specific developmental stages or at a given location. In very general terms, the following rules may apply: (a) various HG epitopes can surround one cell with that of Ca^{2+}-pectate confined to the middle lamella regions, cell corners, and pit fields containing plasmodesmata; (b) arabinan is generally present throughout the wall of dividing cells, whereas galactan is mainly present close to the plasma membrane of expanding cells; (c) RG-II seems to be present throughout the wall, except in the middle lamella region; (d) AG-II epitopes tend to be localized close to the plasma membrane or in walls of particular cell types, for example, tracheary elements (Ridley et al., 2001, refs. therein; Willats et al., 2001a, 2001b; Voragen et al., 2003).

UNRAVELING THE BIOLOGICAL SIGNIFICANCE OF CONSTITUENT POLYMERS

The precisely regulated deposition of pectic polysaccharides suggests that the various constituents of pectin each have their specific functions in determining the properties of the wall (e.g. porosity and water-holding capacity). At this moment, our picture of whether or how individual pectic components contribute to particular properties of the wall is very incomplete. The main reason for this is that our knowledge on the biosynthesis of cell wall polysaccharides, pectin in particular, is still in its infancy (Ridley et al., 2001). The first glycosyltransferase gene involved in polymerizing the backbone of a pectic structural element remains to be identified, although it seems as if important progress has been made very recently (see below). The first enzyme involved in decorating one of the pectic backbones has been identified only a few months ago (Iwai et al., 2002). Because of this, no attempts have been made to interfere with pectin biosynthesis using a reverse genetics approach.

A number of mutants with an altered pectin structure/deposition have been generated (Penfield et al., 2001; Bouton et al., 2002; Iwai et al., 2002; for review, see Voragen et al., 2003), of which the Fuc-deficient Arabidopsis *mur1* is probably best characterized (O'Neill et al., 2001). These mutants contain normal amounts of RG-II, but the two fucosyl residues present in RG-II (see Fig. 1) are replaced by α-L-Gal*p* residues. As a consequence of this, the rate of RG-II dimer formation and the stability of this cross-link are impaired. The *mur1* plants show dwarfed growth, indicating that plant growth depends on pectic polysaccharide organization. The phenotype can be rescued by addition of aqueous borate or L-Fuc. This is in line with experiments in which plants or plant cells were grown under boron-deficient conditions (Fleischer et al., 1999; Ishii et al., 2001). It was observed that boron deficiency results in swollen walls with increased porosity. This situation is reversible upon supplementation of boron, leading to thinner walls and reduced pore size. The phenotype of the wild tobacco (*Nicotiana plumbaginifolia*) *nolac-H18* mutant could not be rescued by the addition of excess borate (Iwai et al., 2002). The plants appear to be mutated in a family 47 glucuronyltransferase (http://afmb.cnrs-mrs.fr/CAZY/). Consequently, RG-II lacks β-Glc*p*A and the attached α-L-Gal*p* in the 2-*O*-methyl-D-Xyl-containing side chain, and the ability to form the borate-diol ester is severely impaired (down to 18% of that observed with wild-type RG-II). Other mutants affected in cell-to-cell adhesion have also been described. Very recently, a family 8 glycosyltransferase (referred to as QUASIMODO1) has been described (Bouton et al., 2002; http://afmb.cnrs-mrs.fr/CAZY/). Arabidopsis plants mutated in this enzyme have a lower galacturonyl content in their walls, and cell adhesion is reduced. The exact substrate specificity of this enzyme still needs to be determined, but a pivotal role in pectin biosynthesis may be anticipated. The fruits of the *Cnr* tomato (*Lycopersicon esculentum*) mutant appear to have mechanically stronger walls, show less cell wall swelling, and also have reduced cell adhesion (Orfila et al., 2002). The galacturonyl content of the walls of these fruits was more or less similar to those of wild-type fruits. However, the *Cnr* fruits seemed to lack regions of unesterified HG, evidenced by antibody labeling and microscopy. It has been suggested that the *Cnr* plants are mutated in a PME isoform.

An alternative approach for determining the biological significance of the various structural elements of pectin is to express fungal pectinases in muro. Such experiments have been done with three enzymes derived from *Aspergillus aculeatus*: endogalactanase (EGAL; Sørensen et al., 2000), endoarabinanase (EARA; Skjøt et al., 2002), and RGL (Oomen et al., 2002). The former two enzymes are capable of "shaving" hairs from the RG-I backbone, whereas the latter can fragment this backbone. EGAL and RGL were successfully expressed in the cell wall of potato tubers using the granule-bound starch synthase I promoter to drive their expression. This was not possible with EARA, because the plants looked sick and did not produce any tubers. Therefore, this enzyme was expressed in Golgi (same promoter, appropriate Golgi signal sequence) to degrade the arabinans before they were deposited in the wall. Monosaccharide compositional analysis of the isolated cell wall mate-

rial of the transgenic tubers showed that the amount of Gal, Ara, and both Gal and Ara was reduced in the EGAL, EARA, and RGL expressers, respectively, when compared with the untransformed controls.

The wild-type and transgenic potato tuber cell walls were also investigated using microscopy in combination with antibody labeling. The LM5 antibody was used to probe the presence of galactan hairs and as a (indirect) diagnostic tool for RG-I. Compared with wild-type potato tubers, the EGAL-expressing tubers had far fewer galactan epitopes recognized by LM5 in their walls, demonstrating that the EGAL effectively removed the galactan from the cell wall. In addition to the complete absence of LM5 epitopes in the primary walls of RGL transformants, a redistribution of the LM5 label to the middle lamella region was observed in some cases. The in muro cleavage of the RG-I backbone with the concomitant redistribution of the LM5 epitope suggests that the galactan hairs do not interact with other cell wall components.

Typically, potato tuber cell walls seem to tolerate a low level of either galactan or arabinan hairs under the growth conditions applied, because both the tubers and the appearance of the cells of the EGAL and EARA transgenic potato tubers look normal (Sørensen et al., 2000; Skjøt et al., 2002). In contrast to this, fragmentation of the RG-I backbone leads to wrinkled tubers with abnormal periderm development and large intercellular spaces in the cortex (Oomen et al., 2002). This strongly suggests that RG-I plays a more pivotal role in cell wall assembly than the hairs.

TOWARD A NEW MODEL FOR PECTIN IN THE WALL

In the previous sections, we have seen that pectin comprises a large collection of pectic molecules, which can differ considerably in their hair style. In the wall, these molecules are cross-linked in different ways forming a larger network. By tuning the biosynthesis/deposition of various pectic molecules and the secretion of wall-modifying enzymes, different microdomains can be formed in the wall, which are expected to provide site-specific properties for facilitating various processes. This last section will deal with how the pectic network might be formed in the wall. In current wall models, a cellulose-xyloglucan network co-exists with a pectin network (McCann and Roberts, 1991). The former is thought to be the load-bearing structure of the wall, whereas the latter determines its porosity. Cosgrove (2000) hypothesized three possible models for the plant cell wall: (a) the "sticky network", (b) the "multi-coat", and (c) the "stratified wall" model. The first model is characterized by xyloglucan tethering microfibrils in one layer and between layers. In the second model, cellulose microfibrils act as a kind of nucleus around which hemicellulose and subsequently pectin are deposited; there is no direct interlinking of microfibrils. The third model shows alternating layers of cellulose/xyloglucan and pectin; xyloglucan tethers microfibrils within one layer. In all of these models, pectin fills the gaps in the cellulose-xyloglucan network, and little consideration is given to the occurrence of microdomains and the formation of the middle lamella region. Our new ideas concerning pectin macromolecular structure, in which pectic molecules are regarded as molecular brushes with different hairstyles (Fig. 2, B–D), have implications for the assembly of the plant cell wall. We propose a new model (Fig. 4), featuring explanations for (a) the formation of the middle lamella and (b) of microdomains, and (c) control of wall thickness. We assume that the molecular brushes are formed as such in Golgi, and that they are not assembled outside the cell by the action of transferases. Contrary to the three models discussed above, pectin has a much more important role than filling the interstices of the cellulose/xyloglucan network. In our view, the wall consists of a cellulose/xyloglucan framework, which is embedded in a matrix of interconnected modular pectins. This will be elaborated below; for a more detailed discussion of the cellulose/xyloglucan network, we refer to Cosgrove (2000). To this end, it is not entirely clear which polysaccharide is deposited first in the cell plate: pectin, xyloglucan, or cellulose. Some papers argue for codeposition of pectin and cellulose in the cell plate (Matar and Catesson, 1988), whereas others argue for pectin first (Shea et al., 1989). For our model, we have assumed that pectin is deposited before cellulose.

Usually, the middle lamella is represented as a separate layer between cells: a Ca^{2+}-pectate gel (high M_r HG with a low degree of esterification). The questions of why these polymers accumulate here and whether these middle lamella HGs are connected to other wall polymers have not really been addressed. We propose that (methyl-esterified) HG-rich molecular brushes (with virtually no arabinan or galactan) are deposited first in the cell plate of two daughter cells. After this, subsequent layers of pectin molecules are deposited. HG hairs can be de-esterified by PMEs, (some of) which are suggested to act in a block-wise or processive fashion. The unesterified regions of HGs (from different cells) become sensitive to Ca^{2+} and can form a gel (indicated with 1 in Fig. 4A). Interestingly, microscopic studies with labeled antibodies have shown that PME and Ca^{2+}-pectate can colocalize to areas of cell-cell contact such as the middle lamella (Morvan et al., 1998). The HG-rich molecular brush (first pectin layer) may represent the apparently pure HG, which can be extracted from the wall material of many plant species using chelating agents. In summary, we hypothesize that the middle lamella is a Ca^{2+}-pectate gel, formed by cross-linking the first layer of pectin to HG-

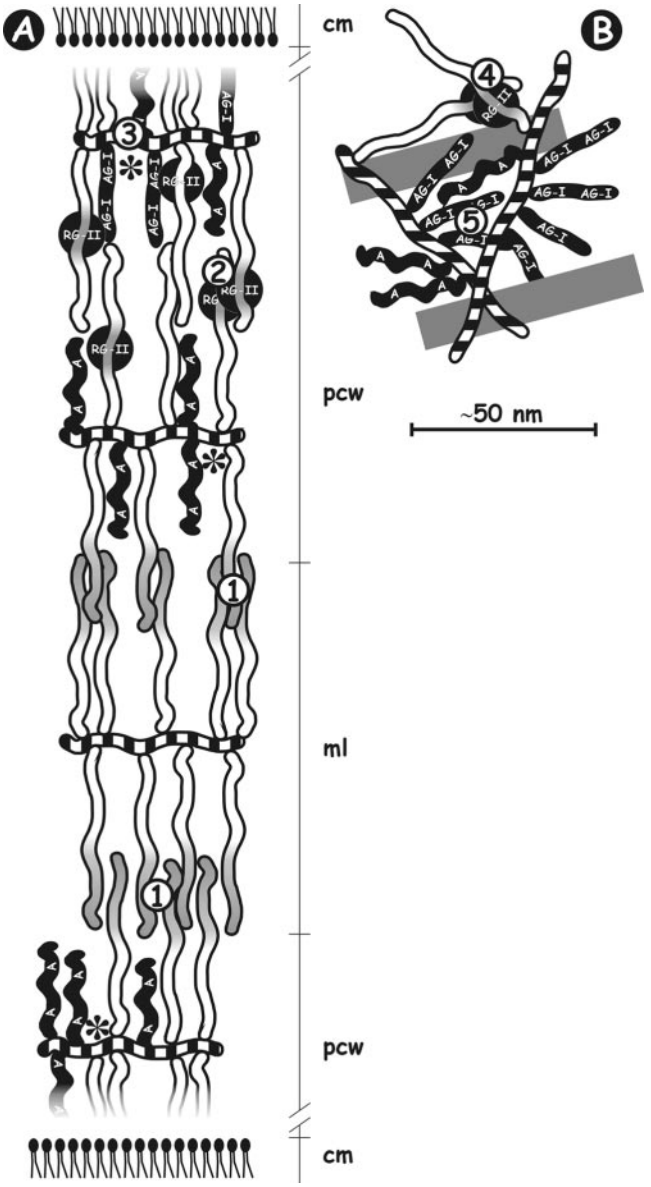

Figure 4. A new model for the pectin network in the middle lamella and the primary cell wall. A, Lateral view of two primary cell walls separated by a middle lamella, connecting two neighboring cells. 1, The middle lamella is formed from the pectin molecules laid down in the cell plate, which are Ca^{2+} cross-linked to HG hairs in subsequently deposited layers of pectin of opposite cells. Pectin methyl esterase can remove the methyl groups from HG, making HG sensitive to Ca^{2+} cross-linking. These pectic macromolecules probably represent the high-galacturonyl pectins that can be extracted from the wall using chelating agents. 2, RG-II cross-linking of successive layers of pectin by boron-diol esters. Many layers of pectin (and cellulose) can be inserted in the wall, until the thickness of the mature primary cell wall has been reached. 3, The RG-I backbone is pressed against a cellulose microfibril. Hairs from the same RG-I molecule will go around both sides of the microfibril, locking them in place and preventing the pectic molecules from escaping from the wall. B, Top view of the wall, looking from the cell membrane to the middle lamella. 4, RG-II cross-linking can occur between two layers of pectin (see 2), but also between pectic molecules within the same layer. 5, AG-I and arabinans fill the gaps in the wall, and may have an important role in controlling wall porosity. Note that, for simplicity, hemicelluloses are not shown in this schematic illustration. Regions without methyl-esterification in HG chains are indicated with shading. Gray bars, cellulose microfibrils (top view); *, cellulose microfibrils (cross section); cm, cell membrane; pcw, primary cell wall; ml, middle lamella. For other symbols, see Figure 1.

containing molecular brushes of the primary cell wall of two neighboring cells. The time point at which the middle lamella is actually formed does not necessarily precede the formation of the primary cell wall. In fact, it has been observed that the middle lamella progressively appears with the primary cell wall (Matar and Catesson, 1988). This model is consistent with observations by others indicating that the reduced presence of unesterified HG correlates with reduced cell adhesion (Orfila et al., 2002) and with the observation that RG-II is absent from the middle lamella region (Ridley et al., 2001).

After the first layer of pectin has been laid down, two processes are expected to run in parallel, leading to the formation of the primary cell wall and the middle lamella. (a) Newly synthesized (in Golgi) cell wall polymers (pectin, but also xyloglucans) are continuously being added on both sides of the Ca^{2+}-pectate gel (or its precursor), which is pushed away from the cell membrane. Eventually, the middle lamella is formed. (b) Cellulose microfibrils are synthesized at the cell membrane and extruded into the extracellular matrix, where they can be cross-linked by xyloglucans. This is also a continuous process, which can be regarded as slowly winding threads around a cell. The cellulose microfibrils are displaced from the plasmalemma by deposition of material synthesized in Golgi, explaining the fact that different lamellae of cellulose microfibrils are encountered in the wall. In Figure 4A, the addition of two extra pectin/cellulose layers is shown, but it should be realized that more layers can be inserted in a similar way. For simplicity, xyloglucans (and other hemicelluloses) are not indicated in the figure.

We hypothesize that the pectin molecules in the second (and following layers) are secured in the wall by two predominant mechanisms. First, there is the physical constraint in which the RG-I backbone of the pectic molecules are pushed against the cellulose microfibres (indicated with 3 in Fig. 4A; see also Fig. 4B), because new material is continuously being added to the wall at the plasmalemma. The hairs fill spaces between the cellulose microfibrils (indicated with 5 in Fig. 4B), thereby restricting the lateral freedom of movement of the pectic brushes. There is some evidence that mainly the pectin molecules highly substituted with side chains associate in one way or another with cellulose (or cellulose/xyloglucan), because high-strength alkaline solutions (in combination with a subsequent enzyme treatment) were required to extract these polysaccharides (Orfila et al., 2002; Oechslin et al., 2003). Also, it has been shown that the pectin molecules with low Ara con-

tent of a mutant wild tobacco line are poorly retained in the wall (Iwai et al., 2001). These results indicate a role for arabinan in anchoring pectin in the wall, although this has not been found in potato tubers with a down-regulated arabinan content (Skjøt et al., 2002). Galactans may play a more predominant role in controlling the pore size of the wall (for review, see Voragen et al., 2003). Second, the various pectic layers in the wall are connected increasing the coherence of the wall. It seems likely that the RG-II borate-diol esters play a predominant role in this (indicated with 2 in Fig. 4A), which is consistent with observations that RG-II occurs throughout the primary cell wall but not in the middle lamella. This is also in agreement with observations that boron-deficient cells have swollen walls, which can repack in thinner, firmer walls upon boron addition (Fleischer et al., 1999; Ishii et al., 2001). Boron cross-linking between pectic brushes within one pectin layer may also be anticipated (see 4 in Fig. 4B). Note that the composition of the pectic molecules in the subsequent layers changes. The RG-II is absent in the middle lamella, but is present throughout the primary wall. AG-I is probably more abundant close to the cell membrane, whereas arabinan is more abundant in the cell wall layers flanking the middle lamella.

In summary, we propose that the pectin network is assembled in a roof tile-like fashion. The RG-I backbone probably runs parallel with the cell membrane, whereas the hairs have a more perpendicular orientation with respect to the RG-I backbone. The HG hairs are presumably longer than the neutral ones and are postulated to overlap partially (as shown in Fig. 4A) or completely with those of the flanking pectic layers to facilitate cross-linking thru RG-II. If we consider the observed length periodicity of HG of approximately 70 to 100 GalA residues (Thibault et al., 1993), then it can be calculated that each pectin lamella has a minimal thickness of 35 to 50 nm (the HG hairs of flanking layers fully overlap) and a maximal thickness of approximately 80 nm (overlapping HG tips; using the generally accepted monosaccharide dimensions of 0.5 nm per residue). In our model, the thickness of one pectin lamella depends on the number of RG-II elements per HG chain, as well as on the exact positioning of the RG-II element within the HG chain. These values are in good agreement with the scale model of the primary plant cell wall proposed by McCann and Roberts (1991). They suggested that the wall was built of various lamellae and that the shortest distance between cellulose microfibrils of different lamellae was approximately 30 nm. The thickness of the middle lamella was measured approximately 50 nm, which is also reasonably consistent with the length periodicity of HG.

The model presented in Figure 4 is consistent with the presence of microdomains in the wall. In our view, the various pectic molecules are anchored in the wall and, consequently, they cannot move around freely. Pectic molecules with galactan hairs, which are usually deposited later in development, would be expected to localize in the pectin layer closest to the cell membrane. More importantly, the model can explain that pectin can build a wall of normal thickness when a cellulose-xyloglucan network is virtually lacking (Wells et al., 1994; His et al., 2001). The tensile strength of such walls is reduced, whereas the porosity is similar to that of normal walls (Shedletzky et al., 1992). In addition, these walls seem to compensate for the lack of cellulose/xyloglucan by depositing pectic molecules with more HG (Shedletzky et al., 1992; Sabba et al., 1999; His et al., 2001). More detailed studies are required to establish whether the wall strength in these cells is determined by Ca^{2+}-, borate-diol ester-, or other kinds of cross-linking. There are also increased levels of protein and phenolics in the walls of 2,6-dichlorobenzonitrile-adapted cells, which could contribute to cross-linking (Sabba et al., 1999).

CHALLENGES IN PECTIN RESEARCH

In this paper, we have provided for the first time, to our knowledge, a detailed picture on how the pectin network may be present in the plant cell wall. Our model is rooted in a putative structure in which HG, XGA, (arabino) galactan, and arabinan occur as side chains to RG-I, forming a kind of molecular brush. By combining different hairs in various fashions, a large number of hairstyles can be made. The forthcoming pectin modules may be deposited in the wall in a time-specific manner, enabling the plant cell to create different micro-environments in the wall and to adapt to varying conditions and needs at a given time point or location. It is clear that a number of things need to be substantiated further. First, the cross-link between RG-I and HG needs to be further established, which will not be an easy task. Second, the distribution of hairs in the RG-I backbone will be an enormous challenge, i.e. do the hairs occur in an at random or in block-wise fashion in RG-I? Third, a number of questions concerning RG-II remain to be answered: How many RG-II elements do HG hairs contain? How are the RG-II elements distributed in the HG backbone? Besides structural details, these investigations are expected to provide clues on the nature of the priming molecules for the biosynthesis of the various hairs. In parallel, a more detailed characterization of glycosyltransferases (when discovered) will provide a clearer picture on the range of different polysaccharide structures that can be expected. With the identification of the first enzymes belonging to the pectin biosynthetic machinery, exciting times in pectin research seem to be ahead of us. Experiments aimed at modifying the expression of these enzymes in plants will help in understanding the biological significance of the various polysaccharides. These studies will no doubt provide important

spin-off to industry, striving to understand/predict pectin functionality in different applications.

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LITERATURE CITED

Bouton S, Leboeuf E, Mouille G, Leydecker M-T, Talbotec J, Granier F, Lahaye M, Höfte H, Truong H-N (2002) Plant Cell **14:** 2577–2590
Braccini I, Grasso RP, Pérez S (1999) Carbohydr Res **317:** 119–130
Brown JA, Fry SC (1993) Plant Physiol **103:** 993–999
Cosgrove DJ (2000) Plant Physiol Biochem **38:** 109–124
Daas PJH, Boxma B, Hopman AMCP, Voragen AGJ, Schols HA (2001) Biopolymers **58:** 1–8
Fleischer A, O'Neill MA, Ehwald R (1999) Plant Physiol **121:** 829–838
Gaspar Y, Johnson KL, McKenna JA, Bacic A, Schultz CJ (2001) Plant Mol Biol **47:** 161–176
Gelineo-Albersheim I, Darvill A, Albersheim P (2001) Book of Abstracts of the Ninth International Cell Wall Meeting, September 2–7, Toulouse, France. CNRS/INRA, Toulouse, p 183
Henrissat B, Coutinho PM, Davies GJ (2001) Plant Mol Biol **47:** 55–72
His I, Driouich A, Nicol F, Jauneau A, Höfte H (2001) Planta **212:** 348–358
Ishii T, Matsunaga T (2001) Phytochemistry **57:** 969–974
Ishii T, Matsunaga T, Hayashi N (2001) Plant Physiol **126:** 1698–1705
Ishii T, Matsunaga T, Pellerin P, O'Neill MA, Darvill A, Albersheim P (1999) J Biol Chem **274:** 13098–13104
Iwai H, Ishii T, Satoh S (2001) Planta **213:** 907–915
Iwai H, Masaoka N, Ishii T, Satoh S (2002) Proc Natl Acad Sci USA **99:** 16319–16324
Lau JM, McNeil M, Darvill AG, Albersheim P (1987) Carbohydr Res **168:** 245–274
Liners F, Thibault J-F, van Cutsem P (1992) Plant Physiol **99:** 1099–1104
Matar D, Catesson AM (1988) Protoplasma **146:** 10–17
McCann MC, Roberts K (1991) *In* CW Lloyd, ed, The Cytoskeletal Basis of Plant Growth and Form. Academic Press, New York, pp 109–129
McNeil M, Darvill AG, Albersheim P (1980) Plant Physiol **66:** 1128–1134
Morvan O, Quentin M, Jauneau A, Mareck A, Morvan C (1998) Protoplasma **202:** 175–184
Oechslin R, Lutz MV, Amado R (2003) Carbohydr Polym **51:** 301–310
O'Neill MA, Eberhard S, Albersheim P, Darvill AG (2001) Science **294:** 846–849
Oomen RJFJ, Doeswijk-Voragen CHL, Bush MS, Vincken J-P, Borkhardt B, van den Broek LAM, Corsar J, Ulvskov P, Voragen AGJ, McCann MC et al. (2002) Plant J **30:** 403–413
Orfila C, Huisman MMH, Willats WGT, van Alebeek G-JWM, Schols HA, Seymour GB, Knox JP (2002) Planta **215:** 440–447
Penfield S, Meissner RC, Shoue DA, Carpita NC, Bevan MW (2001) Plant Cell **13:** 2777–2791
Ridley BL, O'Neill MA, Mohnen D (2001) Phytochemistry **57:** 929–967
Round AN, Rigby NM, MacDougall AJ, Ring SG, Morris VJ (2001) Carbohydr Res **331:** 337–342
Sabba RP, Durso NA, Vaughn KC (1999) Int J Plant Sci **160:** 275–290
Schols HA, Bakx EJ, Schipper D, Voragen AGJ (1995) Carbohydr Res **279:** 265–279
Shea EM, Gibeaut DM, Carpita NC (1989) Planta **179:** 293–308
Shedletzky E, Shmuel M, Trainin T, Kalman S, Delmer D (1992) Plant Physiol **100:** 120–130
Skjøt M, Pauly M, Bush MS, Borkhardt B, McCann MC, Ulvskov P (2002) Plant Physiol **129:** 95–102
Sørensen S, Pauly M, Bush M, Skjøt M, McCann MC, Borkhardt B, Ulvskov P (2000) Proc Natl Acad Sci USA **97:** 7639–7644
Thibault J-F, Renard CMGC, Axelos MAV, Roger P, Crépeau M-J (1993) Carbohydr Res **238:** 271–286
Visser J, Voragen AGJ (1996) Progress in Biotechnology 14: Pectins and Pectinases. Elsevier, Amsterdam
Voragen AGJ, Schols HA, Visser RGF (2003) Advances in Pectin and Pectinase Research. Kluwer Academic Publishers, Dordrecht, The Netherlands
Wells B, McCann MC, Shedletzky E, Delmer D, Roberts K (1994) J Microsc **173:** 155–164
Willats WGT, McCartney L, MacKie W, Knox JP (2001a) Plant Mol Biol **47:** 9–27
Willats WGT, Orfila C, Limberg G, Buchholt HC, van Alebeek G-JWM, Voragen AGJ, Marcus SE, Christensen TMIE, Mikkelsen JD, Murray BS et al. (2001b) J Biol Chem **276:** 19404–19413
Zhan D, Janssen P, Mort AJ (1998) Carbohydr Res **308:** 373–380