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Review

Pectins: structure, biosynthesis, and oligogalacturonide-related signaling

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Abstract

Pectin is a family of complex polysaccharides present in all plant primary cell walls. The complicated structure of the pectic polysaccharides, and the retention by plants of the large number of genes required to synthesize pectin, suggests that pectins have multiple functions in plant growth and development. In this review we summarize the current level of understanding of pectin primary and tertiary structure, and describe new methods that may be useful to study localized pectin structure in the plant cell wall. We also discuss progress in our understanding of how pectin is biosynthesized and review the biological activities and possible modes of action of pectic oligosaccharides referred to as oligogalacturonides. We present our view of critical questions regarding pectin structure, biosynthesis, and function that need to be addressed in the coming decade. As the plant community works towards understanding the functions of the tens of thousands of genes expressed by plants, a large number of those genes are likely to be involved in the synthesis, turnover, biological activity, and restructuring of pectin. A combination of genetic, molecular, biochemical and chemical approaches will be necessary to fully understand the function and biosynthesis of pectin.

Keywords: Cell wall; Homogalacturonan; Oligogalacturonides; Pectin; Polysaccharide; Rhamnogalacturonan

Contents

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1. Structure

1.1. Introduction

A generally accepted primary structure of pectin has emerged from cumulative studies spanning the last 70 years. The pectins used in these studies were typically isolated from plants of economic importance (e.g. citrus, tomato, sugar beet, apple) or from suspension-cultured cells (e.g. sycamore, carrot, spinach, and rose). However, these plants represent only a small fraction of the total number of known flowering plants (~235,000 species), and little is known about the pectin of green algae, liverworts, mosses, and ferns. The characterization of pectin from lower plants, from cell wall mutants, and from plants that grow in extreme environments may reveal hitherto unknown pectin structures and provide further insight into the origins and functions of these complex polysaccharides.

In this section we briefly describe what is currently known about the chemical structures of pectin. We also provide an overview of some new approaches to studying localized pectin structure that may increase our knowledge of the biological functions of pectin.

1.2. The chemical structure of primary cell wall pectins

Pectins are a family of complex polysaccharides that contain 1,4-linked α-D-galactosyluronic acid (Gal*p*A) residues. Three pectic polysaccharides (homogalacturonan, rhamnogalacturonan-I, and substituted galacturonans) have been isolated from primary cell walls and structurally characterized (O'Neill et al., 1990; Visser and Voragen, 1996).

Homogalacturonan (HG) is a linear chain of 1,4-linked α-D-galactopyranosyluronic acid (Gal*p*A) residues in which some of the carboxyl groups are methyl esterified (Fig. 1). HGs may, depending on the plant

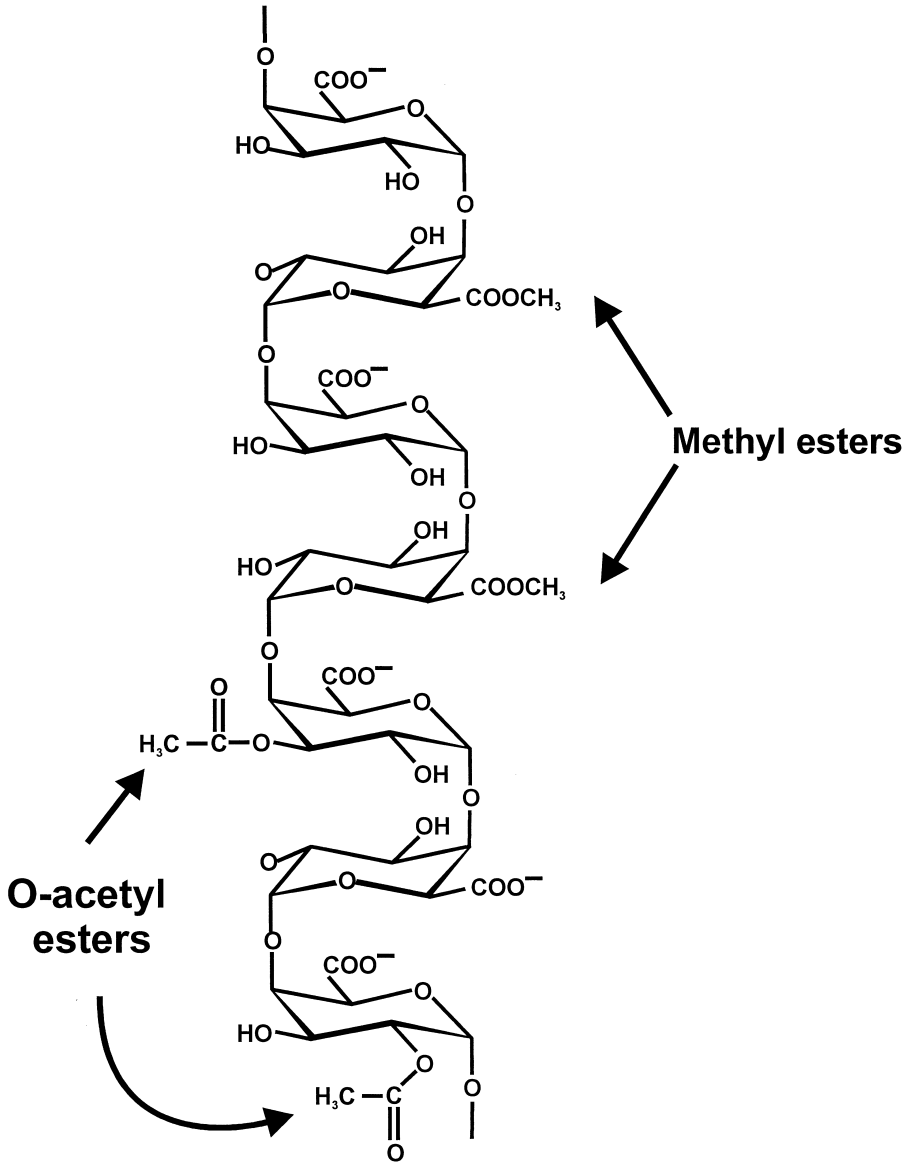

Fig. 1. The primary structure of homogalacturonan. Homogalacturonan is a linear polymer of 1→4 linked α-D-Gal*p*A residues. Some of the carboxylates of the Gal*p*A residues are esterified with methanol. The Gal*p*A residues may also be esterified with acetic acid at C2 and C3. At least three enzymes homgalacturonan galacturonosyltransferase, homogalacturonan methyltransferase, and homogalacturonan acetyltransferase are required for the synthesis of HG (see Table 1 for details).

source, also be partially O-acetylated at C-3 or C-2 (Ishii, 1995, 1997a).

Rhamnogalacturonan-I (RG-I) is a family of pectic polysaccharides that contain a backbone of the repeating disaccharide [→4)-α-D-Gal*p*A-(1→2)-α-L-Rhap-(1→] (Fig. 2). The backbone Gal*p*A residues may be *O*-acetylated on C-2 and/or C-3 (Komalavilas and Mort, 1989). There is no conclusive chemical evidence that the Gal*p*A residues are methyl esterified, however, an enriched RG-I-like wall fraction from flax has been reported to contain methyl esters (Rihouey et al., 1995). The Gal*p*A residues typically are not substituted with mono- or oligosaccharide side chains, although a recent study reported that a single β-D-Glc*p*A residue is linked to C-3 of ~2% of the Gal*p*A in the backbone of sugar beet RG-I (Renard et al., 1999). In contrast, 20–80% of the rhamnosyl (Rha*p*) residues are, depending on the plant source and method of isolation, substituted at C-4 with neutral and acidic oligosaccharide side chains (O'Neill et al., 1990). The predominant side chains contain linear and branched α-L-arabinofuranosyl (Ara*f*), and/or β-D-galactopyranosyl (Gal*p*) residues (Fig. 2), although their relative proportions and chain lengths may differ depending on the plant source (Lerouge et al., 1993). The glycosyl residues α-L-fucosyl (Fucp), β-D-glucuronosyl (Glc*p*A), and 4-*O*-methyl β-D-glucuronosyl (4-*O*-Me GlcpA) may also be present (O'Neill et al., 1990), as may ferulic and coumaric acid (Saulnier and Thibault, 1999).

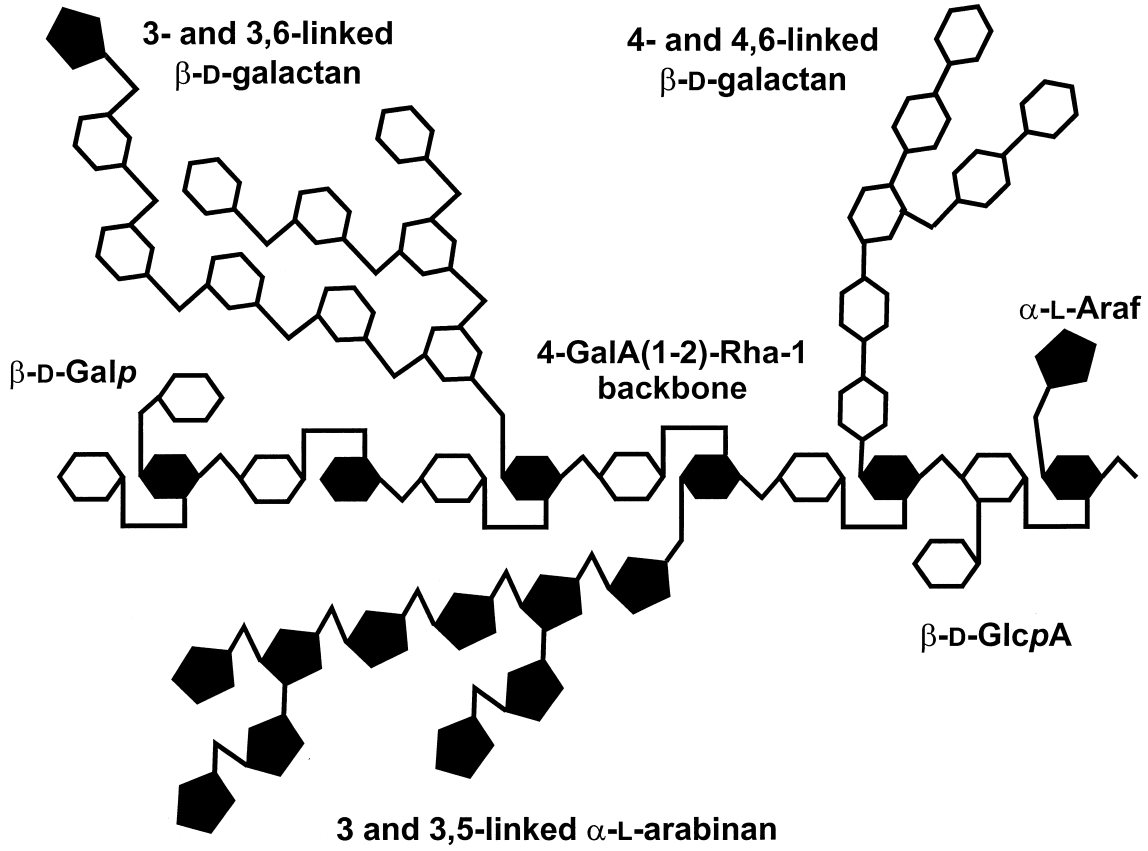

Fig. 2. A model showing the major structural features of rhamnogalacturonan I. The backbone is composed of the disaccharide repeating unit [→4-α-D-Gal*p*A-(→2)-α-L-Rhap-(1→]. Branched and linear oligosaccharides composed predominantly of α-L-Ara*f* and β-D-Gal*p* residues are linked to C4 of some of the Rha*p* residues. Some of the Rha*p* residue may also be *O*-acetylated at C2 and/or C3. More than ten glycosyltransferase activities are required for the biosynthesis of RG-I (see Table 2 for details).

Substituted galacturonans (SG) are a diverse group of polysaccharides that contain a backbone of linear 1,4-linked α-D-Gal*p*A residues (O'Neill et al., 1990). The substituted galacturonan referred to as rhamnogalacturonan II (RG-II) is present in all higher plant primary walls analyzed to date (O'Neill et al., 1990). The demonstration that wine and other fruit juices contain relatively high amounts (20–150 mg/l) of RG-II (Doco et al., 1997), that RG-II binds heavy-metals (Pellerin et al., 1997, Pellerin and O'Neill, 1998; Szpunar et al., 1999; Tahiri et al., 2000), and that RG-II has immunomodulating activities (Shin et al., 1998) has led to a greater interest in the structure of RG-II and to the role of this enigmatic pectic polysaccharide in human nutrition and health.

RG-II is *not* structurally related to RG-I since its backbone is composed of 1,4-linked α-D-Gal*p*A residues rather than the repeating disaccharide [→4)-α-D-Gal*p*A-(1→2)-α-L-Rhap-(1→] (O'Neill et al., 1990). A nonasaccharide (side chain B) and an octasaccharide (side chain A) are attached to C-2 of some of the backbone GalA residues and two structurally different disaccharides (side chains C and D) are attached to C-3 of the backbone (Fig. 3). The locations on the backbone of the side chains with respect to one another have not been established with certainty.

Other substituted galacturonans have been described that are present in the walls of a restricted number of plants. For example, xylogalacturonans (XGA), which contain β-D-xylosyl (Xylp) residues attached to C-3 of the backbone (Kikuchi et al., 1996; Yu and Mort, 1996; Schols et al., 1995; O'Neill et al., 1990; Aspinall, 1980), are present in the walls of reproductive plant tissues (e.g. apple, carrot, cotton, and pine). Apiogalacturonans, which are present in the walls of some aquatic monocotyledons (e.g. *Lemna* and *Zostera*), contain β-D-apiofuranosyl (Api*f*) residues attached to C-2 and C-3 of the backbone either as a single Api*f* residue or as the disaccharide β-D-Api*f*-(1→3′)- β-D-Apif-(1→(Hart and Kindel, 1970a,b; Cheng and Kindel, 1997).

1.3. Advances in the structural analysis of cell wall pectic polysaccharides

1.3.1. Homogalacturonan and rhamnogalacturonan I

The availability of exo- and endoglycanases, and endolyases that fragment HG and RG-I has led to a greater understanding of the structure of these pectic

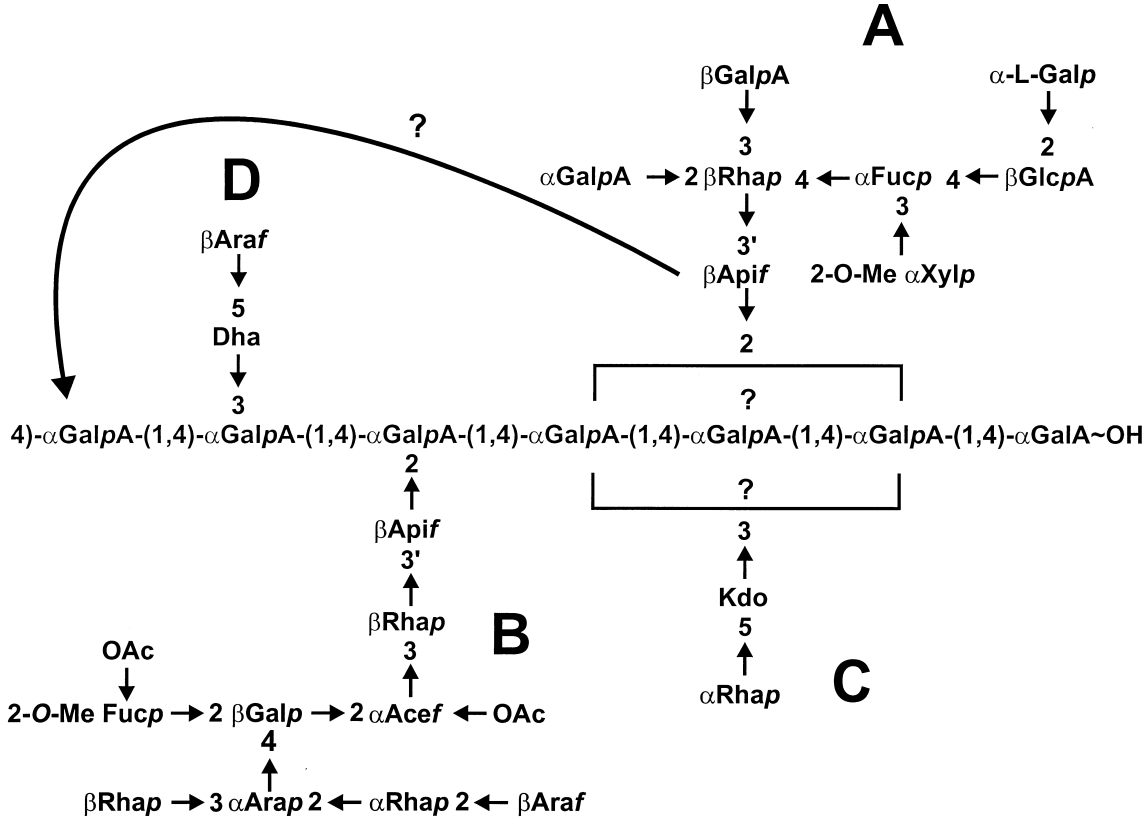

Fig. 3. The primary structure of rhamnogalacturonan II. The backbone of RG-II is composed of at least seven 1→4 linked α-D-Gal*p*A residues. Four structurally different oligosaccharide side chains (A–D) are linked to the RG-II backbone. The anomeric configurations of the glycosyl residues present in side chain B have been determined by high resolution NMR spectroscopy. The anomeric configurations of the glycosyl residues in side chain A are taken from Melton et al., 1986, but have not been confirmed by high resolution NMR spectroscopy. The order of the side-chains has not been unambiguously determined. At least 20 glycosyltransferase activities are required for the biosynthesis of RG-II (see Table 3 for details).

polysaccharides (Prade et al., 1999). For example, rhamnogalacturonase, rhamnogalacturonan lyase, rhamnogalacturonan-rhamnohydrolase, rhamnogalacturonan-galacturonohydrolase, and exo- and endogalacturonases, arabinanases and galactanases have been used to refine the structural features of RG-I (Prade et al., 1999). These enzymes, which have been isolated from fungi and bacteria, also provide insight into the wide range of enzymes that microorganisms secrete to fragment HG and RG-I (De Vries et al., 2000).

The distribution of methyl esters in HG has been investigated using chemical methods (Mort et al., 1993) and by treating walls, or isolated pectin, with pectin methylesterase (PME), endopolygalacturonase (EPGase), and endopectin lyase (PL) in combination with NMR spectroscopy (Grasdalen et al., 1988) and mass spectrometry (Daas et al., 1998; Limberg et al., 2000a). The results of such studies have demonstrated that commercially prepared pectin typically has a less regular pattern of methyl esterification than HGs isolated from cell walls, presumably due to the methods used to extract the pectin from the plant material. Mass spectrometry and anion-exchange chromatography, in combination with EPGase and PL fragmentation, have been used to show that plant PMEs cause blockwise de-esterification of pectin, whereas microbial PMEs typically cause random de-esterification (Limberg et al., 2000b).

1.3.2. Rhamnogalacturonan II

RG-II is present in primary walls predominantly as a dimer that is cross-linked by a 1:2 borate-diol ester (Kobayashi et al, 1996; Ishii et al., 1999). This cross-link is formed between OH-2 and OH-3 of the apiosyl residues in each monomeric RG-II subunit. Two diastereoisomers are formed (see Fig. 4) when methyl β-D-apiofuranoside is reacted with borate at pH 8 (Ishii and Ono, 1999). It is not known whether the naturally occurring dimer contains one or both of the diastereoisomers (O'Neill et al., 1996). Chemical analysis of naturally occurring and in vitro formed dimer suggests that the apiosyl residue of each side chain A, but not those of side chain B (see Fig. 3), are cross-linked by borate (Ishii et al., 1999). However, the possibility cannot be discounted that borate esters may cross-link the apiosyl residues of side chains A and B (Matoh et al., 2000).

RG-II dimer formation is a self-assembly process since it occurs in vitro in the absence of a catalytic protein. The anionic nature and chemical structure of RG-II

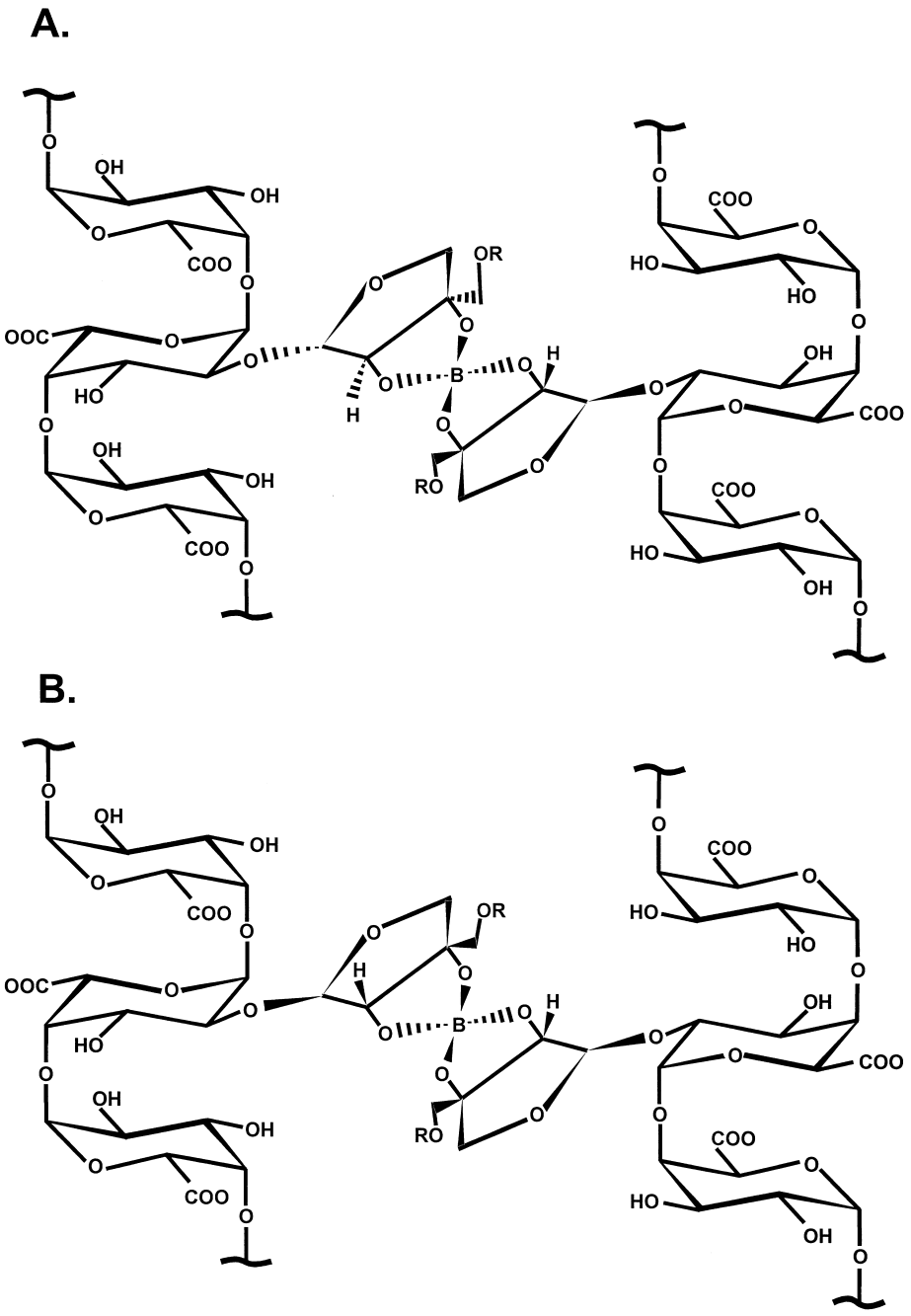

Fig. 4. The borate 1:2 diol ester that cross links two monomeric units of RG-II. The borate ester is formed between OH-2 and OH-3 of the 3′ linked apiosyl residues. "R" represents the oligoglycose that is linked to 3′ of the apiosyl residues. The ester can exist in either of two diastereomeric forms. In A one "R" group is pointing upward and the other is pointing downward. In B both "R" groups are pointing upward.

are likely to be major factors in determining the optimum pH (pH 3.0–4.0) for dimer formation in vitro (O'Neill et al., 1996) since 1:2 borate-diol esters of methyl β-D-apiofuranoside do not form below pH 5 (Ishii and Ono, 1999). The rate and extent of RG-II dimer formation in vitro are increased in the presence of di- and trivalent cations with ionic radii >1.0 Å (Ishii et al., 1999). Calcium at low concentrations (1 mM) is less effective than the larger cations (Ishii et al, 1999). However, higher concentrations (10 mM) of calcium and boric acid do increase the rate of dimer formation in vitro (Matoh and Kobayashi, 1998). The role of Ca^{2+} in dimer formation in vivo is not fully understood but there is evidence that it increases the stability of the RG-II dimer (Fleischer et al., 1999; Kobayashi et al., 1999).

The chemical structure of RG-II has been increasingly refined using high-field NMR spectroscopy in combination with electrospray and matrix-assisted laser desorption mass spectrometry. As a result, some of the previously published structures (Spellman et al., 1983) have been

revised (Vidal et al., 2000). The aceric acid-containing side chain (B in Fig. 3) is most likely a nonasaccharide (Whitcombe et al., 1995; Shin et al 1998; Terrell, M. and Prestegard, J., personal communication) and not a heptasaccharide as had previously been reported (Spellman et al., 1983). Side chain B is linked to *O*2 of the fifth Gal*p*A residue from the reducing end of the backbone, whereas side chain D [β-L-Araf-(1→5)-α-D-Dha-(1→] is most likely linked to *O*3 of the sixth Gal*p*A residue from the reducing end (Vidal et al., 2000). This study also provided evidence that the aceryl acid residue is α linked and that the galactosyl residue is β-linked, thus reversing the previously published anomeric configurations of these residues (Spellman et al., 1983). Somewhat unexpectedly, the terminal Gal*p* residue on side chain A (see Fig. 3) has been shown to have the L rather than the D configuration (Reuhs et al., submitted) thereby confirming that L-galactose is a minor component of primary walls (Baydoun and Fry, 1988).

A preliminary assignment of the ^{1}H and ^{13}C resonances in the NMR spectra of the $NaBH_4$-reduced RG-II monomer have been made and used to propose an NMR solution structure for RG-II (Du Penhoat et al., 1999). The ^{1}H and ^{13}C resonance assignments appear to be based in part on the published, and in some cases incorrectly assigned, anomeric configurations of the glycosyl residues present in RG-II. Inter-glycosidic NOEs and long-range NOEs were measured and interpreted on the basis of these assignments. The specific attachment site of each side-chain was not determined, although the authors suggest that only two arrangements of the side-chains (A, C, B, D or A, D, B, C) are consistent with the NMR spectroscopic data (Du Penhoat et al., 1999). Additional data are required before the validity of this conclusion can be assessed since the unambiguous assignment of all the resonances and the interpretation of NOE data for RG-II is a challenging endeavor. This is largely due to the considerable signal overlap and degeneracy that result from the relatively narrow spectral dispersion of complex carbohydrates (Van Halbeek, 1994). Complete assignment of the resonances in crowded regions of the NMR spectra of RG-II may be achieved by using selective excitation techniques (Freeman, 1992) or by 3D NMR spectroscopy. 3D NMR techniques were developed to facilitate the assignment of resonances in NMR spectra of isotopically-enriched proteins (Marion et al., 1989; Zuiderweg and Fesik, 1989) and have also been used to analyze ^{13}C-labeled complex carbohydrates (Van Halbeek, 1994; Bush et al., 1999). The availability of ^{13}C-enriched RG-II (York, W.S., O'Neill, M.A., Terrell, M., Prestegard, J., unpublished results) now makes 3D NMR spectroscopic analysis of this polysaccharide possible.

Molecular modeling procedures have been used to generate several possible three-dimensional structures for the side-chains of RG-II (Mazeau and Perez, 1998; Perez et al., 2000). The reader should consult Woods (1998) for a concise discussion of the computational methods that are used to model complex carbohydrates. The validity of the RG-II side chain models remain to be established, particularly in light of the evidence showing that in side-chain B the Ace*f*A residue is α- rather than β-linked, and that the 2,4-linked Gal*p* residue is β- and not α-linked. The computer-generated conformations of side-chain A need to be reexamined since they were obtained by assuming that the previously published anomeric configurations of the glycosyl residues (Melton et al., 1986) were correct and that the galactosyl residue was D rather than L.

1.4. The macromolecular organization of HG, RG-I, and RG-II in the primary wall

1.4.1. Are homogalacturonan, rhamnogalacturonan I, and RG-II linked together covalently?

Homogalacturonan, RG-I, and RG-II are solubilized from insoluble walls by treating the walls with aqueous buffers or calcium chelators. The polysaccharides, however, are not separated from each other by size-exclusion chromatography (e.g. Superose 6 and 12) since they elute in the column void volume (molecular masses >200 kDa). Fractions enriched in RG-I, RG-II, and oligogalacturonides are generated by treating the high molecular weight material with EPGase (O'Neill et al., 1990). Thus, it is often assumed that in primary walls HG, RG-I, and RG-II are covalently linked to one another and models have been proposed for the distribution of RG-I and HG in pectin (Matsuhashi et al., 1993).

Homogalacturonan and RG-II are likely to be covalently linked since they both have backbones composed of 1→4-linked α-D-Gal*p*A residues and they are both solubilized by treating walls with EPGase. The backbone of EPGase-solubilized RG-II contains up to 15 1,4-linked α-D-Gal*p*A residues, providing indirect evidence that RG-II is linked to HG (Whitcombe et al., 1995). RG-I is also solubilized by treating walls with EPGase even though there are no glycosyl residues in RG-I that are susceptible to EPGase. However, no oligosaccharides composed of the RG-I backbone disaccharide repeating unit covalently linked to fragments of HG have been isolated, nor is there evidence that HG is glycosidically linked to the side-chains of RG-I. The notion that HG chains are interrupted by the insertion of a single Rhap residue between regions containing 20–30 Gal*p*A residues is almost certainly incorrect (Zhan et al., 1998).

1.4.2. Is pectin cross-linked to other wall components?

Early models of the primary cell wall postulated that xyloglucans and pectic polysaccharides, as well as structural glycoproteins, are covalently linked together

to form a continuous macromolecular network (Keegstra et al., 1973). This view has gradually changed and current models emphasize the predominance of non-covalent linkages between wall polymers (Carpita and Gibeaut, 1993). Nevertheless, there is a substantial body of data showing that RG-II molecules are covalently cross-linked by borate esters.

1.4.3. Borate-ester cross-linked pectin

There is accumulating evidence that borate ester cross-linking of RG-II is required for the formation of a macromolecular pectic network within the plant cell wall (Matoh and Kobayashi, 1998). The function of this network is not understood, although it may have a role in controlling the mechanical properties of the primary wall (Loomis and Durst, 1992; Flesicher et al., 1998, 1999). For example, boron-mediated cross-linking of RG-II in the walls of living plant cells generates a pectin network with a decreased size-exclusion limit for polymers (Fleischer et al., 1999). Such results are consistent with previous studies showing that the size of molecules able to diffuse through the wall is increased by enzymic and chemical fragmentation of wall-bound pectin (Baron-Epel et al., 1988; Ehwald et al., 1992).

Chenopodium album cells divide and grow in the absence of added boron (Fleischer et al., 1998) and their walls contain monomeric RG-II, but no discernible amounts of dimeric RG-II. Thus, borate ester cross-linking of RG-II is unlikely to be required for cell division and growth in these cells. Boron-deficient *C. album* cells, in contrast to their boron-grown counterparts, are unable to reduce their wall pore size and thereby undergo excessive cell enlargement and experience wall rupture during their transition to the stationary phase (Fleischer et al., 1998). Thus, it appears that the size-exclusion or cross-linking effects of the pectic network may become essential for the stability of *C. album* cells only when growth has ceased. In contrast, tobacco cells do not grow in a boron-free medium but do grow in media containing a low amount (10 μg/l) of added boric acid (Matoh et al., 2000). Most (~60%) of the RG-II is present as the monomer in the boron-deficient cells and the walls are swollen suggesting that the cross-linking and organization of the pectin network has been altered (Matoh et al., 2000). The inability to form a borate ester cross-linked pectic network may influence physiologically important processes including the incorporation of polymers into the wall, the access of wall modifying enzymes or proteins to their substrates or ligands, and the transport of polymers from the protoplast into the wall.

Kobayashi et al. (1999) have provided evidence that cyclohexanediaminetetraacetic acid (CDTA) promotes the hydrolysis of the RG-II dimer and that the release of the most tightly bound wall calcium is correlated with the solubilization of wall-bound RG-II. Divalent cations promote soluble dimer formation in vitro (O'Neill et al., 1996; Matoh and Kobayashi, 1998) and together with boric acid prevent the low-pH mediated increase in wall pore size (Fleischer et al., 1999). These results, when taken together with the ability of calcium to stabilize borate complexes in root tips (Wimmer and Goldbach, 1999), suggest that in muro calcium has a role at specific, but as yet unidentified, sites close to the borate ester cross-link.

1.4.4. Anionic cross-links

Pectins are the predominant anionic polymer in the primary walls of dicots and non-graminaceous monocots and thus many of their properties are believed to be determined by ionic interactions. The properties of pectin gels and the mechanism of gelation have been the subject of numerous studies (Grant et al., 1973; Powell et al., 1982; Morris et al., 1982; Thibault and Rinaudo, 1986; Thakur et al., 1997). The pectins used for many of the gelation experiments, however, were often solubilized from plant tissue by degradative treatments (e.g. hot aqueous acid). Thus, the relevance of such studies for the macromolecular properties of cell wall-bound pectin has been questioned (MacDougall et al., 1996).

Pectin gels have also been studied using pectin isolated from cell walls by nondegradative conditions. For example, CDTA treatment of unripe tomato fruits has been shown to result in cell separation and the solubilization of a high methoxy pectin (DM 68%). This pectin is believed to originate from the middle lamella and may have an important role in maintaining cell adhesion (MacDougall et al., 1996). The CDTA-soluble pectin forms elastic gels in the presence of calcium. These gels have a higher affinity for calcium than aqueous solutions of the same polysaccharide (Tibbits et al., 1998). A fraction of the Gal*p*A residues in the gel does not participate in ionic cross-linking but may contribute to gel swelling particularly at low ionic strength (Tibbits et al., 1998). The maximum swelling of the gel and the minimum shear modulus (strength of the gel) occur at pH 3 which is close to the pK_a of the carboxyl group of galacturonic acid (Tibbits et al., 1998). This pH value is also similar to the optimum pH required for borate ester cross-linking of RG-II in vitro (O'Neill et al., 1996). The question of what role RG-II cross-linking has on the properties of CDTA-soluble pectin gels has not yet been addressed.

1.4.5. Other cross-links

A portion of primary wall pectin is not solubilized by treatment with aqueous buffers, calcium chelators, or EPGase, but is solubilized by aqueous base (Bauer et al., 1973; Selvendran, 1985). Thus, the existence of esters that form between the carboxyl group of a 4-linked Gal*p*A residue and a hydroxyl group of another glycosyl residue has been hypothesized (Fry, 1986). The presence of

non-methyl galacturonosyl esters has been implied from chemical analysis of maize cell walls (Kim and Carpita, 1992) and by chemical and infra-red spectroscopic analysis of tobacco cell walls (McCann et al., 1994). Non-methyl esters have also been reported to be present in the walls of spinach (Brown and Fry, 1993) and carrot (Needs et al., 1998), although no fragments of HG containing unusual esters have been isolated and structurally characterized. The trans-esterification of pectin has been reported to be catalyzed by pectin methyl esterase (Hou and Chang, 1996), but additional structural evidence is required to substantiate this claim.

In the walls of the *Chenopodiaceae* (e.g. sugar beet and spinach) some of the pectic arabinosyl and galactosyl residues are esterified with ferulic or coumaric acid and these phenolic residues are potential sites for cross-linking via oxidative coupling (Ishii, 1997b; Saulnier and Thibault, 1999). However, the existence in muro of intermolecular dehydrodiferuloyl cross-linked pectin has not been demonstrated. Hemicellulose-pectin complexes that may be cross-linked by as yet unidentified phenolic material have been reported to be present in the walls of cauliflower (Femenia et al., 1999), asparagus stems (Waldron and Selvendran, 1992), and olive seed hulls (Coimbra et al., 1995).

Pectin and xyloglucan are both solubilized by treating primary walls with alkali (Bauer et al., 1973; Selvendran, 1985; Thompson and Fry, 2000). A portion of the xyloglucan and pectin is assumed to be covalently cross-linked since these polymers co-elute from anion-exchange columns and xyloglucans themselves contain no acidic glycosyl residues (Thompson and Fry, 2000). However, the nature of the covalent linkage between xyloglucan and pectin, if it exists, has not been established. The wall material remaining after 4M KOH treatment is rich in cellulose but may also contain Rhap and Gal*p*A residues and Hyp-rich glycoprotein. Linkages between pectin and wall glycoprotein have been inferred from chemical analysis (Qi et al., 1995; Fu and Mort, 1996; Peronne et al., 1998) and from solid state NMR spectroscopic studies (Pan, 1992) but compelling evidence for these cross-links is lacking.

Although numerous studies have reported cross-linking between pectic and hemicellulosic polysaccharides and between pectin and wall glycoprotein, conclusive evidence for their existence is lacking. Future research should be directed towards the isolation and structural characterization of these putative cross-links.

1.4.6. Biophysical methods for determining the properties and functions of primary wall pectin

The current lack of understanding of the macromolecular organization of pectin in the primary wall is largely due to a paucity of evidence for all but a few of the postulated cross-links and ionic interactions. One interpretation of the available, albeit inadequate, data is that primary wall pectin exists as a continuous three-dimensional cross-linked network. The functional characteristics of this network depend on the chemical structures, the conformations, and the interactions of the individual macromolecules. Thus, techniques are required that can examine primary walls in their native state. Such techniques include solid state NMR spectroscopy, Fourier transform infra-red spectroscopy, and atomic force microscopy.

Solid state ^{13}C NMR spectroscopy is a useful tool for the characterization of synthetic polymers (Andrew and Szczesniak, 1995), biological solids (McDowell and Schaefer, 1996), and plant cell walls (Jarvis and McCann, 2000). Chemical shift data provide information on the local chemical environment and structure whereas local dynamics, including the thermal motion of polymers in the solid state, are derived from various relaxation measurements (Jarvis and McCann, 2000). Such relaxation experiments have led to the general conclusion that pectic galacturonans have a range of thermal mobilities and that highly methyl esterified pectins are more mobile than low esterified pectins (Ha et al., 1997; Jarvis and McCann, 2000; Tang et al., 2000). The most mobile portions of pectin are the galactan and arabinan side chains of RG-I which are suggested to behave as "tethered liquids" (Jarvis and McCann, 2000).

Conventional solid state ^{13}C NMR spectra are typically interpreted primarily in terms of a single polymer chain. Thus, the relationship between the microscopic structure of a polymer, the mobility of a polymer, and its macroscopic properties can only be inferred indirectly. However, an accurate and direct characterization of inter-chain packing is required to understand the mechanical properties of the cell wall. The inter-nuclear distances (10–15 Å) between packed chains can be determined from long-range, weak dipolar couplings using rotational echo double resonance (REDOR) and transferred echo double resonance (TEDOR) NMR (McDowell and Schaefer, 1996; Schaefer, 1998). These technically challenging experiments are complicated because observation of the appropriate dipolar couplings necessitates selective enrichment of the polymers with isotopes (e.g. ^{13}C–^{15}N, ^{13}C–^{2}H, ^{19}F–^{31}P), whose abundance is low in naturally occurring samples. REDOR NMR has been used to investigate the structure and dynamics of cross-links in *Staphylococcus aureus* cell walls (Tong et al., 1997), in mussel byssal plaques (McDowell et al., 1999), in insect cuticle (Merritt et al., 1996), and in the binding sites of several proteins (Schaefer, 1998). REDOR ^{15}N NMR analysis, in combination with double cross-polarization magic angle spinning ^{13}C NMR analysis, of ^{15}N- and ^{13}C-enriched tomato cell walls have provided evidence that some carbonyl carbons, presumably from pectin, are cross-linked to histidine (Pan, 1992). However, additional experimental evidence is required to support this conclusion.

Fourier transform infra-red (FTIR) spectroscopy is a valuable tool for determining the bulk structural features of polysaccharides and cell walls (Jarvis and McCann, 2000; Kacurakova et al., 2000). The IR beam is focused onto a small ($\geqslant$10 μm) sample area using a FTIR microscope and information is obtained for the distribution of wall polysaccharides within a tissue. Information on the mean orientation of pectin and cellulose in the wall can be obtained using polarized IR beams (Jarvis and McCann, 2000).

The IR spectra of polysaccharides are complex. The major classes of cell wall polysaccharide (HG, RG-I, arabinan, galactan, arabinogalactan, xyloglucan, arabinoglucuronoxylan, glucomannan, and galactoglucomannan) each have distinctive absorption bands (Kacurakova et al., 2000). Nevertheless, partial overlap of the absorption bands in the 1200–800 cm^{-1} region of the spectra complicates determining the polysaccharide composition of a wall by IR spectroscopy. The carboxylate and ester bands of pectin at 1607 and 1740 cm^{-1}, respectively, are reasonably well resolved from other absorption bands and their relative intensities provide information on the degree of esterification of HG (Bociek and Welti, 1975). However, the IR spectra of walls containing a large amount of protein are likely to contain a significant amide band (1650 cm^{-1}) and this may overlap with the carboxylate band (Bociek and Welti, 1975).

Scanning probe microscopy encompasses several distinct techniques that have been used to characterize cell surfaces and cell wall polysaccharides (Brant, 1999; Morris et al., 1999). Atomic force microscopy (AFM) is an imaging technique that has been used to visualize plant cell walls (Morris et al., 1997), cellulose (Baker et al., 1997) and pectin (Round et al., 1997). The polysaccharides are imaged directly under butanol and the images are likely to contain less artifacts than electron microscope images which are obtained from rotary-shadowed replicas of polysaccharides (Seymour and Harding, 1987).

AFM images of a sodium carbonate-soluble pectin from tomato show the presence of a mixed population of single chains and aggregates (Round et al., 1997). Somewhat unexpectedly, the AFM images revealed branched structures that have been interpreted as evidence that HG chains are covalently linked to one another or that the neutral-sugar side chains of pectin are considerably longer than previously believed (Round et al., 1997).

Single-molecule force spectroscopy is a technique that has been used to measure the mechanical properties of proteins (Leckband, 2000) and polysaccharides (Brant, 1999). A single polymer chain is tethered at one point to a fixed substrate and at another point to an AFM tip. The application of a stretching force elastically deforms the molecule and may also induce conformational transitions (Marszalek et al., 1998, 1999). The force-extension curves for citrus pectin show two reversible yield domains that correspond to abrupt increases in the length of the polysaccharide chain (Marszalek et al., 1999). The yield domains may result from the conversion of D-Gal*p*A residues in the $^4C_1$ chair to an intermediate boat/skew-boat conformer and then to the $^1C_4$ chair. Such conformational changes are suggested to result in a increase in chain length since the distance spanned by the $^1C_4$ ring conformer is larger than the $^4C_1$ ring conformer (Marszalek et al., 1999). The force-extension curves of cellulose contain no yield domains since the virtual bond length of the glucosyl residues is not increased by a change in ring conformation (Marszalek et al., 1999). Thus, differences in the extensibility of pectin and cellulose may allow plant cells to finely control the extensibility of their walls (Marszalek et al., 1998).

1.4.7. Are there pectin-membrane-cytoskeleton connections?

There is an increasing awareness that the mechanical and biochemical properties of the primary wall influence the growth and development of plant cells and tissues, although the mechanisms by which a cell perceives and responds to changes in wall properties are not understood (Roberts, 1990; Wyatt and Carpita, 1993). The limited available data suggest that the primary wall and the cytoskeleton are connected by membrane associated proteins, although the nature of the connecting proteins and the wall components they interact with are largely unidentified (Miller et al., 1997; He et al., 1996). In contrast, the role of the extracellular matrix in the growth and development of animal cells has been studied in detail (Chothia and Jones, 1997; Deller and Jones, 2000) and models have been proposed to explain how mechanical forces affect the behavior of animal cells (Ingber, 1998).

Homologs of animal cell adhesion molecules (integrins, proteoglycans, vitronectin, and fibronectin) have been detected in plant cell walls and membranes using antibodies and synthetic peptides (Miller et al., 1997). *A. thaliana* plasma membranes contain proteins with homology to animal β-integrins (Laval et al., 1999). Purified plasma membranes from *Arabidopsis* cells have also been reported to contain two types of high-affinity binding sites for the RGD (Arg–Gly–Asp) peptide, a sequence typically required for binding to integrin receptors (Canut et al., 1998). A heptapeptide containing the RGD motif has been reported to cause a loss of wall-membrane attachments (Hechtian threads) in plasmolyzed onion epidermal cells and suspension-cultured *arabidopsis* cells (Canut et al., 1998). However, it was not shown whether the effects of the RGD motif result from an interaction with the cell wall or plasma membrane. RGD peptides have been reported to enhance cell division in soybean (Schindler et al., 1989), to inhibit gravity perception in Chara (Wayne et al., 1992), and to disrupt protoplast adhesion in NaCl-adapted tobacco cells (Zhu et al., 1993), although the mechanisms of action remain unidentified.

Fibronectin-like molecules and wall-associated kinases have been identified in plants and these proteins may bind directly or indirectly to pectic polysaccharides (He et al., 1996; Miller et al., 1997). No direct evidence has been obtained to show that pectic polysaccharides are linked to the plasma membrane, although a narrow zone of the wall adjacent to the plasma membrane is often enriched with unesterified HG (Knox et al., 1990), RG-II (Williams et al., 1996; Matoh et al., 1998), β1→4 linked galactan and β1→6 linked galactans (Jauneau et al., 1998; Sorensen et al., 2000). Such labeling patterns must be interpreted with caution since the pectin located in the region of the wall adjacent to the plasma membrane is likely to be the most recently deposited wall material and may be more accessible to the antibodies than pectin in the rest of the wall.

1.5. The distribution of pectic polysaccharides in the primary wall

Antibodies that recognize specific structural features of primary cell wall pectin are powerful tools for determining the location and distribution of these polysaccharides within a single wall and for following changes in pectin structure during cell and tissue development (Knox, 1997; Vicre et al., 1998; Willats et al., 2000b). Antibodies have been described that are believed to recognize HG with different degrees of esterification including JIM 5 (VandenBosch et al., 1989; Knox et al., 1990); Jim 7 (Knox et al., 1990) and PAM1 (Willats et al., 1999a). Other anti-pectin antibodies are 2F4 that recognizes calcium cross-linked pectin (Liners et al. 1989), and LM5 and LM6 that recognize, respectively, 1→4-linked β-D-galactan (Jones et al., 1997) and 1→5-linked α-L-arabinan (Willats et al., 1998) in the side chains of RG-I. Another series of antibodies includes CCRC-M1 that recognizes polysaccharides containing a terminal non-reducing α-L-fucosyl residue (Puhlmann et al., 1994), CCRC-M2 that recognizes RG-I (Puhlmann et al., 1994), CCRC-M7 that recognizes arabinosylated 1→6-linked β-D-galactan (Steffan et al., 1995), and CCRC-R1 that recognizes RG-II (Williams et al., 1996).

The epitopes recognized by LM5 and LM6 are known with some certainty since these antibodies were generated against specific oligosaccharides coupled to proteins (Jones et al., 1997; Willats et al., 1998). The epitope recognized by CCRC-M1 has been shown, using a series of structurally related oligosaccharides, to contain a terminal 1→2 linked α-L-fucosyl residue (Puhlman et al., 1994). The binding characteristics of PAM1, JIM5, and JIM7 have been compared using pectin of varying degrees and patterns of methyl esterification (Willats et al., 1999a, 2000a). PAM1 binds most effectively to fully de-esterified pectin whereas JIM5 and JIM7 bind to pectin with a wide range of degrees of methyl esterification. Some published studies, however, have been interpreted based on the assumption that JIM5 recognizes deesterified HG and JIM7 recognizes methylesterified HG. Thus, the results of immunolocalization studies using JIM5 and JIM7 must be interpreted with caution (Willats et al., 2000a).

Immunolocalization studies have provided evidence that the localization and structure of pectin may have an impact on plant cell growth and development. For example, the presence of a pectin sheath on the primary walls of developing cotton fibers is correlated with the ability of epidermal cells to differentiate into elongating fiber cells (Vaughn and Turley, 1999), while epidermal cells that do not have a pectin sheath do not elongate. The role of the external pectin-rich layer in cell elongation, however, remains to be determined since epidermal cells in maize coleoptiles (Schindler et al., 1995), flax (Jauneau et al., 1997), and pea stems (Fujino and Itoh, 1998) have an asymmetric distribution of pectin but do not elongate.

Differences in the distribution of pectic epitopes in different cell and tissue types have also been reported in pea cotyledons (McCartney et al., 2000), tomato pericarp (Roy, 1994; Steele et al., 1997; Orfila and Knox, 2000); flax roots and hypocotyls (Jauneau et al., 1997, 1998), potato tubers (Bush and McCann, 1999), Arabidopsis roots (Freshour et al., 1996; Dolan et al., 1997), aspen cambium (Ermel et al., 2000), poplar cambium (Guglielmino, 1997), pollen (Geitmann et al., 1995; Juah and Lord, 1996), *Zinnia* cells (Stacey et al., 1995), suspension-cultured cells (McCann et al., 1994; Williams et al., 1996; Matoh et al., 1998), and cauliflower stems (Femenia et al., 1998). The results of these studies, when taken together, lead to the general conclusion that unesterified HG is localized to the middle lamella, to cell corners, and around air spaces whereas esterified HG is typically present throughout the wall. The immunocytochemistry results also suggest that the presence and location of the arabinan and galactan side chains of RG-I are often correlated with stages of cell and/or tissue development. The appearance of 1→4-linked β-D-galactan and a decrease of 1→5-linked α-L-arabinan in carrot cell walls has been correlated with the transition from cell division to cell elongation (Willats et al., 1999b). The α1,5-arabinan epitopes are also expressed specifically in the central meristem of carrot roots and in proliferating carrot suspension-cultured cells, while the 1,4-galactan epitopes are not expressed in these cells but rather in the root cap and differentiating stele and cortical cells of the carrot root and in non-proliferating induced carrot suspension cultures (Willats et al., 1999a,b). The expression of the arabinan and galactan side chains of RG-I also appears to be developmentally regulated in other plants and tissues. HG and α1,5-arabinan epitopes are present in the pea cotyledon throughout development while the β1,4-galactan-epitope is restricted to a specific stage of cotyledon development

(26–30 days after anthesis) and is present in a thin layer at the plasma membrane face of the cell wall (McCartney et al., 2000). The HG, α1,5-arabinan and β1,4-galactan epitopes in the wall surrounding pit fields in the tomato pericarp are also spatially regulated, with HG present in the pit fields, α1,5-arabinan surrounding the pit fields, and β1,4-galactan absent from the pit field (Orfila and Knox, 2000).

The results of immunocytochemical studies showing the differential labeling of a particular wall epitope may suggest that the synthesis of the epitope is regulated, however, additional experiments are required to determine if the lack of binding of an antibody to a tissue section is due to the absence of the epitope or to the masking of an epitope. For example, Masuda et al. (1984) reported that in elongating carrot walls the ratio of 3,5- to 5-linked arabinosyl residues increased. Thus, increased branching of the 1,5-linked arabinan may account for the reduced labeling of elongating cell walls by the 1→5-linked α-L-arabinan antibody (Willats et al., 1999b). Evidence that the antibody labeling pattern of the tissues is due to the differential expression of arabinan, rather than to the masking of the epitope(s) recognized by the antibody, is not easily obtained by independent methods since it is difficult to excise discrete cell populations and prepare their walls in amounts sufficient for conventional chemical or biochemical analyses.

Another example of a differentially labeled cell wall epitope is an RG-II epitope recognized by polyclonal anti-RG-II antibodies (Matoh et al., 1998). The RG-II epitope is present in dicot and monocot walls, but is not detected in the middle lamella (Matoh et al., 1998). This epitope, however, may be masked by a base-labile component since alkali treatment of the walls typically results in a dramatic increase in the density of labeling (Williams et al., 1996; Matoh et al., 1998). This example again illustrates that care must be taken in interpreting the results of immunolabeling studies. Furthermore, it is difficult to determine whether the changes in the ability of specific antibodies to bind to tissue sections results from a change in the glycosyl residue composition of the wall or to changes in the physical properties of the wall (e.g. permeability). Additional antibodies with well defined epitopes and methods to structurally analyze the walls surrounding single cells are required to address this issue.

The differential expression of a wall carbohydrate epitope may, or may not, indicate that the specific epitope plays a role in plant growth or development. For example, despite the differential expression of the β-D-galactan epitope, the role of 1→4-linked β-D-galactan in plant growth is unclear since the walls of potato tubers transformed with a fungal endo-1,4-β-D-galactanase contain ~30% of the normal amount of galactose and yet the plants have no apparent change in phenotype (Sorensen et al., 2000).

1.6. Probing the structure and function of pectin with cell wall mutants

Our knowledge of the primary structure of wall pectin, its distribution within the wall, and some of its interactions with itself and other wall components has increased dramatically over the last 10 years. In contrast, our understanding of the biological functions of pectic polysaccharides remains inadequate. The increasing availability of cell wall mutants provides new opportunities to study pectin structure and function (Fagard et al., 2000).

Many of the cell wall mutants currently available have been generated using Arabidopsis, although only a limited number of them have been shown to have altered pectin structure or metabolism (Fagard et al., 2000). Arabidopsis mutants (*Mur1–Mur11*) have been described that have reduced levels of Fuc, Ara, Gal, or Rha in their walls (Reiter et al., 1997). These glycosyl residues are typically present in RG-I and RG-II, although Fuc, Ara, and Gal are also components of xyloglucan and arabinogalactan proteins. Thus, some of these mutants are likely to have structural changes in more than one wall component.

Mur1 and *Mur4* have each been shown to be defective in a single gene that is required for the biosynthesis of a specific monosaccharide. For example, the aerial portions of *Mur1* plants are defective in an isoform of GDP-mannose-4,6-dehydratase and thus their walls contain ~5% of the wild type fucose (Bonin et al., 1997). The reduced fucose content is reflected in the almost total absence of fucosyl residues in *Mur1* xyloglucan (Zablackis et al., 1996) and RG-II (Reuhs et al., submitted). Somewhat unexpectedly, ~30% of the β-D-galactosyl residues that would normally be substituted with a L-fucosyl residue are substituted with an α-L-galactosyl residue (Zablackis et al., 1996). Similarly, the 2-*O*-Me α-L-fucosyl and α-L-fucosyl residues in *Mur1* RG-II are replaced with terminal 2-*O*-Me α-L-galactosyl and α-L-galactosyl residues (Reuhs et al., submitted). L-galactosyl residues are believed to be generated from GDP-mannose by GDP-mannose-3,5-epimerase, a enzyme required for the biosynthesis of vitamin C (Wheeler et al. 1998). *Mur4* plants are defective in a membrane-bound UDP-D-xylose 4-epimerase and thus have reduced levels of arabinose in their walls (Burget and Reiter, 1999). *Mur4* plants have no visible phenotype, although the double mutant *Mur4*/*Mur1* has been reported to have an extreme dwarf phenotype (Reiter et al., 1997). The walls of *Mur4*/*Mur1* plants are likely to contain structurally modified RG-II, xyloglucan, and possibly RG-I, although no structural analysis of the individual polysaccharides has been reported.

Mutant plant populations are generated by random mutagenesis of seeds with ethylmethanesulfonate or through T-DNA or transposon-mutagenesis. Typically,

this results in many thousands of M2 lines that must be screened. The identification of wall mutants is labor-intensive since no rapid screening procedures are currently available, although sample throughput may be increased using FTIR spectroscopy (Chen et al., 1998) and immunocytochemical methods (Willats et al., 2000b). Thus, more direct methods for generating transgenic plants with altered pectin structure and/or metabolism are now being exploited.

Antisense mRNA technology has been used to investigate the role of EPGase in the ripening and softening of tomato fruit. Transgenic tomato fruits containing $<1\%$ of the normal EPGase activity ripen normally showing that EPGases are not responsible for ripening-associated softening (Tieman and Handa, 1994). Nevertheless, these fruits have improved storage characteristics since tissue integrity is maintained during fruit senescence. Normal ripening also occurs in transgenic tomato plants with reduced PME activity but there is a significant decrease in tissue integrity during fruit senescence, and the fruits are more susceptible to microbial attack (Tieman and Handa, 1994). Antisense mRNA has also been used to show that in pea root tips the PME-catalyzed de-esterification of pectin has a role in border cell release and in root growth (Wen et al., 1999).

An alternative approach to antisense mRNA approaches to modify the wall is to transform plants with glycanases that are known to fragment cell wall polysaccharides. For example, the walls of transgenic potato tubers expressing a fungal endo-β1,4-galactanase contain reduced amounts of galactose, although plant growth is not affected (Sorensen et al., 2000).

Virus-induced gene silencing of a putative cellulose synthase gene has been used to generate transiently dwarf tobacco plants (Burton et al., 2000). The walls of the infected leaves have a reduced cellulose content, the HG content is increased, and the HG is reported to be less esterified than the HG in the walls of control plants. Pectin also accounts for a greater proportion of the walls of plant cells grown in the presence of the cellulose synthesis inhibitor dichlorobenzonitrile (Shedletzky et al., 1992). Thus, a plant may compensate for reduced cellulose production by increasing the amount of HG in its wall (Burton et al., 2000).

Several Arabidopsis mutants that may yield insights into pectin function have been generated in studies not directly related to primary cell walls. For example, transgenic arabidopsis plants expressing a fungal cutinase exhibit postgenital organ fusion in epidermal tissue that lacks an intact cuticle. These fusions are due in part to pectin-mediated cell-cell adhesion and have been observed in the leaves, stems, and flowers but not in the roots (Sieber et al., 2000). Fusions occur in epidermal cells only early in organ development when the organs are close together and often results in the formation of cell bridges (Sieber et al., 2000). The material in the junctions of the fusions and cell bridges is rich in pectin with low and high degrees of esterification. Thus, in the absence of an intact cuticle, the walls of closely appressed epidermal cells partially fuse by co-polymerization of pectin (Sieber et al., 2000). A second example of abnormal pectin-mediated cell-cell adhesion has been observed in the *Qrt* mutant of arabidopsis. In wild type plants, the pollen mother cell wall consists of an inner callose layer and an outer pectin-rich layer. These layers are both removed during pollen maturation and the four microspores separate before deposition of the exine. Callose is fragmented normally in *Qrt* mutants but the pectin layer persists and the microspores remain attached to one another. The exine is then deposited simultaneously on all four microspores. The nature of the QRT gene product is not known but it is likely to have a role in pectin metabolism (Rhee and Somerville, 1998).

A vitamin C deficient mutant (*Vtc1*) has recently been shown to be partially defective in GDP-mannose pyrophosphorylase (Conklin et al., 1999). This mutation may affect the fucosyl, mannosyl, and L-galactosyl contents of the wall since these glycosyl residues are derived from GDP-mannose (Conklin et al., 1999). The arabidopsis mutant *Cyt1-2* is believed to contain a completely defective GDP-mannose pyrophosphorylase (Conklin et al., 1999). *Cyt1-2* is an embryo-lethal mutant with severely deformed walls, although it has not been shown that the wall defects themselves are directly responsible for the observed phenotype.

The identification of cell wall-related genes in arabidopsis via sequence similarities to *Acetobacter xylinum* and *Agrobacterium tumefaciens* genes involved in cellulose synthesis has met with considerable success (Delmer, 1999; Pear et al., 1996). This approach also has the potential to identify genes involved in the biosynthesis of pectic polysaccharides (Bonin and Reiter, 2000). No bacteria are known that synthesize HG, RG-I, or RG-II. Nevertheless, the glycosyl residues present in pectin are, with the exception of apiose and aceric acid, also present in various bacterial polysaccharides. For example, a 4-linked α-D-Gal*p*A residue and a 2-linked α-L-Rha*p* are present in the hexasaccharide repeating unit of the extracellular polysaccharide synthesized by *Erwinia chrysanthemi* A350 (Gray et al., 2000). The ability to selectively mutate genes involved in cell wall formation and metabolism clearly presents new opportunities for determining the function of pectic polysaccharides in plant growth and development.

2. Biosynthesis

2.1. Introduction

The study of pectin biosynthesis is challenging and progress in this area has been slow. The complex structure

of pectin requires the action of at least 53 different enzymatic activities (Mohnen, 1999). Some of the nucleotide sugar substrates for the pectin biosynthetic enzymes are unknown, most are not commercially available, and the oligo/polysaccharide acceptors required to assay the enzymes must be generated by chemical or enzymatic cleavage of pectin and then purified. In order to understand how the activity of the 53 distinct enzymatic activities required for pectin synthesis (see Tables 1–3) are coordinated, and how that process is regulated, the pectin biosynthetic enzymes must be identified and the corresponding genes cloned. No pectin biosynthetic enzyme has yet been purified to homogeneity, and no pectin biosynthetic glycosyl-, methyl- or acetyltransferase has been cloned. Nevertheless, recent progress in the area of pectin biosynthesis, including the partial purification of some of the pectin biosynthetic enzymes, make it likely that the first genes for pectin biosynthetic enzymes will be identified in the near future. The published literature on pectin biosynthesis has recently been reviewed (Mohnen, 1999), thus only recent developments in our understanding of pectin biosynthesis will be presented here.

2.2. Setting the stage for pectin synthesis

The synthesis of pectin must be regulated in a temporally, spatially, and developmentally specific manner since the pectic polysaccharides are largely absent from many secondary walls while they show cell type and developmental expression in primary walls. A discussion of pectin synthesis must first address where synthesis occurs. Immunocytochemistry using antibodies directed against cell wall carbohydrate epitopes (Hoson, 1991; Moore et al., 1991; Knox, 1992; Staehelin and Moore, 1995) showed that HG and RG-I-like epitopes are present in both the *cis*- and medial-Golgi. Thus, it is likely that the synthesis of these epitopes begins in *cis* Golgi (Lynch and Staehelin, 1992; Zhang and Staehelin, 1992) and continues into the medial Golgi (Moore et al., 1991; Zhang and Staehelin, 1992; Staehelin and Moore, 1995). The esterification of HG appears to occur in the medial and trans Golgi (Vian and Roland, 1991; Liners and Van Cutsem, 1992; Zhang and Staehelin, 1992; Sherrier and VandenBosch, 1994; Staehelin and Moore, 1995), while more extensive branching of pectin occurs in the trans Golgi cisternae (Zhang and Staehelin, 1992; Staehelin and Moore, 1995). Final pectin assembly may occur as the Golgi vesicles are transported to the plasma membrane and the pectin is inserted into the wall, often as a highly methyl esterified polymer (Carpita and Gibeaut, 1993; Liners et al., 1994; Dolan et al., 1997). Some cell types, however, appear to secrete relatively unesterified pectin in the wall. For example, melon callus cells (Vian and Roland, 1991) and slime-secreting clover root epidermal cells (Lynch and Staehelin, 1992) contain unesterified HG in the trans Golgi. Also, unesterified HG epitopes have been found at the plasma membrane-cell wall interface by immunocytochemistry, suggesting that HG can also be inserted into the wall in a relatively unesterified form and that HG is not necessarily synthesized in a highly esterified form (Knox et al., 1990; Casero and Knox, 1995). The extent to which HG is esterified during synthesis thus remains an open question.

Table 1
List of glycosyltransferase activities required for homogalacturonan (HG) biosynthesis

Type of transferase	Working[a] number	Enzyme[b]		Ref.[c] for structure
		Acceptor substrate	Enzyme activity	
Glycosyl-				
D-GalAT	I	*GalAα1,4-GalA	*α1,4-GalAT*	(O'Neill et al., 1990)
D-GalAT	II[d]	GalAα1,2-L-Rha	*α1,4-GalAT*	–
D-XylT	I	GalAα1,4-GalA	*β1,3 XylT*	(Aspinall, 1980; Yu and Mort, 1996; Kikuchi et al., 1996; Schols et al., 1995)
Methyl-				
HG-methyltransferase (HG-MT)		$GalA\alpha1,4\text{-}GalA_{(n)}$		(Goubet et al., 1998; Vannier et al., 1992; Kauss and Hassid, 1967)
Acetyl-				
HG: GalA-3-*O*-acetyltransferase (HG-AT)		$GalA\alpha1,4\text{-}GalA_{(n)}$		(Ishii, 1997a,b; De Vries et al., 1986; Ishii, 1995; Rombouts and Thibault, 1986)

[a] Enzymes that transfer the same type of glycosyl residue to different acceptors are given numbers to facilitate identification of the specific glycosyltransferase activities. Note that the numbers are different from those in (Mohnen, 1999) since the glycosyltransferases have been grouped according to the type of pectic polysaccharide they synthesize.

[b] All sugars are D sugars and have pyranose rings unless otherwise indicated. Glycosyltranferases add to the glycosyl residue on the left* of the indicated acceptor.

[c] Refs. for the structure.

[d] Enzyme that may be required to make an RG-I/HG junction.

Table 2
List of glycosyltransferase activities required for RG-I biosynthesis

Type of glycosyl-transferase	Working[a] number	Parent polymer	Enzyme[b]		Ref.[c] for structure
			Acceptor substrate	Enzyme activity	
D-GalAT	I	RG-I	*L-Rhaα1,4-GalA	*α1,2-GalAT*	(O'Neill et al., 1990; Eda et al 1986; Lau et al., 1985)
D-GalAT	II[d]	RG-I/HG	GalAα1,2-L-Rha	*α1,4-GalAT*	–
L-RhaT	I	RG-I	GalAα1,2-L-Rha	α1,4-L-RhaT	(O'Neill et al.,1990; Eda et al., 1986; Lau et al, 1985)
L-RhaT	II[d]	HG/RG-I	GalAα1,4-GalA	*α1,4-L-RhaT*	–
D-GalT	I	RG-I	L-Rhaα1,4-GalA	*β1,4-GalT*	(O'Neill et al., 1990; Lau et al., 1987)
D-GalT	II	RG-I	Galβ1,4-Rha	*β1,4-GalT*	(O'Neill et al., 1990; Lau et al., 1987)
D-GalT	III	RG-I	Galβ1,4-Gal	*β1,4-GalT*	(Morita. 1965a,b; Aspinall et al., 1967; Stephen. 1983; Aspinall. 1980; O'Neill et al., 1990; Lau et al., 1987)
D-GalT	IV	RG-I	Galβ1,4-Gal	*β1,6-GalT*	(O'Neill et al., 1990; Lau et al., 1987)
D-GalT	V	RG-I/AGP[e]	Galβ1,3-Gal	*β1,3-GalT*	(Carpita and Gibeaut, 1993)
D-GalT	VI	RG-I/AGP	Galβ1,3-Gal	*β1,6-GalT*	(Carpita and Gibeaut, 1993)
D-GalT	VII	RG-I/AGP	Galβ1,6-Galβ1,3-Gal	*β1,6-GalT*	(Carpita and Gibeaut, 1993)
L-AraT	I	RG-I	Galβ1,4-Rha	*α1,3-l-ArafT*	(O'Neill et al., 1990; Lau et al., 1987)
L-AraT	II	RG-I	L-Ara*f*α1,3-Gal	*α1,2-L-ArafT*	(O'Neill et al., 1990; Lau et al., 1987)
L-AraT	III	RG-I	L-Ara*f*α1,2-Ara	*1,5-L-ArafT*	(O'Neill et al., 1990; Lau et al., 1987)
L-AraT	IV	RG-I	L-Rhaα1,4-GalA	*ArafT*	(Lau et al., 1987)
L-AraT	V	RG-I	L-Ara*f*α1,5-Araf	*α1,5-L-ArafT*	(Carpita and Gibeaut, 1993)
L-AraT	VI	RG-I	L-Araα1,5-Araf	*α1,2-L-ArafT*	(Carpita and Gibeaut, 1993)
L-AraT	VII	RG-I	L-Ara*f*α1,5-Araf	*α1,3-L-ArafT*	(Carpita and Gibeaut, 1993)
L-AraT	VIII	RG-I	L-Ara*f*α1,3-Araf	*α1,3-L-ArafT*	(Carpita and Gibeaut, 1993)
L-AraT	IX	RG-I	Galβ1,4-Gal	*α1,3-L-ArafT*	(Morita, 1965a,b; Aspinall, et al., 1967; Stephen, 1983; Aspinall, 1980; O'Neill et al., 1990; Carpita and Gibeaut, 1993)
L-AraT	X	RG-I	L-Ara*f*-1,3-Gal	*1,5-L-ArafT*	(Morita, 1965a,b; Aspinall et al., 1967b; Stephen, 1983b; Aspinall, 1980)
L-AraT	XI	RG-I/AGP	Galβ1,6-Gal	*α1,3-L-ArafT*	(Carpita and Gibeaut, 1993)
L-AraT	XII	RG-I/AGP	Galβ1,6-Gal	*α1,6-L-ArafT*	(Carpita and Gibeaut, 1993)
L-FucT	I	RG-I	Galβ1,4-Gal	*α1,2-L-FucT*	(O'Neill et al., 1990; Lau et al., 1987)
D-GlcAT	I	RG-I	Gal....	*β1,6GlcAT*	(An et al., 1994)
D-GlcAT	II	RG-I	Gal....	*β1,4GlcAT*	(An et al., 1994)
Methyl- RG-I: GlcA-4-*O*-methyltransferase	–	RG-I	GlcAβ1,6-Gal		(An et al., 1994)
Acetyl- RG-I: GalA-3-O/2-*O*-acetyltransferase	–	RG-I	GalAα1,2-L-Rhaα1,4$_{(n)}$		(Lerouge et al., 1993; Ishii, 1997a; O'Neill et al., 1990; Bacic et al., 1988; Komalavilas and Mort, 1989)

[a] Enzymes that transfer the same type of glycosyl residue to different acceptors are given numbers to facilitate identification of the specific glycosyltransferase activities. Note that the numbers are different from those in (Mohnen, 1999) since the glycosyltransferases have been grouped according to the type of pectic polysaccharide they synthesize.

[b] All sugars are D sugars and have pyranose rings unless otherwise indicated. Glycosyltranferases add to the glycosyl residue on the left* of the indicated acceptor.

[c] Refs. for the structure.

[d] Enzyme that may be required to make an HG/RG-I junction.

[e] This enzymatic activity would also be required to synthesize arabinogalactan proteins (AGPs).

Data from biochemical and structural studies of the pectic polysaccharides suggest that HG and RG-II are covalently linked in the wall. It is likely that HG serves as the backbone for RG-II synthesis. It is also possible that HG and RG-I are linked in the wall, however, definitive chemical evidence for that linkage has not been reported.

2.3. Nucleotide sugars, transporters, and a general model for the topology of pectin biosynthesis

The pectin biosynthetic glycosyltranferases studied to date all use nucleotide-sugars as substrates for in vitro enzymatic reactions and it is believed that nucleotide sugars are the immediate substrates for the reactions in

Table 3
List of glycosyltransferase activities required for RG-II biosynthesis

Type of glycosyltransferase	Working[a] number	Parent polymer	Enzyme[b]		Ref.[c] for structure
			Acceptor substrate	Enzyme activity	
D-GalAT	I	HG/RG-II[d]	*GalAα1,4-GalA	*α1,4-GalAT*	(O'Neill et al., 1990)
D-GalAT	II	RG-II	L-Rhaβ1,3′-Apif	*α1,2-GalAT*	(O'Neill et al., 1997; Carpita and Gibeaut, 1993)
D-GalAT	III	RG-II	L-Rhaβ1,3′-Apif	*β1,3GalAT*	(O'Neill et al., 1997; Carpita and Gibeaut, 1993)
L-RhaT	I	RG-II	Apifβ1,2-GalA	*β1,3′-L-RhaT*	(O'Neill et al., 1997; Carpita and Gibeaut, 1993)
L-RhaT	II	RG-II	Kdo2,3GalA	*α1,5-L-RhaT*	(O'Neill et al., 1997; Carpita and Gibeaut, 1993)
L-RhaT	III	RG-II	L-Arapα1,4-Gal	*α1,2- L-RhaT*	(O'Neill et al., 1997; Carpita and Gibeaut, 1993)
L-RhaT	IV	RG-II	L-Arapα1,4-Gal	*β1,3- L-RhaT*	
L-GalT	I	RG-II	GlcAβ1,2-Fuc	*α1,2-L-GalT*	(Reuhs et al., submitted; O'Neill et al., 1997; Carpita and Gibeaut, 1993)
D-GalT	II	RG-II	L-Ace*f*Aα1,3-Rha	*β1,2GalT*	(Vidal et al., 2000; O'Neill et al., 1997; Carpita and Gibeaut, 1993)
L-AraT	I	RG-II	Dha2,3-GalA	*β1,5-L-ArafT*	(O'Neill et al., 1997; Carpita and Gibeaut, 1993)
L-AraT	II	RG-II	Galβ1,2- L-Ace*f*A	*α1,4-L-ArapT*	(Vidal et al., 2000; O'Neill et al., 1997; Carpita and Gibeaut, 1993)
L-AraT	III	RG-II	L-Rhaα1,2-L-Ara	*β1,2- L-ArafT*	(O'Neill et al., 1997; Carpita and Gibeaut, 1993)
L-FucT	I	RG-II	L-Rhaβ1,3′-Api*f*	*α1,4-L-FucT*	(O'Neill et al., 1997; Carpita and Gibeaut, 1993)
L-FucT	II	RG-II	Galβ1,2-L-Ace*A*f	*α1,2-L-FucT*	(Vidal et al., 2000; O'Neill et al., 1997; Carpita and Gibeaut, 1993)
D-ApifT	I	RG-II	GalAα1,4-GalA	β1,2-Api*f*T	(O'Neill et al., 1997; Carpita and Gibeaut, 1993)
D-XylT	I	RG-II	L-Fucα1,4-L-Rha	*α1,3XylT*	(O'Neill et al., 1997; Carpita and Gibeaut, 1993)
D-GlcAT	III	RG-II	L-Fucα1,4-L-Rha	*β1,4GlcAT*	(O'Neill et al., 1997; Carpita and Gibeaut, 1993)
D-KdoT	I	RG-II	GalAα1,4-GalA	*2,3KdoT*	(O'Neill et al., 1997; Carpita and Gibeaut, 1993)
D-DhaT	I	RG-II	GalAα1,4-GalA	*2,3DhaT*	(O'Neill et al., 1997; Carpita and Gibeaut, 1993)
L-AcefA	I	RG-II	L-Rhaβ1,3′-Api*f*	*α1,3AceAfT*	(Vidal et al., 2000; O'Neill et al., 1997; Carpita and Gibeaut, 1993)
Methyl-					
RG-II: xylose-2-*O*-methyltransferase	–	RG-II	D-Xylα1,3-L-Fuc		(O'Neill et al., 1997; Carpita and Gibeaut, 1993)
RG-II: fucose-2-*O*-methyltransferase	–	RG-II	L-Fucα1,2-D-Gal		(O'Neill et al., 1997; Carpita and Gibeaut, 1993)
Acetyl-					
RG-II: fucose-acetyltransferase	–	RG-II	L-Fucα1,2-D-Gal		(O'Neill et al., 1997; Carpita and Gibeaut, 1993)
RG-II: aceric acid-3-*O*-acetyltransferase	–	RG-II	L-Ace*f*Aα1,3-L-Rha		(Vidal et al., 2000; O'Neill et al., 1997; Carpita and Gibeaut, 1993)

[a] Enzymes that transfer the same type of glycosyl residue to different acceptors are given numbers to facilitate identification of the specific glycosyltransferase activities. Note that the numbers are different from those in (Mohnen, 1999) since the glycosyltransferases have been grouped according to the type of pectic polysaccharide they synthesize.

[b] All sugars are D sugars and have pyranose rings unless otherwise indicated. Glycosyltranferases add to the glycosyl residue on the left* of the indicated acceptor.

[c] Refs. for the structure.

[d] GalAT-I may be the same as GalAT-I for HG synthesis if RG-II is synthesized onto an existing HG backbone.

vivo (Mohnen, 1999). The nucleotide sugars required for pectin biosynthesis and progress towards understanding the biochemical pathways by which they are synthesized has recently been reviewed (Mohnen, 1999). The nucleotide sugars are, for the most part (see Hayashi et al., 1988, for a possible exception) believed to be synthesized on the cytosolic side of the Golgi. However, the transferase reactions that catalyze the synthesis and modification of the polysaccharide backbone and side chains are believed to occur in the Golgi. Thus, mechanisms are required to transport the nucleotide sugars across the Golgi membrane. Data from animal systems (Capasso and Hirschberg, 1984) and plants (Munoz et al., 1996; Wang et al., 1997; Neckelmann and Orellana, 1998) suggest that this transport is achieved by nucleotide-sugar:nucleoside monophosphate antiporters. The availability of nucleotide-sugars differentially labeled on both the nucleotide and sugar moieties will facilitate the study of Golgi-localized transporters involved in pectin biosynthesis (Orellana and Mohnen, 1999).

A working model for the topology of pectin synthesis is shown in Fig. 5. It is proposed that the nucleotide-sugars are synthesized on the cytosolic side of the Golgi

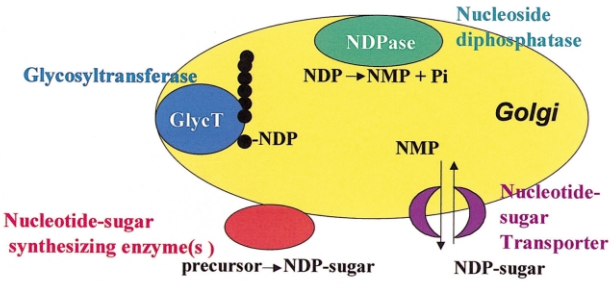

Fig. 5. A model for pectin biosynthesis. The model predicts that pectin biosynthesis is dependent upon the formation of the required nucleotide-sugars by nucleotide-sugar transformation reactions that occur on the cytosyolic side of the Golgi. The nucleotide-sugars are transported into the Golgi by specific nucleotide-sugar:nucleoside monophosphate antiporters. Once inside the Golgi, the nucleotide-sugar is used as a substrate by glycosyltransferases that are specific for the type of nucleotide sugar, the type of oligo/polysaccharide acceptor, and the nature of the anomeric configuration and linkage of the glycosyl linkage formed. The released nucleoside monophosphate is hydrolyzed by a Golgi-localized nucleoside diphosphatase and the nucleoside monophosphate and the inorganic phosphate are transported out of the Golgi by separate transporters (Munoz et al., 1996; Wang et al., 1997; Neckelmann and Orellana, 1998; Sterling et al., submitted).

and transported into the Golgi lumen by specific nucleotide-sugar:nucleoside monophosphate antiporters. The nucleotide-sugar is used as a substrate by the glycosyltransferase and the glycosyl residue is transferred onto a growing polysaccharide chain. The released nucleoside diphosphate (NDP) is hydrolyzed by a Golgi-localized nucleoside-5′-diphosphatase (Orellana et al., 1997) into NMP and inorganic phosphate, making available the nucleoside monophosphate for the nucleotide-sugar:nucleoside monophosphate antiporter. The regulation of pectin biosynthesis could occur during the formation of the nucleotide-sugar, the transport of the nucleotide-sugar into the Golgi, or at the level of the glycosyltransferase or other polymer modifying enzymes. One challenge for the next decade is to identify the molecular components required for each step of pectin biosynthesis. Many questions need to be addressed including: Does the proposed model hold for all of the glycosyl residues found in pectin? Are nucleotide-sugars the immediate glycosyl donors for all the pectin biosynthetic glycosyltransferases or are lipid-linked donors involved? Are all the required nucleotide-sugars made outside the Golgi, or are some made in the Golgi as has been suggested for UDP-Xyl (Hayashi et al., 1988)? Do the levels of nucleotide-sugars in the Golgi fluctuate and, if so, does this level regulate the relative activity of different types of glycosyltransferases? What is the organization of the different glycosyltransferases in the Golgi? How is this organization made and maintained? How is the inorganic phosphate generated by the nucleoside diphosphatase transported out of the Golgi? Does the transport of phosphate establish or maintain an ion gradient in the Golgi that is required for pectin synthesis?

2.4. Synthesis of homogalacturonan

Most effort towards understanding pectin biosynthesis has been directed towards two enzymes believed to be involved in the synthesis of homogalacturonan (HG) in planta: a homogalacturonan-α1,4galacturonosyltransferase (also referred to as polygalacturonate:α1,4-galacturonosyltranferase) and homogalacturonan-methyltransferase (also referred to as pectin methyltransferase) (see Table 1).

2.4.1. α1,4Galacturonosyltransferase (GalAT)

A membrane-bound α1,4galacturonosyltransferase (GalAT) has previously been identified and partially characterized in mung bean (Kauss and Swanson. 1969; Villemez et al., 1966), tomato (Lin et al., 1966), turnip (Lin et al., 1966), and sycamore (Bolwell et al., 1985), and more recently in suspension cultured tobacco cells (Doong et al., 1995), radish roots (H.F. Quigley, and D. Mohnen, unpublished results), enriched Golgi from elongating pea epicotyls (Sterling et al., submitted) and arabidopsis (J. Sterling and D. Mohnen, unpublished results). The GalAT from tobacco has been most extensively characterized. GalAT adds [^{14}C]GalA from UDP-[^{14}C]GalA (Liljebjelke et al., 1995) onto endogenous acceptors in membrane preparations to produce a radiolabeled product of large M_r (i.e. ~105 kDa in tobacco microsomal membranes (Doong et al., 1995) and ⩾500 kDa in pea Golgi (Sterling et al., submitted). The cleavage of up to 89% of the radiolabeled tobacco product into GalA, diGalA and triGalA upon exhaustive hydrolysis with a purified endopolygalacturonase confirmed that the product synthesized was largely HG. Since approximately 90% of the GalA in the primary wall is present in HG (Mohnen et al., 1996), these results suggest that the identified activity may be the GalAT responsible for the elongation of HG. The enzyme was thus named polygalacturonate α-4-galacturonosyltransferase (EC 2.4.1.43) (PGA-GalAT) in accordance with the nomenclature for a comparable enzyme activity in mung bean microsomes (Villemez et al., 1965, 1966; Lin et al., 1966; Kauss and Swanson, 1969). The product synthesized in vitro in tobacco microsomes is ~ 50% esterified and at least 40% of the ester is a C-6 methylester (Doong et al., 1995). These results are consistent with a model of pectin biosynthesis in which the newly synthesized HG is directly, or somewhat belatedly, esterified during synthesis.

Fractionated membranes from pea were recently used to localize GalAT to the Golgi and to demonstrate that the catalytic site of GalAT is on the lumenal side of the Golgi (Sterling et al., submitted). These results provide the first direct enzymatic evidence that a pectin biosynthetic glycosyltransferase (i.e. GalAT) resides in the Golgi.

GalAT was solubilized from membranes and shown to add GalA onto the non-reducing end (Scheller et al.,

1999) of exogenous HG acceptors (oligogalacturonides; OGAs) of a degree of polymerization $\geqslant 10$ (Doong and Mohnen, 1998). Surprisingly, the bulk of the HG is elongated by a single GalA residue in vitro using either solubilized GalAT from tobacco membranes (Doong and Mohnen, 1998) or detergent-permeabilized Golgi from pea (Sterling et al., submitted). Thus, the solubilized GalAT does not express the processivity expected for a polymer synthase. The lack of processivity of the solubilized GalAT may be due to the dissociation of an enzyme complex during solubilization, to the loss of a factor/activator required for processivity, or to the absence of a required substrate(s). Alternatively, the GalAT may not be a processive GalAT but rather a GalAT involved in a priming reaction for HG or in some other pectin biosynthetic reaction. One question that needs to be resolved is: How many contiguous GalA residues are added onto the endogenous acceptor in Golgi or microsomal preparations? In other words, how processive is the membrane-bound GalAT?

The possibility that an acceptor other than oligogalacturonides (OGAs) of DP 10-23 is required to convert the solubilized GalAT into a processive enzyme has been investigated. Specific oligosaccharide acceptor structures have been shown to be required for the processivity of other types of glycosyltransferases (Kojima et al., 1996). For example, a mammalian α2,8-polysialic acid synthase acts processively only when its protein acceptor has a specific α-1,6-linked fucose in its N-linked glycans (Kojima et al., 1996). In this case only one, or a few, sialic acid residues are added to structurally related acceptors without the α-1,6-linked fucose. Studies with GalAT suggest that neither polygalacturonic acid nor pectins of various degrees of esterification (31–90%) stimulate GalAT activity above the rate achieved with OGAs (R.L. Doong and D. Mohnen, unpublished results). GalAT activity is also not stimulated by GalA, diGalA or triGalA as individual acceptors. A question that must still be addressed is: Is endogenous HG in Golgi and in microsomal membranes a better substrate to promote processivity of GalAT in vitro than HG oligomers or commercially available PGA or pectin?

The possibility that high (>1 mM) concentrations of the substrate UDP-GalA might convert solubilized GalAT into a processive mode of action has also been tested. An effect of substrate concentration on the structure of a polymer synthesized by a glycosyltransferase has been reported for the in vitro synthesis of (1→3),(1→4) β-glucan in which the ratio of cellotriosyl to cellotetraosyl sequences increases from 1.5 to 11 as the concentration of UDP-Glc is increased from 5 μM to 30 mM (Buckeridge et al., 1999). The results of preliminary studies with GalAT, however, suggest that high concentrations (>1 mM) of UDP-GalA do not convert in vitro GalAT into a processive mode of action (H.F. Quigley and D. Mohnen, unpublished results).

HG isolated from the wall is partially methyl esterified and acetylated. Thus, it is possible that processivity of GalAT requires the simultaneous action of GalAT, HG-methyltransferase and/or HG-acetyltransferase. The in vitro synthesis of HG by GalAT in microsomal membranes, however, is not stimulated by the methyl donor S-adenosylmethionine (Kauss and Swanson, 1969; Doong et al., 1995) and/or the acetyl donor acetylCoA (D. Mohnen, unpublished results). Thus, no evidence has been obtained to support the idea that in vitro synthesis of HG by membrane bound GalAT requires concurrent methylation and/or acetylation of HG.

An obstacle to the study of GalAT is the time and expense required to synthesize UDP-[^{14}C]GalA. Radiolabeled UDP-GalA can be produced by the enzymatic epimerization of UDP-[^{14}C]GlcA to UDP-[^{14}C]GalA (Liljebjelke et al., 1995). Recently, a procedure for the one step production of UDP-[^{14}C]GalA has been reported (Basu et al., 2000). The procedure is based on the enzymatic oxidation of UDP-[^{14}C]Gal to UDP-[^{14}C]GalA (Rao and Mendicino, 1976; Kelleher and Bhavanandan, 1986). Basu et al. (2000) report a yield of $>90\%$ UDP-[^{14}C]GalA using a polyethyleneimine (PEI-cellulose) column chromatography purification step. We have found, using a slightly modified verison of this procedure, that it is necessary to purify the synthesized UDP-[^{14}C]GalA by HPLC in order to remove contaminants that inhibit GalAT activity. Under these conditions our final yield of UDP-[^{14}C]GalA was 32% (J. Sterling, and D. Mohnen, unpublished results). Further work is required to establish whether the epimerization procedure (approximately 20% yield) or the oxidation procedure (Basu et al., 2000) will prove more useful for the routine synthesis of the radiolabeled UDP-GalA required for studies of pectin biosynthesis.

The purification of GalAT has not yet been reported. Progress towards understanding the role of GalAT in HG synthesis requires that the enzyme be purified and its gene identified.

2.4.2. HG-methyltransferase (HG-MT)

HG-methyltransferase (HG-MT) [the term HG-MT is used in preference to pectin methyltransferase to specify the enzyme that methylates HG rather than RG-I or RG-II] has been identified in microsomal membranes from mung bean (Kauss et al., 1967, 1969; Crombie and Reid, 1998), flax (Vannier et al., 1992; Schaumann et al., 1993), tobacco (Goubet et al., 1998) and soybean hypocotyls (Ishikawa et al., 2000) (see Table 4). The membrane-bound HG-MTs from flax (Bruyant-Vannier et al., 1996; Bourlard et al., 1997a) and from tobacco (Goubet and Mohnen, 1999a) have been solubilized in an active form and the flax enzyme(s) has been partially purified (Bruyant-Vannier et al., 1996). The HG-MT from detergent-treated etiolated soybean hypocotyls is significantly more active (1360 pmol mg^{-1} min^{-1}) than the HG-MT

Table 4
Comparison of catalytic constants and pH optima for HG-methyltransferases involved in, or potentially involved in, pectin biosynthesis[a]

Enzyme[b]	Plant source	Apparent K_m for SAM[c]	pH optimum	V_{max}[d]	Ref
HG-MT[b]	Mung bean	59 μM	6.6–7.0	2.7[e]	(Kauss and Hassid, 1967; Kauss et al., 1969; Kauss et al., 1967)
PMT	Flax	10–30 μM	6.8	n.d.[g]	(Schaumann et al., 1993; Vannier et al., 1992)
PMT (sol)[f]	Flax	0.5 μM	7.1[h] or 5.5[i]	n.d.	(Bourlard et al., 1997a,b; Bruyant-Vannier et al., 1996)
HG-MT	Tobacco	38 μM	7.8	49	(Goubet et al., 1998)
HG-MT (sol)	Tobacco	18 μM	7.8	7.3	(Goubet and Mohnen, 1999a)
PMT-MT	Soybean	230 μM	6.8	1360	(Ishikawa et al., 2000)

[a] Unless indicated, all enzymes are measured in particulate preparations.
[b] All enzymes are thought to be HG-MT. However, since some authors use the previous name, pectin methyltransferase, it is included for clarity.
[c] Apparent K_m for *S*-adenosylmethionine.
[d] V_{max} in pmol min^{-1} mg^{-1} protein.
[e] The V_{max} is our calculation using data from Kauss et al. (1969).
[f] (sol): Detergent-solublized enzyme.
[g] n.d.: Not determined.
[h] From Bruyant-Vannier et al. (1996).
[i] From Bourlard et al. (1997a,b).

from mung bean, flax and tobacco (i.e. 3–49 pmol mg^{-1} min^{-1}, reviewed in Goubet et al., 1998). The apparent K_m of 0.23 mM for *S*-adenosylmethionine (SAM) reported for the soybean enzyme (Ishikawa et al., 2000) is much higher than the values reported for the other HG-MTs, 30–59 μM for membrane-bound HG-MTs (see Table 4 and Goubet et al., 1998) and 0.5–18 μM for solubilized HG-MT.

The low HG-MT activity recovered has hampered the purification of HG-MT or other pectin methyltransferases (Bourlard et al., 1997a). Thus, the increased activity recovered from soybean is noteworthy. The HG-MT from soybean (Ishikawa et al., 2000) was assayed by spotting the radiolabeled product onto paper, followed by paper chromatography to remove the unincorporated radiolabeled SAM. The product immobilized at the base of the paper chromatogram strip was analyzed by scintillation counting. Most other studies of HG-MT have employed a precipitation method to recover synthesized product. The precipitation method may not recover all of the methylated product (Pauly and Scheller, 2000). Alternatively, differences in the recovered HG-MT activity may depend on the plant species, on the amount and type of exogenous pectic acceptors used, or on whether intact membranes, detergent permeabilized membranes, or solubilized enzyme are used.

A question regarding the synthesis and methylation of HG is: How are the activities of GalAT and HG-MT coordinated during the synthesis of HG? The methylesterification of HG in membranes from mung bean (Kauss and Swanson, 1969) and tobacco (Goubet et al., 1998) is increased by the addition of UDP-GalA, a substrate for HG synthesis. The stimulation of HG-MT activity by UDP-GalA would be expected if either HG synthesis, or HG substrate, was required for endogenous methylation of HG. Evidence from studies with intact membranes in which UDP-GalA stimulates HG-MT activity (Kauss and Swanson, 1969; Goubet et al., 1998), and from studies using detergent-permeabilized membranes and solubilized HG-MT in which polygalacturonic acid or partially methylesterified pectin serve as acceptors of HG-MT, support the hypothesis that at least a small stretch of HG is synthesized prior to its methylation by HG-MT in the Golgi. However, the available results regarding the relationship between the action of GalAT and HG-MT need to be confirmed by reconstitution experiments using cloned and/or purified enzymes, and by using plants transgenically modified in the pectin biosynthetic genes.

Tobacco membrane-bound HG-MT has been localized to the Golgi and its catalytic site shown to face the Golgi lumen (Goubet and Mohnen, 1999b). These observations are consistent with earlier reports that mung bean HG-MT resides in a membrane compartment (Kauss and Swanson, 1969), and that a pectin methyltransferase from pea epicotyls is located in the Golgi (Baydoun et al., 1999). Similarly, about half of the pectin methyltransferase activity from flax co-fractionates with Golgi vesicles (Vannier et al., 1992; Bourlard et al., 1997b). The co-localization of the catalytic sites of GalAT and HG-MT in lumen of the Golgi support the notion that these enzymes act together, either sequentially or as a complex, during the synthesis of HG. Two questions that must be addressed are: How many different HG-MTs are required for HG synthesis?, and What is the substrate specificity of the HG-MT(s)? The fact that at least one HG-MT in detergent-permeabilized membranes from flax and soybean shows a preference for partially esterified pectin over polygalacturonic acid (Bourlard et al., 1997b; Ishikawa et al., 2000), suggests

that multiple HG-MTs may exist that either initiate the methylation of HG or enhance the further methylation of HG. Different HG-MTs may also exist that recognize other structural differences in HG (e.g. degree of acetylation or other modifications).

2.4.3. HG-acetyltransferase (HG-AT)

A pectin *O*-acetyltransferase activity has been identified in microsomes from suspension-cultured potato cells (Pauly and Scheller, 2000). Incubation of potato microsomes with [^{14}C]acetyl-CoA resulted in the recovery of a NaCl/ethanol precipitable product. Approximately 8% of the radiolabeled product was specifically solubilized by treatment EPGase and PME indicating that [^{14}C]acetate had been transferred either to HG, or to RG-II or RG-I that was covalently attached to HG. Further work is required to establish whether the described activity is an HG-AT or an acetyltransferase that acetylates one of the other pectic polysaccharides that is covalently linked to HG.

2.5. Synthesis of rhamnogalacturonan-I (RG-I)

2.5.1. RG-I-galactosyltransferase (RG-I-GalT)

Galactosyltransferase activity has been identified in particulate cell free homogenates from mung bean (McNab et al., 1968; Panayotatos and Villemez, 1973). It is possible that RG-I biosynthetic galactosyltransferases (see Table 2) were among those present in the homogenate since the synthesized product contained some β-1,4-galactan and a lesser amount of β-1,3-galactan (Panayotatos and Villemez, 1973). More recently, a β-1,4-galactosyltransferase activity with a pH of optimum of 6.5 has been identified in membrane preparations from mung bean hypocotyls based on sensitivity of the product to fragmentation by endo-β-1,4-galactanase (Brickell and Reid, 1996). Such results are consistent with an RG-I biosynthetic β-1,4-galactosyltransfserase in mung bean microsomal membranes. Galactosyltransferases have also been studied in particulate homogenates (Goubet and Morvan, 1993, 1994) and solubilized enzymes (Goubet, 1994) from flax. The chemical properties of some of the products synthesized in flax are consistent with those expected for GalTs involved in RG-I or arabinogalactan protein synthesis. However, glycosyl-linkage composition analysis of the synthesized product, or proof that the products are cleaved with RG-I-specific enzymes, is required to confirm this interpretation.

An RG-I:β-1,4-galactosyltransferase in suspension-cultured potato cells transfers [^{14}C]Gal from UDP-[^{14}C]Gal onto endogenous acceptor(s) in the membranes (Geshi et al., 2000). The synthesized product was fragmented by a purified endo-β-1,4-galactanase and rhamnogalacturonase A (an endohydrolase that cleaves the glycosidic linkage between the GalA and the Rha in the backbone of RG-I; Azadi et al., 1995), showing that the GalT catalyzed the transfer of galactose to RG-I. The β-1,4-galactosyltransferase had a pH optimum of 6.0–6.5 and required Mn^{2+} for maximum activity. The intact [^{14}C]Gal-labeled product synthesized in potato membranes had an estimated size of >500 kDa. Fragmentation of the intact product using endo-β-1,4-galactanase yielded [^{14}C]Gal and [^{14}C]galactobiose as the primary radiolabeled products. Cleavage of the intact and de-esterified (to remove methyl and acetyl groups from the RG-I backbone) product with rhamnogalacturonase A fragmented the product into material between 50 and 180 kDa in size. Subsequent treatment of the [^{14}C]Gal-labeled fragments with endo-β-1,4-galactanase yielded [^{14}C]Gal and slightly larger fragments that themselves could be cleaved to [^{14}C]Gal by a purified β-galactosidase. Taken together, these results support the conclusion that potato microsomal membranes contain enzymes that both initiate, and elongate, β-1,4-galactan side chains of RG-I.

2.5.2. RG-I-arabinosyltransferase

Arabinosyltransferases putatively involved in the synthesis of RG-I side chains have been recently reviewed (Mohnen, 1999). One advance in this area is the description of an improved procedure for the synthesis and purification of non-radiolabeled and radiolabeled UDP-β-L-arabinopyranose (Pauly et al., 2000). The UDP-Ara is produced by the enzymatic 4-epimerization of UDP-Xyl to UDP-Ara using a partially purified UDP-xylose-4-epimerase from wheat germ (Fan and Feingold, 1970; Feingold and Avigad, 1980). The UDP-Ara is purified by high performance anion exchange chromatography (HPAEC), a method that also allows quantitation of the product (Pauly et al., 2000).

2.5.3. RG-I-methyltransferase (RG-I-MT)

Detergent-solubilized pectin methyltransferase (PMT) from suspension-cultured flax cells uses an RG-I-enriched fraction from flax fibers as an exogenous acceptor (Bourlard et al., 1997a). In the presence of the RG-I fraction, methyltransferase activity was stimulated 1.5- to 1.7-fold above the endogenous acceptor activity and the radiolabeled product recovered had a size similar to the RG-I fraction. While these results suggest that an RG-I methyltransferase activity may have been identified, it has not yet been shown where in RG-I the methylation occurred. Methylation may have occurred on HG tails covalently linked to RG-I. Thus, the identified activity may result from methylesterification of HG rather than methylesterification of the galacturonic acid in the RG-I backbone. Alternatively, the methylation could have occurred on a non-galacturonic substituent. Some of the side chains of RG-I contain 4-*O*-methylGlcA (An et al., 1994). Further work is required to determine the site(s) of methylation and thus, the identity of the enzyme reported.

2.5.4. RG-I-acetyltransferase (RG-I-AT)

An RG-I acetyltransferase has been identified in microsomes from suspension-cultured potato cells (Pauly and Scheller, 2000). Incubation of potato microsomes with [^{14}C]acetyl-CoA resulted in the recovery of a NaCl/ethanol precipitable product that consisted of at least 19% acetylated RG-I based on the release of [^{14}C]acetate by treatment with a purified rhamnogalacturonan *O*-acetyl esterase (Pauly and Scheller, 2000). The product recovered from the microsomes had a mass >500 kDa and was partially fragmented by treatment with rhamnogalacturonan lyase (RGase B). An assay for RG-I-AT was developed based on specific solubilization of the product with RGase B. RG-I-AT has a pH optimum of 7.0 with 80% of activity retained from pH 6.5 to 8.0. The potato RG-I-AT has an apparent K_m for acetyl-CoA of 35 μM and an apparent V_{max} of 54 pmol min^{-1} mg^{-1} protein.

2.6. Synthesis of rhamnogalacturonan-II (RG-II)

2.6.1. RG-II-methyltransferase (RG-II-MT)

Detergent-solubilized pectin methyltransferase (PMT) from suspension-cultured flax cells is able to use RG-II isolated from wine as an exogenous acceptor (Bourlard et al., 1997a). In the presence of RG-II, the methyltransferase activity was stimulated 7-fold above the endogenous acceptor activity and the radiolabeled product had a size similar to RG-II monomers and RG-II dimers. While these results suggest that an RG-II-MT activity (see Table 3) may have been identified, it has not yet been shown where in RG-II the methylation occurred. Methylation of the HG "tails" covalently linked to RG-II, would represent methylesterification of HG rather than methylesterification of RG-II. Alternatively, the activity may represent methyletherification of side chain residues since RG-II side chains contain 2-*O*-methyl xylose and 2-*O*-methyl fucose (Darvill et al., 1978). Further research is required to determine the location of methylation and thus, the identity of the potentially novel enzyme activity.

2.6.2. Pressing needs in pectin biosynthesis research

Progress in understanding how pectin is synthesized requires that genes that encode the biosynthetic enzymes be identified. The genes are essential to generate the tools (e.g. antibodies against the biosynthetic enzymes and transgenic plants modified in pectin biosynthesis) needed to elucidate the structure and function of each enzyme and to determine how the enzymes work together to synthesize the structurally complex pectic polysaccharides. Furthermore, a study of the regulation of the pectin biosynthetic genes may indicate how the fine structure of pectin is altered during plant growth and development. The production of transgenic plants modified in the synthesis of pectin will allow the testing of hypotheses, the establishment of models of pectin biosynthesis, and the elucidation of the function(s) of pectin in the plant.

As noted above, the identification of pectin biosynthetic genes will undoubtedly come, in part, through the purification of the biosynthetic enzymes and the use of amino acid sequences to identify the genes in plant DNA databases. It is also likely that putative pectin mutants will be useful for identifying candidate genes, as will DNA sequence/motif similarity computer searches. Gene families for some of the hemicellulose biosynthetic enzymes such as xyloglucan:α1,2-fucosyltranferase (Perrin et al., 1999) and galactomannan: α1,6-galactosyltransferase (Edwards et al., 1999) have been identified. Some of the putative fucosyltransferase and galactosyltransferase genes may encode enzymes involved in RG-II and/or RG-I synthesis. Once the first genes for pectin biosynthetic enzymes are cloned, it is likely that web-based sequence analysis sites, such as the "carbohydrate active enzyme server (CAZY)" established by Bernard Henrissat (http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html), will be useful aids for the identification of genes for other pectin biosynthetic enzymes. For example, the section of the CAZY site devoted to glycosyltransferases currently contains 51 sequence-based families that are expected to have similar tertiary structure. Thus, once the first pectin galacturonosyltransferase gene (for example) is identified, such sequence/structure-based databases may allow the rapid identification of other putative pectin galacturonosyltransferases. The definitive identification of pectin biosynthetic genes, however, will require proof of enzymatic activity. Thus, one major area of need is the generation of the required nucleotide-sugar and oligo/polysaccharide substrates for the biosynthetic enzymes. This is a challenging problem since many of the chemical or enzymatic methods required to generate the specific fragments needed for use as acceptors for the enzymes are not available. Thus, progress in identifying pectin biosynthetic genes, and in studying the biosynthetic enzymes, will require the combined use of carbohydrate chemistry, biochemistry, molecular biology and genetics.

3. Biological activities of oligogalacturonides

3.1. Introduction

Biologically active carbohydrates that act as signal molecules are called oligosaccharins (reviewed by Côté and Hahn, 1994; Côté et al., 1998). Oligogalacturonides (OGAs), which are linear molecules of two to about twenty α-1,4-D-galactopyranoslyuronic acid (GalA) residues, were the first plant oligosaccharins to be discovered (Bishop et al., 1981; Hahn et al., 1981). OGAs are released upon fragmentation of homogalacturonan (HG) from the plant primary cell wall (reviewed by Côté

et al., 1998). Biological responses to OGAs occur in at least five of the six subclasses of dicotyledonous plants; Magnoliidae, Hamamelidae, Asteridae, Rosidae, Dilleniidae (Côté and Hahn, 1994; Reymond, et al., 1996); in a monocot (Moerschbacher et al., 1999); and a gymnosperm (Asiegbu et al., 1994). A number of different biological responses to OGAs have been reported, and the particular response observed depends on the plant species, the bioassay, and the chemical structure of the OGA used (Côté et al., 1998). A spectrum of modified and unmodified OGAs of various lengths are active in different systems (reviewed by Côté and Hahn, 1994; for recent examples see Reymond et al., 1996; Weber et al., 1996; Simpson et al., 1998; Spiro et al., 1998; Moerschbacher et al., 1999). The biological responses of plants to OGAs can be divided into two broad categories: plant defense, and plant growth and development (Côté and Hahn, 1994).

3.2. Oligogalacturonide-induced responses involved in plant defense

Pathogens enter plant tissues in at least three ways: digesting cell walls, entering through wounds, and invading through natural openings such as stomata. Pectins are one of the first targets of digestion by invading pathogens (Pagel and Heitifuss, 1990). OGAs are released when endopolygalacturonases (EPGases) and endopectate lyases (ePLases) secreted from the pathogen degrade the homogalacturonan in the cell wall (Côté et al., 1998). The OGAs released are a carbon source for the pathogens, but can also be detected by plants as signals to initiate defense responses. A plant may attempt to limit introgression by the pathogen in several ways. For example, the plant may strengthen its cell walls, close its stomata, synthesize phytoalexins and other antibiotic compounds, synthesize pathogenesis-related (PR) proteins and toxic peptides, contain the infection by means of localized cell death resulting from the hypersensitive response (HR), and produce cytotoxic active oxygen species (Low and Merida, 1996; Côté et al., 1998; Bolwell, 1999). Exogenously added OGAs inhibit the light-induced opening of stomata in tomato and *Commelina communis* L. leaves (Lee et al., 1999). Stomatal openings provide access to inner leaf tissues required by many plant pathogens (Agrios, 1997), suggesting that the constriction of stomatal apertures is beneficial for plant defense.

The first response observed after the addition of OGAs that is clearly involved in plant defense is the production of active oxygen species, including H_2O_2, and O_2^- (Low and Merida, 1996). This response, termed the oxidative burst, occurs within a few minutes after the addition of OGAs to suspension-cultured soybean (Legendre et al., 1993a), tobacco (Rout-Mayer et al., 1997; Binet et al., 1998), and tomato (Stennis et al., 1998) cells. Protein synthesis was not required for tomato suspension-cultures to initiate an oxidative burst, suggesting that changes in gene expression are not required (Stennis et al., 1998). Intact tomato leaves have been reported to produce active oxygen species in response to systemically (Orozco-Cardenas and Ryan, 1999) or locally (Lee et al., 1999) applied OGAs.

The oxidative burst is an immediate and localized reaction that is believed to have several roles in plant defense (Low and Merida, 1996; Bolwell, 1999). The quantities of reactive oxygen species produced can be cytotoxic and thus are expected to be antimicrobial. Reactive oxygen species are thought to have direct (through cytotoxicity) and indirect (through signaling) roles in the plant cell death required for the HR. Reactive oxygen species induce the expression of defense related genes (Lamb and Dixon, 1997), and are implicated as second messengers that elicit other defense responses, including systemic acquired resistance (SAR) and the HR (Bolwell, 1999). SAR is the induction of defense mechanisms at locations remote from the original wound or infection site that serve to prepare the plant to defend itself against new attacks by pathogens (Sticher et al., 1997). In addition, reactive oxygen species drive the rapid peroxidase-mediated oxidative cross-linking of cell wall lignins, proteins, and carbohydrates, thereby reinforcing the wall against enzymatic maceration by the pathogen (Côté and Hahn, 1994).

OGAs initiate signaling cascades that activate a plant's defense responses and many examples of OGA-elicited induction of defense related genes and proteins have been reported (Côté and Hahn, 1994). The exogenous application of OGAs elicit the expression of proteinase inhibitors (PIs) in tomato plants (Bishop et al., 1984; Moloshok et al., 1992). These PIs inhibit insect proteases and thereby decrease the digestability of plant proteins and reduce the nutrients available to feeding insects (Orozco-Cardenas et al., 1993). OGAs have been shown to induce phenylalanine ammonia-lyase (PAL), the activity of the first enzyme in the phenylpropanoid pathway, in suspension-cultured carrot (Messiaen and VanCutsem, 1994) and tobacco (Lapous, et al., 1998) cells, and in bean cotyledons (Dixon et al., 1989; Tepper and Anderson, 1990). This biochemical pathway leads to the production of phytoalexins and lignin. The activity of chalcone synthase (CHS), which is part of a branch in the phenylpropanoid pathway leading to the production of flavonoids including some phytoalexins, is induced by OGAs in bean cotyledons (Tepper and Anderson, 1990; Rose et al., 1999). The activity of casbene synthetase, an enzyme required for the synthesis of the phytoalexin casbene, is induced by OGAs in castor bean seedlings (Walker-Simmons et al., 1984). Accordingly, OGA-induced phytoalexin accumulation has been observed in soybean cotyledons (Davis et al., 1986a; Komae et al., 1990), bean cotyledons (Dixon et al., 1989; Tepper and Anderson, 1990), castor bean seedlings (Walker-Simmons

et al., 1984), pea pods (Walker-Simmons et al., 1984), and parsley suspension-cultured cells (Davis and Hahlbrock, 1987).

OGAs have a role in the induction of cell wall strengthening in response to pathogenic infection (Côté and Hahn, 1994). OGAs induce a series of cell wall peroxidase isoenzymes in suspension-cultured castor bean cells (Bruce and West, 1989). In the presence of active oxygen species, such peroxidases catalyze the free-radical mediated cross-linking of lignins, proteins, and carbohydrates, thereby strengthening the wall. OGA treatment of bean seedlings induced the accumulation of hydroxyproline-rich glycoproteins (HRGPs) which may also reinforce the cell wall after free-radical mediated cross-linking (Boudart et al., 1995). In addition, OGAs have been shown to induce lignin deposition in suspension-cultured castor bean cells (Bruce and West, 1989), cucumber hypocotyls (Robertson, 1986), and bean cotyledons (Tepper and Anderson, 1990).

Exogenously added OGAs regulate the expression of some enzymes and polypeptides that are involved in extracellular polysaccharide metabolism. For example, OGAs induce β-1,3 glucanase in suspension-cultured parsley cells (Davis and Hahlbrock, 1987), and chitinase in tobacco leaves (Broekaert and Peumans, 1988). Both of these enzymes fragment fungal cell wall polysaccharides. OGAs also elicit the expression of the mRNA and activity of an endogenous EPGase and of endopolygalacturonase-inhibiting protein (PGIP); two proteins involved in the metabolism of homogalacturonans in the cell wall. The elicited EPGase is present in tomato leaves and may have a role in the SAR response to insect feeding (Orozco-Cardenas and Ryan, 1999). PGIP, which was shown to be elicited by OGAs in suspension-cultured bean cells, binds to fungal EPGases in vitro and slows the rates of their enzymatic reactions (Bergmann et al., 1994). Thus PGIP is thought to expedite the plant's defense response by prolonging the life of the elicitor active OGAs in the apoplasm, and slowing the degradation of cell wall pectins (Cervone et al., 1993).

OGAs have been implicated in HR, but their role is unclear. OGAs may have a role in protecting potato tubers from maceration by the bacterial pathogen *Erwinia carotovora*, which causes potato soft rot (Weber et al., 1996). The protection may be mediated by OGA-induced cell death, but more work is needed to confirm this. Indeed, injection of OGAs into tobacco leaves prior to their inoculation with the pathogenic bacteria *Pseudomanas syringae* pv. syringae, completely inhibited the expected hypersensitive reaction (Baker et al., 1990). In contrast, the hypersensitive reaction is only partially inhibited when wheat leaves are injected with OGAs prior to their inoculation with the wheat rust fungus *Puccina graminis* Pers. f. sp. tritici (Moerschbacher et al., 1999).

3.3. Biological responses involved in plant growth and development

Exogenously added OGAs influence the growth and development of plant tissues (Côté and Hahn, 1994). OGAs inhibit auxin-induced pea stem elongation (Branca et al., 1988) and are also active in the tobacco thin-cell layer (TCL) (Tran Thanh Van et al., 1985; Mohnen et al., 1990), and the tobacco leaf explant (Bellincampi et al., 1993) bioassays. When biologically active OGAs are added to media containing specific phytohormone concentrations, TCLs that would normally form few or no organs form flowers, while TCLs that normally form roots form significantly fewer roots (Eberhard et al., 1989; Marfà et al., 1991). Biologically active OGAs inhibit root formation (Bellincampi et al., 1993) and increase stomata formation (Altamura et al., 1998) on tobacco leaf explants incubated in media with specific phytohormone concentrations. All of the affects OGAs have on tobacco explant morphogenesis could be mimicked if the explants were cultured on media containing a lower auxin concentration (Branca et al., 1988; Eberhard et al., 1989; Altamura et al., 1998).

OGAs are involved in fruit ripening. They have been shown to induce ethylene production in the fruits of tomato (Brecht and Huber, 1988; Campbell and Labavitch, 1991) and citrus (Baldwin and Biggs, 1988). Pectic fragments that elicit ethylene production have been extracted from tomato fruit at the breaker stage of ripeness. This suggests that OGAs, presumably released by EPGase, could be involved in initiating the ripening process (Melotto et al., 1994), since exogenous ethylene initiates the ripening process and the production of ethylene is required for ripening (Theologis et al., 1993; Giovannoni, 1997). The role of OGAs in fruit ripening, however, seems to be complex and is not understood. Tomato fruits expressing antisense EPGase mRNA exhibited a 99% reduction in EPGase activity and a substantial reduction in pectin depolymerization, but were unaffected in their ethylene production and overall ripening (Smith et al., 1990). Moreover, the increase in ethylene production during ripening is detected prior to the increase in EPGase production (Grierson and Tucker, 1983), and elevated ethylene levels can induce the accumulation of EPGase mRNA (Sitrit and Bennett, 1998). The addition of trigalacturonide to ripening tomato fruit tissue inhibits the increase in EPGase mRNA and enzyme activity, as well as fruit ripening (Mignani, et al., 1995). These data indicate that although OGAs are involved in tomato fruit ripening, their role in the process is not clear (discussed in Rose et al., 1998).

In every case reported to date where OGAs regulate the growth and development of plant tissues, with the exception of fruit ripening, their effect is the opposite of the effect of added auxin (Branca et al., 1988; Eberhard et al., 1989; Altamura et al., 1998). Accordingly, OGAs

inhibit the auxin-induced expression of the plant oncogene rolB (Bellincampi et al., 1996), and the auxin-induced division of phloem parenchyma cells (Altamura et al., 1998) in tobacco leaf disk explants. OGAs also induce the tobacco leaf explants to produce extracellular H_2O_2 (Bellincampi et al., 2000). However, the H_2O_2 does not appear to have a role in the OGA induced signal transduction pathway leading to the inhibition of rolB expression (Bellincampi et al., 2000). The mechanism by which OGAs act in opposition to the action of auxin is presently unknown.

3.4. The structural requirements for the biological activity of oligogalacturonides

Many studies attributing biological responses to OGAs have used impure mixtures of OGA oligomers that often contain sugars other than galacturonic acid (Côté and Hahn, 1994). The results of such studies must be considered carefully, since molecules other than OGAs may be responsible for the observed biological activities. Furthermore, studies using mixtures of different sizes of OGA oligomers reveal little about the OGA structure that is required for biological activity. Homogeneous, size-fractionated OGAs are relatively easy to prepare, and have been used in some studies (Spiro et al., 1993, 1998). Thus, only studies using size fractionated OGAs will be discussed in this section.

Most biological responses have been attributed to OGAs with a degree of polymerization (DP) from 10 to 16, with the most active sizes being around DP 12 (Côté and Hahn, 1994). These responses include: the induction of phytoalexins in soybean (Davis et al., 1986a; Komae, et al., 1990), the induction of casbene synthetase in castor bean (Jin and West, 1984), the induction of PAL in suspension-cultured carrot cells (Messiaen and Van Cutsem, 1994), the induction of rolB expression in tobacco leaf explants (Bellincampi et al., 1993), the induction of Ca^{2+} influx in suspension-cultured tobacco cells (Mathieu et al., 1991), and the regulation of organogenesis in tobacco TCL and leaf explants (Marfà et al., 1991; Bellincampi et al., 1993). The same size range of OGAs (i.e. DP ~10-16) having a GalA or a Δ-4,5 unsaturated GalA residue at the non-reducing terminus induce the production of phytoalexins in soybean cotyledons, indicating that 4,5-unsaturation of the non-reducing terminal GalA does not greatly influence the biological activity of the OGAs (Hahn et al., 1981; Davis et al., 1986b; Komae et al., 1990). In contrast, modifications of the reducing terminus including biotinylation, tyramination, C_1 reduction, and C_1 oxidation decrease the biological activity of OGAs 2–6 fold in the tobacco TCL bioassay and 2–32 fold in suspension-cultured tobacco cell extracellular alkalinization bioassays (Spiro et al., 1998). Chemical esterification of the C_6 carboxylates of OGAs greatly diminishes their ability to elicit casbene synthetase in castor bean, whereas subsequent deesterification restores their biological activity (Jin and West, 1984). This shows that, at least in the case of induction of casbene synthetase, free carboxylates are necessary for activity.

The minimum size of OGAs required for most of the biological activities reported, and the minimum size requirement for the formation of a Ca^{2+}-dependent conformation often called the "egg-box" conformation, coincide at a DP of approximately 10 (Kohn, 1975; Côté and Hahn, 1994; Messiaen and Van Cutsem, 1994; Thakur et al., 1997). Millimolar Ca^{2+} is required for biological activity of OGAs in carrot and tobacco cell suspensions (Mathieu et al., 1991; Messiaen and Van-Cutsem, 1994) which suggests that a Ca^{2+}-dependent conformation formed by OGAs with DPs $\geqslant 10$ is required for biological activity in certain bioassays (Côté and Hahn, 1994; Messiaen and VanCutsem, 1994). The polyamines spermidine and spermine are believed to selectively prevent OGAs from adopting the Ca^{2+}-dependent conformation (Messiaen and Van Cutsem, 1999). Physiological concentrations of these polyamines inhibit the biological activity of OGAs in carrot cell suspensions, suggesting that they modulate the biological activity of OGAs by preventing the Ca^{2+}-dependent formation of an active conformation (Messiaen and VanCutsem, 1999).

There are several reports showing that OGAs other than those with DPs from 10 to 17 are biologically active (Côté and Hahn, 1994). For example, OGAs from DP 2 to 30 elicit the expression of proteinase inhibitors (PIs) in tomato seedlings, with the disaccharide being the most active (Farmer et al., 1990; Moloshok et al., 1992). In this system, di- and tri-GalA with Δ-4,5 unsaturated GalA at the non-reducing terminus are active, whereas C_1 reduced oligomers are not. OGAs with a DP from two to six induce ethylene biosynthesis in tomato plants, with the pentasaccharide being the most active (Simpson et al., 1998). Trigalacturonide is active in inhibiting the production of EPGase in ripening tomato fruit tissue (Mignani et al., 1995). Di- and tri- GalA elicit the accumulation of HGRPs in the cell walls of bean seedlings (Boudart et al., 1995). Di- and tri- GalA also suppress the induction of PAL and the hypersensitive response to a fungal pathogen in wheat leaves (Moerschbacher et al., 1999). Nonreducing-end Δ-4,5 unsaturated di-GalA is active in inhibiting tissue maceration in potato tuber tissue infected with the bacterial soft rot pathogen Erwinia carotovora (Weber et al., 1996).

3.5. Presence and fate of biologically active oligogalacturonides in plant tissues

Plant cells and plant pathogens secrete endo- and exo-polygalacturonases into their extracellular matrix.

EPGases are often found at sites where the application of exogenous OGAs elicit a biological response and thus, the EPGases are present at those locations where the release of biologically active OGAs would be expected (Côté et al., 1998; Hadfield and Bennett, 1998). However, a conclusive demonstration that EPGases release biologically active OGAs from a plant's cell wall at physiologically relevant locations and times is lacking. The best evidence to date comes from studies of ripening tomato fruit. Polygalacturonase activity is known to increase during tomato ripening, and pectic oligomers that elicit ethylene have been extracted from tomato fruits at the breaker stage of ripening (Melotto et al., 1994). Purified OGAs with a DP greater than eight initiate ethylene biosynthesis in tomato fruits (Brecht and Huber, 1988; Campbell and Labavitch, 1991). The OGA-enriched material isolated from ripening tomato fruit, and EPGase-generated OGAs have similar chromatographic profiles, suggesting that the active components in the fruit extracts are OGAs. However, the individual OGA oligomers isolated from the fruit cell wall material are contaminated with the neutral sugars present in RG I and RG II.

There have been conflicting reports concerning the movement of OGAs in plants. When crude ^{3}H-labeled pectic fragments (Bishop et al., 1981) or ^{3}H-labeled OGAs (MacDougall et al., 1992) are introduced into the xylem of tomato plants through cut stems, the radiolabel moves through the plant with the transpiration stream. Ninety minutes after being introduced, the label was found throughout the plant (MacDougall et al., 1992). Labeled OGAs recovered from the plants had been fragmented into smaller oligomers, and some were esterified with an unidentified alcohol. In contrast, ^{14}C-labeled pectin and ^{3}H-labeled pectic fragments were immobile when applied to wounds on the leaves of tomato plants (Baydoun and Fry, 1985). The label would have needed to move out of the leaves via the phloem, since xylem flow is transpiration driven and normally flows into leaves. The immobility of the label indicates that OGAs do not move in the phloem, and that biologically active OGAs generated in infected leaves cannot move through the plant and induce SAR. In contrast, OGAs released during infection of the roots or stems could move throughout the plant's xylem and induce SAR (MacDougall et al., 1992).

To function as signal molecules, biologically active OGAs must be present when the physiological responses they induce are required and are likely to be absent when the responses are not required. Therefore, it is likely that OGA signal molecules are removed or inactivated following the activation of a physiological response. Endogenous EPGases are often found in plant tissues where the exogenous application of OGAs would elicit physiological responses. For example, polygalacturonase activity has been found in suspension-cultured tobacco (Mathieu et al., 1998), carrot (Konno et al., 1989) and rose (García-Romera and Fry, 1995) cells, as well as in cotton cotyledons (Zhang et al., 1996). In the tobacco and rose cells, the enzymes do not release biologically active concentrations of OGAs from the plant cell walls, suggesting that the enzymes are maintained in an inactive state or that their substrates are inaccessible (Mathieu et al., 1998; García-Romera and Fry, 1995). On the other hand, exogenously added OGAs are rapidly sequestered into the cell wall or degraded in tobacco (Mathieu et al., 1998) and rose (García-Romera and Fry, 1997) suspension-cultured cells. These findings indicate that endogenous endo- and exo- polygalacturonases capable of degrading biologically active OGAs after initiation of a physiological response are present in suspension-cultured cells (Mathieu et al., 1998; García-Romera and Fry, 1997).

3.6. Oligogalacturonide receptors

Considering the diversity of biologically active OGA structures, and the range of biological activities attributed to these structures, it would not be surprising to find that OGAs are perceived through multiple mechanisms (Côté and Hahn, 1994; Spiro et al., 1998). It is generally believed that biologically active macromolecules are recognized by receptor proteins and that this recognition event elicits a biological response via a signal transduction cascade (Hahn, 1996). The widely variable concentrations of OGAs required for bioactivity (from 10^{-4} to 10^{-7} M) supports the notion that OGAs are recognized by a number of receptors with different affinities (Côté and Hahn, 1994). However, OGAs can bind to each other and to other homogalacturonans in the cell wall. A significant portion of the OGAs added to suspension-cultured tobacco cells was found to be ionically bound to the cell wall (Mathieu et al., 1998). Thus, OGAs may saturate binding sites in the wall before they can be perceived at the plasma membrane. If this is the case, the concentration required for biological activity would not reflect the affinity of the receptor.

The possibility that the cell wall itself could be the 'receptor' for OGAs has been discussed (Mohnen and Hahn, 1993). OGAs may intercalate into the junction zones formed by the self-association of homogalacturonans, thereby releasing some of the tension in the wall (Carpita and Gibeaut, 1993; Rose et al., 1999). Changes in the tension of cell wall polymers may be transmitted to the cytoskeleton and initiate signaling cascades through transmembrane proteins similar to integrins (Schwartz et al., 1995; Miller et al., 1997; Faik et al., 1998).

Suspension-cultured soybean cells have been reported to respond to OGAs in a process of receptor mediated endocytosis in (Horn et al., 1989). An OGA of DP 14,

derivatized with fluorescein via a thiosemicarbazone linkage, bound to the surface of soybean suspension-cultured cells and was internalized. The fluorescence traveled from the plasma membrane to the vacuole over the course of two hours. OGAs labeled with I^{125} after derivatization with tyrosine via a hydrazone linkage were used to measure the binding kinetics. The binding was competitively inhibited by an excess of non-labeled OGA (Horn et al., 1989), and was saturated at a concentration about five times higher than that required for maximum biological activity (Low et al., 1993). To date, no connection has been made between this binding and a biological response elicited by OGAs. It should be noted that the thiosemicarbazone and hydrazone linkages used in these derivatives are chemically unstable (Ridley et al., 1997). Thus, it is not certain whether OGA was covalently linked to all the fluorescence or iodinated signals observed.

A 34 kilodalton (kd) plasma membrane protein that is phosphorylated in response to OGAs with a DP from 13 to 30 has been cloned from potato (Farmer et al., 1991; Reymond et al., 1996). This protein, called remorin, binds to, and is phosphorylated in vitro in response to, OGAs of the same size range (Reymond et al., 1995, 1996). Immunologically similar proteins that are phosphorylated in response to OGAs were found in several widely divergent species of dicots. The OGA size requirement for phosphorylation of remorin does not match the size requirement for any of the known OGA-induced biological responses and remorin has not been implicated in any of the known biological responses elicited by OGAs. Remorin does not have homology to any known receptor proteins, although it does share properties such as plasma membrane-association, binding to polyanions, and phosphorylation, with the 30-kd cell-to-cell movement protein of tobacco mosaic virus (Reymond et al., 1996). Interestingly, viral cell movement proteins bind pectin methylesterase in the wall (Dorokhov et al., 1999) and deletion of the PME-binding region of the movement protein inhibits cell-to-cell viral movement through plasmodesmata (Chen et al., 2000). The hypothesis that remorin plays a role in plant driven transport or signaling through plasmodesmata has been prosposed (Reymond et al., 1996).

3.7. Oligogalacturonide induced signaling cascades

Much information is available concerning the signaling events underlying a plant's responses to OGAs and a coherent picture of these events is now beginning to emerge. Selected elements of the signaling cascades have been studied in suspension-cultured soybean, tobacco, and carrot cells, and in tomato plants, but in no case have all elements been studied in one system. Several potential limitations of these studies should be considered when general conclusions are made. The OGAs used in many of these studies were not size-fractionated or completely pure. Since differently-sized OGAs can elicit unique sets of responses, the responses obtained using mixtures of differently-sized OGAs could result from multiple receptors and/or the induction of multiple signal transduction pathways. Furthermore, cultured plant cells are in vitro systems that are subject to genetic and epigenetic variation and extrapolating results obtained with such cells to the physiology of whole plants can be difficult. Finally, much of the information available is based on the use of pharmacologic inhibitors whose specificity and affects are not well characterized in plants.

3.7.1. Suspension-cultured soybean cells: early signaling events

Cultured soybean cells produce H_2O_2 within seconds to a few minutes after the addition of partially purified pectic fragments (Legendre et al., 1992, 1993a). OGA elicited H_2O_2 production appears to be tightly regulated since the quantity and duration of H_2O_2 production are limited, and are relatively consistent between experiments (Legendre et al., 1993a,b). Furthermore, the cells become desensitized to an additional dose of OGAs for a defined period (refractory period). It is noteworthy that OGA-elicited H_2O_2 production in suspension-cultured soybean cell is not accompanied by transient extracellular alkalinization (Horn et al., 1992), as it is in suspension-cultured carrot and tobacco cells (Messiaen and VanCutsem, 1994; Matheiu et al., 1996b).

Signaling events that require protein phosphorylation and GTP binding proteins (G-proteins) may be required for H_2O_2 production (Fig. 6). The protein kinase inhibitors, K252a and staurosporine block the OGA-induced oxidative burst in a concentration dependent manner, whereas the phosphatase inhibitors, okadaic acid and calyculin A elicit an oxidative burst in the absence of OGAs (Chandra and Low, 1995). This suggests that kinase activity is required for the oxidative burst, and that in the absence of phosphatase activity a basal level of kinase activity can activate the signaling cascades leading to H_2O_2 production in the absence OGAs. The G-protein activator, mastoparan, also elicits an oxidative burst in soybean cells, suggesting that G-protein activation is required for the oxidative burst (Legendre et al., 1992). However, direct G-protein involvement in OGA-elicited oxidative burst has not been demonstrated, and more than one signal transduction pathway leading to oxidative burst is likely to exist in plants (Low and Merida, 1996; Bolwell, 1999). Phospholipase C activation resulting in the release of inositol triphosphate (IP_3) may be part of OGA activated signaling in suspension-cultured soybean cells (Legendre et al., 1993b). OGAs promote a transient increase in the intracellular level of IP_3 prior to the biosynthesis of H_2O_2. Furthermore neomycin sulfate, an inhibitor of

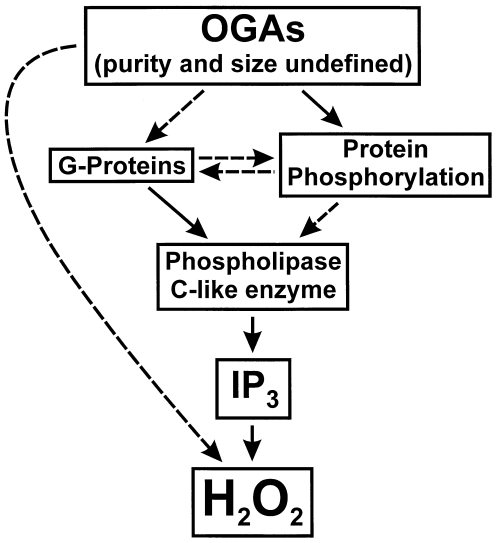

Fig. 6. A working model of OGA-induced signaling in suspension-cultured soybean cells. The solid arrows indicate potential pathways supported by experimental evidence. The dashed arrows indicate hypothetical interactions/pathways that are implied from experimental evidence. The arrows are not meant to suggest that a particular number of biochemical steps are involved in any of the interactions.

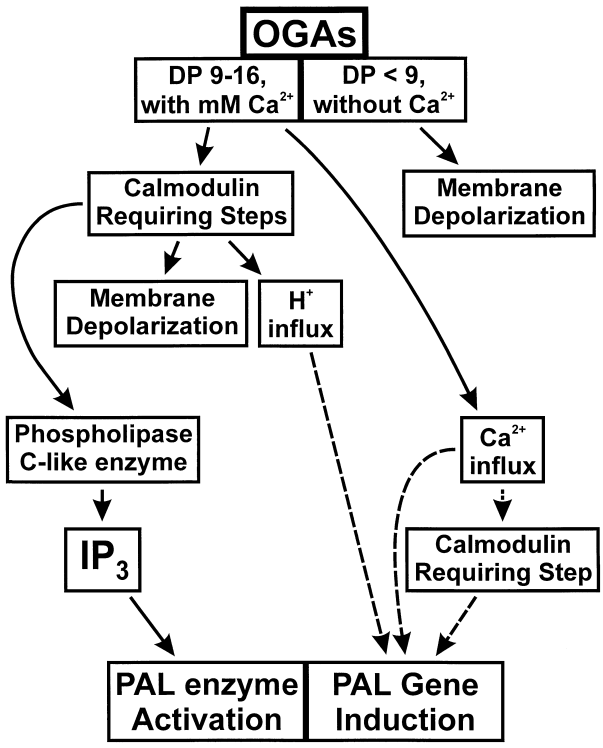

Fig. 7. A working model of OGA-induced signaling in suspension-cultured carrot cells. The solid arrows indicate potential pathways whose existence is supported by experimental evidence. The dashed arrows indicate hypothetical interactions/pathways that are implied from experimental evidence. The arrows are not meant to suggest that a particular number of biochemical steps are involved in any of the interactions.

polyphosphoinositide hydrolysis, partially inhibits the OGA induced biosynthesis of H_2O_2, suggesting that IP_3-independent signaling may also exist.

3.7.2. Suspension-cultured carrot cells: the role of calcium

OGAs with a DP from 9 to 16 induce a transient cytosolic Ca^{2+} mobilization, cytosolic acidification, and plasma membrane depolarization accompanied by an induction of PAL mRNA and enzyme activity in carrot cells (Fig. 7; Messiaen and VanCutsem, 1994). The most active size of OGA is DP 11. Externally supplied Ca^{2+} is required for all of these responses except for membrane depolarization. OGAs with a DP <9 elicit plasma membrane depolarization irregardless of the presence of Ca^{2+}, but do not elicit any of the other responses measured (Messiaen and VanCutsem, 1994). This is a particularly significant result since it suggests that the two different size classes of OGAs interact with the same cells to activate distinct signal transduction pathways.

The calcium channel blockers, verapamil and nifedipine, block OGA (DP 9–16) elicited cytosolic Ca^{2+} mobilization and PAL mRNA induction, but do not block cytosolic acidification or membrane depolarization (Fig. 7, Messiaen and VanCutsem, 1994). Furthermore, when the calcium ionophore A23187 is used to increase the cytosolic free Ca^{2+}, PAL mRNA is induced but cytosolic acidification and membrane depolarization are not. The inhibitor of the ATP-dependent plasma membrane proton-pump, vanadate, causes membrane depolarization and cytosolic acidification, but not PAL induction (Fig. 7; Messiaen and VanCutsem, 1994). Conversely, the activator of the ATP-dependent plasma membrane proton-pump, fusicoccin, causes a slight cytosolic alkalinization and inhibits OGA-elicited cytosolic acidification, membrane depolarization, and PAL mRNA induction. Together, these data suggest that transient cytosolic Ca^{2+} mobilization is required for induction of PAL mRNA induction, whereas transient cytosolic acidification and membrane depolarization do not require Ca^{2+} mobilization and may or may not be involved in PAL mRNA induction (Fig. 7; Messiaen and VanCutsem, 1994).

Neomycin sulfate, an inhibitor of polyphosphoinositide hydrolysis, inhibits the OGA-elicited induction of PAL enzyme activity by about 50%, suggesting that phospholipase C- mediated IP_3 turnover is involved in activation of the enzyme (Messiaen and VanCutsem, 1994). Neomycin sulfate has a small quantitative affect on OGA induced cytosolic Ca^{2+} mobilization, but does not affect cytosolic acidification, plasma membrane depolarization or PAL mRNA induction. The calmodulin inhibitor, calmidazolium, does not affect cytosolic Ca^{2+} mobilization, but blocks OGA elicited induction of cytosolic acidification, membrane depolarization, and PAL mRNA and enzyme activity (Messiaen and VanCutsem, 1994). This suggests that calmodulin is required for

steps affecting all of these responses except cytosolic Ca^{2+} mobilization (Fig. 7).

3.7.3. Suspension-cultured tobacco cells: a role for cytosolic acidification

OGAs with a DP from 10 to 15 (DP 12 is most active) induce a transient K^+ efflux, Cl^- efflux, cytosolic acidification, cytosolic Ca^{2+} mobilization, oxidative burst, plasma membrane depolarization, and induction of PAL mRNA accumulation in suspension-cultured tobacco cells (Fig. 8; Mathieu et al., 1991; Lapous et al., 1998; Spiro et al., 1998). Degradation of the OGA elicitor molecules is not responsible for the transient nature of the responses (Mathieu et al., 1998). The protein kinase inhibitors, staurosporine and 6-dimethylaminopurine, block OGA elicited oxidative burst, cytosolic acidification, and induction of PAL, whereas the protein phosphatase inhibitors, calyculin A and cantharidin induce these responses in the absence of OGAs (Mathieu et al., 1996a,b). Furthermore, OGA-induced changes in the phosphorylation of membrane-associated proteins from suspension-cultured tobacco cells have been detected (Droillard et al., 1997). As with soybean cells, protein kinase activity seems to be required, and in the absence of phosphatase activity a basal level of kinase activity may activate the signaling cascades in the absence of added OGAs.

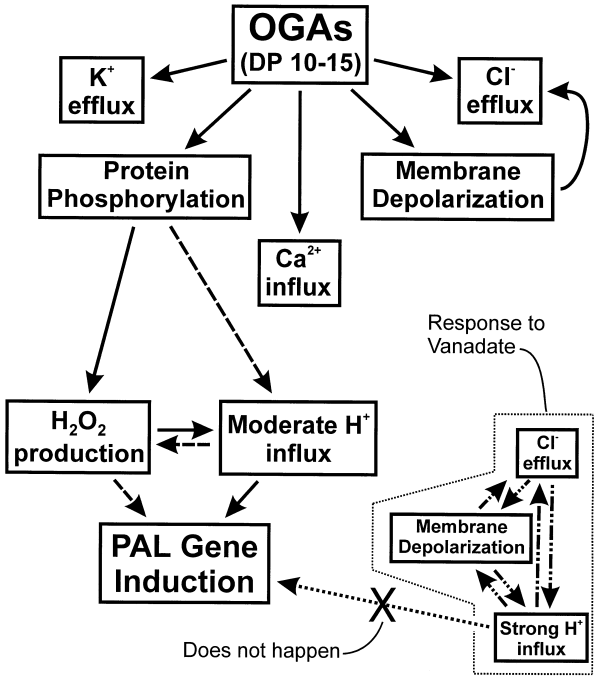

Fig. 8. A working model of OGA-induced signaling in suspension-cultured tobacco cells. The solid arrows indicate potential pathways whose existence is supported by experimental evidence. The dashed arrows indicate hypothetical interactions/pathways that are implied from the experimental evidence. The dot-dashed arrows indicate hypothetical interactions that potentially interconnect the responses induced by vanadate in the absence of OGA's. The arrows are not meant to suggest that a particular number of biochemical steps are involved in any of the interactions.

The inhibitors of the ATP dependent plasma membrane proton pump, vanadate and erythrosine B, cause plasma membrane depolarization, cytosolic acidification, and Cl^- efflux (Fig. 8; Zimmermann et al., 1998). Patch clamp experiments suggest that activation of a voltage-gated anion channel is responsible for the Cl^- efflux. Treatment of tobacco cells with the hydrophobic acid propionate, which moves protons across the plasma membrane with the proton gradient to cause cytosolic acidification, elicits PAL mRNA production. On the other hand, the cytosolic acidification caused by the proton pump inhibitor erythrosin B does not induce PAL mRNA (Lapous et al., 1998). It is suggested that the moderate cytosolic acidification induced by propionate stimulates production of PAL mRNA, while the strong cytosolic acidification induced by vanadate or erythrosine B is inhibitory (Fig. 8). In light of these results, the role of OGA-induced cytosolic acidification in the induction of PAL in carrot suspension-cultured cells merits reexamination.

The oxidative burst inhibitor, iodonium diphenyl, completely inhibited the OGA induced oxidative burst in suspension-cultured tobacco cells and inhibited cytosolic acidification (Rout-Mayer et al., 1997). This suggests that H_2O_2 may induce cytosolic acidification, although inverse experiments involving the addition of exogenous H_2O_2 were not reported.

3.7.4. Tomato wound response: oligogalacturonide signaling in intact plant tissues

When tomato seedlings with their roots excised are placed in a solution containing OGAs, these elicitors travel through the xylem and induce the expression of PIs, EPGase, and oxidative burst throughout the plant (MacDougall et al., 1992; Moloshok et al., 1992; Bergey et al., 1999; Orozco-Cardenas and Ryan, 1999). OGAs from DP 2 to 30 induce the expression of PIs, and other systemic wound response proteins (SWRPs) in these plants (Fig. 9; Moloshok et al., 1992; Bergey et al., 1996, 1999). PIs and SWRPs have been shown to help protect the plant from insect damage by reducing the nutrients available to feeding insects (Orozco-Cardenas et al., 1993). Xylem transport of OGAs is, however, thought to be physiologically insignificant in the normal tomato systemic wound response (SWR) to herbivore attack. Herbivores normally feed on leaves, and OGAs released in leaves are not transported in the phloem and do not move from the leaves to initiate a systemic reaction (Baydoun and Fry, 1985). For this reason, OGAs are thought to constitute a localized signal in the induction of SWR. The peptide hormone, systemin, is transported throughout the plant via the phloem and the xylem after its proteolytic release from its precursor in response to wounding or insect feeding (Doares et al., 1995; Narvaez-

Vasquez et al., 1995; Bergey et al., 1996; Ryan and Pearce, 1998). The signal from systemin propagates very rapidly, perhaps by means of rapid pressure changes in the xylem that are perceived throughout the plant (Malone, 1996; Stratmann and Ryan, 1997). Like OGAs, systemin and wounding induce the expression of PIs, SWRPs, EPGase, and H_2O_2 (Stratmann and Ryan, 1997; Bergey et al., 1999; Orozco-Cardenas and Ryan, 1999). Tomato plants expressing an antisense prosystemin gene respond to OGAs and systemin normally, but do not produce PIs, SWRPs, EPGase, or H_2O_2 in response to wounding, indicating that the activation of systemin is not required for the response to OGAs (Orozco-Cardenas and Ryan, 1999).

Jasmonic acid is required for the induction of H_2O_2, EPGase, PIs, and SWRPs by either OGAs or systemin (Fig. 9; Doares et al., 1995; Howe et al., 1996). Jasmonic acid is synthesized via the octadecanoid pathway from linolenic acid which, presumably, is released from phospholipids by phospholipase A activity (Howe et al., 1996). Tomato plants with a mutation in the octadecanoid pathway do not make jasmonic acid and do not produce PIs in response to OGAs, systemin, or wounding (Howe et al., 1996). Two inhibitors of the octadecanoid pathway, salicylic acid and diethylthiocarbamic acid, severely inhibit the induction of PIs by OGAs, systemin, and wounding (Doares et al., 1995). Wounding, OGAs, systemin, and jasmonic acid all induce tomato plants to produce ethylene and PIs (O'Donnell et al., 1996; Simpson et al., 1998). The induction of PIs is blocked by the ethylene biosynthesis inhibitors, silver thiosulphate and norbornadiene (O'Donnell et al., 1996), suggesting that ethylene is required as a second messenger when wounding, or treatment with OGAs, systemin, or jasmonic acid induce PIs. The ethylene requirement was not readily apparent because the addition of jasmonic acid induces the ethylene biosynthesis required for elicitation (Farmer and Ryan, 1992; O'Donnell et al., 1996; Orozco-Cardenas and Ryan, 1999). Taken together these results indicate that both ethylene and jasmonic acid are required for the induction of PIs by OGAs, systemin or wounding.

A 48 kd myelin basic protein kinase is activated in response to wounding, systemin, and OGAs (Stratmann and Ryan, 1997). The kinase is also activated by the same signals in plants that have a genetic defect in the octadecanoid pathway blocking the normal induction of PIs and SWRPs (Howe et al., 1996) (see Fig. 9). A synthetic analogue of the peptide hormone systemin strongly inhibits activation of the kinase and the induction of SWRPs by systemin (Stratmann and Ryan, 1997). This suggests that the 48 kd myelin basic protein kinase functions between these primary signals and the octadecanoid biosynthetic pathway as part of the signaling cascade leading to the SWR (Stratmann and Ryan, 1997).

Systemin elicits the systemic production of an endogenous EPGase in the leaves of tomato plants (Orozco-Cardenas and Ryan, 1999). This EPGase could generate biologically active OGAs, which act locally in concert with systemin to augment the SWR (Fig. 9; Bergey et al., 1999). Accordingly, the simultaneous addition of OGAs and systemin to suspension-cultured tomato cells produces an oxidative burst at least an order of magnitude larger than when OGAs are added alone (Stennis et al., 1998). This suggests that the two signals could act synergistically in the induction of the SWR in whole plants. Systemin is inactive in eliciting an oxidative burst in the suspension-cultured cells, suggesting that intact plant tissues are required (Stennis et al., 1998). Comparison of the timing and location of EPGase mRNA accumulation with H_2O_2 biosynthesis in whole plants treated with systemin suggests that OGAs

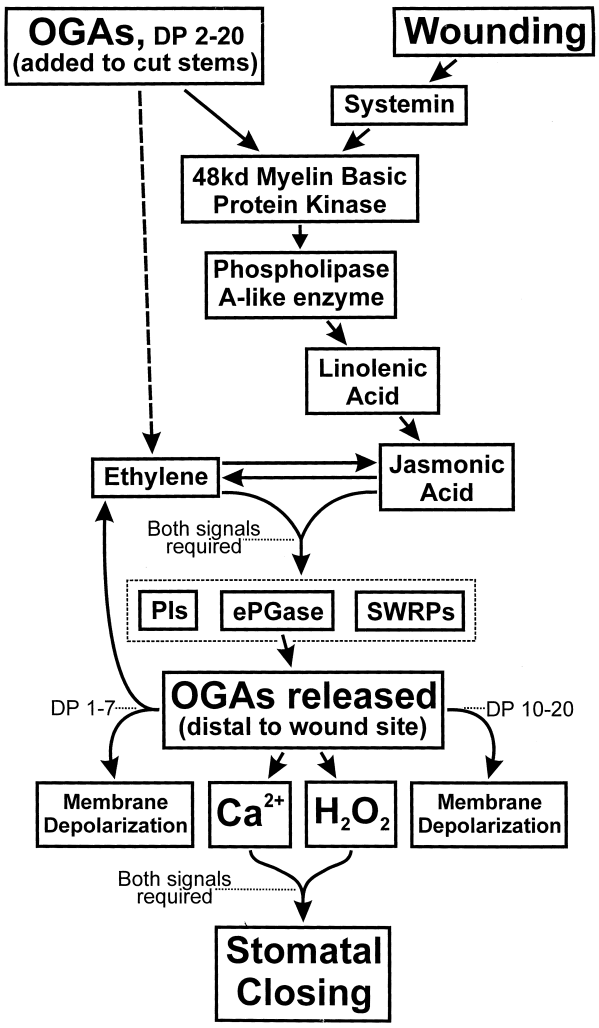

Fig. 9. A working model of OGA-induced signaling in tomato plants. As indicated in the text, the potential existence of some of the interactions shown is based on experiments using tomato tissues other than whole plants. The solid arrows indicate potential pathways whose existence is supported by experimental evidence. The dashed arrows indicate hypothetical interactions/pathways that are implied from experimental evidence. The arrows are not meant to suggest that a particular number of biochemical steps are involved in any of the interactions.

released by this enzyme could be responsible for the systemically induced oxidative burst associated with the SWR (Orozco-Cardenas and Ryan, 1999).

Another response that may be involved in the SWR is the OGA-induced closing of stomata (Fig. 9; Agrios, 1997; Lee et al., 1999). The Ca^{2+} chelater, EGTA, as well as the H_2O_2 scavengers, catalase and ascorbate, completely inhibit OGA induced stomatal closing in tomato leaf epidermal peels (Lee et al., 1999). This suggests that both Ca^{2+} and H_2O_2 signals are required for OGA-induced stomatal closing (Fig. 9).

A unique feature of the induction of SWR by OGAs in tomato plants is that a broad range of OGA sizes are biologically active (Farmer et al., 1990). OGAs with a DP from 1 to 7, as well as those with a DP from 10 to 20 are active in inducing membrane depolarization in tomato leaf mesophyll cells and in inducing PI activity in tomato plants (Thain et al., 1990). However, the membrane depolarization responses elicited by the two size ranges of OGAs are quantitatively and kinetically different. Furthermore, OGAs with a DP from 4 to 6 induce ethylene biosynthesis in tomato plants, whereas other sizes do not (Simpson et al., 1998). In light of these findings, it seems likely that tomato plants recognize OGAs by at least two different mechanisms.

3.7.5. Conclusions and relevance to future work

Oligogalacturonides participate in an information network of branched and interconnecting signal transduction pathways that lead to the more integrated tissue level responses. Plants are able to respond adaptively to changing environmental conditions by means of such information networks. It is of great importance to understand how plants accomplish this remarkable feat. Our understanding of OGA signal transduction is sketchy, and in some cases, conflicting. A variety of OGA signaling elements have been implicated in different plant species, but the role of each element has not been investigated in a single species. A major limitation has been the difficulty of isolating and directly characterizing the components of the OGA signaling cascades, including the receptors. In similar research with animals, the isolation of receptors and other signaling components has allowed interacting signaling elements to be identified.

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