Biosynthesis of Pectin[1]

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The plant cell wall is composed of polysaccharides and proteins. In addition, some cells have walls impregnated with lignin. In all cases, the polysaccharides constitute the major part of the wall. The wall polysaccharides are often classified into cellulose, hemicelluloses, and pectin, and these three types are represented in almost all cell walls in varying proportions. Cellulose is the main load-bearing structure and is composed of β-1,4-linked glucan chains organized in more or less crystalline microfibrils. Hemicelluloses include several different polymers, chiefly xylans, xyloglucans, and (gluco)mannans, which are characterized by having a backbone of β-1,4-linked sugars with an equatorial linkage configuration (Scheller and Ulvskov, 2010). Pectin is the third group of polysaccharides, characterized by relatively high extractability using acid or chelators and a high content of GalUA. Together, the hemicelluloses and pectins constitute the matrix in which cellulose microfibrils are embedded. The interactions between the different polysaccharides ensure the strong yet dynamic and flexible properties of the cell wall.

PECTIN STRUCTURE

Various pectic polysaccharides can be detected in the cell wall, including homogalacturonan (HG), xylogalacturonan (XGA), apiogalacturonan, rhamnogalacturonan I (RGI), and rhamnogalacturonan II (RGII; Fig. 1). In Arabidopsis (*Arabidopsis thaliana*) leaves, the cell wall contains approximately 50% (w/w) pectin but the content varies depending on environment, tissue, and species (Zablackis et al., 1995). The ratio between HG, XGA, RGI, and RGII is also variable, but typically HG is the most abundant polysaccharide, constituting about 65% of the pectin, while RGI constitutes 20% to 35% (Mohnen, 2008). XGA and RGII are minor components, each constituting less than 10% (Zandleven et al., 2007; Mohnen, 2008). The different pectic polysaccharides are not separate molecules but covalently linked domains. Unbranched homopolymer chains of α-1,4-linked D-GalUA are described as HG. The backbone of GalUA residues can be substituted at various positions with other sugar moieties, including Xyl (XGA) and apiofuranose (apiogalacturonan). In XGA, a single Xyl is attached to the O-3 position of some GalUA residues. Additional Xyl residues can be attached to the first Xyl with β-1,4 linkage (Zandleven et al., 2006). XGA is particularly abundant in reproductive tissues but is also present in other tissues, such as Arabidopsis leaves (Zandleven et al., 2007). In aquatic angiosperms such as *Lemna* and *Spirodela*, the galacturonan backbone is decorated with D-apiofuranose at O-2 or O-3 (Hart and Kindel, 1970; Ovodov et al., 1971; Longland et al., 1989).

Besides the simple substitutions in XGA and apiogalacturonan, clusters of complex side chains are also attached onto the O-2 or O-3 position in the galacturonan backbone to form RGII (Fig. 1). These side chains are composed of 12 types of glycosyl residues linked together by at least 22 different glycosidic bonds. Some of the glycosyl residues and glycosidic linkages found in RGII side chains are rare and considered unique in plant polysaccharides (e.g. 2-*O*-methyl-L-Fuc, L-aceric acid, and α-1,3- xylofuranose). Despite its complexity, the structure of RGII is highly conserved among vascular plants (Matsunaga et al., 2004; O'Neill et al., 2004). Frequently, RGII exists as a dimer mediated by borate ion attached to the A branch. Boron is an essential micronutrient for plants, most likely due to an important role of RGII dimerization in ensuring the integrity of the cell wall (O'Neill et al., 2001). Thus, although RGII is a minor component of pectin, it seems to have an essential role, and that may be the main reason why very few mutants with altered RGII have been identified, although many genes must be involved in RGII biosynthesis.

RGI is the only type of pectin not built upon pure galacturonan backbones. Instead, it is a branched polymer with a backbone of disaccharide (α-1,4-D-GalA-α-1,2-L-Rha) repeats. The Rha residues in the backbone can be substituted with β-1,4-galactan,

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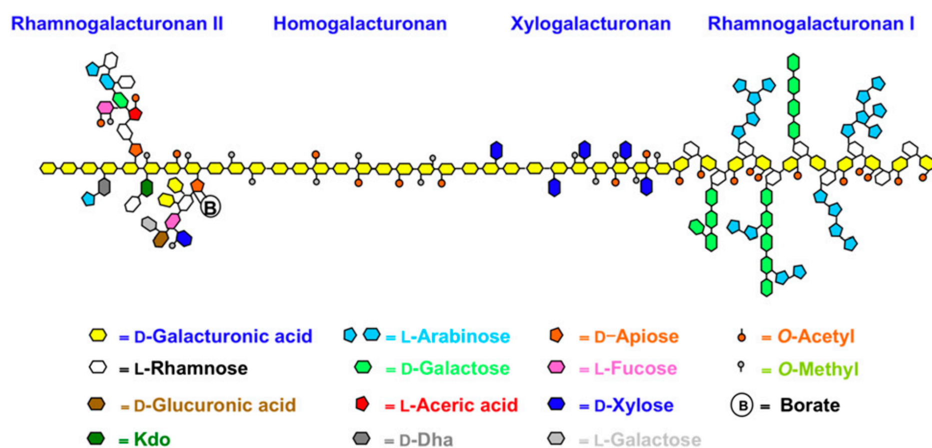

Figure 1. Schematic structure of pectin. Pectin consists of four different types of polysaccharides, and their structures are shown. Kdo, 3-Deoxy-D-manno-2-octulosonic acid; DHA, 3-deoxy-D-lyxo-2-heptulosaric acid. HG and RGI are much more abundant than the other components (see text).

branched arabinan, and/or arabinogalactan side chains. Unlike RGII, the structure of the side chains of RGI can vary greatly among plants. Even a model suggesting that HG and its substituted variants are side chains of RGI was proposed (Vincken et al., 2003). However, the discovery of RGI backbone oligomers with HG and XGA as nonreducing end extensions suggests otherwise (Nakamura et al., 2002; Coenen et al., 2008).

Galacturonic acid in pure galacturonan backbones is further modified by methyl esterification at the C-6 carboxyl position and/or by *O*-acetylation at the O-2 or O-3 position, whereas, in RGI, only the O-3 acetylation has been unambiguously reported (Komalavilas and Mort, 1989; Ishii, 1995, 1997). The degree of methyl and acetyl esterification is variable and affects the physicochemical properties of the pectin, especially the formation of calcium-mediated interactions between HG chains. For example, the dimerization of homopolygalacturonan chains mediated by calcium ions was found to require stretches of at least nine unmodified GalUA residues and could be disrupted by methyl esterification (Liners et al., 1992).

PECTIN FUNCTIONS IN THE WALL

The primary roles of cell walls are to give physical strength to the plant and to provide a barrier against the outside environment. The main role of pectin is to participate in these two functions together with the other polymers. Especially HG and RGII are well known to be involved in strengthening the wall. The mechanical properties of HG and RGII have been reviewed and described thoroughly in other publications (Ryden et al., 2003; Caffall and Mohnen, 2009) and will not be described further here. HG plays additional roles besides pure mechanical support. Plant pathogens cause degradation of pectin, and oligogalacturonides (i.e. α-1,4-linked oligomers of GalUA) are well established to be part of a signaling cascade that senses wall degradation upon pathogen attack (Ridley et al., 2001; D'Ovidio et al., 2004; Kohorn et al., 2009).

MECHANICAL PROPERTIES OF RGI IN THE CELL WALL

Compared with the many studies of HG and RGII function, much less is known about the function of RGI, and it has not been summarized in recent reviews. Treatment of leaf epidermis with arabinanase resulted in hindering of the opening and closing of the stomata (Jones et al., 2003). The proposed hypothesis is that the arabinans work as spatial regulators of the proximity of HG domains. This regulation may prevent the formation of Ca^{2+}-mediated interactions between HG regions; hence, the removal of arabinan may induce stiffening of the cell wall. This hypothesis of the RGI side chains as plasticizers in cell walls that undergo large physical remodeling is in agreement with observations of cell walls functioning under extreme water deficient conditions. Arabinan and galactan are highly mobile polymers compared with the other components of pectin, and this mobility is influenced by the hydration of the cell wall. Therefore, arabinan and galactan are the first polymers to be mobilized upon the hydration of dry cell walls (Tang et al., 1999). Likewise, arabinan and galactan retain mobility relatively longer than other cell wall polymers upon desiccation and may fill the cavities created upon physical rearrangement of the cell wall. Interestingly, an unusually high Ara content (approximately 38 mol %) was observed in the resurrection plant *Myrothamnus flabellifolius* and inferred to represent pectic arabinan (Moore et al., 2006). Seeds also undergo extreme desiccation and may have a high content of arabinans (Shiga and Lajolo, 2006; Gomez et al., 2009). Marama bean (*Tylosema esculentum*), a native bean from southern Africa with a water content down to 5%, has as much as 60% (w/w) Ara in the noncellulosic fraction of the cell wall, practically all from a highly branched arabinan (M. Mosele and J. Harholt, unpublished data). Further evidence for the

role of RGI side chains in the physical properties of the wall comes from the investigation of transgenic plants. Ulvskov et al. (2005) analyzed the mechanical properties of the cell wall of potato (*Solanum tuberosum*) tubers from wild-type and transformant plants with decreased contents of galactan or arabinan. The force needed to induce failure of the cell wall decreased in both types of transgenic tubers. The elastic properties of the tubers were also altered, with a stiffening of the cell wall, as observed in stomatal cells (Jones et al., 2003; Ulvskov et al., 2005). Overall, the data lead us to propose that the components of RGI transmit stresses in the wall and hence play a direct role in wall rheology.

PECTIN AS STORAGE POLYMER

Several cell wall polymers function as storage polymers that are mobilized during seed germination. While mostly hemicelluloses have this role, components of pectin may also serve as storage compounds. Galactan is highly abundant in the cotyledons of certain lupin (*Lupinus*) species (Hirst et al., 1947) and is remobilized during seed germination (Crawshaw and Reid, 1984). Recently, it was shown that polymers containing LM6 epitopes, plausibly arabinan, are remobilized during Arabidopsis seed germination and influence germination negatively if removed before germination (Gomez et al., 2009). Arabidopsis seeds contain up to 40 mol % Ara in the noncellulosic cell wall fraction. The Ara was shown by pulse-chase experiments to be metabolized during germination. Hence, the arabinan in the seeds may have a storage function, but it also has a more direct role in seed germination, as it has been shown that arabinofuranosidase acting on arabinan has an important role in expelling of the seed mucilage upon seed hydration (Arsovski et al., 2009).

BIOSYNTHESIS OF PECTIN

Matrix polysaccharides are assumed to be synthesized in Golgi vesicles, even though it cannot be excluded that some initial steps take place in the endoplasmic reticulum or that some assembly steps take place in the wall. Nevertheless, the evidence for Golgi localization is strong. Several pectic biosynthetic activities have been shown to cofractionate with Golgi markers (Powell and Brew, 1974; Goubet and Mohnen, 1999; Sterling et al., 2001; Nunan and Scheller, 2003; Geshi et al., 2004), and pectic epitopes can be found in the Golgi vesicles but not in endoplasmic reticulum (Andeme-Onzighi et al., 2000). Furthermore, the few pectic biosynthetic enzymes that have been identified have all been shown to be located in the Golgi apparatus. Given the complexity of pectin structures, it is obvious that a large number of enzymes are required to synthesize these polysaccharides. With reasonable assumptions regarding the substrate specificity of the biosynthetic enzymes, Mohnen et al. (2008) have predicted that 67 different glycosyltransferases (GTs), methyltransferases, and acetyltransferases are required. As will be seen below, only three of these enzymes have been unambiguously identified. Pectin biosynthesis has been studied for many years, and HG biosynthesis in vitro was demonstrated already in 1965 (Villemez et al., 1965). Biosynthesis of arabinan and galactan was also demonstrated long ago (McNab et al., 1968; Odzuck and Kauss, 1972; Bolwell and Northcote, 1981). Thus, quite a lot is known about biosynthetic activities in plant tissues, but it is beyond the scope of this review to give a detailed treatise of these fundamental studies. Here, we will mainly focus on the studies that have led to identification of the genes and enzymes involved in the biosynthesis of pectin. For other recent reviews of pectin biosynthesis, see Mohnen (2008), Caffall and Mohnen (2009), and Liepman et al. (2010).

HG

As mentioned above, HG biosynthesis in vitro was reported already 45 years ago. Nevertheless, in spite of a considerable amount of work by different researchers, it took very long before any of the pectin biosynthetic enzymes were identified. A major contribution was the solubilization of the HG synthase from *Nicotiana tabacum* and the development of an acceptor-dependent assay for α-1,4-GalA transferase (Doong and Mohnen, 1998). The in vitro activity indicated that endogenous α-1,4-GalA transferase transfers GalA residues to the nonreducing end of the growing HG chain (Scheller et al., 1999). The work of Mohnen and coworkers ultimately enabled the first successful identification of a pectin biosynthetic enzyme, the HG α-1,4-GalA transferase identified in Arabidopsis by Sterling et al. (2006). The enzyme, designated GALACTURONOSYLTRANSFERASE1 (GAUT1), was expressed in human kidney cells and able to synthesize polygalacturonic acid in vitro. Interestingly, the enriched membrane fraction from Arabidopsis that contained GAUT1 also contained a homologous protein, GAUT7. GAUT7 has not been shown to have activity when expressed heterologously, but the two proteins interact with each other and form a complex in vivo (Mohnen, 2008). GAUT1 is predicted to be a type II membrane protein (i.e. a protein with a single N-terminal transmembrane helix and the main globular domain inside the Golgi lumen). GTs and other carbohydrate active enzymes are cataloged in the CAZy database (www.cazy.org; Cantarel et al., 2009). CAZy has 93 GT families, 42 of which are represented in angiosperms. The GAUT group of proteins belongs to CAZy GT family 8 and has 15 members in Arabidopsis (Fig. 2). One of the members, QUASIMODO1 (QUA1; GAUT8), was actually implicated in pectin biosynthesis prior to the identification of GAUT1

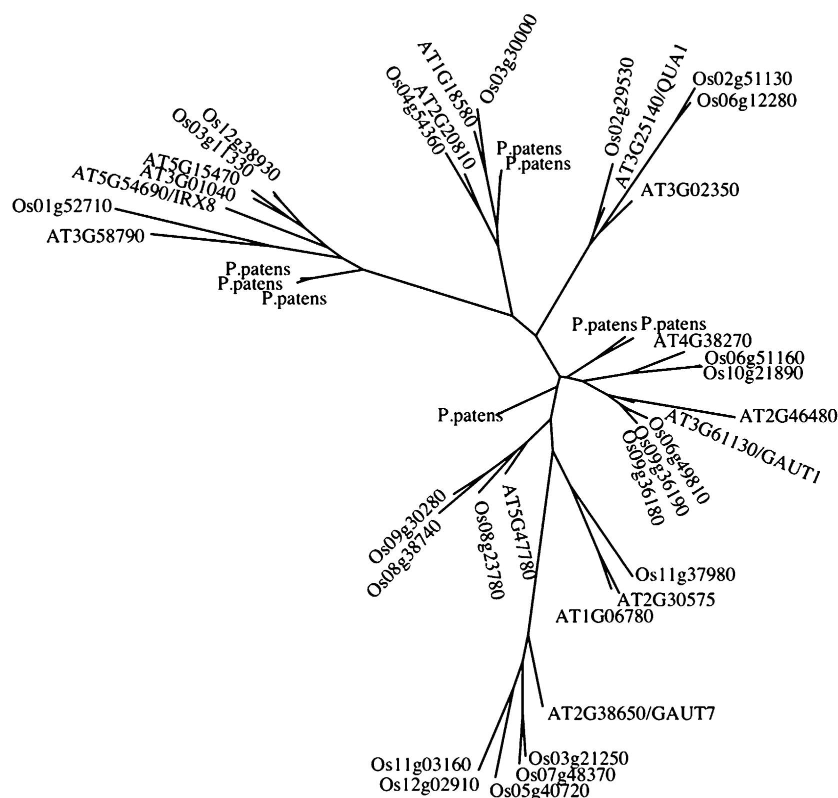

Figure 2. Phylogenetic tree of the GAUT subgroup of CAZy GT8. The tree contains sequences from Arabidopsis, rice, and *P. patens*. The tree was generated using Proml in the Phylip package.

(Bouton et al., 2002). The *qua1* mutant is highly deficient in HG, suggesting an involvement of QUA1 in pectin biosynthesis. However, the activity of QUA1 has not been reported, and although the *qua1* mutant showed decreased activity of HG GalA transferase, it also showed decreased xylan synthase activity (Orfila et al., 2005). Several potential functions have been proposed for another member of the GAUT family, GAUT12 (also known as IRREGULAR XYLEM8 [IRX8]). Loss-of-function mutations in *IRX8* result in decreased xylan content in the wall, especially a decrease in the reducing end oligosaccharide present in xylans of most plants (Pena et al., 2007). This had led to the suggestion that GAUT12 is involved in synthesizing the reducing end oligosaccharide (Pena et al., 2007). The oligosaccharide contains a GalUA residue linked to Xyl with an α-1,4 linkage, and it seems plausible that GAUT12 could synthesize this linkage. Since an obvious GAUT12/IRX8 ortholog is not present in rice (*Oryza sativa*; Fig. 2), an alternative hypothesis was proposed that GAUT12 plays an important role in glucuronoxylan biosynthesis that is specific to dicots (Persson et al., 2007; Caffall et al., 2009). The reducing end oligosaccharide that contains GalA has so far not been demonstrated in grasses; therefore, the two hypotheses are not mutually exclusive. Until the biochemical activity of GAUT12 is clearly determined, it cannot even be excluded that the effect on xylan is pleiotropic. Hence, although it is possible that all GAUT proteins are GalA transferases, it is uncertain how many are involved in pectin biosynthesis. A recent paper by Caffall et al. (2009) provides an excellent overview of the possible functions of the different GAUT proteins in Arabidopsis based on phylogeny and phenotype of insertional mutants. However, redundancy between the individual GAUTs and pleiotropic effects in the mutants complicate the picture of assigning specific activity to each protein and, with the data available at present, to conclude on the individual GAUT activities.

RGI

In vitro pectic arabinosyltransferase activity was reported as early as 1972 (Odzuck and Kauss, 1972). Later, the assays were refined using membrane preparations and verification of the product by specific enzymatic degradation (Bolwell and Northcote, 1981). As for the in vitro HG activity assays described above, the introduction of exogenous acceptors allowed a better characterization of the enzymes (Nunan and Scheller, 2003). This approach was further refined by Ishii et al. (2005a, 2005b), who used very sensitive assays with fluorescently labeled acceptor substrates and succeeded in showing transfer of Ara to both arabinan and galactan acceptors. In these studies by Scheller, Ishii, and others, UDP-L-arabinopyranose was used as substrate and Ara was incorporated into the products in the pyranose configuration. However,

the Ara in pectic arabinan and galactan is almost exclusively in the furanose configuration. UDP-L-arabinofuranose is not commercially available, but it was eventually used as a substrate by Konishi et al. (2006), who were able to demonstrate efficient in vitro transfer of arabinofuranose onto arabino-oligosaccharides. It then became clear that plants have mutases that convert UDP-arabinopyranose to UDP-arabinofuranose. The mutase activity was purified and the protein identified (Konishi et al., 2007). Surprisingly, the mutase had no similarity to bacterial UDP-galactomutase but was found to be a protein previously known as Reversibly Glycosylated Protein (RGP) due to its reversible glycosylation with a variety of UDP-sugars (Langeveld et al., 2002; Drakakaki et al., 2006). RGP is a relatively abundant protein located exterior on the Golgi vesicles. Although RGP may have additional functions, its activity as a mutase is not in doubt, since the heterologously expressed rice protein had clear UDP-arabinomutase activity (Konishi et al., 2007, 2010).

In vitro biosynthesis of galactan was reported in 1968 (McNab et al., 1968). The assays have been further developed with solubilization of the membranes and the use of exogenous acceptors (Geshi et al., 2000, 2002, 2004; Peugnet et al., 2001; Ishii et al., 2004). Activities were found in flax (*Linum usitatissimum*; Peugnet et al., 2001) and potato (Geshi et al., 2002) that could transfer Gal from UDP-Gal onto RGI acceptors, resulting in products that could be digested with galactosidase but not with endo-β-1,4-galactanase. The study of Geshi et al. (2002) showed activity only with acceptors consisting of RGI backbone with short galactan side chains, but not with RGI backbone without any Gal or with galacto-oligosaccharides. In contrast, Ishii et al. (2004) showed galactosyltransferase activity in mung bean (*Vigna radiata*) microsomes using galacto-oligosaccharides. Despite the successful efforts to develop sensitive galactan galactosyltransferase assays, none of the genes involved in the biosynthesis have been identified. Transgenic plants with a decreased content of galactan have been produced by ectopic expression of galactanase, indicating that the viability of galactan-deficient plants is not severely affected (Sorensen et al., 2000; Obro et al., 2009). β-1,4-Galactan constitutes a major part of Gal in primary walls, and galactan-deficient mutants should be readily identifiable in cell wall composition analyses. In our opinion, the lack of identified galactan biosynthetic mutants indicates redundancy both in the GTs that add the initial Gal residues to the RGI backbone and in the GTs that elongate the galactan chain.

In spite of the substantial number of biochemical studies of arabinan and galactan biosynthesis, the knowledge of the specific GTs involved in the biosynthesis of RGI and substituted HGs is sparse. Only two GTs have been published and positively identified as involved in RGI biosynthesis, namely ARABINAN DEFICIENT1 (ARAD1; At2g35100) and XYLOGALACTURONAN DEFICIENT1 (XGD1; At5g33290; Harholt et al., 2006; Jensen et al., 2008). ARAD1 and XGD1 are placed in subgroups B and C of CAZy family GT47, respectively (Fig. 3) according to the nomenclature of Li et al. (2004). Both proteins are apparent type II membrane proteins; based on localization studies of fluorescent fusion proteins, they are located in the Golgi apparatus (Harholt et al., 2006; Jensen et al., 2008; Sakuragi et al., 2010). ARAD1 is a putative arabinosyltransferase. Biochemical analysis of the loss-of-function *arad1* mutants showed a reduced amount of Ara in the cell wall (Harholt et al., 2006). Further characterization showed that the arabinan side chains appeared to be of equal number but smaller than in the wild type. Heterologous expression of ARAD1 in *Escherichia coli*, *Pichia pastoris*, and *Nicotiana benthamiana*, and attempts to demonstrate arabinosyltransferase activity by in vitro or in vivo assays, have so far been unsuccessful in spite of a substantial effort (A. Suttangkakul, J.K. Jensen, N. Geshi, T. Konishi, T. Ishii, and H.V. Scheller, unpublished data).

Subgroup B of CAZy GT47 includes, besides ARAD1, seven Arabidopsis homologs (Fig. 3A). The closest homolog to ARAD1 is designated ARAD2 (At5g44930). ARAD1 and ARAD2 are apparently not redundant, since expression of *ARAD2* under the control of the *35S* promoter in *arad1* does not complement the arabinan deficiency of *arad1* (J.K. Jensen, J. Harholt, and H.V. Scheller, unpublished data). However, immunohistochemical analysis of roots and inflorescence stems in *arad1*, *arad2*, and *arad1 arad2* using the arabinan antibody LM13 showed different labeling from the wild type in all three mutants (Y. Verhertbruggen, J.P. Knox, J. Harholt, and H.V. Scheller, unpublished data). Furthermore, *arad2* labeling had a distinct pattern from those in *arad1* and *arad1 arad2*. The remaining members of CAZy GT47 subgroup B are still unassigned. Cell wall composition analysis of knockout mutants of some of the remaining CAZy GT47B members has not led to the discovery of new cell wall phenotypes (J. Harholt and H.V. Scheller, unpublished data).

GTs involved in the biosynthesis of pectin and hemicelluloses have been hypothesized to function in complexes by several authors (Scheller et al., 2007; Mohnen, 2008; Ralet et al., 2008). It seems logical that such complexes could exist, but actual data to support this are limited. The only strong evidence is the observation, described above, that GAUT1 and GAUT7 are together in a complex involved in the biosynthesis of HG (Mohnen, 2008). Another argument for the existence of complexes is the observation that incorporation of arabinofuranose into polysaccharides takes place via the intermediate UDP-arabinofuranose (Konishi et al., 2006). The furanose form of UDP-Ara has never been found in plants and is energetically unfavorable, suggesting that UDP-arabinofuranose is strongly channeled (i.e. the mutase that converts UDP-arabinpyranose to UDP-arabinofuranose likely delivers the product directly to the arabinosyltransferase). Furthermore, the mutase is a soluble

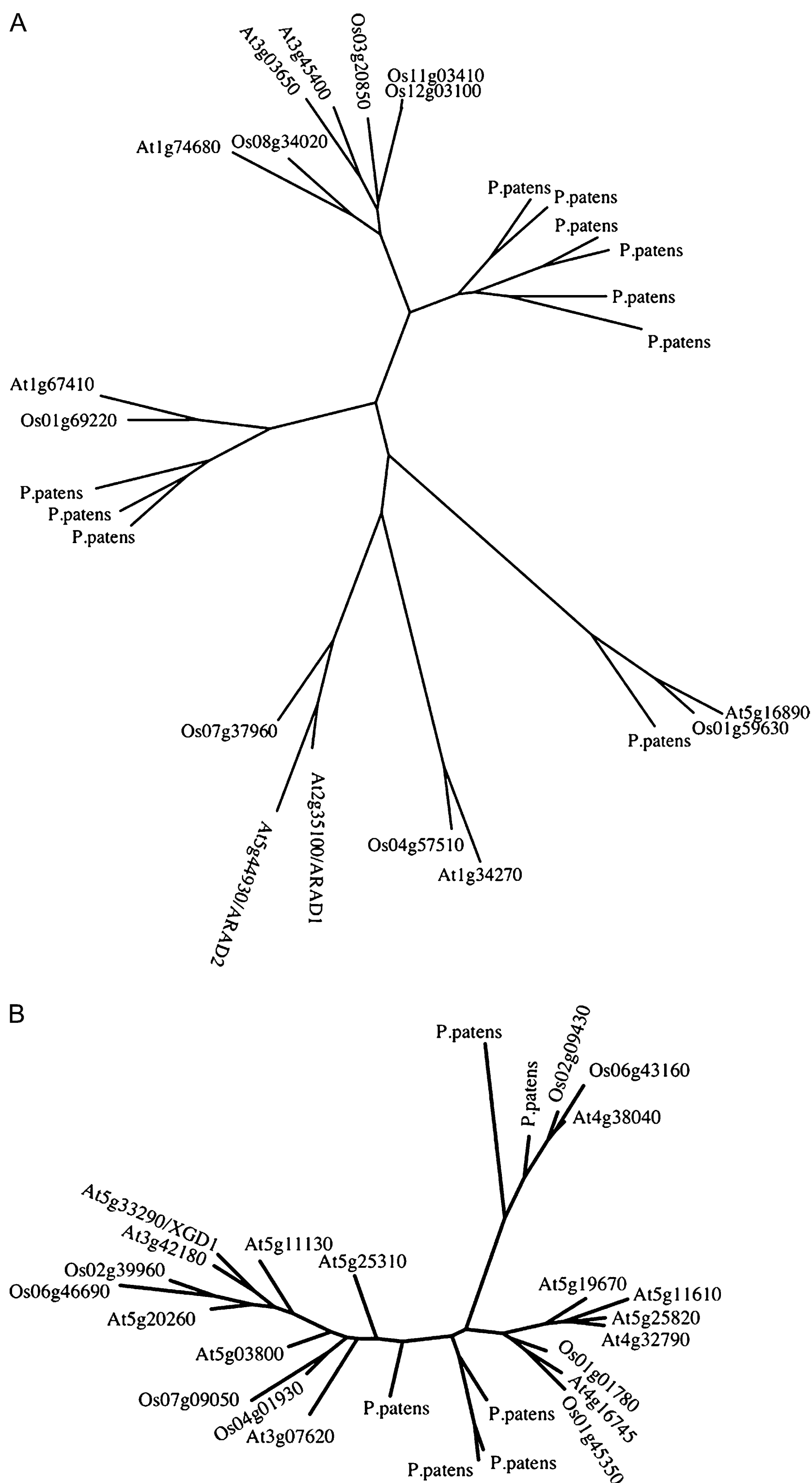

Figure 3. Phylogenetic trees of CAZy GT47 subgroup B (A) and subgroup C (B). The trees contain sequences from Arabidopsis, rice, and *P. patens*. The trees were generated using Proml in the Phylip package.

protein but located on the cytosolic side of Golgi membranes, suggesting that it interacts with integral membrane proteins in the Golgi. These considerations suggest that the enzymes are present in a complex or metabolon. Such interactions between donor sugar biosynthetic proteins and GTs have not been unambiguously demonstrated, but UGE4, an isoform of UDP-Glc 4-epimerase, was shown to be associated with galactosylation of xyloglucan but not of pectic galactan (Nguema-Ona et al., 2006). This apparent channeling of the product of UGE4 supports the hypothesis that donor sugar biosynthetic proteins

could reside along with GTs in complexes. ARAD1 and ARAD2 are both involved in arabinan biosynthesis and therefore also are good candidates for the identification of complex formation. The CAZy GT47B proteins contain conserved C-terminal Cys residues, suggesting that disulfide bonds could play a role in complex formation. ARAD1 and/or ARAD2 were transiently expressed in *N. benthamiana* cells and analyzed by immunoblotting under reducing and nonreducing conditions. Under nonreducing conditions, the M_r of ARAD1 or ARAD2 protein appeared to be larger than expected (C. Søgaard, J. Harholt, and H.V. Scheller, unpublished data). ARAD1/ARAD2, ARAD1/ARAD1, and ARAD2/ARAD2 complexes were indicated by this approach and by bimolecular fluorescence complementation (S. Bernhard, Y. Sakuragi, J. Harholt, and H.V. Scheller, unpublished data).

The XGA xylosyltransferase XGD1 is placed in CAZy GT47 subgroup C (Fig. 3B). Sugar composition and linkage analysis of loss-of-function mutants showed a substantially decreased content of XGA in the mutants (Jensen et al., 2008). Hydrolysis with a specific XGA endohydrolase confirmed the reduced XGA content. Transient expression of *XGD1* in *N. benthamiana* cells and in vitro assays using microsomal preparations showed transfer of Xyl from UDP-Xyl to GalUA oligomers, thereby confirming the function of XGD1 as a XGA xylosyltransferase. Immunolabeling of *xgd1* mutants using the XGA-specific LM8 antibody gave two surprising results. First, LM8 did not detect the major XGA present in vegetative tissues but only epitopes in the root tips and siliques. Second, the LM8-positive XGA was not reduced in the *xgd1* mutant. This indicates that there are orthologous activities to XGD1, possibly encoded by the other members of CAZy GT47C.

RGII

RGII biosynthesis has not been much studied. A group of proteins found to be involved in RGII biosynthesis is the Arabidopsis RGXT family in subgroup B of CAZy GT77. Members of this family with characterized functions include RGXT1, RGXT2, and RGXT3 (Egelund et al., 2006, 2008). These proteins have α-1,3-xylosyltransferase activity and can transfer Xyl from UDP-Xyl onto Fuc. This glycosidic linkage is only present in RGII, suggesting the role of this protein family in pectin biosynthesis. The activity of RGXT1, RGXT2, and RGXT3 has been tested in vitro using exogenously expressed proteins from various systems, including *Baculovirus*-transfected insect cells (Egelund et al., 2006, 2008), *P. pastoris* (Petersen et al., 2009), and transient expression in *N. benthamiana* leaves and *E. coli* (A. Oikawa, A. Suttangkakul, and H.V. Scheller, unpublished data). Besides Fuc, α-methyl-fucoside and α-Fuc-(1,4)-Rha, but not β-methyl-fucoside, function well as acceptors. Compared with other heterologously expressed GTs for which biochemical activity has been observed, the RGXT proteins are remarkably robust and show high activity. RGXT1 and RGXT2 can also add Xyl onto RGII acceptor substrates isolated from *rgxt1* and *rgxt2* mutant plants but not those isolated from the wild type (Egelund et al., 2006). This difference illustrated the subtle defects in RGII extracted from *rgxt* mutants that failed to be detected in sugar analysis of cell walls and physiological characterization of the mutants. Since the RGXT family in Arabidopsis has four members, with three shown to have similar biochemical function in vitro, it is likely that the lack of physiological phenotype is due to functional redundancy. Insertional mutations are available for At4g01220, the only member of this Arabidopsis family that has not been characterized. However, the lack of available mutations in *RGXT3* as well as the close genomic proximity of *RGXT1* and *RGXT2* prevent the generation of quadruple mutants to address the functional redundancy in the family.

RGII, with its complex structure and unique sugar linkages, must require a large number of GTs for its synthesis. However, neither forward nor reverse genetics has led to the identification of any of these. This may be due to redundancy (as for RGXTs) or the lethality of mutations. A cell adhesion mutant in *Nicotiana plumbaginifolia* was reported to be deficient in GlcUA residues of RGII, suggesting that the affected protein, designated NpGUT1 (for glucuronosyltransferase 1), was specifically involved in RGII biosynthesis (Iwai et al., 2002). However, apparent orthologous genes in Arabidopsis, designated *IRX10* and *IRX10L*, are strongly implicated to be involved in xylan biosynthesis, and the corresponding Arabidopsis mutants have no change in RGII structure (Wu et al., 2009). Wu et al. (2009) furthermore presented evidence suggesting that NpGUT1 and IRX10/IRX10L are indeed orthologs. This strongly suggests that the effect on RGII in the *N. plumbaginifolia* mutant was a pleiotropic effect and that NpGUT1 is not directly involved in RGII biosynthesis.

METHYLATION, ACETYLATION, AND FERULOYLATION

Methyl esterification of pectin using *S*-adenosyl-Met as methyl donor has been shown in vitro (Goubet and Mohnen, 1999), but no methyltransferases have been unambiguously identified. A mutant designated *qua2* was shown to be deficient in pectin, and when the mutated gene was identified, it turned out to be a putative methyltransferase (Mouille et al., 2007). The QUA2 enzyme is a member of a large group of putative methyltransferases, but their biochemical activity has not been confirmed and it is not known how many of the enzymes are involved in pectin biosynthesis. Finding a large group of pectin methyltransferases would perhaps not be so surprising, given that the Arabidopsis genome encodes no less than 67 putative pectin methylesterases (CAZy family CE8). Control-

ling the degree of pectin methylation must be very important for plants.

Pectin acetylation with acetyl-CoA as the acetate donor has also been demonstrated in vitro (Pauly and Scheller, 2000). As for methylation, the process appears to take place in Golgi vesicles. Recently, two allelic Arabidopsis mutants have been identified that have decreased levels of pectin acetylation in leaves (Scheller, 2010; Y. Manabe and H.V. Scheller, unpublished data). The mutants, designated *reduced wall acetylation2* (*rwa2*), have inactivation of the At3g06550 locus. The RWA2 protein is homologous to a protein involved in acetylation of the coat polysaccharide in the fungus *Cryptococcus neoformans* (Janbon et al., 2001). Arabidopsis has three additional homologs of the RWA2 protein, and these are most likely involved in acetylation in different tissues. At present, it is unclear if the proteins are indeed acetyltransferases or have another function (e.g. as transporters). The RWA proteins are integral membrane proteins with many transmembrane segments. It is also not clear if the proteins are specific for pectin, but most likely this is not the case. One of the homologs in Arabidopsis and a homolog in poplar (*Populus* species) are highly expressed in tissues with secondary wall synthesis, which contain little pectin. Therefore, the RWA homologs expressed in these tissues may be primarily involved in xylan acetylation rather than pectin acetylation.

In Chenopodiaceae species (e.g. spinach [*Spinacia oleracea*] and sugar beet [*Beta vulgaris*]), RGI side chains are feruloylated by attachment of ferulic acid to O-3 of Ara in arabinans or to O-4 of Gal in galactans (Fry, 1982). Jones et al. (2005) have shown that ferulic acid esterase and arabinanase affect stomatal function in plants outside Chenopodiaceae, and it may be that pectic feruloylation is taxonomically widespread but of low abundance. Recently, genes involved in feruloylation of arabinoxylan have been identified in rice (Mitchell et al., 2007; Piston et al., 2010). These genes belong to a large family encoding CoA-acyl transferases. At the moment, members of this family are the most likely candidates for pectin feruloyl transferases, but no evidence for or against this hypothesis has been presented.

EVOLUTIONARY ASPECTS OF PECTIN BIOSYNTHESIS

The recent sequencing of *Selaginella moellendorffii*, a heterosporus lycopsid, and the availability of the genome sequence of the moss *Physcomitrella patens* give new insight into the evolution of pectin biosynthesis.

The synthesis of HG and probably also the RGII backbone is catalyzed by the *GAUT* group of genes in GT8, as described above. GAUT1 has *S. moellendorffii* and *P. patens* orthologs, in line with the presence of HG in both species (Moller et al., 2007). As described above, GAUT7 in Arabidopsis forms a complex with GAUT1 and is presumably involved in HG biosynthesis as well (Mohnen, 2008). However, there is no GAUT7 homolog in either *S. moellendorffii* or *P. patens*. QUA1 is also involved in HG biosynthesis, even though the exact activity of QUA1 has not been established and pleiotropic effects have been observed (Bouton et al., 2002; Orfila et al., 2005). *P. patens* does not contain any QUA1 ortholog, whereas *S. moellendorffii* does, pointing to a possible gradual evolution of HG biosynthesis, with complex formation requiring GAUT7 in angiosperms as the most recent development.

S. moellendorffii ARAD1 is a close homolog of Arabidopsis *ARAD1*, but no *P. patens ARAD1* ortholog could be found (Fig. 3A). Since arabinan is present in *P. patens* cell walls, other members of the GT47B clade could be anticipated to be involved in arabinan biosynthesis. The Arabidopsis *XGD1* lacks orthologs in both *P. patens* and *S. moellendorffii*. Interestingly, the most similar *S. moellendorffii* gene, *GT47C1*, occupies a separate subclade, which also has a *P. patens* member. The presence or absence of XGA in *S. moellendorffii* and *P. patens* has not been reported. *S. moellendorffii* has orthologous genes to *RGXT*. While Arabidopsis apparently has three or four *RGXT* paralogs (Egelund et al., 2006, 2008) in the RGXT clade, rice, *P. patens*, and *S. moellendorffii* have a single *RGXT* ortholog. Whether RGII is present in mosses has not been determined unambiguously. Some of the rare sugars of RGII have been found in mosses, but in concentrations 100-fold lower than expected if compared with higher plants (Matsunaga et al., 2004). The presence of an RGXT ortholog in *P. patens* suggests the occurrence of at least part of RGII in mosses.

The seeming omnipresence of pectin throughout plant evolution calls for a look at algae cell walls to determine the presence of pectins. The charophycean and chlorophycean algae are known to contain HG in their cell walls (Cherno et al., 1976; Domozych et al., 2007; Estevez et al., 2008). RGI and its side chains of arabinan and galactan or RGII, to our knowledge, have not been identified in algae. Labeling with LM5 and LM6 antibodies, which recognize galactans and arabinans, respectively, has been observed in Chlorophyta, but the epitopes were mainly found in the cytoplasm (Estevez et al., 2008). Genomic sequences are available for bryophytes (*P. patens*), lycophytes (*S. moellendorffii*), and several angiosperm species (e.g. rice and Arabidopsis). However, because the wall polysaccharides found in these species are surprisingly similar, the comparative genome analysis is unlikely to yield much new evidence for putative functions of GTs. In contrast, the known differences in cell walls between charophycean and chlorophycean species and the land plants suggest that a comparative genomic study would be very revealing and could give valuable information on putative functions of GTs and many other aspects of cell wall biosynthesis.

PERSPECTIVES AND FUTURE DIRECTIONS

Pectin biosynthesis requires many enzymes, the large majority of which have not been identified. Reverse genetics of a very large number of GTs has not led to the discovery of many enzymes involved in pectin biosynthesis. Perhaps this is due to redundancy, so that simultaneous mutations in more than one gene homolog are necessary to see significant changes in wall composition or visual phenotype. This then raises the question of what strategies will be most promising for identifying the remaining biosynthetic enzymes. A systematic analysis of the activity of heterologously expressed GTs may be a good strategy. However, there is a paucity of general assays for GT activity. Furthermore, it may well be that many of the pectin biosynthetic enzymes function in complexes and that the individual GT proteins have little or no activity. These technical difficulties suggest that it may be prudent to focus the efforts on the most promising and interesting candidates. Coexpression analysis has proven to be a very powerful method to identify proteins, including GTs, involved in secondary wall biosynthesis (Brown et al., 2005; Persson et al., 2005). The expression profiles for pectin biosynthetic enzymes are bound to be less distinct; nevertheless, such methods may be useful to short list GTs that are likely to be involved in pectin biosynthesis. Expression analysis, or proteomics, of tissues with dominating synthesis of a particular polysaccharide is a related method to identify GTs involved in synthesizing that particular polysaccharide. Cellulose synthase and mannan synthase were identified in this way (Pear et al., 1996; Dhugga et al., 2004). Some plant organs are highly enriched in pectin (e.g. certain fruits). Seeds of some legumes (e.g. lupin) are highly enriched in pectic galactan, which serves as a storage polysaccharide. Hence, deep sequencing of expressed genes in such plant tissues and organs would likely lead to candidates for further analysis. Deep sequencing of developing lupin seeds has been undertaken in order to identify putative candidates for GTs involved primarily in galactan biosynthesis. A finding of potential relevance to galactan biosynthesis was the high expression of members of the C3 group of GT47 (B. Borkhardt and P. Ulvskov, unpublished data). GT47 with its inverting activities could contain galactan galactosyltransferases, and a closer look at GT47C proteins and corresponding mutants would be interesting. Manfield et al. (2004) used microarrays to compare the expression profile of isoxaben-habituated suspension cells with unhabituated cells. Since isoxaben, an inhibitor of cellulose synthesis, leads to increased deposition of noncellulosic polysaccharides, including pectin, putative pectic biosynthetic GTs might be identified, but unfortunately no obvious candidates were identified.

In addition to the challenge of assigning functions to GTs found in CAZy by biochemical or reverse genetic approaches, it is now also clear that there are multiple families of GTs not found in CAZy. Several reports on bioinformatic work leading to the discovery of putative GTs outside CAZy have been published (Egelund et al., 2004; Manfield et al., 2004; Hansen et al., 2009). Of these putative GTs, a family designated DUF266 has subsequently been shown to contain GTs involved in cell wall biosynthesis (Zhou et al., 2009). The DUF266 family is still not part of CAZy, and it exemplifies that some of the major activities missing in our knowledge of pectin biosynthesis could reside outside CAZy.

Pectin has many features, and perhaps perturbations to minor side chains of arabinans and galactans would not be readily observed by the approaches currently utilized in the effort to elucidate pectin biosynthesis. However, it may seem surprising that no candidates for the major galactan biosynthetic enzymes or the enzymes involved in synthesizing the RGI backbone have emerged. The RGI backbone is also a major component of pectin, yet no Rha-deficient mutant affected in a GT has been reported. The biosynthesis of the backbone has not been studied, possibly because the UDP-Rha substrate needed for such studies is not available and is not easy to make. The rhamnosyltransferases should be inverting enzymes, but again we have no good candidates. Interestingly, the reducing end oligosaccharide of xylan contains a Rha residue; hence, there must be a rhamnosyltransferase involved in its synthesis. Several Arabidopsis mutants, *irx7*, *irx8*, and *parvus*, are deficient in synthesizing the oligosaccharide, but no obvious candidate for the rhamnosyltransferase has emerged from those studies (Scheller and Ulvskov, 2010). We think IRX7 is more likely to be a xylosyltransferase, and the other enzymes, IRX8/GAUT12 and PARVUS, are predicted to be retaining rather than inverting, as expected for the rhamnosyltransferase. The α-Rha-1,2-α-GalA linkage in the reducing end of xylan resembles the α-Rha-1,4-α-GalA linkage found in RGI; hence, identification of the rhamnosyltransferase involved in xylan biosynthesis may provide candidates for other rhamnosyltransferases, including those involved in RGI biosynthesis.

RGII contains five Rha residues, all linked in different ways. Several other enzymes are needed for RGII biosynthesis, yet only RGXT has been identified. It seems surprising that not more of these enzymes have been discovered, given that RGII mutants are expected to show a pronounced phenotype. For RGXT, the reason for the weak phenotype is clearly that there are several redundant genes involved. Can this also be the case for all the other GTs required? It seems that there are not enough GTs encoded by the genome of Arabidopsis if each would have perhaps three to four redundant forms. We have previously proposed that the same set of GTs may form complexes with different composition and different biochemical activities (Scheller et al., 2007). This could explain both the extensive redundancy and the somewhat limited number of GTs with unassigned function. It could also explain the difficulty in determining the biochemical

functions of heterologously expressed GTs. The idea that GT activities can be modulated by the presence of other proteins is supported by the classical example of mammalian β-1,4-galactosyltransferase, which changes its acceptor sugar from GlcNAc to Glc in the presence of α-lactalbumin (Ramakrishnan et al., 2002).

With the improved biochemical tools, the ever-increasing amounts of genomic data, and the increased funding for bioenergy research, we can be hopeful that significant advances in our understanding of pectin biosynthesis can be achieved. Pectin is an important product, especially as an ingredient in the food industry. Knowledge of the biosynthesis of pectin should enable tailoring of pectins for specific purposes and could enable the use of pectin-rich raw materials that are currently not used because of their poor functionality. For example, potato and sugar beet pulp are available in large quantities but are not exploited as raw materials for pectin production. Pectin is not a major component of biomass feedstocks for biofuel production, which consist mostly of secondary cell walls with a low pectin content; nevertheless, the fine structure of pectin has an important impact on biomass recalcitrance (Lionetti et al., 2010). It is essential to get a better understanding of the GTs and other enzymes that directly synthesize the pectin polysaccharides, and this must be an important goal of current research efforts. However, one should also be aware that this is only the tip of the iceberg. Other enzymes play important roles in reshaping the polymers after they are synthesized, and plants have an even greater repertoire of carbohydrate-active hydrolases, esterases, and lyases than of synthetic enzymes. Furthermore, the polysaccharides are integrated into the wall by mechanisms that are not understood; surely, self-assembly is an important principle of wall assembly, but unknown factors may play a role as well. Finally, the wall composition is regulated by sensing and signal transduction pathways that we are only beginning to unravel (Hematy et al., 2007; Kohorn et al., 2009). To find out how all these processes work together is the next big challenge.

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