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Arabinogalactan proteins: focus on carbohydrate active enzymes

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Arabinogalactan proteins (AGPs) are a highly diverse class of cell surface proteoglycans that are commonly found in most plant species. AGPs play important roles in many cellular processes during plant development, such as reproduction, cell proliferation, pattern formation and growth, and in plant-microbe interaction. However, little is known about the molecular mechanisms of their function. Numerous studies using monoclonal antibodies that recognize different AGP glycan epitopes have shown the appearance of a slightly altered AGP glycan in a specific stage of development in plant cells. Therefore, it is anticipated that the biosynthesis and degradation of AGP glycan is tightly regulated during development. Until recently, however, little was known about the enzymes involved in the metabolism of AGP glycan. In this review, we summarize recent discoveries of carbohydrate active enzymes (CAZy; http://www.cazy.org/) involved in the biosynthesis and degradation of AGP glycan, and we discuss the biological role of these enzymes in plant development.

Keywords: arabinogalactan proteins, type II arabinogalactan, plant cell wall, carbohydrate active enzymes, glycosyltransferase, glycoside hydrolase

INTRODUCTION

Arabinogalactan proteins (AGPs) are a family of proteoglycans found on the plasma membrane and in the cell walls of diverse species of plants. AGPs are synthesized by several post-translational modifications of proteins in the secretory pathway. The proteins generally contain repetitive dipeptide motifs, e.g., Ala-Pro, Ser-Pro, Thr-Pro, and Val-Pro, which are distinguished from the sequence motifs for extensin type glycosylation [e.g., Ser-(Pro)2,3 known as another major class of O-glycosylation in plants (Kieliszewski, 2001)]. The Pro residues are hydroxylated by prolyl 4-hydroxylases and further O-glycosylated by glycosyltransferases (GTs). Moreover, many AGPs are attached by a glycosylphosphatidylinositol anchor, which attaches AGPs to the plasma membrane, but can be cleaved by phospholipases (Wang, 2001; Schultz et al., 2004). AGPs on the plasma membrane and cell wall may also be processed by proteolytic activities and glycosyl hydrolases or transported by endocytotic multivesicular bodies to wall may also be processed by proteolytic activities and glycosyl phosphatidylinositol anchor, which attaches AGPs to the plasma membrane, but can be cleaved by phospholipases (Wang, 2001; Schultz et al., 2004). AGPs on the plasma membrane and cell wall may also be processed by proteolytic activities and glycosyl hydrolases or transported by endocytotic multivesicular bodies to the vacuole where they are degraded (Herman and Lamb, 1992).

The glycan moieties of AGPs accounts for more than 90% of their total mass, which has been suggested to play an essential role in the function of AGPs, based on studies using synthetic phenylglycoside dyes (β-Yariv reagents) that specifically binds to the β-1,3-galactan moiety of AGPs (Kitazawa et al., 2013) as well as various monoclonal antibodies that recognize different AGP glycan epitopes (Seifert and Roberts, 2007). However, because of its complexity and heterogeneity, little is known about the structure-function relationship of AGP glycan. In fact, various structures have been reported for AGP glycan depending on samples and analytical methods. The common structural feature is a backbone of β-1,3-galactan, which is often substituted at O6 with side chains of β-1,6-galactan decorated further with arabinose, and less frequently also with fucose, rhamnose, and (methyl) glucuronic acid (Figures 1A,B). Tan et al. (2010) proposed that the backbone is composed of a repeat of a β-1,3-galactotriose unit with or without side chains, which is connected by β-1,6-linkages (kinks). This model is based on the AGPs synthesized onto synthetic peptides expressed in tobacco cells and analyzed by NMR (Tan et al., 2004, 2010). In this model, the side chains are rather short and composed of a single Gal decorated by 1–5 other sugars. However, longer β-1,6-galactan side chains have been reported for AGPs from radish root (Haque et al., 2005), wheat flour (Tryfona et al., 2010) and Arabidopsis leaf (Tryfona et al., 2012) based on the linkage and mass spectroscopy analysis.

Knowledge about each enzyme working on an individual step in the biosynthesis and degradation of AGP glycan is useful to understand the role of a particular sugar moiety of AGPs. This review outlines the recent findings for the carbohydrate active enzymes (CAZy; http://www.cazy.org/, Lombard et al., 2014) identified to be responsible for the biosynthesis and degradation of AGPs. The reader is referred to other excellent reviews for other topics with respect to structure, cell biological functions (Seifert and Roberts, 2007), localization, and commercial interests of AGPs (Nothnagel, 1997; Schultz et al., 1998; Majewska-sawka and Nothnagel, 2000; Gaspar et al., 2001; Showalter, 2001; Ellis et al., 2010; Tan et al., 2012).

GLYCOSYLTRANSFERASES INVOLVED IN AGP BIOSYNTHESIS

A large number of functionally distinct GTs are required for the biosynthesis of complex AGP glycan, e.g.,

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GLYCOSYLTRANSFERASES INVOLVED IN AGP BIOSYNTHESIS

A large number of functionally distinct GTs are required for the biosynthesis of complex AGP glycan, e.g.,
The first step in the glycosylation of AGPs is the transfer of Gal to hydroxyproline residues present in the peptide backbone. The Arabidopsis enzyme catalyzing this step was identified (At4g21060, AtGALT2, Basu et al., 2013). This enzyme belongs to the CAZy family GT31 and the recombinant protein expressed in Pichia pastoris demonstrated GalT activity transferring a Gal to hydroxyproline residues in the synthetic AGP peptides. Arabidopsis T-DNA knockout mutants contained reduced levels of γ-arabin precipitable AGPs and microsomes purified from mutants exhibited reduced levels of GalT activity compared to wild type. Since several sugars were altered in the mutant AGPs lines, it is unlikely that the observed phenotype is due to normal plant embryogenesis. How AtGALT31A that is expressed in suspensor cells influences cell division in hypophysis remains unknown.

Figure 1A demonstrates GalT activity transferase activity of heterologously expressed protein in N. benthamiana showed β-1,3-galactosyltransferases (GalTs), β-1,6-GalTs, α-1,3- and α-1,5-arabinosyltransferases, fucosyltransferases, rhamnosyltransferases, glucuronyltransferases, and glucuronic acid methyltransferases. Several GTs identified to date (AtGALT31A and Table 1) are summarized below.

**β-GALACTOSYLTRANSFERASES**

The first step in the glycosylation of AGPs is the transfer of Gal to hydroxyproline residues present in the peptide backbone. The Arabidopsis enzyme catalyzing this step was identified (At4g21060, AtGALT2, Basu et al., 2013). This enzyme belongs to the CAZy family GT31 and the recombinant protein expressed in Pichia pastoris demonstrated GalT activity transferring a Gal to hydroxyproline residues in the synthetic AGP peptides. Arabidopsis T-DNA knockout mutants contained reduced levels of γ-arabin precipitable AGPs and microsomes purified from mutants exhibited reduced levels of GalT activity compared to wild type. The mutant lines showed no detectable growth phenotype under normal growth conditions. Since the GalT activity was not completely abolished in the mutant microsomes, redundant activities encoded by other genes most likely exist. Nevertheless, based on this study, the quantity of AGP-glycans appears to not be crucial for plant development.

Another Arabidopsis GT from family GT14 encoded by At1g32930 was also characterized. Recombinant enzyme expressed in Escherichia coli and Nicotiana benthamiana demonstrated β-1,6-GalT activity elongating β-1,6-galactan and forming 6-Gal branches on β-1,3-galactan of AGP glycans (Dilokpimol et al., 2014). Moreover, Förster resonance energy transfer analysis revealed an interaction between AtGALT29A and AtGALT31A when both proteins are expressed as C-terminal fluorescent fusion proteins in N. benthamiana (Dilokpimol et al., 2014). The protein complex containing heterologously expressed AtGALT29A and AtGALT31A were purified and demonstrated increased levels of β-1,6-GalT activities by the AtGALT29A single enzyme. These results suggest cooperative action between AtGALT31A and AtGALT29A by forming an enzyme complex, which could be an important regulatory mechanism for producing β-1,6-galactan side chains of type II AG during plant development.

**β-GLUCURONOSYLTRANSFERASE**

An Arabidopsis GT from family GT14 encoded by At1g32930 was also characterized. Recombinant enzyme expressed in E. coli and N. benthamiana demonstrated β-1,6-GalT activity elongating β-1,6-galactan side chains of AGP glycans in **in vitro** assays (AtGALT31A; Geshi et al., 2013). AtGALT31A is expressed specifically in the suspensor cells of the embryo proper and T-DNA insertion lines showed abnormal cell division in the hypophysis and arrested further development of embryos. Therefore, functional AtGALT31A is essential for normal plant embryogenesis. How AtGALT31A that is expressed in suspensor cells influences cell division in hypophysis remains unknown.

AtGALT29A (At1g08280) was identified as a gene co-expressed with AtGALT31A. Recombinant enzyme expressed in N. benthamiana demonstrated β-1,6-GalT activities elongating β-1,6-galactan and forming 6-Gal branches on β-1,3-galactan of AGP glycans (Dilokpimol et al., 2014). Moreover, Förster resonance energy transfer analysis revealed an interaction between AtGALT29A and AtGALT31A when both proteins are expressed as C-terminal fluorescent fusion proteins in N. benthamiana (Dilokpimol et al., 2014). The protein complex containing heterologously expressed AtGALT29A and AtGALT31A were purified and demonstrated increased levels of β-1,6-GalT activities by the AtGALT29A single enzyme. These results suggest cooperative action between AtGALT31A and AtGALT29A by forming an enzyme complex, which could be an important regulatory mechanism for producing β-1,6-galactan side chains of type II AG during plant development.
Table 1 | Characterized GTs and GHs, which process AGP-glycans.

<table>
<thead>
<tr>
<th>CAZy Family</th>
<th>Activity</th>
<th>Protein name</th>
<th>Origin</th>
<th>No. of genes in Arabidopsis</th>
<th>CAZy name</th>
<th>Evidence</th>
<th>Comments for enzyme activities and genetic manipulations</th>
</tr>
</thead>
<tbody>
<tr>
<td>GT14</td>
<td>β-glucuronosyltransferase</td>
<td>AtGlcAT14A</td>
<td>Arabidopsis thaliana</td>
<td>1</td>
<td>HE-P</td>
<td>GlcAT activity to β-1,3 and β-1,6-galactan; [GlcA]↓, [Gal]↑, [Ara]↓ in AG and enhanced cell elongation in seedlings in atglcat14a</td>
<td>Knoch et al., 2013</td>
</tr>
<tr>
<td>GT14</td>
<td>β-glucuronosyltransferase</td>
<td>AtGlcAT14B</td>
<td>Arabidopsis thaliana</td>
<td>1</td>
<td>HE-P</td>
<td>GlcAT activity to β-1,3 and β-1,6-galactan; [GlcA]↓, [Gal]↑, [Ara]↓ in AG and enhanced cell elongation in seedlings in atglcat14a</td>
<td>Knoch et al., 2013</td>
</tr>
<tr>
<td>GT14</td>
<td>β-glucuronosyltransferase</td>
<td>AtGlcAT14C</td>
<td>Arabidopsis thaliana</td>
<td>1</td>
<td>HE-P</td>
<td>GlcAT activity to β-1,3 and β-1,6-galactan; [GlcA]↓, [Gal]↑, [Ara]↓ in AG and enhanced cell elongation in seedlings in atglcat14a</td>
<td>Knoch et al., 2013</td>
</tr>
<tr>
<td>GT14</td>
<td>β-glucuronosyltransferase</td>
<td>AtGlcAT14D</td>
<td>Arabidopsis thaliana</td>
<td>1</td>
<td>HE-P</td>
<td>GlcAT activity to β-1,3 and β-1,6-galactan; [GlcA]↓, [Gal]↑, [Ara]↓ in AG and enhanced cell elongation in seedlings in atglcat14a</td>
<td>Knoch et al., 2013</td>
</tr>
<tr>
<td>GT29</td>
<td>β-1,6-galactosyltransferase</td>
<td>AtGALT29A</td>
<td>Arabidopsis thaliana</td>
<td>3</td>
<td>HE-N</td>
<td>β-1,6-GalT activity to β-1,3 and β-1,6-galactan; interaction with AtGALT31A enhances the activity</td>
<td>Dilokpimol et al., 2014</td>
</tr>
<tr>
<td>GT31</td>
<td>Hydroxyproline O-galactosyltransferase</td>
<td>AtGALT32</td>
<td>Arabidopsis thaliana</td>
<td>3</td>
<td>HE-P</td>
<td>GalT activity to hydroxylproline; [Yariv-precipitable AG]↓ in galt2; no detectable growth phenotype under normal growth condition</td>
<td>Basu et al., 2013</td>
</tr>
<tr>
<td>GT31</td>
<td>Hydroxyproline O-galactosyltransferase</td>
<td>AtGALT31A</td>
<td>Arabidopsis thaliana</td>
<td>1</td>
<td>HE-N</td>
<td>β-1,6-GalT activity elongating β-1,6-galactan; mutant is embryo-lethal</td>
<td>Geshi et al., 2013</td>
</tr>
<tr>
<td>GT37</td>
<td>α-1,2-fucosyltransferase</td>
<td>AtFUT4</td>
<td>Arabidopsis thaliana</td>
<td>10</td>
<td>HE-B</td>
<td>FucT activity to AGPs from BY2; [Fuc]↓ in AG in fut4 and fut6; no [Fuc] in AG from fut4/fut6; no detectable growth phenotype under normal growth condition, but reduced root growth under salt stress</td>
<td>Wu et al., 2010; Liang et al., 2013; Tryfona et al., 2014</td>
</tr>
<tr>
<td>GT37</td>
<td>α-1,2-fucosyltransferase</td>
<td>AtFUT6</td>
<td>Arabidopsis thaliana</td>
<td>18</td>
<td>HE-E</td>
<td>FucT activity to AGPs from BY2; [Fuc]↓ in AG in fut4 and fut6; no detectable growth phenotype under normal growth condition, but reduced root growth under salt stress</td>
<td>Wu et al., 2010; Liang et al., 2013; Tryfona et al., 2014</td>
</tr>
<tr>
<td>GT77</td>
<td>Arabinofuranosyltransferase</td>
<td>AtRAY1</td>
<td>Arabidopsis thaliana</td>
<td>2</td>
<td>HE-N</td>
<td>β-ArafT activity to methyl β-1,3-linked Ara in AG of ray1, slower root growth</td>
<td>Gille et al., 2013</td>
</tr>
<tr>
<td>GH3</td>
<td>Exo-β-arabinofuranosidase</td>
<td>RsAraf1</td>
<td>Raphanus sativus</td>
<td>16</td>
<td>HE-E</td>
<td>Cleaves α-linked Araf from AGPs, pectic α-1,5-arabinan, arabinoxylan. Overexpression in Arabidopsis resulted in [Ara]↓ in cell walls, but no growth phenotype</td>
<td>Kotake et al., 2006; Liang et al., 2013; Tryfona et al., 2014</td>
</tr>
<tr>
<td>GH16</td>
<td>Endo-β-1,3-galactanase</td>
<td>FvEn3GAL</td>
<td>Flammulina velutipes</td>
<td>16</td>
<td>HE-E</td>
<td>Cleaves L-Ara p from L-Ara p-nitrophenyl-β-L-Ara p and releases Ara from gum Arabic and larch AG</td>
<td>Fujimoto et al., 2009; Enomoto et al., 2009</td>
</tr>
<tr>
<td>GH16</td>
<td>Endo-β-1,3-galactanase</td>
<td>FsGal1</td>
<td>Fusarium solani</td>
<td>16</td>
<td>HE-E</td>
<td>Cleaves L-Ara p from L-Ara p-nitrophenyl-β-L-Ara p and releases Ara from gum Arabic and larch AG</td>
<td>Fujimoto et al., 2009; Enomoto et al., 2009</td>
</tr>
<tr>
<td>GH18</td>
<td>Endo-β-1,3-galactanase</td>
<td>SjGal1</td>
<td>Streptomyces ammonii</td>
<td>4</td>
<td>HE-E</td>
<td>Cleaves L-Ara p from L-Ara p-nitrophenyl-β-L-Ara p and releases Ara from gum Arabic and larch AG</td>
<td>Fujimoto et al., 2009; Enomoto et al., 2009</td>
</tr>
<tr>
<td>GH30</td>
<td>Endo-β-1,6-galactanase</td>
<td>Tg6GAL</td>
<td>Trichoderma viride</td>
<td>0</td>
<td>HE-E</td>
<td>No detectable growth phenotype under normal growth condition, but reduced root growth under salt stress</td>
<td>Fujimoto et al., 2009; Enomoto et al., 2009</td>
</tr>
<tr>
<td>GH35</td>
<td>Endo-β-1,3,6-galactanase</td>
<td>RbgGAL1</td>
<td>Raphanus sativus</td>
<td>18</td>
<td>PUR, HE</td>
<td>Cleaves β-1,3-galactan in endo fashion</td>
<td>Kato et al., 2004, 2007; Ichinose et al., 2008</td>
</tr>
<tr>
<td>CAZy Family</td>
<td>Activity</td>
<td>Protein name</td>
<td>Origin</td>
<td>No. of genes in Arabidopsis</td>
<td>Evidence</td>
<td>Comments for enzyme activities and genetic manipulations</td>
<td>Selected references</td>
</tr>
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</tr>
<tr>
<td>GH43</td>
<td>Exo-β-1,3-galactanase</td>
<td>Pc1,3Gal43A</td>
<td>Phanerochaete chrysosporium</td>
<td>2</td>
<td>HE-P</td>
<td>Cleaves β-1,3-linked Gal regardless the presence or absence of substituted side chains</td>
<td>Ishida et al., 2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ct1,3Gal43A</td>
<td>Clostridium thermocellum</td>
<td></td>
<td>HE-E</td>
<td></td>
<td>Ichinose et al., 2006b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sa1,3Gal43A</td>
<td>Streptomyces avermitilis</td>
<td></td>
<td>HE-E</td>
<td></td>
<td>Ichinose et al., 2006a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Il1,3Gal</td>
<td>Irpex lacteus</td>
<td></td>
<td>HE-P</td>
<td></td>
<td>Kotake et al., 2009</td>
</tr>
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<td></td>
<td></td>
<td>SGalase1, 2</td>
<td>Streptomyces sp.</td>
<td></td>
<td>HE-E</td>
<td></td>
<td>Ling et al., 2012</td>
</tr>
<tr>
<td>GH54</td>
<td>Exo-α-arabinofuranosidase</td>
<td>NcAraf1</td>
<td>Neurospora crassa</td>
<td>0</td>
<td>HE-P</td>
<td>Broad specificity to α-1,3 and α-1,5-Araf, which includes AGPs, pectic arabinan, arabinoxylan</td>
<td>Takata et al., 2010</td>
</tr>
<tr>
<td>GH78</td>
<td>Exo-α-rhamnosidase</td>
<td>SaRha78A</td>
<td>Streptomyces avermitilis</td>
<td>0</td>
<td>PUR, HE-E</td>
<td>Releases Rha from gum Arabic</td>
<td>Ichinose et al., 2013</td>
</tr>
<tr>
<td>GH79</td>
<td>Exo-β-glucuronidase</td>
<td>AnGlcAse</td>
<td>Aspergillus niger</td>
<td>3</td>
<td>PUR, HE-P</td>
<td>Cleaves both GlcA and methyl GlcA from AG. Methyl GlcA from long β-1,6-galactan is cleaved, but not from short β-1,6-galactan</td>
<td>Haque et al., 2005; Konishi et al., 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NcGlcAse</td>
<td>Neurospora crassa</td>
<td></td>
<td>HE-P</td>
<td>Cleaves GlcA from AG</td>
<td>Konishi et al., 2008</td>
</tr>
<tr>
<td>GH95</td>
<td>Exo-α-1,2-fucosidase</td>
<td>AfcA</td>
<td>Bifidobacterium bifidum</td>
<td>1⁵</td>
<td>PUR, HE-E</td>
<td>Cleaves α-1,2-linked Fuc (linkage present in AG)</td>
<td>Nagae et al., 2007</td>
</tr>
<tr>
<td>GH106</td>
<td>Exo-α-rhamnosidase</td>
<td>Rham</td>
<td>Sphingomonas paucimobilis</td>
<td>0</td>
<td>PUR, HE-E</td>
<td>Broad specificity to α-Rha containing components. Involvement in AG degradation is unclear</td>
<td>Miyata et al., 2005</td>
</tr>
</tbody>
</table>

GTs are only from plants, while GHs are both from plants and microbial origins.

1 HE, activity demonstrated from heterologously expressed protein in: A, A. thaliana; B, tobacco BY2 cell; E, E. coli; N, N. benthamiana; P, P.pastoris; S, Streptomyces cinnamoneus, 1, bacuvirus/insect cells; MA, mutant analysis.

2 Mutant analysis showed reduction of 3-linked Araf in AGPs, but heterologously expressed protein in N. benthamiana showed β-linked Ara to methyl β-Gal product, therefore a role of this protein in AGP glycosylation is not certain since arabinose exists as an α-linked sugar in AGPs (Gille et al., 2013).

3 Characterized Arabidopsis enzymes demonstrated β-xylanase activity toward xylan (Goujon et al., 2003; Mnic et al., 2004).

4 Characterized Arabidopsis enzymes demonstrated xyloglucan endo-transferase activity (Rose, 2002).

5 Characterized Arabidopsis enzymes demonstrated α-1,2-fucosidase activity specifically toward xyloglucan (Léonard et al., 2008; Günl et al., 2011).
is solely a consequence of the reduced levels of GlcA, but most likely related to the dynamic conformational changes of AGP glycans caused in the mutants.

**α-Fucosyltransferase**

Two Arabidopsis GTs from family GT37 were identified as α-1,2-fucosyltransferases involved in the biosynthesis of AGP glycans (AtFUT4 and AtFUT6, encoded by At2g15390 and At1g14080, respectively; Wu et al., 2010). AGPs from tobacco BY2 cells contain no fucose, but heterologous expression of AtFUT4 and AtFUT6 in BY2 cells resulted in fucosylated AGPs. The recombinant enzymes purified from BY2 cells demonstrated fucosyltransferase activity to endogenous AGPs. Single Arabidopsis T-DNA insertion lines, atfut4 and atfut6, contained reduced levels of fucose in AGPs, and the double T-DNA insertion line fut4/fut6 contained no detectable fucose in its AGPs (Liang et al., 2013); however, no obvious phenotype was observed in both types of mutants when they were grown under normal conditions. Differences between wild type and mutants were only seen in seedlings grown under salt stressed condition, where the mutant lines showed reduced root growth compared to wild type (Liang et al., 2013; Tryfona et al., 2014). This was somewhat surprising because the Arabidopsis mur1 mutant, which is defective in β-1,3-linked α-galactan backbone (Bonin et al., 1997), contained a 40% reduction of fucose in root extracts (Reiter et al., 1997) and showed a 50% reduction of cell elongation rate in roots (Van Hengel and Roberts, 2002). The decrease of root cell elongation in mur1 was previously attributed to the lack of fucose in AGPs (Van Hengel and Roberts, 2002), but the findings by Liang et al. (2013) and Basu et al. (2013) refute that hypothesis. The molecular changes behind the mur1 phenotype are hard to pinpoint. Mutants affected in N-glycan fucosylation show reduced root growth under salt stress conditions (Kaufürst-Soboll et al., 2011), and mur2 plants, which lack only xyloglucan fucosylation, have no visible root phenotype (Van Hengel and Roberts, 2002; Vanzin et al., 2002). The mur1 phenotype might be due to under-fucosylated hromagalaturonan II, or to the combination of several cell wall polysaccharides deficient in fucose.

**α-Arabinofuranosyltransferase**

An Arabidopsis GT encoded by At1g70630 (named REDUCED ARABINOSE YARIV1, RAY1; Gille et al., 2013), was characterized as a putative arabinofuranosyltransferase since the mutation caused a reduced level of arabinofuranose (Araf) in its AGPs. This GT belongs to the GT77 family, which also contains XEG113, the mutation of which results in the reduction of β-linked arabinose in extensin (Gille et al., 2009). Therefore, Araf transferase activity that makes β-linkages was expected for RAY1, and indeed, microsomes isolated from Nicotiana benthamiana after expression of recombinant RAY1 demonstrated β-Araf transferase activity to methyl β-Gal. The T-DNA insertion lines contained reduced levels of 3-linked Ara in its AGP fractions compared to AGPs from wild type, and the mutant plants exhibited slower root growth as well as a reduced rosette size and inflorescence. However, β-1,3-Linked Araf has not been reported in AGPs, therefore the involvement of RAY1 in the biosynthesis of AGP glycans remains unclear.

**GLYOSIDE HYDROLASES**

Glycoside hydrolases (GHs) acting on AGPs are potentially very important for the metabolism of these glycoproteins. AGPs from tobacco stylar transmitting tissue are degraded as the pollen tube grows and the released sugars are considered to be used as the carbohydrate resource necessary for the elongation of pollen tubes (Cheung et al., 1995). Similarly, rapid turnover of AGPs are observed in suspension cell culture and millet seedlings (Gibeaut and Carpita, 1991) and a substantial amount of AGPs are considered to be hydrolyzed to free sugars and recycled in the cytosol for the synthesis of new glycans (Gibeaut and Carpita, 1991) or degraded in the vacuole (Herman and Lamb, 1992). The appearance of distinct AGP epitopes in a developmentally regulated manner might be controlled by GHs in the cell walls. Additionally, the occurrence of free AG glycans detached from proteins observed in the cell walls may be a result of GH actions.

For the hydrolysis of AGP glycans, several GHs are required, e.g., β-galactosidases, β-galactanases, α-arabinofuranosidases, β-arabinopyranosidases, β-glucuronidases, α-fucosidases, and α-rhamnosidases. AGP degrading GHs from microbial origin have been relatively well characterized, while only a few plant GHs have been reported to degrade AGP glycans. Below is an overview for those GHs reported to possess hydrolase activity of AGP glycans from both microbial and plant origins (Figure 1B and Table 1).

**β-Galactosidase and β-Galactanase**

A GH16 from the fungus Flammulina velutipes was characterized as an endo-β-1,3-galactanase degrading the AGP galactan β-1,3-galactan backbone (Kotake et al., 2011). The enzyme activity is distinct from other GH16 enzymes, which comprise β-1,3- and β-1,3:1,4-glucanases, xyloglucan endo-transglycosylase and β-agarase activities. Arabidopsis contains 33 proteins in the GH16 family and they are characterized as xyloglucan endo-transglycosylases and their homologs (Kawthai et al., 2013). Several enzymes of microbial origin in GH30 have been characterized as endo-β-1,6-galactanases that hydrolyze β-1,6-galactan side chains of AGP glycans (Kotake et al., 2004; Sakamoto et al., 2007; Ichinose et al., 2008; Takata et al., 2010). These β-1,6-galactanases were originally categorized as part of the GH5 family, but were moved to the GH30 family after additional bioinformatic analysis by (St John et al., 2010). Tv6GAL from Trichoderma viride was the first β-1,6-galactanase cloned and characterized (Kotake et al., 2004). This enzyme specifically recognizes β-1,6-galactan of AGPs and releases galactose and β-1,6-linked galactooligomers with a degree of polymerization from two to five. Efficiency of the hydrolysis of β-1,6-galactan is increased by pretreatment of the AGP substrate with α-L-arabinofuranosidase. The β-1,6-galactanases from Fusarium oxysporum (FoGAL1, Sakamoto et al., 2007), Streptomyces avermitilis (Sa1,6Gal5A, Ichinose et al., 2008), and Neurospora crassa (Nc6GAL, Takata et al., 2010) act in a similar manner, releasing galactose and β-1,6-galactobiose from β-1,6-galactan of AGP. All three enzymes show increased activity on de-arabinosylated AGP, similarly to Tv5GAL. Plants do not have any proteins classified in the GH16 CAZY family.
Several microbial β-1,3-galactosidases from GH43 have been characterized as exo-β-1,3-galactanase that degrades the β-1,3-galactan backbone of AGP glycans (Ichinose et al., 2005, 2006a,b; Kotake et al., 2009). All of these enzymes show similar substrate specificity and degrade β-1,3-linked Gal regardless of the substitution of side chains, which results in free Gal from unsubstituted β-1,3-galactan and side chains attached to β-1,3-linked Gal. Therefore, the GH43 enzymes have been used to release side chains from AGP glycans for structural analysis (Tryfona et al., 2010, 2012; Geshi et al., 2013; Knoch et al., 2013). The GH43 CAZy family contains two uncharacterized Arabidopsis proteins.

In plants, β-1,3-galactosidase has been purified from radish (Raphanus sativus) seed extracts (Kotake et al., 2005). Based on the deduced protein sequence, the enzyme RsBGAL1 was classified to the GH35 CAZy family. This enzyme was expressed heterologously in Pichia pastoris and demonstrated GH activity by degrading β-1,3- and β-1,6-galactan in an exo manner, but not β-1,4-galactan. The efficiency of degradation of AGP glycans by RsBGAL1 alone was limited, but co-treatment with arabinofuranosidase and glucuronidase resulted in the release of up to 90% of the bound sugars from AGPs, indicating the synergy of those GHs in the degradation of AGP glycans.

α-ARABINOFRANOSIDASE AND β-ARABINOPYRANOSIDASE

An α-arabinofuranosidase from the fungus Neurospora crassa with a broad substrate specificity toward AGPs, pectic arabian, and arabinoxylan was identified and classified to the CAZy GH54 family (NcAraf1, Takata et al., 2010). This enzyme was heterologously expressed in Pichia pastoris and demonstrated GH activity by degrading α-1,3- and α-1,5-linked Araf. NcAraf1 has been used extensively for the structural characterization of AGP glycans together with galactosidases, galactanases, and glucuronidases (Tsumuraya et al., 1990; Okimoto et al., 2003; Kotake et al., 2004, 2009; Konishi et al., 2008; Tryfona et al., 2010, 2012). Arabidopsis does not have proteins in the GH54 family.

Plant α-arabinofuranosidase acting on AGP glycans is classified to family GH3. Kotake et al. (2006) purified an α-arabinofuranosidase from radish seeds and named it RsAraf1. The recombinant enzyme expressed in Arabidopsis demonstrated hydrolytic activity on radish AGPs, pectic α-1,5-arabinan and arabinoxylan. Transgenic Arabidopsis overexpressing RsAraf1 showed decreased levels of Ara in the cell wall, but no obvious growth phenotype was observed compared to wild type plants.

β-Arabinopyranosidase generally represents only a minor part of Ara in AGP glycans, but has been reported from acacia, larch and wheat flour AGPs (Aspinall et al., 1958; Groman et al., 1994; Odonnazig et al., 1994; Tryfona et al., 2010). β-Arabinopyranosidase has been identified from Streptomyces avermitilis (Ichinose et al., 2009). The enzyme, named SaArap27A, belongs to family GH27, and the recombinant enzyme expressed in Streptomyces demonstrated the release of L-arabinopyranoside (Arap) from p-nitrophenyl-β-L-arabinopyranoside, as well as the release of L-arabinose from gum Arabic and larch AG. Arabidopsis contains four uncharacterized proteins in the GH27 family.

β-GLUCURONIDASE

Microbial β-glucuronidases are found in family GH79. Two fungal GH79 β-glucuronidases from Neospora crassa (NcGlcAase) and Aspergillus niger (AnGlcAase) have been cloned and recombinant proteins expressed in Pichia pastoris demonstrated β-glucuronidase activity (Konishi et al., 2008). AnGlcAase and NcGlcAase share high homology in their amino acid sequences, but possess slightly different substrate specificity. Both enzymes recognize unsubstituted and 4-methyl substituted β-GlcA on AGPs, but AnGlcAase cleaves both GlcA and 4-methyl GlcA with an equal efficiency, while NcGlcAase preferably cleaves GlcA and only small amounts of 4-methylGlcA. Arabidopsis contains three proteins in the GH79 family.

In plants, β-glucuronidases (GUS) are ubiquitously present and their activity is associated with cell elongation (Sudan et al., 2006). Eudes et al. (2008) partially purified a GUS from Arabidopsis stems, which cleaves p-nitrophenyl-β-D-glucuronide. The corresponding gene was identified and classified to the GH79 family (AtGUS2, At5g07830, Eudes et al., 2008). The T-DNA knockout insertion lines exhibited increased levels of GlcA, whereas plants overexpressing AtGUS2 lacked detectable levels of GlcA in their AGP fractions. The T-DNA insertion mutant of AtGUS2 showed no clear changes in the elongation rate of plant organs, whereas the overexpression lines exhibited increased elongation of roots and stems. The increase of cell elongation observed in the overexpression lines of AtGUS2 resembles similar observations of the atgcat14a T-DNA insertion lines. Although reduced levels of GlcA is observed in both types of plants, the altered profiles of other sugars present in AGP glycans are inconsistent. Therefore, it is unlikely that the increase of cell elongation is solely caused by the reduction of GlcA levels in AGPs.

α-FUCOSIDASE

α-Fucosidases from various prokaryotic and eukaryotic sources have been characterized, and several of them are commercially available. α-Fucosidases are classified into two GH families: GH29 and GH95 (Lombard et al., 2014). α-Fucosidases from GH29 are capable of hydrolyzing various types of linkages, mainly α-1,3/1,4-linked Fuc, whereas GH95 enzymes are active solely on α-1,2-linked Fuc. Only one GH from Arabidopsis is found in each of these GH families. Atg2g28100 (AtFUC1; Zeleny et al., 2006) belongs to GH29 and the recombinant enzyme expressed in Pichia pastoris demonstrated α-1,3/1,4-fucosidase activity (Zeleny et al., 2006). Atg4g34260 (Fuc95A, AXY8; Léonard et al., 2008; Günül et al., 2011) belongs to GH95 and the enzyme heterologously expressed in Nicotiana benthamiana demonstrated α-1,2-fucosidase activity (Léonard et al., 2008). α-1,2-Fuc is present in both xyloglucan and AGP glycans, but Fuc95A (AXY8) acts specifically on α-1,2-Fuc on xyloglucan and not on AGPs (Günül et al., 2011). α-1,2-Fucosidase purified from Xanthomonas manihotis apparently cleaves α-1,2-fucose on AGP glycans and has been used for the product analysis of AGP fucosyltransferases (AtFUT4 and AtFUT6, Wu et al., 2010). Wu et al. (2010) also used α-1,3/4-fucosidase from almond meal for the characterization of the Fuc linkage. Both enzymes are commercially available, but are not classified to CAZy GH families.
α-RAHMNOSIDASE

Microbial α-rhamnosidas are classified into three GH families: GH28, GH78, and GH106 (Fujimoto et al., 2013; Lombard et al., 2014). An α-rhamnosidase from Aspergillus niger in GH28 was identified as specifically degrading pectic rhamnogalacturonan (RgxB; Martens-Uzunova et al., 2006). Several α-rhamnosidases from GH78 have been characterized and recently an α-rhamnosidase from Streptomyces avermitilis expressed in Escherichia coli demonstrated an α-rhamnosidase that releases rhamnose (Rha) from gum Arabic AGPs (SaRaH78A; Fujimoto et al., 2013; Ichinose et al., 2013). GH106 exclusively contains bacterial α-rhamnosidases, of which only one has been characterized to date. This α-rhamnosidase was purified from Sphingomonas paucimobilis EP2001 (Rham; Miyata et al., 2005) and the enzyme expressed in Escherichia coli demonstrated α-rhamnosidase activity on a broad range of substrates containing α-Rha. One of those substrates is α-rhamnosyl-1,4-galactose, but whether the enzyme hydrolyzes α-rhamnosyl-1,4-GlcA, which is found as part of the side chains of AGP glycans, remains unknown. Arabidopsis contains 28 proteins in the GH28 family, but no plant proteins are present in GH78 and GH106. Among Arabidopsis GH28s, only pectin polygalacturonase has been characterized (Torki et al., 2000; Markovic and Janecek, 2001). Functions of other plant GHs in GH28 remain unknown.

CONCLUSIONS

Microbial GHs working on the degradation of plant AGPs have been reported in several studies, but little was known about the enzymes working on the biosynthesis and degradation of AGPs in plants. Recent discovery of plant GTs/GHs working on AGPs, together with the technical development of in-depth structural analysis of complex AGP glycans, has broadened our knowledge for AGP metabolism significantly. On the other hand, the attempt to elucidate the biological role of each sugar moiety or a particular part of AGP glycan structure by investigating knockout mutants or overexpressors of those enzymes did not result in straightforward answers. For instance, a mutation in AtGlcat14a did not result in a sole reduction of GlcA but also exhibited an increase of Gal and a reduction of Ara in AGP glycan as well as enhanced cell elongation in seedlings. Furthermore, the overexpression of AtGUS2 resulted in a reduction of GlcA, Gal and Ara in AGP glycan and seedlings showed increased cell elongation, similarly to atgalcat14a. The developmentally regulated appearance of different AGP glycan epitopes is well known, but the results available thus far are inconclusive concerning the molecular role of a particular part of AGP glycan structures in plant growth and development.

The carbohydrate active enzymes involved in the AGP metabolism have just begun to be identified and characterized. Further investigation of the remaining members in the AGP glycosylation pathway and their role in vivo is needed to understand the role of CAZy enzymes in relation to AGP glycan, the cell wall architecture, and in plant growth and development.

REFERENCES


Knoch et al. Arabinogalactan carbohydrate active enzymes


