



An update on post-translational modifications of hydroxyproline-rich glycoproteins: toward a model highlighting their contribution to plant cell wall architecture

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Plant cell walls are composite structures mainly composed of polysaccharides, also containing a large set of proteins involved in diverse functions such as growth, environmental sensing, signaling, and defense. Research on cell wall proteins (CWPs) is a challenging field since present knowledge of their role into the structure and function of cell walls is very incomplete. Among CWPs, hydroxyproline (Hyp)-rich O-glycoproteins (HRGPs) were classified into three categories: (i) moderately glycosylated extensins (EXTs) able to form covalent scaffolds; (ii) hyperglycosylated arabinogalactan proteins (AGPs); and (iii) Hyp/proline (Pro)-Rich proteins (H/PRPs) that may be non-, weakly- or highly-glycosylated. In this review, we provide a description of the main features of their post-translational modifications (PTMs), biosynthesis, structure, and function. We propose a new model integrating HRGPs and their partners in cell walls. Altogether, they could form a continuous glyco-network with non-cellulosic polysaccharides via covalent bonds or non-covalent interactions, thus strongly contributing to cell wall architecture.

Keywords: arabinogalactan protein, extensin, hydroxyproline, O-glycosylation, proline-rich protein

INTRODUCTION

Plant cell walls are composite structures mainly composed of polysaccharides, namely cellulose, hemicelluloses and pectins, containing also a large set of proteins involved in the cell dynamics through diverse functions such as growth, environmental sensing, signaling, and defense (Fry, 2004). Research on cell wall components emerged in the nineteen sixties (Lampart and Northcote, 1960; Rees and Wight, 1969) and is still a very active field with continuous advances on the nature, structure and functions of polysaccharides (Carpita and Gibeau, 1993; Willats et al., 2006; Scheller and Ulvskov, 2010) and of proteins (Rose and Lee, 2010; Albenne et al., 2013). However, the question of how these components are connected to make a functional matrix is still a matter of debate (Keegstra et al., 1973; Park and Cosgrove, 2012; Wang et al., 2012).

Among cell wall proteins (CWPs), hydroxyproline (Hyp)-rich O-glycoproteins (HRGPs) are complex macromolecules with various structures and functions. Identified several decades ago, HRGPs were classified into three categories: (i) moderately glycosylated extensins (EXTs); (ii) hyperglycosylated

arabinogalactan proteins (AGPs); and (iii) Hyp/Pro-rich proteins (H/PRPs) that may be non-, weakly- or highly-glycosylated. Each HRGP sub-family is characterized by repetitive consensus sequences which determine the way they are glycosylated according to the so-called Hyp-O-glycosylation code (Kieliszewski, 2001; Tan et al., 2004; Estevez et al., 2006). From a functional point of view, HRGPs are also very diverse. AGPs are implicated in a variety of physiological processes including cell expansion, reproductive development, embryogenesis, signaling, and defense (Seifert and Roberts, 2007). EXTs are mostly described as structural proteins able to form covalent scaffolds (Qi et al., 1995; Brady et al., 1996; Cannon et al., 2008; Velasquez et al., 2012). Finally, H/PRPs are the less documented HRGPs and little is known about their structure and function. They seem to be associated to development and defense against biotic and abiotic stresses (Bradley et al., 1992; Bernhardt and Tierney, 2000; Battaglia et al., 2007). Hybrid and chimeric HRGPs also exist, enlarging the diversity of this superfamily. As previously defined, hybrid HRGPs are composed of HRGP modules from different families, and chimeric HRGPs are composed of one or

more HRGP modules within a non-HRGP protein (Showalter et al., 2010). An expert bioinformatics analysis of the *Arabidopsis thaliana* genome identified 166 HRGPs classified in 85 AGPs, 59 EXTs, 18 H/PRPs, and 4 AGP/EXT hybrid proteins (Showalter et al., 2010). Besides, related to HRGPs but not classified in any of its three sub-families, some allergen proteins containing Hyp residues substituted by arabinogalactans (AGs) were identified in *Artemisia vulgaris* and *Ambrosia artemisiifolia* (Léonard et al., 2005, 2010).

Despite the great interest that plant biologist have had in HRGPs for more than 50 years, many questions about their mode of action in cell walls are still unanswered and HRGP research is still very challenging. In this review, we provide an update on (i) their post-translational modifications (PTMs) which consist in Pro-hydroxylation and O-glycosylation on serine (Ser) and Hyp residues and (ii) their roles in cell walls. We also focus on new insights into HRGP supramolecular assembly and propose a model including most recent data on covalent and non-covalent networks connecting HRGPs and polysaccharides.

EXTENSINS (EXTs)

EXTs AS STRUCTURAL MOLECULES IN PLANT CELL WALLS

EXTs are modular, highly repetitive HRGPs showing similar features as collagen that contain Tyr cross-linking motifs. Unlike collagen, EXTs also undergo plant specific post-translational O-glycosylation on Ser-(Hyp)_{n≥2} motifs. EXTs are represented in the *A. thaliana* genome by 59 members, some are classical EXTs while others are EXT-like chimeras and hybrid-EXTs that also contain other domains. Despite the high number of proteins with EXT domains in plant cell walls (Lampert et al., 2011), we know little about their exact functions and how this protein diversity is coordinated during plant development. There are several reasons that may explain our current lack of understanding of the EXT biology: (i) a high similarity in their protein sequences that make their characterization at the molecular level very difficult; (ii) the highly repetitive nature of their sequences since they are modular proteins, large in size and with complex chemical structures that carry several PTMs. Consequently, the biochemical characterization of a single EXT protein is still today very challenging; (iii) large number of EXTs and EXTs-related proteins encoded in known plant genomes; and (iv) several EXT genes are expressed at the same time in the same plant tissues (see Genevestigator database, <https://www.genevestigator.com>). In addition, most of the available EXT mutants analyzed until now show no clear phenotype. Few exceptions are the mutants *atext3* (embryo lethal), *atext6*, *7*, *10*, *12* (shorter root hairs) and *lrx1*, *2* (root hair morphogenesis) that showed clear phenotypes (see Table 1).

PTMs OF EXTs AND THE ENZYMES INVOLVED

Structural O-glycoproteins containing EXT domains that are ultimately secreted into plant cell walls are first shaped by several and complex PTMs that include: (i) signal peptide processing (in the ER), (ii) hydroxylation of Pro into Hyp residues, (iii) O-glycosylation on Hyp and Ser residues (in the ER-Golgi apparatus) and finally, (iv) Tyr cross-linking to promote the formation of a covalent network (in the cell wall). In the last few years,

great progress has been made in our knowledge of the molecular players that act on the EXT biosynthetic pathway with the identification of several enzymes involved in their PTMs (summarized in Table A1). Hydroxylation of peptidyl-Pro is catalyzed by prolyl 4-hydroxylases (P4Hs) providing reactive hydroxyl groups for further O-glycosylation. Plant P4Hs are membrane-bound enzymes that belong to a family of 2-oxoglutarate-dependent dioxygenases (Hieta and Myllyharju, 2002; Koski et al., 2007, 2009). Partial *in vitro* and *in vivo* characterization of plant P4Hs (see Table A1) has been carried out in several plant model systems (Hieta and Myllyharju, 2002; Tiainen et al., 2005; Yuasa et al., 2005; Keskiäho et al., 2007; Vlad et al., 2007, 2010; Asif et al., 2009; Velasquez et al., 2011, 2012, in revision; Parsons et al., 2013). Most P4Hs are able to hydroxylate with different affinities several types of substrates containing collagen-like, polyproline EXT-type as well as AGP-like sequences. On the other hand, structural information on plant P4Hs is scarce since only one P4H from *Chlamydomonas reinhardtii* (CrP4H1) has been crystallized (Koski et al., 2007, 2009) and few P4Hs were characterized *in vivo* (Velasquez et al., in revision). Recent evidence showed that in *A. thaliana*, P4H5 forms homo-/hetero-dimers with P4H2 and P4H13 in the Golgi, suggesting the existence of P4H complexes required for proper Pro hydroxylation. It is plausible that more than one type of P4H complex would be formed in the ER-Golgi compartment, and in the case of the hetero-complexes, the presence of specific P4Hs (e.g., AtP4H5) may be required either for the correct recruitment or the scaffolding of the other P4Hs (e.g., AtP4H2) (Velasquez et al., in revision).

Hydroxylated EXTs are usually O-glycosylated with chains of up to four linear Ara residues on each Hyp (Velasquez et al., 2011; Ogawa-Ohnishi et al., 2013). The usual arabinoside structure linked to each Hyp unit is composed of β-L-Araf-(1,2)-β-L-Araf-(1,2)-β-L-Araf-(1,3)-α-L-tAraf. A fifth arabinose unit was reported in some tissues (Lampert, 1973). Specifically, three groups of arabinosyltransferases (AraTs) HPAT1-HPAT3 (GT8 CAZy family), RRA1-RRA3 (GT77 family), and XEG113 (GT77 family) have recently been implicated in the sequential addition of the innermost three L-Ara residues (Egelund et al., 2007; Gille et al., 2009; Velasquez et al., 2011; Ogawa-Ohnishi et al., 2013). The AraT that would transfer the fourth (1,3)-α-L-Araf moiety was identified very recently as Extensin Arabinose Deficient transferase (ExAD) within the GT47 family (Petersen et al. *in preparation*). In addition, one novel peptidyl-Ser galactosyltransferase named as SGT1 has been reported to add a single α-Galp residue to each Ser residue in Ser-(Hyp)₄ motifs of EXTs and it would belong to a new family of CAZy (Saito et al., 2014). Glycosylated EXTs are cross-linked, at least *in vitro*, by putative type-III peroxidases (PERs) at the Tyr residues (Schnabelrauch et al., 1996; Jackson et al., 2001; Price et al., 2003) forming *intra*- and *inter*-EXT linkages (Cannon et al., 2008; Lampert et al., 2011). Thus, EXTs are able to form a three-dimensional glycoprotein network that possibly interacts with other cell wall components like pectins (Nuñez et al., 2009; Dick-Perez et al., 2011). Although the *in vivo* molecular mechanism of the covalent cross-link is unknown, there is evidence of PER-catalyzed oxidative coupling of Tyr residues *in vitro* that mediates the insolubilization of the

Table 1 | Examples of EXTs and EXT-related proteins characterized in the last years.

Protein/Gene name	Tissue or sub-cellular localization	Assumed function /Phenotype of mutants	References
EXTs			
AtEXT1 (At1g76930)	Roots and Inflorescences	Cell wall formation/Induction in response to mechanical wounding, pathogen infection, senescence and at abscission zones, and treatment with hormones (methyl jasmonate, salicylic acid, auxin, brassinosteroids)	Merkouropoulos and Shirsat, 2003
AtEXT3 (At1g21310)	Embryo	Cell wall formation/Embryo-lethal mutant. Incomplete cross wall assembly	Hall and Cannon, 2002; Cannon et al., 2008
AtEXT6 (At2g24980) AtEXT7 (At4g08400) AtEXT10 (At5g06640) AtEXT11 (At5g49080) AtEXT12 (At4g13390)	Root hairs	Cell wall formation/Short root hair	Velasquez et al., 2011
AtMOP10 (At5g05500)	Root hairs	Cell wall formation/Short root hair	Velasquez et al., 2011
AtEXT-LIKE (At4g26750)	Root hairs	Cell wall formation/Short root hair	Velasquez et al., 2011
SIEXT1	Trichoblasts	-/Induced by ethylene	Bucher et al., 2002
BnExtA	External and internal phloem of the main stem	-/Greatest expression in regions where a maximum tensile stress is exerted	Shirsat et al., 1996
NtEXT1.4	Stems, Roots and Carpels	-/Cells under mechanical stress: emergence of lateral roots, junction stem/petiole, fusion of carpels. Induction by mechanical stress in roots and stems	Hirsinger et al., 1999; Salvà and Jamet, 2001
NsEXT1.2A	Stems and Roots	-/Expression in the root transition zone, in stem inner and outer phloem and in cortical cells at the stem/petiole junction. Induced by wounding	Guzzardi et al., 2004
LRXs			
AtLRR-EXT (At4g29240)	Root hairs	Cell wall formation /Short root hair	Velasquez et al., 2011
AtLRX1 (At1g12040)	Root hairs	Cell wall formation/Morphogenesis of root hair	Baumberger et al., 2001
AtLRX2 (At1g62440)	Root hairs	<i>atlr2</i> acts synergistically with <i>atlr1</i> . <i>atlr1/atlr2</i> show osmophilic aggregates and local disintegration of the cell wall	Baumberger et al., 2003
VcISG (Inversion-Specific Glycoprotein)	Extracellular matrix	–	Ertl et al., 1992
ZmPex1/ZmPex2/SIPEX (Pollen extensin-like)	Callose portion of the pollen tube cell wall	–	Rubinstein et al., 1995; Stratford et al., 2001

Dc, *Daucus carota*; *Dca*, *Dianthus caryophyllus*; *La*, *Lupinus albus*; *Ns*, *Nicotiana sylvestris*; *Nt*, *N. tabacum*; *Sl*, *Solanum lycopersicon*; *Vc*, *Volvox carteri*; *Zm*, *Zea mays*.

proteins (Schnabelrauch et al., 1996; Jackson et al., 2001; Price et al., 2003). Recently, six apoplasmic type-III PERs were identified as putative candidates for the cross-linking of EXTs specifically in the root hairs of *A. thaliana* (Velasquez et al., in revision). Structural proteins with polyproline sequences like collagen can also be Tyr-cross-linked by the action of a PER not only *in vitro* but also *in vivo* (Edens et al., 2001) suggesting that EXTs and

collagen, as extracellular building blocks, would share structural features and functions.

Root hair as models to study EXT functions and related GTs

Root hairs have been used as a single-cell model to study cell wall biosynthesis in general and specifically EXTs during tip-growth (Park et al., 2011; Velasquez et al., 2011). Mutants deficient in

the synthesis of a single wall polymer specifically in the root hair are generally impaired in growth because their cell wall structure is severely compromised (Diet et al., 2006; Cavalier et al., 2008; Ringli, 2010; Park et al., 2011; Pena et al., 2012; Velasquez et al., 2012). In this framework of interconnected wall polymers (Cosgrove, 2005; Dick-Perez et al., 2011), cross-linked EXTs have a key role during cell expansion and growth (Cannon et al., 2008; Ringli, 2010; Lampport et al., 2011; Velasquez et al., 2011). EXT domains seem to be important during polarized cell expansion since several EXT-related mutants have shorter root hairs such as classical *ext6*, *ext7*, *ext10* and *ext12* (Velasquez et al., 2011, 2012) and *lrx1* and *lrx2* mutants (Baumberger et al., 2001, 2003; Ringli, 2010).

Impact of O-glycosylation on EXT function

It is accepted that O-glycans increase HRGP solubility, resistance to proteolytic degradation and thermal stability (Kieliszewski et al., 1989; Ferris et al., 2001; Shpak et al., 2001; Kieliszewski et al., 2011; Lampport et al., 2011). Most of the mutants that correspond to glycosyltransferases (GTs) known to glycosylate EXTs (Table A1) have been related to root hair drastic phenotypes, highlighting that even minor changes in the O-glycosylation status of EXTs affect EXT function during polarized cell expansion (Velasquez et al., in revision). In addition, it was found that both O-glycosylation types present in EXTs (Hyp-O-arabinylation and Ser-O-galactosylation) are required and have additive effects for correct EXT function in root hair growth (Velasquez et al., in revision). The known roles of EXTs in cell wall assembly, cell shape and growth raise the question about the function of each individual EXT molecule (Hall and Cannon, 2002; Cannon et al., 2008; Velasquez et al., 2011). Some examples of already characterized EXT or EXT-related genes are presented in Table 1. Recently, it was reported that EXTs can form, at least *in vitro*, a tridimensional covalent network through Tyr-linkages mediated by EXT PERs between individual EXT molecules and also via self-recognition and alignment of hydrophilic O-glycosylated Ser-(Hyp)_{3–4} repeats and hydrophobic peptide-cross-linking modules (Cannon et al., 2008). Thus, the ordered EXT monomer assembly in plant cell walls would

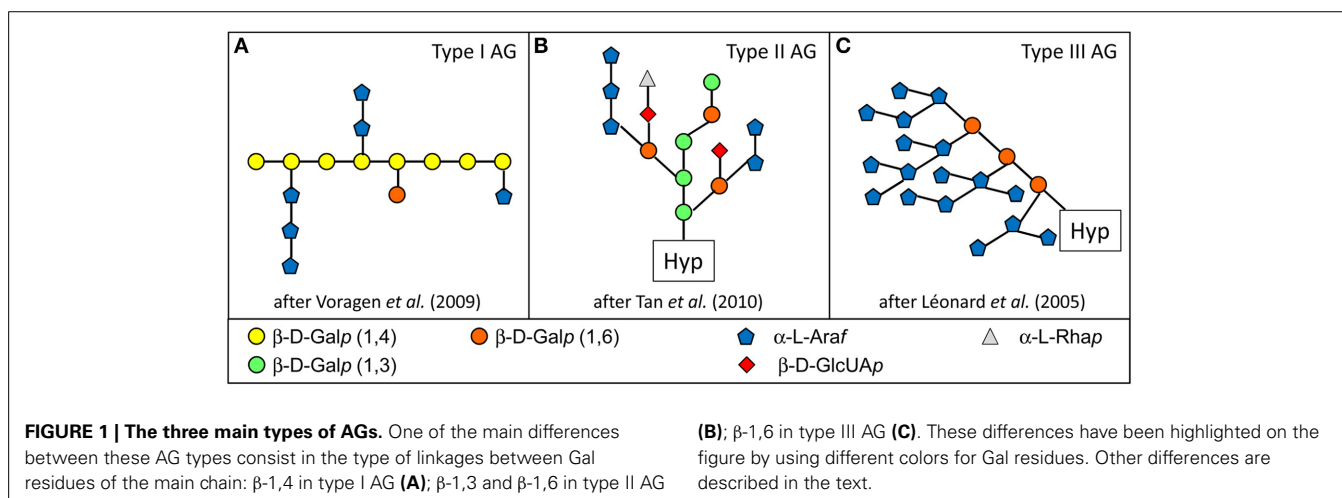
involve a zipper-like endwise association via cross-linking at the ends of the molecules (Kieliszewski et al., 2011; Lampport et al., 2011). Recently, molecular dynamics and homology modeling experiments suggested that classical EXTs would be able to form a putative triple helix structure by lateral staggered alignment (Cannon et al., 2008) and Tyr cross-linking, similar to that present in collagen (Velasquez et al., in revision). It is also proposed that EXTs interact with pectins by a simple acid-base reaction forming a supramolecular ionic structure in the nascent cell wall (Valentin et al., 2010), which would serve as a template for further cell wall deposition (Cannon et al., 2008; Lampport et al., 2011). In addition, covalent EXT-pectin cross-links were also suggested (Qi et al., 1995; Nuñez et al., 2009). However, it is unclear how EXT monomers are secreted and assembled into the glyco-network and how EXT-pectin interactions are controlled in a coordinated way during new cell wall formation. In addition, pectin methyl esterases de-esterify galacturonic acid residues in homogalacturonans and liberate acidic charges for ionic interactions (Micheli, 2001) with positively-charged domains in molecules like EXTs.

ARABINO GALACTAN PROTEINS (AGPs)

Many articles reporting the state of the art concerning AGP structure, function and biosynthesis have been published recently (Seifert and Roberts, 2007; Ellis et al., 2010; Tan et al., 2012; Lampport and Várnai, 2013; Nguema-Ona et al., 2013; Knoch et al., 2014). AGPs are HRGPs containing a high proportion of sugars, up to 90%. They are characterized by repetitive X(Pro)_n motifs in which X is mostly alanine (Ala) or Ser. In this review, we focus on specific aspects concerning (i) the characterization of their O-glycan moiety and (ii) their interactions with cell wall polysaccharides.

STRUCTURE OF O-GLYCANS OF AGPs

A remarkable work performed on proteins deriving from synthetic genes and produced in cell suspension cultures has allowed to characterize AGP O-glycans covalently linked to [Ser(Hyp)]_n and [Ala(Hyp)]_n motifs (Tan et al., 2010). It has been possible to precisely define the structure of type II AGs by combining monosaccharide and linkage analyses to mass spectrometry and



NMR. An example of type II AG is given in **Figure 1B**. Type II AGs contain a β -D-Galp backbone formed by a succession of three β -1,3 linked D-Galp interrupted by a β -1,6 linkage causing a reverse turn. Gal residues of side chains can be substituted with α -L-Araf, α -L-Rhap or Me-GlcP A (Tan et al., 2010; Tryfona et al., 2012). The chelation of Ca^{2+} ions could occur at the level of GlcA residues located in close proximity. It should be noted that different variants of this basic structure exist, for example β -1,6 side chains can vary in length from 1 over 20 Gal residues (Tryfona et al., 2012). Type II AGs react positively with the β -glucosyl and β -galactosyl Yariv reagents, but not with the α -glucosyl and α -galactosyl Yariv reagents (Kitazawa et al., 2013). The β -galactosyl Yariv reagent has been shown to recognize the β -1,3-Gal main chains of type II AGs. They are different from previously described type I AGs which constitute lateral branches of RGI (Vöragen et al., 2009). Type I AGs are formed by a linear chain of β -D-Galp (1,4) with lateral chains of α -L-Araf (1,5 attached to Gal O-3) and β -D-Galp (attached to Gal-O-6) (**Figure 1A**). Type II AGs also differ from type III AGs found on allergens like the *Artemisia vulgaris* Art v 1 (Léonard et al., 2005). The structure of type III AGs has been determined by combining the results of linkage analysis, NMR and enzymatic degradation. They are formed by a short linear chain of β -D-Galp (1,6). They only contain Gal and Ara residues, and they have large branched Ara chains. The linkage analysis indicates the presence of terminal Araf, 5-Araf, 3,5-Araf, 2,5-Araf, 2,3,5-Araf and 3,6-Galp. This Hyp O-glycan was shown to consist of Hyp₁Gal₃Ara_{5–28} series by MALDI-TOF MS. Type III AGs react with the β -glucosyl Yariv reagent suggesting that Art v 1 is an AGP. As for type II AGs, type III AGs probably exist in various forms and only a consensus model can be proposed (**Figure 1C**). Another kind of type III AGs has been later described for Amb a 4, an allergen of *Ambrosia artemisiifolia* (Léonard et al., 2010). It differs from the that of Art v 1 by the presence of different Hyp₁Gal₁Ara_{5–20} series with a lower amount of Gal, the presence of more α -Araf (1,5) and less α -Araf (1,3).

The existence of different types of AGs linked to AGPs (types II and III) raises the questions of (i) the role of the amino acid sequence and (ii) the presence of different types of GTs in plants to ensure the appropriate O-glycosylation of HRGPs (Léonard et al., 2005).

INTERACTIONS OF AGPs WITH POLYSACCHARIDES

The question of how AGPs are connected to other cell wall components and the involvement of their carbohydrate moieties in the interactions is of great importance, but still poorly documented. It has been assumed that AGPs could act as covalent cross-linkers in polysaccharide networks. Several lines of evidence suggested associations between AGPs and pectins. More than 40 years ago, it was hypothesized that Rha residues on type II AG side chains could be attached to RGI (Keegstra et al., 1973). Since then, several studies have reported the existence of strong associations between AGPs and pectins from different plant tissues, including grape (Pellerin et al., 1995), carrot (Immerzeel et al., 2006) or sugar beet (McKenna et al., 2006). Pectins were shown to co-localize with AGPs in pollen tubes (Li et al., 1995; Jauh and Lord, 1996; Mollet et al., 2002). Besides, enzymatic treatment of cell wall

fractions with pectin-degrading enzymes allowed for an increased release of AGPs (Immerzeel et al., 2006; Lamport et al., 2006). One study also suggested the existence of AGP/xylan complexes (Kwan and Morvan, 1995). However, all these AGP/polysaccharide complex analyses were either indirect or achieved on preparations containing a mixture of AGPs, thus preventing a detailed characterization of the interactions. The first in depth structural study of an AGP polysaccharide complex involving pure AGP was only recently reported (Tan et al., 2013). It was shown that two isoforms of a purified *A. thaliana* AGP, At3g45230, are covalently attached to pectins and hemicelluloses. Linkages have been demonstrated between: (i) RGI/homogalacturonan and the Rha residue in the AGP type II AG domain and (ii) arabinoxylan and either a Rha residue of RGI or an Ara residue in the type II AG domain. A model was proposed for this complex called Arabinoxylan Pectin Arabinogalactan Protein1 (APAP1). The *apap1* mutant showed an increased extractability of pectin and xylan, suggesting a structural role for APAP1 (Tan et al., 2013). However, since APAP1 was isolated from suspension culture media, it could correspond to a simplified structure with pectin and xylan domains smaller than expected in plant cell walls. Larger APAP1 complexes may exist in cell walls, but their extraction is undoubtedly the bottleneck preventing their characterization. Large AGP/pectin/xylan complexes should also be found in other plants, corroborating all prior studies indirectly suggesting their existence (Tan et al., 2013).

Present knowledge on AGP/polysaccharide interactions indicates that some AGPs may serve as cross-linker in cell walls and act as polysaccharide plasticizers as previously assumed (Lamport, 2001; Lamport et al., 2006). Chimeric proteins containing AGP domains were also suggested to interact with polysaccharides. In particular, SOS5 (SALT-OVERLY SENSITIVE 5), a Fasciclin-AGP, was assumed to interact with pectins, thus mediating mucilage adherence (Griffiths et al., 2014). SOS5 interacting partners were not identified. Further efforts will be necessary to highlight the contribution of AGPs to cell wall architecture and to give more insight into its molecular basis.

HYP/PRO-RICH PROTEINS

Like EXTs and AGPs, H/PRPs belong to the HRGP superfamily and some of them are chimeric proteins. As mentioned above, little is known about the O-glycosylation of H/PRPs and their interactions with polysaccharides. With regard to O-glycosylation, information is only available for H/PRPs having X(Pro/Hyp)_n≥2X motifs. This type of domain can be associated with a short N-terminal AGP domain, a histidine (His)-stretch and a C-terminal PAC (Proline-rich protein and AGP, containing Cys) domain like in the *A. thaliana* AtAGP31 (Liu and Mehdy, 2007; Hijazi et al., 2012). Up to now, twelve such proteins have been identified in *A. thaliana*, *Daucus carota*, *Gossypium hirsutum*, *Nicotiana glauca*, *N. tabacum*, *Phaseolus vulgaris*, *Capsicum annuum* and *Petunia hybrida* (Hijazi et al., 2014).

STRUCTURE OF THE O-GLYCANS OF H/PRPs OF THE ATAGP31 TYPE

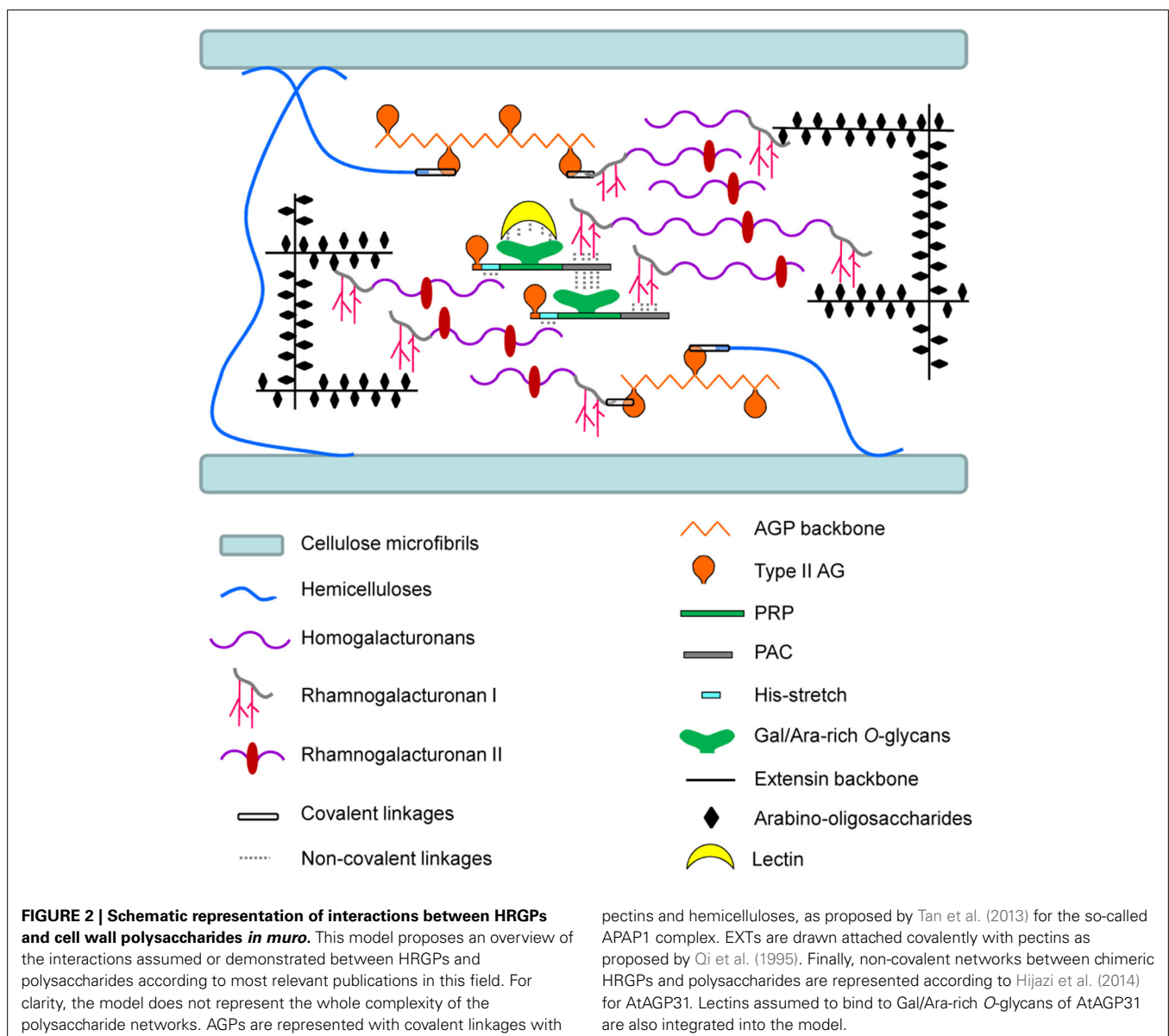
O-glycosylated amino acid motifs of the H/PRP domain of AtAGP31 have been characterized by mass spectrometry: Lys(A la/Ser)HypVal, Lys(Pro/Hyp)(Hyp/Pro)(Thr/Val), Thr(Pro/Hy

p)(Hyp/Pro)Val, and Tyr(Pro/Hyp)(Hyp/Pro)Thr (Hijazi et al., 2012). The monosaccharide linked to Hyp is an hexose which is most probably a Gal based on the monosaccharide analysis of the purified protein (53.2% Gal, 39.5% Ara, 2.2% Xyl, 1.9% Fuc, 1.8% Glc, 1.3% Man, 0.3% GlcUA). It should be noted that this global analysis includes *O*-glycans linked to the AGP domain of AtAGP31 and *N*-glycans linked to its PAC domain. The *O*-glycan linked to the H/PRP domain of AtAGP31 is not recognized by the β -D-glucosyl Yariv reagent, but it interacts with the Peanut Agglutinin (PNA), a lectin having a high affinity for Gal residues (Hijazi et al., 2012). It was called Gal/Ara-rich motif (Hijazi et al., 2012). *Nicotiana alata* NaPRP4 shares the same type of H/PRP domain and a PAC domain with AtAGP31 (Sommer-Knudsen et al., 1996). The predominant monosaccharide of this *O*-glycoprotein is Gal (83%) whereas Ara, GlcNac, Man, Xyl are in minor amounts (7, 4, 4, 1% respectively). The

linkage analysis has shown the presence of terminal Araf (6%), terminal Galp (48%), 1,3-Galp (4%), 1,6-Galp (14%), 1,3,6 Galp (25%), 1,2-Manp (1%) and Xylp (1%). Altogether, H/PRPs with X(Pro/Hyp)_n≥2X motifs are *O*-glycosylated with Gal-Ara-rich glycans which seems to be slightly different from the previously described type I, II and III AGs. Further characterization, especially by NMR will be required to fully describe these structures.

INTERACTIONS OF H/PRPs WITH POLYSACCHARIDES

H/PRPs are assumed to be cross-linked in cell walls, but direct evidence is still lacking (Bradley et al., 1992; Brisson et al., 1994; Frueauf et al., 2000). Nothing is known about the possible roles of *O*-glycosylations. AtAGP31 was recently proposed to be involved in non-covalent interactions networks (Hijazi et al., 2014). Consistently and unlike HRGPs which are covalently insolubilized in cell walls, AtAGP31 is easily extracted



from cell walls of etiolated hypocotyls (Hijazi et al., 2012). It should be noted that NaPRP4 is not insolubilized in cell walls as well (Sommer-Knudsen et al., 1996). AtAGP31 was shown to interact *in vitro* with RGI type I AG branches through its PAC domain and with methyl-esterified polygalacturonic acid, probably through its His-stretch. Protein/protein interactions were also assumed for AtAGP31, with (i) self-recognition between its PAC domain and its H/PRP domain *O*-glycans, and (ii) interaction with cell wall lectins. It was proposed that the AtAGP31 multi-domain organization results in complex supra-molecular scaffolds with different cell wall components, thus contributing to the strengthening of cell walls of quickly growing organs like etiolated hypocotyls. Such non-covalent networks have not been described before for HRGPs. A similar behavior may exist for proteins sharing features with AtAGP31 (Hijazi et al., 2014). However, as mentioned above, except NaPRP4 whose glycosylation has been characterized (Sommer-Knudsen et al., 1996), these proteins were not described at the molecular level and their interactions with cell wall polysaccharides were not investigated. TTS-1 and TTS-2 (Transmitting Tissue-Specific) from *N. tabacum*, and DcAGP1 from *D. carota* were shown to display an ellipsoidal shape and to self-assemble into higher-order structures using microscopy techniques (Baldwin et al., 2000, 2001; Wu et al., 2001). Interestingly, the deglycosylation of TTS disrupts its ability to aggregate, suggesting a regulation of self-association by its level of *O*-glycosylation (Wu et al., 1995). Self-assembly in a head-to-tail fashion through interactions between the *O*-glycans of H/PRP domain and the PAC domain can be proposed for proteins like TTS and DcAGP1, similarly to AtAGP31.

CONCLUDING REMARKS AND FUTURE DEVELOPMENTS

In this review, we have focused on some structural features of HRGP *O*-glycans and we have highlighted their possible interactions *in muro* through covalent glycosidic linkages or non-covalent interactions. As proposed in the model shown in **Figure 2**, HRGPs could serve as cross-linkers in cell walls, connecting non-cellulosic polysaccharides, thus forming a continuous network. Large covalent complexes connecting AGP, hemicelluloses and pectins, as proposed in APAP1, are represented (Tan et al., 2013). However, the relevance of such covalent complexes in cell walls need to be confirmed. EXTs appear to form covalent linkages with pectins as reported (Qi et al., 1995; Nuñez et al., 2009). The precise moieties involved in these linkages have not been identified so far. Finally, chimeric HRGPs with H/PRP and PAC domains like AtAGP31 may form non-covalent networks with a set of cell wall components, including polysaccharides and lectins (Hijazi et al., 2014). It can be speculated that these protein/polysaccharide networks contribute to the cell wall architecture, by reinforcing the polysaccharide scaffold and by controlling its porosity. A recent high-resolution solid-state NMR study elucidating the 3D-architecture of the polysaccharides and proteins *in muro* revealed that the structural proteins in the primary cell wall are separated from the polysaccharides by more than one nanometer (Wang et al., 2012). This corroborates the assumption that *O*-glycans acts as spacers between HRGP backbones and cell wall polysaccharides.

These new features render even more complex the cell wall architecture. Plant cell walls contain a variety of complex macromolecules, possibly interconnected, resulting from a sophisticated metabolism. A tremendous set of carbohydrate active enzymes is required to achieve (i) polysaccharide synthesis and assembly, (ii) protein glycosylation, and (iii) possible polysaccharide/protein linkages. Non-cellulosic polymer synthesis occurs in the Golgi (Mohnen, 2008; Brown et al., 2011), and HRGP synthesis starts in the ER and continues in the Golgi (Basu et al., 2013; Knoch et al., 2014). An important issue is now to determine in which sub-cellular compartment covalent HRGP/polysaccharide complexes are formed and by which mechanism. Is there a code for establishing these links or are they occurring randomly? Which enzymes are involved? Answering these questions constitutes a real challenge toward a better understanding of cell wall biosynthesis and architecture. Further studies will also be necessary to elucidate the molecular basis of HRGP functions in cell walls and their involvement in physiological processes like cell plate formation or root hair cell expansion (Cannon et al., 2008; Velasquez et al., 2011).

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fpls.2014.00395/abstract>

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