Pergamon

PII: S0031-9422(97)00724-3

Phytochemistry, Vol. 47, No. 4, pp. 483–497, 1998

Printed in Great Britain
0031-9422/98 $19.00+0.00

REVIEW ARTICLE NUMBER 126

HYDROXYPROLINE-RICH PLANT GLYCOPROTEINS

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(*Received* 27 *June* 1997; *in revised form* 29 *July* 1997)

Key Word Index—Hydroxyproline-rich glycoproteins; Extensin; Proline/hydroxyproline-rich glycoproteins; Arabinogalactan-proteins; Solanaceous lectins; Cell wall; HRGP; P/HRGP; AGP.

Abstract—This review summarizes the structures of the four major groups of hydroxyproline-rich glycoproteins from plants; extensins, proline/hydroxyproline-rich glycoproteins, arabinogalactan-proteins, and solanaceous lectins. Similarities and differences within and between the groups are discussed.

INTRODUCTION

In this review we present current knowledge of a broad group of glycosylated proteins which are characterized by the proline/hydroxyproline content of the protein backbone and the fact that the glycosyl components contain arabinose and galactose as the major monosaccharides. These glycoproteins and proteoglycans, known as hydroxyproline-rich glycoproteins (HRGPs), are widely distributed in the plant kingdom. They are ubiquitous in the land plants and have also been reported in a number of unicellular algae. They are believed to have arisen early during the evolution of plants. Because of their antiquity and distribution, these molecules are believed to play a fundamental role in function of plants. We have focused on a subgroup of the HRGPs, the proline/hydroxyproline-rich glycoproteins (P/HRGPs). We also survey recent work on the two other main groups (extensins and arabinogalactan-proteins, AGPs) and refer the reader to earlier reviews for more detailed background.

Our current understanding of the structure of each of the sub-groups of HRGPs is incomplete. Progress in establishing detailed structures is limited by several technical problems. First, isolating a pure sample of a molecule from a mixture of closely-related glycosylated proteins in the plant extract or exudate is difficult. Secondly, it is challenging to establish the sequence of the protein backbone, the detailed structure of the carbohydrate side-chains and their exact site of linkage to the protein backbone. The protein backbones, being heavily glycosylated and rich in proline/hydroxyproline, are not readily amenable to direct protein sequencing. If sequence of the amino acids is obtained, it often includes a high proportion of Pro/Hyp, Ser, Thr and Ala. There are multiple codons for each of these amino acids which has made cDNA cloning based on PCR very difficult. cDNA cloning is also complicated by deletions, rearrangements, and hybrid formation during preparation of the libraries.

Another major challenge is that of obtaining complete structural information for the carbohydrate substituents. This is extremely difficult and has only been achieved in a few cases. The problems relate to limitations in analysis and sequencing of carbohydrates and the fact that HRGPs generally have very large and heterogenous glycosyl substituents. It is relatively straightforward to get a monosaccharide composition and linkage analysis from a purified sample. However, this information can usually be interpreted in a number of ways and is often consistent with several different structures. To get a definitive structure requires the glycans to be released from the protein backbone, purified, and analysed separately. In the case of HRGPs, significant proportions of carbohydrate are attached through *O*-glycosidic linkages to Hyp. Since Hyp-glycosyl links are difficult to cleave, it is often necessary to break the glycosylated protein into glycopeptides and analyse each of the glycopeptides individually. Without complete structural information, it is difficult to design definitive experiments to address the question of function.

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CLASSIFICATION OF HYDROXYPROLINE-RICH GLYCOPROTEINS (HRGPs)

The HRGPs can be classified into four main groups: Extensins, AGPs, P/HRGPs (this group is sometimes referred to as proline-rich proteins, PRPs) and solanaceous lectins (for reviews see [1–5]). These are not necessarily discrete classes of molecules but represent a family of (glyco)proteins/proteoglycans forming a continuum of diverse molecules ranging from basic, minimally glycosylated proteins (e.g. PRPs) to acidic, highly glycosylated proteoglycans such as the AGPs [5, 6]. In some cases a single glycoprotein may have characteristics of several groups. Although the boundaries between the different classes of hydroxyproline-rich glycoproteins are indistinct, they are discussed in four major groupings to allow the available information to be structured;

HRGPs (hydroxyproline-rich glycoproteins) is a generic term covering all molecules that are rich in Hyp/Pro (generally $\geqslant 5\%$). The major sub-groups have the following main structural features:

Extensins describes Hyp-rich, basic glycoproteins containing several repeat sequences, the most abundant of which is the pentapeptide $Ser(Hyp)_4$. They are normally highly glycosylated with Hyp-linked arabinosyl chains (Ara_{1-4}) as the predominant oligosaccharide and Ser-linked, single Gal-units also present [7–12].

AGPs (arabinogalactan-proteins) describes Hyp-containing, highly glycosylated glycoproteins or proteoglycans. They contain arabinogalactan chains and the linkage between the carbohydrate and the protein backbone is generally through Hyp-Gal. The protein backbone is usually neutral to acidic and often contains Hyp/Pro-Ala or Ala-Hyp/Pro motifs. (A variety of definitions have been used in the literature for AGPs, for review see [13–16].)

P/HRGPs (proline/hydroxyproline-rich glycoproteins) is used as a broad classification covering molecules that are rich in Pro and/or Hyp but cannot be classified as extensins, AGPs, or solanaceous lectins. The amino acid motifs Pro-Pro-Xaa-Yaa-Lys, Pro-Pro-Xaa-Lys, Pro-Pro-Xaa-Yaa-Pro-Pro or related sequences are typically present [17]. Very few P/HRGPs have been isolated as (glyco)proteins; most have only been characterized as cDNAs. This group also includes proteins referred to as proline-rich proteins (PRP) that are only minimally glycosylated.

Solanaceous lectins are hydroxyproline-rich lectins from the *Solanaceae*. They are normally chimeric glycoproteins consisting of a carbohydrate-binding lectin-domain and an extensin-like domain.

Appropriate classification of a HRGP may require analysis of both the gene and the glycosylated gene product as neither the Hyp-content nor the glycosylation pattern can be deduced from the nucleotide sequence. Plant proteins that contain only a few residues of hydroxyproline per protein molecule are not classified as hydroxyproline-rich molecules. For example neither the copper-containing cupredoxin isolated from cucumber peelings which has a short, 23 amino acid long C-terminal containing hydroxyproline and serine [18], nor a group of chitinases found in sugar beet and various *Nicotiana* species [19, 20] are HRGPs.

EVOLUTIONARY RELATIONSHIPS BETWEEN HRGPs

An evolutionary relationship between HRGPs has been proposed by a number of authors [21, 22]. This theory is supported by examination of the three-letter nucleotide codon for proline, CCX. If the first base of this codon is interchanged with other bases, the codon encodes serine, alanine, and threonine, three of the most common amino acids in HRGPs [5]. Minimal changes in the code could in this way have led to different HRGPs evolving from an ancestral nucleotide sequence which encoded a proline-rich protein, indeed all these proteins may have a common ancestry originating from the C/G-rich areas of the genome [23]. If these theories on the evolutionary relationships between the hydroxyproline-rich molecules are correct, it should, in principle, be possible to trace this family of proteins back to a small number of protein modules or archetypes [5, 24, 25]. AGPs exist in embryophytes at least as far back in evolutionary development as the liverworts [26], PRPs and $Ser(Hyp)_4$ repeats exist in proteins from gymnosperms [11, 27], and $(Ser\text{-}Hyp)_n$ motifs have been found in the unicellular alga *Chlamydomonas* [28]. On this basis, [5] proposed a phylogeny for the HRGP-family. Consistent with this theory, but on a biochemical level, is the relationship between the evolutionary age of unicellular algae and their hydroxyproline-content [29].

Genetic length polymorphisms have been observed in P/HRGP-encoding genes from soybean. Different cultivars of soybean contain deletions (or insertions) of tandem repeats within the coding regions of these genes in a manner which conserves the reading frame [30]. This indicates that the basic repeat units can be conserved while insertions or deletions take place. This could be a possible mechanism for the evolution, or transfer, of repeat units between different groups of genes. Domain conservation has also been found in cell wall HRGPs from distantly related species of *Chlamydomonas* [31].

The presence of the $Ser(Pro)_4$-repeat, but not the Pro-Pro-Xaa-Yaa-Lys repeat, in algae could indicate that the latter motif evolved more recently. This type of repeat sequence is also present in gymnosperms [27] and therefore possibly evolved early in the development of embryophytes. The presence of the Pro-Pro-Xaa-Yaa-Lys motif as part of the repeats in some extensins could have arisen by fusion of the two sequence motifs. Alternatively the P/HRGPs could have evolved from extensins by deletion of the $Ser(Pro)_4$-repeats. A number of HRGPs also have palindrome and palindrome-like sequences. The sig-

nificance of the palindrome sequence is not known but they may play a role in self-assembly [5].

Glycine-rich proteins (codon GGX) could also be evolutionary related to the proline-rich proteins (codon CCX) by inversions and duplications [32]. This hypothesis is supported by the analysis of a cDNA from tomato in which one strand hybridizes on Northern blots to an extensin-encoding transcript and the other strand hybridizes to a glycine-rich encoding transcript [33]. A gene from *Arabidopsis thaliana* encodes a product rich in both proline and glycine [34] but no gene product encoded by the reverse coding strand of a nucleotide sequence that encodes an extensin has yet been identified.

COMMON FEATURES OF HRGPs

Hydroxyproline

The common building block of the Pro/Hyp-rich proteins is the imino acid proline. Hydroxyproline is formed by postranslational hydroxylation of proline in proteins by prolyl hydroxylases [2]. The prolyl hydroxylases are well-characterized from a number of plant species [23, 35]. The enzyme is tetrameric and consists of two catalytically active α-subunits with a M_r of 65.10^3 and two β-subunits with a M_r of 60.10^3 [36]. Plant prolyl hydroxylases also require molecular oxygen, ferro-ions, ascorbate, and α-ketoglutarate for activity and seem to have *in vitro* properties similar, but not identical, to their mammalian counterparts; the plant enzyme is capable of hydroxylating polyproline [2]. This observation led to the theory that the plant prolyl hydroxylase requires a polyproline II conformation for activity [37, 38]. However, the specificity *in vivo*, deduced by sequencing of proline- and hydroxyproline-containing peptides, shows a sequence-dependent hydroxylation pattern; Lys-Pro, Tyr-Pro, and Phe-Pro are never hydroxylated, whereas hydroxylated sequences include Ser-$(Hyp)_4$, Hyp-Val, Ala-Hyp, Hyp-Ala, and other sequences depending on species [5, 36]. This variety in sequences in which proline is hydroxylated might indicate the existence of multiple forms of prolyl hydroxylase, each with their own sequence specificity [5, 36]. This is consistent with two-dimensional (2D) SDS-PAGE analysis of purified prolyl hydroxylase from french bean which showed four major spots for both the α- and β-subunits [36].

Glycosylation patterns

Hydroxyproline can be *O*-glycosylated. This modification is unique to plants and Chlorophycean algae [7, 39]. Glycosylation can vary from less than 1% by weight of the molecule, in the case of some P/HRGPs, [40] up to 98% by weight in some AGPs [41, 42]. The nature of the glycosyl substituents can vary from the addition of a single arabinosyl or galactosyl residue up to a 75 residue glycan [12]. The glycosylation pattern of hydroxyproline may depend on the primary structure of the protein backbone; for example in a P/HRGP from Douglas Fir arabinosylation of hydroxyproline occurs primarily at contiguous hydroxyproline residues [12]. *O*-glycosylation may also occur through serine and to lesser extent through threonine [5, 8]. *N*-glycosylation as well as *O*-glycosylation on the same molecule is much less common but has been reported in a P/HRGP from french bean [43] and a P/HRGP from styles of some tobacco species [44, 45].

Covalent intermolecular cross-links

The presence of tyrosine in HRGPs gives the possibility of both inter- and intra-molecular linkage through the formation of isodityrosine. Isodityrosine has been detected in higher plants and in *Chlamydomonas* [46–49], whereas dityrosine has only been detected in unicellular algae [49]. The cross-linking through isodityrosine in higher plants may involve certain sequence motifs such as Val-Tyr-Lys and catalysis by a peroxidase [5]. An extensin peroxidase selectively cross-links HRGPs containing either Xaa-Hyp-Val-Tyr-Lys or Val-Tyr-Lys motifs *in vitro* [50]. Some P/HRGPs are not substrates for peroxidases which cross-link extensin *in vitro* [5, 50]. The non-cross-linking HRGPs can usually be extracted with salt, as are other ionically-bound wall components. A number of other covalent protein–protein, protein–carbohydrate, and protein–lignin linkages have been proposed, but none have yet been confirmed *in vivo* [4, 47, 51–53]. However, a covalent cross-link between extensin and pectin has been shown in cultured cells from cotton [54].

EXTENSINS

Structure of the protein backbone and glycosylation

Extensins are characterized by a Ser$(Hyp)_4$-repeat or related sequences (Table 1). They are highly glycosylated, containing 50–60% (w/w) carbohydrate. The monosaccharides of extensin from flowering plants are normally arabinose (90–97 mol %) and galactose (3–7 mol %) [9, 10, 55]. The arabinose mainly occurs as Hyp-$Ara_{1\text{–}4}$ [7, 9–12] and the galactose as Ser-Gal [8]. Extensins are secreted to the cell wall as monomers that are believed to be held in the wall initially through ionic forces, but are then insolubilized, probably through isodityrosine-linkages [48, 56, 57]. Intra-molecular isodityrosine linkages have been found in extensin [58], but an inter-molecular counterpart has not been demonstrated *in vivo*. However, inter-molecular cross-linking of extensin through isodityrosine linkages has been achieved *in vitro* using a crude, salt-extractable peroxidase preparation from tomato [59]. The formation of isodityrosine *in vitro* has been confirmed using a purified cell wall peroxidase from peanut [60].

Extensins exist as a polyproline II helix, a left-

Table 1. Some extensin repetitive motifs

Sequence	Source	References
SOOOO	Volvox, Douglas fir	[11, 200]
SPPPPKH	Soybean	[65]
SPPPPKK	Rape	[68]
SPPPPKKPYYPP	Tobacco	[71]
SPPPPSP	Bean, Almond	[87, 121]
SPPPPSPKYVYK	Tomato	[33, 92]
SPPPPSPSPPPP	Bean cells, *N. alata*	[67, 79]
SPPPPYYYH	Rape, soybean	[65, 70]
SPPPPYYYK	Bean cells, tomato, potato	[33, 79, 92, 201]
SOOOOTOVYK	Tomato	[55]
SPPPPTPVYK	Carrot, petunia, Tobacco	[4, 71, 99]
SOOOOVYK	Tomato	[55]
SPPPPVYK	Rape, soybean, Sunflower, *N. sylvestris*	[65, 68, 78, 84]
SPPPPVYSPPPP	Bean	[87]
SPPPPVHSPPPPVA	Tomato	[88]
SPPPPVK	Rape	[68, 70]
SPPPPVKSPPPP	Maize	[73]
SOOOOVKP	Tomato	[55]

handed helix with three residues per turn and a pitch of 9.36 Å [2, 9]. Electron microscopy of rotary shadowed samples of salt-extractable extensin 'precursor-monomers' shown thin, kinked rods of approximately 70–100 nm in length [9, 61, 62]. The kinks may result from intra-molecular isodityrosine-linkages [62]. The polyproline II structure seems to be stabilized by the carbohydrate (arabinosylation of the hydroxyproline residues), as deglycosylation of extensin results in partial distortion of unwinding of the helix [9]. This has been confirmed by electron microscopy and by gel filtration which indicate that the native, glycosylated extensin monomer has a Stokes radius of 89 Å, whereas deglycosylated extensin is much more compact with a Stokes radius of approximately 11 Å [63]. As glycosylation of extensin seems to impose a certain conformation on the protein backbone, glycosylation is probably important for the structural role of extensin in the plant cell wall [64].

Function

The extensins are generally believed to function as structural proteins (e.g. [4, 5]). Expression of extensins seems, to a certain degree, to be tissue-specific and developmentally regulated (e.g. [4, 65–77]). Synthesis of most, but not all, extensins can be induced by wounding, fungal attack, and/or treatment with elicitors (e.g. [33, 65, 78–92]). Spatial and tissue-specific differences in accumulation of extensin transcripts are found in bean hypocotyls in both compatible and incompatible fungal infections; this implies that induction of extensin expression is linked to specific race–cultivar interactions [93]. A highly cross-linked network of extensins might provide anchorage for lignification and in this way create a barrier impenetrable to fungal hyphae [2, 22, 94]. The highly basic extensins could also interact with acidic blocks of pectin, each extensin molecule 'zipping' together three or four pectin molecules; this would provide a simple, reversible means of non-covalent protein–polysaccharide cross-linking in the cell wall [48, 95–97]. Extensins have also been detected in the plasma membrane by immunological techniques and may function as wall-to-membrane linkers [98].

PROLINE/HYDROXYPROLINE-RICH GLYCOPROTEINS (P/HRGPs)

This group includes molecules that have a high content of Pro and Hyp, but which do not have the characteristic features of extensins, AGPs or solanaceous lectins.

Structure of the protein backbone

Like the extensins, the P/HRGPs also contain certain protein sequence motifs (Table 2). The occurrence of these motifs in individual P/HRGPs can vary from only one or two repeats up to constituting most of the protein backbone. One of the most commonly reported motifs is Pro-Pro-Xaa-Yaa-Lys, where Xaa

Table 2. Some P/HRGP motifs

Sequence	Source	References
PPRHK PP	Pea	[103]
OOVHK	Sugar beet	[117]
PPVHK PP	Carrot	[99]
T PVHK PP	Pea	[103]
PPIHK PP	Carrot	[99]
POVVK PO	Douglas fir	[27]
T OVYK	Sugar beet	[117]
POVYK PO	Soybean	[122]
POVYK	Bean	[43]
PPVYK	Soybean	[21, 40, 105, 202]
K PPVYK	Soybean	[202]
OOVYK PO	Douglas fir	[27]
PPVEK	Soybean	[21, 40, 202]
K POVEK	Bean	[43]
K POIYK PO	Soybean	[203]
PPIYK	Soybean	[40, 106]
PPTYK P	Rice, sorghum	[102, 112]
K PPTPK P	Maize	[118, 119]
POTEK PO	Soybean	[122]
PPHEK PP	Soybean, pea	[100, 104]
PPHVK PP	Tomato	[204]
K PPGAK	Antirrhinum	[205]
K PPATK PP	Sorghum	[102]
PPYV PP	Maize	[107]
P PPAT PPP	Cotton	[206]
PPTP RP	Sugar beet, maize	[107, 108]
K PPQ K	Pea	[103, 111]
K PPV K PP	*N. alata/tobacum*	[109, 110]
PPV K PP	Bean	[207]

is normally Val, His, Thr, or Ala and Yaa is normally Tyr, Thr, Glu, or Pro (in amino acid sequences derived from nucleotide data, Pro and Hyp cannot be distinguished). This sequence is often expanded with one lysine and/or one or two proline residues to (Lys)-Pro-Pro-Xaa-Yaa-Lys-(Pro-Pro) [27, 99–104]. This 'extended' general motif can also be present in proteins that have consecutive Pro-Pro-Xaa-Yaa-Lys repeats throughout the molecule [40, 105, 106]. As a variation on the Pro-Pro-Xaa-Yaa-Lys repeat, one gene from maize encodes a Pro-Pro-Tyr-Val-Pro-Pro repeat [107]. The same gene also predicts a Pro-Pro-Thr-Pro-Arg-Pro repeat which is also present in the N-terminal region of a putative chitinase encoded by a genomic clone isolated from sugar beet [108]. However, the proline-rich region of these sequences has only been found in genomic clones and the expression of these sequences has not yet been demonstrated by isolation of the corresponding mRNA or peptides. Other genes encode a Lys-Pro-Pro-Xaa-Lys-(Pro-Pro) motif, where Xaa is normally Gln, Val, Thr, or Ala [109–111]. The P/HRGPs that do not consist almost entirely of the repetitive motifs tend to have the motifs 'clustered' (e.g. [99, 102, 107, 109, 110, 112]). This could indicate that the motif has a structural function. Molecular modelling shows that tandem repeats of proline-rich peptides ($\approx$5–10-mers) frequently form β-turn helices [113]. This is confirmed by studies of synthetic peptides based on the repeat sequence of a mussel adhesive protein: Ala-Lys-Pro-Ser-Tyr-Hyp-Hyp-Thr-Tyr-Lys [114, 115] which forms an extended chain of S-shaped reverse β-turns [116].

Glycosylation patterns in P/HRGPs

As much of the information on this group is derived from cDNA sequences, there is little information on the glycosylation of the P/HRGPs. Only a few P/HRGPs have been isolated and characterized as glycoproteins. The properties of these P/HRGPs are summarized in Tables 3 and 4. The glycosylation ranges from $<3\%$ [40] to approximately 70% [44] of the glycoprotein. Only arabinose (and possibly glucose) was detected in the soybean P/HRGPs described by Datta *et al.* (1989) [40], whereas both arabinose and galactose (86 and 14 mol %, respectively) were found in the Douglas fir P/HRGP described by Kieliszewski *et al.* (1992) [27]. The P/HRGP from sugar beet described by Li *et al.* (1990) [117] also contains arabinose, but the content of other monosaccharides was not reported. In the few known cases, the P/HRGPs contain higher proportions of non-glycosylated hydroxyproline than the extensins (32–73 mol % *vs* 3–12 mol %, [9, 10, 27]), and less hyp-$Ara_{3,4}$ than the extensins. The carbohydrate fraction of the P/HRGP from french bean characterized by Millar *et al.* (1992) [43] contains mostly arabinose and galactose (47 and 40 mol %, respectively) with smaller amounts of *N*-acetylglucosamine and mannose (5 mol % of each), indicating *N*-linked glycosylation. However, the content of arabinose and galactose indicates substantial *O*-linked glycosylation through hydroxyproline and/or serine. *N*-linked glycosylation is also present in a style-specific P/HRGP isolated from some *Nicotiana* species [44, 45]. This *N*-glycosylation is consistent with the presence of Asn-X-Ser/Thr sites in the protein sequence encoded by a style-specific cDNA from *Nicotiana alata* [44, 109].

Possible relationships between extensins and P/HRGPs

The P/HRGP repeat Pro-Pro-Val-Tyr-Lys is present within the repeated sequence Ser-Pro-Pro-Pro-Pro-Val-Tyr-Lys of a number of extensins from sunflower [78], oilseed rape [68], and tomato [55]. Part of the extensin repeat from carrot [99] containing the sequence Thr-Pro-Val-Tyr-Lys could be changed into Pro-Pro-Val-Tyr-Lys by a single base-pair conversion from ACA (Thr) to CCA (Pro) [21]. The sequence repeat Ser-Pro-Pro-Pro-Pro-Val-Lys of the stylar P/HRGPs from tobacco [109, 110] is found in extensins from rape and maize [68, 70, 73]. When the P/HRGP-repeats occur in extensins, they only do so

Table 3. Properties of some purified P/HRGPs*,†

Name	30 kDa protein	Sb 33 kDa protein	28 kDa protein	33 kDa protein	TTS-protein	PHRGP	P1	42 kDa glycoprotein
Plant	Soybean	Soybean	Soybean	Soybean	Tobacco	Douglas Fir	Sugar beet	French bean
Tissue	Seedlings	Cell culture	Seedlings	Seedlings	Style	Susp. culture	Susp. culture	Susp. culture
Protein (% w/w)	—	—	>97	>97	65	79	60	58‡
CHO (% w/w)			<3	<3	35	21	40	42‡
Most abundant amino acids (mol %)	Hyp:17 Pro:19 Lys:15 Val:15 Glx:10	Hyp:20 Pro:23 Val:17 Lys:16 Tyr:13	Hyp:20 Pro:22 Lys:17 Val:16 Glx:10	Hyp:20 Pro:22 Lys:17 Val:17 Tyr:12	—	Pro:21 Hyp:28 Val:17 Lys:11 Ile: 9	Hyp:34 Pro: 6 Val:11 Lys:10 Ser:10	Hyp:7 Pro:14 Val:15 Glx:11 Gly:10
Monosaccharide composition (mol %)	—	—	Ara	Ara	Gal 70 Glc 17 GalA 6.5 Ara 6	Ara:86 Gal:14	Ara	Ara:47 Gal:40 GlcNAc: 5 Man: 5
Apparent MW, kDa								
Native	30	33	28	33	50–100	97	—	42
Deglycosylated	—	—	—	(30)	27	—	40	31
Reference	[122]	[105]	[40]	[40]	[45, 208, 209]	[27]	[117]	[43, 194]

* The "identity" of these HRGPs as P/HRGPs is based on detection of P/HRGP-motifs during peptide sequencing.
† See also Table 4 (GaRSGP and the 120 kDa glycoprotein).
‡ Reported as 47 res. CHO/100 res. amino acids.
—: not reported.

Table 4. HRGPs and cDNA clones encoding HRGPs from pistils of *N. alata*

Name	GaRSGP	120 kDa glycoprotein	AGP RT25	AGP RT35	*NaPRP3*[a]
Tissue	Transmitting tract of style	Transmitting tract of style	Style	Stigma	Transmitting tract of style
Protein (% w/w)	25–35	65	<10	<10	—
CHO (% w/w)	65–75	35	>90	>90	
Most abundant amino acids (mol %)	Lys 14 Pro 12 Hyp 9 Val 8 Ser 7 Thr 7	Hyp 21 Pro 12 Ser 8 Lys 7 Ala 7 Leu 6	Ala 20 Hyp 18 Ser 15 Gly 9 Thr 7 Val 6	Asx 15 Glx 12 Ala 11 Thr 7 : Hyp 5 Pro 4	Pro/Hyp 30 Ser 17 Leu 8 Ala 7 Lys 5 Val 4
Carbohydrate (mol %)	Gal 83 Ara 7 GlcNAc 4 Man 4 Xyl 2 Glc 1	Gal 37 Ara 63	Gal ~60 Ara ~30	Gal 52 Ara 45 Rha 2 Glc 1 Xyl <1	—
Apparent M_r (kDa)					
Native	45–120	120	>90	>90	—
Deglycosylated	28–30	78	—	—	—
Predicted M_r of backbone (kDa)[b]	28	43	10	17	14
pI	⩾10	>9	—	—	—
Predicted pI[b]	10.5	—	6.8	7.5	10.6
Corresponding clone	*NaPRP4*	*NaPRP5*	*AGPNa1*	*AGPNa3*	*NaPRP3*
Class	P/HRGP	P/HRGP	AGP	AGP	Extensin
Reference	[44, 109]	[124, 199]	[142, 145]	[142, 145]	[67]

[a] Only isolated as a cDNA clone.
[b] Predicted from cDNA clone.
—, not reported.

as part of the extensin-repeats and not 'on their own' in separate parts of the molecule. Also, some of the P/HRGPs contain one Ser-$(Pro)_4$ motif only, normally located close to the C-terminus (e.g. [118, 119] (maize); [102] (sorghum)). Some of the information on the relationships between extensins and P/HRGPs is clouded by the finding that recombinations or hybrid clones of different HRGPs are frequently formed during preparation of cDNA libraries (e.g. [120, 121]; Drs. C.-G. Chen, K. Hauser, and C. Schultz, personal communication). Therefore, if a cDNA encoding a putative extensin or P/HRGP hybridizes to more than one transcript on a Northern blot, the homogeneity of the clone should be verified by comparing Northern-blots that have been probed independently with both the 3′- and the 5′-ends of the clone. It is also helpful to have analyses of both cDNAs and genomic clones to resolve whether the sequence truly represents a single protein sequence or whether a cloning artefact has been introduced.

Function

P/HRGPs are in general presumed to be cell wall proteins (e.g. [22, 44, 105]), and the highly repetitive nature of some of the P/HRGPs (e.g. [40]) suggests a structural role. The expression of many P/HRGPs is tissue-specific and temporally-regulated. For example P/HRGPs are expressed in developing tissues of soybean [21, 40, 89, 105, 122, 123], sexual tissues of *N. alata* and *N. tabacum* [44, 45, 109, 110, 124], and in various tissues of maize [107, 118, 119, 125, 126]. A number of P/HRGPs are expressed specifically in the cell walls of legume nodules [100, 103, 104, 111].

Expression of some P/HRGPs is associated with infection and wounding. For example P/HRGPs are found in maize roots as a response to the presence of a symbiotic, mycorrhizal fungus [127]. A number of P/HRGPs (and putative P/HRGPs) are expressed in response to wounding and bacterial, fungal, and viral pathogen infection [4, 22, 43, 83, 89, 120, 128–131]. Most of these studies have been on dicots, but accumulation of P/HRGPs in response to wounding has also been reported in a monocot, maize [126]. This, however, might be a restricted phenomenon as hydroxyproline does not accumulate in response to wounding in barley, oats, wheat, or rice [132, 133]. More direct evidence for a role in plant defence is that some P/HRGPs are insolubilized in cell walls as quickly as 2–5 min after wounding or elicitor treat-

ment by peroxide-mediated oxidative cross-linking [134, 135]. This rapid insolubilization may act to strengthen the wall and hinder pathogen invasion [134, 135]. Like the extensins, these P/HRGPs might also act as nucleation sites for lignin deposition [94]. Another possible role for the P/HRGPs in defence against pathogen attack could be to act as microbial agglutinins. Highly glycosylated HRGPs isolated from cell walls of potato tubers and tobacco callus can agglutinate avirulent, but not virulent strains, of potential bacterial and fungal pathogens [136–138]. These agglutinins show no lectin activity and their agglutination capacity, *in vitro*, is greatly reduced by high concentrations of salt, suggesting an ionic interaction between the basic P/HRGPs and the negatively charged bacterial lipopolysaccharides [138]. The virulent bacterial strains, that were not agglutinated by the P/HRGPs, produce an extracellular polysaccharide slime [139]. Furthermore, a chitin-binding P/HRGP from french bean binds to hyphae of a pathogenic fungus of bean [43].

ARABINOGALACTAN-PROTEINS (AGPs)

Structure of the protein backbone

Most arabinogalactan-proteins (AGPs) differ from the extensins and the P/HRGPs by having a neutral to acidic protein backbone and a protein content typically between 2 and 10% [41], although some fractions obtained from gum arabic contain only 0.3% protein [42]. Because of the high carbohydrate content they are often described as proteoglycans. AGPs are widely distributed throughout the plant kingdom and are found in flowering plants from every taxonomic group tested and in gymnosperms, mosses, and liverworts [1, 26, 41]. AGPs are detected in almost all tissues from higher plants, in leaves, stems, trunks, roots, floral parts (AGPs have been found in the styles of all angiosperms surveyed), seeds and they are also often secreted in large amounts by suspension cultured cells (e.g. [1, 16, 41, 140]).

The protein backbones of AGPs are normally rich in hydroxyproline, alanine, serine, threonine, and glycine [2, 4, 41]. However, a few AGP-like glycoproteins have been isolated that contain only small amounts of hydroxyproline [141, 142]. Because of the high content of carbohydrate and the problem that there are often many closely related AGPs in a single tissue extract, obtaining complete sequence from a pure deglycosylated protein backbone has been difficult. Another problem is the often extreme 'stickiness' of some AGPs, so that even under highly dissociating conditions, the AGP may be associated with another (glyco)protein [142, 143]. Even when sequences of individual proteins have been obtained, the redundancy of the codons for the most common amino acids in AGPs have made cloning based on PCR difficult. In a few cases the technical difficulties of cloning have been overcome to give complete cDNA clones [142, 144–146] or genomic clones [147]. These cDNAs all encode proteins consisting of three domains as shown in Fig. 1. cDNA sequences or genomic clones, which because of the composition of the deduced protein sequence probably encode AGPs, have also been published [148–153].

Common motifs in the AGP backbone are Ala-Hyp or Hyp-Ala, which may be diagnostic of AGPs [4, 147, 154]. However, whether these motifs are non-random and significant to the function of AGPs remain to be resolved. Sequences encoding these motifs are also present in cDNAs cloned from pear- and *N. alata*-cell suspension culture [144, 146], and from *N. alata* styles [145] which encode the protein backbones of AGPs. The same motifs are found in cDNAs which might encode AGPs from tomato fruits [152] and xylem from loblolly pine [151]. These cDNAs also encode several Ser-Hyp repeats (the prolyl hydroxylation has been confirmed by partial peptide mapping [144, 145]) but no other characteristic motifs. The Hyp-Ala, Ala-Hyp, and the Ser-Hyp motifs are also found in peptides from an AGP isolated from maize suspension cultured cells [6]. The Ser-Hyp motif might play a role in the partial polyproline II helix observed in AGP from *Lolium* suspension culture [155]. However, recent reports indicate that some protein backbones are relatively poor in Hyp/Pro and Ala.

Glycosylation patterns in AGPs

The carbohydrate moiety usually accounts for more than 90% by weight of the molecule. The major sugar residues are arabinose and galactose, although rhamnose, glucuronic acid, and other monosaccharide residues are present as minor components in some cases [41]. The carbohydrate chains are normally *O*-linked to hydroxyproline, serine, and in some cases threonine [41, 156, 157]. In many AGPs the majority of the carbohydrate moiety is composed of a 1,3-β-linked galactosyl backbone that is extensively branched at C(O)6 with chains of 1,6-β-linked galactosyl residues substituted with 1,3-linked- and terminal-arabinosyl and other residues [41, 51, 158, 159].

Two structural models have been proposed for AGPs. One is the 'wattle blossom' model [41] in which several polysaccharide chains are attached to Hyp-residues on the protein backbone to generate a spheroidal molecule. Another model depicts gum arabic AGP as a 'twisted hairy rope' where short triarabinosides and longer chains of galactose and glucuronorhamnoarabinogalactans are linked to closely interspaced Hyp-residues [160]. Both are consistent with the incomplete analytical data. Establishing a complete structure will involve fragmentation of the molecule to small glycopeptides, separation of the glycopeptides and detailed sequence and linkage analysis of each.

An often used diagnostic feature of AGPs is their ability to interact with the β-glucosyl Yariv reagent,

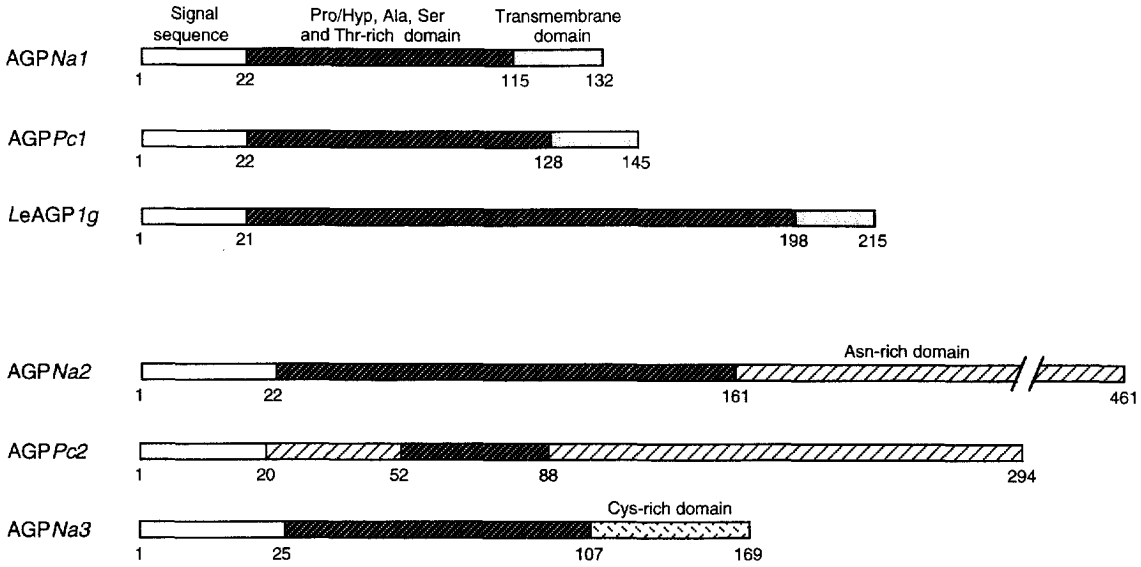

Fig. 1. Schematic representation of the domain structures of some nucleotide sequences encoding AGPs. The numbers below the sequences indicate the amino acid residue number in the deduced protein sequence. The AGP*Na*1 and AGP*Na*3 cDNA clones were isolated from styles of *N. alata* [142, 145]; AGP*Na*2 from suspension-cultured cells of *N. alata* [144]; and AGP*Pc*1, AGP*Pc*2 from suspension cultured cells of *Pyrus communis* [144, 146]. The genomic *Le*AGP1*g* clone was isolated from *Lycopersicon esculentum* [147]. Modified from [14] with permission.

forming orange-red complexes or precipitates [161]. The basis of this widely used diagnostic test is unknown. The specific interaction between AGP and β-glucosyl Yariv reagent is used to quantify AGPs by radial gel diffusion [162] and to detect different types of AGPs separated by crossed electrophoresis [163]. Size-determination of AGPs is dependent on the method used. An AGP estimated to have a M_r within a narrow range of around 75.10^3 by gel permeation chromatography can appear as a smear from 110 to more than 200 when analysed by SDS-PAGE [164]. Alternative methods for size determination of AGPs are ultracentrifugation and light scattering.

Location of AGPs within plant tissues

Due to the extreme solubility of most AGPs, their cellular location is often difficult to determine precisely [4]. Most AGPs are secreted extracellularly [2], but AGPs are also present in plasma membranes and intravacuolar multivesicular bodies [164–170] and the cell walls of vascular bundles and sclerenchyma cells [167], cell suspension cultures [171], pollen, and pollen tubes [172–174]. Most work on the structure and composition of AGPs has focused on the secreted AGPs from gummy exudates, suspension cultured cells, and from stylar canal or transmitting tract cells of styles.

Function of AGPs

Although no general function for AGPs has been established, recent work has indicated that some AGPs might act as signalling molecules and/or be involved in cell differentiation, development, positioning, death [15, 166, 167, 175–180], cell expansion [181] and cell proliferation [171, 182] as well as somatic embryogenesis [183]. AGPs may also have a function in adhesion between cell walls and plasma membranes [184] and in cell expansion as a wall-loosening factor [185, 186]. Further, in the case of extracellularly secreted AGPs in styles and stigmas of flowers, AGPs could have a role in pollen tube growth either as adhesives or nutrients or they might simply provide a hydrated matrix that can support the germination and growth of pollen tubes [187].

SOLANACEOUS LECTINS

Lectins with binding specificity for oligomers of *N*-acetylglucosamine have been isolated from a number of Solanaceous plants. The best characterized are those from tubers of potato (*Solanum tuberosum*), seeds of thorn-apple (*Datura stramonium*), and fruits of tomato (*Lycopersicon esculentum*). The lectin from potato tubers consists of a non-glycosylated, cysteine- and glycine-rich, carbohydrate binding domain and a highly glycosylated, hydroxyproline-rich domain [188]. The *Datura* lectin has a similar structure [189]. The potato lectin contains the extensin-like Ser-$(Hyp)_4$ motif [190], consistent with the polyproline II helix conformation of the hydroxyproline-rich domain of the lectin [191]. Potato lectin contains approximately 50% carbohydrate which is composed of 95 mol % arabinose and 5 mol % galactose [188]. The structure of the carbohydrate moiety is similar to that of the extensins; it consists mainly of tri- and tetra-β-arabinofuranosides (with additional small amounts of mono- and di-β-arabinofuranosides) *O*-linked to hyd-

roxyproline and of single α-galactopyranoside residues *O*-linked to serine [188, 192].

In addition to the 'classical' potato-lectin, a novel chitin-binding glycoprotein with a distinctively different amino acid and carbohydrate composition has been isolated from potato tubers [193]. This new lectin has a composition which seems closer to the chitin-binding P/HRGP from bean [43, 194], with which it is also immunologically cross-reactive [193]. This chitin-binding lectin is expressed in response to wounding as opposed to the 'classical' lectin, which seems to be developmentally regulated [193, 195]. It is found both in association with the cell wall and in the cytoplasm and vacuole, which is an unusual localization for a HRGP [193, 196].

The function of the Solanaceous lectins has not been unequivocally established, although a role in defense, in which the lectin acts by binding to chitin present on the surface of the pathogens, seems likely [4, 193]. However, these lectins might also be involved in cell–cell interaction, sugar transport, stabilization of storage proteins, control of cell division, and wound healing [2, 4].

HRGPs OF THE FEMALE SEXUAL TISSUES OF *NICOTIANA ALATA*

The pistils of many flowering plants are a rich source of HRGPs [197]. As an example of the diversity of HRGPs from a single tissue in a single species, we here review HRGPs isolated and characterized from styles of one plant. The most intensively studied source is *N. alata* from which several HRGPs have been isolated and analysed as glycoproteins and also described by cDNA sequencing. Five different HRGPs have been characterized from *N. alata* stylar tissues (Table 4) and many others are present in extracts but have not been characterized.

Arabinogalactan-Proteins (AGPs)

Two AGPs have been isolated as single molecular species from styles of *N. alata*. One, AGP*Na*3 [142] is specific to the stigma and the other, AGP*Na*1 [145], is expressed both in styles and other tissues such as leaves. Both contain >90% carbohydrate, with the monosaccharides Ara and Gal in the linkage arrangement typical of other AGPs. AGP*Na*1 has a C-terminal region which has no proline/hydroxyproline and which has the features of a transmembrane helix. This is typical of the backbones from the few other AGP backbone proteins known. AGP*Na*3 differs from other AGPs in having a low content of Hyp and Cys-rich C-terminal domain which is in some way similar to the Cys-rich C-terminal domain of the solanaceous lectins. Both these AGPs are secreted to the extracellular matrix.

The 120 kDa glycoprotein

This glycoprotein is, like AGP*Na*1 and AGP*Na*3, secreted to the extracellular fluid. This extracellular location is unusual for a basic HRGP which are most commonly secreted and bound to the acidic cell wall. The function of the 120 kDa glycoprotein is not known but as it is found in pollen tubes growing through styles it may be involved in pollen tube growth [198]. A role in defence has also been suggested [124]. The 120 kDa glycoprotein can be classified as a P/HRGP. The sequence includes several characteristic ProProXaaLys motifs and also contains several Ser/Pro_{1-3} repeats and two Ser/Pro_4 motifs [199], which ara characteristic of the extensins. The carbohydrate linkage analysis is consistent with the carbohydrate being arranged in short 1,2-linked Ara*f* chains and chains typical of AGPs, i.e. 1,3-Gal backbones, with side-branches of shorter 1,6-Gal chains, some of which terminate in Ara*f*. Thus this glycoprotein has features of both P/HRGPs, extensins and AGPs.

The galactose-rich style-specific cell wall glycoprotein (GaRSGP)

This glycoprotein is associated with cell walls and is specific to the stylar transmitting tissue [44, 109]. The protein backbone includes the motif Pro-ProXaaLys which is typical of the H/PRGPs. It is quite distinct from the 120 kD glycoprotein but they have several stretches of up to 10 amino acids in common. The carbohydrate fraction is unusual in that it consists mainly of galactose residues in 1,6-, 3,6- and terminal-linkages. It includes a carbohydrate epitope which is common with an epitope of the 120 kD glycoprotein. This cross-reactivity illustrates the limitations of using immunological techniques to identify individual HRGPs.

This glycoprotein also specifically binds certain divalent heavy metal ions, a property which has not been noted for any other HRGPs [17]. Whether this property is relevant to the function of the molecule is not known.

Extensins

The cell walls of the transmitting tissue of the style are expected to include extensins and the cDNA encoding an extensin has been isolated (*NaPRP3*; [67]). The sequence is unusual in that it is relatively short (151 aa) and lacks tyrosine and thus would not be cross-linked through dityrosine linkages. This extensin has not been studied as a glycoprotein.

Although the inventory of the HRGPs in the style so far described is incomplete, we have learnt that one tissue can include many distinct HRGPs, that these different molecules can share both protein and carbohydrate epitopes, and that the cellular locations of the different HRGPs may differ. Establishing the detailed structures of the individual members of this group will

give the background for experimental design aimed at understanding function.

CONCLUDING REMARKS

There are a number of general questions regarding this group of molecules which cannot be answered from our current knowledge. Why does the plant synthesize so many closely related molecules? Different functions for different molecules with separate locations within tissues can be imagined, e.g. AGPs secreted into the extracellular milieu; extensins and P/HRGPs in the wall. Why is such diversity generated for each location? The requirement for so many members of the family within the plant implies specificity of function which is not yet understood. Designing experimental approaches to the question of function is difficult if the full structure of the molecule is not known. Whereas for some HRGPs we have complete information on the amino acid sequence of the protein backbone and an understanding of the linkage arrangements for some of the simpler carbohydrate substituents (e.g. the arabinose chains in extensins), generally neither the structure of the side-chains nor the point of glycosylation on the protein backbone are known.

Questions arise as to whether function is related to the specific protein sequences, the nature and/or order of the glycosyl substituents or the three-dimensional structure, or (as is likely) all these aspects: how can the question of function be addressed? Two classical approaches are: (1) To transform plants with antisense constructs so as to down-regulate the transcript encoding the protein backbone of a single molecule and to examine the transformants for changed phenotype; and (2) to isolate the molecule in a pure and native form and test it in a bioassay. Both approaches have major limitations for examining the function of the HRGPs. Down-regulation by antisense transformation may well down-regulate not only the target protein backbone, but also related proteins bearing common motifs. The limitation for a bioassay approach is in knowing what property the bioassay should be designed to measure. The questions of pathways for biosynthesis which involve the post-translational modification or hydroxylation of certain proline residues and specific glycosylation is another incomplete aspect of our knowledge.

The value of focusing studies of different HRGPs on one tissue of one plant is illustrated by studies of HRGPs of *N. alata* styles. An alternative model system is the *Arabidopsis* which offers simple genetics. Certainly understanding the genetics of the synthesis of this whole class of molecules will be an important approach for the future. However, is seems likely that the glycosyl components of the HRGPs play a role in function and at present we have a poor understanding of the control of hydroxylation of the proline residues and of glycosylation. Therefore, the genetic approach using a model system, such as *Arabidopsis*, will be valuable, but will need to be complemented by a second model system in which sufficient material can be isolated for direct analysis and functional testing. We are now at the beginning of a field of study which, in its diversity, complexity and importance, is probably analogous to the study of the animal extracellular matrix. We can expect, as knowledge of this class of molecules is established, that we will get a new dimension to an understanding of such fundamental plant processes as cellular identity, differentiation, and defence.

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