Characterization of carrot arabinogalactan proteins

Peter Immerzeel

Promotoren: Prof. dr. S.C. de Vries
Hoogleraar in de Biochemie
Wageningen Universiteit

Prof. dr. A.G.J. Voragen
Hoogleraar in de Levensmiddelenchemie
Wageningen Universiteit

Co-promotor: Dr. H.A. Schols
Universitair docent, departement Agrotechnologie en Voedingswetenschappen, Wageningen Universiteit

Samenstelling promotiecommissie:

Prof. dr. L.H.W. van der Plas (Wageningen Universiteit)
Prof. dr. C. Mariani (Radboud Universiteit Nijmegen)
Dr. Ir. M.A.J. Toonen (PRI, Wageningen)
Dr. M. Kreuger (Syngenta, Enkhuizen)

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Contents

Outline

This thesis describes research on arabinogalactan proteins (AGPs) that were isolated from different carrot tissues. AGPs are proteoglycans that are widely distributed in the plant kingdom and are present in the cell wall, at the plasma membrane and are secreted in the medium when cells are grown in culture (Nothnagel, 1997). In general, it appears that AGPs are involved in plant development (Showalter, 2001). Complete plants can be regenerated via somatic embryogenesis from cells grown in culture (reviewed by Mordhorst et al., 1997), a phenomenon first described for carrot (Steward, 1958; Reinert, 1958). AGPs have been studied for their role in the formation of somatic embryos but their precise biological function at the molecular level remains unknown. Addition of AGPs to carrot cell cultures increased the number of somatic embryos formed (Kreuger and van Holst, 1993). De Jong et al. (1992) showed that secreted acidic chitinase could rescue the development of a carrot somatic embryo variant when grown at the non-permissive temperature. Chitinases hydrolyse the linkage between acetylglucosamine (GlcNAc) or glucosamine (GlcN) residues. The knowledge that both AGPs and chitinase were involved in embryogenesis was combined in experiments where AGPs were incubated with chitinase (van Hengel, 1998). It was shown that incubation of carrot seed AGPs with chitinase before the addition to wild-type carrot protoplasts resulted in an increased formation of somatic embryos.

The aim of the research presented here was to establish the composition of the carbohydrate part of AGPs that have biological activity during somatic embryogenesis of plants. The results of the isolation, purification and partial characterization of AGPs that were isolated from different carrot tissues are described in this thesis.

In the introduction (chapter 1) an overview of cell wall polymers is presented with emphasis on protein containing polymers. The chemical characteristics, some proposed biological functions and the interaction of AGPs with other cell wall polymers are described in this chapter.

In chapter 2 data are presented of experiments showing that AGPs increase the number of somatic embryos when compared to non-supplemented cultures. When AGPs were pretreated with chitinase, the number of embryos obtained was higher when compared to untreated AGP extracts. Before and after incubation of AGPs with

chitinase, different elution profiles for AGPs were observed by chromatography. The experiments in chapter 2 were done with a crude, total AGP extract and showed that at least some AGPs were sensitive to degradation by chitinase. A logical approach therefore was to try and purify a GlcN containing AGP fraction after degradation with chitinase followed by characterization of the hydrolysis products.

In chapter 3 the purification is described for AGP fractions that were obtained from seeds and cell culture medium, potentially including GlcN containing AGPs. Chemical characterisation showed that different AGP fractions were present in both seeds and medium with respect to both molecular weight and carbohydrate composition. Although GlcN was detected in the AGP seed extract before further purification, it could not be found in any of the purified fractions. Therefore the planned chitinase treatments of AGPs were replaced by a more extended chemical analysis of the carbohydrate- and protein structures of carrot AGPs.

In chapter 4 the research is described in which carrot cell culture medium AGPs are analyzed and compared to AGPs isolated from the cell walls of a carrot suspension culture. This work showed that different AGPs occur in medium and cell culture cell wall fractions with respect to carbohydrate composition. AGPs with a high percentage of galacturonic acid (GalA) were detected in medium and buffer isolated cell wall AGPs. After incubation of both GalA rich AGP fractions with pectin methylesterase and polygalacturonase, homogalacturonan hydrolysis products were detected. This indicated that the GalA present in AGP extracts is, at least partly, organized as homogalacturonan structures. Homogalacturonan is a major component of the cell wall polysaccharide pectin. In order to prove that GalA rich AGP fractions were also present in other carrot tissues, carrot tap roots were extracted.

In chapter 5 AGPs are described that were isolated from three sequential cell wall extracts from carrot tap root. An AGP fraction with a high GalA content was obtained. With pectin methylesterase and polygalacturonase hydrolysis products of the GalA rich AGP fraction were observed. This finding indicated that GalA is also present as homogalacturonan in a small fraction of buffer-extracted tap root cell wall AGPs.

In the concluding remarks of this thesis (chapter 6) the function of AGPs are discussed with respect to the findings from this research. Different cell wall models are presented and focus on how AGPs and pectin might be linked. The presence of homogalacturonan in AGP fractions is discussed in view of the biological function of an AGP-pectin linkage.

Chapter 1

Introduction

Components of the cell wall

The extracellular matrix of plant cells is composed of a wall that gives shape, strength and protection to plants. The function of a cell is related to the composition and structure of its cell wall (Pennell, 1998). As stated by Scheible and Pauly (2004) the main components of a cell wall are polysaccharides, most likely because plants are able to generate a large amount of sugars during photosynthesis. In contrast with the extracellular matrix of animal cells, proteins, such as structural glycoproteins and enzymes, are present in limited amounts in plant cell walls. From the outside to the inside of the cell, the middle lamellae, the primary cell wall and, in some cell types, a secondary cell wall can be distinguished.

An important component of cell walls is cellulose that is arranged in microfibrils and gives tensile strength to the wall. Cellulose microfibrils are found both in primary and secondary cell walls. Pectins are branched polymers that can establish a network by covalent- or ionic interactions with other pectins or other polymers. Borate diesters of rhamnogalacturonan II are identified, ionic interactions with calcium and ester linkages through phenolic dimers such as diferulic acid. Pectins are mainly present in the middle lamellae and the primary cell wall and play important roles in the adhesion of adjacent cells (Carpita and Gibeaut, 1993). Besides cellulose and pectins other polysaccharides are present in the cell wall that are indicated as hemicellulosic polysaccharides. Arabinoxylans, xyloglucans, mannans, glucomannans and arabinogalactans belong to the hemicelluloses. In the primary walls of dicots and non-graminaeceous monocots, xyloglucan is the quantitatively predominant hemicellulosic polysaccharide. Xyloglucan coats the surface of cellulose microfibrils and establishes cross-links between the cellulose microfibrils. Some of the above-mentioned hemicelluloses also occur as part of other molecules. For instance arabinogalactan is attached to the amino acids of arabinogalactan proteins (AGPs).

Although proteins are present in the cell wall in smaller amounts when compared to the carbohydrates, they have important roles in the formation, extension and maintenance of the cell wall. Hydroxyproline was discovered as a major amino acid constituent of cell wall hydrolysates of cell cultures (Lamport and Northcote, 1960). Many hydroxyproline rich glycoproteins (HRGPs) have been described in a wide variety of plants and algae. Other proteins in the cell wall include various enzymes, expansins and wall-associated kinases (WAKs) (Johnson et al., 2003). Some of these enzymes are vital for in situ wall assembly and remodelling during development. Others are involved in pathogen responses or stress responses. The

phenolic compounds in plant cells can be divided into two groups: high molecular weight lignin and low molecular weight hydroxycinnamic acids e.g. ferulic acid and *p*-coumaric acid. The linkage pattern of lignin is irregular due to the non-enzymatic nature of the polymerisation of the monolignol units. Lignins give pressure strength to the wall and protect the wall against biological attack. Ferulic acid is the most abundant hydroxycinnamic acid in the plant world (Mathew and Abraham, 2004) and may have a role in the cross-linking of cell wall polymers.

Hydroxyproline rich glycoproteins

Structural proteins of the cell wall are rich in the amino acids proline (Pro), hydroxyproline (Hyp) (formed by posttranslational hydroxylation of proline), serine (Ser), threonine (Thr) and glycine (Gly). These proteins are members of the family of hydroxyproline rich glycoproteins (HRGPs) or belong to the glycine rich proteins (GRPs). The HRGPs comprise a group of glycoproteins and proteoglycans that are rich in Hyp/Pro (generally > 5%) and include extensins, AGPs and proline rich proteins (PRPs) (Sommer-Knudsen et al., 1998). The molecules that belong to the HRGPs or the GRPs should be viewed as a phylogenetic continuum based on their protein part and, in the case of HRGPs, also on their carbohydrate part. HRGP backbones can be glycosylated and arabinofuranosyl and galactopyranosyl residues are the most abundant sugars (Johnson et al., 2003). Glycosylation ranges from basic, minimally glycosylated proteins (e.g. PRPs) to acidic and highly glycosylated proteoglycans such as AGPs (Gaspar et al., 2001). A typical feature of structural cell wall proteins is the presence of repetitive peptide motifs (Serpe and Nothnagel, 1999).

GRPs show glycine contents up to 60 or 70% of all amino acid residues (Ringli et al., 2001). GRPs are located in the vascular tissue of many higher plants and besides a structural function, some glycine rich proteins are known to have a RNA-binding capability. GRPs are, unlike the proteins belonging to the HRGPs, not glycosylated (Ringli et al., 2001).

PRPs contain essentially one repetitive amino acid motif: variations of (Pro-Pro-Xaa-Yaa-Lys)$_n$ (Xaa can be Val, His, Thr or Ala; Yaa can be Tyr, Thr, Glu or Pro) and are lowly glycosylated (reviewed in Sommer-Knudsen et al., 1998). Sugar residues found on PRPs are reported to contain mainly arabinose. The highly repetitive nature of some PRPs suggests a structural role (Sommer-Knudsen et al., 1998). When PRPs are cross-linked to extensins, a heteropeptide framework locking cellulose microfibrils

within the cell wall can be formed, as suggested by Carpita and Gibeaut (1993). Some of the PRPs are nodulins and are specifically found in the cell walls of nitrogen fixing root nodules.

Extensins were thought to play a role in controlling cell elongation and extension. When the plant cell reaches its final size, extensins are formed in the primary cell wall to block further expansion (Nothnagel, 1997). Extensins are di-tyrosine cross-linked and water insoluble (Kido et al., 1996). Extensins contain carbohydrates for approximately 50% of the mass with mostly arabinosides (90 to 97 mol% Ara_{1-4}) *O*-linked to Hyp. Some single galactopyranosyl residues may be attached to Ser (Johnson et al., 2003). The arrangement of the arabinosides possibly determines how these glycoproteins interact with other polymers of the wall; it also instructs them how to assemble into the wall (Cassab, 1998). Extensins have been proposed to be structural cell wall proteins that may also play a significant role in plant defence (Sommer-Knudsen et al., 1998).

In general lectins have the ability to bind proteins or glycoproteins of different origin. Lectins are normally chimeric glycoproteins consisting of a carbohydrate binding lectin domain and an extensin-like domain. The solanaceous lectins represent a unique class of hydroxyproline rich plant lectins. These lectins occur only in solanaceous plants, they have the ability to agglutinate oligomers of *N*-acetylglucosamine, they occur predominantly extracellularly and they have an unusual amino acid and carbohydrate composition (reviewed in Showalter, 1993). Because hydroxyproline is a major constituent, solanaceous lectins are grouped with other HRGPs. Potato lectin contains approximately 50% carbohydrate in the form of arabinose (95%) and galactose (5%) (Sommer-Knudsen et al., 1998). The function of the solanaceous lectins has not been unequivocally established although a role in defence, in which the lectins act by binding to chitin present on the surface of pathogens is proposed (Sommer-Knudsen et al., 1998).

Arabinogalactan proteins

In general three criteria have defined AGPs: structural characteristics are a hydroxyproline rich protein backbone and the presence of type II arabinogalactan chains, an operational characteristic is the ability to bind to a synthetic phenylazo dye (*ß*-glucosyl Yariv phenylglycoside) (Du et al., 1996). However, some exceptions have been described: there have been arabinogalactan chains found on proteins that do not bind Yariv phenylglycosides and some AGPs are Hyp deficient (Baldwin et al.,

1993). The current view of AGPs is of a family of molecules with different protein backbones that are glycosylated with a wide variation of carbohydrate chains. Most protein backbones of AGPs differ from the extensins and the PRPs by being neutral to acidic. The protein content typically varies between 2 and 10% on weight basis (Sommer-Knudsen et al., 1998). Because of the high carbohydrate content they are often described as proteoglycans. Proteoglycans in general exist as complex mixtures of glycosylated variants (glycoforms). AGPs form the major component of water-soluble HRGPs (Kido et al., 1996).

AGPs are widely distributed in the plant kingdom, probably occurring in all tissues of every plant from bryophytes to angiosperms (Majewska-Sawka and Nothnagel, 2000). AGPs have been located on the plasma membrane, in the cell wall and intercellular spaces (Baldwin et al., 2001). Gum arabic is one of the best-known AGPs and is secreted by *Acacia senegal* upon wounding of the tree (Cassab, 1998). AGPs are also secreted in large amounts by cells in suspension cultures (Serpe and Nothnagel, 1999). It is not certain that these AGPs are normally targeted beyond the cell wall. An alternative possibility is that culture medium AGPs are destined for the cell wall but end up in the medium upon failure to be assembled in the cell wall under culture conditions (Serpe and Nothnagel, 1999).

While certain structural characteristics are common features of AGPs, considerable variation occurs among these macromolecules in both their polypeptide and carbohydrate components (Serpe and Nothnagel, 1999). The molecular weight for AGPs varies between 60 and 300 kDa (Majewska-Sawka and Nothnagel, 2000) but also arabinogalactan-peptides as small as 22 kDa have been isolated (Fincher et al., 1974). When the number of amino acid residues is limited to 10 or 13, the molecule is designated as an arabinogalactan-peptide (AG-peptide) (Schultz et al., 2002).

Based on transmission electron microscopic imaging of AGPs, some AGPs are globular, whereas others are rodlike molecules (Showalter, 2001). Two models of molecular organization correspond to these observed shapes: “the wattle blossom” model (Fig. 1.1A) that predicts a round shape of AGPs (Fincher et al., 1983) and “the twisted hairy rope” model (Fig. 1.1B) that predicts a rodlike structure (Qi et al., 1991).

Knowledge of the protein moieties of AGPs was mostly obtained from purifying AGPs, deglycosylating them and analyzing their respective core proteins by amino acid sequence analysis (Showalter, 2001). AGPs have iso-electric points in the range of pH 2 to 5 (Showalter, 1993) and include a high proportion of proline/hydroxy-

proline, serine, threonine and alanine (Clarke et al., 1979). There are multiple codons for each of these amino acids (Sommer-Knudsen et al., 1998).

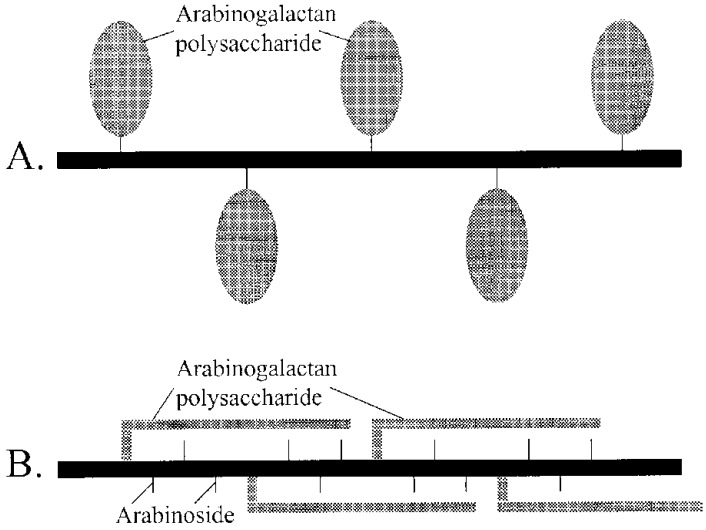

Figure 1.1 Two proposed models for AGPs. The black line indicates the protein backbone.
(A) 'Wattle blossom model'; several type II AG chains are attached to the protein backbone that results in a spheriodal molecule. (B) the 'twisted hairy rope'; short tri-arabinosides and chains of arabinogalactans are *O*-glycosidically linked to hydroxyproline of the protein backbone (Showalter, 2001).

Chen et al. (1994) published the first DNA sequence of a cDNA encoding the protein core of a pear cell suspension AGP. Genomic DNA sequencing projects have identified a large number of putative AGP protein backbone encoding genes (Gaspar et al., 2001). At the molecular level, Mau et al. (1995) and Du et al. (1996) have divided AGP core polypeptides into 'classical' and 'non-classical'. In general, 'classical' AGP sequences encode a polypeptide with at least three distinct domains. First an N-terminal signal sequence not found in the mature proteins, a central Pro/Hyp rich region, and finally, a C-terminal hydrophobic region that functions as a signal for attachment to membranes via GPI anchors (Baldwin et al., 2001; Gaspar et al., 2001; Showalter, 2001). There are at least ten classical AGPs found in *Arabidopsis* with protein backbones of between 85 and 151 amino acids (Gaspar et al., 2001). Non-classical AGPs are defined as having regions that are atypical of AGPs, for example Hyp poor AGPs or regions rich in Asn or Cys residues in addition to regions containing Pro/Hyp (Gaspar et al., 2001; Showalter, 2001). None of the known non-classical AGPs contains a hydrophobic C-terminal domain (Majewska-Sawka and Nothnagel, 2000). As proposed by Gaspar et al. (2001) this nomenclature

might be reconsidered, as the number of sequenced genes that encode AGP protein cores with diverse domain structures is increasing. AGPs could be more accurately indicated by their specific domain or function in the future.

After the synthesis of the AGP protein core some post-translational modifications can occur. Post-translational modifications include hydroxylation of prolyl residues, the *O*-glycosylation of Hyp residues and, for classical AGPs, the addition of a glycosylphosphatidylinositol (GPI) anchor at the C-terminal end. In animals, yeast and protozoa, GPI-anchors provide means to anchor proteins to the cell surface (Schultz et al., 1998). The occurrence of GPI-anchors is not restricted to AGPs (Borner et al., 2003). The hydroxylation of some, but not all proline residues by prolylhydroxylases takes place in the endoplasmatic reticulum (ER). The enzymes responsible for the biosynthesis of the glycan chains of glycoconjugates and polysaccharides are glycosyl transferases (Serpe and Nothnagel, 1999). Glycosylation of Hyp residues occurs in the ER/Golgi apparatus. Given the complexity of carbohydrates present on AGPs, there are likely to be many different glycosyl transferases for the assembly of the polysaccharide chains (Gaspar et al., 2001).
Based on pulse-chase experiments with radioactive sugars several authors concluded that AGPs are rapidly synthesized, secreted to the cell surface and fairly rapidly turned over (Nothnagel, 1997). It has been suggested that once assembled, the carbohydrate components of some AGPs are subsequently processed extracellularly by glycosidases (Kreuger and van Holst, 1996).

Arabinose (Ara) and galactose (Gal) are the main sugars present in AGPs. The sugar moiety of AGPs can also contain other sugars such as L-rhamnose, D-mannose, D-xylose, D-glucose, L-fucose, D-glucosamine and the uronic acids D-glucuronic acid and D-galacturonic acid (Serpe and Nothnagel, 1999). Plant arabinogalactans (AGs) can be classified as type I and type II. In type I arabinogalactans, galactosyl residues occur predominantly in (1,4)-*ß*-D-galactopyranosyl linkages (Serpe and Nothnagel, 1999). Type I arabinogalactans are associated with pectic polysaccharides. On AGPs arabinose and galactose residues are organised in type II arabinogalactans (Serpe and Nothnagel, 1999). The galactosyl residues in type II arabinogalactans occur predominantly in a backbone of (1,3)-*ß*-D-galactopyranosyl linkages that is substituted at O-6 by side chains of (1,6)-*ß*-D-galactopyranosyl linkages (Gaspar et

al., 2001). Most of the arabinosyl residues are attached at carbon atom 3 of some of the galactosyl residues in the AG side chains (Nothnagel, 1997). In 1983 Churms et al. stated that AGPs derived from *Acacia senegal* are composed of carbohydrate subunits. Several groups have demonstrated, based on susceptibility to periodate oxidation, that the (1,3)-*ß*-D-galactopyranosyl backbone comprises a repetitive structure of about seven galactopyranosyl residues, interspersed by a periodate-sensitive linkage that is postulated to be either (1,5)-*α*-L-arabinofuranosine or (1,6)-*ß*-D-galactopyranosyl (Gaspar et al., 2001).

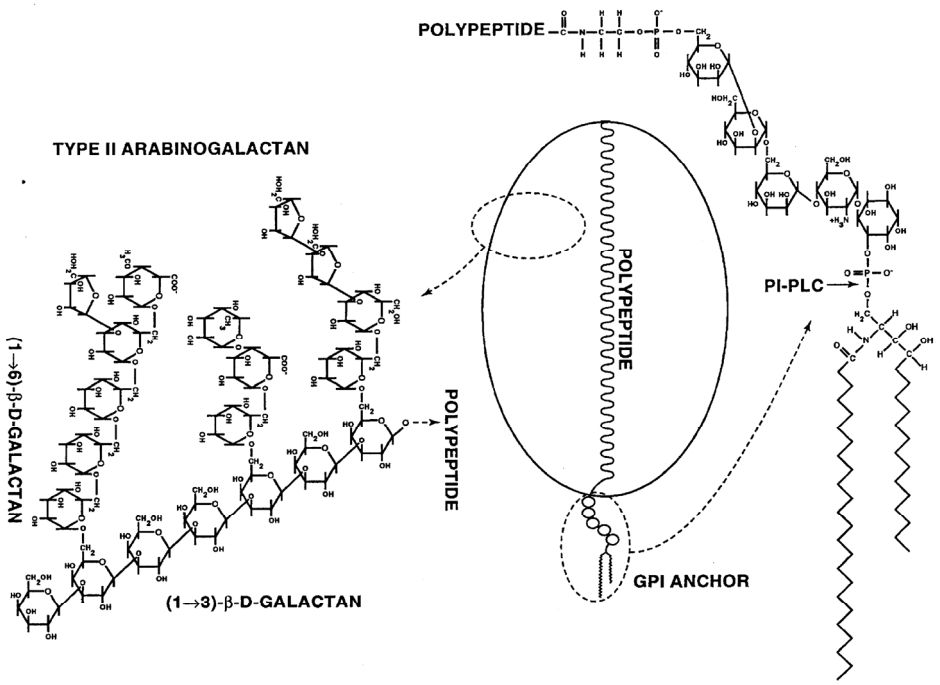

Figure 1.2 A model of a classical arabinogalactan protein with GPI anchor.
The wavy line represents the core polypeptide to which a GPI anchor is attached at the C-terminal end. The carbohydrate structures found on the (1-3)-*ß*-D-galactan backbone are structurally characterized, however the placement is hypothetical (Majewska-Sawka and Nothnagel, 2000).

The size of type II AGs varies among different AGPs, with estimates falling in the range of 30-150 sugar residues (Serpe and Nothnagel, 1999). Large type II AG chains are *O*-glycosidically linked with their reducing end to Hyp residues in the protein backbone. Glycosylation with AGs may also occur through Ser and to a lesser extent through Thr residues (Sommer-Knudsen et al., 1998). Figure 1.2 shows a model of a classical AGP molecule with GPI-anchor and the hypothetical chemical structure of a carbohydrate side chain. Besides the residues shown in this model,

AGPs contain more sugar residues such as GlcA and Rha that are attached to the (1-6)-*ß*-D-galactan side chains (Nothnagel, 1997). Therefore in nature more complex chains can be expected. Short arabinose oligosaccharide chains can be attached to Hyp (Johnson et al., 2003; Showalter, 2001) and therefore the glycosylation of hydroxyproline occurs as galactosylation and as arabinosylation.
The exact location of the carbohydrate chains on the protein core of native AGPs remains to be elucidated. There is increasing evidence that the sequence of hydroxyproline residues in the protein backbone of AGPs determines the glycosylation pattern. The hypothesis that predicts arabinosylation of contiguous Hyp residues and galactosylation of clustered non-contiguous Hyp is called the Hyp contiguity hypothesis (Kieliszewski and Lamport, 1994). By using synthetic genes to express single repetitive glycopeptide motifs of AGPs and extensins in tobacco cells, evidence for the Hyp contiguity hypothesis was derived (Kieliszewski, 2001). The protein of an AGP isolated from a tomato cell suspension was cloned by Gao et al. (1999). Subsequently a construct was made in which the AGP-protein was fused to a green fluorescent protein tag. The expression of this construct in tobacco cells allowed cellular localisation and AGP purification by chromatography (Zhao et al., 2002).
Besides *O*-glycosylation, asparagine residues can be *N*-glycosylated in the endoplasmatic reticulum with *N*-acetylglucosamine as the first attached sugar. *N*-glycosylation has been detected in HRGPs from French bean (Millar et al., 1992) and tobacco (Sommer-Knudsen et al., 1996).

AGPs can be distinguished from other members of the HRGP family by precipitation with Yariv phenylglycoside, although exceptions are known (Clarke et al., 1979; Fincher et al., 1983; Sommer-Knudsen et al., 1996; Nothnagel, 1997). Figure 1.3A shows the structure of Yariv phenylglycoside with *ß*-D-glucose as terminal sugars (*ß*-D-Glc)$_3$. This is the most commonly used phenylglycoside and where Yariv phenylglycoside is mentioned in the literature, it normally refers to this specific molecule.
Yariv phenylglycoside can be used for the isolation of AGPs by selective precipitation, for diagnostic staining of AGPs in tissues and gels and for investigating the effect of cross-linking AGPs in planta (Schultz et al., 2000). Yariv phenylglycoside is thought to form stacks of 10-50 molecules (Woods et al., 1978) in which the carbohydrate part is located outside the stack and can non-covalently bind to AGPs (Fig. 1.3B).

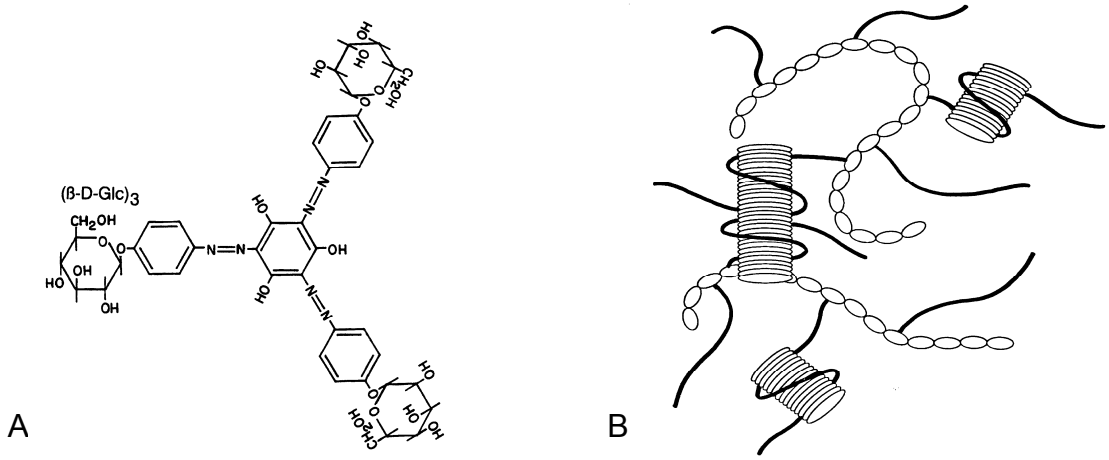

Figure 1.3 Chemical structure of Yariv phenylglycoside and interaction with AGPs.
(A) structure of Yariv phenylglycoside, chemical name: 1,3,5-tri-(*p*-glycosyloxyphenylazo)-2,4,6-trihydroxybenzene (Nothnagel, 1997); (B) the interaction of Yariv with AGPs, the Yariv is depicted as round stacks whereas the AGPs are depicted as protein chains (open) with carbohydrate side-chains (black lines) (Kreuger, 1996).

Both the protein and carbohydrate components of AGPs are required for complex formation (Johnson et al., 2003) and therefore type II arabinogalactan isolated from Larch did not react with Yariv phenylglycoside (Redgwell et al., 2002). When (*ß*-D-Glc)$_3$ is replaced by another set of sugars, e.g. (*α*-D-Gal)$_3$ or (*ß*-D-Man)$_3$, the phenylglycoside does not form a complex with AGPs (Nothnagel, 1997). Non complex-forming Yariv can be used as a negative control in experiments where Yariv phenylglycoside is used to study the effect of AGP complex-formation in living cells.

Biological functions of AGPs

Glycosylation of proteins usually has a function in the maintenance of protein stability. For most glycoproteins their biological function is primarily associated with the polypeptide rather than the carbohydrate part. In contrast, the carbohydrate part of most AGPs is proposed to be the active part (Kieliszewski, 2001). Therefore the function of the AGPs might be related with their carbohydrate decoration, as suggested by Serpe and Nothnagel (1999). Indirect evidence for the importance of the carbohydrate part of AGPs came from studies where a specific prolyl hydroxylase inhibitor was used. Extensins and AGPs became abnormally glycosylated and growth inhibition of mung-bean radicles and soybean cell cultures was observed (Serpe and Nothnagel, 1994).

Although the precise functions of AGPs are not yet established, several lines of evidence indicate that AGPs participate in the regulation of plant growth and development (Serpe and Nothnagel, 1999; Showalter, 2001). The suggestion of involvement of AGPs in development was first inferred by the observation that the composition and concentration of AGPs is related to certain aspects of plant development (Nothnagel, 1997). The complicating factor to relate AGP structure to its function is the difficulty to purify a single AGP species. Recently, a complete type II AG structure was characterized from a fusion protein that was encoded by a synthetic gene (Tan et al., 2004). However, the exact localisation of AGs on the protein is still difficult because hydrolysis of the protein gives insight in the ratio of glycosylated and unglycosylated Hyp residues but the original location of these residues is lost.

Several groups have added Yariv phenylglycoside to cell cultures and intact tissues to disrupt AGP function. In suspension cultured rose cells Serpe and Nothnagel (1994) observed a Yariv-induced inhibition of cell growth in a concentration-dependent manner. Willats and Knox (1996) noticed a reduction in root- and shoot growth and an altered morphology in *Arabidopsis thaliana* seedlings upon addition of Yariv. The perturbation of AGPs with Yariv phenylglycoside also interferes with wall assembly at the pollen tip and thereby blocking growth of lily pollen tubes (Jauh and Lord, 1996). Gao and Showalter (1999) found that suspension-cultured Arabidopsis cells die within 3 days after application of Yariv phenylglycoside and this death occurs not by simple necrosis but by induction of programmed cell death. The ability of Yariv phenylglycoside to inhibit cell division in cell suspension cultures and to inhibit cell expansion in cell cultures and roots provides support for the action of AGPs at the cellular level (Showalter, 2001).

Besides Yariv phenylglycosides, antibodies have been used to characterize cells in a specific developmental stage, to purify AGPs by precipitation on a column and for tissue localization of AGPs. Due to the strong glycosylation of AGPs, antibodies are mainly raised against the carbohydrate part. With immuno localization studies, tissue differentiation in roots (Knox et al., 1991) and flowers (Pennell and Roberts, 1990; Pennell et al., 1991) was studied. Transcripts of transmitting tissue-specific (TTS) AGPs are very abundant in transmitting tract tissue and immunocytochemistry with antibodies directed against the TTS-1 core polypeptide confirmed an abundance of this glycoprotein in the extracellular matrix of the transmitting tissue (Cheung et al.,

1993). AGPs in the pistil may act as glue or lubricants and it is proposed that these AGPs guide pollen tubes to the ovary (Cheung and Wu, 1999).

With the growing number of complete AGP sequences available genetic studies have been initiated to determine AGP expression and function. One example is the *rat1* mutant of Arabidopsis that shows resistance to *Agrobacterium tumefaciens* (Gaspar et al., 2004). The phenotype of *rat1* correlates with downregulation of AtAGP17 in roots due to a mutation in the promotor of this Lys-rich AGP gene. It was suggested that AGP17 could have a role in binding of the bacterium to the root surface or that AGP17 is involved in a signalling pathway that affects the ability of *Agrobacterium* to bind to the root surface (Gaspar et al., 2004). Another example comes from a study on a root-specific non-classical AGP from Arabidopsis, AtAGP30 (van Hengel and Roberts, 2003). The *AtAGP30* knock out showed impairment of in vitro root formation in tissue cultures while in planta timing of seed germination was affected (van Hengel and Roberts, 2003). AtAGP30 is a close homologue of DcAGP1, an AGP that was isolated from a carrot cell culture and characterized by Baldwin et al. (2001). Acosta-García and Vielle-Calzada (2004) used a combination of enhancer detection tagging and RNA interference posttranscriptional silencing to show that AGP18 defines the sporophytic to gametophytic transition in *Arabidopsis thaliana*. A cytological study of AGP18-silenced plants showed that the functional megaspore fails to enlarge and mitotically divide. AGP18 encodes a classical arabinogalactan protein.

In conclusion, it appears that the genetic approaches suggest that individual AGP genes are involved in a variety of developmental processes. These observations support the previously reported roles of AGPs derived from inhibition studies using Yariv phenylglycoside or the application of mixtures of AGPs to cultured cells.

Plant embryogenesis

Plant zygotic embryogenesis spans the period of plant development that ranges from the fertilised egg cell, the zygote, to the mature desiccated embryo present in a protective seed (reviewed by Mordhorst et al., 1997). A specific feature of plants is that embryos can also develop from normal somatic cells. When no intermediate culturing is required and the formation of somatic embryos is initiated from differentiated tissue the process is named “direct somatic embryogenesis”. Direct somatic embryogenesis can be observed on the leaves in some species such as *Bryophyllum* and *Malaxis* (reviewed by Mordhorst et al., 1997). The system that comprises in vitro culturing of callus in the presence of growth regulators is called

"indirect somatic embryogenesis" (reviewed by Mordhorst et al., 2005). Indirect somatic embryogenesis was first described by Steward (1958) and Reinert (1958) in cell cultures of carrot. Since, carrot somatic embryogenesis has become one of the most-studied model systems and has been used in attempts to elucidate the physiological, biochemical and molecular mechanisms that underlie plant embryogenesis (Satoh, 1998). A practical application of somatic embryogenesis is the large-scale propagation of plants. Plant species, which are capable of expressing their embryogenic potential regardless of the type of explant include *Daucus carota* and *Medicago sativa.* For many other species embryonal or juvenile tissue has to be used as explant (reviewed by von Arnold et al., 2002). Indirect somatic embryogenesis is a multi-step regeneration process starting with the formation of single embryogenic cells, pro-embryogenic masses (PEMs), followed by somatic embryo formation, maturation, desiccation and seedling growth (von Arnold et al., 2002). PEMs are small and tightly adhering cell masses of 10-20 cells. Auxin, such as 2,4-dichlorophenoxyacetic acid (2,4-D) is required for proliferation of PEMs but is inhibitory for the development of PEMs into somatic embryos (de Vries et al., 1988).

Plant cells grown in suspension culture secrete an array of macromolecules including polysaccharides, proteins, glycoproteins and other molecules that accumulate in the culture medium (Bauer et al., 1973; Talmadge et al., 1973). The resulting medium is called conditioned medium and the macromolecules it contains are generally considered to be representative of those forming the cell wall (Serpe and Nothnagel, 1999). In most crops, the embryogenic potential decreases with prolonged culture and is eventually lost (von Arnold et al., 2002). It was shown that, when non-embryogenic cultures were treated with growth medium conditioned by highly embryogenic cultures, the cultures became embryogenic (Hari, 1980). A protein that is able to stimulate the formation of somatic embryos in embryogenic cultures of *Daucus carota* has been identified as an endochitinase (de Jong et al., 1992). The temperature-sensitive variant *ts11* in which somatic embryogenesis at a non-permissive temperature is blocked at the globular stage was investigated. Addition of the endochitinase to *ts11* embryogenic cultures rescues embryogenesis and promotes embryo formation (de Jong et al., 1992). In accordance, an endochitinase from sugar beet stimulates early development of somatic embryos in *Picea abies* (Egertsdotter and von Arnold et al., 1998). The precise role of the chitinases in the formation of somatic embryos is not clear. They are possibly involved in the release

of signalling molecules that originate from substrate molecules found in conditioned medium (van Hengel, 1998). Endochitinases need at least three contiguous *ß*-(1-4) linked GlcNAc residues for hydrolytic activity (Molano et al., 1979; Usui et al., 1990). GlcNAc containing compounds in plants include lipochitooligo-saccharides (LCOs), AGPs and *N*-glycosylated proteins. LCOs are a class of signalling molecules that promote division of plant cells. LCO signals secreted by Rhizobium (Nod factors) induce cell divisions in the root cortex, leading to the formation of nodules that can be colonised by bacteria (Spaink et al., 1991). Rhizobial Nod factors were also found to stimulate somatic embryos of *Daucus carota* to proceed to the late globular stage (De Jong et al., 1993) and were therefore proposed to be able to substitute the effect of chitinase in the formation of somatic embryos.

AGPs also have a role in the development of somatic embryos. Addition of Yariv phenylglycoside to a carrot cell culture blocks embryogenesis (Thompson and Knox, 1998). The addition of seed AGPs to a non-embryogenic cell culture (Kreuger and van Holst, 1993) or carrot protoplasts (van Hengel, 1998) can stimulate the formation of embryos and shows that certain AGPs are essential for embryogenesis. The age of the cell line can affect the types of AGPs present in the conditioned medium (Serpe and Nothnagel, 1999). As the age increases, the AGPs secreted into the conditioned medium become different from those present in the mother tissues from which the culture was derived (Serpe and Nothnagel, 1999). Other evidence for the presence of structurally different AGPs came from studies where purified AGPs were added to embryogenic cell cultures and the finding that certain AGPs will stimulate, whereas others will inhibit somatic embryogenesis (Toonen et al., 1997). Besides AGPs, chitinase and LCOs other molecules are known to influence the process of indirect somatic embryogenesis. Phytosulfokines were shown to enhance embryogenesis in carrot cell cultures (Hanai et al., 2000) whereas a 4-hydroxybenzyl alcohol was shown to inhibit carrot somatic embryogenesis (Kobayashi et al., 2000).

AGPs have a carbohydrate binding capacity

AGPs were shown to be involved in a variety of different biological processes and it is possible that AGPs function as signalling molecules. AGPs can also be involved in a linkage to other cell wall polymers and have a more structural function. It remains possible that the higher-order structure of Yariv phenylglycoside mimics a cell wall polymer that is the natural interactive partner for some AGPs (Serpe and Nothnagel, 1999). A cell wall polymer that often has been suggested to have interaction with

AGPs is pectin (Baldwin et al., 1993). Pectin is found in the middle lamellae and the primary cell wall and these branched molecules have a cell adhesive function. Pectin consists of several structural elements and is rich in galacturonic acid, rhamnose, arabinose and galactose. The most characteristic structural element of pectin is homogalacturonan that consists of a chain of *α*-(1-4) linked GalA. In rhamnogalacturonan I, dimers of GalA and Rha form the alternating sequence of the backbone. Arabinans, galactans and type I arabinogalactans are present as side branches that are linked to some of the rhamnose sugars of rhamnogalacturonan I. Also rhamnogalacturonan II has a highly branched structure and the backbone consist of approximately 8 GalA residues. The backbone of rhamnogalacturonan II is substituted with branches containing 10 different glycosyl residues (Mollet et al., 2000). Another structural element of pectins is xylogalacturonan that consists of a branched galacturonan.

At this moment no consensus exists on the macromolecular structure of pectin. Although the individual structural elements of pectin are well characterised, for the organization of these elements a few models exist. Vincken et al. (2003) presented a new model in which the homogalacturonan is placed as side chains of the rhamnogalacturonan (Fig. 1.4).

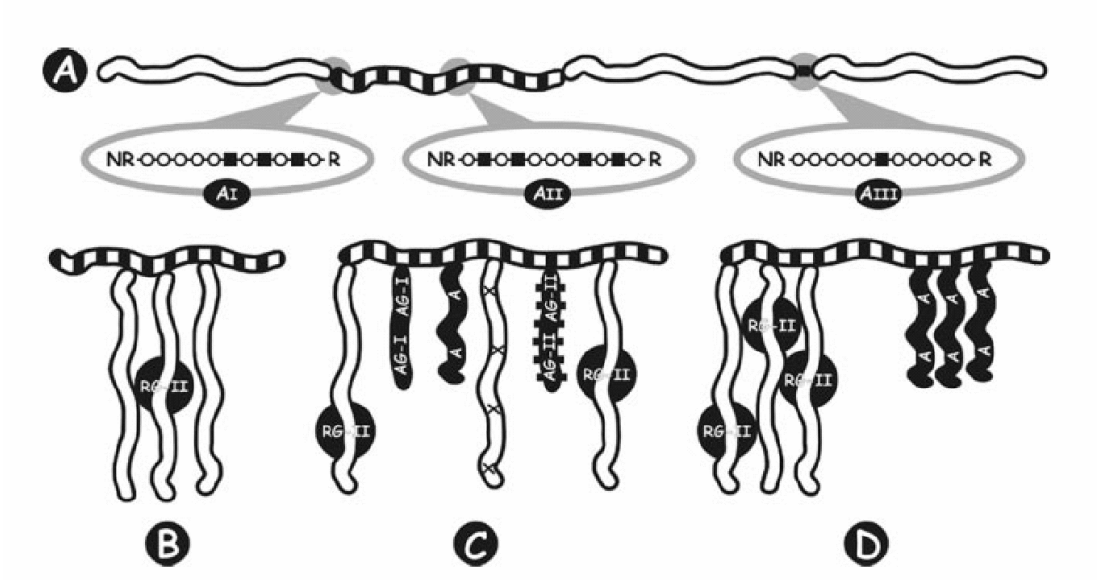

Figure 1.4 Different models of the macromolecular structure of pectin.
(A) Homogalacturonan (white) is interspersed by rhamnogalacturonan I (alternating black-white). The side chains of rhamnogalacturonan I are not shown. (B, C, D) Recent models in which rhamnogalacturonan I (alternating black-white) is the main backbone to which homogalacturonan chains (white), arabinogalactans (AG-I, AG-II), arabinans (A) and rhamnogalacturonan II chains (RG-II) are attached (Vincken et al., 2003).

Several investigators have found that AGPs often co-purify with pectin. When pectins are purified and characterized occasionally some fractions show the presence of type II arabinogalactans (Asgar et al., 2004; Carpita, 1989; Iraki et al., 1989; Shea et al., 1989). One interpretation for this phenomenon is that at least some AGPs can interact with pectins, most likely by ionic interactions through the carbohydrate part (Showalter, 2001) or directly with basic amino acid residues found in some AGPs. Baldwin and co-workers have provided some support for this idea in their study of the Hyp-poor carrot AGP, which appears to show some degree of copper ion mediated binding of pectin in blotting experiments performed in vitro (Baldwin et al., 1993). However, the observed binding appears weak. Pennell et al. (1992) found that a cell wall AGP-related molecule is tightly bound to pectin. Other HRGP molecules are known to have interactions with other cell wall polymers. A covalent cross-link between extensin and pectin has been shown in cultured cells from cotton (Qi et al., 1995). Interactions with AGPs and other polymers might also occur without the carbohydrate part. Basic proline rich AGP from carrot may have binding properties to carbohydrates due to cysteine (Baldwin et al., 2001). Tyr residues in AGPs could facilitate cross-linking by iso-dityrosine bridges (Waffenschmidt et al., 1993).

In conclusion, AGPs are highly complex molecules that can act as signalling molecules and may exhibit cross-linking properties with other polymers in plant cell walls. A combination of molecular-genetic and biochemical approach is now needed to supplement the cell-biological approaches in order to assign a specific function.

Chapter 2

N-Acetylglucosamine and glucosamine-containing arabinogalactan proteins control somatic embryogenesis

Arjon J. van Hengel[1], Zewdie Tadesse, Peter Immerzeel[2,3], Henk A. Schols[3], Ab van Kammen, Sacco C. de Vries[2]

[1]Present address: Department of Cell Biology, John Innes Centre, Colney Lane, Norwich NR4 7UH, United Kingdom

[2]Laboratory of Biochemistry, Department of Agrotechnology and Food Sciences, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

[3]Laboratory of Food Chemistry, Department of Agrotechnology and Food Sciences, Wageningen University, Bomenweg 2, 6703 HD Wageningen, The Netherlands

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Abstract

In plants, complete embryos can develop not only from the zygote, but also from somatic cells in tissue culture. How somatic cells undergo the change in fate to become embryogenic is largely unknown. Proteins, secreted into the culture medium such as endochitinases and arabinogalactan proteins (AGPs) are required for somatic embryogenesis. Here we show that carrot (*Daucus carota*) AGPs can contain glucosamine and *N*-acetyl-D-glucosaminyl and are sensitive to endochitinase cleavage. To determine the relevance of this observation for embryogenesis, an assay was developed based on the enzymatic removal of the cell wall from cultured cells. The resulting protoplasts had a reduced capacity for somatic embryogenesis, which could be partially restored by adding endochitinases to the protoplasts. AGPs from culture medium or from immature seeds could fully restore or even increase embryogenesis. AGPs pretreated with chitinases were more active than untreated molecules and required an intact carbohydrate constituent for activity. AGPs were only capable of promoting embryogenesis from protoplasts in a short period preceding cell wall reformation. Apart from the increase in embryogenesis, AGPs can reinitiate cell division in a subpopulation of otherwise non-dividing protoplasts. These results show that chitinase-modified AGPs are extracellular matrix molecules able to control or maintain plant cell fate.

Introduction

Zygotic plant embryos pass through characteristic globular-, heart-, and torpedo-shaped stages before being desiccated in the mature seed. Somatic embryos pass through the same stages, but lack a desiccation phase and develop directly into plantlets. The carrot (*Daucus carota*) cell line *ts11* is a temperature-sensitive variant in which somatic embryogenesis is arrested in the globular stage at non-permissive temperatures (Lo Schiavo et al., 1990). The phenotype of *ts11* is pleiotropic and may result from a secretory defect (Baldan et al., 1997). It is remarkable that the addition of a carrot class IV endochitinase (designated EP3) allows further development of the embryos into plantlets (De Jong et al., 1992; Kragh et al., 1996). Endochitinases (1, 4-*N*-acetyl-*ß*-D-glucosaminide glycanhydrolase, EC 3.2.1.14) hydrolyze *ß*-(1–4) linkages between at least three adjacent *N*-acetyl-D-glucosaminyl (GlcNAc) residues in chitin polymers (Molano et al., 1979; Usui et al., 1990). Endochitinases also hydrolyze *ß*-(1–4) linkages in partially deacetylated chitin (chitosan) and show variable efficiency in hydrolyzing chitin oligomers (Brunner et al., 1998). Plant cell

walls are devoid of chitin or chitosan polymers, suggesting that plants contain other, uncharacterized targets for endochitinase activity.

AGPs are proteoglycans that can occur attached to membranes or in cell walls. In tissue cultures AGPs are secreted into the culture medium from which they can be selectively precipitated with 1,3,5-tri-(*p*-glycosyloxyphenylazo)-2,4,6-trihydroxybenzenes or Yariv reagent (Kreuger and Van Holst, 1993). Applying Yariv reagent to suspension cultures of rose cells inhibited culture growth due to suppression of cell division. After transfer to medium without Yariv reagent, cell division and culture growth were restored (Serpe and Nothnagel, 1994). Arabidopsis roots grown in the presence of Yariv reagent were found to have only one-third of the length of roots grown without this compound. The reduction of length resulted from cells in the elongation zone that were found to be bulbous rather than elongated (Willats and Knox, 1996). These experiments show that cell expansion can be perturbed by the addition of Yariv reagent.

A role for AGPs in plant development was initially proposed based upon their striking temporal and spatial localization patterns as visualized by the use of monoclonal antibodies (Knox et al., 1989, 1991). Direct addition of mature carrot seed AGPs to a weakly embryogenic cell line caused re-initiation of embryogenic cell formation (Kreuger and Van Holst, 1993). This resulted in the presence of clusters of small cytoplasm rich rapidly dividing cells, in line with the reverse effect reported for the addition of Yariv reagent to rose cells (Serpe and Nothnagel, 1994). AGPs that contained an epitope recognized by the monoclonal antibody JIM8 were isolated from carrot cell-conditioned medium (McCabe et al., 1997). These JIM8 AGPs were added to a cell population unable to form somatic embryos and devoid of the JIM8 cell wall-bound epitope. This treatment restored the formation of somatic embryos from these cells. Therefore, a role for the JIM8 epitope containing AGPs in cell-to-cell communication during somatic embryogenesis was proposed.

AGPs consist of a small protein backbone contributing less then 10% of the mass of the AGP molecule. More than 90% of the AGP consists of carbohydrate with arabinosyl and galactosyl residues as the major sugar constituents (Nothnagel, 1997). In AGPs the polysaccharides are *O*-linked to the protein core and have highly complex side chains with different terminal residues. Individual monosaccharides in AGPs can be linked in different ways (Mollard and Joseleau, 1994), further increasing the structural complexity of the molecule. The presence of a small amount of glucosamine (GlcN) in AGPs was previously noted by van Holst et al. (1981).

Later, it was found that certain AGPs contain a glycosylphosphatidylinositol (GPI) membrane anchor attached to the carboxy terminus of the protein backbone in which a single GlcN residue was detected (Youl et al., 1998; Svetek et al., 1999). However, the occurrence of oligomers of GlcNAc or GlcN has not previously been reported (Van Holst et al., 1981; Komalavilas et al., 1991; Baldwin et al., 1993; Mollard and Joseleau, 1994; Serpe and Nothnagel, 1994, 1996; Smallwood et al., 1996). However, based on these observations and the notable absence of GlcNAc or GlcN in all other wall carbohydrates, it appears that AGPs are one of the few plant cell wall molecules that may contain GlcNAc or GlcN in a form that could be a target for endochitinase activity. Our results suggest that AGPs from developing seeds and embryogenic suspension cultures indeed contain GlcNAc and GlcN residues. In addition, we show that these AGPs indeed contain cleavage sites for endochitinases and that these sites may have biological significance in somatic embryogenesis. We also demonstrate that EP3 endochitinases increase the formation of embryogenic cell clusters and somatic embryos from wild-type carrot protoplasts. Thus, the effect of endochitinases is not restricted to the *ts11* variant (De Jong et al., 1993) and may exert its effect through hydrolytic activity on AGPs.

Results

AGPs from embryogenic cell lines contain GlcN and GlcNAc

Carrot suspension cells in basal medium with 2,4-dichlorophenoxyacetic acid (2,4-D) were labeled with ^{14}C-GlcNAc. After 1 week, 20% of the total radioactivity could be recovered from the medium by precipitation with Yariv, suggesting incorporation into AGPs. Although about 70% of the label was retained in the cells, less than 2% was found in the insoluble cell wall fraction (data not shown). Because Yariv precipitation can result in coprecipitation of pectins, the pectin-specific monoclonal antibodies JIM5 and JIM7 were used to determine the possible occurrence of pectins in labeled and unlabeled AGP preparations obtained from suspension cell cultures. Immunodetection of pectin epitopes was performed before and after treatment with pectinase. Dot-blot analysis showed that after pectinase treatment, no pectin epitopes could be detected anymore. AGP-specific epitopes that are recognized by the monoclonal antibodies JIM8, MAC207, and MAC254 remained unaltered upon reprecipitation of the AGPs after pectinase treatment (data not shown). Over 80% of the radioactivity originally present in the AGPs was retained in the re-isolated pectin-free AGP fraction, indicating that the radioactivity was incorporated in the AGPs

secreted into the medium. It cannot be excluded that some cleavage of AGPs occurred during the pectinase incubation. However, the resulting AGP preparations eluted as a series of defined peaks after high performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) were fully active in the somatic embryogenesis bioassay, and therefore, all AGPs as used in this study were treated with pectinase before use.

Because GlcNAc occurs in extracellular glycoproteins with *N*-linked carbohydrates, it was of importance to verify the absence of such labeled proteins from the AGP preparations. Electrophoresis of AGPs before and after pectinase treatment, followed by silver staining of polyacrylamide gels, did not reveal any contaminating proteins in the AGP fraction (data not shown).

To determine whether ^{14}C-label in the newly formed AGPs occurred in GlcN and in GlcNAc, labeled AGPs were incubated with 2 M trifluoroacetic acid (TFA) for 45 min at 100°C or alternatively for 60 min at 120°C. AGP degradation was monitored by thin-layer chromatography (TLC) followed by autoradiography and densitometric scanning of the autoradiographs (Fig. 2.1).

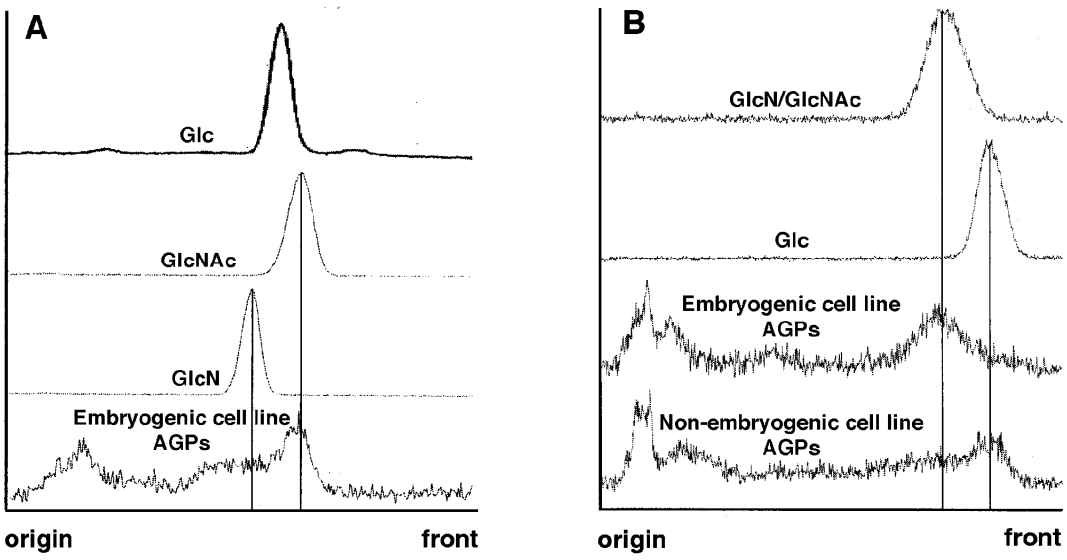

Figure 2.1 Optical density scans of autoradiograms of TLCs.
The scans represent the mobility of the TFA-degraded AGPs of an embryogenic and a nonembryogenic cell line labeled with 10^{-7} M ^{14}C-GlcNAc, compared with the mobility of D-[1-^{14}C] labeled Glc, GlcN, and GlcNAc references. A, AGPs and references degraded by means of incubation in 2 M TFA at 100°C for 45 min. B, AGPs and references degraded by means of incubation in 2 M TFA at 120°C for 60 min.

The AGP sample incubated at 100°C for 45 min contained labeled compounds migrating to a position coinciding with GlcNAc. Label was also found at positions corresponding to GlcN and Glc. However, under these conditions AGP degradation was not complete (Fig. 2.1A). After incubation of AGPs at 120°C for 60 min, most of the label was found at the position of GlcN. Under these conditions all GlcNAc is deacetylated to GlcN. AGPs from a nonembryogenic cell line contained less aminated sugars and instead had more label converted into monosaccharides such as Glc (Fig. 2.1B). Because other major components of AGPs such as Ara and Gal have the same mobility as Glc on TLC, the possibility remains that deacetylation, deamination, and interconversion into other hexoses had occurred.

To determine which aminated and acetylated hexoses are present in AGPs, unlabeled AGPs were isolated from embryogenic suspension cultures by Yariv precipitation, and were hydrolyzed, acetylated, and subsequently analyzed by gas-liquid chromatography (GLC) and by gas chromatography-mass spectrometry (GC-MS). The following molar percentages of neutral sugars were found: Ara, 30.4%; Gal, 59.6%; Rha, 6.0%; Glc, 1.6%; Xyl, 1.3%; Man, 0.9%, and GlcN, 0.2%. The presence of GlcN was confirmed by GLC and GC-MS data. The mass spectrum of the compound in the AGP hydrolysate that co-eluted with a standard of GlcNAc precisely matched the spectrum of GlcN having mass signals at 84, 102, 144, and 318 *m/z* (Fox et al., 1989). No galactosamine or mannosamine were found in the AGP hydrolysate. Thus, we have demonstrated unambiguously the presence of GlcN as the only aminated sugar present in AGPs from embryogenic carrot suspension cultures. Therefore, the aminated and acetylated sugar that was found by TLC analysis can only have been GlcNAc.

AGPs from immature carrot seeds contain a cleavage site for plant endochitinases

After having shown that GlcN and GlcNAc occur in AGPs, medium AGPs or AGPs from immature carrot seeds were incubated with endochitinase. However, after HPAE-PAD chromatography of these AGPs, no changes in elution profile were observed when compared with untreated preparations. It is apparent that no endochitinase cleavage product was produced in sufficient quantity to be detected by HPAE-PAD (data not shown). Immature seed AGPs were then incubated with a mixture of pure endogalactosidase, endoarabinofuranosidase, and exoarabino-furanosidase of fungal origin. This resulted in a limited number of discrete oligosaccharides resolved by HPAE-PAD (Fig. 2.2A). The peaks visible in Figure

2.2A with retention times of 1 min 30 s, 2 min 40 s, 4 min 30 s, and 8 min 40 s (marked by asterisks in Fig. 2.2A) were observed also in AGP preparations that were treated with pectinase only. These peaks may thus represent oligosaccharides present in the starting material or minor AGP species.

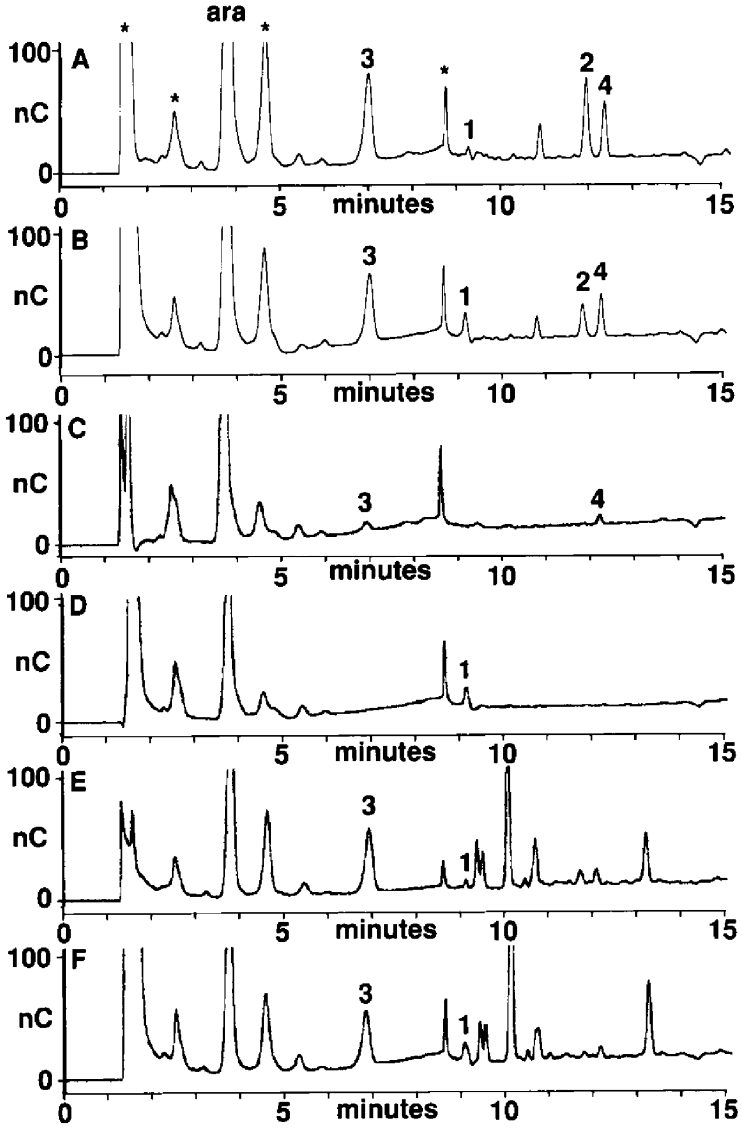

Figure 2.2 HPAE-PAD chromatography of AGP-derived oligosaccharides.
HPAE-PAD chromatograms of AGPs that were isolated from immature seeds treated with pectinase and then incubated with AGP degrading hydrolases in the presence or absence of EP3 endochitinase. Oligosaccharides that change in relative amount upon incubation with EP3 endochitinase are numbered as 1 (retention time of 9 min 10 s), 2 (retention time of 11 min 50 s), 3 (retention time of 7 min), and 4 (retention time of 12 min 20 s). Peak height is expressed in nanocoulomb (nC). A, AGPs incubated with endogalactosidase, endo-, and exoarabinofuranosidase. B, AGPs incubated with endogalactosidase, endo-, and exoarabinofuranosidase in combination with EP3 endochitinase. C, AGPs incubated with exoarabinofuranosidase. D, AGPs incubated with exoarabinofuranosidase in combination with EP3 endochitinase. E, AGPs incubated with endoarabinofuranosidase. F, AGPs incubated with endoarabinofuranosidase in combination with EP3 endochitinase.

The peak with a retention time of 3 min 50 s represents free Ara. Upon inclusion of endochitinases in the incubation mixture, a subtle shift in the pattern of oligosaccharides 1, 2, 3, and 4 occurred. There appeared to be an increase in an oligosaccharide numbered 1 (Fig. 2.2B, retention time of 9 min 10 s), a decrease in an oligosaccharide numbered 2 (retention time of 11 min 50 s), and a slight decrease in two other oligosaccharides numbered 3 and 4 (retention times of 7 min and 12 min 20 s, respectively). Oligosaccharide 1 did not result from the added endochitinase, endogalactosidase, endo-, and exoarabinofuranosidase preparations, since these only showed a single peak with a retention time of 1 min 30 s after HPAE-PAD, whereas incubation of young flower AGP preparations failed to show oligosaccharides at the position of peak 1 (A.J. van Hengel and S.C. de Vries, unpublished data). In interpreting the relatively small changes in these patterns after endochitinase incubation, it must be taken into account that the AGPs used for analysis are isolated from living plant material and no control was possible over hydrolytic degradation prior to isolation. Upon isolation of AGPs from immature seeds between 4 and 21 d after pollination (DAP) this became evident in the form of an increase in susceptibility for endochitinase activity of oligosaccharide 2 (A.J. van Hengel and S.C. de Vries, unpublished data) with developmental time, suggesting a change in composition or a higher level of preincubation with seed hydrolases. Incubation of AGPs with exoarabinofuranosidase only (Fig. 2.2C) indicates that removal of terminal arabinosyl residues generates the expected peak of free Ara, but unexpectedly, also the two oligosaccharides marked 3 and 4. Both were found to disappear after incubation with a mixture of exoarabinofuranosidase and the endochitinase (Fig. 2.2D), whereas oligosaccharide 1 appeared. Oligosaccharide 3 appears much more prominently after incubation of AGPs with endoarabino-furanosidase only (Fig. 2.2E), suggesting that it arose from residual endo-activity in the exofuranosidase preparation. Oligosaccharide 3 was found to be slightly reduced in area after incubation with a mixture of endoarabinofuranosidase and endochitinase (Fig. 2.2F), whereas oligosaccharide 1 appeared. Oligosaccharide 2 (Fig. 2.2A) only appeared after incubation with all three fungal hydrolases, suggesting that it is not the product of a single hydrolytic reaction.

These results show that the product of endochitinase activity, oligosaccharide 1, can be generated from different oligosaccharides. They also show that arabinofuranosidase treatment of immature seed AGPs can make sufficient endochitinase cleavage sites available to be detectable by HPAE-PAD.

Immature seed AGPs promote somatic embryogenesis and can be activated by chitinases

Next we asked whether endochitinase-mediated cleavage of AGPs serves a biological function. The first experiment was to include AGPs in the medium of suspension cultures with a low-to-moderate embryogenic potential. Compared with unsupplemented controls, no significant effect on somatic embryogenesis was observed after addition of carrot immature seed AGPs (Table 2.1).

Table 2.1 The effect of AGPs on somatic embryogenesis from cells.
Effect of pectinase-treated AGPs isolated from immature seeds at 21 DAP on the no. of somatic embryos formed from suspension cells. The effect of addition of AGPs is expressed as the no. of globular-, heart-, and torpedo-stage embryos obtained per 10,000 suspension cells. Statistical analysis was done by means of *F* tests on the average of the mean. The overall effect of a treatment was regarded as significantly different when calculated *P* values were ≤ 0.05. Bioassay conditions are described in "Materials and Methods."

Compound	Concentration µg/ mL	Mean No. of embryos per 10,000 suspension cells	SE[a]	*n*[b]	*P* Values compared with control
Control	–	18	2.6	2	-
AGPs	3.0	22	2.6	2	0.811

[a] The SE is included. [b] The no. of independent experiments (*n*) was obtained in two individual assays.

After preparation of protoplasts from these cells, the number of somatic embryos drops up to 20-fold (Table 2.2). Adding immature seed AGPs at a concentration comparable with that normally found in the medium of suspension cells (1–3 µg/mL after 7 d of subculturing) resulted in a 30-fold increase in the number of somatic embryos formed (Table 2.2; Fig. 2.3). Therefore, the loss of embryogenic potential due to cell wall removal is mainly caused by the concomitant removal of AGPs (Tables 2.1 and 2.2). At an increased AGP concentration, protoplasts produced up to 5-fold more embryos than the cells from which they were derived. Secreted AGPs of an embryogenic culture were added to carrot protoplasts of the same cell line. This resulted in an increase of the number of somatic embryos that was fully comparable with the effect of immature seed AGPs. Thus, AGPs can also confer embryogenic potential to previously nonembryogenic cells, in line with previous observations (Kreuger and van Holst, 1993). It is surprising that the addition of immature seed AGPs preincubated with EP3 endochitinases reduced the number of embryos that

developed to the level observed when EP3 chitinases were added alone. This suggested that cleavage of GlcNAc-containing oligosaccharide side chains by EP3 chitinase results in inactivation of AGPs. In contrast, when AGPs were preincubated with EP3 endochitinase and were then re-isolated by Yariv precipitation, the promotive effect of AGPs was not only completely restored, but was even increased by more than 50% when compared with non-chitinase-treated AGPs (Table 2.2). These results showed that endochitinase treatment renders AGPs more effective in promoting somatic embryogenesis, and that an inhibiting compound, not precipitable by Yariv reagent, might be produced.

Table 2.2 The effect of chitinases and AGPs on somatic embryogenesis from protoplasts.
Partial restoration of somatic embryogenesis from protoplasts at an optimal concentration of EP3 chitinase and full restoration by 21 DAP seed AGPs at physiological concentrations. Effect of EP3 endochitinase pre-treatment of immature seed AGPs with and without re-isolation of AGPs by Yariv precipitation. The effect of addition of AGPs is expressed as the no. of globular-, heart-, and torpedo-stage embryos obtained per 10,000 protoplasts. Statistical analysis was done by means of *F* tests on the average of the mean. The overall effect of a treatment was regarded as significantly different when calculated *P* values were ≤ 0.05. Enzyme treatment of AGPs and bioassay conditions are described in "Materials and Methods." All AGP preparations were pretreated with pectinase.

Compound and treatment	Conc. µg/mL	Mean No. of embryos per 10,000 protoplasts	SE[a]	*n*[b]	*P* values compared with control
Control	–	1	0.7	2	
AGPs	0.3	5	3.5	2	0.131
	3.0	31	13	2	0.016
	15	42	0.5	2	0.000
	30	>100	nd[c]	2	nd
AGPs from culture medium	15	40	–	1	–
EP3	0.2	8	3.8	4	0.020
AGPs + EP3	15 + 0.2	6		1	–
AGPs + EP3/AGPs re-isolated	15 + 0.2	68	2.5	8	0.000 (0.030)[d]

[a] The SE is included. [b] The no. of independent experiments (*n*) was obtained in two individual assays. [c] nd, Not determined. [d] *P* value when compared to 15 µg/mL AGPs added without EP3 treatment is in parentheses.

In all assays described so far, AGPs were added shortly after protoplast preparation and before cell wall regeneration was complete. AGPs were not effective in promoting embryo formation when added 1 d after protoplast isolation (Table 2.3). AGP epitopes rapidly reappear on the surface of protoplasts after enzymatic digestion of cell surface polysaccharides (Pennell et al., 1989), and within 24 h protoplasts have synthesized a new cell wall. Therefore, AGPs are only fully active before cell wall regeneration is complete. AGPs isolated from manually dissected

endosperm of immature seeds were found to be highly active (Table 2.3), demonstrating that AGPs that promote somatic embryogenesis are mainly found in the endosperm. AGPs isolated from gum arabic or from immature seeds at 11 DAP were not active, demonstrating that there is species and temporal specificity in the embryo-forming activity of AGPs.

Table 2.3 The effect of AGPs and AGP-derived oligosaccharides on somatic embryogenesis.
AGPs were isolated from immature seeds at 21 DAP and were added immediately or 1 d after protoplast isolation. AGPs derived from carrot endosperm, gum arabic, or from immature seeds at 11 DAP were isolated and added to protoplasts. Effects of BaOH treatment of AGPs, endogalactosidase, endo-, and exoarabinofuranosidase (endoG, endoA, and exoA, respectively) treatment of AGPs were compared with activation of AGPs by EP3 endochitinase. The results in this table were obtained in duplicate assays, but in single experiments, and therefore did not allow statistical analysis by *F* tests. The results are expressed as the no. of somatic embryos formed in a dish containing 100,000 protoplasts with AGPs, divided by the no. of somatic embryos formed in control dishes without AGPs that accompanied each experiment. The no. of somatic embryos in the control dishes varied between seven and 11. In all experiments the final concentration of AGPs used was 15 µg/mL. Enzyme treatment of AGPs and bioassay conditions are described in "Materials and Methods." All AGP preparations were pretreated with pectinase.

Compound and treatment	Fold increase in embryogenesis
Control	1
AGPs	38
AGPs added after 1 d	1
AGPs from endosperm	153
AGPs from gum arabic	3
AGPs 11 DAP	4
AGPs x EP3/AGPs re-isolated	61
AGPs x BaOH	36
AGPs x exoA + endoA + endoG	2

Barium hydroxide hydrolysis, which cleaves polypeptide linkages (Lamport and Miller, 1971) and releases oligosaccharides *O*-linked to hydroxy-Pro and free oligosaccharides derived from any linkages to other amino acids, did not reduce the embryo-forming effect of AGPs (Table 2.3). This shows that the effect of AGPs on embryogenesis requires its intact carbohydrate, but not its intact polypeptide constituent. AGPs treated with EP3 chitinase gave an approximately 60-fold increase in embryogenesis, which was comparable with the increase reported in Table 2.2. In contrast, treatment with the same mixture of hydrolases employed for the HPAE-PAD analysis (Fig. 2.2) rendered AGPs completely ineffective. Therefore, AGP side chains with intact arabinogalactan carbohydrate moieties are essential for the effect on

somatic embryogenesis (Table 2.3), whereas hydrolytic activation with endochitinases appears essential for full embryo-forming activity of the AGPs.

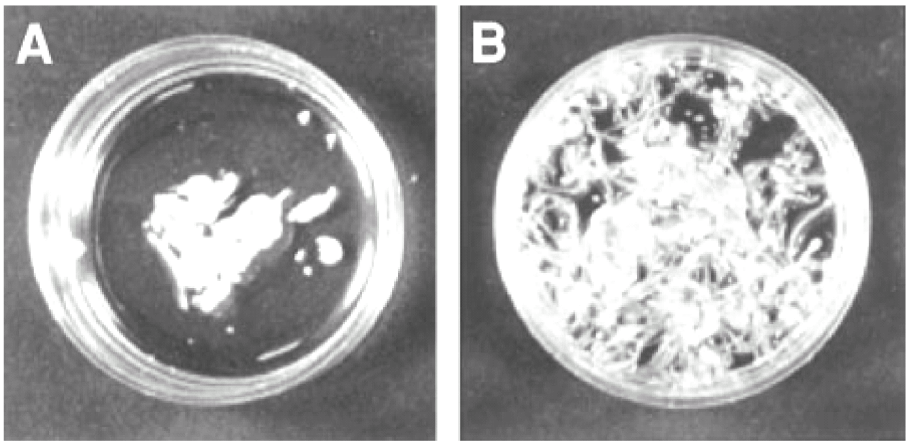

Figure 2.3 Restoration of somatic embryogenesis from protoplasts by seed AGPs.
A, Callus and an occasional somatic embryo formed in unsupplemented control culture. B, Plantlets formed after addition of 3 µg/mL of immature 21 DAP seed AGPs, pretreated with pectinase.

Early effects of immature seed AGPs on protoplasts

To determine whether protoplasts showed a morphologically recognizable effect after addition of AGPs we employed cell tracking of protoplast-derived cells. Cell tracking involves analysis of daily repeated video recordings made from the same area of a dish containing immobilized carrot protoplasts (Toonen and de Vries, 1997). When following the development of a population of protoplasts by cell tracking, four different possible developmental patterns can be distinguished, commencing from an initially fairly uniform population of protoplast-derived cells (F. Guzzo and S.C. de Vries, unpublished data). Protoplast-derived cells can divide without expanding to much more then their original size, resulting in small compact clusters; they can divide and simultaneously enlarge, resulting in loosely attached clusters of vacuolated cells; they can enlarge, but not divide, resulting in large vacuolated cells, or they can neither divide, nor enlarge and remain unchanged in morphology during the period of analysis. In Figure 2.4, examples of these four patterns are shown. Somatic embryos only derive from cells that follow pattern 1 (F. Guzzo and S.C. de Vries, unpublished data).

In Table 2.4 the results of the cell tracking experiments on protoplasts obtained from two embryogenic suspension cultures are summarized. Samples of protoplasts were immobilized and cultured with and without immature seed AGPs. The results are

presented as a percentage of the total number of protoplasts that follow any of the four possible developmental patterns as shown in Figure 2.4.

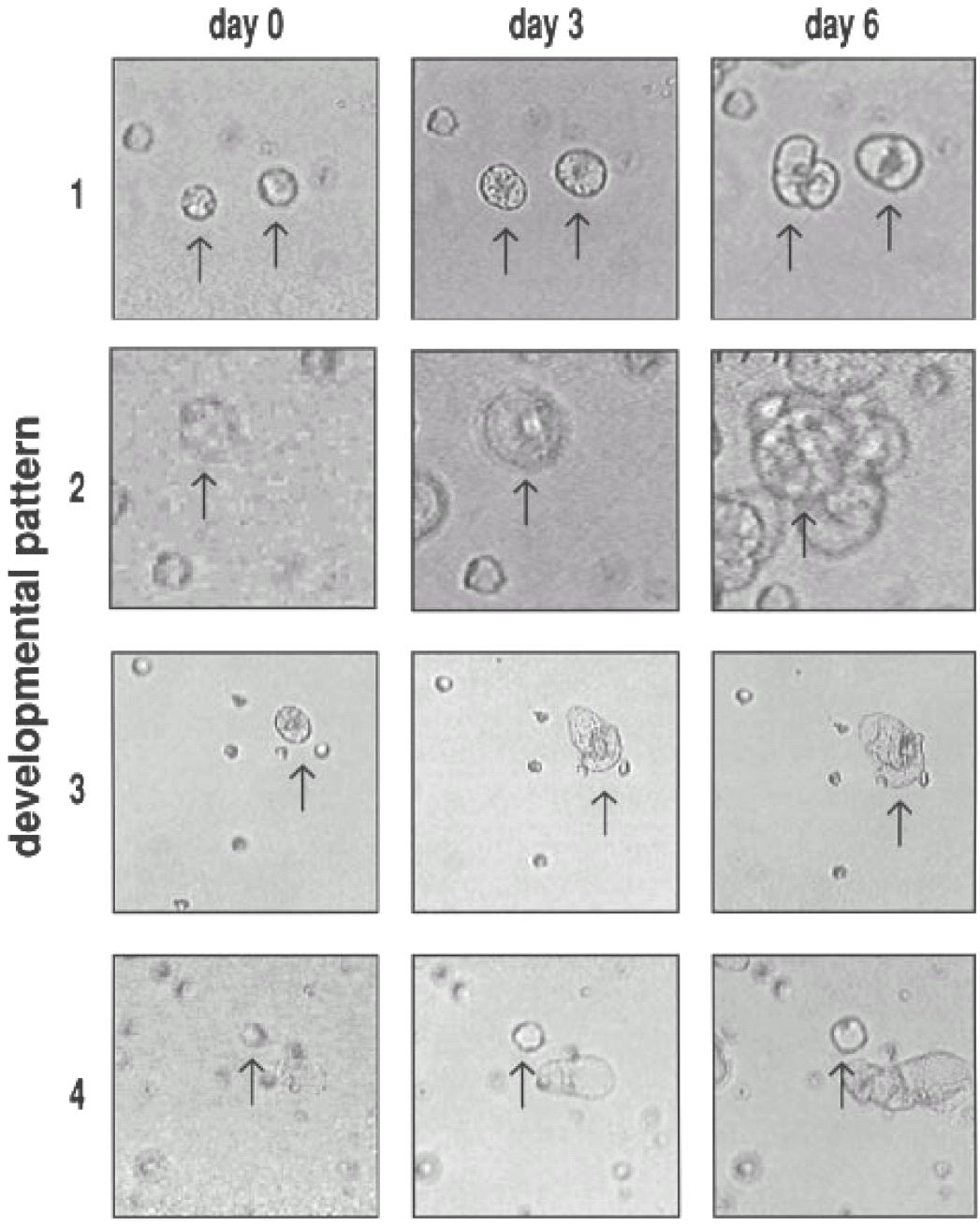

Figure 2.4 Development of immobilized carrot protoplasts.
Development of individual carrot protoplasts was analyzed by means of video cell tracking. After comparison of images as obtained after 0, 3, and 6 d, four patterns of development were identified: 1, cells that only divide and do not enlarge; 2, cells that enlarge and divide; 3, cells that only enlarge; and 4, cells that do not divide or enlarge. Arrows indicate cells representing the developmental pattern indicated.

For each culture treatment more than 600 individual protoplasts were recorded. The particular pattern followed was determined from the video tapes at d 6 to ensure that all cells that could have responded had indeed done so. Without any addition about 5% of the protoplasts followed pattern 1 (division without elongation) and the same percentage of protoplasts was found to follow pattern 3 (elongation). Thirteen percent followed pattern 2 (division in elongated cells), whereas three-quarters of the protoplasts remained unchanged (pattern 4). Addition of AGPs resulted in a statistically significant (P = 0.04) decrease in cells following pattern 4, resulting in a redistribution of cells over the other three patterns of development. A subtle difference was noted in that after addition of AGPs more cells entered into pattern 1, suggesting that AGPs are more effective in triggering cells into a rapid cell division mode than they are in promoting cell elongation.

Table 2.4 The effect of AGPs on the development of individual carrot protoplasts as analyzed by video cell tracking.
Development of protoplasts of embryogenic carrot cell lines. The protoplasts were placed into four different categories: 1, dividing protoplasts; 2, dividing and enlarging protoplasts; 3, enlarging protoplasts; and 4, protoplasts that do not divide nor enlarge. Protoplasts were cultured in the presence or absence of 2,4-D with or without AGPs isolated from immature 21 DAP carrot seeds. Protoplasts that follow either of the four developmental pathways are represented as a percentage of the total no. of analyzed protoplasts of one treatment. The overall effect of addition of 2,4-D or 21 DAP AGPs was assessed by means of F tests using the absolute nos. of embryos formed after addition of 2,4-D or AGPs, and the absolute no. of embryos formed in controls without any additions. All AGP preparations were pretreated with pectinase.

	Developmental Patterns							
Compound	1,	*P* values compared with control	2,	*P* values compared with control	3,	*P* values compared with control	4,	*P* values compared with control
No additions	5.0	–	13.1	–	5.0	–	76.9	–
AGPs	10.6	0.06	15.8	0.14	8.8	0.11	64.8	0.04
2,4-D	7.8	0.12	17.1	0.04	10.4	0.04	64.7	0.04
2,4-D + AGPs	14.9	0.04[a]	17.5	0.91[a]	10.3	0.99[a]	57.4	0.16[a]

[a] The overall effect of addition of AGPs in cultures containing 2,4-D was compared with control cultures containing solely 2,4-D. (P values less than 0.05 were regarded as indicative for significant differences).

The increase in the number of cells following pattern 1 after addition of AGPs was, however, not statistically significant (P = 0.06). The addition of 2,4-D had an effect comparable with that of AGPs, also decreasing the number of cells following pattern 4. These cells entered into either one of the other patterns of development with a

slight preferential increase for cells that elongate only (pattern 3). Addition of 2,4-D and AGPs gave an additive effect on the decrease in the number of cells following pattern 4. In following the fate of the cells that had shifted to the other three patterns of development, an additive effect between 2,4-D and AGPs was observed for cells entering into the rapid division mode without elongation (pattern 1). No additive effect was seen for cells following the developmental patterns 2 or 3.

We conclude that the biological effect of AGPs is to reactivate cells to enter into division and to a lesser extent to promote elongation. The resulting increase in cells that follow pattern 1 is, however, not statistically relevant. Because in a comparable population of cells AGPs increase embryogenesis over 25-fold (Tables 2.1–2.3), the primary effect of AGPs is seen in embryogenesis and not in cell division or cell elongation.

Discussion

Several questions remained after the initial finding that plant endochitinases were able to rescue somatic embryogenesis in a carrot line unable to complete embryo development (de Jong et al., 1992). The first and most important of these was to identify a plant-derived substrate for these enzymes. Staehelin et al. (1994) and Goormachtig et al. (1998) showed that plant chitinases were able to cut and inactivate bacterial lipochitooligosaccharides (LCOs), suggesting a role for chitinases in controlling the level of chitin-based signaling molecules. Although LCOs from bacterial origin mimicked the effect of chitinases on *ts11* embryogenesis (de Jong et al., 1993), biologically active LCO-like molecules have so far not been found in higher plants. Previous results showed that the gene encoding the 32-kD carrot chitinase was expressed in the endosperm during zygotic embryogenesis (van Hengel et al., 1998), suggesting that a potential substrate would be present in developing seeds. In conifer cultures an extracellular enzyme activity, possibly coinciding with chitinase activity, was reported to cause hydrolysis of AGPs (Domon et al., 2000).

Our evidence that AGPs are candidate molecules to function as a substrate for endochitinases consists of the presence of GlcNAc and GlcN in secreted AGPs from embryogenic cell cultures, the presence of chitinase cleavage sites in seed and secreted AGPs, and the observation that chitinase treatment enhances the embryo-promoting activity of AGPs.

A second related question was whether the activity of chitinases is restricted to embryogenesis in the carrot cell variant *ts11*. In this work we show that chitinases are

also able to increase somatic embryogenesis from wild-type protoplasts. Taken together, our data suggest a general role for chitinases and AGPs in plant embryogenesis.

AGPs contain GlcNAc, GlcN, and cleavage sites for endochitinases

The occurrence of GlcNAc or GlcN in AGPs has not been reported before in studies on the total sugar composition of AGPs (Van Holst et al., 1981; Komalavilas et al., 1991; Baldwin et al., 1993; Mollard and Joseleau, 1994; Serpe and Nothnagel, 1994, 1996; Smallwood et al., 1996). The carrot AGPs characterized here only contained about 0.2% of aminated sugars in the form of GlcN and GlcNAc, in line with the value found by van Holst et al. (1981). It is well possible that GlcNAc has been overlooked in most analyses, especially in view of the tissue and stage specificity of AGP epitopes (Knox et al., 1989, 1991). Because a subset of secreted AGPs contain GPI anchors with a single GlcN residue (Youl et al., 1998), the possibility exists that our GC-MS analysis has detected such AGPs in carrot culture media. Secreted rose cell AGPs contain between two and six times more GlcN as expected, based on the presence of the GPI anchor alone. No preferential release of GlcN occurred after incubation with glycoamidase A, suggesting that the GlcN was not present as *N*-glycans in rose AGPs (Svetek et al., 1999). Several tobacco chitinases, including the class I type, are capable of degrading partially re-acetylated chitin (chitosan), chitin oligomers of at least three residues, but not oligomers of GlcN (Brunner et al., 1998). The carrot EP3 class IV endochitinase employed here is related to class I enzymes and is also able to use chitosan as substrate (van Hengel, 1998). Therefore, it is unlikely that GPI anchors themselves are the substrate for endochitinase activity. It is evident that the occurrence of endochitinase cleavage sites in AGPs is a relatively rare event, because only very few differences in AGP-derived oligosaccharides were observed after endochitinase treatment. Generating partially degraded AGPs has also proven to be a useful tool for controlled degradation in structural studies of AGPs (Gleeson and Clarke, 1979; Tsumuraya et al., 1984, 1990; Saulnier et al., 1992). The enzymes and the combinations that we have used imply that GlcNAc is present in side chains of AGPs (van Hengel, 1998). However, these studies do not provide enough information to allow a precise identification of the AGP oligosaccharides that contain an endochitinase cleavage site. In addition to EP3 endochitinases, *ß*-galactosidase and *α*-arabinofuranosidase activity is present in the conditioned medium of carrot suspension cultures (Konno and Katoh, 1992; Konno et

al., 1994). A potential problem, therefore, is that AGPs may always be partially processed prior to isolation and therefore variable in composition. In vivo, hydrolytic enzymes of fungal and plant origin have been shown to be capable of degrading AGPs, suggesting that a stepwise AGP degradation mechanism occurs by means of individual hydrolytic enzymes (Nothnagel, 1997).

AGPs and cell identity

The temporal and spatial expression of AGP epitopes present on the cell surface and the proposed functions of AGPs in plant development suggest that in plants the identity of cells or tissues might be reflected by the AGPs present in the cellular matrix (Nothnagel, 1997). If so, the production of AGPs that reflect cellular identity must be correlated to cell differentiation. This hypothesis is supported by the observation that MAC207 epitopes, present on a large number of AGPs, are lost from cells involved in sexual reproduction and are absent in early zygotic embryos where the MAC207 epitope reappears after the embryos reach the heart stage (Pennell et al., 1989). An almost inverse pattern was found using the JIM8 monoclonal antibody. In oil seed rape, AGPs containing JIM8 epitopes were localized in gametes, anthers, ovules, and in the early embryo (Pennell et al., 1991). Taken together, the presence of MAC207 and JIM8 epitopes demonstrates that the expression of certain AGP epitopes is tightly connected to flower and embryo development and suggests that AGPs might be involved in the regulation of cell differentiation.

The presence of JIM8 epitopes was shown to have a polar localization in the cell wall of individual carrot suspension cells (Pennell et al., 1992; McCabe et al., 1997). The function of this JIM8 reactive material is unknown. It was previously suggested that cells containing the JIM8 epitope represent an intermediary cell type in somatic embryogenesis (Pennell et al., 1992). However, the development of living cells decorated with the JIM8 antibody by cell tracking revealed that the JIM8 cell wall epitope does not coincide with the ability of single suspension cells to form somatic embryos (Toonen et al., 1996). The release of compounds containing the JIM8 epitope from JIM8-labeled cells was suggested to function as a soluble signal that may activate non-JIM8 decorated cells to enter into the embryogenic pathway. The removal of the cell population carrying JIM8 epitopes resulted in a decrease in the embryogenic potential in the remaining cell culture (McCabe et al., 1997). Addition of the JIM8 epitope containing soluble signals might then compensate for the lack of this cell population. Apart from a more structural role in ensuring a proper membrane-

cell wall connection, AGPs have been proposed to function as signaling molecules (Bacic et al., 1988). We have shown here that AGPs can be activated after chitinase treatment, suggesting that entire AGPs themselves are signaling molecules rather than small chitinaceous molecules derived from them. The effects of AGPs on embryogenesis were observed at concentrations that did not exceed nanomolar ranges, which seems to be in line with a signaling function rather than a structural role. EP3 endochitinases alone can partially restore the embryogenic potential in wild-type suspension cell protoplasts. It is likely, but unproven, that this effect is mediated through endogenous AGPs.

Schultz et al. (1998) have outlined potential mechanisms for the involvement of GPI-membrane anchored AGPs in signal transduction pathways. It remains possible that the entire AGP molecule, as well as oligosaccharides derived from it, perform different signaling roles. Thus, depending on the environment and ambient presence of enzymes such as endochitinases, AGPs may give rise to a variety of molecules capable of redirecting fate and controlling proliferation of plant cells. This would be fully in line with our observations that AGPs can simultaneously reactivate non-dividing and nonexpanding cells and can form somatic embryos from dividing plant cells.

Materials and methods

Plant material and isolation of chitinases and AGPs

Embryogenic carrot (*Daucus carota* L. cv Trophy) suspension cultures were initiated and maintained as described (De Vries et al., 1988). Nonembryogenic cultures arose from embryogenic ones after subculture for approximately 1 year. EP3 endochitinases were isolated as described (Kragh et al., 1996) or produced as single isozymes in the Baculovirus insect cell expression system. No difference was observed in catalytic activity between the plant and insect cell-produced enzymes. AGPs were isolated from carrot suspension cultures 7 d after subculturing or from immature carrot seeds (Novartis Seeds, Enkhuizen, The Netherlands) by precipitation with Yariv reagent as described (Kreuger and Van Holst, 1993). Pectin-free AGP fractions were obtained by incubating 1 mg of AGPs in 50 mM NaAc, pH 5.0, containing 10 units of pectinase (Sigma, St. Louis) for 16 h followed by a second AGP isolation. The AGP concentration was determined by the radial gel diffusion method (Van Holst and Clarke, 1985).

Labeling of suspension cultures and degradation of labeled AGP fractions

Carrot suspension cells (2 mL packed cell volume) were cultured for 1 week in 50 mL of B5 medium containing 0.2 µM 2,4-D. The cells were washed with and transferred to B5 medium with or without 0.2 µM 2,4-D and were grown in the presence of [1-^{14}C]GlcNAc. Medium samples were taken after 2, 3, 4,

and 7 d. Cell wall fractions were obtained using the method described by Brown and Fry (1993). The degradation of the labeled AGP fractions was done by incubation with 2 M TFA for 45 min at 100°C or 60 min at 120°C. After degradation the samples were analyzed by TLC (*n*-butanol:acetic acid:water, 6:2:2) next to the reference compounds D-[1-^{14}C] Glc, D-[1-^{14}C]GlcN, and [1-^{14}C]GlcNAc that had been subjected to the same degradation reactions. Detection and quantification of label was done using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

AGP carbohydrate composition analysis

A solution of AGPs was hydrolyzed in 2 M TFA (1 h at 121°C) using inositol as internal standard. The released sugars were converted into their alditol acetates (Englyst et al., 1982) and were analyzed by GLC on an SPB-1701 capillary column (30 m x 0.32 mm, 0.25-µm film thickness, Supelco, Bellefonte, PA) in a GC8000 Top gas chromatograph. The temperature program was run from 80°C to 180°C at 20°C/min, 180°C to 250°C at 1.5°C/min, and at 250°C for 3 min. Identification of the compounds was confirmed by GC-MS using a SPB-1701 capillary column (30 m x 0.32 mm, 0.25-µm film thickness, Supelco) in a gas chromatograph (HP 6890, Hewlett-Packard, Palo Alto, CA) coupled to a mass-selective detector (HP 5973, Hewlett-Packard) and using an HP Chem Station (Hewlett-Packard). The temperature program was identical to the one used for GLC measurements.

Enzymatic hydrolysis of AGPs and chromatography

Samples of 80 µg of AGPs were incubated for 24 h in 10 mM MES [2-(*N*-morpholino)-ethanesulfonic acid], pH 5.5, supplemented with 80 to 200 ng of EP3 chitinases and/or with 0.050 units of exoarabinofuranosidase, 0.024 units of endoarabinofuranosidase, and 0.030 units of endogalactosidase, all three of which were produced by *Aspergillus niger*. Analysis of AGPs and enzymatically degraded AGPs was performed by HPAE-PAD, using the CarboPac PA-100 column (Dionex, Sunnyvale, CA). The flow rate was 1 mL/min and the eluent consisted of 10% (v/v) 0.5 M NaOH: water in combination with a linear salt gradient starting at t = 3 min with 0% NaAc and ending at t = 18 min with 80% (v/v) 0.5 m NaAc.

Bioassay

Protoplasts were obtained from suspension cultures 3 d after subculturing. The cells were collected and incubated overnight in 1% (w/v) macerozyme and 2% (w/v) cellulose (both from Yakult Biochemicals, Tokyo) in 50 mM citrate- HAc, pH 4.8, and 0.3 M mannitol. Protoplasts were sieved through a 50-µm nylon mesh and washed three times in 100 mM $CaCl_2$ and 0.3 M mannitol. After 2 h the protoplasts were washed once more and transferred to B5 medium with 0.3 M mannitol to initiate somatic embryogenesis. After the formation of heart- and torpedo-stage embryos, the plant material was transferred to fresh B5 medium without mannitol for further embryo development into plantlets. Aliquots of 30 µg of AGPs and 400 ng of EP3 were added to 100,000 freshly isolated carrot protoplasts in 2 mL of B5 medium containing 0.3 M mannitol. Enzyme treatment of AGPs to be used in bioassays was performed by incubation of 100 µg of AGP and 200 ng of EP3 in 1 mL of 20 mM citrate buffer, pH 5.5, for 16 h, followed by a re-isolation of AGPs. In the controls the enzymes were replaced by water. Aliquots of 500 mg of AGPs were incubated in 1 mL of 0.1 M barium hydroxide for 6 h at

100°C. The hydrolysate was neutralized by adding 1 N H_2SO_4 until the pH was stable at 7.0. After a 15-min centrifugation at 12,000 x *g* the precipitated $BaSO_4$ was discarded and the supernatant containing the hydrolyzed AGPs was used in the bioassays. Hydrolyzed AGPs were used in the bioassay in concentrations that were based on the amount of AGPs from which the hydrolysate was derived. Samples in which AGPs were omitted, but were otherwise treated the same way were added to protoplasts. In none of these controls were more somatic embryos observed than in unsupplemented controls.

Cell tracking

Immobilization of protoplasts obtained from two different embryogenic cell lines and subsequent video cell tracking was performed as described before for single cells (Toonen and De Vries, 1997), with the difference that the medium used contained 0.3 M mannitol. 2,4-D was added to the phytagel top layer to give a final concentration of 2 µm. AGPs in 1 mL of B5 medium were poured on top of the phytagel layers to give a final concentration of 13 µg/mL. Statistical analysis was done by using the SAS System based upon a generalized linear model (Aitkin et al., 1991). The overall effect of treatment was assessed by means of *F* tests and significant differences were expressed with *P* values less than 0.05.

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Chapter 3

Different arabinogalactan proteins are present in carrot (*Daucus carota*) cell culture medium and in seeds

Peter Immerzeel[1,2], Henk A. Schols[2], Alphons G. J. Voragen[2] and Sacco C. de Vries[1]

[1]Laboratory of Biochemistry, Department of Agrotechnology and Food Sciences, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

[2]Laboratory of Food Chemistry, Department of Agrotechnology and Food Sciences, Wageningen University, Bomenweg 2, 6703 HD Wageningen, The Netherlands

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Abstract

Arabinogalactan proteins (AGPs) were isolated by Yariv phenylglycoside precipitation from the medium of carrot (*Daucus carota* L.) cell cultures and from carrot seeds. The isolates showed a different composition of AGPs. The medium AGPs contained an arabinose poor AGP fraction that had relatively high levels of glucuronic acid and rhamnose. In contrast the seed AGPs only contained arabinose and galactose rich AGP fractions that had low levels of glucuronic acid. Linkage analysis on all fractions showed that most of the arabinose residues were terminally linked and that almost all galactose was present in the 1,3-, 1,6- and 1,3,6- form. The strongly branched type II arabinogalactans are characteristic of the carbohydrate part of AGPs. AGP characteristic amino acid residues as Hyp, Pro, Glx, Ser, Gly, Asx, Ala, Leu and Thr were detected in three different fractions.

Introduction

Arabinogalactan proteins (AGPs) are proteoglycans found in all higher plants that appear to be present in all stages of plant development and in all tissues investigated (Serpe and Nothnagel, 1995; Gaspar et al., 2001). AGPs are found linked via glycosylphosphatidylinositol (GPI) anchors to the outer surface of the plasma membrane, bound to the cell wall or as secretions in intercellular spaces, culture media or the environment (Serpe and Nothnagel, 1999; Showalter, 2001). AGPs are found to be associated with processes as diverse as somatic embryogenesis, xylem development in primary roots, tip growth of pollen tubes and programmed cell death in cell cultures (Majewska-Sawka and Nothnagel, 2000). Exogenously added AGPs or Yariv-mediated interference with AGP functioning often show clear effects at the cellular level, however, the underlying molecular mechanisms of such AGP activities are still unsolved (Showalter, 2001). Therefore, the precise biological functions of AGPs remain largely unknown.

AGPs belong to the hydroxyproline rich glycoprotein (HRGP) family that also includes the extensins, Pro/Hyp rich glycoproteins (PRPs) and the solanaceaous lectins (Gaspar et al., 2001). The HRGP family comprises a continuum of molecules; from the non- or minimally glycosylated PRPs, the moderately glycosylated extensins, through to the AGPs, where the carbohydrate moiety usually accounts for more than 90% by weight of the molecule (Sommer-Knudsen et al., 1998). Due to their high carbohydrate content AGPs are often referred to as proteoglycans (Sommer-Knudsen et al., 1998). Most AGPs can be specifically precipitated with Yariv

phenylglycoside (Nothnagel, 1997). However, some examples are known of molecules that have structural properties of AGPs but do not or hardly bind to Yariv phenylglycoside (Nothnagel, 1997). The AGPs that have been investigated at the structural level have been isolated from the medium of cultured cells, plant exudates, xylem and stylar transmitting tract tissue (Serpe and Nothnagel, 1995; Showalter, 2001). The molecular size of soluble AGPs ranges from 60 to 300 kDa (Majewska-Sawka and Nothnagel, 2000) although arabinogalactan-peptides are described by Fincher et al. (1974) that have an average molecular weight of 22 kDa. Mainly arabinose and galactose are present in the AGP carbohydrate side chains (Bacic et al., 1987). These are organized in type II arabinogalactans that contain a 1,3-*ß*-D-galactopyranosyl backbone, 1,3,6-*ß*-D-galactopyranosyl branching points and 1,6-*ß*-D-galactopyranosyl side chains (Serpe and Nothnagel, 1999; Showalter, 2001). They carry *α*-arabino-furanosides as terminal residues and minor amounts of Glc, Rha, Xyl, Fuc and uronic acids (Baldwin et al., 1993). Type I arabinogalactans found in plants and micro-organisms differ from type II arabinogalactans in respect to their galactosyl residues that occur predominantly in 1,4-*ß*-D-galactopyranosyl linkages (Serpe and Nothnagel, 1999). The size of type II arabinogalactans that are *O*-linked to the protein core varies between 30 and 50 sugar residues (Serpe and Nothnagel, 1999). Variation in the size, linkage and sequence of the arabinogalactans creates a wide chemical and structural diversity. Arabinose can also be linked to the protein core as small neutral oligosaccharides (Zhao et al., 2002). The proteins that are translated for classical AGPs contain a N-terminal secretion sequence that is not present on the mature protein, a central domain rich in Pro/Hyp and a C-terminal hydrophobic domain (Gaspar et al., 2001). According to Showalter (2001) AGPs should be classified as non-classical when the amino acid composition deviates from the classical AGPs, for instance when the protein core is Cys rich or Asn rich. Hydroxyproline-deficient AGPs that have been isolated (Baldwin et al., 1993; Mollard and Joseleau, 1994) should also be regarded as non-classical. The C-terminal hydrophobic domain functions as a signal for the attachment of a glycosylphosphatidylinositol (GPI) anchor (Gaspar et al., 2001). Classical C-terminal GPI anchor containing AGPs are found at the outer surface of the plasma membrane and are thought to interact with other molecules of the extracellular matrix (Zhao et al., 2002).

Plant embryogenesis can be mimicked in vitro and was first described for suspension cultured carrot cells (Mordhorst et al., 1997). Cell lines produce a specific set of

AGPs that are released into the medium and are able to stimulate the formation of embryos in carrot suspension cultures (Kreuger and van Holst, 1993) and in Norway spruce (Egertsdotter and von Arnold, 1995). With the use of crossed-electrophoresis it has been shown that the pattern of medium-isolated AGPs is related to the developmental stage of the cell culture (Kreuger and van Holst, 1993). In addition to AGPs other carbohydrates and proteins are also present in the cell culture medium. de Jong et al. (1992) describe the extracellular protein 3 (EP3) endochitinase that was able to promote the formation of embryos of genetically modified carrot cultures at the non-permissive temperature. In previous experiments we have shown that carrot seed AGPs contained potential cleavage sites for the EP3 endochitinase. These results were based on the finding that both the EP3 endochitinase as well as seed AGPs were able to increase the number of somatic embryos formed from protoplasts. Seed AGPs pre-treated with EP3 endochitinase were optimal in activity (van Hengel et al., 2001). It was also suggested that only a limited subset of AGPs contained such an endochitinase-sensitive carbohydrate moiety. To identify such a carbohydrate moiety and to increase our knowledge about the chemical composition of seed and medium AGPs, we fractionated AGPs from seeds and embryogenic cultures. The different fractions obtained were analyzed on molecular size, chemical composition and some fractions were incubated with AGP-degrading enzymes. Our results suggest that AGPs from the different fractions are quite different in composition. The small amount of GlcN detected in the total seed AGP extract was, however, not retained in any of the further purified AGP fractions. This makes it unlikely that the previously proposed presence of GlcN in AGP preparations is an integral part of the AGP carbohydrate moiety.

Results

Carrot AGPs were isolated with Yariv phenylglycoside from the medium of a non-embryogenic and an embryogenic cell culture and from commercially available seeds. The monosaccharide composition was largely the same for the AGPs from the embryogenic and the non-embryogenic culture (Table 3.1). Surprisingly, the total sugar content of the non-embryogenic culture was about 2.5 times lower than that of the embryogenic culture. The differences between the medium and seed AGPs is mainly caused by a lower amount of uronic acids in the seed AGPs.

Arabinose and galactose are the most abundant sugar residues in all AGPs with Ara:Gal ratios of about 1 : 2. High arabinose and galactose content is indicative of

AGPs (Sommer-Knudsen et al., 1998). Other neutral sugars are present in low amounts. Although glucosamine is not a common constituent of AGPs, minor amounts of amino sugars, especially GlcN, have been detected in several AGPs (Akiyama and Kato, 1981; Serpe and Nothnagel, 1995; van Hengel et al., 2001). Seed AGPs contain trace amounts of glucosamine (0.2 mol%), which was confirmed with GC–MSD. The presence of trace amounts of glucosamine in seed AGP preparations was confirmed in different cultivars and seed batches (data not shown). Glucosamine could not be detected in medium AGPs.

Table 3.1 Monosaccharide composition (mol%) of medium AGPs and seed AGPs.
NC: non-embryogenic cell culture; EC: embryogenic cell culture; tr: trace amounts (<1%)

	medium AGPs		seed AGPs
	NC	EC	
Rha	5	5	tr
Ara	23	24	34
Xyl	0	tr	tr
Man	0	tr	tr
Gal	50	49	59
Glc	4	1	tr
GlcN	0	0	tr
UA	19	19	4
Total sugar (w/w %)	32	81	75

All three AGP preparations were subjected to size exclusion chromatography to determine the size distribution. Figure 3.1 shows chromatograms of AGPs that were isolated from an embryogenic cell culture, a nonembryogenic cell culture and seeds. The SEC columns employed separate carbohydrates between approximately 200 and 0.2 kDa. The AGPs that were isolated from an embryogenic cell culture and the seed AGPs eluted in two distinct fractions. AGPs that were isolated from a non-embryogenic cell culture eluted in one peak (Mw about 45 kDa when compared with a pectin standard). Pectin was used for an indication of the size distribution as there are no AGP standards known. The interaction with the negatively charged column material and the negatively charged pectins will differ from the AGPs that have less charged sugar residues. Therefore the molecular weight of the AGPs might be somewhat underestimated. The material that elutes at 33 min is an unknown compound eluting at low Mw (approximately 400 Da) that shows absorption at a wavelength of 254 nm (data not shown). The ratio of the two populations of the

embryogenic cell culture (100 and 45 kDa) can vary strongly among different embryogenic cell lines examined (data not shown). The elution profile of seed AGPs shows for both fractions a higher molecular weight; 105 and 75 kDa, respectively.

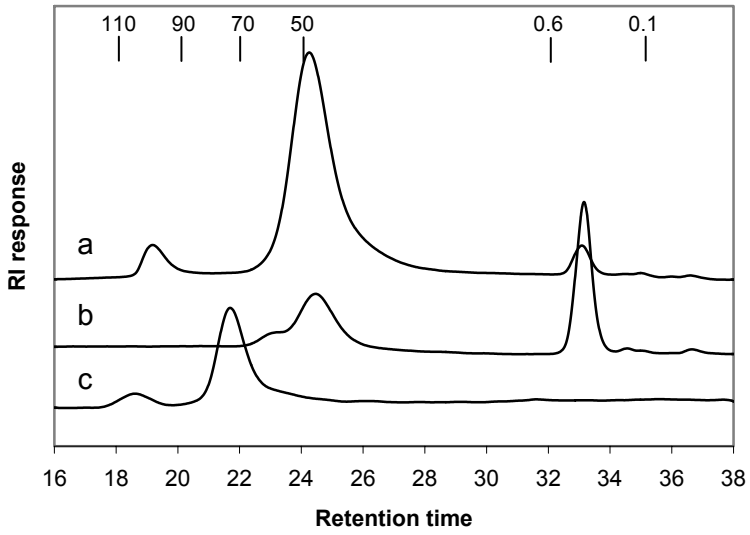

Figure 3.1 HPSEC elution profiles of different AGP extracts.
(a) Elution profile of an embryogenic cell culture, (b) elution profile of a non-embryogenic cell culture and (c) elution profile of seed AGPs. Pectins were used as molecular weight standards (kDa).

The question arose whether both fractions indeed represent AGPs or other high molecular weight compounds. Since AGPs are known to aggregate in vitro (Sommer-Knudsen et al., 1998; Showalter, 2001), we could also not exclude the possibility that the different fractions have the same AGP composition. Therefore, embryogenic culture AGPs and seed AGPs were separated on a semi-preparative scale to allow a more detailed analysis of both fractions. The elution patterns of medium AGPs and seed AGPs again showed the presence of two high molecular weight fractions in figures 3.2 and 3.3.

The two high molecular weight fractions that were present using the analytical column were also base-line resolved using the preparative column and allowed us to pool the two different fractions. A fraction designated AGPmIV was pooled because it showed a high UV_{254} absorption which might indicate that this fraction contains a higher protein concentration (Fig. 3.2; dashed line). In the seed AGPs a fraction designated AGPsII was pooled. This fraction contained material being eluted between the main peaks having an intermediate molecular mass.

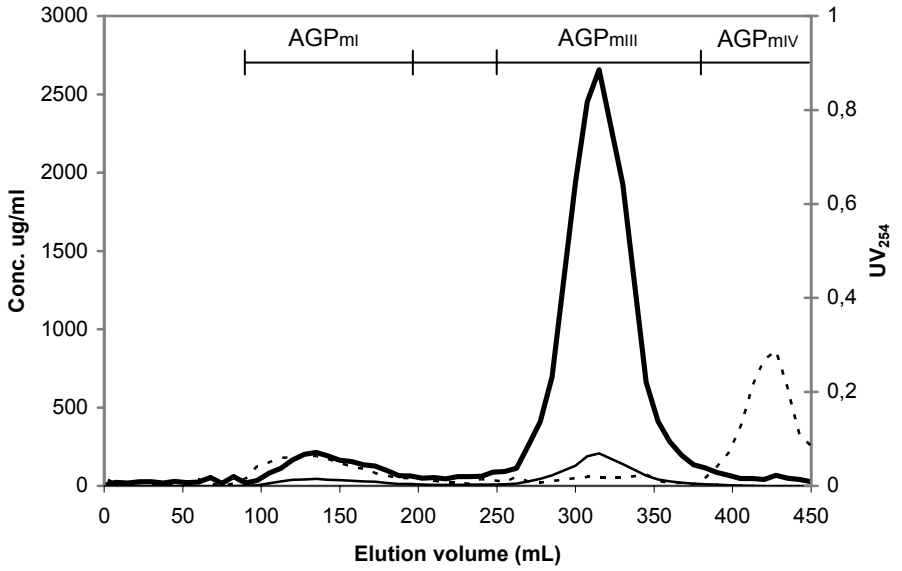

Figure 3.2 Preparative SEC elution profiles of embryogenic cell culture medium AGPs on Sephacryl S-500; Thick line: neutral sugars; thin line: uronic acids; dashed line: UV_{254}

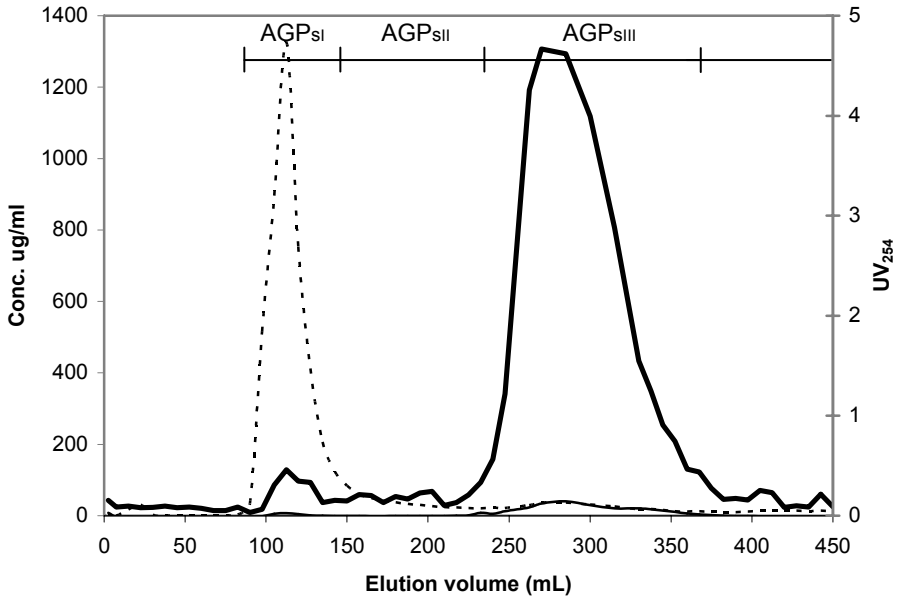

Figure 3.3 Preparative SEC elution profiles of seed AGPs on Sephacryl S-500
Thick line: neutral sugars; thin line: uronic acids; dashed line: UV_{254}

Table 3.2 shows the monosaccharide composition of the different fractions. For two fractions sufficient material was available for an additional total protein determination. AGPmI show low amounts of arabinose (3 mol%) resulting in an Ara:Gal ratio of 1:18. There is a high concentration of glucuronic acid in this fraction. AGPs with a high glucuronic acid content and low arabinose concentration have been previously isolated from rose cell walls (Serpe and Nothnagel, 1995). However, this is an unusual sugar composition for AGPs in which arabinose and galactose are the main carbohydrates as reported before (Nothnagel, 1997).

Table 3.2 Monosaccharide composition (mol%) of different fractions of embryogenic cell culture medium AGPs and seed AGPs obtained after size exclusion chromatography on Sephacryl S-500. AGPmI, AGPmIII, AGPmIV: medium AGPs, fractions I, III and IV resp.; AGPsI, AGPsII, AGPsIII: seed AGPs, fractions I, II and III resp.; tr: trace amounts (<1%);
ND: not determined

	medium AGPs			seed AGPs		
	AGPmI	AGPmIII	AGPmIV	AGPsI	AGPsII	AGPsIII
Rha	11	5	5	1	2	1
Ara	3	28	26	37	30	33
Xyl	5	0	1	0	tr	tr
Man	5	0	1	tr	tr	tr
Gal	56	60	60	47	59	61
Glc	0	0	1	2	3	0
GlcN	0	0	0	0	0	0
GalA	tr	0	tr	8	tr	tr
GlcA	20	6	6	4	5	5
Total sugar (w/w %)	74	89	62	14	36	83
Total protein (w/w %)	ND	2	ND	ND	ND	9

Fraction AGPmIII contains high amounts of arabinose and galactose and has an Ara:Gal ratio of 1:2.2. Rhamnose and glucuronic acid are present at lower levels. The total uronic acid content in all three medium fractions together is lower than the amount of uronic acids in the starting material (19 mol%, Table 3.1) as the main fraction (AGPmIII) contains 6 mol% GlcA. Uronic acids as shown in Table 3.1 were hydrolyzed with sulphuric acid and the neutral sugars were hydrolyzed with TFA, which is known to be not as strong as sulphuric acid. As the data of the UA content and the data of the neutral sugars are merged in Table 3.1 the amount of uronic acids might be somewhat overestimated. The total amount of protein in this fraction is 2% (w/w). The sugar composition of AGPmIV is comparable with that of AGPmIII. Fraction AGPsI contains a very low amount of sugars (14% w/w) and shows a high UV_{254} absorption (Fig. 3.3). This might indicate a relatively high protein content for

this fraction or implies incomplete dialysis. The main neutral sugars are arabinose and galactose, present in an Ara:Gal ratio of 1:1.3. Other neutral sugars are present in low amounts and galacturonic acid is present at 8 mol%. $AGPs_{I}$ differs from $AGPs_{II}$ and $AGPs_{III}$ in the Ara : Gal ratio and the presence of galacturonic acid. $AGPs_{II}$ and $AGPs_{III}$ show a comparable carbohydrate composition. Most of the dry weight material from the seed extracts is located in fraction $AGPs_{III}$. The total sugar content of $AGPs_{III}$ is 83% (w/w) and the total protein content is 9% (w/w). Arabinose and galactose are the main contents in a ratio of 1:1.9. The low amount of glucosamine (0.2 mol%) that was detected in the crude Yariv extract of the seeds (Table 3.1) was not traceable in any of the three fractions. If GlcN was present in low molecular weight compounds they would not have been recovered here.

The chemical composition of $AGPm_{I}$ is unusual for AGPs because of the low arabinose content and the relative high glucuronic acid content. In order to prove that all fractions contain AGPs, a Yariv radial diffusion gel was prepared to test the reactivity for all fractions. This gel showed for all fractions a precipitation with Yariv (Fig. 3.4), which is indicative for AGPs (Nothnagel, 1997). For all fractions an identical amount of material was used. The smaller halo obtained for the first fractions of medium and seed AGPs might be caused by the higher molecular weight of the AGPs that impedes the diffusion through the gel.

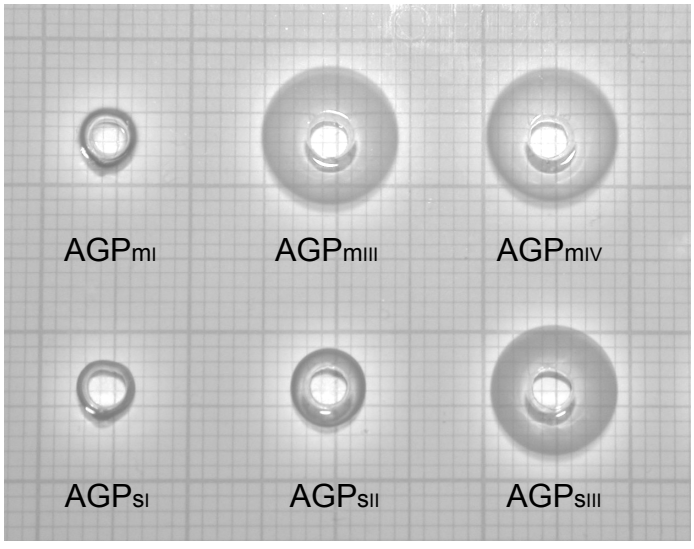

Figure 3.4 Yariv phenylglycoside radial diffusion gel.
$AGPm_{I}$, $AGPm_{III}$, $AGPm_{IV}$ medium AGPs; $AGPs_{I}$, $AGPs_{II}$, $AGPs_{III}$ seed AGPs

AGPmI, AGPmIII, AGPsI and AGPsIII were subjected to linkage analysis (Table 3.3). Arabinose was found to be present in the furanose form and occurs mainly as terminal sugar. In addition 1,5- linked arabinose was detected in all fractions at different concentrations. In seed AGPs 1,3,5-arabinose is detected in very small amounts and is completely absent in medium AGPs. Galactose is detected in the pyranose form and is mainly present as 1,3-, 1,6- and 1,3,6- linked residues, unevenly distributed over all fractions. In all fractions galactose was also present as terminal residues, the highest amounts were observed in AGPmI. In medium AGPs trace amounts of galactose were found in the 1,4,6- form. Rhamnose and xylose were detected as terminal residues at high levels in AGPmI. These results show that the carbohydrates that are present in the AGP fractions are linked in a manner characteristic for type II arabinogalactans (Serpe and Nothnagel, 1999). The ratio of terminal-linked sugar residues and branched sugar residues for AGPmI showed a high value of 2.6 and values approaching 1 for the other fractions.

Table 3.3 Sugar linkage composition (mol%) of the main fractions of embryogenic culture medium AGPs and seed AGPs obtained after size exclusion chromatography on Sephacryl S-500. AGPmI, AGPmIII: medium AGPs, fractions I and III; AGPsI, AGPsIII: seed AGPs, fractions I and III; tr: trace amounts (<1%)

		medium AGPs		seed AGPs	
Residue	Linkage	AGPmI	AGPmIII	AGPsI	AGPsIII
Rha*p*	terminal	12	5	tr	tr
Ara*f*	terminal	2	32	33	36
	1,5-	tr	4	6	6
	1,3,5-	0	0	tr	tr
	unmethylated	0	0	tr	0
Xyl*p*	terminal	11	tr	0	0
Man*p*	unmethylated	tr	0	0	tr
Gal*p*	terminal	12	5	5	5
	1,3-	34	8	13	13
	1,6-	16	12	4	4
	1,3,6-	11	34	34	35
	1,4,6-	tr	tr	0	0
	unmethylated	tr	tr	0	tr
Glc*p*	terminal	0	0	tr	0
	1,4-	tr	0	1	0
Ratio t-linkages/branching points		2.6	1.2	1.1	1.1

Amino acid analysis was performed on $AGPm_{I}$, $AGPm_{III}$ and on $AGPs_{III}$ that were present in sufficient amounts. $AGPm_{I}$ contained many Pro, Glx, Ser, Gly, Asx and Ala residues (hydroxyproline not determined) whereas $AGPm_{III}$ had high concentrations of Hyp, Pro, Ala, Gly, Ser and Leu (Table 3.4). $AGPs_{III}$ had relatively high concentrations of Hyp, Ser, Ala, Glx, Gly and Thr residues. AGP-indicative amino acids such as Hyp, Pro, Ala, Ser, Gly and Thr were present in the fractions at different concentrations.

Table 3.4 Amino acid composition (mol%) of $AGPm_{I}$, $AGPm_{III}$, and $AGPs_{III}$; tr: trace amounts (<1%); ND: not determined; Ornithine was not included in the table

	medium AGPs		seed AGPs
Residue	$AGPm_{I}$	$AGPm_{III}$	$AGPs_{III}$
Asx	9	5	5
Thr	4	5	7
Ser	9	7	16
Glx	10	5	11
Pro	15	17	5
Gly	9	8	8
Ala	7	10	15
Val	5	5	6
Ile	4	4	4
Leu	5	7	2
Tyr	3	3	tr
Phe	4	3	2
Lys	4	1	3
His	4	4	5
Arg	3	4	2
Hyp	ND	9	9

Two AGP fractions were incubated with galactosidase, arabinofuranosidase and endo arabinanase to determine whether the carbohydrate part of the AGPs could be used as substrate by the enzymes. Incubation of $AGPm_{III}$ with galactosidase and arabinofuranosidase resulted in a slight decrease of the molecular weight and the formation of break down products (Fig. 3.5.1b). The break down products elute between 33 and 37 min. The peak eluting at 35 min is caused by the buffer solution used for the enzymes. Incubation with arabinanase (Fig. 3.5.1a) had no observable effects on this fraction. When seed AGPs (fraction III) were incubated with galactosidase and arabinofuranosidase a substantial part of the AGP aliquot is degraded (Fig. 3.5.2b), even resulting in the formation of some oligomeric fragments. For seed AGPs no degradation was observed when this fraction was incubated with

endo arabinanase (Fig. 3.5.2a). The observation that the exo-enzymes were able to degrade the polysaccharides in an endo-fashion is probably due to very minor impurities of the enzymepreparations used.

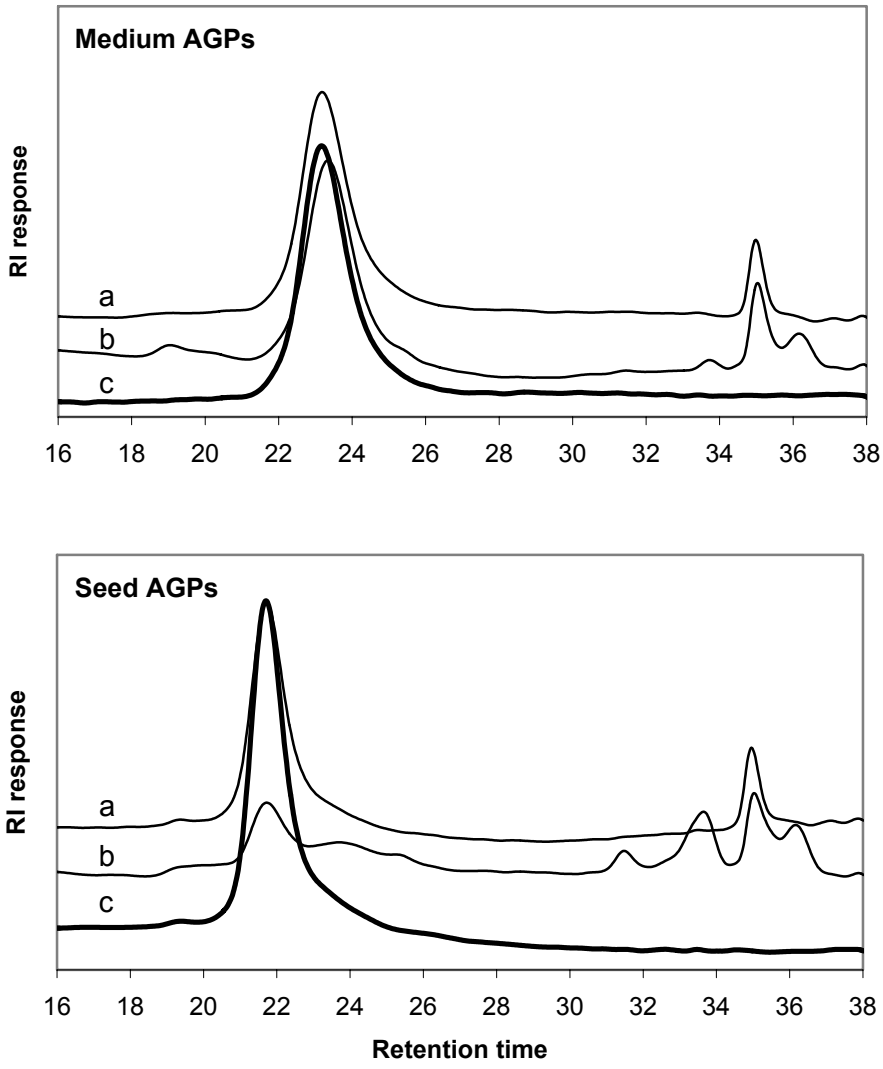

Figure 3.5 HPSEC elution profiles of medium AGPs fraction III (1) and of seed AGPs fraction III (2) before (c) and after incubation with *α* 1-5 arabinanase (a) and *ß* galactosidase and *α* arabinofuranosidase (b). For molecular weight calibration see figure 3.1.

The enzyme incubation experiments showed that AGPs that have been extracted from seeds are more degradable with galactosidase and arabinofuranosidase when compared with medium AGPs. When medium and seed AGPs were incubated with a high concentration of the commercial protein hydrolyzing enzyme (Pronase E) (Connolly et al., 1987) only the seed AGPs showed the formation of high molecular

weight breakdown products (data not shown). The experiments with hydrolytic enzyme incubations indicate a high sensitivity for seed AGPs for both the carbohydrate part and the protein part of the molecule. Medium AGPs have a low substrate affinity for galactosidase and arabinofuranosidase.

Discussion

During this study AGPs were analysed that were isolated from medium and seeds. Medium AGPs from an embryogenic cell culture and seed AGPs could be separated into two main fractions that showed different molecular weight and chemical composition. In the different fractions it is not clear whether the protein core of the AGPs is identical or that different protein cores are present. It is known for Arabidopsis for instance, that many different protein cores for AGPs are present (Schultz et al., 2000). In order to determine the glycosylation patterns of a specific protein, Zhao et al. (2002) labelled a specific AGP protein backbone from tomato with a fluorescent protein. These labelled AGPs could be traced in planta and allowed fractionation on the basis of the protein core. As we have no data available about the protein sequence of the AGPs, we are restricted to draw general conclusions about the obtained data of the isolates.

The high molecular weight fraction AGPmI was found to contain low amounts of arabinose and high amounts of glucuronic acid and rhamnose. Serpe and Nothnagel (1995) found a cell wall AGP fraction in rose cell cultures with a low Ara : GlcA ratio and decided to designate this fraction as a glucuronogalactan protein (GGP). Rhamnose is present in high amounts in the same fraction. We can rule out the possibility that rhamnose is related to pectin as galacturonic acid is only present in trace amounts. All rhamnose residues are terminally linked to the carbohydrate chains. The 45 kDa fraction (AGPmIII) of the medium AGPs has high amounts of arabinose and galactose and low levels of rhamnose and glucuronic acid. The protein content is 2% (w/w) and this implies a remarkable small protein core for an AGP, even when the molecular weights of the AGPs are somewhat underestimated as pectins were used for the Mw standard. Therefore this fraction should be indicated as AG peptides (Schultz et al., 2000). From the monosaccharide composition data it can be concluded that the two main peaks present on HPSEC elution patterns contain macromolecules with a different carbohydrate composition. Therefore it is unlikely that a substantial part of the AGPs in the high molecular weight fraction

(100 kDa) are aggregates of the AG peptides present in the low molecular weight fraction (45 kDa).

The seed AGPs also contain two fractions and showed a higher molecular weight (105 and 75 kDa) when compared with the medium AGPs. The low molecular weight fraction has a protein content of 9% (w/w). In all seed fractions arabinose and galactose are the main sugars. The Ara : Gal ratio distinguishes the high and the low molecular weight fraction. Although present in the starting material, GlcN could not be detected in any of the fractions from the seed AGPs. It is not likely that the GlcN detected was part of a GPI-anchor. Although every anchor contains one GlcN residue, the AGPs with GPI-anchors also would have been detected after fractionation. N-linked glycans represent a family of glycans with a trimannosyl core and contain two contiguous GlcNAc residues. However, for the function of chitinase at least three contiguous GlcNAc residues are required. When the amino acid sequence is known, the occurrence of N-glycans can be predicted. To our knowledge no such sequences are known for carrot AGPs but we cannot exclude this possibility. Another possibility for the loss of GlcN detection is that GlcN was present in low molecular weight compounds that were not pooled after fractionation. The oligosaccharides of AGPs that changed in relative amount upon incubation with EP3 endochitinase (van Hengel et al., 2001) could be characterized in order to show that they contain glucosamine. Alternatively, the substrate activity of EP3 endochitinase should be studied in more detail. We can not rule out the possibility that the activity of EP3 endochitinase as observed on AGPs (van Hengel et al., 2001) is due to side activity of the enzyme. This means that, besides the ability of the enzyme to hydrolyze glycosidic bounds between GlcNAc, bounds between other sugar residues could also be hydrolyzed. In that case the reduction in size of the AGPs after enzyme incubation is responsible for the increase in embryo formation after addition of enzyme-treated AGPs to carrot protoplasts.

Linkage analysis data confirmed that all fractions contained type II arabinogalactans. However, the fraction AGPmI contained one third of the typical 1,3,6-*ß*-D-Gal*p* linkages typical for AGPs when compared with the other three fractions. Together with the high ratio of terminal linked sugar residues and branched sugar residues (2.6) we can conclude that this fraction contains less branched galactan chains and more unbranched oligosaccharides that are attached to the protein core. All fractions showed precipitation with Yariv phenylglycoside. The halo that was formed from the AGPmI fraction proved that these proteoglycans are still able to interact with Yariv

despite the low arabinose concentration and the different galactose linkage composition. Hydroxyproline was detected in the two main AGP fractions from medium and seed. Other AGP characteristic amino acids were present in all fractions tested. Enzymatic degradation of carbohydrates and proteins showed that the seed AGPs were more degraded by hydrolytic enzymes than the AGPs that were isolated from the medium. This characteristic can be explained by a glycosylation pattern where both the protein part and the carbohydrate part are easily accessible for hydrolyzing enzymes. Since the protein size of this seed AGP fraction is about seven times larger than the medium AG peptides, it is possible that the arabinogalactans are less closely packed on the seed-derived AGPs. Additional protein analysis could reveal data on the sequence of the proteins that are present in the AGP fractions. The hydroxyproline distribution of the protein cores and the corresponding attachment of the arabinogalactans depending on the amino acid sequence (Zhao et al., 2002) could prove this hypothesis. The amount of amino acids detected for AGPmIII is higher than the calculated total amount of amino acids in the protein core. Therefore it is likely that different protein cores are present in the obtained fractions. Additional research is required for a better insight of the complete structure of individual AGP molecules. A prerequisite will be the isolation of AGP molecules with an identical protein backbone.

Materials and methods

Plant material

Carrot (*Daucus carota* L. cv Autumn King/Trophy; Syngenta Seeds BV, Enkhuizen, The Netherlands) cell cultures were maintained as described in de Vries et al. (1988). One-week-old suspension cultures grown in 500 mL of Gamborg's B5 medium, containing 0.2 µM 2,4-dichlorophenoxyacetic acid were used as a source for AGPs.

Arabinogalactan protein isolation

Suspension cells were removed by filtration and the medium was centrifuged at 13,900 x *g* for 20 min. The AGPs from the supernatant were precipitated by adding 30 mg Yariv phenylglycoside to 1 L medium and NaCl at a final concentration of 0.15 M (Kreuger and van Holst, 1993). The Yariv–AGP complex was precipitated at 4°C overnight, centrifuged at 13,900 x *g* for 20 min, washed three times with 0.15 M NaCl and dissolved in water. Sodium hydrosulphite was added to decompose the *ß*-glucosyl Yariv phenyl glycoside. The solution was heated to 50°C until the red color disappeared, dialyzed extensively against water at 4°C and freeze-dried.

Carrot seeds (*Daucus carota* L. cv Yukon) were kindly provided by Dr Marc Kreuger (Syngenta Seeds BV, Enkhuizen, The Netherlands). Seeds (200 g) were ground in a cryo-mill to a fine powder that was re-suspended in 1 L distilled water at 4°C. After centrifugation at 13,900 x *g* for 20 min the resulting pellet was extracted once with 1 L water at 4°C. The two extracts were pooled and the solubilized AGPs present in the extract were precipitated with 150 mg Yariv phenylglycoside. The Yariv–AGP complex was further processed as described for the medium AGPs.

Sugar analysis

Neutral sugar compositions were determined by gas chromatography according to Englyst and Cummings (1984). A solution of 0.1 mg AGPs was dried and hydrolysed in 1 mL trifluoroacetic acid (TFA) (2M) for 1 h at 121°C using inositol as internal standard. For the determination of glucosamine, allose was used as internal standard. After hydrolysis the released sugars were converted into their alditol acetates and analyzed by GLC on a J & W (J&W Scientific Inc., Folsom, CA) DB-225 capillary column (15 m x 0.53 mm, 1.0 µm film thickness) in a GC8000 Top gas chromatograph (Thermo Electron Corporation, Milan, Italy). The temperature programme was run from 60 to 180ºC at 20 ºC/min, 180 to 210ºC at 2.5ºC/min and at 210ºC for 6 min. For detection a flame ionization detector was used.

Confirmation of small amounts of GlcN was performed with a GC–MS using a SPB-1701 capillary column (30 m x 0.32 mm, 0.25 µm film thickness; Supelco, Bellefonte, PA) in a gas chromatograph (HP 6890; Hewlett-Packard, Palo Alto, CA) coupled to a mass-selective detector (HP 5973; Hewlett-Packard) and using a HP Chem Station (Hewlett-Packard). The temperature program was run from 80 to 180ºC at 20ºC/min, 180 to 250ºC at 1.5ºC/min and at 250ºC for 3 min (van Hengel et al., 2001). The uronic acid content was determined by the automated colorimetric *m*-hydroxydiphenyl assay (Blumenkrantz and Asboe-Hansen, 1973; Thibault, 1979) using an auto-analyser (Skalar Analytical BV, Breda, The Netherlands). Galacturonic acid was used as a standard.

To discriminate between glucuronic acid and galacturonic acid AGPs were subjected to methanolysis (de Ruiter et al., 1992). AGP aliquots (0.1 mg) were dried at 40ºC under vacuum and hydrolysed with 2M HCl in dry methanol for 16 h at 80ºC. The monomeric sugars were analyzed by High-Performance-Anion-Exchange-Chromatography (HPAEC) using a Thermo-separations HPLC system (Thermo Separations Products Inc., San Jose, CA) equipped with a Dionex ED40 Pulsed Amperometric Detection (Dionex Corporation, Sunnyvale, CA) with a Dionex CarboPac PA10 column and CarboPac PA-1 Guard column as described before (de Ruiter et al., 1992).

The total sugar content is derived from data obtained from the different sugar residues present in the fractions as determined by GLC and HPAEC.

Protein analysis

Total protein content was determined by a semi-automated micro-Kjeldal method (Roozen and van Boxtel, 1979). All nitrogen was assumed to originate from proteins and the conversion factor used was 6.25 x N.

Amino acids were analyzed after hydrolysis of 5 mg AGPs with 6M HCl at 110ºC for 24 h using AOAC (1990) methodology (method no. 982.30). Separation was carried out on an amino acid analyzer with a cation exchange column. Detection of the amino acids was carried out after post-column

derivatization with ninhydrin at 440 and 570 nm. Cysteine, methionine and tryptophan were not included for the analysis. The determination of hydroxyproline was carried out according to the colorimetric method as described in Huszar et al. (1980).

Sugar linkage analysis

Methylation without a carboxyl reduction was performed with dimsyl anion and methyl iodide in DMSO (Sandford and Conrad, 1966). AGPs were hydrolyzed with 2 M TFA for 2 h and the monomers were converted into their partially methylated alditol acetates. The partially methylated alditol acetates were identified by GC–MS as described (Oosterveld et al. 2002). For quantification the samples were run on a GLC using flame ionization detector (FID) detection.

High-performance size-exclusion chromatography

High-performance size-exclusion chromatography (HPSEC) was performed on three columns of Tosoh Bioscience TSK gel in series (G4000 PWXL, G3000 PWXL, G2500 PWXL), in combination with a PWXL-guard column (Tosoh Bioscience Inc., South San Francisco, CA). Elution took place at 30ºC with 0.2M $NaNO_3$ at a flow rate of 0.8 ml/min (Kabel et al., 2002). The column effluent was monitored with a refractive index detector (Spectra System RI 150) (Thermo Electron Corporation, Milan, Italy). Pectins having molecular weight values in the range of 1000–100,000 Da (as determined by viscometry) were used for calibration of the molecular weight. The neutral sugar percentage of the pectins was less than 10mol%.

Preparative size-exclusion chromatography

Preparative size-exclusion chromatography was performed on a Sephacryl S-500 column (100 x 2.6 cm) using a Hiload System (Amersham Biotech) (Oosterveld et al., 2002). AGPs (±110mg) were applied to the column gel and eluted with 50 mM NaAc pH 5.0 at a flow rate of 2.5 mL/min and fractions were collected during 3 h over 180 tubes. The fractions were analyzed for neutral sugar (Tollier and Robin, 1979) and uronic acid (Blumenkrantz and Asboe-Hansen, 1973; Thibault, 1979) using an auto-analyser (Skalar Analytical BV) with arabinose and galacturonic acid as standards, respectively. Proteins were measured spectrophotometrically at 254 nm with a Shimadzu UV1601 spectrophotometer (Shimadzu, Tokyo, Japan). Pooling the content of tubes together created three fractions. The formed fractions were dialyzed and freeze-dried prior to further analysis.

Radial Yariv gel diffusion

An 1% agarose gel with 0.15 M NaCl and 0.03 g/L Yariv phenylglycoside was prepared according to van Holst and Clarke (1985). The wells were filled with AGPs (8 mL of 5 g/L) and incubated overnight at room temperature in a box with high relative humidity to avoid drying of the gel. The Yariv phenylglycoside (1,3,5-tri[4-*ß*-D-glucopyranosyl-oxyphenylazo]-2,4,6-trihydroxybenzene) was prepared as described by Yariv et al. (1962).

Enzymatic degradation

The incubations were performed at 25ºC for 16 h as described by Huisman et al. (1999) and references herein. AGPs at a concentration of 5 g/L in 0.05 M NaAc, pH 5.0 were incubated with *ß*-galactosidase (0.22 µg/mL substrate solution) together with *α*-arabinofuranosidase (0.27 µg/mL substrate solution). Incubations were also performed with endo *α*-1,5-arabinanase (14.6 µg/mL substrate solution). *ß*-galactosidase and *α*-1,5-arabinanase were purified from *Aspergillus aculeatus*, *α*-arabinofuranosidase was purified from *Aspergillus niger*. The amount of enzymes used was calculated to be sufficient for complete substrate digestion in 6 h. After the incubation the AGPs were heated for 10 min at 100ºC to inactivate the enzymes. The AGP digests were analyzed by HPSEC.

Acknowledgements

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Chapter 4

Arabinogalactan proteins isolated from carrot cell culture cell walls and medium show galacturonic acid rich fractions

Peter Immerzeel[1,2], Sacco C. de Vries[1], Henk A. Schols[2], Alphons G. J. Voragen[2]

[1]Laboratory of Biochemistry, Department of Agrotechnology and Food Sciences, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

[2]Laboratory of Food Chemistry, Department of Agrotechnology and Food Sciences, Wageningen University, Bomenweg 2, 6703 HD Wageningen, The Netherlands

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Abstract

Arabinogalactan proteins (AGPs) from medium and cell walls of an embryogenic carrot (*Daucus carota* L.) cell culture were analyzed. The cell walls of the culture were sequentially extracted with water, EDTA buffer solution and a cold sodium hydroxide solution. AGPs were isolated from the cell wall extracts and from the medium with Yariv phenylglycoside and analyzed for molecular weight distribution and carbohydrate composition. Size exclusion chromatography showed that all the Yariv phenylglycoside fractions were composed of two main populations with an estimated molecular weight of 50 kDa and 100 kDa respectively. The carbohydrate composition showed different ratios for arabinose and galactose in the various AGP fractions. The medium AGPs and the cell wall AGPs that were isolated from the chelating agent and sodium hydroxide extracts contained relatively high galacturonic acid (GalA) concentrations of 13 mol%, 17 mol% and 14 mol% respectively. Medium AGPs and EDTA buffer extracted AGPs were further fractionated with copper ions into a GalA rich and a GalA poor fraction. On a Yariv radial diffusion gel both GalA rich and GalA poor fractions formed a halo indicating that both fractions contained AGPs. The GalA rich fractions were incubated with pectin methylesterase and polygalacturonase. This enzyme incubation released GalA fragments from the AGP fractions as monitored by HPAEC and MALDI-TOF MS.

The research described in this chapter suggests that medium AGPs resemble AGPs extracted from cell walls both in size and in sugar composition. Both medium and cell culture cell wall AGPs are covalently linked to pectin containing a homogalacturonan structural element.

Introduction

Under in vitro conditions somatic embryogenesis can be induced from embryogenic cell cultures of various plants (reviewed by Mordhorst et al., 1997; Satoh, 1998). Carrot cell culture media contain secreted proteins that can influence somatic embryogenesis in carrot cultures. One group of molecules that is secreted in the medium of plant cell cultures are AGPs. These proteoglycans have a protein core that contains hydroxyproline and are glycosylated with type II arabinogalactans and arabinosides (Serpe and Nothnagel, 1999). Besides hydroxyproline and proline also serine, threonine and alanine are present in AGPs (Clarke et al., 1979). The carbohydrate part of AGPs varies between 90 and 98% on weight basis (Sommer-Knudsen et al., 1998). Arabinose and galactose are the predominant sugars in

AGPs. Other sugars that have been detected in AGPs include rhamnose, mannose, xylose, glucose, fucose, and uronic acids (Fincher et al., 1983). AGPs show a variety in both carbohydrate and protein composition AGPs have been detected with high amounts of glucuronic acid and low amounts of arabinose (Serpe and Nothnagel, 1995) or hydroxyproline deficient (Baldwin et al., 1993). A characteristic of AGPs is their ability to bind to Yariv phenylglycoside (Nothnagel, 1997). Yariv phenylglycoside is used for detection of AGPs in tissues and isolation of AGPs from liquids. The molecular weight of AGPs shows variation from 60 kDa to 300 kDa but also small arabinogalactan-peptides with a molecular weight of 22 kDa have been detected (Majewska-Sawka and Nothnagel, 2000; Fincher et al., 1974). Some AGPs are known to contain a glycosylphosphatidylinositol (GPI) anchor (Youl at al., 1998). GPI anchors enable AGPs to reside at the outer layer of the plasma membrane and are also found on other proteins (Borner et al., 2003).
With the aid of crossed electrophoresis it was shown that different organs contain different AGPs (van Holst and Clarke, 1986; Cassab, 1986). It was also shown that distinct AGP epitopes occurred at particular stages of development (Knox et al., 1989; Pennell et al., 1991). It was suggested that AGPs could have apparent roles in somatic embryogenesis (Pennell et al., 1992). Later it was shown that when AGPs were added to cell cultures it was possible to increase the number of somatic embryos formed (Kreuger and van Holst, 1993). Other studies have shown that, depending on the type of AGP, somatic embryogenesis can be stimulated or repressed (Toonen et al., 1997). AGPs isolated from carrot seeds as well as from the medium of embryogenic cell cultures were found to be active in promoting the formation of somatic embryos (van Hengel et al., 2001).

Isolation and structural characterization of AGPs from plant cell walls has been conducted for rose cells, styles from tobacco and cabbage leaves (Serpe and Nothnagel, 1999). Structural comparison of rose cell wall AGPs obtained from suspension cultured cells and cell membrane AGPs indicated that some of these molecules were probably related, while other AGPs were restricted to the cell wall (Serpe and Nothnagel, 1999). Sofar, little information is available on the composition of carrot cell wall AGPs. To answer the question whether arabinogalactan proteins that are present in medium and in cell walls of carrot cultured cells are related to each other, different fractions were analyzed and their molecular weight distribution and carbohydrate composition was compared.

Results

The medium of an embryogenic cell culture was ultrafiltrated and Yariv phenylglycoside was used to precipitate the AGPs present in the medium (Table 4.1). The suspension cells from the same culture were collected, freeze-dried, ground and subjected to sequential cell wall extractions.

Table 4.1 Yield of the extracts of carrot cell culture cell walls.
Fractions were obtained with Yariv phenylglycoside precipitation and copper nitrite precipitation. The total amount of material that forms a precipitation with Yariv (Y+) is calculated after the precipitation of a part of the cell wall extract. The percentages of Y+Cu- (Yariv fraction that forms no complex with copper ions) and Y+Cu+ (Yariv fraction that forms a complex with copper ions) are based on the total yield after dialyzing and freeze drying. Nd: not determined. WES: water extractable solids; ChSS: chelating agent soluble solids; DASS: dilute alkali soluble solids. The percentage between brackets shows the total sugar content (w/w) of the different fractions.

Extract	Yield	Y+	Y+Cu-	Y+Cu+
Medium		33 mg	64% (86%)	78% (48%)
Cell wall material	13.0 g			
WES	0.3 g	27 mg	nd	nd
ChSS	0.9 g	44 mg	76% (57%)	68% (45%)
DASS	0.4 g	7 mg	nd	nd
Residue	3.0 g	nd	nd	nd

The total yield of the different AGP preparations as precipitated from the medium, extracted with water (WES), chelating agent (ChSS) and finally with a dilute alkaline solution (DASS) is shown in table 4.1. The total amount of material recovered was 35% based on dry weight of the starting material (13 g). Cell wall extracts were partly used for precipitation with Yariv and the total amount of material that is Yariv positive (Y+) is calculated and shown in table 4.1. After drying and grinding the cells, most soluble AGPs either present in the walls or in transit in the secretory pathway at the time of isolation are expected to be present in the WES fraction. This probably explains the relatively high amount of AGPs in this fraction. Therefore, the AGPs that were extracted from the ChSS and DASS fractions were expected to more closely resemble the medium AGPs in case of a direct continuum between wall and medium. To characterize the AGPs present in the medium and in the different cell wall fractions, the material was analysed with respect to molecular weight distribution and carbohydrate composition. The molecular weight of the Yariv precipitated material was determined with HPSEC (Fig. 4.1). All fractions contain two high molecular

weight peaks (50 kDa and 100 kDa) and a low molecular weight peak that elutes after 31 min.

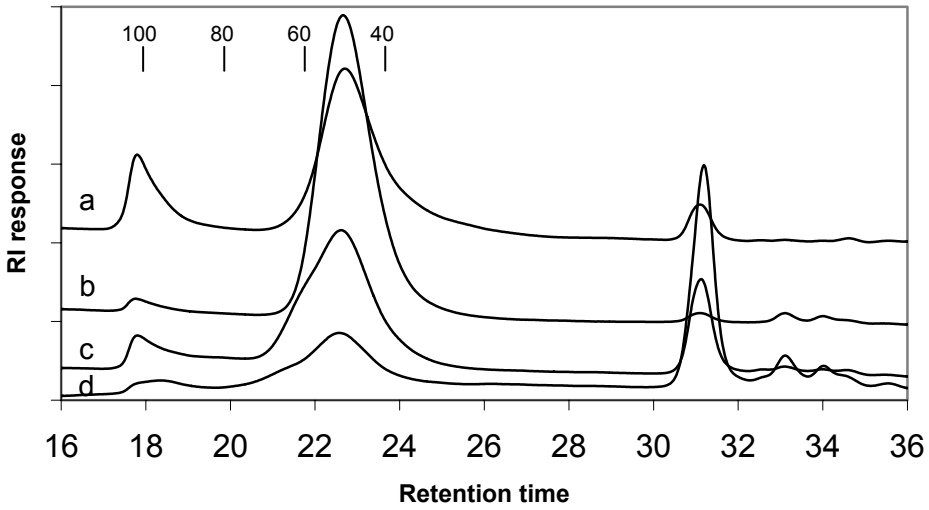

Figure 4.1 HPSEC elution profiles of Yariv precipitated material.
(a) medium; (b) WES fraction; (c) ChSS fraction; (d) DASS fraction. Pectins were used as molecular weight standards (kDa).

In previous work we showed that the two high molecular weight populations present in medium AGPs, contain two chemically different AGP species (Immerzeel et al., 2004). Therefore it is not likely that the 100 kDa population (Fig. 4.1) was identical to dimerization of AGPs from the 50 kDa population. In this study we combined the 50 and the 100 kDa population due to material constraints. The low molecular weight material peak (31 min) was caused by salts that were not removed during dialysis. The HPSEC elution patterns do not indicate substantial variation in the molecular weight of the AGPs from different fractions.

For determining the sugar composition of every fraction, material was hydrolyzed with a water free hydrochloric acid/methanol solution. The analysis of the obtained monomers with HPAEC allows distinction between galacturonic acid and glucuronic acid. The medium AGPs (mediumY+) show a high amount of arabinose and galactose in a ratio of 1:3 (table 4.2). Galacturonic acid, glucuronic acid and rhamnose are present in lower amounts. In contrast, the polysaccharides present in the medium that did not precipitate with Yariv (Medium Y-) show high amounts of glucose, xylose and galactose. Other sugars are present in much lower concentra-

tions. The occurrence of glucose and xylose may indicate the presence of xyloglucans, the principal hemicellulose of the primary cell walls of dicotyledonous plants.

Table 4.2 Sugar composition (mol%) of medium isolated AGPs and fractions from culture cell walls obtained after water extraction (WES), chelating agent extraction (ChSS) and alkaline extraction (DASS). Y+: precipitated with Yariv; Y-: not precipitated with Yariv; tr: trace amounts < 1 mol%

	Sugar composition								
Fraction	Rha	Ara	Xyl	Man	Gal	Glc	GalA	GlcA	total sugar w/w %
MediumY+	6	17	2	1	50	2	13	9	86
MediumY-	2	8	21	4	19	37	6	3	56
WES	3	16	4	5	30	21	11	9	35
WESY+	4	26	tr	tr	57	2	4	7	93
WESY-	3	11	8	7	16	34	12	9	23
ChSS	6	21	4	2	11	4	50	2	53
ChSSY+	4	20	2	3	43	3	17	8	81
ChSSY-	6	20	5	5	7	6	51	0	32
DASS	5	13	6	4	8	14	48	3	13
DASSY+	5	23	2	2	44	3	14	8	49
DASSY-	7	22	9	2	11	9	38	2	26
Residue	5	21	10	2	12	29	20	tr	57

The sugar composition of water extracted material containing AGPs (WESY+) show a high amount of arabinose and galactose in a ratio of 1:2. Other neutral sugars are present at low levels. Glucuronic acid is present at 7 mol% comparable to that of all other Yariv-precipitable fractions. The level of galacturonic acid is low (4 mol%), when compared to the other AGP containing fractions. The sugar compositions of ChSSY+ and DASSY+ show a high similarity. Both fractions contain a high amount of galactose, arabinose and galacturonic acid. The glucuronic acid concentration is 8 mol% and other sugars are present in lower amounts. All Yariv precipitable extracts show high amounts of arabinose and galactose although the ratio was found to be different for medium AGPs (1:3) and cell wall AGPs (1:2). WESY+ contains a low percentage of GalA (4 mol%) where the other AGP fractions show percentages in the range of 13 to 17 mol%. Due to the low level of GalA in WESY+ the relative molecular percentages of arabinose and galactose are relative high when compared to the other three AGP preparations. The residue has high amounts of glucose,

galacturonic acid and arabinose. Polysaccharides that will remain in the residue are cellulose, xyloglucans, other hemicelluloses and pectins that were not extracted with the buffer and alkaline extraction. These results suggest that the sugar composition of AGPs as found in the medium is broadly similar to that found in the cell walls. The main difference appears to be in the total WESY+ fraction mainly due to the low level of GalA.

The fractions containing AGPs from medium and ChSS were further fractionated with the aid of copper ions, a method to distinguish between polymers based on their galacturonic acid content. The WES fraction was not included for further analysis as AGPs from this fraction can originate from the cell wall or the cytoplasm. AGPs extracted from the DASS fraction were also left out for further analysis because of the low amount of material available. The remaining fractions (MediumY+ and ChSSY+) were dissolved in water and precipitated with copper nitrate. The amount of material recovered with the copper is listed in table 4.1. The total percentage of recovered material exceeds 100%, which indicates that the fractions still contain contaminations, presumably in the form of copper ions.

Sugar analysis was performed to determine the carbohydrate composition of the AGP sub-populations. Table 4.3 shows the sugar analysis of AGPs that were isolated from the medium and buffer extracted cell wall material and fractionated with copper.

Table 4.3 Sugar composition (mol%) of medium isolated AGPs and chelating agent extraction (ChSS) containing AGPs. Y+Cu-: precipitated with Yariv, not with copper; Y+Cu+: precipitated with Yariv and with copper; tr: trace amounts < 1 mol%

	Sugar composition								
Fraction	Rha	Ara	Xyl	Man	Gal	Glc	GalA	GlcA	total sugar w/w %
MediumY+Cu-	5	23	2	1	55	tr	5	9	86
MediumY+Cu+	7	7	3	4	34	2	30	13	48
ChSSY+Cu-	4	28	tr	tr	53	2	7	7	57
ChSSY+Cu+	6	15	4	3	36	4	26	7	45

The table shows a clear difference in the levels of galacturonic acid as present in the material that did not precipitate with copper and the material that did. The neutral sugar composition of MediumY+Cu- AGPs shows high amounts of galactose and arabinose in a ratio of about 1:2. Other sugars are present at lower levels. The

fraction that precipitated with copper (MediumY+Cu+) has a galactose:arabinose ratio of 1:5. Other neutral sugars are present in somewhat higher concentrations. In the AGPs that were purified from the ChSS fraction also a different sugar composition was observed for the copper positive and the copper-negative fraction. The fraction ChSSY+Cu- shows a high amount of galactose and arabinose with a ratio of 1:2. Galacturonic acid and glucuronic acid are both present at 7 mol%. Neutral sugars are present at low or trace amounts. The fraction that did precipitate with copper shows a four times higher GalA concentration (26 mol%) when compared to the copper-negative fraction. The glucuronic acid concentrations of both fractions are 7 mol%. Galactose and arabinose are the predominant neutral sugars in a ratio of 1:3. The other neutral sugars have a higher concentration when compared to the ChSS AGPs that did not precipitate with copper. Figure 4.2 shows the elution profiles of medium AGPs before and after the precipitation with copper.

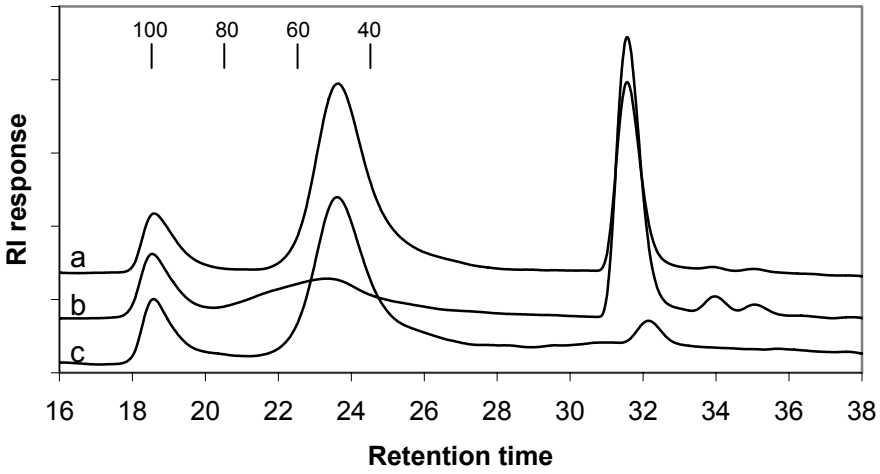

Figure 4.2 HPSEC elution profiles of medium AGPs before and after copper precipitation. (a) Copper negative; (b) copper positive; (c) untreated material. Pectins were used as molecular weight standards (kDa).

The elution profiles of the crude AGP extract and the material that did not precipitate with copper show the same profile (Fig. 4.2). The material that did precipitate with copper ions shows a peak with a mass of 100 kDa and a population having a broad distribution (40 kDa - 80 kDa) (Fig. 4.2). The 100 kDa population from the crude AGP extract as shown in figure 4.2 has a copper sensitive population and a population that does not precipitate. Figure 4.3 shows the elution profiles of ChSS AGPs before and after the precipitation with copper.

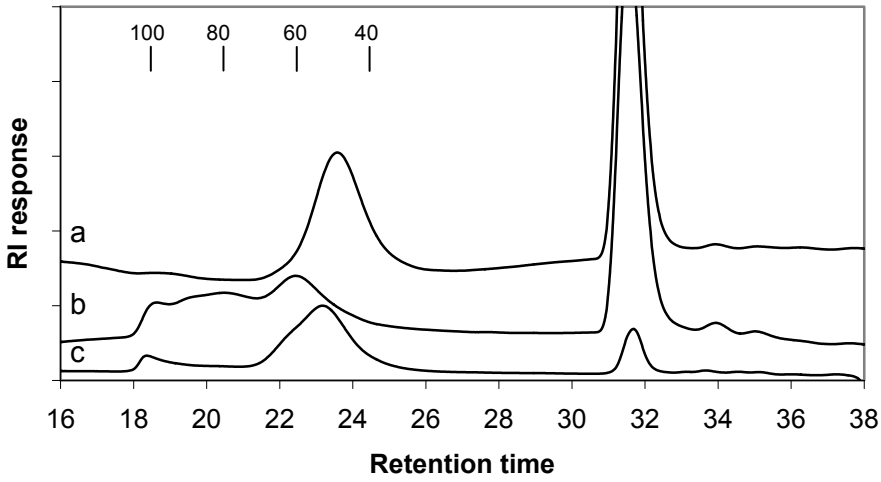

Figure 4.3 HPSEC elution profiles of ChSS AGPs before and after copper precipitation. (a) Copper negative; (b) copper positive; (c) untreated material. Pectins were used as molecular weight standards (kDa).

The profile shows that material that did not precipitate with copper eluted in a single population with an estimated molecular weight of 50 kDa (Fig. 4.3). The material that did precipitate with copper shows a broad molecular weight distribution from 60 kDa till 100 kDa (Fig. 4.3). We therefore conclude that GalA rich AGPs have a broad molecular weight distribution and that GalA poor AGPs are restricted to a relative low molecular weight population (Fig. 4.3) or to two distinct populations (Fig. 4.2).
To confirm that both copper negative and -positive fractions contained AGP molecules after the precipitation with copper, a Yariv phenylglycoside gel was prepared. Figure 4.4 shows the Yariv radial diffusion gel of medium and ChSS isolated AGPs.

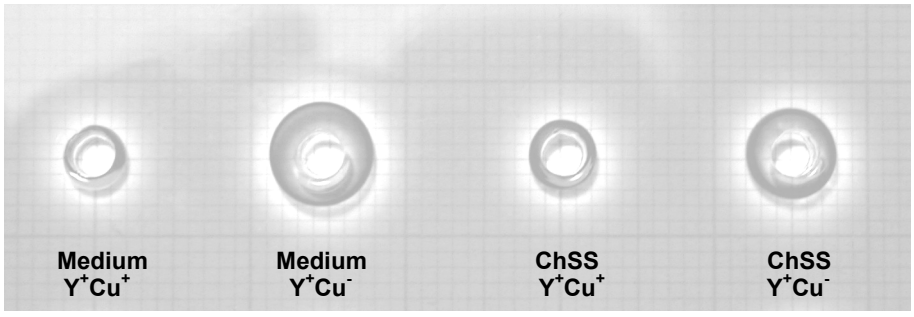

Figure 4.4 Yariv radial diffusion gel for medium and ChSS AGPs after copper fractionation.

All fractions were positive in this Yariv test as shown by the halos formed. This means that the Y+Cu+ fractions that contain a high percentage of galacturonic acid indeed still consist of AGPs. The presence of GalA in AGP fractions is of high interest as GalA is known to be a major sugar in pectins and interaction of AGPs with pectins has been suggested to occur (Morvan et al., 2003). This would imply that the copper positive AGP fraction would consist of AGPs linked to pectin. An alternative hypothesis is that GalA residues are not present as part of pectins but scattered on the AGP carbohydrate moiety as is suggested for glucuronic acid (reviewed by Nothnagel, 1997).When pectic structures would be attached to AGPs they should be sensitive to degradation by pectin hydrolases such as polygalacturonases. Therefore the Y+Cu+ fractions were incubated with a combination of pectin methylesterase and polygalacturonase. Pectin methylesterase releases methanol from the galacturonic acid residues. Homogalacturonan can be hydrolysed by polygalacturonase at non-esterified regions and the hydrolysis results in the formation of galacturonic acid monomers, but also dimers and trimers can be formed.

The effect of these two enzymes was monitored by HPSEC after 4 and 21 hours. The elution profiles after 4 and 21 hours show the same elution pattern and therefore the degradation was considered as complete. Figure 4.5 shows the elution pattern before and after 21 hours of incubation with pectin degrading enzymes of medium AGPs.

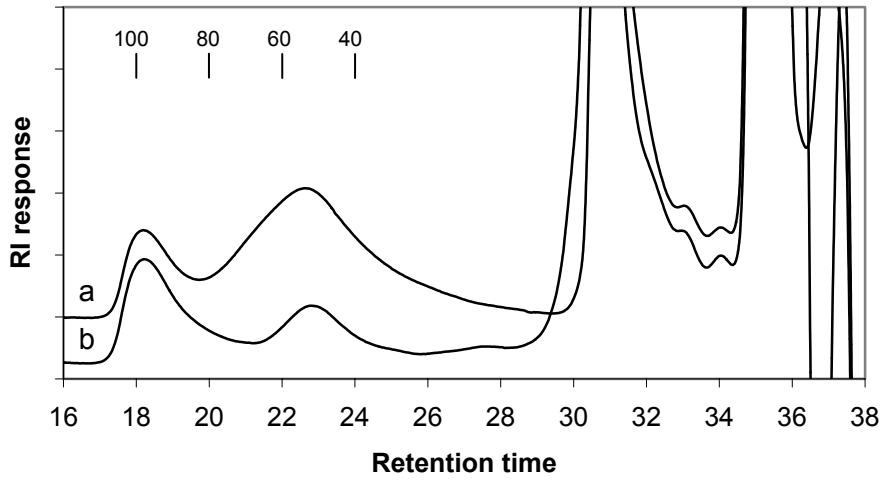

Figure 4.5 HPSEC elution profiles of MediumY+Cu+ AGPs before (a) and after (b) enzyme incubation with pectin methylesterase and polygalacturonase. Pectins were used as molecular weight standards (kDa).

The material with a molecular mass of 100 kDa did not seem to be affected by the enzyme treatment. AGPs with a molecular weight in the range of 40 kDa till 80 kDa were polygalacturonase and pectin methylesterase sensitive. The ChSSY+Cu+ fraction showed, after enzyme incubation, a reduction of material with a molecular weight of 70 kDa to 90 kDa as depicted on figure 4.6. Two fractions remain with a molecular weight of 60 kDa and 100 kDa. In both HPSEC elution profiles peaks are visible with a low molecular weight (retention time around 30 min). The buffer in which the AGPs were dissolved and also the degradation products formed are responsible for these peaks.

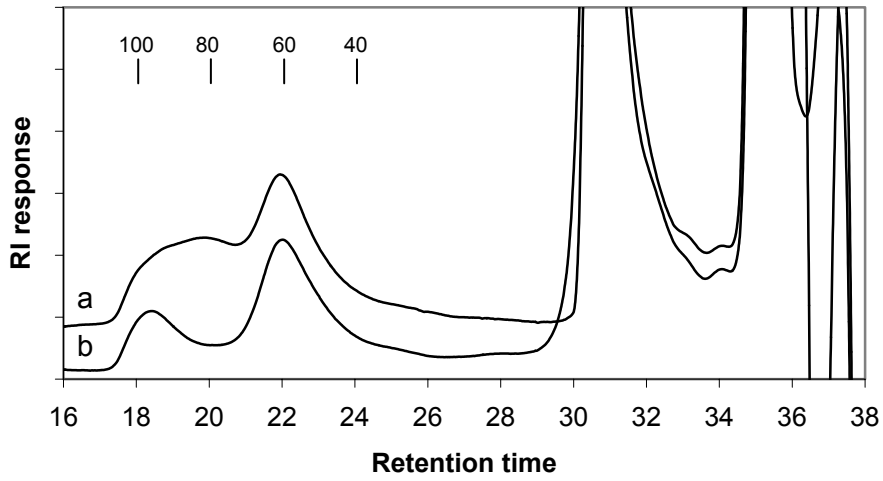

Figure 4.6 HPSEC elution profiles of ChSSY+Cu+ AGPs before (a) and after (b) enzyme incubation with pectin methylesterase and polygalacturonase. Pectins were used as molecular weight standards (kDa).

The pectin hydrolyzing enzymes were not able to completely degrade the copper positive fractions. Polygalacturonase needs at least four contiguous unmethylated GalA residues for hydrolytic activity. It is possible that the GalA residues in AGPs with different molecular weight are organized in homogalacturonan with a different pattern in methylesterification. To avoid activity repression of polygalacturonase by methylesters, pectin methylesterase was included in the incubation. The amount of enzymes used was calculated to be sufficient for complete demethylation. However it might be possible that not all methyl groups were removed due to sterical hindrance of the pectin methylesterase causing differences in the activity of polygalacturonase. From this point of view the GalA residues in the population of medium AGPs (ca. 50

kDa) and the population of ChSS AGPs with a molecular weight of ca. 80 kDa contained a lower level of methylesters. However the degree of methylation before and after the enzyme incubation is not known.

The enzyme incubated AGP fractions were analyzed on an anion exchange column to confirm the presence of GalA residues and to determine the degree of polymerization of the hydrolysis products. Figure 4.7 shows the presence of GalA residues with different degrees of polymerisation for both AGP isolates. The HPAEC elution patterns from the analysis show for both AGPs material that elutes between 2 and 7 minutes that can be considered as contaminants.

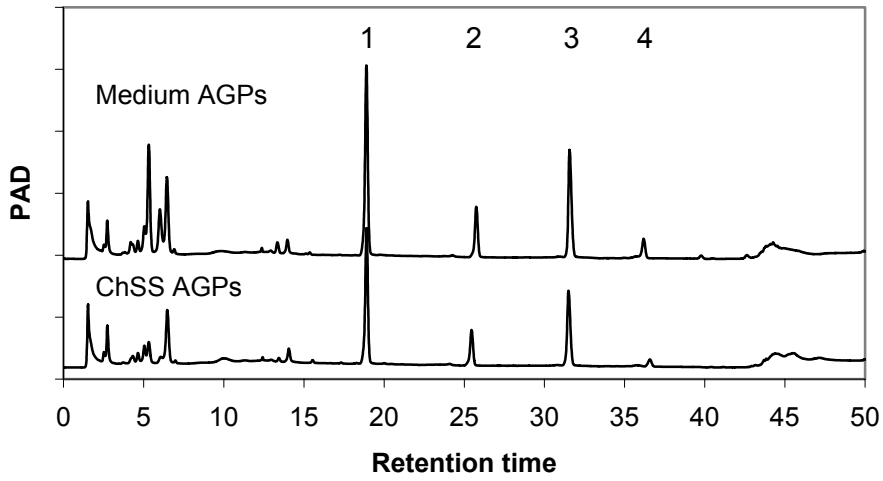

Figure 4.7 HPAEC profiles of degradation products from medium AGPs and ChSS AGPs. (1) $(GalA)_1$; (2) $(GalA)_2$; (3) $(GalA)_3$; (4) $(GalA)_4$.

The presence of GalA dimers, -trimers and -tetramers in the enzyme incubated material was confirmed by MALDI-TOF MS analysis (data not shown).

Discussion

The AGPs present in medium and different cell wall extracts all showed two high molecular weight fractions. Total carbohydrate analysis of the AGP fractions showed a high arabinose and galactose content for all fractions, which is characteristic for AGPs (Bacic et al., 1987). AGPs that were isolated with water from the cell wall material showed higher amounts of arabinose and galactose due to a low amount of galacturonic acid present. The purification of AGPs present in medium and the ChSS cell wall extract led to the discovery of galacturonic acid rich AGP fractions.

Galacturonic acid containing AGPs have been reported before (reviewed by Nothnagel, 1997) but the purification of AGPs with copper ions in this study revealed a very high amount of galacturonic acid. In contrast to galacturonic acid, glucuronic acid has been detected in high amounts in rose cell culture AGPs (Serpe and Nothnagel, 1995). Glucuronic acid (GlcA) is known to occur as terminal residues at the side branches of type II AGs or as single residues within the side chain of type II AGs (Fig. 4.8). However no chemical structures concerning the organization of high amounts of GalA in AGPs are currently available. The use of pectin methylesterase and polygalacturonase showed that GalA is present as homogalacturonan elements in medium and ChSS isolated AGPs. Homogalacturonan is a well-known pectin component. The linkage between AGPs and pectins has been suggested before (Baldwin et al., 1993; Oosterveld et al., 2002). At this moment it is not clear how the linkage is accomplished and which part of the AGPs and pectin are involved for the linkage. Due to the isolation procedure used, ionic interaction between AGPs and pectins seems unlikely.

Figure 4.8 Oligosaccharides characterized from the ß-(1-6) galactan side chain of type II AGs (reviewed by Nothnagel, 1997).

α-L-Ara*f*-(1-3)-*ß*-D-Gal*p*-(1-

α-L-Ara*f*-(1-3)-α-L-Ara*f*-(1-3)-*ß*-D-Gal*p*-(1-

ß-D-(4-*O*-methyl-Glc*p*A)-(1-6)-*ß*-D-Gal*p*-(1-6)-*ß*-D-Gal*p*-(1-6)-*ß*-D-Gal*p*-(1-6)-D-Gal

ß-D-(4-*O*-methyl-Glc*p*A)-(1-6)[*α*-L-Ara*f*-(1-3)]-*ß*-D-Gal*p*-(1-6)-D-Gal

α-L-Rha*p*-(1-4)-*ß*-D-Glc*p*A-(1-6)-*ß*-D-Gal*p*-(1-

α-L-Fuc*p*-(1-2)-*α*-L-Ara*f*-(1-

The high amount of galacturonic acid favors an AGP-pectin linkage in which homogalacturonan is involved. However, the sugar composition of both medium and ChSS AGPs also shows the presence of rhamnose (Table 4.3). The presence of rhamnose indicates that also other pectin structures can be present such as rhamno-galacturonan I.

In the cell all polysaccharides, except cellulose, are synthesized in the Golgi apparatus and transported to the cell wall by the secretory pathway (Gibeaut and Carpita, 1994). AGPs and other proteoglycans are believed to be synthesized at the

rough endoplasmatic reticulum and Golgi apparatus before secretion to the cell wall by vesicles (reviewed by Nothnagel, 1997). The secretion of material to the outside of the plasma membrane by exocytosis is in contrast to the formation of cellulose polymers that are produced at the plasma membrane (Delmer and Amor, 1995). It is not known if AGPs and pectins are transported to the plasma membrane in the same vesicles. If this would occur it might even be envisaged that linkage of AGPs and pectins already occurs in these vesicles rather than in the cell wall.

The conditions under which the pectin containing AGPs were formed were artificial as the cells were growing in culture medium. Carrot tap roots will be isolated to isolate AGPs. When carrot tap roots also contain high levels of galacturonic acid it can be excluded that the detection of galacturonic acid is due to in vitro growth conditions of the cells.

Materials and methods

Plant material and the isolation of cell wall material

Carrot (*Daucus carota* L. cv Autumn King/Trophy, Syngenta Seeds BV, Enkhuizen, The Netherlands) cell cultures were maintained as described in de Vries et al. (1988). One-week-old suspension cultures grown in 500 mL of Gamborg's B5 medium, containing 0.2 µM 2,4-dichlorophenoxyacetic acid were used as a source for AGPs. Suspension cells were collected by filtration. The medium was centrifuged at 13,900 x *g* for 20 min, ultrafiltrated with a Millipore Bio-A filter (10 kDa cut-off) and the retentate was stored at -20°C before isolation of AGPs. The suspension cells were freeze dried and ground in a mortar before isolation of cell wall material. Water extractable solids (WES) of the cell wall material were obtained after two extractions of one hour with 300 mL deionized water at 4°C. The water insoluble solids were removed from the extract by filtration and centrifugation (19,000 *x g* for 30 min at 4°C). The two extracts were combined. The water insoluble solids were resuspended in 300 mL EDTA buffer (50 mM EDTA, ammonium oxalate 50 mM, sodium acetate 50 mM, pH 5.2) and stirred at 70°C for 2 hours to obtain the Chelating agent Soluble Solids (ChSS) fraction (de Vries et al., 1981). The second EDTA extraction was performed in 300 mL buffer under the same conditions. The two ChSS extracts were pooled and dialysed. The pellet was washed twice with water for 30 min at 4°C to remove the buffer. The water was discharged. The Dilute Alkali Soluble Solids (DASS) were obtained by isolating the cell wall material two times with 300 mL sodium hydroxide (50 mM) for 1 h at 4°C. Before the extract was dialyzed, the pH was adjusted to pH 7.4 with acetic acid. The residue was neutralised (pH 5.9) and dialyzed. All extracts and the residue were freeze-dried prior to further analysis.

Isolation of arabinogalactan proteins with Yariv phenylglycoside and Cu^{2+}-ions

Yariv phenylglycoside was synthesized as described by Yariv et al. (1962). The AGPs from the culture medium were precipitated by adding 100 mg Yariv phenylglycoside to 1 L medium and 0.15 M NaCl (Kreuger and van Holst, 1993). The Yariv-AGP complex was precipitated at 4°C overnight, centrifuged at 13,900 x *g* for 20 min, washed 3 times with 0.15 M NaCl and dissolved in water. In order to remove the Yariv phenylglycoside from the AGPs, $Na_2S_2O_4$ was added to degrade the Yariv molecules. The solution was heated to 50°C until the red colour disappeared, dialysed extensively against deionized water at 4°C and freeze-dried.

The fractions WES (150 mg), ChSS (300 mg) and DASS (150 mg) were dissolved to a 0.5 mg/mL solution with deionized water. NaCl was added to a final concentration of 0.15 M. Precipitation of AGPs was accomplished by adding Yariv phenylglycoside to the solutions (40 mg to WES and DASS; 80 mg to ChSS). The AGP-Yariv complex was precipitated at 4°C overnight, centrifuged at 13,900 x *g* for 20 min. The supernatant was kept aside (Yariv negative material: Y-) and the pellet was washed three times with 0.15 M NaCl and dissolved in water (Yariv positive material: Y+). The Y+ and Y- fractions were purified from the Yariv phenylglycoside as described for the medium AGPs.

Yariv positive material (Y+) (25 mg) was dissolved in 5 mL water and treated with aqueous copper nitrate (7% solution) for 1 h and centrifuged (12,000 x *g* for 15 min at 20°C) to precipitate pectic material (Renard et al., 1997). The pellet contains Yariv positive material together with GalA-rich material (Y+Cu+); the supernatant contains Yariv positive material without GalA-rich material (Y+Cu-). Ethylenediamine tetra-acetic acid (EDTA) (0.07 M, pH 5) was added to the fractions to remove the copper ions. The fractions were first dialysed against EDTA and sodium acetate and thereafter with deionized water.

Analytical methods

Sugar analysis was performed with methanolysis (de Ruiter et al., 1992). Fractions (0.1 mg) were dried at 40°C under vacuum and hydrolysed with 2 M HCl in dry methanol for 16 h at 80°C. The methylgroup was removed from the sugars with 2 M trifluoroacetic acid (TFA). The monomeric sugars were analysed by high-performance anion-exchange chromatography (HPAEC) on a Thermo Separations HPLC system (Thermo Separations Products, Inc, San Jose, CA) equipped with a Dionex CarboPac PA-10 column (Dionex Corporation, Sunnyvale, CA), a CarboPac Guard column and a Dionex borate trap. Detection was performed with a Dionex ED40 Pulsed Amperometric Detector (PAD). Sugars were separated using the following gradient (1 mL/min): 0 min: 15 mM NaOH; 45 min: 15 mM NaOH; 75 min 800 mM NaOH; 80 min 800 mM NaOH; 81 min 15 mM NaOH; 100 min 15 mM NaOH. The total sugar content is derived from data obtained from the different sugar residues present in the fractions as determined by HPAEC.

Enzymatic degradation of AGPs

Fractions (5 mg/mL) were incubated with pectin methylesterase and polygalacturonase. Pectin esterase was cloned and purified from *Aspergillus niger* and polygalacturonase was cloned and purified from *Kluyveromyces fragiles*. The incubation with purified enzymes took place in sodium acetate buffer (50 mM, pH 5.0) for 21 hours at 30 °C (Vierhuis et al, 2003) and the reaction vials were

rotated 'head over tail'. The enzymes were inactivated by heating for 10 minutes at 100°C. The amount of enzymes used was calculated on basis of the activity of the enzyme and the amount of galacturonic acid present in the fraction. With HPSEC the process of the enzyme incubation was monitored after 4 and 21 hours. All digests were analyzed by HPAEC and MALDI-TOF MS.

High-Performance Size-Exclusion Chromatography (HPSEC)

HPSEC was performed on three Tosoh Bioscience TSK PWXL gel columns in series (G4000, G3000, G2500) (Tosoh Bioscience, Inc, South San Francisco, CA), in combination with a PWXL-guard column (Kabel et al., 2002). Elution took place at 30 °C with 0.2 M $NaNO_3$ at a flow rate of 0.8 mL/min. The column effluent was monitored using a Finnigan Spectrasystem RI 150 detector (Thermo Electron Corporation, Milan, Italy). Pectins having molecular weight values in the range of 1,000 – 100,000 Da (as determined by viscometry) were used for calibration of the columns. The neutral sugar percentage of the pectins was less than 10 mol%.

High-Performance Anion-Exchange Chromatography (HPAEC)

HPAEC was used to analyse the breakdown products after enzyme treatment on a Thermo Separations HPLC system with a Dionex CarboPac PA-1 column and a Dionex CarboPac Guard column. Breakdown products were separated using the following gradient (1 mL/min): 0 min: 0.1 M NaOH; 5 min: 0.1 M NaOH; 40 min: 0.1 M NaOH, 0.5 M NaAc; 45 min: 0.1 M NaOH, 1 M NaAc; 50 min: 0.1 M NaOH, 1 M NaAc; 51 min: 0.1 M NaOH; 65 min: 0.1 M NaOH . The eluate was monitored using PAD detection.

MALDI-TOF MS

For MALDI-TOF MS analysis, the fraction was desalted and 1 µL of the fraction was transferred to a MALDI sample plate and mixed with 1 µL of matrix and dried at room temperature. The matrix solution was prepared by dissolving 9 mg 2,5-dihydroxybenzoic acid in 1 mL water. Mass spectra were obtained from all fractions using a Bruker Ultraflex workstation. The mass spectrometer was calibrated with a mixture of maltodextrines (Kabel et al., 2002).

Yariv radial diffusion gel

An 1% agarose gel with 0.15 M NaCl and 0.03 g/L Yariv phenylglycoside was prepared according to van Holst and Clarke (1985). The wells were filled with extracted material (8 µL of 5 g/L) and incubated overnight at room temperature in a box with high relative humidity to avoid drying of the gel. The Yariv phenylglycoside (1,3,5-tris[4-*ß*-D-glucopyranosyl-oxyphenylazo]-2,4,6-trihydroxybenzene) was prepared as described by Yariv et al. (1962).

Chapter 5

Arabinogalactan proteins from carrot tap root cell walls are linked to pectin

Peter Immerzeel[1,2], Mieke M. Eppink, Sacco C. de Vries[1], Henk A. Schols[2], Alphons G. J. Voragen[2]

[1]Laboratory of Biochemistry, Department of Agrotechnology and Food Sciences, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

[2]Laboratory of Food Chemistry, Department of Agrotechnology and Food Sciences, Wageningen University, Bomenweg 2, 6703 HD Wageningen, The Netherlands

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Abstract

Tap roots of carrot were ground and pectins and other polysaccharides sequentially extracted with water, EDTA buffer solution and a cold sodium hydroxide solution. Arabinogalactan proteins (AGPs) were isolated from these extracts by precipitation with Yariv phenylglycoside. Copper ions were used to separate the Yariv fractions obtained into AGP fractions with a high and a low level of galacturonic acid (GalA). All fractions were analyzed to determine their sugar composition and molecular weight distribution. Incubation of the EDTA isolated GalA rich AGP fraction with pectin methylesterase and polygalacturonase resulted in the formation of galacturonic acid monomers, dimers and trimers. This finding shows that a small fraction of carrot tap root cell wall AGPs is linked to homogalacturonan containing pectins.

Introduction

Arabinogalactan proteins (AGPs) are proteoglycans and members of a large family of hydroxyproline rich glycoproteins (HRGPs) (Zhang et al., 2003). The total carbohydrate part represents often more than 90 percent of the molecule on weight basis (Majewska-Sawka and Nothnagel, 2000). Arabinose and galactose are the predominant sugar residues present. In many AGPs the protein core contains high amounts of the amino acids hydroxyproline, serine, threonine and alanine (Majewska-Sawka and Nothnagel, 2000). The large, branched and heterogeneous patterns of glycosylation make it difficult to purify an AGP fraction with identical protein and carbohydrate composition, essential for determining the structure. AGPs can be specifically precipitated with ß-glucosyl Yariv phenylglycoside (reviewed by Nothnagel, 1997). AGPs are widely distributed in the plant kingdom and they can be found in all plant tissues (Serpe and Nothnagel, 1995). At the cellular level AGPs are mainly located at the plasma membrane and in the cell wall (Serpe and Nothnagel, 1999). Some AGPs contain glycosylphosphatidylinositol (GPI) anchors that keep AGPs in the outer layer of the plasma membrane, exposed to the cell wall (reviewed by Baluška et al., 2003). Large amounts of AGPs are found in the medium of cell cultures. There are indications that AGPs have a role in plant growth and developmental processes but the underlying molecular mechanisms are not known (Showalter, 2001). AGPs are thought to be involved in cell adhesion (Cosgrove, 1997) and might be functioning as cell surface extracellular matrix binding proteins (Pennell et al., 1989). Identification of AGP interacting molecules may help clarify the function of AGPs as suggested by Nothnagel (1997). Addition of Yariv to intact plants

or cells has been shown to interfere with cell division in cell cultures, inhibition of carrot root growth and inhibition of lily pollen tube growth (Zhang et al., 2003).
Because AGPs are precipitating with Yariv phenylglycoside, it has been suggested that there exists a natural interactive partner for AGPs (Serpe and Nothnagel, 1995). Numerous reports showed that AGPs were present in cell wall preparations (Kohorn, 2000) indicating that the structure of AGPs has great potential to bind to other components of the cell wall. Pectins are often proposed as AGP interacting cell wall polymers.

Pectin is made up of a group of pectic polysaccharides rich in galacturonic acid, rhamnose, arabinose and galactose and 13 other monosaccharides in lower amounts (Vincken et al., 2003). Pectins are localized in the middle lamella and primary cell wall and are, like AGPs, branched molecules. Pectin may have homogalacturonan (HG) structural elements and regions where the alternating sequence of a galacturonic acid residue and a rhamnose residue form repeating disaccharides (rhamnogalacturonan I). Different side branches can be present on rhamnogalacturonan I in pectins such as arabinans, galactans and type I arabinogalactans (AGs). In type I AGs the galactose is *ß*-(1-4) linked whereas in type II AGs the galactose chains consist of *ß*-(1-3) linked galactose with side chains at the C(6) position (Serpe and Nothnagel, 1999). Type II AGs are known to be a distinctive part of arabinogalactan proteins. Type II AG linkages have been detected in pectin extracts but it is not clear if these polymers are linked to pectin or that AGPs were co-extracted with the pectin (Vincken et al., 2003). Another structural element of pectin is rhamnogalacturonan II that has a highly branched structure. It contains the same homopolymer backbone as the homogalacturonan but it is substituted with 10 different glycosyl residues and among them are some very rare sugars (Mollet et al., 2000). Enzyme studies with pectin methyl esterase and polygalacturonase revealed that rhamnogalacturonan II may be attached to homogalacturonan. Another structural element of pectins is xylogalacturonan that consists of a branched galacturonan with xylose side chains (Vincken et al., 2003). Much of the structural elements of pectins are known but to date no complete structure of pectin is available (Vincken et al., 2003).
When pectins were isolated from cell wall material there were indications that AGPs might be included as well. These indications were based on AGP-specific carbohydrates and proteins that were found in pectin extracts. Baldwin et al. (1993)

mentioned that pectic fractions often contained strongly bound AGPs as detected by monoclonal antibodies. Oosterveld et al. (2002) found an AGP containing pectin fraction extracted from hop. Enzyme studies on this fraction suggested a possible linkage of AGPs and pectin although no proof for the type of linkage was presented. Redgwell et al. (2002) isolated a type II AG rich fraction from coffee beans. This fraction also contained rhamnogalacturonan and galacturonic acid and Redgwell et al. (2002) speculated that the AG was part of an AGP that might be linked to pectin. Hairy regions from pectin isolated from the pulp of grape berries contained type II arabinogalactans (Saulnier and Brillouet, 1988, and references therein). Besides the finding of AGP-specific carbohydrates, research on pectin also resulted in some cases in the isolation of pectic fractions that contained proteins (Rombouts and Thibault, 1986; Schols and Voragen, 1994). However, the proteins in these pectin fractions were sometimes regarded as contaminants and were not further investigated in detail. From another point of view, when AGPs were precipitated with Yariv phenylglycoside, sometimes pectin specific carbohydrates were found in the extract (Serpe and Nothnagel, 1995). AGPs from red wine were purified and fractionated and two AGP fractions showed glucuronic- and galacturonic acid in association with 2- and 2,4- linked rhamnose indicating the presence of AG-rhamnogalacturonan fragments (Pellerin et al., 1995). It was further suggested that complexes of AGPs and rhamnogalacturonan were present in cabbage leaf extracts (Kido et al., 1996), although no further details were presented on the nature of the linkage between AGPs and pectic structural elements.

In many publications speculations can be found on the occurrence of an AGP-pectin interaction, but details on the linkage between AGPs and pectins are scarce. One of the possible candidates for linkage is ionic interaction. Dot blotting experiments were performed in which cell wall polysaccharide samples were dried onto nitrocellulose and subsequently incubated with purified soluble AGPs. These experiments showed that carrot AGP exhibited calcium dependent binding to a pectin fraction (Baldwin et al., 1993). An AGP fraction isolated from carrot cell cultures (DcAGP1) contained a basic histidine rich domain and has pectin binding properties as proposed by Baldwin et al. (2001). There are also suggestions that AGPs are able to form covalent linkages with other cell wall polymers. For instance Kwan and Morvan (1995) describe ether linkages between polymers of type II AGs and ß-(1,4) xylans. The AG II chain can be linked with the reducing end to the protein part of the AGP or to the carbohydrate part of the pectin. It might be possible that the AG II chain of AGPs is

linked to the pectic galacturonic acid by ester linkages but at this moment this type of interaction is not proven.

More knowledge about the interaction of AGPs with pectins will help to understand the chemical compositions found in AGP and pectin extracts. It might also give further insight into the biological function of AGPs. During previous work (chapter 4) GalA rich AGP fractions from carrot cell culture cell walls and medium were identified. In the present study cell wall material from carrot tap roots was isolated and fractionated by Yariv phenylglycoside- and copper precipitation to determine that GalA rich AGP fractions occur in intact plant tissues and are not restricted to tissue cultured material.

Results

The dry matter content of the carrot tap roots was 9.1%. Alcohol insoluble solids (AIS) were isolated from the roots and 30.7% of the ground carrot was recovered, based on the dry weight content of the carrot (Table 5.1). This means that the weight loss of nearly 70% was caused by the removal of low molecular weight material. When the yield of the AIS fraction is calculated on fresh weight, the yield is 27.9 g/kg. This value is within the range of other values found in the literature for carrot tap roots: 24 g/kg (Voragen et al., 1983; Massiot et al., 1988) and 35 g/kg fresh weight (Ng et al., 1998). Table 5.1 shows the yields of the extracts after grinding carrot tap roots and the sequential extraction with water (WES), EDTA buffer (ChSS), a sodium hydroxide solution (DASS) and the residue. The highest yield of cell wall extractable material was obtained with EDTA buffer (7.9% carrot dry weight) whereas the WES and DASS fractions represent 0.3% and 1.7% respectively. The residue is expected to contain high amounts of cellulose and makes up 12.5% of the total dry weight. The total recovery (WES, ChSS, DASS and the residue) is 22.4%. The difference between the total recovery and the AIS fraction is probably caused by losses due to the extra steps in the extraction procedure. After dialysis and freeze drying the extracts were re-dissolved in water to allow precipitation with Yariv phenylglycoside that specifically binds to AGPs (Nothnagel, 1997). A high amount of the WES fraction (65% w/w) can be precipitated with Yariv phenylglycoside. In the ChSS fraction a lower percentage was isolated with Yariv phenylglycoside (12%). The DASS fraction contains a low amount of Yariv extractable material (2%). The highest total amount of Yariv positive material was found in the ChSS fraction.

Table 5.1 Yield of the extractions of carrot taproot cell walls (based on 100 g dry weight).
Dry matter content: 9.1%. Fractions were obtained after precipitation with Yariv phenylglycoside and copper ions. Percentages based on w/w. Y+: percentage of the yield fraction that forms a precipitation with Yariv; Y+Cu-: percentage of the Yariv precipitate that forms no pellet with copper; Y+Cu+: percentage of the Yariv precipitate that also forms a pellet with copper; the percentage between brackets shows the total sugar content (w/w) from the different fractions. nd: not determined

Extract	Yield	Y+	Y+Cu-	Y+Cu+
AIS	30.7%	nd	nd	nd
WES	0.3%	65%	95% (44%)	43% (9%)
ChSS	7.9%	12%	84% (57%)	15% (62%)
DASS	1.7%	2%	nd	nd
Residue	12.5%	nd	nd	nd

Copper ions are known to precipitate with pectic material (Kravtchenko et al., 1992; Renard et al.,1997) and form a stronger complex when compared to calcium ions. Copper nitrite was used to separate the Yariv extracts into a fraction with a high and with a low amount of galacturonic acid. Table 5.1 shows the weight percentages of Yariv positive material after copper treatment that was present in the supernatant (Y+Cu-) or the pellet (Y+Cu+) as based on the yields after dialysis and freeze-drying. The WES isolates were probably not completely desalted as expressed by the high values of the recovered fractions. The assumption that some copper ions were still present was confirmed by the low total sugar concentration data for WESY+Cu+ (9% w/w). These results indicate that the WES AGP fraction with a high amount of uronic acids (WESY+Cu+) is a rather small fraction. Most of the AGPs that were isolated with water do not have interaction with copper. Material that was isolated from the cell walls with EDTA buffer (ChSS) shows precipitation with copper ions although the highest percentage was present in the supernatant. The total sugar content for ChSSY+Cu- and ChSSY+Cu+ (Table 5.1) indicates that these fractions were better desalted than the WESY+Cu+ fraction. The Yariv extractable material from DASS was a minor fraction (2% w/w) and has been excluded from further separation due to the limiting amounts available.

The fractions were separated on size exclusion columns to obtain the molecular weight distribution of the different extracts. Figure 5.1 shows the elution pattern of the WES extract and the different sub-fractions. The WES extract contained mainly AGPs that did not precipitate with copper ions.

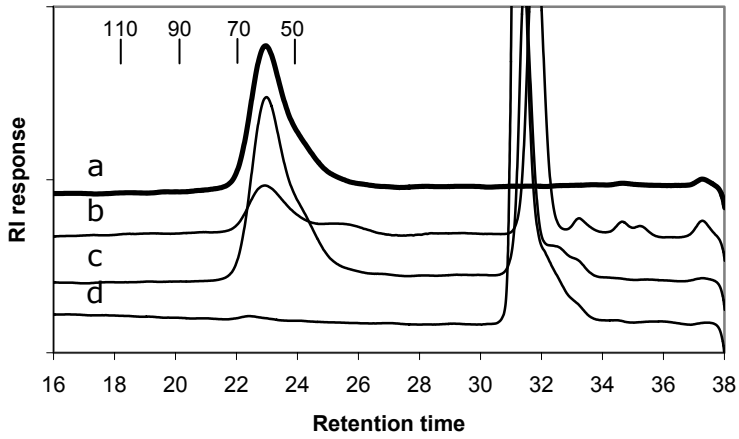

Figure 5.1 HPSEC chromatograms of (a) WES extract, (b) WESY-, (c) WESY+Cu-, (d) WESY+Cu+ . Pectin was used as a molecular weight standard (kDa).

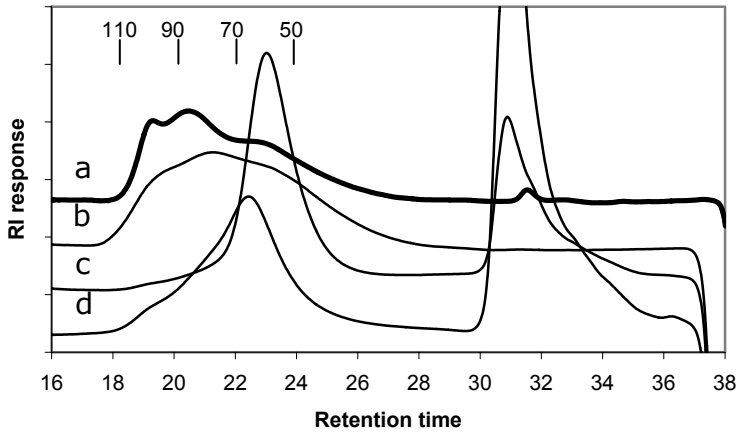

Figure 5.2 HPSEC chromatograms of (a) ChSS extract, (b) ChSSY-, (c) ChSSY+Cu-, (d) ChSSY+Cu+. Pectin was used as a molecular weight standard (kDa).

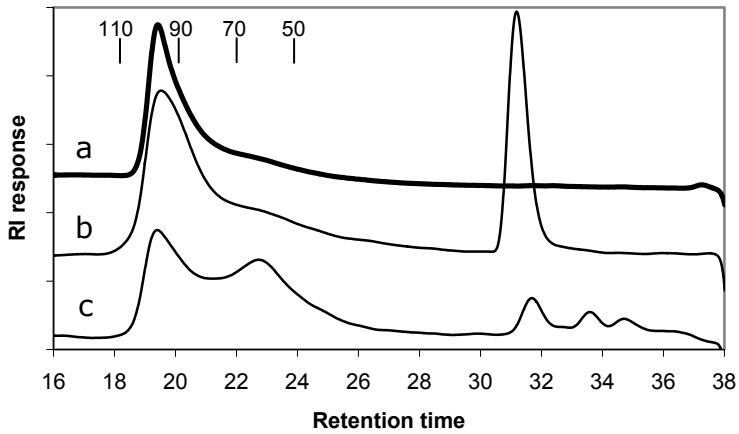

Figure 5.3 HPSEC chromatograms of (a) DASS extract, (b) DASSY-, (c) DASSY+. Pectin was used as a molecular weight standard (kDa).

The total WES extract and WESY+Cu- material eluted at the same time of about 23 min (60 kDa) (Fig. 5.1). The peak for the WESY- fraction shows the same elution time as found for the WES and WESY+Cu- peak. No high molecular weight material was detected with HPSEC for WESY+Cu+ (Fig. 5.1). The concentration of carbohydrates was rather low in this fraction. Salts that were not removed during dialysis cause the low molecular weight peak (32 min.). The elution pattern of the EDTA buffer extracted cell wall material shows a much broader molecular weight distribution than the one of the WES extracts (Fig. 5.2). Yariv negative material (ChSSY-) has a broad elution pattern as well. The ChSSY+Cu- fraction elutes in one peak with an estimated Mw of 60 kDa. The Yariv and copper positive material shows a broader distribution of the Mw (ranging from 50 till 110 kDa). The alkaline extract shows one peak of about 100 kDa (Fig. 5.3) and a broad distribution of lower molecular weight material. The Yariv positive material eluted in two fractions. The fraction with the lower molecular weight (60 kDa) has the same Mw as the WES and ChSS Y+Cu- fractions (Fig. 5.1 and 5.2). The high Mw fraction is about 100 kDa. As with the other elution patterns the Yariv negative material showed a comparable profile as the total extract.

Table 5.2 shows the sugar composition of the WES, ChSS and DASS extracts, the Yariv and copper purified fractions. The WES extract and the sub-fraction WESY+Cu- show a comparable carbohydrate composition. It was already concluded that the major part of the WES material was present in the WESY+Cu- fraction. High amounts of arabinose and galactose are present in a ratio of 1:2 while other sugars are present in rather low amounts. The WESY+Cu+ fraction also contains high amounts of arabinose and galactose and other sugars in low amounts. The concentration of galacturonic acid (11 mol%) is about four times higher than the copper negative fraction. As the glucuronic acid concentration is higher in the copper negative fraction it is obvious that the precipitation with ions is related with galacturonic acid residues. The WESY- fraction shows a high percentage of arabinose, galactose, galacturonic acid and glucuronic acid. The determination of the protein content was restricted to the crude cell wall extracts due to the limited amount of material available for the sub-fractions. The protein content in the WES extract is 7%.

The carbohydrate composition of EDTA buffer extracted material (ChSS) is shown in table 5.2. With EDTA mainly pectin is isolated from cell wall material. After removing a relative small fraction of AGPs (12%) the ChSSY- fraction was obtained and this fraction showed a high amount of galacturonic acid (64 mol%). Besides galacturonic

acid mainly arabinose, galactose and rhamnose are present in ChSSY-. The sugar composition of ChSSY- is in agreement with literature data. Massiot et al. (1988) isolated pectin from alcohol insoluble carrot cell wall material under acidic and basic conditions. The extracts found yielded around 61 to 66 mol% galacturonic acid and the main neutral sugars found were galactose, arabinose and rhamnose (Massiot et al., 1988). Apparently the different method of extraction shows the same yield of the carrot pectins.

Table 5.2 Sugar composition (mol%) of fractions from large carrot cell walls obtained after water extraction (WES), chelating agent extraction (ChSS), alkaline extraction (DASS) and the residue. Y+: precipitated with Yariv; Y-: not precipitated with Yariv; Cu+: precipitated with copper; Cu-: not precipitated with copper; tr: trace amounts <1 mol%; nd: not determined

	Sugar composition								total w/w %	
Fraction	Rha	Ara	Xyl	Man	Gal	Glc	GalA	GlcA	sugar	protein
WES	0	26	1	tr	59	1	6	6	66	7
WESY+Cu-	0	29	1	tr	59	2	3	6	44	nd
WESY+Cu+	0	26	8	6	38	9	11	2	9	nd
WESY-	0	19	3	2	43	7	16	11	19	nd
ChSS	5	8	tr	tr	8	tr	77	0	99	11
ChSSY+Cu-	2	30	tr	0	60	2	3	3	57	nd
ChSSY+Cu+	3	24	1	0	48	4	17	3	62	nd
ChSSY-	8	13	tr	0	13	1	64	tr	71	nd
DASS	8	16	tr	tr	17	1	56	1	80	12
DASSY+	7	16	1	0	21	2	51	3	52	nd
DASSY-	9	19	tr	tr	21	1	47	tr	69	nd
Residue	6	14	9	5	14	19	32	2	27	nd

When ChSS extracted cell wall material is selectively precipitated with Yariv and separated with copper, a striking difference is obtained between the ChSSY+Cu+ fraction and the ChSSY+Cu- fraction. The copper negative fraction contains high amounts of arabinose and galactose in a ratio of 1:2 with other neutral sugars present in low amounts. Galacturonic acid is present at only low levels (3 mol%). The ChSSY+Cu+ fraction contains lower amounts of arabinose and galactose although their ratio remains to be 1:2. The galacturonic acid content is 17 mol%. The total sugar content for the total extract (99% w/w) is probably overestimated since the protein content is determined at 11%. The neutral sugar composition for DASS shows mainly rhamnose, arabinose and galactose. Other neutral sugars are present

in low amounts. Galacturonic acid is present at 56 mol%. The protein concentration is found to be 12%. The DASSY+ fraction contains high amounts of arabinose and galactose in a ratio of 1:1.3. A high percentage of galacturonic acid is present (51 mol%). The separation of the DASS fraction into a Yariv positive and a Yariv negative fraction led to fractions that showed relative small differences in the sugar composition. Summarized we can conclude that with the use of copper ions it is possible to separate the extracts into a high galacturonic acid containing AGP fraction and a low galacturonic acid containing AGP fraction. The residue is expected to contain cellulose, hemicellulose and pectins that were not extracted with the EDTA buffer and the sodium hydroxide solution. A remarkable high amount of galacturonic acid was still present (32 mol%) and the amount of glucose measured was 19 mol%.

The sugar analysis indicated a galacturonic acid poor fraction and a galacturonic acid rich fraction for the WES and ChSS fractions. The galacturonic acid from the Y+Cu+ fractions could be present as single residues that are distributed over the AGP carbohydrate moiety or grouped together in homogalacturonan structures. Previous work on cell culture AGPs (Chapter 4) showed that at least some of the galacturonic acid rich AGPs were sensitive for homogalacturonan degrading enzymes. The material was incubated with pectin methylesterase and polygalacturonase that respectively demethylates- and hydrolyses galacturonic acid of the homogalacturonan part of pectin.

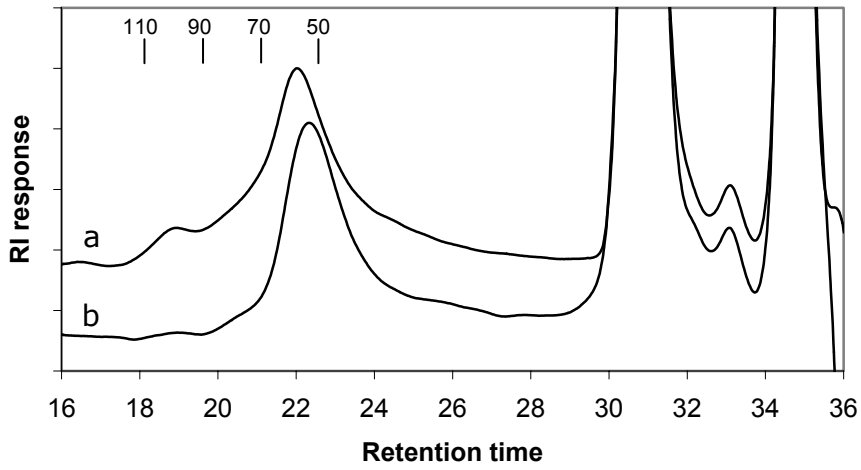

Figure 5.4 HPSEC chromatogram of the EDTA buffer isolated fraction CHSSY+Cu+ before (a) and after (b) incubation with polygalacturonic acid hydrolyzing enzymes.

The same enzyme combination was used in this work to show whether homogalacturonan structures were present. The effect of the incubation was monitored by HPSEC. The amount of WESY+Cu+ material for the enzyme incubation was limited. To exclude non-specific activity of the pectic degrading enzymes on the AGPs, the galacturonic acid poor WESY+Cu- fraction was used for the enzyme incubation and served as a negative control. No degradation of the WESY+Cu- fraction after enzyme incubation was observed (data not shown). Incubation of the ChSSY+Cu+ fraction with the homogalacturonan degrading enzymes resulted in the breakdown of high molecular weight material and a small shift of the peak at ca. 22 min to a lower Mw (Fig. 5.4). This indicates that at least part of the galacturonic acid present in the ChSSY+Cu+ fraction is organized as homogalacturonan. The HPSEC elution profiles did not show breakdown products with a low molecular weight. Therefore the fraction was analyzed with HPAEC for low molecular weight components after enzyme incubation. Figure 5.5 shows the HPAEC chromatogram of the ChSSY+Cu+ fraction after enzyme incubation.

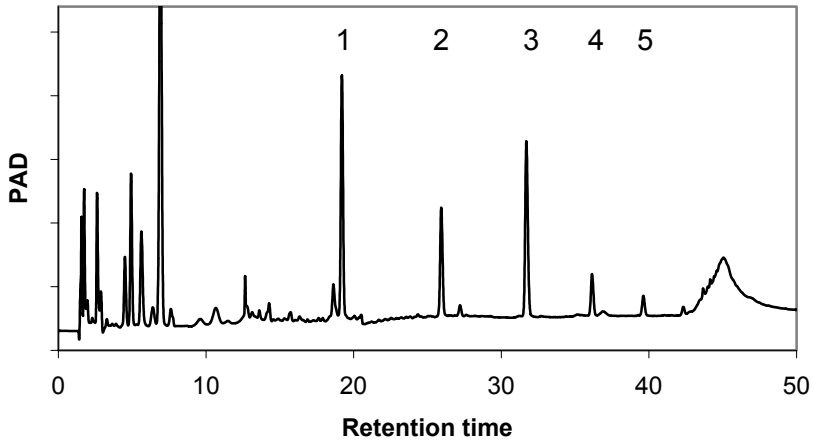

Figure 5.5 HPAEC after incubation of the EDTA buffer isolated fraction ChSSY+Cu+ with polygalacturonic acid hydrolyzing enzymes. (1) $GalA_1$; (2) $GalA_2$; (3) $GalA_3$; (4) $GalA_4$; (5) $GalA_5$

Hydrolysis products as monomers, dimers, trimers and low amounts of tetramers and pentamers were identified. The detection of galacturonic acid fragments supports the finding that part of the galacturonic acid present in the ChSSY+Cu+ fraction is organized as homogalacturonan.

These experiments show that carrot tap root isolated AGPs contain pectin structural elements and that this observation is not restricted to cell culture isolated AGPs.

Discussion

In this work, evidence was obtained for the interaction of AGPs with cell wall polymers such as pectin. Most AGPs have a low isoelectric point, meaning that they carry a considerable negative charge that allows nonspecific ionic binding with other molecules (Baldwin et al., 2001). The negative charge of AGPs may also enable divalent cations to mediate nonspecific binding between AGPs and other anionic molecules as suggested by Baldwin et al. (1993). Interactions of AGPs with other polymers can also be established by a covalent ether linkage (Kwan and Morvan, 1995). In this research we showed that in carrot tap root cell walls some AGP fractions contain pectic structures and that ionic linkage of these structures is unlikely because of the extraction procedure used.

The EDTA buffer extracted material showed the highest total amount of galacturonic acid rich AGPs and is therefore the most suitable extract for the study of AGP-pectin linkages. Selective precipitation of AGPs with copper ions showed to be very useful to separate AGP fractions on glucuronic acid content. The only drawback of the use of copper nitrate is the firmness of the complex formed. This makes purification by dialysis of the fractions difficult as expressed by the low total sugar content for the WES AGPs.

To prove the hypothesis that galacturonic acid is organized as homogalacturonan in the ChSS AGP fraction, this fraction was hydrolyzed with pectin methylesterase and polygalacturonase. The same enzymes were used in previous research (Chapter 4) on cell culture isolated AGPs. Also in tap root isolated AGPs degradation was observed. As homogalacturonan is a well known part of pectin, this finding suggest that also an AGP fraction isolated with EDTA buffer from carrot tap root cell walls is linked to pectin. Although both cell culture isolated AGPs and tap root isolated AGPs showed sensitivity to pectin degrading enzymes, a different pattern was observed on HPSEC elution profiles after enzyme incubation. In cell culture AGP fractions only a part of the fraction showed sensitivity, leaving the other populations untouched. Degradation was observed in medium AGPs at ca. 50 kDa and in ChSS AGPs at ca. 80 kDa. In tap root AGPs degradation was observed in a range of 60 to 110 kDa. A small shift of the peak at ca 22 min was observed that could indicate that the enzyme was able to release most or all of the galacturonic acid present on the AGPs, resulting in a pectin free AGP fraction with an estimated molecular weight of ca. 50 kDa. At this moment we have no further details on the type of the covalent linkage between AGPs and pectin.

It is not likely that the AGP-pectin fraction characterized in this study is composed as a pectic structural element that functions as attachment site for multiple AGP molecules. The molecular weight of such a complex is expected to exceed the molecular weight of the values obtained for the AGP-pectin fraction in this research. It might be possible that such a complex of multiple AGP-pectin exists in muro and is fragmented during the extraction procedure.

This research showed that pectin-associated AGPs, previously found in carrot suspension cells, are indeed present in carrot tap root cell walls. Besides the presence of homogalacturonan in EDTA buffer extracted AGPs, a xylose and GalA rich fraction was found in WES AGPs. This finding points towards the presence of xylogalacturonan in this particular fraction. Additional carbohydrate linkage analysis and the use of antibodies, specific for AGPs and pectin structures, would be in favor to unravel the type of the linkage of both macromolecules. Enzymatic studies to partly degrade pectin specific regions could show sequential degradation of an AGP-pectin complex.

Materials and methods

Isolation of cell wall material

Carrots tap roots (*Daucus carota* L.) (approximately 1 kg fresh weight, purchased at the local market) were cleaned and ground at 4°C. Water Extractable Solids (WES) were obtained after two extractions of one hour with 2.5 L deionized water at 4°C. The solid material was removed from the extract by filtration and centrifugation (19,000 x *g* for 15 min at 4°C). Before dialysis, the WES fraction was adjusted to pH 5 with acetic acid and boiled for 5 min. By boiling proteins present are denatured and endogenous enzymes are inactivated. The water insoluble solids were resuspended in 3 L EDTA buffer (50 mM EDTA, ammonium oxalate 50 mM, sodium acetate 50 mM, pH 5.2) and stirred at 70 °C overnight to obtain the Chelating agent Soluble Solids (ChSS) (de Vries et al., 1981). The second EDTA extraction was performed in 2 L buffer under the same conditions. Before dialysis of the ChSS extract the volume was reduced in a rotating evaporator. The pellet was washed twice with water for 30 min at room temperature. The water was discharged. The Dilute Alkali Soluble Solids (DASS) were obtained by isolating the cell wall material two times with 1.5 L sodium hydroxide (50 mM) for 1 h at 0°C. Before the extract was dialyzed, the pH was adjusted to pH 5.7 with acetic acid. The residue was neutralised (pH 4.6) and dialyzed. All extracts and the residue were freeze-dried prior to further analysis. An additional extraction with 800 mL acetone was applied on the fractions ChSS and DASS to reduce the amount of carotene. After this purification step the material was air dried and homogenized.

An alcohol insoluble solid fraction (AIS) was made by grinding 100 g carrot tap roots followed by three times extraction with 1 L ethanol (final concentration 70%) at 50°C for 1 h. After the extraction the residue was purified with acetone, dried and homogenized.

Precipitation with Yariv phenylglycoside and Cu^{2+}-ions

Yariv phenylglycoside was synthesized as described by Yariv et al. (1962). The fractions (WES, ChSS, DASS) were dissolved in Millipore water (150 mg in 300 mL) and NaCl was added to a final concentration of 0.15 M. Precipitation of AGPs was accomplished by adding Yariv phenylglycoside to the solutions (80 mg to WES and 40 mg to ChSS, DASS). The amount of Yariv phenylglycoside added to the WES fraction was double as the amount of AGPs present were expected to be higher.

The AGP-Yariv complex was precipitated at 4°C overnight, centrifuged at 16,000 x *g* for 20 min. The supernatant was kept aside (Yariv negative material: Y-) and the pellet was washed three times with 0.15 M NaCl and dissolved in water (Yariv positive material: Y+). In order to remove the Yariv phenylglycoside from the AGPs and degrade the Yariv molecules, $Na_2S_2O_4$ was added to both pellet and supernatant solutions and heated to 50°C until the red colour disappeared. The AGP precipitates and the supernatants were dialyzed against Millipore water and freeze dried.

Yariv positive material (Y+) (100 g) was dissolved in 10 mL water and treated with aqueous copper nitrate (7% solution) for 1 h and centrifuged (12,000 x *g* for 15 min at 20°C) to precipitate pectic material (Renard et al., 1997). The pellet contains Yariv positive material together with pectic material (Y+Cu+); the supernatant contains Yariv positive material without pectin (Y+Cu-). Ethylenediamine tetra-acetic acid (EDTA) (0.07 M, pH 5) was added to remove the copper ions. The fractions were first dialyzed against EDTA and sodium acetate and thereafter with Millipore water.

Analytical methods

Sugar analysis was performed using methanolysis (de Ruiter et al., 1992). Fractions (0.1 mg) were dried at 40°C under vacuum and hydrolyzed with 2 M HCl in dry methanol for 16 h at 80°C. The methyl was removed from the sugars with 2 M trifluoroacetic acid (TFA). The monomeric sugars were analyzed by high-performance anion-exchange chromatography (HPAEC) on a Thermo Separations HPLC system (Thermo Separations Products, Inc, San Jose, CA) equipped with a CarboPac PA-10 column (Dionex Corporation, Sunnyvale, CA), a CarboPac Guard column and a Dionex borate trap. Detection was performed with a Dionex ED40 Pulsed Amperometric Detector (PAD). Sugars were separated using the following gradient (1 mL/min): 0 min: 15 mM NaOH; 45 min: 15 mM NaOH; 75 min 800 mM NaOH; 80 min 800 mM NaOH; 81 min 15 mM NaOH; 100 min 15 mM NaOH. The total sugar content is derived from data obtained from the different sugar residues present in the fractions as determined by HPAEC. Total protein content was determined by a semi-automated micro-Kjeldal method (Roozen and van Boxtel, 1979). All nitrogen was assumed to originate from proteins and the conversion factor used was 6.25 x N.

Enzymatic degradation of AGPs

The fractions (5 mg/mL) were incubated with pectin-methyl esterase and polygalacturonase. Pectin methyl esterase originated from *Aspergillus niger* and polygalacturonase originated from *Kluyveromyces fragiles* (Schols et al., 1995). The incubation with purified enzymes took place in sodium acetate buffer (50 mM, pH 5.0) for 18 hours at 30°C (Vierhuis et al., 2003) and the reaction vials were rotated 'head over tail'. The amount of enzymes used was calculated on basis of the activity of the enzyme and the amount of galacturonic acid present in the fraction. The hydrolysis of all GalA

linkages was calculated to be completed after 6 hours. The enzymes were inactivated by heating for 10 minutes at 100°C. The digests were analyzed by HPSEC and HPAEC.

High-Performance Size-Exclusion Chromatography (HPSEC)

HPSEC was performed on three Tosoh Bioscience TSK PWXL gel columns in series (G4000, G3000, G2500) (Tosoh Bioscience, Inc, South San Francisco, CA) in combination with a PWXL-guard column (Tosoh Bioscience). Elution took place at 30°C with 0.2 M $NaNO_3$ at 0.8 mL/min (Kabel et al., 2002). The eluate was monitored using a Finnigan Spectrasystem RI 150 detector (Thermo Electron Corporation, Milan, Italy). Size fractionated pectins (1,000 – 100,000 Da, as determined by viscometry) were used for calibration of the columns. The neutral sugar percentage of the pectins was less than 10 mol%.

High-Performance Anion-Exchange Chromatography (HPAEC)

HPAEC was used to analyze the breakdown products after enzyme treatment on a Thermo Separations HPLC system with a Dionex CarboPac PA-1 column and a Dionex CarboPac Guard column. Oligomeric products were separated using the following gradient (1 mL/min): 0 min: 0.1 M NaOH; 5 min: 0.1 M NaOH; 40 min: 0.1 M NaOH, 0.5 M NaAc; 45 min: 0.1 M NaOH, 1 M NaAc; 50 min: 0.1 M NaOH, 1 M NaAc; 51 min: 0.1 M NaOH; 65 min: 0.1 M NaOH . The eluate was monitored using PAD detection.

Chapter 6

Concluding remarks

Background

Arabinogalactan proteins (AGPs) have been studied for over forty years. As a result the overall structural characteristics of AGPs, a hydroxyproline rich protein to which type II arabinogalactans are attached, are well-known. To determine the biological function of AGPs, different approaches have been used. Yariv phenylglycoside that interacts specifically with AGPs has been used to localize AGPs in tissues and to specifically precipitate AGPs from culture media and the soluble fraction of cell walls. When Yariv phenylglycoside was added to cell cultures their growth was disrupted. It was therefore concluded that AGPs, when immobilised with Yariv, were not able to carry out their function resulting in the observed biological effect (Serpe and Nothnagel, 1994). Monoclonal antibodies studies have been undertaken to determine the cellular localization of AGPs (Majewska-Sawka and Münster, 2003). Affinity chromatography has been used for the isolation of AGPs based on the properties of their carbohydrate epitopes (Baldwin et al., 1993). With the availability of more advanced carbohydrate analysis techniques and genetic data, notable progress has been achieved in the elucidation of the side chain structures and the determination of the biological function of AGPs respectively. NMR spectroscopy has enabled to obtain the complete structure of an arabinogalactan. Bacic et al. (1987) suggested that small repetitive subunits make up larger arabinogalactan polysaccharides. The suggestion that these subunits are linked by a periodate sensitive sugar proposed to be either (1-5)-*α*-L-Ara*f* or (1-6)-*ß*-D-Gal*p* (reviewed in Gaspar et al., 2001) was supported by the detection of a single *ß*-(1-6)-D-Gal*p* residue in the main AG chain (Tan et al., 2004). The Hyp contiguity hypothesis has been formulated that predicts the site of attachment of carbohydrate chains to the protein core depending on the hydroxyproline sequence in the protein backbone (Kieliszewski, 2001). An important finding with implications for the function of AGPs was the detection of glycosylphosphatidylinositol (GPI) anchors that connect proteins to the outer plasma membrane at some AGPs (Youl et al., 1998). GPI anchors can be cleaved by phospolipase C in a signal-dependent manner (Baluška et al., 2003) implicating that plants are able to actively control the release of AGPs from the membrane. Genetic studies have revealed data on AGP protein sequences. The first AGP encoding gene that was cloned corresponded to an AGP purified from a pear suspension culture (Chen et al., 1994). Based on that sequence AGP genes and putative AGP encoding genes have been identified. Genetic approaches such as reported by Acosta-Garcia and Vielle-Calzada (2004) who showed the involvement of a single classical AGP encoding

gene, AGP18, in Arabidopsis female gametogenesis are promising to determine the function of AGPs.

Research motive

This study on the characterization of AGPs was initiated after the finding that AGPs and chitinase were both involved in the somatic embryogenesis of carrots. AGPs have been found that stimulate the formation of somatic embryos (Kreuger and van Holst, 1993) whereas other AGPs can repress the formation of somatic embryos when AGPs are supplied to cell cultures (Toonen et al., 1997). One of the proteins found in carrot cell culture medium was able to stimulate the formation of embryos in a cell line with a mutated carrot variant that did not form embryos at a non-permissive temperature (de Jong et al., 1993). The protein was characterized as an endochitinase that is able to hydrolyse glycosidic linkages of chitin and chitosan. Plants are however not known to produce chitin and chitosan. It was suggested that a plant-derived GlcNAc or GlcN containing oligomer was present in the medium of cultures that could function as a chitinase substrate (van Hengel, 1998). AGPs have previously also been reported to contain low amounts of GlcN (van Holst et al., 1981) and carrot seed AGPs showed sensitivity to endochitinase (van Hengel, 1998). Also NOD-factor like signal molecules that showed sensitive to chitinase have been isolated that were able to promote early somatic embryo development in Norway Spruce (Dyachok et al., 2002).

In this project AGPs were isolated from carrot cell culture medium and the cell walls of different carrot tissues and partially characterized. The initial goal was to characterize a GlcN containing AGP derived carbohydrate fragment involved in somatic embryogenesis that was susceptible to endochitinase activity.

Characterization of carrot AGPs

AGPs were isolated with Yariv phenylglycoside from seeds of different carrot varieties, the conditioned medium of different cell cultures, the cell wall material of a cell culture and from cell walls of carrot tap roots. Therefore we compared all different compositions obtained over a long period. Carbohydrate analysis showed that all crude AGP extracts had high levels of arabinose, galactose and uronic acids (Table 6.1). The embryogenic cell culture (EC) medium data from chapter 2 show a 1:2 ratio for arabinose and galactose. This ratio was observed for most of the crude AGP extracts in this research. The uronic acid content was not determined for EC medium

and therefore the relative contribution of the neutral sugars is probably overestimated. The data for the total amount of uronic acid shows a large deviation with values between 4 mol% and 54 mol%. Rhamnose and glucose are present in all fractions and xylose and mannose are absent or only present at very low levels. Fucose was not detected in any of the AGP fractions. The sugar composition for DASS tap root AGPs strongly deviates from the composition of other fractions and showing a high amount of galacturonic acid and low amounts of arabinose and galactose. Two well characterized AGPs from *L. multiflorum* and *N. alata* showed high amounts of arabinose and galactose in a ratio of 1:2 (reviewed by Nothnagel, 1997). Other sugars are found in AGPs at different levels (Clarke et al., 1979).

Table 6.1 Sugar composition (mol%) of carrot AGPs isolated with Yariv phenylglycoside from medium, seeds and the cell walls of a cell culture and tap roots. Medium EC: AGPs isolated from a embryogenic cell culture; Medium NC: AGPs isolated from a non-embryogenic cell culture; Seeds: AGPs isolated from seeds; AGPs were isolated from cell wall material with respectively water (WES), EDTA buffer (ChSS) and a sodium hydroxide solution (DASS); UA: uronic acids; tr: trace amounts < 1 mol%; nd: not determined

	Sugar composition									
Fraction	Rha	Ara	Xyl	Man	Gal	Glc	GlcN	GalA	GlcA	total UA
Cell culture										
Medium EC[a]	6	30	1	tr	60	2	tr	nd	nd	nd
Cell culture/seeds										
Medium NC[b]	5	23	0	0	50	4	0	nd	nd	19
Medium EC[b]	5	24	tr	tr	49	1	0	nd	nd	19
Seeds[b]	tr	34	tr	tr	59	tr	tr	nd	nd	4
Cell culture										
Medium[c]	6	17	2	1	50	2	nd	13	9	22[e]
WES[c]	4	26	tr	tr	57	2	nd	4	7	11[e]
ChSS[c]	4	20	2	3	43	3	nd	17	8	25[e]
DASS[c]	5	23	2	2	44	3	nd	14	8	22[e]
Tap roots										
ChSS[d]	3	29	tr	0	54	2	nd	9	3	12[e]
DASS[d]	7	16	1	0	21	2	nd	51	3	54[e]

[a] data derived from chapter 2; [b] data derived from chapter 3; [c] data derived from chapter 4; [d] data derived from chapter 5; [e] value calculated from GalA and GlcA

Size exclusion chromatography showed that most fractions were composed of two high molecular weight fractions. When the crude AGP extracts were further purified by size-fractionation or their ability to precipitate with copper ions, different

carbohydrate compositions were established for the fractions obtained. In chapter 3 the separation and the subsequent analysis of two populations present in EC medium and seed AGP fractions showed structurally different AGPs. However, all AGP fractions could be precipitated with Yariv phenylglycoside, all AGP samples contained hydroxyproline and linkage analysis showed the presence of type II AGs proving that AGPs were present in all fractions. The carbohydrate analysis showed that the two AGP fractions with different molecular weight had different sugar compositions (table 6.2). In EC medium, an arabinose poor fraction was present with a high amount of glucuronic acid (Medium I). Arabinose poor AGPs with a high amount of glucuronic acid have also been isolated from the cell walls from rose cell cultures (Serpe and Nothnagel, 1995).

Table 6.2 Sugar composition (mol%) of carrot AGPs isolated with Yariv phenylglycoside from medium and the cell walls of a cell culture. Medium: AGPs isolated from a embryogenic cell culture; Seeds: AGPs isolated from seeds; ChSS: AGPs isolated from cell wall material with EDTA buffer; UA: uronic acids; tr: trace amounts < 1 mol%; nd: not determined

	Sugar composition								
Fraction	Rha	Ara	Xyl	Man	Gal	Glc	GalA	GlcA	total UA
Cell culture/seeds									
MediumI[a]	11	3	5	5	56	0	tr	20	20[c]
MediumII[a]	5	28	0	0	60	0	0	6	6[c]
SeedsI[a]	1	37	0	tr	47	2	8	4	12[c]
SeedsII[a]	1	33	tr	tr	61	0	tr	5	5[c]
Cell culture									
MediumY+Cu-[b]	5	23	2	1	55	tr	5	9	14[c]
MediumY+Cu+[b]	7	7	3	4	34	2	30	13	43[c]
ChSSY+Cu-[b]	4	28	tr	tr	53	2	7	7	14[c]
ChSSY+Cu+[b]	6	15	4	3	36	4	26	7	33[c]

[a] data derived from chapter 3; [b] data derived from chapter 4; [c] value calculated from GalA and GlcA

The deviations in the sugar composition for the two seed AGP fractions were not as extreme as in medium AGPs although differences were present in the arabinose, galactose and galacturonic acid content.

When the medium and ChSS fractions were precipitated with copper ions, the sugar composition showed large difference between AGPs that precipitated with copper

ions and the AGPs that did not precipitate (Table 6.2). The two AGP fractions that did precipitate with copper showed a much higher amount of galacturonic acid, reduced levels of arabinose and galactose and slightly higher levels of the other neutral sugars.

Besides fractionation and copper precipitation no other purification techniques were used in our study. Baldwin et al. (1993) isolated polysaccharides and proteoglycans from a carrot cell culture by ethanol precipitation. The anti-AGP monoclonal antibody MAC207, that is known to recognize an arabinose-containing AGP epitope, was used to purify AGPs from the crude extract. The purified AGPs that bound to Yariv phenylglycoside showed high amounts of alanine, serine and proline but no hydroxyproline. The deglycosylated protein had an apparent mass of 30 kDa. In a later study the cDNA sequence of the core protein DcAGP1 was obtained predicting this AGP encoding gene consisted of 242 amino acids and having a molecular weight of 25.6 kDa (Baldwin et al., 2001). The carbohydrate composition showed high amounts of arabinose (19 mol%), galactose (40 mol%) and a remarkable high amount of glucose (25 mol%). Other sugars were present at lower levels (Baldwin et al., 1993).

Both the purification of AGPs from carrot cell culture medium based on molecular weight and interaction with copper ions as described in this thesis and the purification based on AGP epitopes using antibodies (Baldwin et al., 1993) yielded fractions with a different carbohydrate composition. This indicates that there is a large variation in the glycosylation of AGPs in carrot cell cultures. Therefore the experiments in chapter 2 in which a crude AGP extract was incubated with chitinase or added to a cell culture to observe the effect on embryo formation makes the conclusion whether all, or just a small AGP fraction is involved, quite difficult to make. When functional and structural studies on AGPs are performed, a better approach would be to include the purification and sequence analysis of the protein cores of the AGPs involved. With recombinant DNA techniques AGPs have been linked to a green fluorescent protein tag that allowed cellular localisation of AGPs when expressed in tobacco cells and AGP purification by chromatography (Zhao et al., 2002). Also synthetic genes were used to encode AGP-like proteins with hydroxyproline present in different sequences. After expression of these constructs in tobacco cells the glycosylation patterns of these proteins were studied (Tan et al., 2003). These studies allow characterization of AGPs with an identical protein core.

Glucosamine containing AGPs

In EC medium AGPs (chapter 2) and seed AGPs (chapter 3) a low amount of glucosamine was detected (0.2 mol% for both fractions). The presence of GlcN was also found at low levels in three batches of seeds from different varieties (data not shown). In chapter 3 the experiments are described on the purification of seed AGPs in three fractions with a different molecular weight. After the fractionation of the crude seed AGPs it was not possible to detect GlcN in one of the fractions obtained. There could be several reasons for this result.

The crude, GlcN containing seed AGP fraction showed two high molecular weight populations. These two high molecular weight populations and the material with an intermediate molecular weight were recovered. Although probably more than 95% of all carbohydrate was recovered in these three fractions, there is still a possibility that the GlcN containing AGP fraction was lost during this procedure. Another explanation for the loss of GlcN detection in purified AGP fractions is the assumption that a non-AGP component was present in crude AGP extracts. The non-AGP component could have been co-precipitated with AGPs and Yariv phenylglycoside due to weak interactions. This component was apparently lost during the separation on the chromatography column. The indication that the component should contain at least three adjacent GlcN residues comes from a study with endochitinase in which AGPs showed sensitivity. The chitinases used in the experiments for chapter 2 were either purified from plant cell cultures or after heterologous expression in insect cells. Since both enzyme preparations gave essentially the same result on AGPs, contamination of chitinases with other carbohydrate degrading enzymes is unlikely. Therefore the chitinase-sensitive component in seed AGPs is likely to be coprecipitated with Yariv and consists of only a very small fraction of the material isolated.

Galacturonic acid containing AGPs

The treatment of AGPs from carrot medium and cell wall fractions with copper ions led to the purification of AGPs with a high amount of galacturonic acid (Table 6.2). Copper ions proved to be very useful in the separation of AGP extracts into fractions with high and low galacturonic acid content. Copper ions form a stronger complex with homogalacturonan than calcium ions (Schlemmer, 1989; Renard et al., 1997). The fact that AGPs were able to precipitate with copper ions was an indication that the galacturonic acid was, at least partly, organised as homogalacturonan. With homogalacturonan degrading enzymes (pectin methylesterase and polygalac-

turonase) the organisation of galacturonic acid in contiguous sequences was shown. Sofar, the occurrence of homogalacturonan on AGPs has not been described in detail although galacturonic acid rich AGPs are known to occur.

Galacturonic acid containing AGPs have been described in fibres of flax and red wine. Two AGP-like polymers (2YP1, 2YP2) have been solubilised from flax cell wall material with cellulase (Girault et al., 2000). These polymers showed a strong interaction with Yariv phenylglycoside, contained amino acids characteristic for AGPs but were deficient in hydroxyproline. The protein content was estimated to be 0.1% for 2YP1 and 0.025% for 2YP2 on weight basis. The sugar composition of the AGP-like polymers is shown in table 6.3.

Table 6.3 Sugar composition (mol%) of flax cel wall AGPs (Girault et al., 2000).
UA: uronic acids; tr: trace amounts < 1 mol%

	Sugar composition								
Fraction	Rha	Ara	Xyl	Man	Gal	Glc	GalA	GlcA	total UA
2YP1	4	3	tr	2	66	6	13	7	20[a]
2YP2	8	2	tr	2	61	2	19	7	26[a]

[a] value calculated from GalA and GlcA

Other AGPs are known that contain galacturonic acid (Pellerin et al., 1995). These AGPs purified from red wine showed glucuronic acid and galacturonic acid in association with 2- and 2,4- linked rhamnose.

The interaction of AGPs with pectin has been suggested earlier. Ionic interactions have been proposed (Baldwin et al., 1993) but also covalent interactions with pectin (Morvan et al., 2003). Morvan et al. (2003) presented a model in which rhamnogalacturonan I is ester linked to the carbohydrate part of AGPs. However no structural evidence was presented for the points of attachment on AGPs or pectins. The occurrence of homogalacturonan containing AGPs is an indication that such covalent pectin-AGP interactions are present in carrot cell walls. Ionic interactions with AGPs and pectins are unlikely to have survived the isolation procedure.

The AGPs that contained high amounts of galacturonic acid were synthesized under in vitro conditions using cell cultures. Although in vitro setups are often used to study a large array of biological processes and to isolate biomolecules, it remains an

artificial environment for the cells. To determine whether galacturonic acid rich AGPs were also present in roots, carrot tap root cell walls were extracted with a purification procedure optimized for pectins. From such pectin-enriched fractions galacturonic acid containing AGP fractions were purified. With homogalacturonan degrading enzymes the presence of homogalacturonan was confirmed. It is not known whether, besides the homogalacturonan, other structural elements of pectin like rhamnogalacturonan I or II are present. The results for AGPs in tap roots suggest that AGPs with structural elements of pectin are not restricted to cell cultures and are likely to be part of a common plant cell wall structure.

Interaction of AGPs with pectin

Several suggestions concerning the interaction between AGPs and pectins have been proposed. Ionic interactions were suggested by Baldwin et al. (1993) who found that AGPs showed calcium mediated binding with pectin on dot blots. Also ionic interactions without calcium have been suggested as some AGPs possess a protein core with positively charged amino acids. Baldwin et al. (2001) isolated DcAGP1 from a carrot cell culture and this AGP contained 11 positively charged amino acid residues out of a stretch of 14 amino acids. It was hypothesized that the positive charges confer pectin-binding properties to the AGP. Besides ionic interaction of AGPs with pectins, also covalent linkages have been proposed. The following cross links in pectin have been suggested by Fry (1986): ester links from the carboxylic acid groups of some of the galacturonic acid residues to other polysaccharides, crosslinks between phenolic acids esterified to side chain residues of the pectin and other polymers and ionic interactions between acidic groups of pectins and basic groups of proteins. In our research we have isolated cell wall fractions with EDTA from which galacturonic acid rich AGP fractions were derived. It is not likely that ionic interactions were responsible for the linkage of pectin structures to AGPs as ionic interactions would have been disrupted by the extraction with EDTA. Therefore it is more likely that the linkage between AGP and pectins is covalent. Fry (1986) has suggested ester links from pectic galacturonic acid residues to other polysaccharides. For AGPs that were present in the medium or isolated from cell wall material that was isolated with EDTA, ester linkages are possible. However, in chapter 5 it is described how cell wall material that was isolated with sodium hydroxide showed very high amounts of galacturonic acid in AGP fractions. Ester linkages would not have been able to resist the alkali treatment. It is possible that

both ester and ether linkages are present in the proposed AGP-pectin network. In the early cell wall model of Keegstra et al. (1973) a linkage was suggested between pectin and the cell wall protein extensin. However, Pope (1977) showed that the glycoprotein involved in the model was an AGP instead of a extensin (reviewed by Mort, 2002). The model of Keegstra proposed that all cell wall polymers were interconnected into an extensive network. Recent cell wall models of primary cell walls suggest that there are no covalent linkages between pectins and other cell wall polymers (McCann and Roberts, 1991; Carpita and Gibaut, 1993). In the model of Carpita and Gibaut (1993) a network of cellulose and xyloglucan is proposed, embedded in an independent pectin matrix. However, Morvan et al. (2003) presented a model, based on the model of Albersheim's group (Keegstra et al., 1973), in which type II AG structures are linked to pectin (Fig. 6.1).

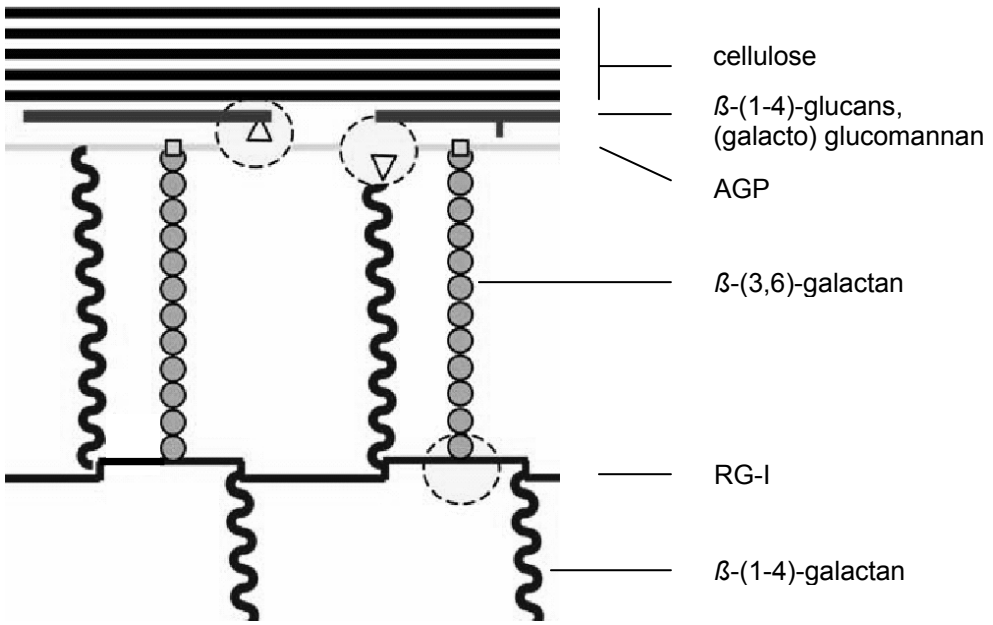

Figure 6.1 Part of the cell wall model that was based on the analysis of secondary walls of flax fibres (Morvan et al., 2003). A cellulose microfibril is shown to which glucans and (galacto) glucomannans are attached by hydrogen bounds. *ß*-(1-4)-galactan chains are linked to RG-I and are forming the pectin in this model. The pectins may interact with the *ß*-(3,6)-galactan chains of the AGPs. The circle represents an ester linkage; the triangle in a circle represents an ester linkage that is established by a glutamic acid residue.

The suggestions proposed for the cross-linking of AGPs and pectin include ester linkages, in which the uronic acids of pectins are involved or glutamic acid of the proteins (Morvan et al., 2003). As shown in their model (Fig. 6.1) such a complex can

be attached to cellulose microfibrils suggesting that AGPs and pectins can form a complex with cellulose (Fig. 6.1). Girault et al. (2000) have isolated AGP-like polymers from cell wall material with cellulose, providing support to the model that AGP-pectin structures are attached to cellulose.

Fasciclin-like AGPs (FLAs) have, besides glycosylated regions, putative cell adhesion domains known as fasciclin domains (Johnson et al., 2003). FLAs precipitate with Yariv phenylglycoside, indicating that they share structural characteristics with AGPs. Johnson (2003) has proposed a model in which FLAs interact through their fasciclin domain to form aggregates that interfere with the cross linking of cell wall polysaccharides. FLAs may regulate cell expansion through interactions with pectins or other cell wall polysaccharides. As fourteen of the FLAs are predicted to be GPI anchored (Johnson et al., 2003) the aggregates reside at the plasma membrane. The model of Johnson (2003) was used to show how a pectin linked AGP with GPI anchor could be located in the cell wall (Fig. 6.2). As GPI anchors can be cleaved by phospolipase C in a signal-dependant manner (Baluška, et al., 2003) this would allow plants to actively control plasma membrane-cell wall interactions.

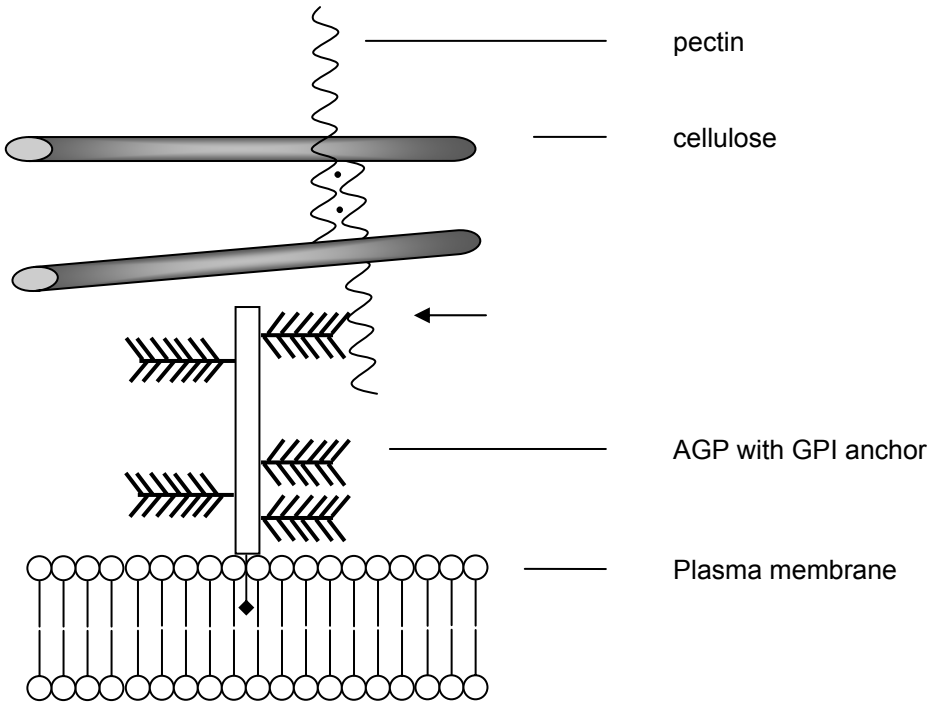

Figure 6.2 Proposed model of an AGP with GPI anchor that is linked to pectin in a primary cell wall (derived from Johnson, 2003). The AGP is schematically drawn as a rectangle to which carbohydrate chains are attached. The arrow points to the location where type II AG and pectin are linked. Not drawn to scale.

Zhao et al. (2002) have shown by fluorescence microscopy that plasma membrane located AGPs in tobacco cells after plasmolysis were also present in the Hechtian threads, connections between the cell wall and the membrane. It is not known if these kinds of membrane-cell wall connections are indeed established by AGPs in the same way as wall associated kinases (WAKs) are able to connect the plasma membrane to the cell wall. Gens et al. (2000) have suggested that AGPs and WAKs show co-localisation. Besides AGPs, WAKs are linked to pectin as suggested by Kohorn (2000) and Anderson et al. (2001).

The nature and amount of the linkages between AGPs and pectins are likely to be developmentally regulated. In this study AGPs were isolated from different carrot tissues and culture medium but no variation in time was included. It would be interesting to isolate AGPs from a cell culture at different developmental stages. The analysis of the AGP-pectin complex could show differences in the relative amount of this complex compared to the total amount of AGPs present. The study of AGP-pectin linkages should primarily focus on the nature of the linkage. As the residues involved in the linkage are forming a small amount of the total mass of the polymers involved, large amounts of material are needed for linkage studies (reviewed Mort, 2002).

The enzyme incubations in our study showed that not all galacturonic acid rich AGP fractions were sensitive to pectin methylesterase and polygalacturonase. It is important to first characterise the different AGPs that precipitate with copper after fractionation on charge with ion exchange chromatography.

When galacturonic acid rich AGP fractions are obtained, enzymes could be used to partly degrade the pectin and the type II AG chain of AGPs. It will be a challenge to partly degrade the AGP-pectin complex to such an extent that the complex with the linkage is small enough to obtain interpretable data from NMR spectroscopy studies.

The research described in this thesis showed that in carrot tissues and medium, AGPs can be found that have a large variety in sugar composition. It remains a difficult task to characterize complete carbohydrate structures of individual AGP species due to this variety. As AGPs are strongly glycosylated it is plausible that the function of AGPs is related with the carbohydrate part. The finding of pectin containing AGP fractions underlines the importance of the carbohydrate part of AGPs. However, carbohydrate analysis alone would be insufficient to address the function of AGPs in plants. The most promising research approach to unravel AGP

functions appears to combine molecular approaches with analytical chemistry. Only then it can be hoped that the function of the growing number of DNA sequences encoding potential AGPs can be determined.

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Summary

Arabinogalactan proteins (AGPs) are highly glycosylated proteins. Besides galactose and arabinose the carbohydrate part of AGPs contains other neutral sugars and uronic acids. AGPs are widely distributed in the plant kingdom, probably occurring in all tissues of every plant. Yariv phenylglycoside is a synthetic molecule and can form a complex with AGPs and this property of Yariv is used to isolate AGPs. Exposure of cell cultures or seedlings to Yariv phenylglycoside indirectly showed that AGPs have a biological function. For the study of embryogenesis cell cultures have been used. The cells of a cell culture can differentiate, form embryos and finally develop into plants. The growth conditions can be changed and the effect on the development of the embryos can be investigated. By adding AGPs to cell cultures the amount of embryos formed can be manipulated.

This research showed that especially AGPs that were derived from carrot seeds were able to increase the number of embryos formed. Pre-treatment of the seed AGPs with chitinase increased the number of embryos formed when compared to the untreated AGPs. Chitinase is an enzyme able to hydrolyse the glycosidic bounds between acetylglucosamine and glucosamine. For the determination of an AGP that showed sensitivity to chitinase and the formed products, AGPs have been isolated from carrot cell culture medium and carrot seeds. In the AGPs that were isolated from the cell culture no glucosamine could be detected. The seed AGP extract contained a very low concentration of glucosamine. After fractionation of the seed AGP extract it was not possible to detect glucosamine in the obtained fractions. It could be possible that the glucosamine containing compound present in crude AGP extracts was coprecipitated with Yariv phenylglycoside during the isolation of AGPs.

AGPs that were isolated from carrot cell cultures and the cell walls of different carrot tissues show in general different molecular weight fractions. Linkage analysis of the carbohydrates of two main fractions and additional protein analysis showed that both fractions posses the chemical characteristics of AGPs. Analysis of AGPs that were isolated with Yariv shows a large variation in sugar composition, depending on the purification procedure used. When AGPs from carrot cell culture medium and cell wall fractions were further purified with copper ions, galacturonic acid rich fractions were identified. Homogalacturonan hydrolysing enzymes were used to test whether the galacturonic acid was organised as homogalacturonan or present in the side chains of the AGPs. Homogalacturonan is a characteristic structural element of

pectin. Oligogalacturonans were found after incubation of the galacturonic acid rich AGP fractions with polygalacturonase and pectin methylesterase. This result indicates that the galacturonic acid present in AGPs is organised as homogalacturonan. AGPs that were isolated from carrot tap root cell walls also showed a galacturonic acid rich fraction that was sensitive to homogalacturonan hydrolysing enzymes.

The chemical analysis of carrot cell wall AGPs from two different tissues showed that a small fraction of AGPs is present that contains galacturonic acid in the form of homogalacturonan. The interaction of AGPs and pectin has been suggested earlier and could be due to non-covalent interactions or a covalent linkage. The galacturonic acid rich AGP fractions were isolated from the cell walls with EDTA buffer and it is very unlikely that the interaction between AGPs and pectin found in this study is accomplished by ionic interactions.

This research has shown that AGP-pectin complexes exist in carrot tissues and this finding could be a starting point for a more precise determination of the linkage between AGPs and pectin.

Samenvatting

Arabinogalactaan eiwitten (AGPs) zijn eiwitten met een hoog gewichtspercentage aan suikerpolymeren. De suikerpolymeren van AGPs bestaan naast galactose en arabinose uit andere neutrale suikers en uronzuren. AGPs komen algemeen in het plantenrijk voor en worden in alle delen van de plant aangetroffen. Voor het isoleren van AGPs kan Yariv phenylglycoside worden toegepast dat een complex kan vormen met AGPs. Yariv phenylglycoside is een synthetisch molecuul en door het toevoegen ervan aan bijvoorbeeld celculturen of jonge kiemplanten is indirect aangetoond dat AGPs een biologische functie bezitten. Celculturen kunnen worden toegepast voor de studie naar de functie van AGPs in de embryogenese. Onder bepaalde groeicondities kunnen cellen van een celcultuur zich differentiëren tot planten en door de condities te variëren kan het effect hiervan op de embryo's worden onderzocht. Door het toevoegen van AGPs aan celculturen kan het aantal embyo's dat wordt gevormd gemanipuleerd worden.

Het onderzoek dat is beschreven in dit proefschrift laat zien dat met name AGPs geïsoleerd uit zaden van de peen toegevoegd aan een celcultuur in staat zijn om het aantal embryo’s te laten toenemen. Een voorbehandeling van zaad AGPs met chitinase verhoogt het aantal gevormde embryo's. Chitinase is een enzym dat glycosidische bindingen tussen acetylglucosamine en glucosamine kan hydrolyseren. Vervolgens werd gezocht naar een AGP die gevoelig is voor chitinase en de identificatie van een fragment dat door incubatie met chitinase ontstaat. Hiertoe werden AGPs geïsoleerd uit het medium van een celcultuur en uit de zaden van de peen. In het AGP extract uit het medium kon geen glucosamine worden aangetoond. Het AGP extract van de zaden bevatte een zeer lage concentratie glucosamine. Nadat de zaad AGP fractie met glucosamine verder werd gefractioneerd kon geen glucosamine meer worden aangetoond. Het is mogelijk dat een kleine hoeveelheid chitinase gevoelig materiaal dat glucosamine bevat met de eerste AGP extractie is gecoprecipiteerd.

Een isolatie van AGPs met Yariv uit medium van peen of de celwanden van diverse peen weefsels bevat doorgaans verschillende gewichtsfracties. Bindingsanalyse van de suikers in twee grote fracties en eiwit analyse van een medium en zaad AGP extract heeft aangetoond dat beide gewichtsfracties de chemische kenmerken van AGPs bezitten. Suikeranalyse van AGPs laat zien dat er, afhankelijk van de verdere zuivering, een grote variatie kan bestaan in AGPs die worden geisoleerd met Yariv

phenylglycoside. De analyse van AGPs welke werden geïsoleerd uit medium en uit celwand fracties van een peen celcultuur en een verdere zuivering met koperionen resulteerde in de karakterisatie van galacturonzuur rijke AGP fracties. Om aan te tonen of het galacturonzuur voorkomt in de zijketens van de AGPs of als hoofdketen in de vorm van homogalacturonaan zijn enzymen toegepast die homogalacturonaan kunnen hydrolyseren. Homogalacturonaan is karakteristiek voor het celwand-polymeer pectine. Na incubatie van galacturonzuurrijke AGP fracties met endopolygalacturonase en pectine methylesterase konden oligogalacturoniden worden aangetoond. Dit resultaat bewijst dat galacturonzuur in deze AGP fracties voorkomt als homogalacturonaan. Vervolgens werden AGPs geïsoleerd uit celwandfracties van de winterpeen waarbij ook een galacturonzuurrijke AGP fractie kon worden gezuiverd. Deze galacturonzuurrijke fractie was gevoelig voor de incubatie met homogalacturonaan hydrolyserende enzymen.

De resultaten van de chemische analyse van AGPs laten zien dat er een kleine fractie AGPs aanwezig is in de celwanden van verschillende peen weefsels dat gebonden is aan homogalacturonaan. De interactie tussen AGPs en pectine is vaker gesuggereerd en voorgesteld als een non-covalente interactie of covalente binding. Door de isolatie van celwandfracties met EDTA buffer kan worden uitgesloten dat de AGP-pectine interactie in de onderzochte fracties tot stand komt door ionische bindingen. Met dit onderzoek is een begin gemaakt met de karakterisering van de binding tussen AGPs en pectine.

Nawoord

Bij het tot stand komen van dit proefschrift heb ik van veel mensen hulp gehad. Ik wil mijn promotor Sacco de Vries bedanken voor het vertrouwen dat hij in mij had door het geven van een kans om aan dit project te werken. De wijze waarop een onderzoeker systematisch te werk moet gaan heeft hij mij duidelijk gemaakt en ik heb daar veel van geleerd. Van mijn co-promotor Henk Schols heb ik de leerzame werkbesprekingen, het snelle nakijkwerk van mijn schrijfwerk en de persoonlijke dynamiek altijd erg gewaardeerd. Henk heeft mij laten groeien in het onderzoek van koolhydraten. Halverwege het project werd Fons Voragen als tweede promotor bij het project betrokken die altijd bruikbare opmerkingen had op mijn manuscripten.
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Peter

Curriculum Vitae

Peter Immerzeel werd op 1 december 1969 geboren te Middelburg. Na het volgen van lager en middelbaar technisch onderwijs met het materiaal hout als rode draad, werd na een stage aan het Houtinstituut TNO te Delft in 1991 het besluit genomen om door te studeren in de houtkunde. De studie aan de Internationale Agrarische Hogeschool Larenstein te Velp werd halverwege verwisseld voor de studie bosbouw aan de toenmalige Landbouwuniversiteit van Wageningen. Van 1993 tot 1997 werd de studie bosbouw afgelegd met de oriëntatie chemische houttechnologie. Een eerste afstudeervak werd gedaan bij Reyes Sierra-Alvarez met als onderwerp de biologische afbreekbaarheid van inhoudstoffen van naaldhout. Een tweede afstudeervak werd gedaan bij AnneMie Emons naar de structuur en de chemische samenstelling van de celwanden van de wortelharen van heggewikke. Tijdens een stage op de Universiteit van Missouri (Columbia, USA) werd bij Tobias Baskin gewerkt aan de structuur van Arabidopsis celwanden. In september 1997 behaalde hij het doctoraal diploma en werd vervolgens een half jaar gewerkt bij de sectie houtkunde van de Vakgroep Bosbouw aan de Landbouwuniversiteit Wageningen als toegevoegd onderzoeker. Daarna heeft hij een jaar gewerkt als houtkundig adviseur bij de Stichting Robinia in Wageningen.

In het najaar van 1999 werd aangevangen met het promotieonderzoek bij de vakgroep Moleculaire Biologie en dat heeft geleid tot dit proefschrift.

Sinds december 2004 is hij postdoc op het Max Planck Instituut voor Moleculaire Plantenfysiologie in Golm (Berlijn) en werkt daar in de onderzoeksgroep van Markus Pauly aan celwanden.