Running head title: β-Gal-Yariv binds to β-1,3-galactan

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β-Galactosyl Yariv reagent binds to the β-1,3-galactan of arabinogalactan-proteins

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ABSTRACT

Yariv phenylglycosides, 1,3,5-tri-(p-glycosoxyphenylazo)-2,4,6-trihydroxybenzene, are a group of chemical compounds, which selectively bind to arabinogalactan-proteins (AGPs), a type of plant proteoglycan. Yariv phenylglycosides are widely used as cytochemical reagents to perturb the molecular functions of AGPs, as well as for the detection, quantification, purification, and staining of AGPs. However, the target structure in AGPs to which Yariv phenylglycosides bind has so far not been determined. Here, we identify the structural element of AGPs required for the interaction with Yariv phenylglycosides by stepwise trimming of the arabinogalactan moieties using combinations of specific glycoside hydrolases. Whereas the precipitation with Yariv phenylglycosides (Yariv reactivity) of radish root AGP was not reduced after enzyme treatment to remove α-L-arabinofuranosyl and β-glucuronosyl residues and β-1,6-galactan side chains, it was completely lost after degradation of the β-1,3-galactan main chains. In addition, Yariv reactivity of gum arabic, a commercial product of acacia AGPs, increased rather than decreased during the repeated degradation of β-1,6-galactan side chains by Smith degradation. Among various oligosaccharides corresponding to partial structures of AGPs, β-1,3-galactooligosaccharides longer than β-1,3-galactoheptaose exhibited significant precipitation with Yariv in a radial diffusion assay on agar. A pull-down assay using oligosaccharides crosslinked to hydrazine beads detected interaction of β-1,3-galactooligosaccharides longer than β-1,3-galactopentaose with Yariv phenylglycoside. To the contrary no interaction with Yariv was detected for β-1,6-galactooligosaccharides of any length. Therefore we conclude that Yariv...
phenylglycosides should be considered specific binding reagents for β-1,3-galactan chains longer than five residues, and seven residues are sufficient for crosslinking, leading to precipitation of the Yariv phenylglycosides.
INTRODUCTION

Arabinogalactan-proteins (AGPs) are a type of plant proteoglycans consisting of a hydroxyproline-rich core protein and large arabinogalactan (AG) moieties (Fincher et al., 1983; Nothnagel, 1997). Although there are many molecular species of AG differentiated by their core-proteins, the AG moieties commonly comprise β-1,3-galactan main chains and β-1,6-galactan side chains, to which L-arabinose (L-Ara) and other auxiliary sugars such as glucuronic acid (GlcA), 4-O-methyl-GlcA (4-Me-GlcA), L-fucose, L-rhamnose, and xylose are attached (Fincher et al., 1983; Nothnagel, 1997; Seifert and Roberts, 2007). A commercial product of AGPs prepared from the acacia tree is known as gum arabic and utilized as a food stabilizer. In the Japanese herbal remedy Juzen-Taiho-To, AGs from Astragalus membranaceus are the active ingredient (Kiyohara et al., 2002; Majewska-Sawka and Nothnagel, 2000). In intact plants, AGPs are implicated in various physiological events and serve as extracellular constituents and signaling molecules. For instance, an AGP from stylar transmitting tissue attracts pollen tubes and stimulates their elongation in tobacco (Nicotiana tabacum) (Cheung et al., 1995).

Yariv phenylglycosides {chemical name, 1,3,5-tri-(p-glycosyloxyphenylazo)-2,4,6-trihydroxybenzene} are a group of chemical compounds, which were initially developed as carbohydrate antigens for the purification of anti-glycoside antibody and sugar-binding protein (Yariv et al., 1962, 1967a). It then turned out that Yariv phenylglycosides specifically precipitate AGPs (Yariv et al., 1967b; Jermyn et al., 1975). The specific interaction of AGPs with Yariv
phenylglycosides forming brown-red precipitate is called Yariv reactivity and has been recognized as an important criterion in the definition of AGPs, even though a number of AGPs do not exhibit Yariv reactivity. Nevertheless, the structure involved in the interaction with Yariv phenylglycoside is presumed to be conserved in many AGPs. The interaction of Yariv phenylglycosides with AGP depends on the glycosyl residues attached to the phenylazo-trihydroxybenzene core. In particular $\beta$-glucosyl Yariv phenylglycoside ($\beta$-Glc-Yariv) and $\beta$-galactosyl Yariv phenylglycoside ($\beta$-Gal-Yariv) bind to AGPs, whereas $\alpha$-Glc-Yariv and $\alpha$-Gal-Yariv do not bind to AGPs (Jermyn et al., 1975; Larkin 1977, 1978; Nothnagel and Lyon, 1986). Because of the specific interaction with the $\beta$-glycosyl Yariv phenylglycosides ($\beta$-Yarivs), AGPs were formerly called “$\beta$-lectins” (Jermyn et al., 1975; Nothnagel and Lyon, 1986; Glesson and Jermyn, 1979).

The $\beta$-Yarivs are useful tools for staining, detection, and quantification of AGPs. Using $\beta$-Glc-Yariv, “$\beta$-lectins” were shown to exist in angiosperm, gymnosperm, fern, moss, and liverwort, illustrating the wide distribution of AGPs in the plant kingdom (Jermyn et al., 1975; Clarke et al., 1978). In addition, $\beta$-Yarivs are also used as chemical reagents in the purification of AGPs. A non-classical AGP, xylogen that is a signaling molecule inducing the differentiation to tracheary elements, has been purified from the culture medium of Zinnia ($Zinnia$ $elegans$) cells by precipitation with $\beta$-Glc-Yariv (Motose et al., 2004). As the treatment with $\beta$-Yarivs causes perturbation of various physiological processes in plants, $\beta$-Yarivs are reliable cytochemical reagents to explore AGP functions. Application of $\beta$-Yarivs to cultured cells of Arabidopsis ($Arabidopsis$
*thaliana* induced programmed cell death, demonstrating the involvement of AGPs in the determination of cell fate (Gao and Showalter, 1999). In tobacco cultured cells, the treatment with \( \beta \)-Yarivs has indicated a possible role of AGPs in the orientation of cortical microtubules and the polymerization of F-actin (Sardar et al., 2006).

Although Yariv phenylglycosides have been extensively utilized in studies of AGPs over 40 years, the identification of the target structures on AGPs required for \( \beta \)-Yariv reactivity, remains elusive (Nothnagel, 1997; Seifert and Roberts, 2007). It has been proposed that \( \beta \)-Yarivs bind to the hydroxyproline-rich core protein based on the observation that deglycosylation treatment with hydrogen fluoride did not abolish the Yariv reactivity of gum arabic and a tobacco AGP (Akiyama et al., 1984). To the contrary, other reports have asserted the importance of the carbohydrate moieties for Yariv reactivity (Komalavias et al., 1991). However, with regard to the specific carbohydrate structure required for interaction with \( \beta \)-Yarivs, the results were not always consistent: neither \( \alpha \)-L-arabinofuranosyl residues nor \( \beta \)-1,6-galactan side chains were found to be involved in Yariv reactivity of AGPs from *Gladiolus*, radish, and grape (Glesson and Clarke, 1979; Tsumuraya et al., 1987; Saulnier et al., 1992); partial acid hydrolysis to remove \( \alpha \)-L-arabinofuranosyl residues diminished Yariv reactivity of a rose AGP (Komalavias et al., 1991); mugwort pollen *O*-glycans consisting of a \( \beta \)-1,6-galactan core and branched \( \alpha \)-L-arabinofuranosyl side chains precipitated with \( \beta \)-Glc-Yariv (Leonard et al., 2005). Accordingly, it has also been suggested that Yariv reactivity depends on the overall physical and chemical properties rather than a specific structural feature of AGPs.
In the present study, we demonstrate that the peptide component of AGPs is not required for Yariv reactivity. By sequentially trimming the AG moieties of AGPs with sets of specific glycoside hydrolases we show that β-Gal-Yariv binds to the β-1,3-galactan main chains of radish root AGP. We confirm that β-1,6-galactan side chains are not necessary for Yariv reactivity and we identify β-1,3-galactopentaose (β-1,3-Gal5) as the smallest carbohydrate structure to interact with β-Gal-Yariv, while we show that β-1,3-galactoheptaose (β-1,3-Gal7) or longer β-1,3-galactosyl chains are required for the formation of insoluble precipitate with Yariv phenylglycoside. Based on the computational modeling, a possible interaction mechanism between β-Gal-Yariv and β-1,3-galactan is suggested.

RESULTS

Yariv Reactivity of AGP Core-protein

We first investigated whether the hydroxyproline-rich core protein is responsible for the interaction with Yariv phenylglycosides as proposed by Akiyama et al. (Akiyama et al., 1984). The β-1,3-galactan main chains are attached to Hyp residues of the core-protein via O-glycosidic linkages (Tan et al., 2004). The peptide linkages are susceptible to base-treatment, but the glycosyl linkages are resistant and therefore smaller AG molecules linked to Hyp residues (Hyp-AG) can be released by base-hydrolysis of AGP. Base-treatment of gum arabic and consecutive gel-permeation chromatography yielded three Hyp-AG fractions, designated F2, F3, and F4 (Fig. 1A).
Yariv reactivity of the Hyp-AG fractions was assessed by radial gel diffusion assay, and was quantified based on the area of the halo formed in agarose gel containing β-Gal-Yariv (van Holst and Clarke, 1985). These fractions showed clear Yariv reactivity, although the reactivity was relatively weak compared to native gum arabic (fractions F2, F3 and F4 were 28, 23 and 23% respectively, as reactive with β-Gal-Yariv as the intact gum arabic based on sugar weight, Figs. 1B and 3). Since these fractions contain AG linked to Hyp, these results indicate that the core protein may not be required for Yariv reactivity.

Effect of Sequential Enzymatic Carbohydrate Trimming on Yariv Reactivity of Radish AGP

To investigate the carbohydrate component of AGPs that is important for Yariv reactivity, we sequentially trimmed the carbohydrate moieties of radish root AGP. Like in many other AGPs (Tryfona et al., 2010; 2012), the carbohydrate moieties of radish root AGP consist of β-1,3-galactan main chains and β-1,6-galactan side chains, to which L-Ara and 4-O-Me-GlcA are attached (Fig. 2) (Tsumuraya et al., 1988). The trimming was accomplished with the aid of four arabinogalactan-specific glycoside hydrolases, α-L-arabinofuranosidase from Neurospora crassa (NcAraf1), β-glucuronidase from Aspergillus niger (AnGlcAase), endo-β-1,6-galactanase from Trichoderma viride (Tv6GAL), and exo-β-1,3-galactanase from Irpex lacteus (Il1,3GAL) (Takata et al., 2010; Konishi et al., 2008; Kotake et al., 2009). All proteins were expressed in Pichia yeast (Pichia pastoris) and purified by chromatography. Trimmed AGP1, 2, 3, and 4

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were prepared by combinational digestion of native AGP with these enzymes and subsequent gel-permeation chromatography to remove mono- and oligosaccharides released from the AGP. Structural analysis confirmed that α-L-arabinofuranosyl residues were removed in AGP1 resulting from treatment of native AGP with NcAraf1; all α-L-arabinofuranosyl residues and about half of the uronosyl residues were lost in AGP2 produced by treatment with NcAraf1 and AnGlcAase; β-1,6-galactan side chains were reduced to short stubs in AGP3 digested with NcAraf1, AnGlcAase, and Tv6GAL; AGP4, the product of hydrolysis by all four enzymes including the exo-β-1,3-galactanase, lacked long main chains and thus most of its AG moiety (Fig. 1; Table 1). Yariv reactivity of the trimmed AGPs was monitored with the radial gel diffusion assay as described above. Surprisingly, amongst the trimmed AGPs, only trimmed AGP4 lost Yariv reactivity toward β-Gal-Yariv, whereas trimmed AGP1, 2, and 3 retained the reactivity (Fig. 3). Compared with native and trimmed AGP1 and 2, trimmed AGP3 exhibited relatively high Yariv reactivity based on sugar weight (Table 2). None of the trimmed AGPs showed any reactivity toward α-Gal-Yariv (Fig. S1).

Mono- and oligosaccharide fractions, including L-Ara, 4-Me-GlcA, Gal, and β-1,6-Gal$_2$ released from the AGP during the trimming did not exhibit any Yariv reactivity at all (Fig. S1). The results suggested that β-Gal-Yariv binds to the β-1,3-galactan main chains but not to α-L-arabinofuranosyl and 4-Me-glucuronosyl residues and β-1,6-galactan side chains.

**Effect of Removal of β-1,6-Galactan Side Chains on Yariv Reactivity**
We next examined the effect on Yariv reactivity of the degradation of β-1,6-galactan side chains of gum arabic, a commercial mixture of AGP, by Smith degradation, to exclude the possible influence of the large number of short stubs of β-1,6-galactan side chains attached to β-1,3-galactan main chains left even after enzymatic trimming in AGP3 (Fig. 3). Smith degradation can break sugar residues with vicinal hydroxyl groups, and therefore O-3-linked hexoses including β-1,3-galactosyl residues would survive this treatment, while terminal sugars and hexoses in any other linkage such as β-1,6-galactosyl residues would be degraded. As a result, several cycles of Smith degradation are capable of removing the β-1,6-galactan side chains of gum arabic while preserving the β-1,3-galactan main chains (Tsumuraya et al., 1990). In the present study, β-galactan I, II, III, and IV were prepared from gum arabic by single, double, triple, and quadruple Smith degradation, respectively. On the basis of structural analysis, the proportion of β-1,3-galactan main chains in native gum arabic and β-galactan I, II, III, and IV were calculated to be 31, 49, 58, 91, and 93%, respectively (Fig. S2) (Akiyama et al., 1984; Kotake et al., 2009; Tsumuraya et al., 1990; Defaye and Wong, 1986). Although most of the short stubs of β-1,6-galactan side chains were removed in β-galactan IV, we could not obtain β-1,3-galactan completely lacking β-1,6-galactosyl branches, as the fourth Smith degradation was not effective in further degrading β-1,6-galactan side chains.

Yariv reactivity of gum arabic was not diminished by the repeated Smith degradation treatments (Fig. 4; Table 2). On the contrary, β-galactans III and IV, which have a high proportion of β-1,3-galactan, exhibited significantly higher reactivity than native gum
Yariv reactivity of β-galactan IV was lost by treatment with endo-β-1,3-galactanase from winter mushroom, FvEn3Gal (Kotake et al., 2011). β-Galactan IV also failed to interact with α-Gal-Yariv (Fig. S1). Taken together these data strongly support the hypothesis that β-Gal-Yariv binds to the β-1,3-galactan main chains but not to β-1,6-galactan side chains. Our findings also suggest that neither branching residues of β-1,3-galactan nor remnant β-1,6-galactan side chains are important for Yariv reactivity, since β-galactans III and IV, which have considerably fewer β-1,6-galactosyl branches than intact gum arabic (Table 2) exhibited higher Yariv reactivity.

Yariv Precipitation with β-1,3-Galactooligosaccharides

To address the question of how many β-1,3-galactosyl residues are necessary for Yariv precipitation activity, we next examined the Yariv reactivity of a series of β-1,3-galactooligosaccharides (β-1,3-Galₙ). The oligosaccharides β-1,3-Gal₄, β-1,3-Gal₅, β-1,3-Gal₆, β-1,3-Gal₇, β-1,3-Gal₈, and β-1,3-Gal₉ were prepared from β-galactan IV by partial acid hydrolysis and fractionation on gel-permeation chromatography (Fig. 4). Methylation analysis showed that β-1,3-Gal₇, β-1,3-Gal₈, and β-1,3-Gal₉ still have 0.5, 0.8, and 1.1 β-3,6-galactosyl branches (→3,6Galp→) per molecule on average, which are derived from remnant side chains of β-galactan IV (Table S1). Among the oligosaccharides, only β-1,3-Gal₇, β-1,3-Gal₈, and β-1,3-Gal₉ exhibited precipitation activity with β-Gal-Yariv and β-Glc-Yariv, whereas the shorter oligosaccharides,
β-1,3-Gal₄, β-1,3-Gal₅, β-1,3-Gal₆ did not precipitate with either of the two Yariv phenylglycosides tested (Table 3, Figs. 6 and S3). Compared with β-galactan IV, β-1,3-Gal₈ and β-1,3-Gal₉ exhibited relatively high Yariv reactivity (Figs. 3 and 6), which probably results from rapid diffusion of these oligosaccharides in the agarose gel during the assay. Similarly to β-galactan IV, β-1,3-Gal₉ lost Yariv reactivity when treated with FvEn3Gal.

Other oligosaccharides such as β-1,6-Gal₆, β-GlcA-1,6-β-Gal-1,6-Gal, and α-L-Araβ-1,3-β-Gal-1,6-Gal corresponding to partial structures of β-1,6-galactan side chains of AGPs did not exhibit any Yariv reactivity (Fig. S3), demonstrating that β-1,6-galactan side chains and attached α-L-arabinofuranosyl and β-glucuronosyl residues are not necessary for Yariv reactivity.

**Detection of Interaction of β-1,3-Galₙ with β-Gal-Yariv**

The formation of an insoluble precipitate in the agarose gel likely results from crosslinking of the glycan and Yariv, and therefore may require binding of at least two Yariv phenylglycosides to each oligosaccharide. It is also conceivable that a weak interaction of a short β-1,3-Galₙ with Yariv phenylglycoside may not be detected. In order to detect weak interactions with Yariv phenylglycoside, a pull-down assay using β-1,3-Gal₅, β-1,3-Gal₆, β-1,3-Gal₇, β-1,3-Gal₈, and β-1,3-Gal₉ crosslinked to hydrazide beads was performed. The beads were suspended in β-Gal-Yariv solution at the concentration from 6.25 to 400 µg ml⁻¹, and Yariv phenylglycoside co-precipitated with beads was observed. β-1,3-Gal₆-, β-1,3-Gal₇-, β-1,3-Gal₈-, and β-1,3-Gal₉-beads formed
an apparent red-color precipitate (Fig. 7). Since the β-1,3-Gal₆-beads did not precipitate β-Gal-Yarov except at the higher β-Gal-Yarov concentrations (above 100 µg ml⁻¹), the interaction between β-Gal-Yarov and β-1,3-Gal₆-beads may be weak compared with longer β-1,3-Gal₅-beads. β-1,3-Gal₅-beads did not precipitate β-Gal-Yarov at all. Given that a galactosyl residue at the reducing end of the β-1,3-Gal₆ is lost in the crosslinking reaction with the bead hydrazide group we conclude that β-1,3-Gal₅ is able to interact with β-Gal-Yarov. None of β-1,3-Gal₅-beads precipitated α-Gal-Yarov. Consistent with the radial gel diffusion assay findings, no interaction between β-1,6-Gal₇₈-beads (a mixture of β-1,6-Gal₇-beads and β-1,6-Gal₈-beads) and β-Gal-Yarov was detected. Combining all the data from the base hydrolysis of the peptide backbone, the stepwise trimming of the carbohydrate component of AGPs and the pull down assay, we propose that β-Yarivs should be considered specific binding reagents for β-1,3-galactan chains longer than five residues.

**Computational Modeling of β-1,3-Galactan**

There are only few reports on the conformation of β-1,3-galactan; it is known however, that β-1,3-glucan has a right-handed helical structure (Sletmoen and Stokke, 2008). In order to address the question of what mechanism might be responsible for the interaction between β-Yarivs and β-1,3-galactan, molecular dynamics (MD) simulations for β-1,3-galactan were carried out by means of the AMBER11 software package with the all-atom GLYCAM06 force field. In the computer simulations, a straight β-1,3-galactan consisting of 16 galactosyl residues was put into a virtual water box, and
the stable conformation was predicted based on the physical movement of the molecule. The simulations suggested that β-1,3-galactan itself forms a right-handed helical structure that has seven or eight galactosyl residues per turn (Fig. 8, Fig. S4). The helix is somewhat looser than those reported for β-1,3-glucan and larch AG, which both have six galactosyl residues per turn (Chandrasekaran and Janaswamy, 2002). The modeled β-1,3-galactan was flexible in water, undergoing repeated minor conformational changes. Although the modeled structure did not reveal a specific binding site for β-Yarivs, the inside of the helix is relatively hydrophobic, because, as is the case for the β-1,3-glucan helix, OH groups of galactosyl residues other than C-2 OH are facing outward in water (Sletmoen and Stokke, 2008; Miyoshi et al., 2004).

DISCUSSION

The present study for the first time indicates that the β-1,3-galactan main chains of AGPs are a target structure for β-Gal Yariv. This provides an explanation for Yariv binding to most AGPs and AGs, not only the radish root AGP and gum arabic studied here, since the β-1,3-galactan main chain is a conserved carbohydrate structure of AGPs and AGs (Fincher et al., 1983; Nothnagel, 1997; Seifert and Roberts, 2007; Ellis et al., 2010). The core protein is not the target structure, since Hyp-AGs released from gum arabic by degradation of the peptide linkages of the core proteins retained Yariv reactivity. Most importantly, sequential trimming of the carbohydrate component of AGPs with arabinogalactan specific glycoside hydrolases, while keeping the core protein intact abolished Yariv reactivity of AGPs (trimmed AGP4). On the contrary,
β-1,3-galactan (β-galactan IV), and β-1,3-Gal₉ precipitated with β-Gal-Yariv but this property was abolished when these molecules were broken down with the FvEn3Gal enzyme. The present results are consistent with previous reports suggesting that neither α-L-arabinofuranosyl residues nor β-1,6-galactan side chains are involved in Yariv reactivity (Glesson and Clarke, 1979; Tsumuraya et al., 1987; Saulnier et al., 1992). However, several other reports have proposed the interaction of β-Yarivs with core proteins or α-L-arabinofuranosyl residues of AGPs. It is unclear why these results are inconsistent with ours, but we suggest that deglycosylation with hydrogen fluoride partially left β-1,3-galactans of the AGPs in the work of Akiyama et al. (1985). Acid hydrolysis of the AG moieties of rose AGP to remove α-L-arabinofuranosyl residues might partially degrade β-1,3-galactans as well as α-L-arabinofuranosyl residues, diminishing the Yariv reactivity (Komalavias et al., 1991).

Our computational modeling proposed a helical structure for β-1,3-galactan that has seven or eight galactosyl residues per turn. The inside cavity of the helix is relatively hydrophobic and therefore a candidate site for hydrophobic interaction with the phenylazo-trihydroxybenzene core of β-Yarivs. A previous equilibrium sedimentation analysis has demonstrated strong self-association of Yariv phenylglycosides in water (Yariv et al., 1962; Wood et al., 1978). Further Yariv hydrophobic interactions may therefore cause the formation of large insoluble β-1,3-galactan-Yariv complexes. Our modeling did not explain the specific interaction of β-1,3-galactan with β-glucosyl or β-galactosyl residues of Yariv phenylglycosides, but we would like to propose that these likely bind to Gal residues in the helix. To demonstrate the molecular mechanism for
Yariv reactivity, physico-chemical analysis of the interaction between β-1,3-galactan and β-Gal-Yariv will now be important.

There are also alternative proposed structures of some AGPs to our model of long chain β-1,3-galactan. Tan et al. (2010) propose a repetitive main chain of two β-1,3-galactosyl residues and a kink of β-1,6-linked Gal, whereas others postulate a kink (of unknown nature) in the main chain spaced at seven or more Gal residues (Ellis et al., 2010; Churms et al., 1983; Bacic et al., 1987). Although we did not examine the Yariv reactivity of such a kinked main chain, a kink spaced at seven or more residues might not disrupt the helical structure of the main chain, particularly if it is a kink of β-1,6-linked Gal since the β-1,6-linkage is generally flexible compared with other types of linkages (Rees et al., 1971). On the other hand, the present results do not appear consistent with structural models for AG moieties that have a short kinked main chain but are nevertheless precipitated with β-Gal-Yariv (Tan et al., 2010; Xu et al., 2010). Additionally, in the case of mugwort pollen, O-glycans were precipitated by β-Glc-Yariv although they have been reported to lack β-1,3-galactan (Leonard et al., 2005). We cannot exclude the possibility that some AGPs have a target structure for β-Yarivs other than β-1,3-galactan, but our data suggests that re-investigation for the presence of β-1,3-galactan main chains longer than β-1,3-Gal7 in these AGs is necessary.

Yariv reactivity requires a longer β-1,3-galactosyl chain to make an insoluble precipitate in the agarose gel than it does to bind to the oligosaccharide in the pull down assay. β-1,3-Gal7 is the smallest oligosaccharide precipitable by Yariv. On the other
hand β-1,3-Gal₆-beads beads are reactive in the Yariv pull-down assay, suggesting β-1,3-Gal₅ is the smallest carbohydrate structure for Yariv-interaction, as β-1,3-Gal₆-beads have lost a galactosyl residue at the reducing end. Just as precipitation of proteins with antibodies requires a multivalent interaction, longer oligosaccharides may be required for multiple Yariv glycoside interaction and consequent precipitation.

In view of the fact that β-Yarivs stain tissues of various higher plants including gymnosperm, fern, moss, and liverworts (Jermyn and Yeow, 1975; Clarke et al., 1978), we propose that β-1,3-galactan, rather than other AG glycan structures or certain features of the core protein, is the widely distributed characteristic distinguishing AGPs in higher plants. The former name “β-lectin” for AGPs is indicative of their interaction with β-glucosyl and β-galactosyl residues (Pennell et al., 1989). These residues can be found on several cell wall polysaccharides such as cellulose (β-1,4-glucan), β-1,3:1,4-glucan, callose (β-1,3-glucan), xyloglucan, pectic β-1,4-galactan, and AGP. It is therefore conceivable that the AGP β-1,3-galactan may interact with any of these polysaccharides. Consistent with this hypothesis, several groups have reported co-purification of AGPs with other cell wall polysaccharides (Baldwin et al., 1993; Serpe and Nothnagel, 1995). Since most AGPs are secreted and first localize on the cell surface with their glycosylphosphatidylinositol (GPI) anchors (Fincher et al., 1983, Sherrier et al., 1999), they may play a role in the modification of cell wall architecture on the cell surface through the interaction of β-1,3-galactan with cell wall polysaccharides. The proposed interaction between β-1,3-galactan and other cell wall components should provide important hints in the elucidation of the many functions of
AGPs.

**MATERIALS AND METHODS**

**Preparation of released Hyp-AGs**

Base-treatment of gum arabic was performed according to the previous studies (Tan et al., 2004; 2010). Peptide linkages of core proteins included in gum arabic were hydrolyzed in 0.44 M NaOH at 105°C for 18 h. The hydrolysate was neutralized with acetic acid to pH 7.0, and then Hyp-AGs were purified by gel-permeation chromatography. The sugar content in the fractions was determined by the phenol-sulfuric acid method (Dubois et al., 1956). The Hyp-AG fractions, F2, F3, and F4, were used for radial gel diffusion assay for Yariv reactivity.

**Trimming of AGP**

NcAraf1, AnGlcAase, Tv6GAL, and Il1,3GAL were prepared as described (Takata et al., 2010; Konishi et al., 2008; Kotake et al., 2009). The purity of the enzymes has been confirmed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Radish root AGP was digested with NcAraf1, AnGlcAase, Tv6GAL, and Il1,3GAL in 25 mM acetate buffer (pH 4.0) at 37°C for 24 h. The AGP was applied onto a Sephadex G-15 column (2.4 x 23 cm, GE Healthcare) to separate it from released mono- and oligosaccharides. The structures of the trimmed AGPs were determined by methylation analysis with a Shimadzu gas chromatograph GC-6A fitted with a column (0.28 mm x 50 m) of Silar-10C (Hakomori, 1964; Albersheim et al., 1967). Content of uronic acid
was measured by a modified carbazole–sulfuric acid method (Galambos, 1967). In the hydrolysis of \( \beta \)-galactan IV and \( \beta \)-1,3-Gal\(_9\), recombinant FvEn3Gal, was used (Kotake et al., 2011).

**Assay of Yariv reactivity**

Yariv reactivity was determined by radial gel diffusion assay (van Holst and Clarke, 1985). The carbohydrate sample was applied to a gel plate containing 0.004% (w/v) \( \beta \)-Gal-Yariv, 75 mM NaCl, 0.01% (w/v) sodium azide, and 1% (w/v) agarose. The relative reactivity was quantified based on the halo area using gum arabic (Sigma, St. Louis, MO) as the standard, and expressed based on equal sugar amount.

**Smith degradation**

Smith degradation was performed as described previously (Tsumuraya and Hashimoto, 1987; Goldstein et al., 1956). Oxidation of gum arabic with 100 mM metaperiodate was carried out in the dark at 7°C for 4 days, and terminated by addition of 1,2-ethanediol. After neutralization with NaOH, the products were reduced with NaBH\(_4\) for 12 h and treated with 1 M trifluoroacetic acid (TFA) for 12 h. The products were washed with 70% (v/v) ethanol, ethanol, acetone, and petroleum ether.

**Preparation of oligosaccharides**

We prepared \( \beta \)-1,3-Gal\(_4\), \( \beta \)-1,3-Gal\(_5\), \( \beta \)-1,3-Gal\(_6\), \( \beta \)-1,3-Gal\(_7\), \( \beta \)-1,3-Gal\(_8\), and \( \beta \)-1,3-Gal\(_9\), from \( \beta \)-galactan IV by partial acid hydrolysis with 40 mM TFA at 100°C for
1 h, and purified them by gel-permeation chromatography (Fig. 5). Oligosaccharides \( \beta \)-1,6-Gal\(_4\), \( \beta \)-1,6-Gal\(_5\), \( \beta \)-1,6-Gal\(_6\), \( \beta \)-1,6-Gal\(_7\)\(_-8\), and \( \beta \)-1,6-Gal\(_9\)\(_-8\) were prepared from radish root AGP by treatment with NcAraf1, AnGlcAase, and Il1,3GAL, and separated on Bio-Gel P-2 column (Fig. S5). To confirm purity, the \( \beta \)-1,3-Gal\(_n\) and \( \beta \)-1,6-Gal\(_n\) were coupled at their reducing terminals with (ABEE) according to the method of Matsuura and Imaoka (Matsuura and Imaoka, 1988) and analyzed on a high-performance liquid chromatography (HPLC) system equipped with a TSKgel Amide-80 (4.6 x 250 mm; Tosoh, Tokyo, Japan) column (Fig. 5 and S5) as described previously (Kotake et al., 2004). Molecular mass of the oligosaccharides was ascertained by matrix-assisted laser desorption/ionization-time-of flight mass spectrometry, and their structures were examined by methylation analysis. Other oligosaccharides were prepared as described (Okemoto et al., 2003; Kuroyama et al., 2001).

**Pull-down assay**

\( \beta \)-1,3-Gal\(_5\), \( \beta \)-1,3-Gal\(_6\), \( \beta \)-1,3-Gal\(_7\), \( \beta \)-1,3-Gal\(_8\), \( \beta \)-1,3-Gal\(_9\), and \( \beta \)-1,6-Gal\(_7\)\(_-8\) crosslinked to beads were prepared with a GlycoBlot Glycan purification and labeling kit according to the manufacturers’ instruction (Sumitomo Bakelite, Tokyo, Japan). In brief, 50 nmole of each oligosaccharide were crosslinked to hydrazide groups on the beads, and free oligosaccharides were washed out from the beads. In the pull-down assay, a portion (app. 5 µl) of the beads was suspended in \( \beta \)-Gal-Yariv solution ranging from 6.25 to 400 µg ml\(^{-1}\) at 25°C for 4 h. After washing the beads with 1 ml of water thrice, co-precipitated Yariv phenylglycoside was observed.
Modeling

MD simulations were carried out by using the AMBER11 software package (Case et al., 2010) with the all-atom GLYCAM06 force field (Kirschner et al., 2008) for β-1,3-galactan. The electrostatic interactions were calculated with the particle mesh Ewald method and the cutoff was 8 Å. Using the LEAP module in AMBER 11, the structure was immersed in a truncated octahedral water box with a solvation shell of 12 Å thickness using the TIP3P model for water. The minimization procedure for solvated β-1,3-galactan consisted of two steps. In the first stage, the β-1,3-galactan was kept fixed, and positions of the water were minimized. The solvated structures were then subjected to 500 steps of steepest descent minimization followed by 500 steps of conjugate gradient minimization. During this minimization process, the β-1,3-galactan was kept fixed in its starting conformation using harmonic constraints with a force constant of 500 kcal/mol/Å². In the second stage, the entire system was minimized by 1000 steps of steepest descent minimization followed by 1500 steps of conjugate gradient minimization without the restraints. The minimized structure was then subjected to 20 ps of MD, using a 2 fs time step for integration. During the MD simulation, the system was gradually heated from 0 to 300 K using 10 kcal/mol/Å² weak positional restraints on the β-1,3-galactan. The SHAKE algorithm was used in which all bonds involving hydrogen are constrained. After the system was heated at constant volume with weak restraints on the complex, MD was performed for 10 ns
with a time step of 2 fs under constant volume/constant temperature (NVT ensemble) at 300 K. SHAKE was used to constrain bonds involving hydrogen, and the temperature was kept at 300 K with Anderson dynamics.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Table S1.** Structure of β-1,3-galactooligosaccharides.

**Supplemental Figure S1.** Yariv reactivity of trimmed AGPs, released mono- and oligosaccharides, and β-galactans.

**Supplemental Figure S2.** Average structures of native gum arabic and the β-galactans.

**Supplemental Figure S3.** Yariv reactivity of β-1,3-Gal<sub>n</sub> and other oligosaccharides.

**Supplemental Figure S4.** Three dimensional structure of β-1,3-galactan.

**Supplemental Figure S5.** Preparation of β-1,6-Gal<sub>n</sub>.
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FIGURE LEGENDS

Figure 1. Yariv reactivity of Hyp-AGs. (A) Hyp-AGs released from gum arabic by base-treatment were purified by gel-permeation chromatography. On the basis of the comparison of the elution profiles for base-treated gum arabic (open circle) and a mixture of gum arabic and Gal (closed circle), Hyp-AG fractions, F2, F3, and F4, were identified. The sugar content in the fractions was determined by the phenol-sulfuric acid method. $V_0$ and $V_i$ indicate void volume and inner volume, respectively. (B) Yariv reactivity of F2, F3, and F4 was examined by radial diffusion assay.

Figure 2. Structures of AG moieties of native and trimmed AGPs. Radish root AGP was subjected to sequential trimming with AG specific enzymes. Based on the results of methylation analysis, the structures of AG moieties of native AGP (A) and the trimmed AGP3 (B) and AGP4 (C) were inferred (Table 1) (Tsumuraya et al., 1988). The $\beta$-1,3-galactan main chain is shown in pink and $\beta$-1,6-galactan side chains are blue.

Figure 3. Yariv reactivity of trimmed AGPs. Yariv reactivity of trimmed AGPs was examined by radial diffusion assay. The amount of sugar applied is indicated at the bottom. No reactivity toward $\alpha$-Gal-Yariv was observed (Fig. S1).

Figure 4. Yariv reactivity of $\beta$-galactans. $\beta$-Galactans resulting from single ($\beta$-galactan I), double ($\beta$-galactan II), triple ($\beta$-galactan III), and quadruple Smith degradation
(β-galactan IV) of acacia gum were subjected to Yariv radial diffusion assay. Yariv reactivity of β-galactan IV treated with FvEn3Gal is also shown. The average structures of the β-galactans are shown in Fig. S2. No reactivity toward α-Gal-Yariv was observed (Fig. S1).

**Figure 5.** Preparation of β-1,3-Galₐₐ. A, β-1,3-Galₐ₀ oligosaccharides prepared from β-galactan IV by partial acid hydrolysis were separated by gel-permeation chromatography on Bio-Gel P2 column. β-1,3-Gal₇ (B), β-1,3-Gal₈ (C), and β-1,3-Gal₉ (D) were further purified on the same column. In the Yariv assay, the fractions indicated by arrowheads were used. E, To confirm purity, purified β-1,3-Galₐ₀ were coupled at their reducing terminals with ABEE and analyzed on the HPLC system.

**Figure 6.** Yariv reactivity of β-1,3-Galₐ₀. The chain length of β-1,3-galactan necessary for β-Gal-Yariv reactivity was investigated. In the assay of β-1,3-Gal₈ and β-1,3-Gal₉, smaller amounts of samples were used due to their high reactivity. None of them exhibited Yariv reactivity toward α-Gal-Yariv (Fig. S3).

**Figure 7.** Interaction of β-Gal-Yariv with oligosaccharides. For pull-down assays, β-1,3-Gal₅, β-1,3-Gal₆, β-1,3-Gal₇, β-1,3-Gal₈, β-1,3-Gal₉, and β-1,6-Gal₇₈ crosslinked to beads were suspended in β-Gal-Yariv solution ranging from 6.25 to 400 µg ml⁻¹ in concentration. As a control experiment, the beads were also suspended in 400 µg ml⁻¹ α-Gal-Yariv solution.
Figure 8. Computational modeling. The conformation of β-1,3-galactan consisting of 16 galactosyl residues in water was predicted by MD simulations. The simulations for the β-1,3-galactan were performed in a truncated octahedral TIP3P water box with a solvation shell of 12 Å thickness. Side (A) and top views (B) are shown. Note that the modeling may not correctly predict the conformation of the ends of the β-1,3-galactan chain, because of the edge effect.
Table 1. Structure of native and trimmed AGPs

<table>
<thead>
<tr>
<th>Residue</th>
<th>Native AGP</th>
<th>Trimmed AGPs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mole%</td>
<td>1</td>
</tr>
<tr>
<td>Ara/f→</td>
<td>19.2</td>
<td>1.0</td>
</tr>
<tr>
<td>→2Araf→</td>
<td>0.3</td>
<td>0.0</td>
</tr>
<tr>
<td>→5Araf→</td>
<td>3.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Fucp→</td>
<td>0.4</td>
<td>0.0</td>
</tr>
<tr>
<td>Galp→</td>
<td>7.4</td>
<td>16.5</td>
</tr>
<tr>
<td>→3Galp→</td>
<td>12.4</td>
<td>8.7</td>
</tr>
<tr>
<td>→6Galp→</td>
<td>13.9</td>
<td>29.1</td>
</tr>
<tr>
<td>→3,6Galp→</td>
<td>31.2</td>
<td>18.3</td>
</tr>
<tr>
<td>Uronic acids&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.2</td>
<td>11.3</td>
</tr>
<tr>
<td>Total&lt;sup&gt;c&lt;/sup&gt;</td>
<td>100.0</td>
<td>84.9</td>
</tr>
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</table>

<sup>a</sup>Proportions of methylated sugars are expressed in mol%.

<sup>b</sup>Content of uronic acids was determined by a modified carbazole-sulfuric acid method using GlcA as the standard.

<sup>c</sup>Total sugar content was determined by the phenol-sulfuric acid method and is expressed in % of that of native AGP.
Table 2. Relative Yariv reactivity of polysaccharides

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative reactivity (%)&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Gum arabic from acacia tree</td>
<td>100</td>
</tr>
<tr>
<td>β-Galactan I (gum arabic treated by single Smith degradation)</td>
<td>94</td>
</tr>
<tr>
<td>β-Galactan II (gum arabic treated by double Smith degradation)</td>
<td>81</td>
</tr>
<tr>
<td>β-Galactan III (gum arabic treated by triple Smith degradation)</td>
<td>248</td>
</tr>
<tr>
<td>β-Galactan IV (gum arabic treated by quadruple Smith degradation)</td>
<td>250</td>
</tr>
<tr>
<td>Native AGP from radish roots</td>
<td>72</td>
</tr>
<tr>
<td>Trimmed AGP1 (treated with NcAraf1)</td>
<td>72</td>
</tr>
<tr>
<td>Trimmed AGP2 (treated with NcAraf1 and AnGlcAase)</td>
<td>73</td>
</tr>
<tr>
<td>Trimmed AGP3 (treated with NcAraf1, AnGlcAase, and Tv6GAL)</td>
<td>102</td>
</tr>
<tr>
<td>Trimmed AGP4 (treated with NcAraf1, AnGlcAase, Tv6GAL, and II1,3GAL)</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Relative Yariv reactivity is expressed in % of that toward gum arabic based on the halo area.
Table 3. Relative Yariv reactivity of galactooligosaccharides

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative reactivity (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-1,3-Gal&lt;sub&gt;4&lt;/sub&gt;</td>
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</tr>
<tr>
<td>β-1,3-Gal&lt;sub&gt;5&lt;/sub&gt;</td>
<td>0</td>
</tr>
<tr>
<td>β-1,3-Gal&lt;sub&gt;6&lt;/sub&gt;</td>
<td>0</td>
</tr>
<tr>
<td>β-1,3-Gal&lt;sub&gt;7&lt;/sub&gt;</td>
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</tr>
<tr>
<td>β-1,3-Gal&lt;sub&gt;8&lt;/sub&gt;</td>
<td>68</td>
</tr>
<tr>
<td>β-1,3-Gal&lt;sub&gt;9&lt;/sub&gt;</td>
<td>100</td>
</tr>
<tr>
<td>β-1,6-Gal&lt;sub&gt;6&lt;/sub&gt;</td>
<td>0</td>
</tr>
<tr>
<td>β-1,6-Gal&lt;sub&gt;7&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>β-1,6-Gal&lt;sub&gt;8&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Relative Yariv reactivity is expressed in % of that toward β-1,3-Gal<sub>9</sub> based on the halo area.

<sup>b</sup>The fraction contained both β-1,6-Gal<sub>7</sub> and β-1,6-Gal<sub>8</sub>.

<sup>c</sup>The fraction was a mixture of β-1,6-Gal<sub>n</sub> higher than β-1,6-Gal<sub>8</sub>. 
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Figure 6. Yariv reactivity of β-1,3-Galn. The chain length of β-1,3-galactan necessary for β-Gal-Yariv reactivity was investigated. In the assay of β-1,3-Gal8 and β-1,3-Gal9, smaller amounts of samples were used due to their high reactivity. None of them exhibited Yariv reactivity toward α-Gal-Yariv (Fig. S3).
Figure 7. Pull-down assay of β-Gal-Yariv with β-1,3-Galₙ-beads. β-1,3-Gal₅, β-1,3-Gal₆, β-1,3-Gal₇, β-1,3-Gal₈, β-1,3-Gal₉, and β-1,6-Gal₇+₈ cross-lined to beads were suspended in β-Gal-Yariv solution ranging from 6.25 to 400 μg ml⁻¹. As a control experiment, the beads were also suspended in 400 μg ml⁻¹ α-Gal-Yariv solution.
Figure 8. Computational modeling. The conformation of β-1,3-galactan consisting of 16 galactosyl residues in water was predicted by MD simulations. The simulations for the β-1,3-galactan were performed in a truncated octahedral TIP3P water box with a solvation shell of 12 Å thickness. Side (A) and top views (B) are shown. Note that the modeling may not correctly predict the conformation of the ends of the β-1,3-galactan chain, because of the edge effect.