β-Galactosyl Yariv Reagent Binds to the β-1,3-Galactan of Arabinogalactan Proteins

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Yariv phenylglycosides [1,3,5-tri(α-D-glycosyl)phenylazo]-2,4,6-triiodoanisole are a group of chemical compounds that 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 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 (Raphanus sativus) 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 (Acacia senegal) 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 cross linked to hydrazine beads detected an 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 cross linking, leading to precipitation of the Yariv phenylglycosides.

Arabinogalactan proteins (AGPs) are a type of plant proteoglycans consisting of a Hyp-rich core protein and large arabinogalactan (AG) moieties (Fincher et al., 1983; Nothnagel, 1997). Although there are many molecular species of AGP 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-Ara and other auxiliary sugars, such as GlcA, 4-O-methyl-GlcA, L-Fuc, L-Rha, and Xyl, are attached (Fincher et al., 1983; Nothnagel, 1997; Seifert and Roberts, 2007). A commercial product of AGPs prepared from the acacia (Acacia senegal) 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 (Majewksa-Sawka and Nothnagel, 2000; Kiyohara et al., 2002). 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 [1,3,5-tri(α-D-glycosyl)phenylazo]-2,4,6-triiodoanisole are a group of chemical compounds that 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

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precipitate AGPs (Yariv et al., 1967b; Jermyn and Yeow, 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 phenylazotrihydroxybenzene core. In particular, β-glucosyl Yariv phenylglycoside (β-Glc-Yariv) and β-galactosyl Yariv phenylglycoside (β-Gal-Yariv) bind to AGPs, whereas α-glucosyl Yariv and α-galactosyl Yariv (α-Gal-Yariv) do not bind to AGPs (Jermyn and Yeow, 1975; Larkin, 1977, 1978; Nothnagel and Lyon, 1986). Because of the specific interaction with the β-glycosyl Yariv phenylglycosides (β-Yarivs), AGPs were formerly called “β-lectins” (Jermyn and Yeow, 1975; Gleeson and Jermyn, 1979; Nothnagel and Lyon, 1986).

The β-Yarivs are useful tools for staining, detection, and quantification of AGPs. Using β-Glc-Yariv, β-lectins were shown to exist in angiosperm, gymnosperm, fern, moss, and liverwort, illustrating the wide distribution of AGPs in the plant kingdom (Jermyn and Yeow, 1975; Clarke et al., 1978). In addition, β-Yarivs are also used as chemical reagents in the purification of AGPs.

A nonclassical AGP, xylogen, which 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 β-Glc-Yariv (Motose et al., 2004). As the treatment with β-Yarivs causes the perturbation of various physiological processes in plants, β-Yarivs are reliable cytochemical reagents to explore AGP functions. Application of β-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 β-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

**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 (white circles) and a mixture of gum arabic and Gal (black circles), Hyp-AG fractions F2, F3, and F4 were identified. The sugar content in the fractions was determined by the phenolsulfuric acid method. V₀ and Vᵢ indicate void volume and inner volume, respectively. B, The Yariv reactivity of F2, F3, and F4 was examined by radial diffusion assay.

![Figure 1](https://plantphysiol.org)

**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 I; Tsumuraya et al., 1988). The β-1,3-galactan main chain is shown in pink, and β-1,6-galactan side chains are shown in blue.
identification of the target structures on AGPs required for β-Yariv reactivity remains elusive (Nothnagel, 1997; Seifert and Roberts, 2007). It has been proposed that β-Yarivs bind to the Hyp-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 (Komalavilas et al., 1991). However, with regard to the specific carbohydrate structure required for interaction with β-Yarivs, the results were not always consistent: neither α-L-arabinofuranosyl residues nor β-1,6-galactan side chains were found to be involved in Yariv reactivity of AGPs from Gladiolus spp., radish (Raphanus sativus), and grape (Vitis vinifera; Gleeson and Clarke, 1979; Tsumuraya et al., 1987; Saulnier et al., 1992); partial acid hydrolysis to remove α-L-arabinofuranosyl residues diminished Yariv reactivity of a rose (Rosa spp.) AGP (Komalavilas et al., 1991); and mugwort (Artemisia vulgaris) pollen O-glycans consisting of a β-1,6-galactan core and branched α-L-arabinofuranosyl side chains precipitated with β-Glc-Yariv (Léonard 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 this 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, we identify β-1,3-galactopentaose (β-1,3-Galα) as the smallest carbohydrate structure to interact with β-Gal-Yariv, and we show that β-1,3-galactoheptaose (β-1,3-Galβ) or longer β-1,3-galactosyl chains are required for the formation of insoluble precipitate with Yariv phenylglycoside. Based on 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 Hyp-rich core protein is responsible for the interaction with Yariv phenylglycosides, as proposed by 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; 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 on an agarose gel containing β-Gal-Yariv (van Holst and Clarke, 1985). These fractions showed clear Yariv reactivity, although the reactivity was relatively weak compared with 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; Fig. 1B). 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 the 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. As 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-methyl-GlcA are attached (Fig. 2; Tsumuraya et al., 1988). The trimming was accomplished with the aid of four AG-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; Konishi et al., 2008; Kotake et al., 2009; Takata et al., 2010). All proteins were expressed in Pichia pastoris and purified by chromatography. Trimmed AGP1, AGP2, AGP3, and AGP4 were prepared by combinational digestion of native AGP with these enzymes and subsequent gel-permeation chromatography to remove monosaccharides 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 one-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 I). Yariv reactivity of the trimmed AGPs was monitored with the radial gel diffusion assay as described above. Surprisingly, among the trimmed AGPs, only trimmed AGP4 lost Yariv reactivity toward β-Gal-Yariv, whereas trimmed AGP1, AGP2, and AGP3 retained the reactivity (Fig. 3). Compared with native and trimmed AGP1 and AGP2, trimmed AGP3 exhibited relatively high Yariv reactivity based on sugar weight (Table II). None of the trimmed AGPs showed any reactivity toward α-Gal-Yariv (Supplemental Fig. S1). Monosaccharide and oligosaccharide fractions, including l-Ara, 4-O-methyl-GlcA, Gal, and β-1,6-Gal, released from the AGP during the trimming, did not exhibit any Yariv reactivity at all (Supplemental Fig. S1). These results suggested that β-Gal-Yariv binds to the β-1,3-galactan main chains but not to α-l-arabinofuranosyl and 4-O-methyl-glucuronosyl residues and β-1,6-galactan side chains.

### Table II. Relative Yariv reactivity of polysaccharides

| Substrate                                 | Relative Reactivity |
|-------------------------------------------|---------------------|
| Gum arabic from acacia tree               | 100                 |
| β-Galactan I (gum arabic treated by single Smith degradation) | 94                  |
| β-Galactan II (gum arabic treated by double Smith degradation) | 81                  |
| β-Galactan III (gum arabic treated by triple Smith degradation) | 248                 |
| β-Galactan IV (gum arabic treated by quadruple Smith degradation) | 250                 |
| Native AGP from radish roots              | 72                  |
| Trimmed AGP1 (treated with NcAraf1)       | 72                  |
| Trimmed AGP2 (treated with NcAraf1 and AnGlcAase) | 73                  |
| Trimmed AGP3 (treated with NcAraf1, AnGlcAase, and Tv6GAL) | 102                 |
| Trimmed AGP4 (treated with NcAraf1, AnGlcAase, Tv6GAL, and Il1,3GAL) | 0                   |

*Relative Yariv reactivity is expressed in percentage of that toward gum arabic based on the halo area.

Effect of the 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 (OH) groups; 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 this 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,

![Figure 4. Yariv reactivity of β-galactans. β-Galactans resulting from single (β-galactan I), double (β-galactan II), triple (β-galactan III), and quadruple (β-galactan IV) Smith degradation of acacia gum were subjected to Yariv radial diffusion assay. The Yariv reactivity of β-galactan IV treated with FvEn3Gal is also shown. The average structures of the β-galactans are shown in Supplemental Figure S2.](https://plantphysiol.org)
the proportions 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 (Supplemental Fig. S2; Akiyama et al., 1984; Defaye and Wong, 1986; Tsumuraya et al., 1990; Kotake et al., 2009). 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 II). 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 arabic and β-galactan I based on sugar weight. Yariv reactivity of β-galactan IV was lost by treatment with endo-β-1,3-galactanase from winter mushroom (Flammulina velutipes), FvEn3Gal (Kotake et al., 2011). β-Galactan IV also failed to interact with α-Gal-Yariv (Supplemental 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 II), 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-Gal₆). 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 by gel-permeation chromatography (Fig. 5). 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-1,3,6-galactosyl branches (→3,6Gal→) per molecule on average, which are derived from remnant side chains of β-galactan IV (Supplemental 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, and β-1,3-Gal₆, did not precipitate with either of the two Yariv phenylglycosides tested (Fig. 6; Table III; Supplemental Fig. 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 the rapid diffusion of these oligosaccharides on the agarose gel during the assay. Like β-galactan IV, β-1,3-Gal₈ lost Yariv reactivity when treated with FvEn3Gal.

Other oligosaccharides, such as β-1,6-Gal₄, β-GlcA-1,6-β-Gal-1,6-Gal, and α-L-Ara-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 (Supplemental 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 the Interaction of β-1,3-Gal₇ with β-Gal-Yariv

The formation of an insoluble precipitate on the agarose gel likely results from cross linking of the glycan and Yariv and, therefore, may require the binding of at least two Yariv phenylglycosides to each oligosaccharide. It is also conceivable that a weak interaction of a short β-1,3-Gal₇ with Yariv phenylglycoside may not be detected. In order to detect weak interactions with Yariv phenylglycoside, a pull-down assay using β-1,3-Gal₆, β-1,3-Gal₇, β-1,3-Gal₈, and β-1,3-Gal₉ cross linked to hydrazide beads was performed. The beads were suspended in β-Gal-Yariv solution at concentrations from 6.25 to 400 μg mL⁻¹, and Yariv phenylglycoside coprecipitated 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-Gal₉ beads did not precipitate β-Gal-Yariv except at the higher β-Gal-Yariv concentrations (above 100 μg mL⁻¹), the interaction between β-Gal-Yariv and β-1,3-Gal₉ beads may be weak compared with longer β-1,3-Galₙ beads. β-1,3-Gal₈ beads did not precipitate β-Gal-Yariv at all. Given that a galactosyl residue at the reducing end of the β-1,3-Gal₉ is lost in the cross-linking reaction with the bead hydrazide group, we conclude that β-1,3-Gal₉ is able to interact with β-Gal-Yariv. None of β-1,3-Galₙ beads precipitated α-Gal-Yariv. Consistent with the radial gel-diffusion assay findings, no interaction between β-1,6-Gal₇,G₈,G₉ and β-1,6-Gal₇,G₈,G₉ (a mixture of β-1,6-Gal₇, and β-1,6-Gal₈,G₉) and β-Gal-Yariv 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 a 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 simulations (SMD) were performed (Figure 8) as a complement to the radial gel-diffusion experiment. The SMD showed a significant change in the β-1,3-galactan conformation during the simulation (Supplemental Movie S1). Similar results have been observed for β-1,3-galactans in other studies (Sletmoen and Stokke, 2008). These results suggest that the observed interaction between β-Yariv and β-1,3-galactan is due to the conformational change of the β-1,3-galactan during the radial gel-diffusion experiment.

Table III. Relative Yariv reactivity of galactooligosaccharides

| Substrate | Relative Reactivity |
|-----------|---------------------|
| β-1,3-Gal₄ | 0                   |
| β-1,3-Gal₅ | 0                   |
| β-1,3-Gal₆ | 0                   |
| β-1,3-Gal₇ | 13                  |
| β-1,3-Gal₈ | 68                  |
| β-1,3-Gal₉ | 100                 |
| β-1,6-Gal₄ | 0                   |
| β-1,6-Gal₅,₆ | 0                 |
| β-1,6-Gal₇₋₈,₉ | 0               |

*Relative Yariv reactivity is expressed in percentage of that toward β-1,3-Gal₇ based on the halo area. This fraction contained both β-1,6-Gal₄ and β-1,6-Gal₅₋₉. This fraction was a mixture of β-1,6-Gal₄, higher than β-1,6-Gal₅₋₉.*
DISCUSSION

This study indicates, to our knowledge for the first time, 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 AG-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 results presented here are consistent with previous reports suggesting that neither α-L-arabinofuranosyl residues nor β-1,6-galactan side chains are involved in Yariv reactivity (Gleeson 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. (1984). 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 (Komalavilas 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 phenylazotrihydroxybenzene core of β-Yarivs. A previous equilibrium sedimentation analysis has demonstrated strong self-association of Yariv phenylglycosides in water (Yariv et al., 1962; Woods et al., 1978). Further Yariv hydrophobic interactions, therefore, may 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 propose that these likely bind to β-Gal-Yariv with oligosaccharides. For pull-down assays, β-1,3-Gal₆, β-1,3-Gal₇, β-1,3-Gal₈, and β-1,6-Gal₁₋₈ cross linked 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.

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 (B) views 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.
Gal residues in the helix. To demonstrate the molecular mechanism for Yariv reactivity, physicochemical 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 (Churms et al., 1983; Bacic et al., 1987; Ellis et al., 2010). 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 and Scott, 1971). On the other hand, our 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 (Léonard et al., 2005). We cannot exclude the possibility that some AGPs may have a target structure for β-Yarivs other than β-1,3-galactan, but our data suggest that reinvestigation for the presence of β-1,3-galactan main chains longer than β-1,3-Gal, in these AGs is necessary.

Yariv reactivity requires a longer β-1,3-galactosyl chain to make an insoluble precipitate on the agarose gel than it does to bind to the oligosaccharide in the pull-down assay. β-1,3-Gal₃ is the smallest oligosaccharide precipitable by Yariv. On the other hand, β-1,3-Gal₄, beads are reactive in the Yariv pull-down assay, suggesting that β-1,3-Gal₅ is the smallest carbohydrate structure for Yariv interaction, as β-1,3-Gal₄, beads have lost a galactosyl residue at the reducing end. Just as the precipitation of proteins with antibodies requires a multivalent interaction, longer oligosaccharides may be required for multiple Yariv glyco-side interaction and consequent precipitation.

In view of the fact that β-Yarivs stain tissues of various higher plants, including gymnosperm, fern, moss, and liverwort (Jermy 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. Therefore, it is conceivable that the AGP β-1,3-galactan may interact with any of these polysaccharides. Consistent with this hypothesis, several groups have reported copurification 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 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 previous studies (Tan et al., 2004, 2010). Peptide linkages of core proteins included in gum arabic were hydrolyzed in 0.44 N 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

NcAraf, AnGlcAase, Tv6GAL, and III3GAL were prepared as described (Konishi et al., 2008; Kotake et al., 2009; Takata et al., 2010). The purity of the enzymes has been confirmed by SDS-PAGE. Radish (Raphanus sativus) root AGP was digested with NcAraf, AnGlcAase, Tv6GAL, and III3GAL 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 × 23 cm; GE Healthcare) to separate it from released monosaccharides and oligosaccharides. The structures of the trimmed AGPs were determined by methylation analysis with a Shimadzu GC-6A gas chromatograph fitted with a Silar-10C column (0.28 mm × 50 m; Hakomori, 1964; Albersheim et al., 1967). The content of uronic acid was measured by a modified carbazole-sulfuric acid method (Galambos, 1967). In the hydrolysis of β-galactan IV and β-1,3-Galα, 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, 1983). The carbohydrate sample was applied to a gel plate containing 0.004% (w/v) β-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) as the standard and expressed based on equal sugar amount.

Smith Degradation

Smith degradation was performed as described previously (Goldstein et al., 1956; Tsumuraya et al., 1987). Oxidation of gum arabic with 100 mM metaperiodate was carried out in the dark at 7°C for 4 d and terminated by the addition of 1,2-ethanediol. The products were washed with 70% (v/v) ethanol, ethanol, acetone, and petroleum ether. The products were coupled at their reducing terminals with NcAraf1, AnGlcAase, and Il1,3GAL and separated on a Bio-Gel P-2 column (Supplemental Fig. S5). To confirm purity, β-1,3-Galα, and β-1,6-Galβ, were coupled at their reducing terminals with p-amino benzoic acid ethyl ester according to the method of Matsuzawa and Imaoka (1988) and analyzed on an HPLC system equipped with a TSKgel Amide-80 (4.6 × 250 mm; Tosoh).
column (Fig. 5; Supplemental Fig. 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 (Kuroyama et al., 2001; Okemoto et al., 2003).

**Pull-Down Assay**

\(\beta-1,3\text{-Gal}\), \(\beta-1,3\text{-Gal}\), \(\beta-1,3\text{-Gal}\), \(\beta-1,3\text{-Gal}\), and \(\beta-1,6\text{-Gal}\) cross linked to beads were prepared with a BlotGlyco glycan purification and labeling kit according to the manufacturer’s instructions (Sumitomo Bakelite). In brief, 50 nmol of each oligosaccharide was cross linked to hydrazide groups on the beads, and free oligosaccharides were washed out from the beads. In the pull-down assay, a portion (approximately 5 \(\mu\)L) of the beads was suspended in \(\beta\)-Gal-Yariv solution ranging from 6.25 to 400 \(\mu\)g mL \(^{-1}\) at 25°C for 4 h. After washing the beads with 1 mL of water three times, coprecipitated 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 \(\beta\)-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 AMBER11, 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 \(\beta\)-1,3-galactan consisted of two steps. In the first stage, the \(\beta\)-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 \(\beta\)-1,3-galactan was kept fixed in its starting conformation using harmonic constraints with a force constant of 500 kcal mol \(^{-1}\) Å \(^{-2}\). In the second stage, the entire system was minimized by 1,000 steps of steepest descent minimization followed by 1,500 steps of conjugate gradient minimization without the constraints. The minimized structure was then subjected to 20 picoseconds of MD, using a 2-femtosecond time step for integration. During the MD simulation, the system was gradually heated from 0 to 300 K using 10 kcal mol \(^{-1}\) Å \(^{-2}\) weak positional restraints on the \(\beta\)-1,3-galactan. The SHAKE algorithm was used, in which all bonds involving hydrogen are constrained. After the system was heated at a constant volume with weak restraints on the complex, MD was performed for 10 nanoseconds with a time step of 2 femtoseconds under constant volume/constant temperature (number of particles, volume, and thermal 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 Figure S1.** Yariv reactivity of trimmed AGPs, released monosaccharides and oligosaccharides, and \(\beta\)-galactans.

**Supplemental Figure S2.** Average structures of native gum arabic and the \(\beta\)-galactans.

**Supplemental Figure S3.** Yariv reactivity of \(\beta\)-1,3-Gal, and other oligosaccharides.

**Supplemental Figure S4.** Three-dimensional structure of \(\beta\)-1,3-galactan.

**Supplemental Figure S5.** Preparation of \(\beta\)-1,6-Gal\(_\alpha\).

**Supplemental Table S1.** Structures of \(\beta\)-1,3-galactooligosaccharides.

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