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Structural analysis of galactoarabinan from duckweed

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ABSTRACT

A highly branched galactoarabinan named DAG1 (Mw ~ 4.0×10^4 Da) was purified from *Lemna aequinoctialis* 6000 *via* 70% (v/v) ethanol extraction, followed by size-exclusion chromatography on Bio-Gel P2 and Superdex 75. Methylation analysis showed that DAG1 consisted of *t*-Ara*f*, (1 → 5)-Ara*f*, (1 → 2,5)-Ara*f*, (1 → 3)-Gal*p*, and (1 → 3,6)-Gal*p* in a relative proportion of approximately 6:4:3:3:3, suggesting an arabinogalactan/galactoarabinan polysacchairde. With the aid of arabinan degrading enzymes, the structure of DAG1 repeating unit was further characterized by ELISA with specific monoclonal antibodies and Yariv reagent assay. Analyses indicated that the proposed repeating unit of DAG1 had a backbone composed of seven α-(1 → 5)-L-arabinofuranose residues where branching occurred at O-2 with either terminal arabinoses or arabinogalactan side chain. The arabinogalactan side chain was composed of six β-(1 → 3)-D-galactopyranose residues, half of which were ramified at O-6 with terminal arabinoses and the last galactose was terminated with arabinose.

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1. Introduction

Arabinose and galactose are important constituents of plant cell wall. So far, most polysaccharides rich in arabinose and galactose are classified as arabinogalactan (AG) (Showalter, 2001; Yapo, 2011). Two types of AGs are distinguished. Type I AG contains a β-(1 → 4)-D-galactan backbone, which can be substituted at O-3 by side chains containing α-(1 → 5)-L-linked arabinofuranose residues (Carpita & Gibeaut, 1993). In comparison to type I AG, type II AG has β-(1 → 3)-D-linked galactopyranose residues that form a backbone substituted at O-6 by β-1,6-galactan side chains, usually terminating in Ara*f*, Rha*p*, and Gal*p* residues (Ellis, Egelund, Schultz, & Bacic, 2010; Fincher, Stone, & Clarke, 1983). Short oligoarabinosides are also found as side chains of type II AG (Lee et al., 2005). In plant cell wall, either type I or type II AGs are often linked covalently to the backbone of rhamnogalacturonan I (RG-I) or present as free polymers (Ridley, O'Neill, & Mohnen, 2001). Type II AG can also be attached to hydroxyproline residues of many plant cell wall polypeptides to form arabinogalactan protein (AGP) (Showalter, 2001).

Occasionally, there are reports that galactoarabinans (GAs) are found in plant cell wall. GAs have been identified in RG-I pectic polysaccharides from sugar beet pulp, potato tubers, and blackgram native and fermented products (Harholt, Scheller, & Orfila, 2004; Sakamoto & Sakai, 1995; Tharanathan, Changala Reddy, Muralikrishna, Susheelamma, & Ramadas Bhat, 1994). GAs reported have a backbone chain of α-(1 → 5)-L-linked arabinan, substituted at O-3 by single Gal*p* units and/or short β-(1 → 4)-D-linked galactan side chains (average DP ≥ 4) (Yapo, 2011).

These observations indicate the complexity and diversity of arabinose and galactose rich polysaccharides in the context of cell wall architecture. The structure of arabinose and galactose rich polysaccharide is poorly characterized, and this is mainly due to the great heterogeneity of glycan structures (Estevez, Kieliszewski, Khitrov, & Somerville, 2006). The glycan structure can also be different depending on the tissue type and developmental stage, adding to the great heterogeneity of these molecules and therefore limiting their detailed characterization (Tryfona et al., 2012; Tsumuraya, Ogura, Hashimoto, Mukoyama, & Yamamoto, 1988). In this paper, we add another example to complexity of arabinose and galactose rich polysaccharides and report a new structural element to GAs.

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2. Materials and methods

2.1. Plant materials

The duckweed plants (*Lemna aequinoctialis* 6000) were maintained in Schenk and Hildebrandt (SH) medium (Schenk & Hildebrandt, 1972) in a growth chamber at 23 °C under a 16-h-light/8-h-dark condition at 110 μmol photons m^{-2} s^{-1} irradiance. For chemical composition analysis, duckweed samples were harvested after 16-h light treatments, washed with dH_2O to remove the medium, freeze-dried and ground into powder with liquid nitrogen.

2.2. Extraction and purification of polysaccharide

Powdered duckweed was extracted at 80 °C for 12 h twice with methanol–chloroform (1:1, v/v) and twice with methanol–acetone (1:1, v/v) sequentially. Residues were then suspended in 70% (v/v) aqueous ethanol at 80 °C for 5 h, and the mixture was centrifuged at 5000 rpm for 10 min. The solid material was extracted twice under the same ethanol condition. The supernatants were combined, concentrated by rotary evaporation, re-dissolved in dH_2O, centrifuged to remove sediments, and lyophilized to yield the ethanol soluble material (ESM).

ESM was dissolved in distilled water and fractionated by size-exclusion chromatography on a Bio-Gel P2 column (3 × 90 cm), eluted with 0.15 M NaCl solution at 0.1 mL min^{-1}. The eluates (4-mL per tube) were collected and assayed for distribution of total sugar by phenol-sulfuric acid (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and UV absorbance at 280 nm. The appropriate collections were combined, concentrated, dialyzed against dH_2O and freeze-dried to give four fractions: ESM1, ESM2, ESM3, and ESM4. ESM1 was then applied to a pre-loaded Superdex 75 column (16/60, Amersham Biosciences) attached to the AKTA system eluted with 0.15 M NaCl solution at 1 mL min^{-1}. The eluates were (2-mL per tube) were collected and assayed for distribution of total sugar to give DAG1 and DAG2 two fractions. DAG1 was investigated for further structure. The procedure for the extraction and purification of DAG1 from duckweed is shown in Fig. 1.

2.3. Determination of molecular size

The molecular size of DAG1 was estimated by high performance size-exclusion chromatography (HPSEC) at room temperature. HPSEC was performed on an high performance liquid chromatography (HPLC, Dionex) system fitted with Shodex OHpak SB-803 and SB-806 columns connected in series and monitored with a differential refractometer (RI, Dionex). The elution solvent was 50 mM sodium nitrate at an isocratic flow rate of 0.5 mL min^{-1}. The column was calibrated with 270,000, 150,000, 80,000, 40,000, 25,000, and 10,000 Dalton dextrans from Sigma.

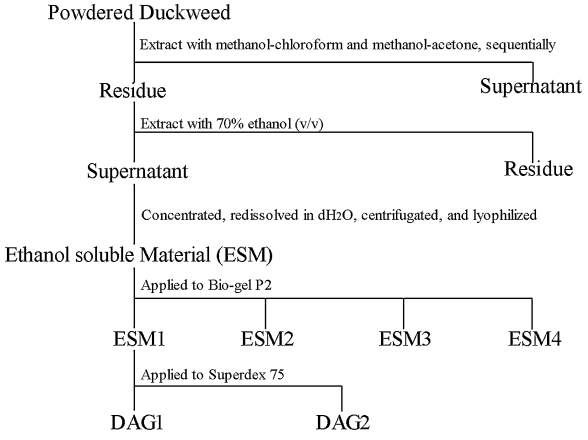

Fig. 1. Extraction and purification scheme for DAG1 from duckweed.

2.4. Monosaccharide composition

Monosaccharide composition was determined by HPLC as previously described (Zhang et al., 2009). In brief, DAG1 sample was hydrolyzed in 2 M trifluoroacetic acid (TFA, 0.5 mL) at 120 °C for 2 h. TFA was removed by evaporation under vacuum and the hydrolysates were derivatized with 1-phenyl-3-methyl-5-pyrazolone (PMP) and 0.3 M NaOH at 70 °C for 30 min. The generated PMP-derivates were analyzed by a Waters HPLC system equipped with a Hypersil ODS-2 C18 column (4.6 × 250 mm; Thermo) and a 2489 Uv/Vis detector.

2.5. ^1H NMR spectroscopy

^1H NMR spectrum was recorded on a Bruker 600 spectrometer operating at 600.13 MHz. DAG1 sample was examined as solution in D_2O at 65 °C in a 5 mm OD tube. All the data were analyzed using standard Bruker software. The chemical shifts are expressed in δ (ppm) values.

2.6. Glycosyl linkage analysis

Glycosyl linkage analysis was performed using a modification of the Hakamori method (Hakomori, 1964): 2 mg of DAG1 was permethylated with iodomethane, followed by TFA hydrolysis (2 M at 120 °C, 2 h), reduced with sodium borodeuteride (10 mg mL^{-1} in 1 M NaOH overnight at room temperature), and acetylated with acetic anhybride/concentrated TFA (100 °C, 1 h). Partially methylated alditol acetates were separated on a DB-5 column using an Agilent 7890A chromatograph, and detected by electron-impact ionization mass spectrometry with a 5975C mass selective detector (mass-to-charge ratio of 50–350). The temperature program was as our previous method (Yu et al., 2014).

2.7. Enzyme-linked immunosorbent assays (ELISA)

In this study, four rat monoclonal antibodies (LM5, LM6, LM13, and LM16) and one mouse monoclonal antibody (CCRC-M7) were used in this study.

For ELISA assay, after DAG1 was coated onto microtitre plates (Costa, 3599) at 50 μg mL^{-1} at 4 °C overnight, plates were washed and then 200 μL per well of 3% (w/v) milk protein in phosphate-buffered saline (MP/PBS) was added to block the plates at room temperature for 2 h. The plates were washed and 100 μL per well of 20-fold dilutions of primary antibodies were added. After 2 h incubation at room temperature, plates were washed and wells were incubated with 100 μL per well of anti-rat or anti-mouse IgG coupled to horseradish peroxidase (HRP) at 1000-fold dilution in MP/PBS for 1 h at room temperature. After extensive washing, microtitre plates were developed with 150 μL per well of HRP substrate. The reaction was stopped by the addition of 50 μL of 2 M H_2SO_4 to each well and the absorbance of each well at 450 nm was determined. For enzyme pre-treatments, DAG1 immobilized on the plates was incubated with 100 μL per well of the arabinan-degrading enzyme solutions for 1 h at room temperature before blocking with MP/PBS and then washed immediately. These enzymes include (1) 50 μg mL^{-1} arabinanase from *Cellvibrio japonicas* (Megazyme, Bray, Ireland) in sodium acetate buffer (50 mM, pH 5.5) or (2) 50 μg mL^{-1} α-arabinofuranosidase from *Aspergulus niger*

(Megazyme, Bray, Ireland) in sodium acetate buffer or (3) a combined enzyme including the above two enzymes for 1 h at room temperature.

2.8. Assay of Yariv activity

Yariv reagent activity was determined by dot assay. For dot assay, 1 μL aliquots of adjusted DAG1 solution in dH_2O were applied to a nitrocellulose membrane (Hybond-N^+ RPN303B, Amersham Biosciences, UK) and dried for 1 h. Nitrocellulose membranes were blocked with 3% (w/v) MP/PBS for 30 min followed by incubation with 20 μg mL^{-1} β-Glc-Yariv reagent in PBS for 1 h at room temperature. The Yariv activity was estimated based on the halo area and dot intensities using gum arabic (Sigma) as the positive control. Prior to application to nitrocellulose membrane, DAG1 was pre-treated with a series of arabinan-degrading enzymes as above.

3. Results and discussion

3.1. Fractionation of DAG1 from duckweed

L. aequinoctialis strain 6000 (*L. aequinoctialis*), which has high starch content and rapid growth ability, was obtained through large scale screening of more than 100 species strains of duckweed distributed in 20 provinces and municipalities in China (data not shown). These provinces and municipalities included Shandong, Shanxi, Guangdong, Guangxi, Hebei, Henan, Jiangsu, Jiangxi, Zhejiang, Fujian, Shanxi, Hainan, Hubei, Hunan, Sichuan, Guizhou, Beijing, Tianjin, Shanghai, and Chongqing (Supplemental Fig. 1). *L. aequinoctialis* strain 6000 was collected from Lixian in Hunan Province.

For *L. aequinoctialis* cultured under the conditions as described in Section 2, chlorophyll, lipid and flavone account for 18.6% of the dry weight (data not shown). In order to analyze the carbohydrate polymers, dry duckweed was treated with methanol–chloroform and methanol–acetone sequentially to remove chlorophyll, lipid and flavone firstly. The residues were extracted with 70% (v/v) ethanol at 80 °C to get the ethanol-soluble materials (ESM), which could be expected to contain oligosaccharides, 70% ethanol-soluble polysacchairdes, and flavone. The ESM was fractionated on Bio-gel P2, giving fractions ESM-1, ESM-2, ESM-3 and EMS-4 (Fig. 2A), in 11.0%, 21.5%, 43.9% and 17.9% yield, respectively. ESM-1 was rich in carbohydrate polymers and was then purified on Superdex 75 to give fractions DAG1 and DAG2 (Fig. 2B), in 31.8% and 53.5% yield, respectively. ESM-2, ESM-3, ESM-4 and DAG2 had high UV absorption and low carbohydrate contents, whereas DAG1 was rich in carbohydrate and was further analyzed in this study.

3.2. Molecular weight and sugar composition of DAG1

Fraction DAG1 showed a homogeneous profile and the molecular mass was estimated to be 30 kDa when analyzed by HPSEC-RI with a polydispersity of 1.029 (Fig. 3). Sugar analysis indicated that DAG-1 was composed of arabinose, galactose and glucose in a molar ratio of 7.0:2.9:0.1, suggesting an arabinogalactan/galactoarabinan polysaccharide.

3.3. Glycosyl linkage compositions

Linkage analysis showed that DAG1 was a highly branched polysaccharide, with 32.1% (mol%) of non-reducing end-unit of Ara*f* (t-Ara*f*) (Table 1). A proportion (21.2%) of interchain 5-linked arabinosyl residues were also present and 16.7% of 2,5-linked arabinosyl residues indicated that approximately 44% of the 5-linked residues were branched at O-2 (Table 1). According to the predominant ^1H

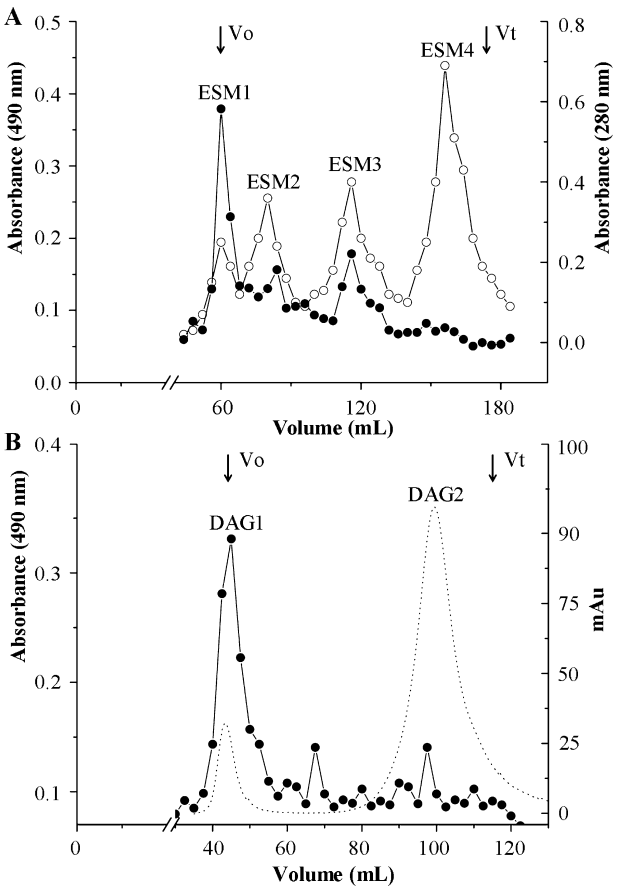

Fig. 2. Separation of DAG1 from ethanol soluble materials (ESM) by size-exclusion chromatography. (A) Elution profile of ESM on Bio-Gel P2 column and (B) elution profile of ESM1 on Superdex 75 column (total sugars, ●; UV absorbance, ○; conductivity, ----).

NMR anomeric proton signals of DAG1 at δ 5.10–4.95 (Supplemental Fig. 2), the great majority of the arabinosyl units are in the α-Ara*f* form (Habibi, Mahrouz, & Vignon, 2005; Mandal et al., 2011). Galactose was found in two methylated products arising from 3-linked, and 3,6-linked galactopyranose residues, which revealed the presence of linkages characteristic for type II arabinogalactan. They

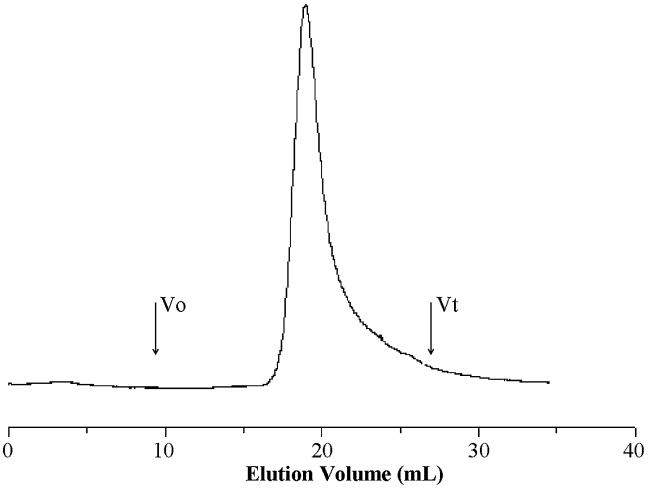

Fig. 3. Elution profile of DAG1 polysaccharide using HPSEC with a refractive index (RI) detector.

Table 1
Linkage types based on analysis of partially O-methylalditol acetates obtained from methylated DAG1.

Partially O-methyladitol acetate	Relative ratio[a]	Linkage type[b]
2,3,5-Me_3-Ara*f*	32.1 ± 3.6	Ara*f*-(1→
2,3-Me_2-Ara*f*	21.2 ± 3.1	→5)-Ara*f*-(1→
3-Me-Ara*f*	16.7 ± 2.9	→2,5)-Ara*f*-(1→
2,4,6-Me_3-Gal*p*	14.7 ± 1.7	→3)-Gal*p*-(1→
2,4-Me_2-Gal*p*	14.3 ± 1.6	→3,6)-Gal*p*-(1→

[a] Results are given as the mean molar percentage ± variance of two samples, where 0 = <0.05. Values are scaled to monosaccharide composition analysis.
[b] Based on derived O-methylalditol acetates.

constituted 29.0% of all derivatives and the ratio of 3- and 3,6-linked galactopyranose residues was 1:1 (Table 1), thus indicating that half of the 3-linked galactosyl residues in the 1,3-galactan chain was branched at O-6. No anomeric proton signals for galactopyranose residues were observed in the ^{1}H NMR spectra (Supplemental Fig. 2), perhaps for the relatively low amount or being masked by the highly amount of substitutions.

In all, the glycosyl linkage composition of DAG1 is relatively simple, with t-Ara*f*, 5-Ara*f*, 2,5-Ara*f*, 3-Gal*p*, and 3,6-Gal*p* in a relative ratio of 6:4:3:3:3. To facilitate the discussion of DAG1 structure, we suggest that the repeating unit of DAG1 contains six t-Ara*f*, four 5-Ara*f*, three 2,5-Ara*f*, three 3-Gal*p*, and three 3,6-Gal*p*. However, it is difficult to deduce whether 1,5-arabinan or 1,3-galactan present as the backbone only by glycosyl linkage result.

3.4. Arabinan chain structure

Monoclonal antibodies directed to cell wall polysaccharide epitopes have been shown to be useful tools for identifying structural features present in isolated polysaccharide fractions (Verhertbruggen et al., 2009; Yu, Zhou, & Knox, 2011). Herein, DAG1 structural features were further analyzed by ELISA with a set of arabinose and galactose related monoclonal antibodies. Fig. 4 shows ELISAs of LM5, LM6, LM13, LM16, and CCRC-M7 binding to DAG1. The LM5 antibody is specific to more than three contiguous units of β-(1 → 4)-galactosyl residues (Jones, Seymour, & Knox, 1997) and the CCRC-M7 antibody is proposed to recognized trimer or larger of β-(1 → 6)-galactosyl carrying one or more Ara residues (Steffan, Kovác, Albersheim, Darvill, & Hahn, 1995). No LM5 and CCRC-M7 epitopes were detected in DAG1 (Fig. 4),

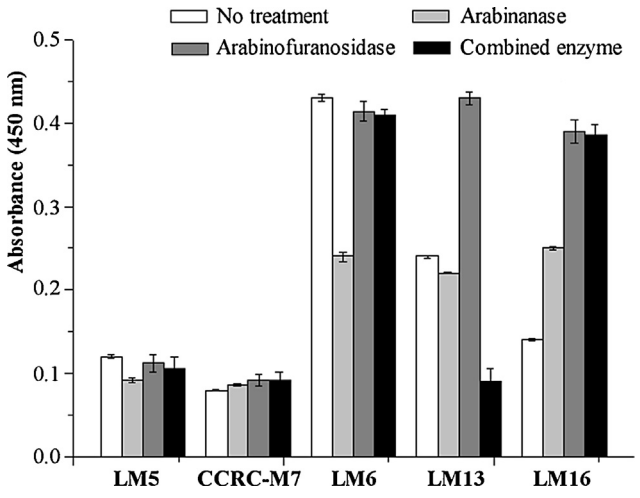

Fig. 4. ELISA analysis of the binding of cell wall monoclonal antibodies to DAG1 and related polysaccharides.

confirming the linkage analysis data that no 4-linked or 6-linked galactopyranose were observed (Table 1). LM6, LM13, and LM16 comprise a set of antibodies that can detect arabinan and related structures. LM6 and LM13 are specific to 1,5-arabinan with LM13 showing greater selectivity toward longer even perhaps larger than tetra-arabinoligosaccharide (Verhertbruggen et al., 2009; Willats, Marcus, & Knox, 1998). LM16 is proposed to recognize a galactosyl residue/galactan stub between the arabinan side chains and the galactan backbone (Verhertbruggen et al., 2009). LM6 displayed strong recognition of DAG1 and LM13 showed a weak binding. LM16 showed even lower binding to DAG1 compared to LM13.

In order to explore the fine structure of arabinan, immobilized DAG1 was hydrolyzed with arabinan-degrading enzymes. The *C. japonicus* arabinanase exhibits an endo-mode of action and significant exo-activity against α-1,5-linked arabino-oligosaccharides with a d.p. of ≥4 (McKie et al., 1997; Proctor et al., 2005). The *A. niger* arabinofuranosidase is proposed to optimally hydrolyze the terminal, α-1,3-linked and α-1,2-linked arabinosyl side chains but has very low activity toward 1,5-linked arabinosyl (Verhertbruggen et al., 2009). The binding of LM6 was reduced by the action of arabinanase, with antibody signals being <60% of the no-enzyme control, whereas arabinofuranosidase and the combined enzyme (a combination of arabinanase and arabinofuranosidase) had no significant impact on the binding of LM6 to DAG1. Pre-treatments of arabinofuranosidase increased the LM13 binding significantly whereas the LM13 epitope was lost by the action of combined enzyme. LM13 epitope was also slightly reduced by the action of arabinanase. LM16 epitope was increased with the actions of these enzymes, with signals being >50% of the no-enzyme control in arabinofuranosidase and combined enzyme treatments (Fig. 4).

These observations indicated that the repeating unit of DAG1 comprised seven contiguous units of α-(1 → 5)-arabinosyl residues, which was ramified at O-2 with terminal arabinose and galactose-rich chains. In this α-1,5-arabinan chain, at least four contiguous non-substituted α-1,5-linked arabinosyl residues was the substrate of arabinanase, action of which leads to relatively shorter and highly substituted arabinan with less recognition by LM6 and LM13. Removal of the terminal arabinose with arabinofuranosidase could produce less ramified α-1,5-arabinan chain, which contributed to the higher recognization of DAG1 by LM6 and LM13. The combined enzyme could hydrolyze the α-1,5-arabinan chain thoroughly until the loss of the LM13 epitopes. Arabinan degrading with either arabinanase or arabinofuranosidase revealed the LM16 galactan stub.

3.5. Backbone of DAG1

β-1,3-Galactooligosaccharides longer than β-1,3-galactopentose has been demonstrated to specifically bind β-glucosyl Yariv reagent, a red colored dye which has been used to precipitate and purify type II arabinogalactan (Kitazawa et al., 2013; Yariv, Rapport, & Graf, 1962). DAG1 showed clear Yariv reactivity, although the reactivity was relatively weak compared with the gum arabic control (Fig. 5). This result indicated the presence of pentamer or lager of β-(1 → 3)-galactosyl units in DAG1, which confirmed the Linkage analysis results that each repeating unit of DAG1 had six β-(1 → 3)-galactosyl units with one half being substituted at O-6.

To test whether arabinan or galactan as the backbone, DAG1 was hydrolyzed using the arabinan-degrading enzymes and applied to nitrocellulose membranes, and then the Yariv activity was determined. If DAG1 had β-(1 → 3)-galactosyl residues as the backbone, the arabinose-rich substitutions could restrict the access of β-1,3-galactan to Yariv reagent, and arabinan degrading would increase the Yariv reactivity and had no significant impact on the molecular weight of DAG1. Otherwise, if α-1,5-arabinan was present as the

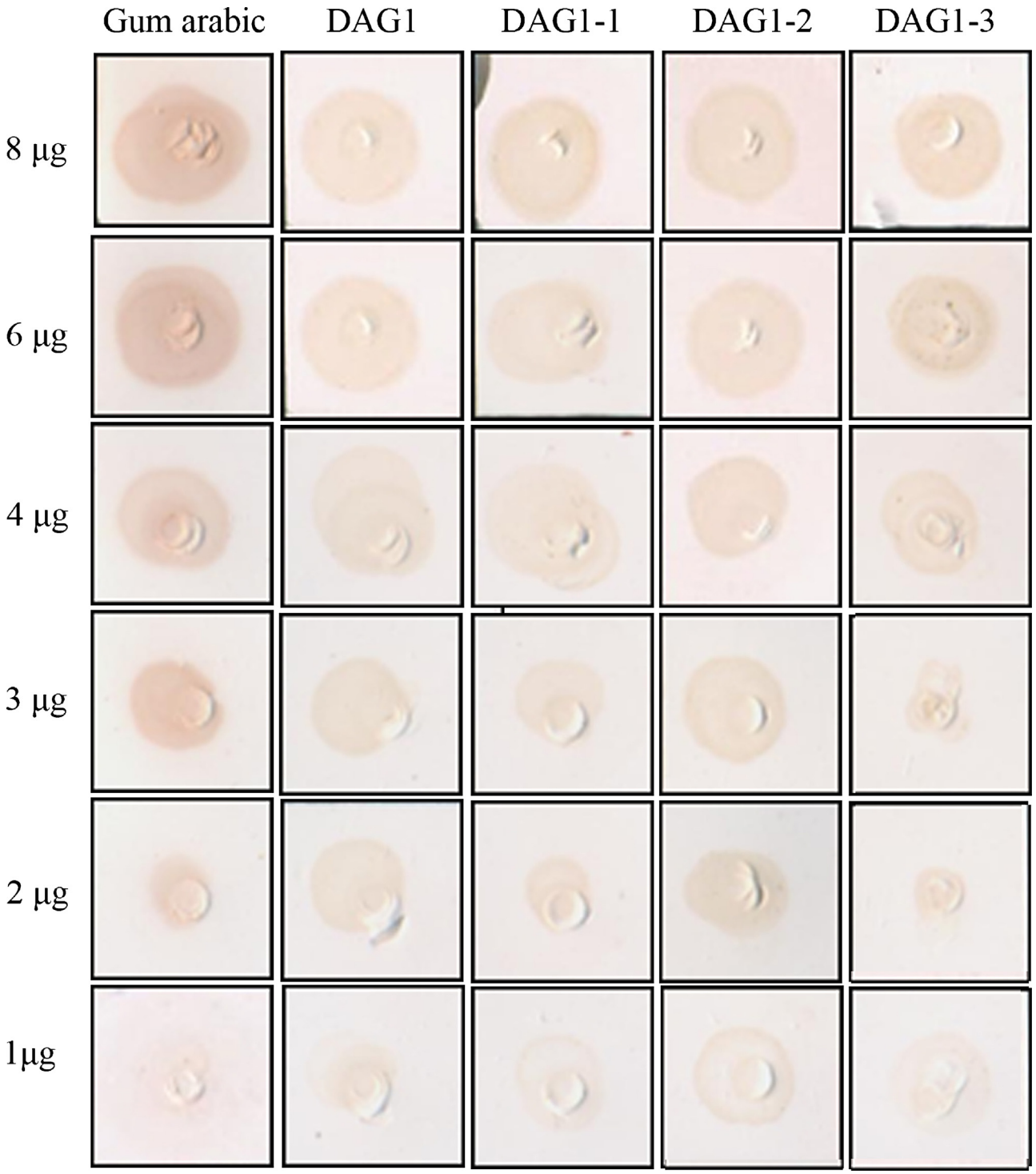

Fig. 5. Dot blots analysis of the binding of β-glucosyl Yariv reagent to DAG1 and related polysaccharides. DAG1-1, DAG1 treated with arabinanase; DAG1-2, DAG1 treated with arabinofuranosidase; DAG1-3, DAG1 treated with a combination of arabinanase and arabinofuranosidase.

backbone, action of arabinanase could hydrolyze DAG1 polysaccharide into smaller molecule, which might have low affinity to the nitrocellulose membranes. Indeed, the action of arabinofuranosidase increased the Yariv activity markedly (Fig. 5), indicating that the β-1,3-galactan was highly ramified by terminal arabinose. However, pre-treatments with arabinanase and the combined enzyme slightly decreased the Yariv activity (Fig. 5). So we deduced that the β-1,3-galactan ramified with terminal arabinose was present as the side chain of α-1,5-arabinan in DAG1. It is generally accepted that one polysaccharide molecule rich in galactose and arabinose belongs to type I or type II arabinogalactan. In this study, we showed that one new galactoarabinan molecule contained α-1,5-arabinosyl residues as the backbone and β-1,3-galactosyl residues as the side chain. The presence of peculiar galactoarabinan structural element suggests that the structural variation within the plant cell wall is large.

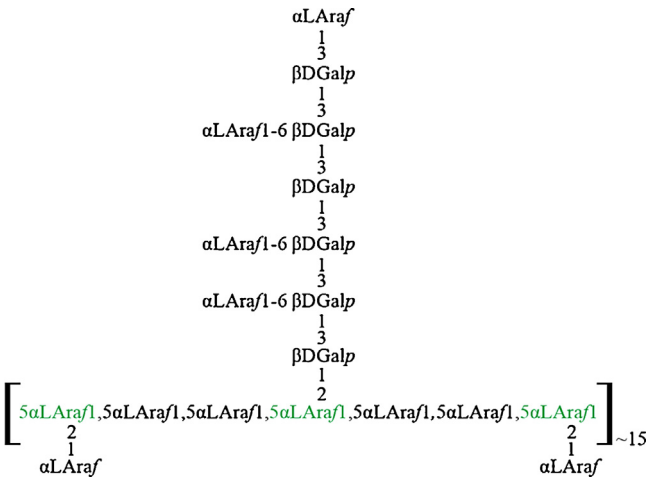

Fig. 6. Schematic structure of DAG1 repeating unit.

4. Conclusion

In sum, a new galactoarabinan was isolated from 70% ethanol extract of *L. aequinoctialis* and purified by size-exclusion chromatography. This polymer was composed of *t*-Ara*f*, (1 → 5)-Ara*f*, (1 → 2,5)-Ara*f*, (1 → 3)-Gal*p*, and (1 → 3,6)-Gal*p* in a relative proportion of approximately 6:4:3:3:3. On the basis of the cell wall antibody ELISA and Yariv reagent assay, the proposed structure of the repeating unit of the present galactoarabinan molecule was established as Fig. 6. This galactoarabinan molecule has α-1,5-arabinan as the backbone and the β-1,3-galactan highly ramified with terminal arabinose is attached to the O-6 of the backbone arabinose. This work has implications for understanding arabinogalactan/galactoarabinan complexity in the context of cell wall architectures and in relation to cell wall functions in plant development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.10.044.

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