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## Enzymic transfer of $\alpha$ -L-arabinopyranosyl residues to exogenous 1,4-linked $\beta$ -D-galacto-oligosaccharides using solubilized mung bean (*Vigna radiata*) hypocotyl microsomes and UDP- $\beta$ -L-arabinopyranose

Received: 7 September 2004 / Accepted: 25 January 2005 / Published online: 22 April 2005  
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**Abstract** A single  $\alpha$ -L-arabinopyranosyl ( $\alpha$ -L-Ara *p*) residue was shown, by a combination of chemical and spectroscopic methods, to be transferred to *O*-4 of the nonreducing terminal galactosyl (Gal) residue of 2-aminobenzamide (2AB)-labeled galacto-oligosaccharides when these oligosaccharides were reacted with UDP- $\beta$ -L-arabinopyranose (UDP- $\beta$ -L-Ara *p*) in the presence of a Triton X-100-soluble extract of microsomal membranes isolated from mung bean (*Vigna radiata*, L. Wilezek) hypocotyls. Maximum-(1  $\rightarrow$  4)-arabinopyranosyltransferase activity was obtained at pH 6.0–6.5 and 20°C in the presence of 25 mM Mn<sup>2+</sup>. The enzyme had an apparent *K*<sub>m</sub> of 45  $\mu$ M for the 2AB-labeled galactoheptasaccharide and 330  $\mu$ M for UDP- $\beta$ -L-Ara *p*. A series of 2AB-labeled galacto-oligosaccharides with a degree of polymerization (DP) between 6 and 10 that contained a single  $\alpha$ -L-Ara *p* residue linked to the former nonreducing terminal Gal residue were generated when the 2AB-labeled galactohexasaccharide (Gal<sub>6</sub>-2AB) was reacted with UDP- $\beta$ -L-Ara *p* in the presence of UDP- $\beta$ -D-Gal *p* and the solubilized microsomal fraction. The mono-arabinosylated galacto-oligosaccharides are not acceptor substrates for the galactosyltransferase activities known to be present in mung bean microsomes. These results show that mung bean hypocotyl microsomes contain an enzyme that catalyzes the transfer of Ara *p* to the nonreducing Gal residue of galacto-oligosaccharides and suggest that the presence of a  $\alpha$ -L-Ara *p* residue on the former terminal Gal residue prevents galactosylation of galacto-oligosaccharides.

**Keywords** 2-Aminobenzaminated galacto-oligosaccharide · Arabinopyranose ·  $\alpha$ -(1  $\rightarrow$  4)-arabinopyranosyltransferase · Galactan · Rhamnogalacturonon I · *Vigna*

**Abbreviations** 2AB: 2-Aminobenzamide · Ara *p*: Arabinopyranosyl · AraT: Arabinopyranosyltransferase · DP: Degree of polymerization · Gal: Galactose · HPAEC: High-performance anion-exchange chromatography · LC-ESI-MS: Liquid chromatography-electrospray-ionization mass spectrometry · NMR: Nuclear magnetic resonance · RG-I: Rhamnogalacturonan I

### Introduction

Arabinose is quantitatively a major component of numerous plant polysaccharides including rhamnogalacturonan I (RG-I), arabinogalactan types I and II, and arabinoxylan (Ridley et al. 2001; O'Neill and York 2003). In these polysaccharides the arabinosyl residues exist predominantly in the  $\alpha$ -L-arabinofuranosyl ( $\alpha$ -L-Ara *f*) form (Carpita and Gibeaut 1993).  $\alpha$ -L-arabinopyranosyl ( $\alpha$ -L-Ara *p*) residues have been reported to be quantitatively minor components of arabinans isolated from several plants (Karácsonyi et al. 1975; Capek et al. 1983; Kiyohara et al. 1987; Swamy and Salimath 1991; Odonmažig et al. 1994). Soybean pectin contains galactan carrying an arabinopyranosyl residue at the nonreducing terminal residue (Huisman et al. 2001). A single  $\alpha$ -L-Ara *p* residue is also present in the aceric acid-containing side chain of rhamnogalacturonan II (O'Neill et al. 2004).

Immunolocalization studies using antibodies that recognize (1  $\rightarrow$  4)- $\beta$ -D-galactan or (1  $\rightarrow$  5)- $\alpha$ -L-arabinan have shown that the distribution of these side chains differs in the walls of different cell types and may be spatially and developmentally controlled (Knox 2002). Arabinan-deficient tobacco callus is brittle and exhibits

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weaker cell adhesion than wild-type callus (Iwai et al. 2002). Cell wall arabinan in the guard-cell would maintain the wall flexibility (Jones et al. 2003). Nevertheless, the function of the oligosaccharides with arabinosyl residues remains unclear.

Uridine-5'-diphospho-arabinopyranose (UDP-Ara *p*) has been used as glycosyl donor for in vitro studies of arabinose-containing plant polysaccharides. Previous studies using this UDP-sugar and crude membrane preparation from mung bean (*Vigna radiata*) shoots (Odzurk and Kauss 1972), French bean (*Phaseolus vulgaris*) callus and hypocotyls (Bolwell and Northcote 1981; Rodgers and Bolwell 1992) provided evidence that arabinosyl residues from UDP-Ara incorporated into polysaccharides. However, neither the structures of the acceptor polysaccharides were fully characterized nor were the ring form, anomeric configuration, and glycosidic linkage of the incorporated arabinosyl residues determined. Nunan and Scheller (2003) have shown that [<sup>14</sup>C] Ara is transferred from UDP-β-L-[<sup>14</sup>C] arabinopyranose onto exogeneous (1 → 5)-linked α-L-arabino-oligosaccharides by enzymes present in detergent-soluble extracts of mung bean hypocotyl microsomal membranes. These authors also provided evidence that the newly incorporated arabinosyl residue exists in the pyranose ring form. However, as far as we are aware, there are no reports describing plant glycosyltransferases that catalyze the formation of a glycosidic bond between a α-L-Ara *p* and a β-D-Gal *p* residue.

We, therefore, carried out this investigation to determine the activity of arabinopyranose transfer to β-D-Gal *p* residue in plant extracts and to characterize the products formed. We describe that galacto-oligosaccharides containing a single α-L-Ara *p* residue in the nonreducing end are generated by reacting UDP-β-L-Ara *p* with 2-aminobenzamide (2AB)-labeled (1 → 4)-linked β-D-galacto-oligosaccharides in the presence of a detergent-solubilized microsomal membrane fractions of mung bean hypocotyls. We characterize the structure of the mono-arabinosylated oligosaccharides by NMR spectroscopy, MS, and sugar analysis and report some enzymic properties of the arabinopyranosyltransferase.

## Materials and methods

### Chemical materials and plant

Mung bean (*Vigna radiata* L. Wilezek) seeds were purchased from Tokita Seed and Plant (Saitama, Japan). 2AB-labeled galacto-oligosaccharides with a degree of polymerization (DP) between 2 and 7 were prepared as described (Ishii et al. 2004). UDP-β-L-arabinopyranose (UDP-Ara *p*) and UDP-β-D-galactopyranose (UDP-Gal) were obtained from Peptide Inst. Inc. (Osaka, Japan) and Kyowa Hakko Co. (Tokyo, Japan), respectively. Mes, Hepes, and Triton X-100 were obtained from Dojindo Laboratory (Kumamoto, Japan), and Sigma-Aldrich, respectively. α-L-arabinofuranosidase

and endo-polygalacturonase (EPG; Megazyme, Ireland) were purchased from Biocon (Nagoya, Japan). All other chemicals and reagents used were purchased from Wako Pure Chemicals (Osaka, Japan).

### Preparation of microsomal membranes and solubilization of microsomal membrane

Mung bean (*Vigna radiata*) seeds were soaked overnight in water and then placed on moist rock fiber and grown for 3 days in the dark at 25°C. The hypocotyls (1.5–2 cm in length from the cotyledons) were used for the preparation of the microsomal membranes as described (Nunan and Scheller 2003). The microsomal membranes (25 mg ml<sup>-1</sup> protein) were treated with Triton X-100 (final concentration 0.5%, w/v) containing 150 mM KCl. The suspensions were vortexed and kept on ice for 30 min. The suspensions were then centrifuged for 20 min at 4°C and 100,000 g using a Beckman-Coulter MLA-130 ultracentrifuge rotor. The supernatant was collected and used immediately for subsequent reactions. Total protein in the microsomal membranes and in the solubilized microsomal fraction were determined with bovine serum albumin as a standard using a Protein assay reagent kit and a DC Protein assay reagent kit (Bio Rad, Hercules, CA, USA), respectively, according to the manufacturer's instructions.

### Assay procedure for α-(1 → 4)-arabinopyranosyltransferase

Arabinopyranosyltransferase activity was measured at 25°C for 20 min unless otherwise specified in a standard reaction mixture (total volume, 30 μl) containing solubilized microsomal fraction (80–100 μg protein), 10 μM 2AB-labeled galactoheptasaccharide (Gal<sub>7</sub>-2AB), 2 mM UDP-β-L-Ara *p*, 40 mM Mes-KOH (pH 6.5), 25 mM MnCl<sub>2</sub>, 20 mM NaF, and 160 mM sucrose. The reaction was terminated by the addition of acetic acid (1.5 M, 30 μl) and then boiled for 1 min. The reaction mixture was centrifuged, and the supernatant was diluted with water to about 300 nM solution, and then analyzed by HPAEC with a linear gradient of sodium acetate (0 mM for 2 min, to 250 mM at 60 min in 100 mM NaOH), at a flow rate of 1.0 ml min<sup>-1</sup>. The products were detected by their fluorescence (λ<sub>ex</sub> = 330 nm, λ<sub>em</sub> = 420 nm). Enzyme activity is expressed as pmol Ara transferred min<sup>-1</sup>(mg protein)<sup>-1</sup>, based on the concentration curve of 2AB-Gal<sub>4</sub> as the calibration standard. The apparent *K*<sub>m</sub> and *V*<sub>max</sub> values of AraT as the crude enzyme for the Gal<sub>7</sub>-2AB were determined at 25°C for 20 min using Gal<sub>7</sub>-2AB (1–100 μM) and the same concentrations of other components as used in the standard assay mixture. The apparent *K*<sub>max</sub> and *V*<sub>max</sub> values for UDP-Ara were determined at 25°C for 20 min with UDP-Ara (20–1,000 μM) and the same concentrations of other components as used in the standard assay

mixture. The production of Ara<sub>1</sub>Gal<sub>7</sub>-2AB was quantified by HPAEC. The enzyme activity toward oligosaccharides with different DPs was determined at 25°C for 20 min by using 2-AB-labeled galacto-oligosaccharides with DP 3–7 (100 μM) and the same concentrations of other components as used in the standard assay mixture. The effect of various cations on the activity was examined by incubations in 25 mM final concentration and the same concentrations of other components as used in the standard assay mixture at 25°C for 20 min. Enzyme assays were performed, at least, in duplicate.

#### Analysis of reaction product

Transfer reactions were performed in 40 mM Mes-KOH, pH 6.5 (30 μl) containing UDP-Ara (2 mM), Gal<sub>6</sub>-2AB or Gal<sub>7</sub>-2AB (0.5 mM), detergent solubilized microsomal fraction (80–100 μg protein), and the same concentrations of the components as used in the standard assay mixture for 2 h at 25°C, and then kept for 16 h at 4°C. At this time the starting galacto-oligosaccharides converted into mono-arabinosyl galacto-oligosaccharides in almost 90% yield. The reaction mixture was then heated for 1 min in a boiling-water bath, centrifuged and the supernatant collected. The products from 50 separate reactions were combined and applied to a Bio-Gel P-2 (1.6 cm×90 cm) and a Bio-Gel P-4 (1.6 cm×90 cm, Bio-Rad) column connected in series and eluted with water at 40°C at a flow rate of 0.4 ml min<sup>-1</sup>. The fluorescence-positive fractions were collected and freeze-dried. A solution of the residue in water (100 μl) was extracted with toluene (100 μl×10) to remove Triton X-100, and the aqueous fraction then freeze-dried. The residue was dissolved in ammonium acetate and separated by normal-phase liquid chromatography (LC) with UV detection at 254 nm. The UV-positive fractions (retention time 17 min and 19 min for Ara<sub>1</sub>Gal<sub>6</sub>-2AB and Ara<sub>1</sub>Gal<sub>7</sub>-2AB, respectively) were collected manually, concentrated by rotary evaporator, and freeze-dried. These procedures were repeated three times to obtain a sufficient amount of the products for NMR analysis (0.3–0.5 mg). The purified oligosaccharides were analyzed by electrospray-ionization mass spectrometry (ESI-MS), <sup>1</sup>H and <sup>13</sup>C NMR spectroscopy and by glycosyl residue composition and glycosyl linkage analyses. Normal-phase LC with UV (254 nm) and fluorescence (λ<sub>ex</sub> = 330 nm, λ<sub>em</sub> = 420 nm) detection was performed using a Phenomenex Luna NH<sub>2</sub> column (150×4.6 mm Shimadzu GLC, Tokyo, Japan) eluted at 0.4 ml min<sup>-1</sup>, at 30°C. The column was eluted as follows: eluent A, 50 mM ammonium acetate (pH 4.5) and eluent B, aqueous 90% (v/v) acetonitrile, and a linear gradient of eluent B from 73%(v/v) to 55%(v/v) in 70 min. Normal phase LC-ESI-MS analysis was performed at 0.4 ml min<sup>-1</sup> by connecting the outlet of the column to a LCQ classic mass spectrometer (Thermo electron, Waltham, MA, USA). Electrospray-ionization mass spectra were recorded in the positive-ion mode

with a spray voltage of 3.5 KV, a capillary voltage of 5.0 V and a capillary temperature of 200°C. Spectra were obtained between *m/z* 260 and 2,000. For NMR spectroscopic analysis, the transfer product was dissolved in 99.96% isotopically enriched D<sub>2</sub>O and then freeze-dried. The residue was dissolved in 99.96% enriched D<sub>2</sub>O. One- and two-dimensional <sup>1</sup>H and <sup>13</sup>C spectra were recorded at 30°C and 800 MHz with a Bruker Avance 800 NMR spectrometer using the pulse sequences and software provided by the manufacturer (Ishii et al. 2005). The glycosyl residue and glycosyl linkage compositions of the oligosaccharide products were determined as described previously (York et al. 1986). Portions of the oligosaccharides were treated for 2 h at 40°C with α-L-arabinofuranosidase (0.1 unit) in 25 mM Na-acetate, pH 4.6. After heating the reaction mixture in boiling water the products were analyzed by HPAEC.

#### UDP-Ara and UDP-Gal coincubation with Gal<sub>6</sub>-2AB

For preparing the reaction product for structural analysis Gal<sub>6</sub>-2AB (0.1 mM) was coincubated with UDP-Ara *p* (2 mM) and UDP-Gal (2 mM) and the same concentrations of other components as used in the standard assay mixture under the same conditions as those of the large scale preparation of transfer products. The products from ten separate reactions were combined and subjected to the same procedures as those of the large-scale preparation of the transfer products. The purified oligosaccharides were analyzed by normal phase LC-ESI-MS as described above.

#### Isolation of rhamnogalacturonan I

Alcohol-insoluble residue was prepared from the hypocotyls (1.5–2 cm in length from the cotyledon). The tissues were saponified with 0.1 N NaOH for 4 h at 4°C and then digested with EPG for 16 h at 35°C (Ishii et al. 2001).

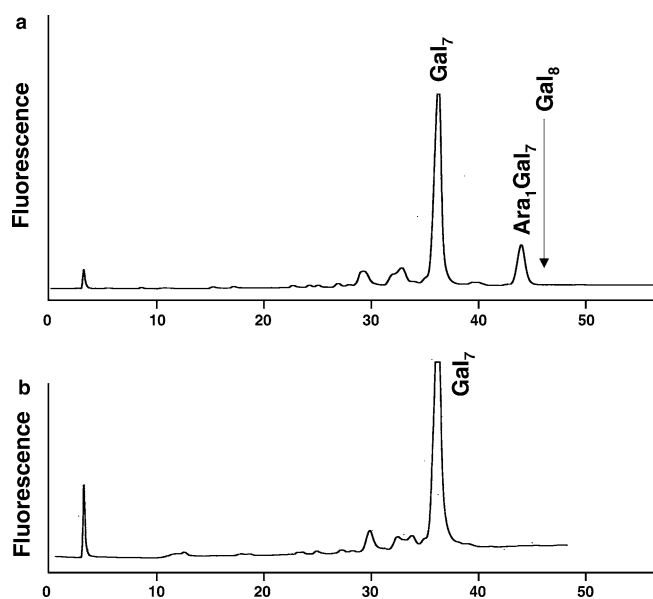
The EPG-soluble fraction was digested with α-amylase and dialyzed with molecular cut off 1,000 dialysis tube and then freeze-dried. The EPG-soluble, α-amylase treated material was separated with a Superdex 75 column (1.6 cm×40 cm) eluted with 50 mM HCOONH<sub>4</sub> (pH 5.0). The void volume fraction was manually collected and dialyzed, and then freeze-dried.

## Results

Mung bean microsomes contain enzymes that transfer arabinose from UDP-β-L-Arap onto galacto-oligosaccharides

Reacting 2AB-labeled galacto-heptasaccharide (Gal<sub>7</sub>-2AB) and UDP-β-L-Ara *p* with a detergent-solubilized microsomal fraction from mung bean hypocotyls

generated a product whose elution time differed from that of standard Gal<sub>7</sub>-2AB (35.1 min) and Gal<sub>8</sub>-2AB (44.0 min; Fig. 1a). The LC-ESI-MS spectrum of the newly formed product contained an ion at  $m/z$  1,427 that corresponds to the  $[M + Na]^+$  ion of a compound that is composed of one pentose, six hexoses, a hexitol, and 2AB (Fig. 2a). The product ion spectrum of the sodium adduct ion at  $m/z$  1,427 contained ions at  $m/z$  1,295, 1,133, 971, 809, and 647. These series of ions are the  $Y_n$  ions (Costello and Vath 1993), and correspond to the sequential loss of a pentose, and a series of hexoses (Fig. 2b). The product ion mass spectrum also contained ions at  $m/z$  1,127, 965, 803, and 641 that correspond to the  $Z_n$  ions that result from the loss of 2AB-Gal, 2AB-Gal<sub>2</sub>, 2AB-Gal<sub>3</sub> and 2AB-Gal<sub>4</sub> (Fig. 2b). These results are consistent with the existence of the oligosaccharide Ara<sub>1</sub>Gal<sub>7</sub>-2AB. The results of ESI-MS-MS analysis of the product formed by reacting Gal<sub>6</sub>-2AB with UDP- $\beta$ -L-Ara *p* in the presence of the detergent-solubilized microsomal fraction were consistent with the formation of Ara<sub>1</sub>Gal<sub>6</sub>-2AB. No peak corresponding to Ara<sub>1</sub>Gal<sub>7</sub>-2AB was detected when Gal<sub>7</sub>-2AB and UDP- $\beta$ -L-Ara *p* were reacted together in the absence of the solubilized microsomal fraction or in the presence of the heat-denatured microsomal extract (Fig. 1b), or when UDP- $\beta$ -L-Ara *p* was omitted from the reaction mixture. Taken together these results provide strong evidence that an enzyme that transfers a single arabinose to the nonreducing terminal galactosyl residue of 1,4-linked  $\beta$ -D-galacto-oligosaccharides is present in mung bean hypocotyl microsomes.



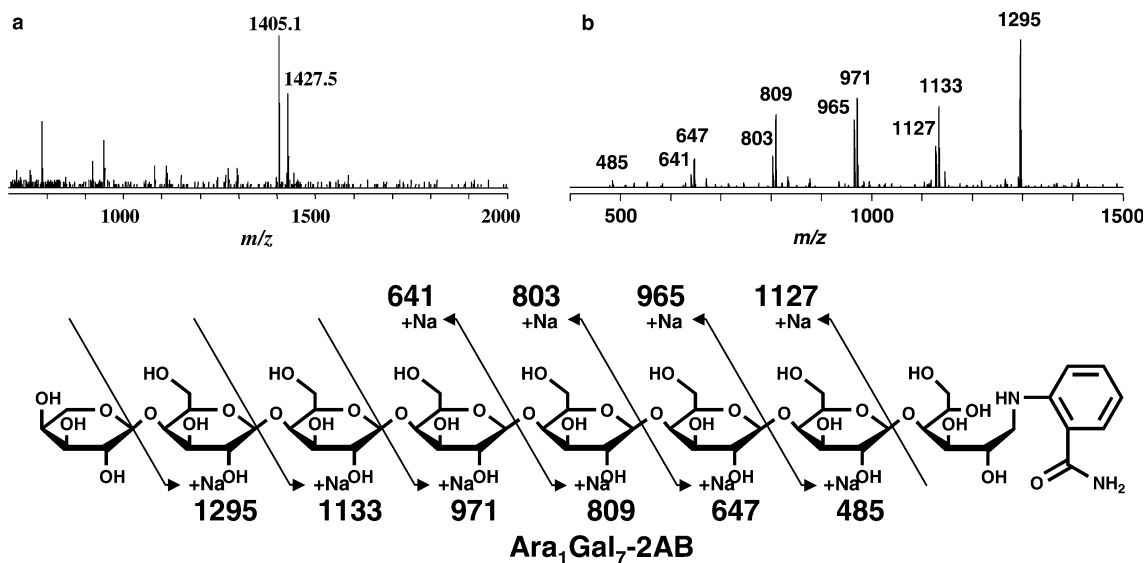
**Fig. 1** HPAEC profiles of the products formed when Gal<sub>7</sub>-2AB and UDP-Ara are reacted together at 25°C for 20 min with the Triton X-100 solubilized extract of the microsomal fractions from mung bean (*Vigna radiata*) under the standard conditions. **a** The reaction mixture contained Gal<sub>7</sub>-2AB (100  $\mu$ M), UDP-Ara (2 mM) and the solubilized microsomal fraction (100  $\mu$ g protein). **b** Denatured one. The elution position of Gal<sub>8</sub>-2AB is indicated by an arrow

The primary structures of enzymically formed Ara<sub>1</sub>Gal<sub>6</sub>-2AB and Ara<sub>1</sub>Gal<sub>7</sub>-2AB

The primary structures of the enzymically formed Ara<sub>1</sub>Gal<sub>6</sub>-2AB and Ara<sub>1</sub>Gal<sub>7</sub>-2AB (Fig. 3) were determined by <sup>1</sup>H and <sup>13</sup>C NMR spectroscopy and by glycosyl residue and glycosyl linkage composition analyses. Ara<sub>1</sub>Gal<sub>6</sub>-2AB and Ara<sub>1</sub>Gal<sub>7</sub>-2AB in amounts sufficient for these analyses were generated by reacting Gal<sub>6</sub>-2AB and Gal<sub>7</sub>-2AB (0.5 mM) with UDP- $\beta$ -L-Ara *p* (2 mM). The products from fifty separate reactions were combined and purified by size-exclusion and normal-phase liquid chromatography and this procedure was repeated three times to obtain a sufficient amount of the products for NMR analysis (see “Materials and methods”).

Ara<sub>1</sub>Gal<sub>6</sub>-2AB and Ara<sub>1</sub>Gal<sub>7</sub>-2AB were shown, by glycosyl residue composition analysis, to contain Ara:Gal in the molar ratio of 1:3.5 and 1:4.5, respectively (Table 1). Glycosyl linkage composition analysis gave 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol (derived from 4-linked galactopyranosyl residue) and 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylarabinitol in the molar ratio of 5.2:1 and 6.5:1 for Ara<sub>1</sub>Gal<sub>6</sub>-2AB and Ara<sub>1</sub>Gal<sub>7</sub>-2AB, respectively (Table 1). The arabinitol derivative must be formed from a nonreducing terminal arabinopyranosyl residue because it contains *O*-methyl groups at *O*-2, *O*-3, and *O*-4. No arabinose was released when Ara<sub>1</sub>Gal<sub>6</sub>-2AB was treated with a commercial arabinofuranosidase that is known to hydrolyze terminal  $\alpha$ -L-Ara *f* residues (data not shown). These results strongly suggest that an arabinopyranosyl rather than an arabinofuranosyl residue is linked to the galacto-oligosaccharides.

Additional evidence for the ring form and anomeric configuration of the arabinosyl residue linked to the galacto-oligosaccharides was obtained by <sup>1</sup>H and <sup>13</sup>C NMR spectroscopic analyses of Ara<sub>1</sub>Gal<sub>6</sub>-2AB. The signals in the <sup>1</sup>H NMR spectrum of this product (Fig. 4) were assigned (see Table 2) by 2D-double quantum filtered correlation spectroscopy (DQFCOSY), and 2D-total correlation spectroscopy (TOCSY). The doublet at  $\delta$ 4.555 ( $J_{1,2}$  7.6 Hz) is assigned to the H-1 resonance of the nonreducing terminal arabinopyranosyl residue (Ara *p*). This chemical shift value and the magnitude of the  $J_{1,2}$  coupling constant are consistent with a  $\alpha$ -linkage (Glushka et al. 2003). The chemical shift values of the H-1 of the former reducing Gal residue (residue A) and the H-1s of the internal galactosyl residues (B, I, E, and T) and the magnitude of their  $J_{1,2}$  coupling constants (7.9 Hz) are consistent with a  $\beta$ -linkage (Agrawal 1992). The signals corresponding to H-2, H-3, and H-4 of the Ara and Gal residues were assigned using DQFCOSY and TOCSY. The two H-5s of the Ara residue and H-5 and two H-6s of the Gal residue were assigned by HSQC and HMBC analyses. The anomeric configuration and ring conformation of an arabinopyranosyl residue is readily determined by measuring its <sup>3</sup> $J_{H,H}$  scalar coupling constant (Glushka et al. 2003). The values of <sup>3</sup> $J_{H1,H2}$  and <sup>3</sup> $J_{H2,H3}$  are > 7 Hz, which is characteristic of a *trans*-diaxial arrangement of H-1, H-2 and H-3 in a



**Fig. 2** Electrospray-ionization mass spectra of the enzymatically formed Ara<sub>1</sub>Gal<sub>7</sub>-2AB. **a** Positive-ion mode ESI mass spectrum of Ara<sub>1</sub>Gal<sub>7</sub>-2AB. **b** Positive-ion mode MS/MS spectrum of the product ion ([Ara<sub>1</sub>Gal<sub>7</sub>-2AB + Na]<sup>+</sup>, *m/z* 1427) of Ara<sub>1</sub>Gal<sub>7</sub>-2AB

**Table 1** Characterization of arabinosyltransferase transferred products

Compound	Ara <sub>1</sub> Gal <sub>6</sub> -2AB	Ara <sub>1</sub> Gal <sub>7</sub> -2AB
Molecular weight		
[M + Na] <sup>+</sup>	1,265	1,427
Calculated mass	1,242	1,404
Glycosyl composition (mole ratio)		
Ara	1.0	1.0
Gal	3.5	4.5
Glycosyl linkage (mole ratio)		
T-Ara <i>p</i> <sup>a</sup>	1.0	1.0
4-Gal <sup>b</sup>	5.2	6.5

<sup>a</sup>T-Ara *p* means terminal arabinopyranosyl residue

<sup>b</sup>4-Gal means 4 linked galactopyranosyl residue

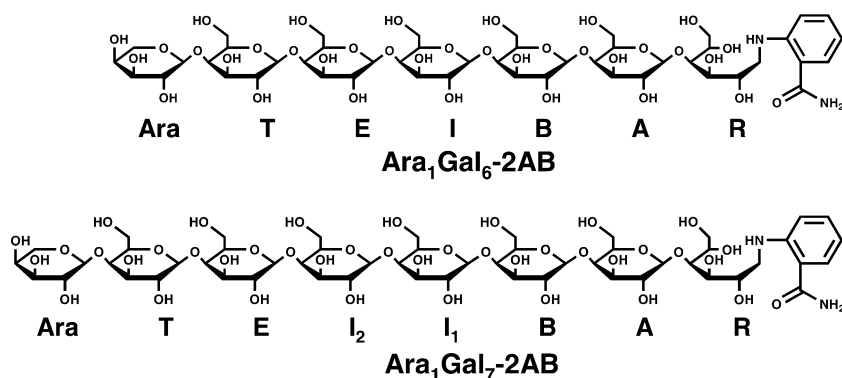
pyranose ring with a <sup>4</sup>C<sub>1</sub> chair conformation, and thus confirms the α-Ara *p* configuration. The <sup>13</sup>C NMR spectra of the 2AB-labeled Ara<sub>1</sub>Gal<sub>6</sub> and Ara<sub>1</sub>Gal<sub>7</sub> were assigned using HSQC and HMBC spectroscopy (see Table 3). The HMBC spectrum gave a cross peak between H-1 of Ara *p* and C-4 of Gal residue T, thereby, confirming that the terminal arabinose is linked to O-4 of this Gal residue (Fig. 5). Taken together, these results show that mung bean hypocotyls contain α-(1 → 4)-arabinopyranosyltransferase (AraT) activity and that this enzyme transfers Ara *p* from UDP-β-L-Ara to O-4 of the terminal Gal residue of exogenous 2AB-labeled galacto-oligosaccharides.

#### Characterization of α-(1 → 4)-arabinopyranosyltransferase

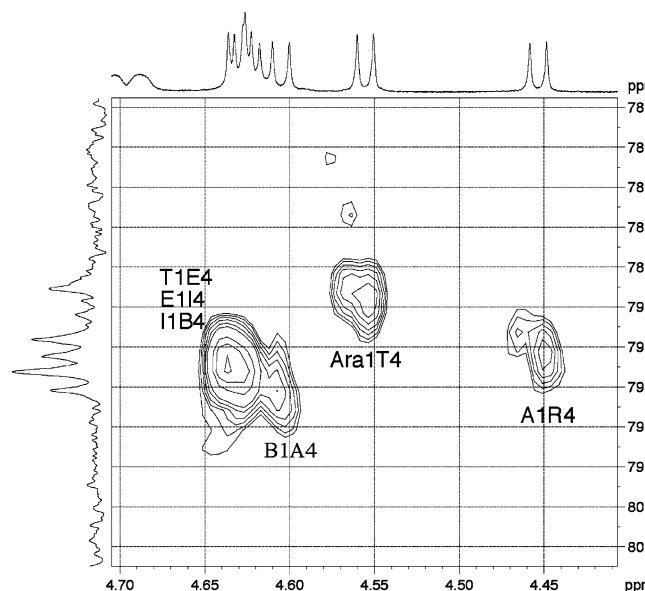
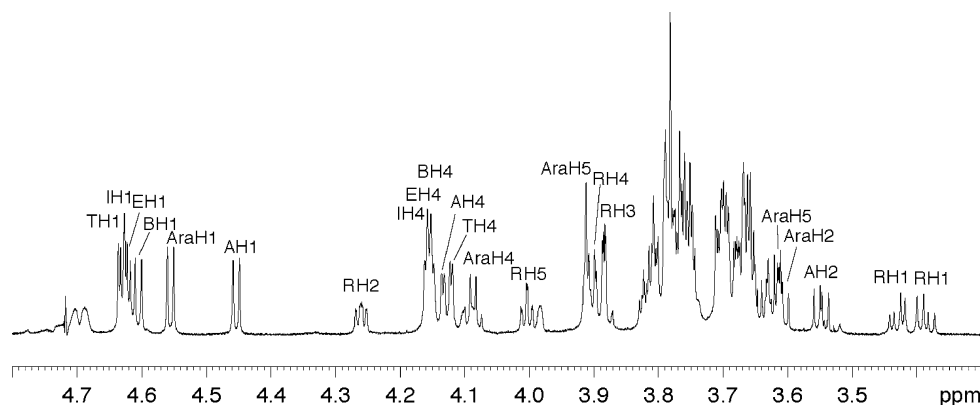
The transfer of Ara from UDP-β-L-Ara to Gal<sub>7</sub>-2AB increased with time and was linear over 20 min (Fig. 6a), was dependent on the protein concentration (Fig. 6b) and

was maximum at pH 6.5 (Fig. 6c). Maximal enzyme activity was obtained at 20°C (Fig. 6d). The activity increased in the presence of Mn<sup>2+</sup> (5.3), but not in the presence of Mg<sup>2+</sup> (0.9), Ca<sup>2+</sup> (0.6), Co<sup>2+</sup> (1.2), Cu<sup>2+</sup> (1.3), and Zn<sup>2+</sup> (1.1; numbers in parenthesis indicate the relative activity to none as 1.0). The apparent *K<sub>m</sub>* values for the Gal<sub>7</sub>-2AB and for UDP-β-L-Ara were 45 μM and 330 μM, respectively. The apparent *V<sub>max</sub>* values for Gal<sub>7</sub>-2AB and for UDP-β-L-Ara were 200 pmol min<sup>-1</sup>(mg protein)<sup>-1</sup> and 250 pmol min<sup>-1</sup>

**Fig. 3** Structure of Ara<sub>1</sub>Gal<sub>6</sub>-2AB and Ara<sub>1</sub>Gal<sub>7</sub>-2AB



**Fig. 4**  $^1\text{H}$  NMR spectrum of the enzymatically formed  $\text{Ara}_1\text{Gal}_6\text{-2AB}$  2AB-labeled  $\text{Ara}_1\text{Gal}_6$  is composed of galactitol (*R*), five internal galactosyl residues (*A*, *B*, *I*, *E*, and *T*), and a nonreducing terminal arabinopyranosyl residue (*Ara p*). Signals of 2AB are not shown. (see Fig. 3)



**Fig. 5** Contour plot of a portion of the HMBC spectrum of  $\text{Ara}_1\text{Gal}_6\text{-2AB}$ .  $\text{Ara}_1\text{T}_4$  is the cross-peak observed between H-1 of terminal Ara residue (*Ara*) and C-4 of former terminal Gal residue (*T*)

( $\text{mg protein}^{-1}$ ), respectively.  $\text{Gal}_6\text{-}$  and  $\text{Gal}_7\text{-2AB}$  are more effective acceptors than  $\text{Gal}_4\text{-}$  and  $\text{Gal}_5\text{-2AB}$  (Fig. 7). Virtually no arabinose was transferred to  $\text{Gal}_3\text{-}$  and  $\text{Gal}_2\text{-2AB}$  (Fig. 7). The oligosaccharides with DP more than 8 were not determined because it was not possible to separate completely oligosaccharides with DP 8, 9 and 10 from a mixture by size-exclusion chromatography with BioGelP-2 and P-4 columns.

Galactosyltransferase activity to the galacto-oligosaccharides containing terminal arabinopyranosyl residue

In a previous study we showed that mung bean microsomes contain an enzyme that catalyzes the transfer of Gal from UDP- $\beta\text{-D-Gal p}$  to  $\text{Gal}_7\text{-2AB}$  to from  $\text{Gal}_8\text{-2AB}$  through  $\text{Gal}_{13}\text{-2AB}$  (Ishii et al. 2004). We now provide evidence that  $\text{Ara}_1\text{Gal}_6\text{-2AB}$ ,  $\text{Ara}_1\text{Gal}_7\text{-2AB}$ ,  $\text{Ara}_1\text{Gal}_8\text{-2AB}$ , and  $\text{Ara}_1\text{Gal}_9\text{-2AB}$  are formed (see Fig. 8) when  $\text{Gal}_6\text{-2AB}$  reacts with UDP- $\beta\text{-D-Gal p}$

together with UDP- $\beta\text{-L-Ara p}$  and the solubilized microsomal fraction. ESI-MS-MS analysis showed that the pentosyl residue is attached to the nonreducing terminal Gal residue of the galacto-oligosaccharides. No peaks were detected that correspond to 2AB-labeled galacto-oligosaccharides that contain more than one pentosyl residue. Moreover, no galactosyl residues were added to  $\text{Ara}_1\text{Gal}_7\text{-2AB}$  when this derivative was reacted with UDP-Gal and the solubilized microsomal fraction (data not shown). Such results show that  $\text{Ara}_1\text{Gal}_7\text{-2AB}$  is not an acceptor substrate for galactosyltransferase (GalT) and suggests that the presence of *Ara p* prevents the elongation of galactan chains.

#### Mung bean hypocotyls contain nonreducing terminal *Ara p* Residue

Rhamnogalacturonan I was isolated from EPG solubilized fraction of mung bean hypocotyls. Glycosyl linkage analysis gave 2-linked and 4-linked Rha residues (Table 4), showing that the polysaccharide was RG-I and that the side chains are attached to the backbone at *O*-4 of the Rha residues (Ridley et al. 2001). The presence of 4-linked Gal and 5-linked *Ara f* in molar ratio of 1:12, indicated that the side chain contained a short linear (1  $\rightarrow$  4)-linked galactan and a long linear (1  $\rightarrow$  5)-linked arabinan. Terminal *Ara p* and terminal *Ara f* were detected in molar ratio of 2:7, indicating that terminal arabinopyranosyl residues are present in the side chain of RG-I.

#### Discussion

We have shown that mung bean microsomes contain an enzyme that transfers the arabinopyranosyl residue from UDP- $\beta\text{-L-arabinopyranose}$  onto 1,4-linked  $\beta\text{-D-galacto-oligosaccharides}$ . Our chemical and spectroscopic data show unequivocally that a single  $\alpha\text{-L-arabinopyranosyl}$  residue is linked to *O*-4 of the terminal Gal residue of the galacto-oligosaccharide. Such results are consistent with a previous report showing that mung bean hypocotyls contain arabinopyranosyltransferases (Nunan and Scheller 2003).

**Table 2**  $^1\text{H}$  Chemical shifts ( $\delta$ , ppm) and first-order coupling constants (Hz) for compounds Ara<sub>1</sub>Gal<sub>6</sub>-2AB and Ara<sub>1</sub>Gal<sub>7</sub>-2AB

Compound	Residue	$^1\text{H}$ Chemical shifts (ppm) <sup>a, b</sup>					
		H-1	H-2	H-3	H-4	H-5	H-6
Ara <sub>1</sub> Gal <sub>6</sub> -2AB	R	3.386 (5.2), 3.430 (8.0)	4.259 (8.0)	3.684 (3.5)	3.899 (1.0)	4.004	3.776 (7.0), 3.677 (4.0)
	A	4.454 (7.9)	3.549 (9.9)	3.708 (3.0)	4.133 (<1)	3.703 <sup>c</sup>	3.823 <sup>c</sup> , 3.781 <sup>c</sup>
	B	4.605 (7.9)	3.653 (10.0)	3.771 <sup>c</sup> (3.0)	4.153 <sup>c</sup> (<1)	3.695 <sup>c</sup>	3.815 <sup>c</sup> , 3.781 <sup>c</sup>
	I	4.626 (7.9)	3.651 <sup>c</sup> (10.0)	3.756 <sup>c</sup> (3.0)	4.158 <sup>c</sup> (<1)	3.692 <sup>c</sup>	3.810 <sup>c</sup> , 3.776 <sup>c</sup>
	E	4.623 (7.9)	3.649 <sup>c</sup> (10.0)	3.756 <sup>c</sup> (3.0)	4.155 <sup>c</sup> (<1)	3.693 <sup>c</sup>	3.808 <sup>c</sup> , 3.775 <sup>c</sup>
	T	4.632 (7.9)	3.652 <sup>c</sup> (10.0)	3.747 <sup>c</sup> (3.0)	4.121 (<1)	3.695 <sup>c</sup>	3.788, 3.771
	Ara	4.555 (7.6)	3.609 (10.0)	3.666 (3.0)	4.087 (<1)	3.915, 3.614	–
Ara <sub>1</sub> Gal <sub>7</sub> -2AB	R	3.430 (5.2), 3.387 (8.0)	4.261 (8.0)	3.890 (3.5)	3.899 (1.0)	4.005	3.765 (7.0), 3.684 (4.0)
	A	4.452 (8.0)	3.551 (9.9)	3.701 (3.0)	4.135 (<1)	3.654 <sup>c</sup>	3.813 <sup>c</sup> , 3.761
	B	4.607 (8.0)	3.654 <sup>c</sup> (10.0)	3.748 <sup>c</sup> (3.0)	4.153 <sup>c</sup> (<1)	3.696 <sup>c</sup>	3.816 <sup>c</sup>
	I <sub>1</sub>	4.630 <sup>c</sup> (8.0)	3.658 <sup>c</sup> (10.0)	3.752 <sup>c</sup> (3.0)	4.154 <sup>c</sup> (<1)	3.703 <sup>c</sup>	3.821 <sup>c</sup>
	I <sub>2</sub>	4.624 <sup>c</sup> (8.0)	3.660 <sup>c</sup> (10.0)	3.757 <sup>c</sup> (3.0)	4.155 <sup>c</sup> (<1)	3.709 <sup>c</sup>	3.814 <sup>c</sup>
	E	4.624 <sup>c</sup> (8.0)	3.673 <sup>c</sup> (10.0)	3.761 <sup>c</sup> (3.0)	4.155 <sup>c</sup> (<1)	3.703 <sup>c</sup>	3.815 <sup>c</sup>
	T	4.632 (8.0)	3.660 <sup>c</sup> (10.0)	3.761 <sup>c</sup> (3.0)	4.122 (<1)	3.703 <sup>c</sup>	3.817 <sup>c</sup>
	Ara	4.555 (7.6)	3.611 (10.0)	3.658 (3.0)	4.034 (<1)	3.908, 3.620	–

<sup>a</sup> $^1\text{H}$  shifts are quoted from methyl proton of internal 2-methyl-2-propanol (1.230 ppm) at 800 MHz at 30°C

<sup>b</sup> $^1\text{H}$  chemical shift and coupling constant assignments are based on 1D,  $^1\text{H}$ , DQFCOSY, and TOCSY spectra

<sup>c</sup>Interchangeable, uncertain

**Table 3**  $^{13}\text{C}$  chemical shifts ( $\delta$ , ppm) for compounds Ara<sub>1</sub>Gal<sub>6</sub>-2AB and Ara<sub>1</sub>Gal<sub>7</sub>-2AB

Compound	Residue	$^{13}\text{C}$ chemical shifts (ppm) <sup>a</sup>					
		C-1	C-2	C-3	C-4	C-5	C-6
Ara <sub>1</sub> Gal <sub>6</sub> -2AB	R	47.49	68.75	71.63	79.16	71.69	64.23
	A	104.79	73.01	74.77	79.42	75.93	62.62 <sup>b</sup>
	B	106.04	73.52	75.02 <sup>b</sup>	79.32	76.21 <sup>b</sup>	62.62 <sup>b</sup>
	I	106.04	73.52	75.02 <sup>b</sup>	79.32	76.21 <sup>b</sup>	62.62 <sup>b</sup>
	E	106.04	73.52	75.02 <sup>b</sup>	79.32	76.21 <sup>b</sup>	62.62 <sup>b</sup>
	T	106.04	73.52	75.11 <sup>b</sup>	78.91	76.31 <sup>b</sup>	62.60 <sup>b</sup>
	Ara	106.24	73.17	74.13	70.20	68.18	–
Ara <sub>1</sub> Gal <sub>7</sub> -2AB	R	47.47	68.72	71.60	79.13	71.66	64.21
	A	104.77	72.99	74.75	79.40	75.92	62.42 <sup>b</sup>
	B	106.03	73.50	75.00 <sup>b</sup>	79.22	76.20 <sup>b</sup>	62.42 <sup>b</sup>
	I <sub>1</sub>	106.03	73.50	75.00 <sup>b</sup>	79.32	76.20 <sup>b</sup>	62.42 <sup>b</sup>
	I <sub>2</sub>	106.03	73.50	75.00 <sup>b</sup>	79.32	76.20 <sup>b</sup>	62.42 <sup>b</sup>
	E	106.03	73.50	75.00 <sup>b</sup>	79.32	76.20 <sup>b</sup>	62.42 <sup>b</sup>
	T	106.03	73.50	75.09 <sup>b</sup>	78.90	76.29 <sup>b</sup>	62.58 <sup>b</sup>
	Ara	106.23	73.16	74.24	70.05	68.16	–

<sup>a</sup> $^{13}\text{C}$  chemical shifts are quoted from methyl carbon of 2-methyl-2-propanol (31.30 ppm) at 150 MHz at 30°C and their assignments are based on 1D  $^{13}\text{C}$ , HSQC and HMBC spectra

<sup>b</sup>Interchangeable, uncertain

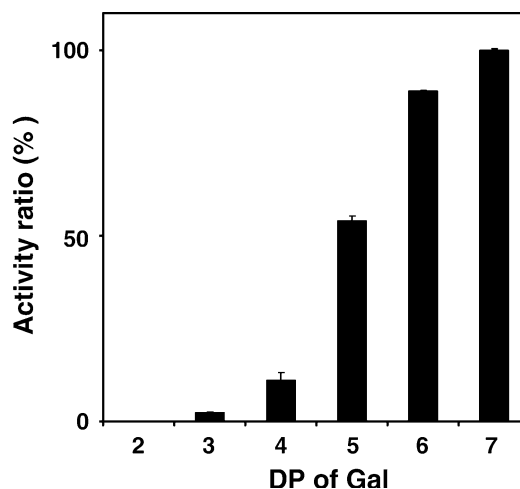
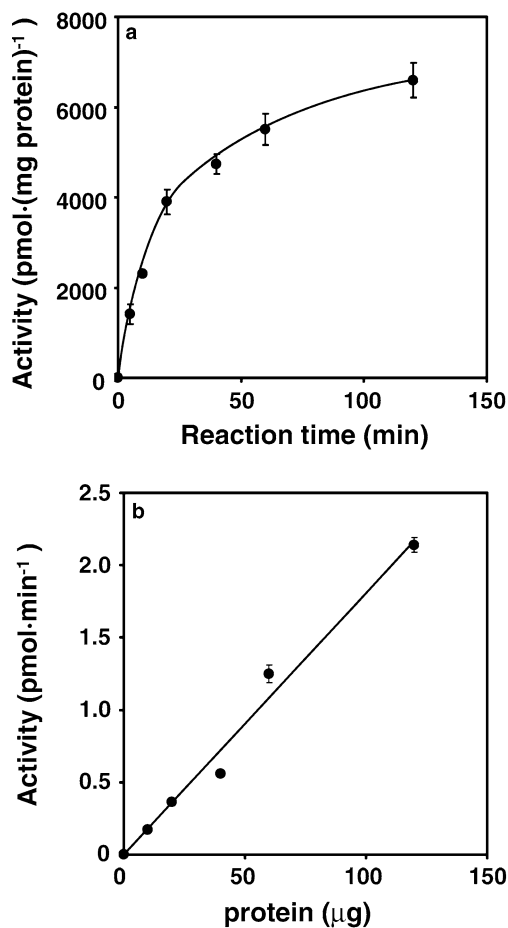
Studies of the biosynthesis of arabinose-containing plant polysaccharides are complicated by the fact that most of the arabinose residues exist in the  $\alpha$ -L-Ara *f* form (Carpital and Gibeaut 1993) yet the only readily available glycosyl donor for in vitro studies is UDP- $\beta$ -L-Ara *p*. Early studies using this UDP sugar and crude membrane preparations provided evidence for the incorporation of arabinosyl residues into polysaccharides (Odzurk and Kauss 1972; Bolwell and Northcote 1981). However, neither the structures of the acceptor polysaccharides were fully characterized nor were the ring form, anomeric configuration, and glycosidic linkage of the incorporated arabinosyl residues determined. Our data together with the results of Nunan and Scheller (2003) show that mung bean microsomes contain enzymes that add a single  $\alpha$ -L-Ara *p* residue to exogenous  $\alpha$ -L-(1  $\rightarrow$  5) linked arabinooligosaccharide and  $\beta$ -D-(1  $\rightarrow$  4) linked galactooligosaccharides. RG-I isolated from 3-day-old mung bean

hypocotyls contained nonreducing terminal Ara *p* residues. Some terminal Ara *p* residues detected here might be derived from the arabino-oligosaccharides. Some plant species have been reported to synthesize polysaccharides that do contain quantitatively small amounts of this pyranosyl residue. For example, terminal Ara *p* residues have been detected in arabinans from white willow (Karácsonyi et al. 1975), marsh mallow (Capek et al. 1983), and Mongolian larchwood (Odonmažig et al. 1994). Ara *p* residues are believed to terminate some of the galactose-containing side chains of soybean RG-I (Huisman et al. 2001). Thus, we believe our data is consistent with the notion that Ara *p*-containing oligosaccharides are formed naturally in plants (Nunan and Scheller 2003) and that this process is likely to account for some of Ara *p* that are present in RG-I of mung bean hypocotyls.

Present results show that mung bean hypocotyls contain an enzyme that catalyzes the formation of a

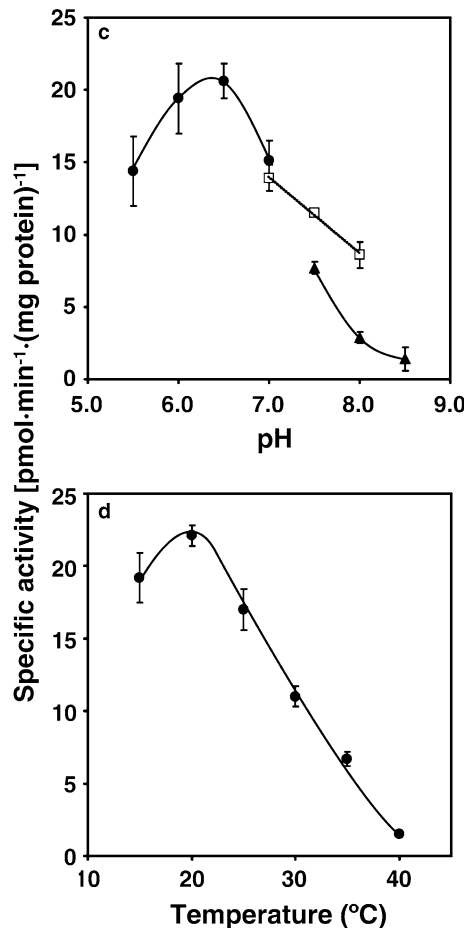
glycosidic bond between  $\alpha$ -L-Ara *p* and *O*-4 of the nonreducing terminal Gal of 1,4-linked  $\beta$ -D-galacto-oligosaccharides. Such a reaction is likely to be catalyzed by an  $\alpha$ -(1  $\rightarrow$  4)-arabinopyranosyltransferase. However, there will be some possibilities that Ara *p*-containing oligosaccharides form. One possibility is that plants contain other enzymes that convert Ara *p* to Ara *f*, in addition to transferase. Thus, the enzymes that convert UDP- $\beta$ -L-Ara *p* to UDP- $\beta$ -L-Ara *f*, if they exist, may be labile and loose activity when they are solubilized with

**Fig. 6** Activity of AraT in the Triton X-100 soluble fraction from mung bean hypocotyl microsomes. **a** Time course of the formation of Ara<sub>1</sub>Gal<sub>7</sub>-2AB from Gal<sub>7</sub>-2AB. The membrane extract (100  $\mu$ g protein) was incubated with 100  $\mu$ M Gal<sub>7</sub>-2AB and 2 mM UDP-Ara at 25°C in the presence of 25 mM MnCl<sub>2</sub> under the standard conditions. **b** Effect of membrane-protein concentration on the formation of Ara<sub>1</sub>Gal<sub>7</sub>-2AB from Gal<sub>7</sub>-2AB. The reaction was allowed to proceed at 25°C for 20 min using 10  $\mu$ M Gal<sub>7</sub>-2AB and 2 mM UDP-Ara under standard conditions. **c** Effects of pH on the formation of Ara<sub>1</sub>Gal<sub>7</sub>-2AB from Gal<sub>7</sub>-2AB. Mung bean membrane extract (100  $\mu$ g protein) was incubated with 10  $\mu$ M Gal<sub>7</sub>-2AB and 2 mM UDP-Ara at 25°C for 20 min under the standard conditions. The buffers used to control the reaction pH were 40 mM Mes-KOH (filled circle), Hepes-KOH (open square) and Tris-HCl (filled triangle). **d** Effects of temperature on the formation of Ara<sub>1</sub>Gal<sub>7</sub>-2AB from Gal<sub>7</sub>-2AB. Mung bean membrane extract (100  $\mu$ g protein) was incubated with 10  $\mu$ M Gal<sub>7</sub>-2AB and 2 mM UDP-Ara at 25°C for 20 min under the standard conditions



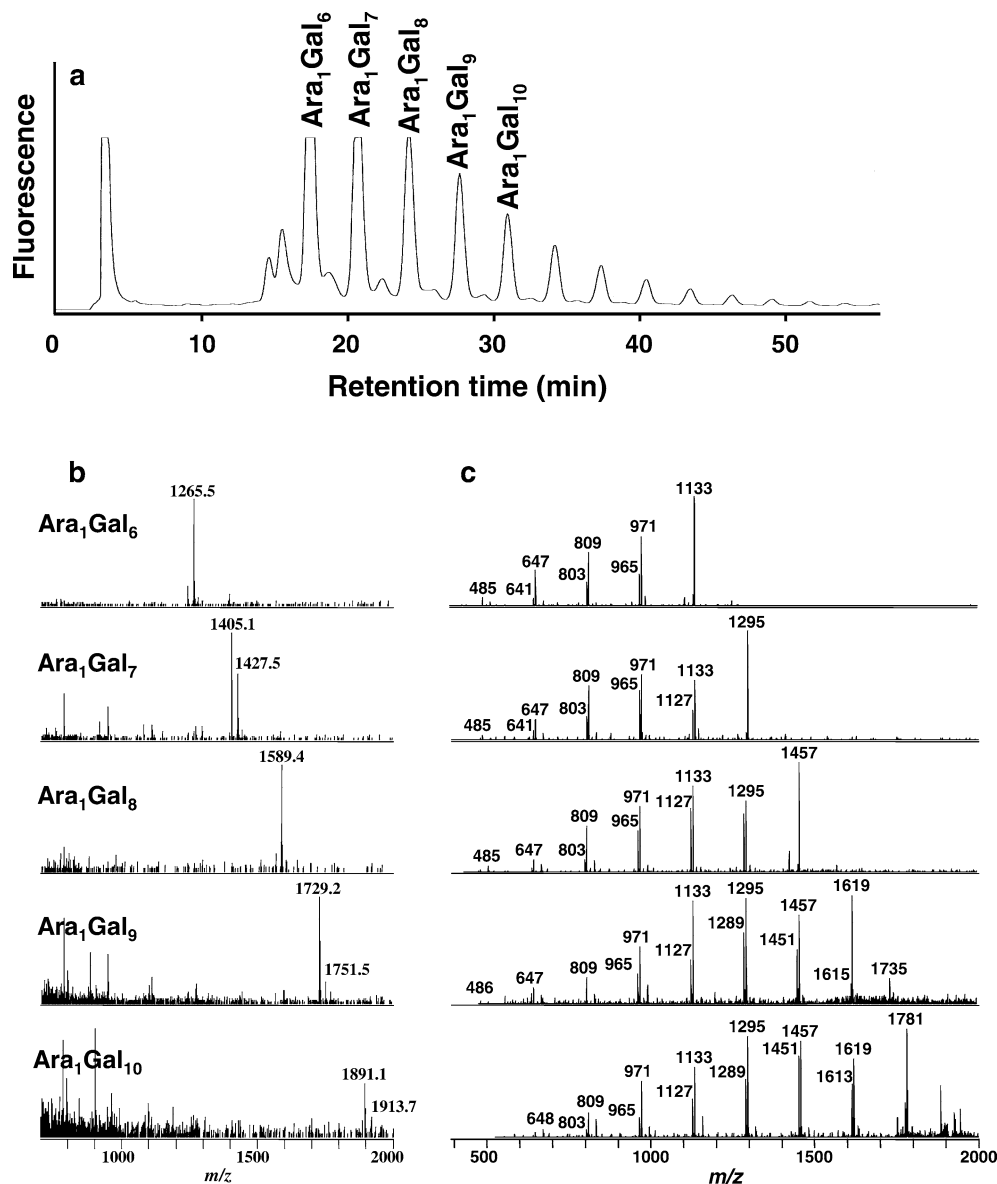
**Fig. 7** Effects of the DP of the galacto-oligosaccharide acceptors on solubilized microsomal AraT activity. The reaction was conducted with solubilized microsomes (100  $\mu$ g protein), 2 mM UDP-Ara and 2AB-labeled galacto-oligosaccharides (100  $\mu$ M) with DPs between 3 and 7 at 25°C for 20 min. The transferase activities are shown relative to that of the Gal<sub>7</sub>-2AB. Data are the average of duplicates

detergent. In this study only Triton X-100 was used for solubilization of the membranes. However, when a range of different detergents were used to study transfer





**Fig. 8** HPEAC profile and ESI-MS spectra of the products formed when Gal<sub>6</sub>-2AB (100 μM), UDP-Ara (2 mM) and UDP-Gal (2 mM) are reacted with solubilized microsomal fractions (100 μg protein). **a** Fluorescence detection of the enzymatically formed 2AB-labeled mono-arabinosylated galacto-oligosaccharides. **b** Positive-ion mode ESI mass spectra of 2AB-labeled mono-arabinosylated galacto-oligosaccharides with DP 6–10. **c** Positive-ion mode MS/MS spectra of the sodium adduct ion [M + Na]<sup>+</sup> of 2AB-labeled mono-arabinosylated galacto-oligosaccharides with DP 6–10

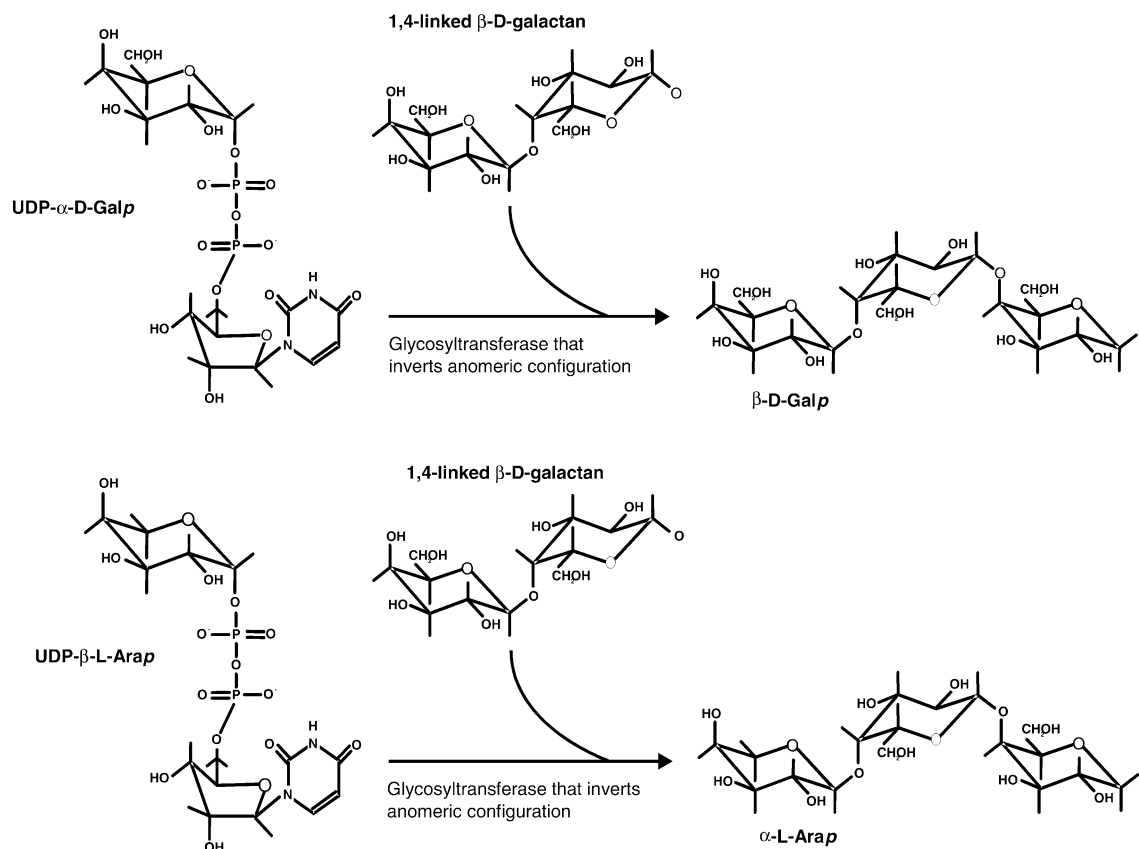


**Table 4** Glycosyl-linkage analysis of RG-I from 3-day-old mung bean

(*Vigna radiata*) hypocotyls

Glycosyl	Linkage	mole %
residue Ara	T-Ara <i>f</i>	7
	T-Ara <i>p</i>	2
	5-Ara <i>f</i>	36
	2,5-Ara <i>f</i>	3
	3,5-Ara <i>f</i>	4
	2,3,5-Ara <i>f</i>	2
Xyl	T-Xyl	3
	Rha	12
Gal	2-Rha	9
	T-Gal	12
	3-Gal	4
	4-Gal	3
	3,6-Gal	3

of arabinose to arabino-oligosaccharides only Ara *p* was detected in the product (Nunan and Scheller 2003). Indeed, reacting crude microsomal membranes of wheat (*Triticum aestivum*) with UDP-β-L-[<sup>14</sup>C] Ara *p* results in the generation of endogenous polysaccharides that contain [<sup>14</sup>C] Ara *f* residues. However, only Ara *p* residues are transferred to exogenous acceptors using detergent-soluble extracts of these membranes (Porchia et al. 2002). Studies with bacteria have shown that UDP-L-Ara *f* and UDP-L-Ara *p* are interconverted by a UDP-galactopyranose mutase (Houseknecht and Lowary 2001). However, the reaction favors the formation of the thermodynamically more stable pyranose because only quantitatively small amounts of UDP-Ara *f* are formed when the mutase is reacted with UDP-Ara *p* (Zhang and Liu 2001). Moreover, there is no evidence showing that such mutases exist in plants. As neither UDP-L-arabinofuranose nor mutase were available, it was not possible



**Fig. 9** Reaction scheme for formation of galacto-oligosaccharide and mono-arabinosylated galacto-oligosaccharide

to determine the activity using UDP-L-arabinofuranose as a donor. Another possibility is the involvement of galactopyranosyltransferase. UDP- $\beta$ -L-Ara *p* and UDP- $\beta$ -D-Gal *p* differ only in the nature of the substituents at C-5 of the glucose (Fig. 9). L-Ara *p* has two protons whereas D-Gal *p* has a proton and a hydroxymethyl group. Thus, a galactosyltransferase may be capable of forming both a  $\beta$ -D-Gal *p* and a  $\alpha$ -L-Ara *p* glycosidic linkage because it does not readily distinguish between UDP- $\beta$ -D-Gal *p* and UDP- $\beta$ -L-Ara *p*. However, it would be not possible to distinguish arabinopyranosyltransferase activity from the apparent activity due to that galactopyranosyltransferase may be able to transfer Ara *p* from UDP-Ara *p* when the activity is determined by using detergent-solubilized protein fractions.

In conclusion, an  $\alpha$ -(1  $\rightarrow$  4)-arabinopyranosyltransferase activity has been solubilized from microsomal membranes prepared from mung bean hypocotyls. It transfers a single arabinopyranosyl residue from UDP- $\beta$ -L-arabinopyranose onto 2AB-labeled galacto-oligosaccharides. The transferred arabinose is  $\alpha$ -L-arabinopyranosyl form linked to O-4 of the terminal galactopyranosyl residue of 2AB-galacto-oligosaccharides. The nonreducing terminal  $\alpha$ -L-arabinopyranosyl residue of galacto-oligosaccharides terminates galactan chain elongation. Using biochemical and bioinformatic strategies, identification and purification of  $\alpha$ -(1  $\rightarrow$  4)-

arabinopyranosyltransferases will be helpful for understanding the function of arabinopyranosyl residue in galactan.

**Acknowledgements** We thank Dr. Malcolm A. O'Neill and Dr. Debra Mohnen (Complex Carbohydrate Research Center, University of Georgia, Athens GA, USA) for critical reading the manuscript. We also thank Dr. Yuki Ito and Ms. Kyoko Oshima for preparing the galacto-oligosaccharides and the manuscript, respectively. This work was supported by the program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) and Research grant (no. 200101) of the Forestry and Forest Products Research Institute (to TI).

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