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A β-(1 → 3)-arabinopyranosyltransferase that transfers a single arabinopyranose onto arabino-oligosaccharides in mung bean (*Vigna radiate*) hypocotyls

Tadashi Ishii [a,*], Teruko Konishi [a], Yuki Ito [a], Hiroshi Ono [b], Mayumi Ohnishi-Kameyama [b], Ikuko Maeda [b]

[a] *Forestry and Forest Products Research Institute, P.O. Box 16, Tsukuba Norin Kenkyu, Danchi-Nai, Ibaraki 305-8687, Japan*
[b] *National Food Research Institute, Tsukuba, Ibaraki, 305-8642, Japan*

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Abstract

Arabinopyranosyltransferase (Ara*p*T) activity that results in the transfer of a single arabinopyranose (Ara*p*) residue from UDP-β-L-arabinopyranose (UDP-Ara*p*) to exogenous (1 → 5)-linked α-L-arabino-oligosaccharides labeled with 2-aminobenzamide (2-AB) at their reducing ends was identified in a particulate preparation obtained from 3-day-old mung bean (*Vigna radiate* L. Wilezek) hypocotyls. The transferred Ara residue was shown to be β-(1 → 3)-linked to *O*-3 of the non-reducing terminal Ara*f* residues of the oligosaccharide using nuclear magnetic resonance spectroscopy together with glycosyl composition and glycosyl linkage composition analyses. The 2AB-labeled arabino-octasaccharide was the most effective acceptor substrate analyzed, although arabino-oligosaccharides with a degree of polymerization between 4 and 7 were also acceptor substrates. Maximum Ara*p*T activity was obtained at pH 6.5–7.0, and 20 °C in the presence of 25 mM Mn^{2+} and 0.5% Triton X-100.

Keywords: *Vigna radiate*; Arabinogalactan; Arabinan; Arabino-oligosaccharide; Pectin; Rhamnogalacturonon I; β-(1 → 3)-Arabinopyranosyltransferase

1. Introduction

L-Arabinose is a predominant glycosyl residue of plant cell wall polysaccharides. Linear (1 → 5)-linked α-L-arabinosyl residues are often present as side-chains that are linked to the backbone of the pectic polysaccharide rhamnogalacturonan I (RG-I) (Ridley et al., 2001). Arabinose (Ara) may also exist as branched side chains composed of 2,5-and 3,5-linked L-arabinosyl residues (Carpita and Gibeaut, 1993). Arabinoxylan contains a single α-L-arabinosyl residue at *O*-3 and/or *O*-2 of the xylosyl residues (O'Neill and York, 2003). In these polysaccharides the arabinosyl residues exist predominantly in the α-L-arabinofuranosyl (Ara*f*) form (Carpita and Gibeaut, 1993). α-L-Arabinopyranosyl (Ara*p*) residues are quantitatively minor components of arabinans isolated from several plants (Aspinall et al., 1968; Capek et al., 1983; Kiyohara et al., 1987; Swamy and Salimath, 1991). 3-*O*-β-L-Arabinopyranosyl-L-arabinose was isolated from partial acid hydrolyzates of larch wood arabinogalactan (Odonmažig et al., 1994). Arabinopyranose has also been found in the non-reducing end of pectic galactan (Huisman et al., 2001). A single α-L-Ara*p* residue is also present in the acetic acid-containing side chain of rhamnogalacturonan II (RG-II) (O'Neill et al., 2004).

UDP-L-arabinopyranose (UDP-Ara*p*) is a readily available nucleotide sugar that has been used for in vitro studies of the biosynthesis of arabinose-containing plant polysaccharides. Arabinosyltransferase (AraT) activity has been demonstrated by measuring the amount of [^{14}C]-labeled arabinose incorporated from UDP-[^{14}C]-arabinose onto

* Corresponding author. Tel.: +81 29 873 3211; fax: +81 29 874 3720.
E-mail address: tishii@ffpri.affrc.go.jp (T. Ishii).

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endogenous and exogenous acceptor substrates (Odzuck and Kauss, 1972; Bolwell and Northcote, 1981; Rodgers and Bolwell, 1992). However, the structures of the acceptor polysaccharides and the synthesized products were not fully characterized. Nunan and Scheller (2003) demonstrated that [^{14}C]-Ara is transferred from UDP-β-L-[^{14}C]-arabinopyranose onto exogenous (1 → 5)-linked α-L-arabino-oligosaccharides by solubilized microsomal membranes of mung bean hypocotyls. These authors provided evidence that the newly incorporated arabinosyl residue exists in the pyranose ring form but did not establish its linkage position or anomeric configuration.

We therefore carried out this investigation to characterize in detail the structure of the products formed when UDP-β-L-Ara*p* and 2-aminobenzamide (2AB)-labeled (1 → 5)-linked α-L-arabino-oligosaccharides are reacted with microsomal membrane fractions of mung bean hypocotyls. We show that these membranes contain arabinopyranosyltransferase (Ara*p*T) activity that results in the transfer of a single β-L-arabinopyranose to *O*-3 of the Ara*f* at the non-reducing end of the arabino-oligosaccharides and describe some of the enzymatic properties of the Ara*p*T.

2. Results

2.1. Arabinosyltransferase activity in microsomes

Reacting 2AB-labeled arabino-heptasaccharide (**1**) and UDP-β-L-Ara*p* for 2 h with a microsomal fraction from mung bean hypocotyls generated one major product, **2** (see Fig. 1(a)). Prolonged incubation (18 h at 4 °C) gave **2** in a yield of about 90%. The positive ion LC–ESI–MS spectrum of **2** contained an ion at *m*/*z* 1217 that corresponds to the $[M + Na]^+$ ion of an oligosaccharide derivative that is composed of seven pentoses, a pentitol, and 2AB. The product ion spectrum of the sodium adduct ion at *m*/*z* 1217 contained ions at *m*/*z* 1085, 953, 821, 689, 557, and 425. These are *Yn* ions (Costello and Vath, 1993) that correspond to the sequential losses of pentose residues. Such results are consistence with the existence of the octasaccharide derivative **2**. No compound **2** was formed when UDP-Ara*p* or microsomes were omitted from the reaction mixture or when heat-denatured microsomes were used (Fig. 1(a)). These observations show that microsomes of mung bean hypocotyls contain an arabinosyltransferase that transfers a single arabinose to arabino-oligosaccharides.

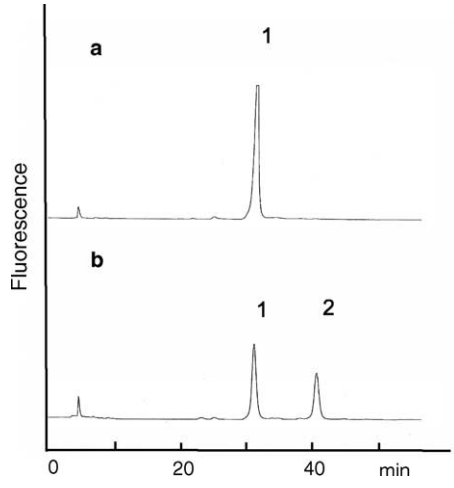

Fig. 1. HPLC profiles of the products formed when Ara$_7$-2AB (**1**) and UDP-Ara are reacted together at 20 °C for 2 h with the microsome fractions from mung bean (*Vigna radiate*) under the standard assay conditions. (a) Denatured enzyme. (b) The reaction mixture contained Ara$_7$-2AB (**1**) (0.5 μM), UDP-Ara (2 mM), and the microsome fractions (40 μg protein).

2.2. The primary structure of enzymically formed *Arap*Ara$_7$-2AB

The primary structure of **2** (Fig. 2) was determined by ^{1}H and ^{13}C NMR spectroscopy and by glycosyl residue and glycosyl linkage composition analyses. Product **2** was obtained in amounts sufficient for these analyses by reacting **1** (0.5 mM) with UDP-β-L-Ara*p* (2 mM). The products from four separate reactions were combined and purified by size-exclusion chromatography and normal-phase LC (see Section 4).

Product **2** was shown, by glycosyl residue composition analysis, to contain only Ara residues. Glycosyl linkage composition analysis of **2** gave 1, 5-di-*O*-acetyl-2, 3, 4-tri-*O*-methylarabinitol (derived from T-Ara*p*), 1, 3, 4-tri-*O*-acetyl-2, 5-di-*O*-methylarabinitol (derived from 1,3-linked Ara*f*), and 1, 4, 5-tri-*O*-acetyl-2, 3-di-*O*-methylarabinitol (derived from 5-linked Ara*f*) in the molar ratios of 1.0:1.2:6.1. The 1, 5-di-*O*-acetyl-2, 3, 4-tri-*O*-methylarabinitol must be formed from a non-reducing terminal arabinopyranosyl residue because it contains *O*-methyl groups at *O*-2, *O*-3, and *O*-4. A non-reducing terminal arabinofuranosyl residue would contain *O*-methyl groups at *O*-2, *O*-3, and *O*-5. No arabinose was released when **2** was treated with a α-L-arabinofuranosidase that is known to hydrolyze (1 → 5)-, (1 → 2)-, and (1 → 3)-linked terminal α-L-Ara*f* residues (data not shown). These results when taken together strongly suggest that an arabinopyranosyl rather than an arabinofuranosyl residue is linked to the arabino-oligosaccharide.

Additional evidence for the ring form and anomeric configuration of the arabinosyl residue linked to the arabino-oligosaccharide was obtained by ^{1}H and ^{13}C NMR spectroscopic analyses of **2**. The signals in the ^{1}H NMR spectrum of **2** (Fig. 3) were assigned by 2D-double quantum filtered correlation spectroscopy (DQFCOSY), and 2D-total correlation spectroscopy (TOCSY) (see Table 1) and ^{1}H spectra of 2AB-labeled arabino-oligosaccharides (Ishii et al., 2005a). The doublet at δ 5.002 ($J_{1,2}$ 3.8 Hz) is assigned to the H-1 resonance of the non-reducing terminal arabinopyranosyl residue (Ara*p*). This chemical shift value and

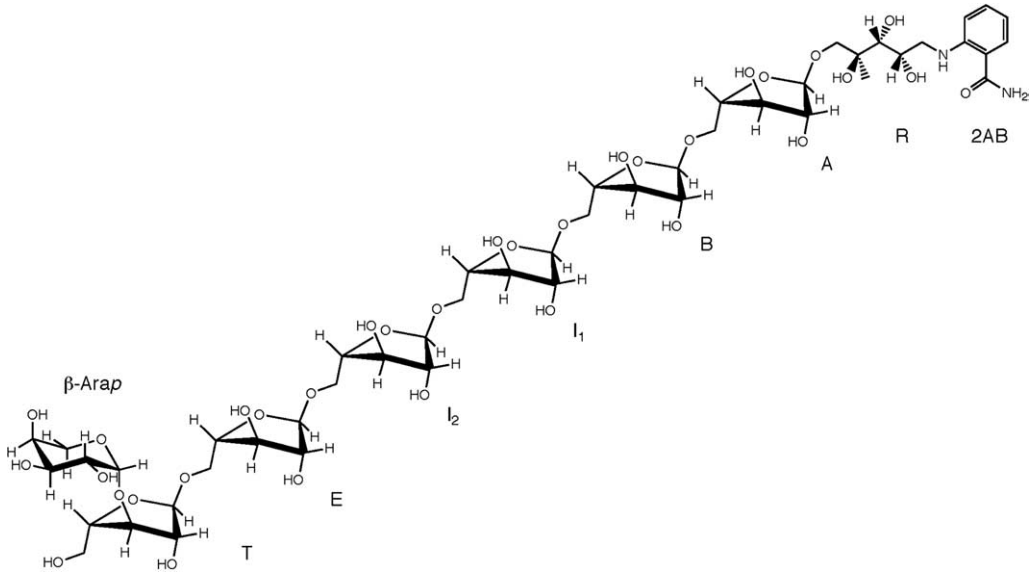

Fig. 2. The primary structure of Ara*p*Ara$_7$-2AB (**2**).

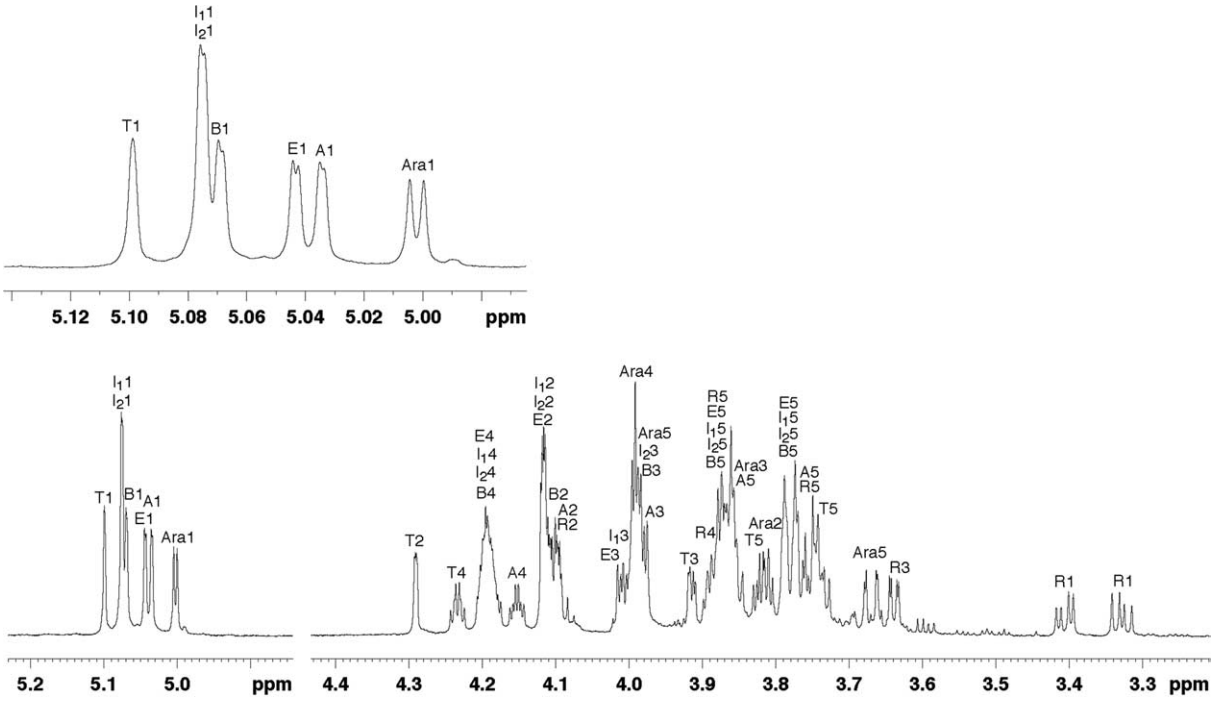

Fig. 3. ^{1}H NMR spectrum of the enzymatically formed Ara*p*Ara$_7$-2AB (**2**). 2AB-labeled Ara*p*Ara$_7$ (**2**) is composed of arabinitol (R), six internal arabinosyl residues (A, B, I$_1$, I$_2$, E and T), and a non-reducing terminal arabinopyranosyl residue (Ara*p*) (see Fig. 2). Signals of 2AB group are not shown.

magnitude of the $J_{1,2}$ coupling constant are consistent with a β-linkage (Mizutani et al., 1989). The chemical shift values of the former reducing Ara residue (residue A) and the H-1s of the internal arabinosyl residues (B, I$_1$, I$_2$, E, and T) and the magnitude of their $J_{1,2}$ coupling constants (1.1–1.4 Hz) are consistent with a α-linkage (Mizutani et al., 1989). The signals corresponding to H-2, H-3, H-4, and H-5 of the Ara*p* residue were assigned using DQFCOSY and TOCSY. The two H-5s of the Ara*p* and the internal of Ara*f* residues were confirmed by HSQC and HMBC analyses. The anomeric configuration and ring conformation of an arabinopyranosyl residue is readily determined by measuring its $^3J_{H,H}$ scalar coupling constant. The HMBC spectrum gave a cross-peak between H-1 of Ara*p* and C-5 of the same Ara*p*, confirming that the terminal arabinose is in the pyranose form (Fig. 4). The values of $^3J_{H1,H2}$ and $^3J_{H2,H3}$ are 3.8

Table 1
^{1}H NMR spectroscopic data for Ara*p*Ara$_7$-2AB (**2**) (δ in ppm, J in Hz)

Compound	Residue	^{1}H chemical shifts (ppm)[a] and first-order coupling constants (Hz)[a]					
		H-1 ($J_{1a,2}$) ($J_{1b,2}J_{1a,1b}$)	H-2 ($J_{2,3}$)	H-3 ($J_{3,4}$)	H-4	H-5a ($J_{4,5a}$)	H-5b ($J_{4,5b}J_{5a,5b}$)
Ara*p*Ara$_7$-2AB	R	3.406, 3.328 (5.2) (8.1, 13.0)	4.099 (2.0)	3.638 (8.0)	3.893	3.867 (2.5, 6.0)	3.750 (5.0, 12.0)
	A	5.034 (1.2)	4.096 (2.0)	3.977 (5.9)	4.152	3854 (3.3, 5.9)	3.759 (5.9, 12.0)
	B[b]	5.069 (1.1)	4.108 (2.0)	3.988 (6.2)	4.188	3.889 (3.2, 6.0)	3.788 (6.0, 12.0)
	I_1[b]	5.075 (1.1)	4.116 (2.0)	4.004 (6.2)	4.199	3.889 (3.2, 6.0)	3.788 (6.0, 12.0)
	I_2[b]	5.075 (1.1)	4.114 (2.0)	3.996 (6.2)	4.199	3.879 (3.2, 6.0)	3.774 (6.0, 12.0)
	E	5.043 (1.4)	4.118 (2.0)	4.011 (6.2)	4.179	3.888 (3.5, 6.0)	3.779 (6.0, 12.0)
	T	5.100 (broad singlet)	4.291 (2.1)	3.914 (5.5)	4.233	3.822 (3.9, 6.9)	3.737 (6.9, 12.0)
	Ara	5.002 (3.8)	3.810 (9.5)	3.861 (3.2)	3.992	3.984 (1.3)	3.669 (2.0, 12.0)

[a] ^{1}H chemical shift and coupling constant assignments are based on 1D, DQFCOSY, and TOCSY spectra.
[b] Assignments of residues B, I^1 and I^2 are interchangeable.

and 9.5 Hz, respectively, which are characteristic of a *cis–trans* arrangement of H-1, H-2, and H-3 in a pyranose ring with a $^4C_1$ *cis–trans* chair conformation, and thus confirms the presence of β-Ara*p*. The ^{13}C NMR spectrum of **2** was assigned using HSQC and HMBC spectroscopic analyses (see Table 2). The HMBC spectrum gave a cross-peak between H-1 of Ara*p* and C-3 of Ara*f* residue T, thereby confirming that the terminal arabinopyranose is linked to *O*-3 of Ara*f* residue (Fig. 4). Taken together, these results show that mung bean hypocotyls contain β-(1 → 3)-arabinopyranosyltransferase (Ara*p*T) activity and that this enzyme transfers L-Ara*p* from UDP-β-L-Ara*p* to *O*-3 of the terminal Ara*f* residue of exogenous 2AB-labeled arabinooligosaccharides.

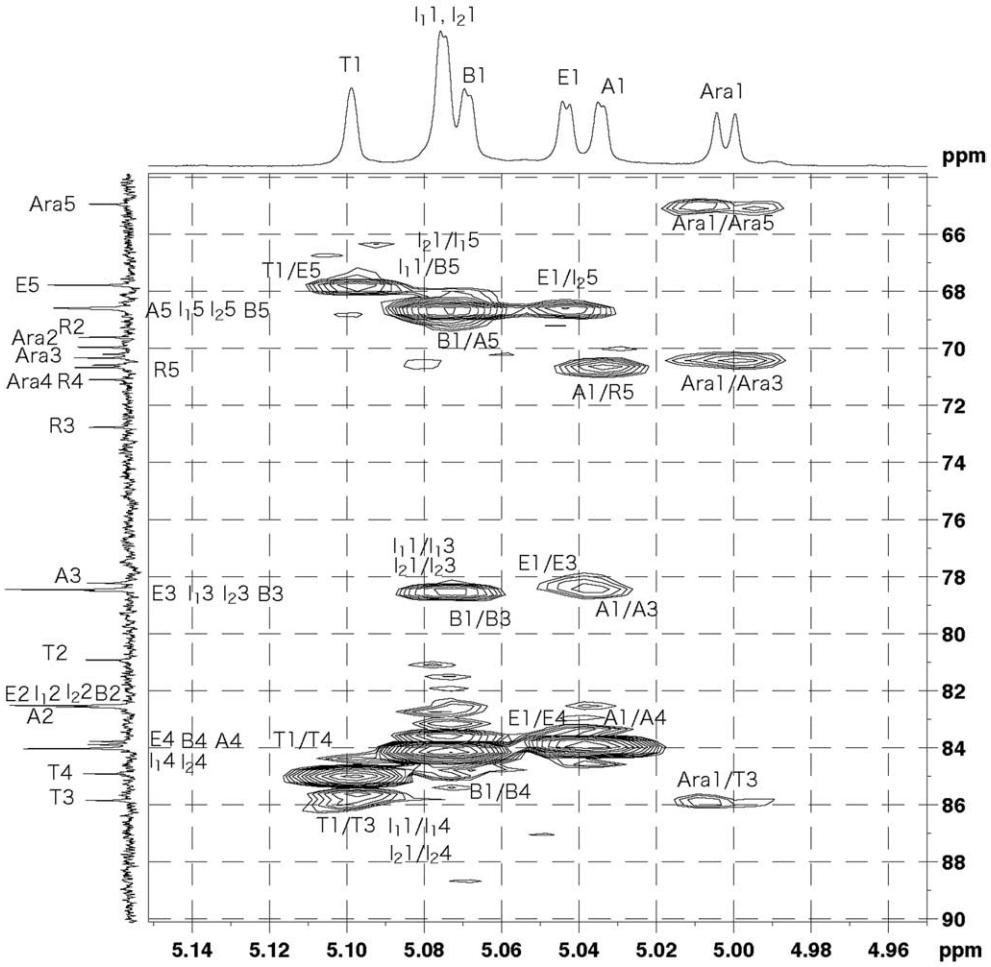

Fig. 4. Contour plot of a portion of the HMBC spectrum of Ara*p*Ara$_7$-2AB (**2**). Ara1/T3 is the cross-peak observed between H-1 of the terminal Ara*p* residue (Ara*p*) and C-3 of the former terminal Ara*f* residue (T). Ara1/Ara 5 is the cross-peak observed between H-1 and H-5 of the terminal Ara*p* residue (Ara*p*).

Table 2
^{13}C NMR spectroscopic data for Ara*p*Ara$_7$-2AB (**2**) (δ in ppm)

Compound	Residue	^{13}C chemical shifts (ppm)[a]				
		C-1	C-2	C-3	C-4	C-5
Ara*p*Ara$_7$-2AB	R	47.37	69.61	72.76	71.09	70.59
	A	109.21	82.56	78.20	83.87[b]	68.58
	B	109.21	82.50	78.43	84.02	68.58
	I_1	109.21	82.50	78.43	84.02	68.58
	I_2	109.21	82.50	78.43	84.02	68.58
	E	109.21	82.50	78.50	83.86[b]	67.78
	T	109.21	80.90	85.84	84.89	63.11
	Ara	101.51	69.96	70.33	71.09	64.94

[a] ^{13}C chemical shift assignments are based on 1D ^{13}C, HSQC and HMBC spectra.
[b] Interchangable, uncertain.

2.3. Characterization of β-(1 → 3)-arabinopyranosyltransferase

The transfer of Ara*p* from UDP-β-L-Ara*p* to Ara$_7$-2AB increased with time and was linear over 2 h (Fig. 5(a)), was dependent on the protein concentration (Fig. 5(b)), and was maximum between pH 6.5 and 7.0 (Fig. 5(c)). Maximal enzyme activity was obtained at 20 °C (Fig. 5(d)). The activity increased in the presence of Mn^{2+} (6.3), Mg^{2+} (1.6) and Co^{2+} (2.1), but not in the presence of Ca^{2+} (0.7), Cu^{2+} (0.18), and Zn^{2+} (0.08) (numbers in parenthesis indicate the relative activity to that obtained without addition of divalent cations). The apparent K_m values for **1** (5–400 μM of Ara$_7$-2AB) with 2 mM UDP-β-L-Ara*p* and for UDP-β-L-Ara*p* (10–1000 μM of UDP-Ara) with 15 μM **1** were 200 and 550 μM, respectively (Fig. 6). The apparent V_{max} values for **1** and for UDP-β-L-Ara*p* were 1700 and 480 pmol min^{-1} (mg protein)$^{-1}$ (Fig. 6). Ara$_8$-2AB and Ara$_7$-2AB are more effective acceptors than Ara$_6$-and Ara$_5$-2AB (Fig. 7). Virtually no arabinose was transferred to Ara$_3$- and Ara$_1$-2AB. Approximately 20% of the total Ara*p*T activity was recovered in the supernatant obtained by centrifuging (100,000*g* for 1 h) microsomes (100 μL; protein, 2 mg) that had been kept for 30 min at 0 °C in 40 mM Mes-KOH (pH 6.5) containing 0.75% Triton X-100, indicating that the enzyme is partially solubilized by the detergent.

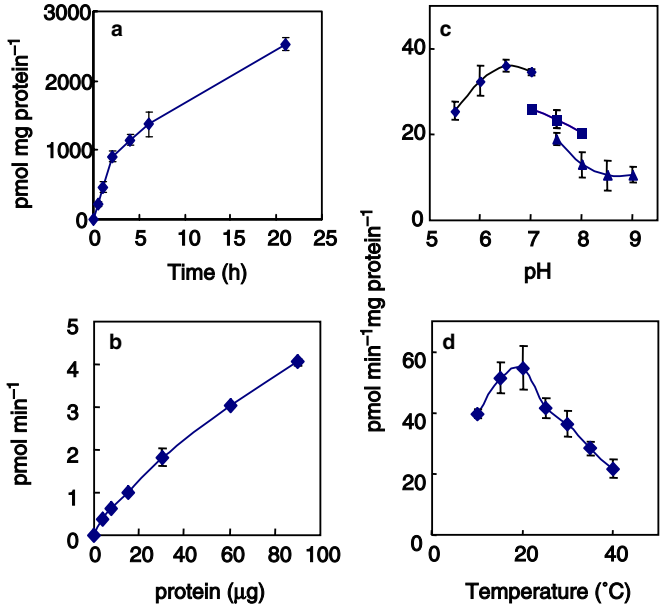

Fig. 5. Activity of Ara*p*T in the microsome membrane fractions of mung bean hypocotyls. (a) Time course of the formation of Ara*p*Ara$_7$-2AB (**2**) from Ara$_7$-2AB (**1**). Microsomal membranes (protein, 30 μg) were incubated with 100 μM Ara$_7$-2AB (**1**) under the standard assay conditions. (b) Effects of membrane-protein concentration on the formation of Ara*p*Ara$_7$-2AB (**2**). The reaction was allowed to proceed at 20 °C for 30 min using 10 μM Ara$_7$-2AB (**1**) and 2 mM UDP-Ara under standard conditions. (c) Effects of pH on the formation of Ara*p*Ara$_7$-2AB (**2**). The membrane (protein 30 μg) was incubated with 10 μM Ara$_7$-2AB (**1**) and 2 mM UDP-Ara at 20 °C for 30 min under standard conditions. The buffers used to control the reaction pH were 40 mM Mes-KOH (pH 5.5–7.0, ●), Hepes-KOH (pH 7.0–8.0, ■) and Tris–HCl (pH 7.5–9.0, ▲). (d) Effects of temperature on the formation of Ara*p*Ara$_7$-2AB (**2**). The membrane (protein 30 μg) was incubated with 10 μM Ara$_7$-2AB (**1**) and 2 mM UDP-Ara at 20 °C for 30 min under standard conditions (For details, see assay conditions Section 4).

3. Discussion

The chemical and spectroscopic data show unequivocally that **2** contains a β-L-arabinopyranosyl residue that is linked to *O*-3 of the terminal Ara*f* residue of the arabino-oligosaccharide. Such results are consistent with a previous report showing that arabinopyranose is transferred from UDP-β-L-[^{14}C]-arabinopyranose onto exogenous (1 → 5)-linkedα-L-arabino-oligosaccharide by solubilized microsomal membranes of mung bean hypocotyls (Nunan and Scheller, 2003). These results were somewhat unexpected since most of the arabinosyl residues in plant polysaccharides exist in the furanose form. However, β-L-arabinopyranosyl-(1 → 3)-L-arabinose was isolated from partial acid hydrolyzates of larchwood arabinogalactan (Odonmažig et al., 1994). AraT activity determined here may involve in biosynthesis of arabinan side chain of RG-I and arabinopyranosyl residues presented in arabinogalactan.

No elongation of α-L-(1 → 5)-linked arabinan backbone by 5-linked arabinofuranosyl residues was observed when UDP-Ara*p* and 2AB-labeled arabino-oligosaccharides were incubated with the microsomal membrane fractions

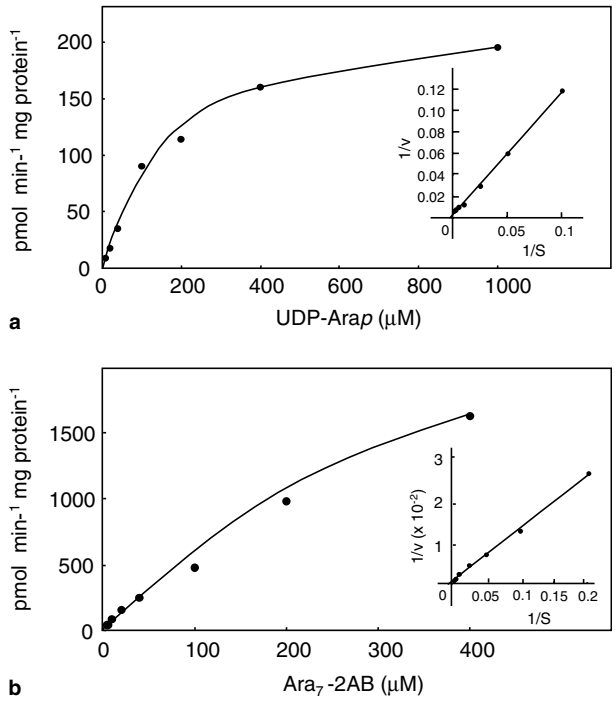

Fig. 6. Effect of substrate concentration of AraT activity. (a) Effect of concentration of UDP-Ara*p* on AraT activity. The enzyme activity was measured under standard assay conditions with 15 μM Ara_7-2AB (**1**) by incubation of the microsomes with varying concentrations of UDP-Ara*p* 10–1000 μM. (b) Effect of concentration of Ara_7-2AB (**1**) on AraT activity. The enzyme activity was measured under standard assay conditions with 1 mM UDP-Ara*p* by incubation of the microsomes with varying concentrations of Ara_7-2AB (5–400 μM).

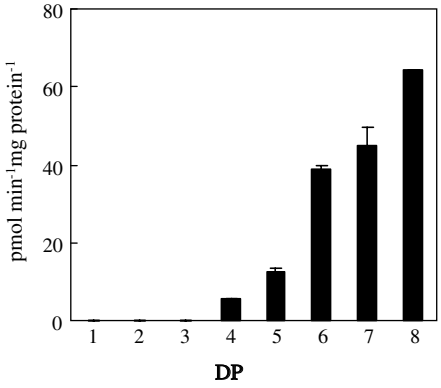

Fig. 7. Effects of the DP of the arabino-oligosaccharide acceptors on Ara*p*T activity.

of mung bean. In a previous paper we reported that a single α-L-arabinopyranosyl residue was transferred to 0-4 of the nonreducing terminal galactosyl residue of 2AB-labeled galacto-oligosaccharides when UDP-Ara*p* and the galacto-oligosaccharides were incubated with the solubilized microsomal membrane fractions of mung bean (Ishii et al., 2005b). These results may indicate that arbinosyltransferase does not convert UDP-Ara*p* to UDP-arabinofuranose (UDP-Ara*f*) as suggested earlier (Fry and Northcote, 1983) and that UDP-Ara*p* is directly incorporated by arabinosyltransferase to form arabinopyranosyl residue. This possibility could be tested by using UDP-Ara*f* as a donor substrate.

There was a large difference in V_{max} values for Ara_7-2AB (1700 pmol min^{-1} (mg protein)$^{-1}$) and UDP-Ara*p* (480 pmol min^{-1} (mg protein)$^{-1}$). This would be due to the assay conditions. The kinetics for UDP-Ara*p* were done using 15 μM of Ara_7-2AB, which has much less saturating levels of Ara_7-2AB (Fig. 6(b)). The available Ara_7-2AB and UDP-Ara*p* for kinetics was limited because a large quantity of Ara_7-2AB and UDP-Ara*p* was used for production of Ara*p* Ara_7-2AB. AraT activity has previously been detected in microsomal membrane fractions and solubilized membrane fractions. For example, Bolwell and Northcote (1981) reported that a partially purified AraT from French bean membrane fractions had an apparent K_m of 178 ± 45 μM for UDP-Ara. Nunan and Scheller (2003) reported that AraT from mung bean had maximum activity at pH 6.5 and 20 °C.

In conclusion, this process is likely to account for some of the Ara*p* that are present in RG-I and arabinogalactan.

4. Experimental

4.1. Analytical methods

Normal phase LC–ESI–MS analysis was performed at 0.4 mL min^{-1} by connecting the outlet of the column to a LCQ classic mass spectrometer (Thermoelectron, 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 2000. For NMR spectroscopic analysis, the transfer product (0.2 mg) was dissolved in 99.96% isotopically enriched D_2O and then freeze-dried. The residue was dissolved in 99.96% enriched D_2O. One-and two-dimensional ^1H and ^{13}C spectra were recorded at 303 K and 800 MHz with a Brucker Avance 800 NMR spectrometer with a cryoprobe using the pulse sequences and software provided by the manufacturer (Ishii et al., 2002). One-dimensional ^{13}C spectra were recorded with a Brucker Avance 600 NMR spectrometer. 2-Methyl-2-propanol was used as internal reference (δ_H 1.230, δ_C 31.30).

4.2. Materials

Mung bean (*Vigna radiate* L. Wilezek) seeds were purchased from Marutane CO. (Kyoto, Japan) and grown as described (Ishii et al., 2004). Arabino-oligosaccharides with a degree of polymerization (DP) between 2 and 8 and α-L-arabinofuranosidase from *Aspergillus niger* were purchased from Megazyme (Wicklow, Ireland). Arabino-oligosaccharides were labeled at their reducing end with 2AB as described (Ishii et al., 2002). UDP-β-L-arabinopyranose (UDP-Ara*p*) was obtained from

Peptide Inst. Inc. (Osaka, Japan). Hepes, Mes, and Triton X-100 from Dojindo Lab. (Kumamoto, Japan), and Sigma–Aldrich (Tokyo, Japan), respectively. All other chemicals and reagents used were purchased from Wako Pure Chemicals (Osaka, Japan).

4.3. Preparation of microsomal membranes

Microsomal membrane fractions of mung bean hypocotyls were prepared as described (Nunan and Scheller, 2003). Total protein in the microsomal membranes was determined with bovine serum albumin as a standard using a Protein assay reagent kit (Bio Rad, Hercules, CA), according to the manufacturer's instruction.

4.4. Assay procedure for β-(1 → 3)-arabinopyranosyltransferase

AraT activity was measured at 20 °C for 30 min unless otherwise specified in a standard reaction mixture (total volume, 30 μL) containing microsomal membranes (40 μg protein), 15 μM 2AB-labeled arabinoheptasaccharide (**1**) (Ara_7-2AB), 2 mM UDP-β-L-Ara*p*, 40 mM Mes-KOH (pH 6.5), 25 mM $MnCl_2$, 20 mM NaF, 0.5% Triton X-100 and 160 mM sucrose. The reaction was terminated by the addition of AcOH (1.5 M, 30 μL) and the suspension boiled for 1 min. The reaction mixture was centrifuged, and the supernatant analyzed by normal-phase liquid chromatography (LC) using fluorescence ($\lambda x = 330$ nm, $\lambda em = 420$ nm) for detection. Normal-phase LC was performed using a Phenomenex Luna NH_2 column (4.6 × 150 mm, Shimadzu GLC, Tokyo, Japan) eluted at 0.4 mL min^{-1} 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) CH_3CN, and a linear gradient of eluent B from 84% (v/v) to 73% (v/v) in 50 min. Enzyme activity is expressed as pmol Ara transferred min^{-1} (mg protein)$^{-1}$, based on the concentration curve of 2AB-Ara_3 as the calibration standard. The apparent K_m and V_{max} values of AraT as the crude enzyme for the Ara_7-2AB (1) were determined at 20 °C for 30 min using Ara_7-2AB (1) (5–400 μM), UDP-Ara (2 mM), and the same concentrations of other components as the standard assay mixture. The apparent K_m and V_{max} values for UDP-Ara were determined at 20 °C for 30 min with UDP-Ara (10–1000 μM), Ara_7-2AB (1) (15 μM), and the same concentrations of other components as the standard assay mixture. The production of **2** was quantified by normal-phase LC. The enzyme activity toward the oligosaccharides with different DPs was determined at 20 °C for 1 h by using 2AB-labeled arabino-oligosaccharides with DP 1-8 (20 μM) and the same concentration of other components as the standard assay mixture. The effect of various cations on the AraT activity was examined by incubation at cation final concentration at 25 mM and the same concentrations of other components as the standard assay mixture at 20 °C for 30 min. All enzyme assays were performed at least, in duplicate.

4.5. Enzyme assays and analysis of enzyme reaction product

Enzyme reactions were performed in 40 mM Mes-KOH, pH 6.5 (900 μL) containing UDP-Ara (2 mM), Ara_7-2AB (1) (0.5 mM), microsomal fractions (400 μg protein), and the same concentrations of the components as the standard assay mixture for 2 h at 20 °C, and then kept for 18 h at 4 °C. At this time **2** formed 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 four separate reactions were combined and applied to a Bio-Gel P-2 (1.6 × 90 cm) and a Bio-Gel P-4 (1.6 × 90 cm, Bio-Rad, Hercules, CA) column connected in series and eluted with water at 40 °C at flow rate 0.4 mL min^{-1}. 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 was then freeze-dried. The residue was dissolved in 50 mM ammonium acetate, pH 4.5 and separated by normal-phase liquid chromatography (LC) with UV detection at 254 nm. The UV-positive fractions (retention time 41 min for **2**) was collected manually, concentrated by rotary evaporation, and freeze-dried. The purified oligosaccharide was analyzed by electrospray-ionization mass spectrometry (ESI-MS), ^1H and ^{13}C NMR spectroscopy and by glycosyl residue composition and glycosyl linkage analyses (Tables 1–3 and Figs. 3 and 4). The glycosyl residue and glycosyl linkage compositions of the oligosaccharide products were determined as described previously (York et al., 1986). Acid hydrolysis of the product with 2M TFA was performed at 121 °C for 15 min. Portions of the oligosaccharides were treated for 2 h at 40 °C with α-L-arabinofuranosidase (0.1 U) in 50 mM NaOAc, pH 5.0. After heating the reaction mixture in boiling water the products were analyzed by LC.

Table 3
^1H and ^{13}C NMR spectroscopic data for 2-aminobenzamide group (δ in ppm, coupling constants J in Hz)

			H-3	H-4	H-5	H-6	
2-Aminobenzamide			6.923 $^3J_{3,4}$ 8.4	7.444 $^3J_{4,5}$ 7.1	6.784 $^3J_{5,6}$ 7.8	7.559	
	C-1	C-2	C-3	C-4	C-5	C-6	C=0
2-Aminobenzamide	117.71	150.18	114.6	135.36	118.25	130.89	176.03

See legends of Tables 1 and 2.

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