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A XYLOGLUCAN IN ETIOLATED SEEDLINGS OF *PINUS RADIATA*

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Abstract—The changes in cell wall carbohydrates and starch were investigated in hypocotyls and cotyledons during growth of etiolated seedlings of *Pinus radiata*. Starch was depleted within two weeks with an accompanying rise in a hemicellulosic xyloglucan component. This was purified by fractional precipitation. Fractionation, methylation analysis, partial hydrolysis and chromium trioxide oxidation showed that this component was a fucogalactoxyloglucan of similar structure to those reported from dicots. It was the major hemicellulose in two-week-old hypocotyls and cotyledons. Other cell wall polysaccharides for which evidence was obtained by methylation analysis were a xylan, a glucomannan and an arabinan. © 1997 Elsevier Science Ltd

INTRODUCTION

The primary cell wall hemicelluloses of dicots and monocots have been fairly well characterized, but gymnosperm primary cell walls have received less attention. Lorences and Zarra [1] reported briefly on the hemicellulose changes during growth of *Pinus pinaster* seedlings. Their results showed an increase in non-cellulosic cell wall glucose followed by a decrease, when expressed as glucose per hypocotyl. It was suggested that these changes could be reflecting the turnover of a xyloglucan. More recently, the purification and methylation analysis of a xyloglucan from *P. pinaster* hypocotyls has been described [2]. This xyloglucan was purified by ion-exchange chromatography and iodine precipitation. Methylation analysis suggested a structure resembling that of a typical dicotyledonous fucogalactoxyloglucan, but the proportion of mannose was higher than would be expected from dicots. Gel permeation chromatography suggested that the extra mannose corresponded to a low M_r mannan and that it was absent from the higher M_r portion of their purified sample.

We have studied the production of hemicelluloses in dark-grown *P. radiata* seedlings and report here on the isolation of a xyloglucan by fractional precipitation and its partial structural characterisation as a fucogalactoxyloglucan. The mannose content was shown to vary according to the purification steps employed and was reduced to 1% in one preparation, supporting the conclusion of Acebes *et al.* [2] for their *P. pinaster* xyloglucan. Further studies reported here involve partial hydrolysis and studies on anomeric configuration. These results, previously unpublished for gymnosperm xyloglucans, support a close similarity to dicot xyloglucans. A preliminary conference report on this work has been presented [3].

RESULTS AND DISCUSSION

Changes in polysaccharide composition with seedling growth

Pinus radiata seedlings were grown and harvested as described in Experimental. One-week-old and two-week-old plants were each cut to give upper and lower hypocotyl segments, as well as cotyledons. This harvesting procedure provided hypocotyl segments of four different physiological ages, since the upper hypocotyl is younger than the lower hypocotyl in plants of a given age. In one-week-old plants, the upper hypocotyl was actively elongating, while elongation growth had virtually ceased in the lower half of the hypocotyl at this age. Two-week-old seedlings were incapable of further hypocotyl elongation.

Segments were extracted successively with acetone, hot 0.5% ammonium oxalate and 10% KOH (see Experimental, procedure a). For a range of segments, oxalate extraction removed, on average, 92% of the starch and a substantial proportion of pectic substances (57 and 49% of total arabinose and galactose, respectively). On the other hand, most of the hemicellulose (93% of total xylose, 94% of non-starch glucose and 96% of mannose) appeared in the KOH fraction.

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Table 1. Monosaccharide composition of hemicellulose preparations from hypocotyl segments of different ages (weight %)

	1-week		2-weeks	
	Upper	Lower	Upper	Lower
Fuc	6	4	3	3
Ara	15	7	7	6
Xyl	41	38	35	33
Man	6	8	11	15
Gal	16	11	12	10
Glc	16	32	31	32

Starch in pine seedlings originates very early in germination [4], but it is rapidly depleted during growth in the dark. In one-week-old seedlings, starch constituted 64% of the non-cellulosic polysaccharide of the upper, elongating, hypocotyl regions, as well as their cotyledons. In the lower, non-elongating, region of the hypocotyl, however, starch was depleted to 5%, while the corresponding figures for the two-week-old seedlings were 14% (upper hypocotyl) and 1% (lower hypocotyl). The depletion of starch and cessation of elongation growth were accompanied by a change in hemicellulose composition (Table 1). In the younger, upper region of the one-week-old hypocotyls, the hemicellulose fraction contained only 16% glucose, but in the lower region and in both regions of the two-week-old hypocotyls, the proportion of glucose was almost doubled. The increase in hemicellulosic glucose was accompanied by a decrease in arabinose and galactose. Lorences and Zarra [1] reported much higher proportions of glucose in their 7- to 10-day-old *P. pinaster* hypocotyls, but this was not accompanied by high xylose and may have included residual starch.

The mannose content of both cotyledons and hypocotyls continued to increase after elongation had ceased, reaching 15% of the total hemicellulose fraction from the lower portion of two-week-old hypocotyls. This presumably reflected an increase in secondary wall glucomannan. A further batch of hypocotyls was extracted by procedure b, with an initial cold K-P_i buffer treatment, yielding a hemicellulose preparation (fraction C) with only 4% mannose. This result suggests that the bulk of the mannose component was water-soluble, as in a galactoglucomannan with a relatively high galactose content [5].

Isolation of hemicellulose fractions

In order to characterise the hemicellulosic glucan component, further fractionations were carried out on a number of KOH extracts of hypocotyls and cotyledons. Four extracts with high glucan and low starch content were selected for this study. Fraction A was from two-week-old cotyledons. Extracts from the lower, relatively starch-free, portions of one-week-old and two-week-old hypocotyls were combined as fraction B to provide adequate material for structural studies. These fractions and fraction C (above) were then further fractionated as outlined below; analytical data on the subfractions are presented in Table 2. Together with other work reported in this paper, these data show that the glucan component is a xyloglucan.

Fraction A was first treated with barium hydroxide to precipitate the glucomannan component. As shown by the analytical data in Table 2, however, the xyloglucan was precipitated at the same time (fraction A1), leaving a xylan-rich fraction in solution (fraction A2, precipitated by ethanol). Further fractionation of the xyloglucan and glucomannan was attempted with cetyltrimethylammonium bromide (CTAB) and borate [6], but no significant change in composition was achieved.

Fraction B was then fractionated with CTAB and borate, without prior barium hydroxide treatment. The fraction precipitated by CTAB alone was evidently pectin-rich (40% uronic acid), while further addition of borate to the supernatant yielded a precipitate B2, containing principally xyloglucan and glucomannan, but with 1% starch and 4% uronic acid (data not shown in Table 1). Xylan remained in the supernatant and could be precipitated with ethanol.

Since fraction B2 was still impure, an alternative approach was used to produce a more pure xyloglucan, starting with fraction C, which already had a low mannose content. Fraction C was dissolved in calcium chloride solution and treated with iodine as described by Gaillard [7]. This produced an enrich-

Table 2. Yield * and composition (mol %) of hemicellulose fractions

Fraction	Yield (%)	Rha	Fuc	Ara	Xyl	Man	Gal	Glc
A1 $Ba(OH)_2$	58	(5)†		4	32	13	9	37
A2 EtOH to 80%	10	(5)		14	67	3	5	7
B2 Borate, pH10	45	(6)		5	27	14	13	36
C1 $CaCl_2$ insol.	9	1	2	40	19	14	10	15
C2 I_2/KI	67	0	5	7	43	3	10	33
C2-3 Borate, pH10	37	0	6	3	36	4	11	40
C2-3a EtOH to 40%	30	0	8	2	37	4	10	40
C2-4a EtOH to 40%	2	0	4	2	43	1	6	44

* % of total non-starch carbohydrate in KOH fraction (A, B or C).

† Values in parentheses represent Rha plus Fuc not separated by GC.

ment of xyloglucan in fraction C2 (Table 2). Xylan in this fraction was largely removed by subsequent CTAB/borate treatment. Fraction C2-4a was largely xyloglucan, with only 1% mannose (Table 2). The major fraction, C2-3, still contained 4% mannose, but had less xylose, no detectable starch and only 2% uronic acid. Further fractionation of C2-3 with EtOH produced no significant change in monosaccharide composition (fraction C2-3a).

Monosaccharide composition of xyloglucan

The most purified xyloglucan fractions still contained mannose which, according to the methylation analysis, was predominantly 4-linked and probably derived from a contaminating glucomannan. No further purification of the xyloglucan was attempted but its approximate molar composition was estimated by making a correction for the contribution of a galactoglucomannan to the values in Table 2. For these calculations, the molar ratios for the galactoglucomannan were taken as Gal–Glc–Man = 1:1:3, as has been reported for many water-soluble galactoglucomannans from *Pinus* species [5]. Alkali-soluble glucomannans of softwoods (including one isolated from *P. radiata* wood [8]) have a lower galactose content, but the difference is insufficient to affect the conclusions regarding the xyloglucan structure. For fraction C2-3a, the resulting mol ratios were: Fuc 2.1, Ara 0.5, Xyl 9.6, Gal 2.3, Glc 10.0. Fraction B2 gave corresponding ratios of 1.9, 1.5, 8.7, 2.6, 10.0.

For the purified xyloglucan preparations, the corrected molar ratios were close to those published for *P. pinaster* [2], for xyloglucan oligosaccharides isolated from suspension-cultured Douglas fir cells [9], and for many dicots [10], although the xylose content was somewhat higher. Previously studied xyloglucans from gymnosperm wood [11, 12] lack fucosyl residues. Methylation analysis of fraction B2 (see below) suggested that the high arabinose content of this fraction was associated with an arabinan contaminant. In fraction C2-3a, which had undergone different purification steps, the arabinose content was lower, much of it being insoluble in calcium chloride solution and, therefore, removed during the purification procedure.

Structural features of xyloglucan

A sample of fraction B2 was methylated once by the Hakomori procedure. The product was only partially soluble in chloroform, giving a soluble portion, B2-I, and an insoluble portion. The latter was remethylated by the Hakomori procedure, giving a fully methylated product B2-II. Both these permethylated fractions were hydrolysed and their composition analysed by GC-mass spectrometry of the derived alditol acetates.

Data for fraction B2-II are shown in Table 3. In fraction B2-I, xylose and glucose were undermethylated and the arabinose content was high (14%). The methylation pattern in B2-I (data not shown) suggested the presence of an arabinan that was fully methylated after one Hakomori treatment. This left only 2.4% arabinose in fraction B2-II. The linkages of fraction B2-II were in accordance with those of known fucogalactoxyloglucans. The 4-linked mannose probably represents a glucomannan or galactoglucomannan component.

Table 3. Methylation analysis of xyloglucan

	Methylated positions	Mol % in fraction B2-II
Arabinose	2,3,5-	1.0
	2,3-	1.4
Xylose	2,3,4-	12.7
	2,3-/3,4-	11.9
Fucose	2,3,4-	5.0
Galactose	2,3,4,6-	4.0
	3,4,6-	8.9*
Glucose	2,3,4,6-	1.5
	2,3,6-	8.9*
	2,3-	33.9
Mannose	2,3,6-	10.6
	2,3-	trace

* Quantified by GC-MS.

Partial hydrolysis of samples from fractions A1 and B2 (Table 2) yielded, in each case, a disaccharide which behaved as cellobiose in several chromatographic systems (see Experimental). No other disaccharides were found and all sugar residues apart from glucose were quantitatively released as the monomers (data not shown). This suggests the presence of (1-4)-linked β-glucosyl residues in the backbone of the polymer.

The anomeric configuration of glucose was established as β- by the finding of cellobiose in the partial hydrolysates. Other anomeric configurations were determined by chromium trioxide oxidation [13] of a peracetylated portion of fraction C2-3 (see Experimental). During 3 hr oxidation, *ca* 100% of the fucose survived, but only 21% of the galactose, indicating that they were liked α- and β-, respectively. Xylose and glucose gave equivocal results (49 and 83% survival, respectively). High resistance to oxidation of β-glucosyl residues has been reported in certain glucans [14]. Under identical conditions, using a standard carob galactomannan, galactose and mannose survived 88 and 11%, respectively. These data are in full accord with the structures reported for fucogalactoxyloglucans from dicots [10] and also complement the linkage analyses of other gymnosperm xyloglucans [2, 9].

EXPERIMENTAL

Plant material. Pinus radiata Don. seeds were germinated and the seedlings grown in a pumice-peat potting mixt. at 25° in the dark, watered with tap H_2O. Germination was usually apparent after one week and

growth ceased two weeks later, by which time the hypocotyls averaged 7 cm in length. One group of seedlings was harvested one week after germination, by cutting through the hypocotyls just above the potting mix. The hypocotyls had reached 4 cm length at this stage. A second group of plants was harvested at two weeks (hypocotyl length 7 cm). Each harvested plant was quickly cut transversely just below the cotyledons and also half-way up the hypocotyl, thus providing three segments: cotyledons (including also the unexpanded shoot), upper hypocotyl and lower hypocotyl. These were immediately transferred to liquid N_2 and kept frozen until required for extraction of polysaccharides.

Extraction of polysaccharides. All polysaccharide prepns were obtained as dry powders by precipitating from soln by slow addition of EtOH, followed by successive washing with several changes of EtOH, MeOH and hexane (solvent exchange [15]), and were stored in a desiccator. The following two procedures were used to obtain polysaccharides from plant tissue. (a) Frozen tissue was ground in a mortar in liquid N_2, then extracted at 0° with Me_2CO (4×, 10 ml g^{-1} fr. wt). The residue was suspended in 0.5% aq. ammonium oxalate (NH_4ox) at 10 ml g^{-1} fr. wt, stirred for 1 hr at 75° and centrifuged. The residue was washed with 0.5% NH_4ox and the polysaccharides pptd with 4 vols EtOH and dried. The NH_4ox residue was extracted with 10% KOH containing 0.5% $NaBH_4$ (10 ml g^{-1} fr. wt), for 1 hr at room temp. After centrifuging, the residue was washed with 10% KOH and the comb. supernatants neutralised with HOAc. The polysaccharide material ('hemicellulose') was pptd with EtOH and dried. (b) Frozen tissue was extracted successively with K-P_i buffer, $CHCl_3$–MeOH and Me_2CO, as described in ref. [16]. The final Me_2CO residue was then extracted with hot NH_4ox and then 10% KOH as in procedure (a).

Fractionation of polysaccharides. (1) *$Ba(OH)_2$.* Hemicellulose fr. A (from 2-week-old cotyledons, 46.6 mg) was treated with $Ba(OH)_2$ according to the method of ref. [17] to give a pptd fr. A1. Further treatment of the supernatant with 4 vol of EtOH caused the pptn of a xylan-rich fr.

(2) *Cetyl trimethylammonium bromide (CTAB).* Fr. A1 was treated successively with CTAB and borate, according to the method of ref. [6] to give three frs. A fourth fr. was pptd from the final supernatant by addition of 4 vols of EtOH. All frs were dissolved in HOAc [6], then reprecipitated with EtOH, washed and dried. Fr. B (combined hypocotyl frs, 20.4 mg) was similarly treated with CTAB to yield the pectin-rich fr. B1 and then with borate (pH 10) to yield fr. B2 (xyloglucan). Fr. C2 (see below) was likewise treated with CTAB/borate to yield the xyloglucan-enriched fr. C2-3 (pptd with borate, pH 10).

(3) *I_2CaCl_2.* Fr. C (see text, 89 mg) was dissolved in 3.7 M $CaCl_2$ and treated with I_2 according to the method of ref. [7]. The supernatant was then treated with 4 vols of EtOH to give the pptd fr. C3 (see Table 1). Fr. C2, pptd by I_2/KI, was further fractionated by the CTAB procedure.

(4) *EtOH.* Fr. C2-3, redissolved in H_2O at pH 7, and also the supernatant (C2-4) from the CTAB/borate treatment of fr. C2, were both subjected to an EtOH fractionation according to the procedure of ref. [18]. The frs pptd at 40% EtOH (C2-3a and C2-4a, respectively, in Table 1) were enriched in xyloglucan.

Analytical methods. PC was performed in two solvent systems: solvent A, PrOH–EtOAc–H_2O (7:1:2); solvent B, PrOH–EtOAc–H_2O (32:57:13). GC was performed with FID on the following columns: (A) 2 m × 2 mm OV-225 (3% on Varaport 30, 100–120 mesh); (B) 2.5 m × 3 mm SP2340 (3% on Supelcoport 100–120); (C) 3 m × 3 mm ECNSS-M (3% on Chromosorb W, AW-HMDS); (D) 60 m × 0.5 mm SE-30 SCOT column. For GC-MS, column A was coupled to a mass spectrometer operating in the EI mode (ionization potential 70 eV).

Total carbohydrate was determined by the PhOH–H_2SO_4 method, as modified in ref. [19]. Total uronic acid was determined by the *m*-hydroxydiphenyl method [20]. Determination of starch was based on the amyloglucosidase and glucose oxidase method of ref. [21]. Monosaccharide analysis was by the method of ref. [22], except that HNO_3 (0.5 M containing 0.5% urea, 4 hr at 100°) was used for hydrolysis of polysaccharides [23]. Alditol acetates were sepd on column A (isothermal, 206°) or column B (isothermal, 230°). Partial hydrolysis was performed with 2 M TFA, 1 hr at 100° [22]. The partial hydrolysate was subjected to PC in solvents A and B, and the spot migrating as authentic cellobiose was eluted, trimethylsilylated [24] and identified by GC of the TMSi derivative on column D (isothermal, 210°). Methylation analysis was by the Hakomori method as described in ref. [25]. Partially methylated alditol acetates were sepd on column A (isothermal, 206° or temp. programmed 130° to 210° at 1° min^{-1}, with a 70 min hold at 160°), column B (temp. programmed 180° to 230° at 2° min^{-1}, with 10 min hold at 200°) and column C (isothermal, 150°). Standards were prepd from known oligo- and polysaccharides, identification confirmed by GC-MS and quantitation was by measurement of peak areas. Anomeric configurations were determined by oxidation of the fully acetylated polysaccharide with CrO_3 [13].

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REFERENCES

1. Lorences, E. P. and Zarra, I., *Physiologica Plantarum*, 1986, **67**, 377.
2. Acebes, J. L., Moral, R. and Zarra, I., *Phytochemistry*, 1993, **33**, 1343.
3. Little, J. W. L., Fenemor, D. R. and Andrew,

I. G., *Proceedings of the Australian Biochemistry Society*, 1980, **13**, 26.
4. Ching, T. M., *Plant Physiology*, 1966, **41**, 1313.
5. Timell, T. E., *Advances in Carbohydrate Chemistry*, 1965, **20**, 409.
6. Barker, S. A., Stacey, M. and Zweifel, G., *Chemistry and Industry*, 1957, 330.
7. Gaillard, B. D. E., *Nature*, 1961, **191**, 1295.
8. Harwood, V. D., *Svensk Papperstidning*, 1973, **76**, 377.
9. Thomas, J. R., McNeil, M., Darvill, A. G. and Albersheim, P., *Plant Physiology*, 1987, **83**, 659.
10. Hayashi, T., *Annual Review of Plant Physiology and Molecular Biology*, 1989, **40**, 139.
11. Schreuder, H. R., Cote, W. A. and Timell, T. E., *Svensk Papperstidning*, 1966, **69**, 641.
12. Ramalingham, K. V. and Timell, T. E., *Svensk Papperstidning*, 1964, **67**, 512.
13. Lindberg, B., Lindqvist, B., Lonngrenn, J. and Nimmich, W., *Carbohydrate Research*, 1977, **8**, 443.
14. Woolard, G. R., Rathbone, E. B. and Novellie, L., *Carbohydrate Research*, 1976, **51**, 239.
15. Green, J. W., *Methods in Carbohydrate Chemistry*, 1963, **3**, 95.
16. English, P. D., Jurale, J. B. and Albersheim, P., *Plant Physiology*, 1971, **47**, 1.
17. Meier, H., *Methods in Carbohydrate Chemistry*, 1965, **5**, 45.
18. Whistler, R. L. and Sanella, J. L., *Methods in Carbohydrate Chemistry*, 1965, **5**, 34.
19. Immers, J., *Journal of Chromatography*, 1964, **15**, 252.
20. Blumenkrantz, N. and Asboe-Hansen, G., *Analytical Biochemistry*, 1973, **54**, 484.
21. Messer, M. and Dahlqvist, A., *Analytical Biochemistry*, 1966, **14**, 376.
22. Albersheim, P., Nevins, D. J., English, P. D. and Karr, A., *Carbohydrate Research*, 1967, **5**, 340.
23. Jermyn, M. A. and Isherwood, F. A., *Biochemical Journal*, 1956, **64**, 123.
24. Sweeley, C. C., Bentley, R., Makita, M. and Wells, W. W. J., *Journal of the American Chemical Society*, 1963, **85**, 2497.
25. Lindberg, B., *Methods in Enzymology*, 1972, **28**, 178.