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Long-Term Effects of Growth Regulators and Nitrogen Sources on Proliferation and Turnover of Cell Wall Polysaccharides in Suspension Culture of Kidney Bean (*Phaseolus vulgaris* L.)

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강낭콩의 현탁배양시 증식과 세포벽 다당류 전환에 미치는 생장조절제 및 질소원의 장기간 효과

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To underatand *in vitro* regulation of differentiation, the effects of growth regulators and nitrogen source on metabolism of cell wall polysaccharides in suspension culture of kidney bean (*Phaseolus vulgaris* L.) were investigated. The suspension cells (cell clusters) were directly induced from the epicotyl segments of the seedlings, which were cultivated in MS medium supplemented with 1.0 mg/L of 2,4-D and 0.5 mg/L of kinetin. When compared with cell wall sugar contents of the epicotyl segments, the cellulose content of the suspension-cultured cells decreased; while the pectin and hemicellulose content increased; suggesting increases of rhamnogalacturonan I and arabinogalactan II during the dedifferentiation, respectively. The effects of growth regulators(2,4-D, 1.0 mg/L and kinetin, 0.5 mg/L) and nitrogen source (potasium nitrate, 19.0 mg/L and ammonium nitrate, 16.5 g/L) in the medium on the proliferation and the turnover of the cell wall polysaccharides were investigated for 30 days. In the medium with growth regulators and without nitrogen source, the proliferation rate was extremely high (16 folds). Growth regulators and nitrogen source increased the pectin content. Analysis of neutral sugar composition of pectin fraction showed that nitrogen source enhanced rhamnose level remarkably, suggesting that rhamnogalacturonan I was the one most likely synthesized. In hemicellulose fraction, growth regulators reduced arabinose level, suggesting that arabinogalactan II was degraded. And nitrogen source reduced galactose level, suggesting that xyloglucan was also degraded.

Key words; cell wall polysaccharide, growth regulator, kidney bean (*Phaseolus vulgaris*), nitrogen source, suspension culture

The cell walls of higher plants are exoskeletons which are principally composed of polysaccharides (Lamport, 1960). The cell wall polysaccharides turned over extensively during cell growth and development of higher plants (Labavitch, 1982 ; Taiz, 1984 ; Masuda, 1990 ; Sakurai, 1991 ; Hoson, 1993). Among these polysaccharides, the crystalline cellulose plays an important role in the regulation of cell shape and rigidity. Massive synthesis of cellulose takes place during growth and development, but the mechanism of cellulose synthesis and its regulation remain unclear (Delmer, 1987) and in dispute (Okuda et al., 1993 ; Delmer et al., 1993). Recently, Albersheim et al. (1997) suggested that the structure of cell wall polysaccharide may define its mode of synthesis.

Cell wall consists of several species of non-cellulosic matrix polysaccharides. It is well known that the pectic polysaccharide, rich in galacturonic acid, rhamnose, arabinose, and galactose, includes rhamnogalacturonan I (RG I), arbinan, galactan, arabinogalactan I, homogalacturonan and rhamnogalacturonan II (RG II), and that the hemicellulosic polysaccharide also includes xylans, glucomannans, mannans, galactomanans, glucuronomannan, xyloglucan, callose, β1,3, β1,4 glucan and arabinogalactan II. In contrast to pectins, the hemicelluloses are greatly different in cell types and in different species. In most cell types, one hemicellulose predominates, with others present in smaller amounts (Brett and Waldron, 1990).

During growth and development of plant cells, various *in vitro* measurements of wall extensibility have been made, some of which are correlated with *in vivo* growth rates (Preston, 1982). Wall extensibility is influenced greatly by growth substances, which may exert some of their short-term effects by altering the pH of the wall. Long-term changes in growth rate involve changes in cell wall synthesis and in the cross-linking of wall polymers (Brett and Waldron, 1990). The structural turnover may be related to the control of growth (Brummer and Parich, 1983).

A large amount of nitrogen source (potasium nitrate, 19.0 g/L and ammonium nitrate, 16.5 g/L), following carbon source (sucrose, 30 g/L) supplemented in the medium, is essential for plant growth (Nielsen and MacDonald, 1978). It is well known that the use of nitrogen fertilizer in practical agriculture affects the utilization of carbohydrates in plant cells (Noggle and Fritz, 1983). Until now, the effect of nitrogen source on metabolism of cell wall polysaccharides is not well understood in respect to *in vitro* regulation of maturation.

To learn more *in vitro* regulation of differentiation, the effects of growth regulator and nitrogen source on metabolism of cell wall polysaccharides in the suspension-cultured cells of a dicotyledonous kidney bean (Phaseolus vulgaris L.), which is more actively growing than those of the monocotyledonous plants generally, were investigated.

MATERIALS AND METHODS

Plant materials and cell culture

The seeds of kidney bean (*Phaseolus vulgaris* L. cv. Kangnangkong No. 1) were soaked in running tap water for 1 day and then, germinated in a mixture (1:1, v/v) of sand and vermiculite. After 7 days of germination, the epicotyl segments of the seedlings were sterilized with 70% ethanol for 30 s and with 1% sodium hypochlorite solution for 10 min. The epicotyl segments at 2 mm × 3 mm were excised and then, transferred into a 100-mL flask containing 25 mL of a liquid MS (Murashige and Skoog, 1962) medium supplemented with 1.0 mg/L of 2,4-D and 0.5 mg/L of kinetin. Before use, the medium was adjusted to pH 5.8 with 1 N KOH, and then, autoclaved at 121℃ for 15 min. The culture condition was under a white fluorescent light (15 μE m^{-1} s^{-1}) in a shaking incubator (25 ± 1℃) with rotation at 120 rpm. After 30 days of culture, the suspension cells (clusters) were sedimented by standing for 10 min. The dedifferentiated suspension cells were designated as Susp. I. The suspension cells (388 ± 3 mg FW, n=3) were transferred into a 100 mL-flask containing 25 mL of the medium supplemented with growth regulators (2,4-D, 1.0 mg/L and kinetin, 0.5 mg/L) and nitrogen source [potasium nitrate (KNO_3), 19.0 g/L and ammonium nitrate (NH_4NO_3), 16.5 g/L] or without. The cells were cultured for 30 days (long-term) under the same condition described above.

After 30 days of culture, the suspension-cultured cells were precipitated and pH of the media was measured. Data from one experiment with triplicated samples are given. The cells were washed three times with deionized water by centrifugation for 10 min at 250 *g* and one gram was weighed immediately. The proliferated suspension cells were designated as Susp. II. The epicotyl segments and the Susp. I and II were fixed respectively in 5 mL of methanol at 65℃ for 15 min and stored at room temperature until use.

Cell wall fractionation

Fractionation was performed by a modified method of Sakurai et al. (1987). The samples in methanol were centrifuged for 10 min at 1,000 *g* and then, hydrated in deionized water for 10 min. The samples were homogenized in deionized water with a mortar and pestle. The homogenate was boiled for 10 min to inactivate any glycanase and centrifuged for 10 min at 1,000 *g*. The residue was washed twice with deionized water and with acetone by centrifugation for 10 min at 1,000 *g*. The residue was washed with a mixture (1:1, v/v) of methanol and chloroform by centrifugation for 10 min at 1,000 *g*. The residue was again washed three times with deionized water. The crude cell wall materials in test tube were treated with 2 mL (10 units) of porcine pancreatic α-amylase (Type I-A, Sigma, St. Louis, MO, USA) in 50 mM sodium acetate buffer (pH 6.8) for 2 h at 37℃ to remove starch. The treated cell wall materials were washed three times with deionized water.

Pectic substances from cell walls were extracted three times, each for 15 min, with 50 mM EDTA in 50 mM sodium phosphate buffer (pH 6.5) at 95℃. The extract was designated as pectin fraction. Next, hemicellulosic substances were extracted three times with 17.5% NaOH containing 0.02% sodium borohydride for 18 h at 25℃. The extract was designated as hemicellulose fraction. The residue was washed three times, each with 0.03 M acetic acid and a mixture (1:1, v/v) of ethanol and diethyl ether to remove any phenolic compounds. The washed residue was dried for one day at 25℃ and two days at 40℃. The dried material was designated as cellulose fraction.

Measurements of sugar contents

The total sugar amount of each fraction was determined by a phenol-sulfuric acid method (Dubois et al., 1956). Before determination, the cellulose fraction was hydrolyzed with 7.5 M sulfuric acid over ice bath and 1 M sulfuric acid for 1 h at 100℃. The sugar content of each fraction was represented as % to total sugar amount of cell wall fraction. Uronic acid amount in pectin and hemicellulose fractions was determined by an m-hydroxydiphenyl method (Blumenkranz and Asboe-Hansen, 1973). Data from one experiment with triplicated samples are given.

Analysis of neutral sugar component of non-cellulosic (pectin and hemicellulose) fractions

The alditol acetate derivatives of non-cellulosic polysaccharides were analyzed by gas-liquid chromatography (GLC). The hemicellulose fraction was neutralized with a half volume of glacial acetic acid. The pectin and neutralized hemicellulose fractions were dialyzed against deionized water with seamless cellulose tubing (18/32) for 2 days. A portion (3 mL) of each fraction was placed in a screw-capped tube and dried with a stream of filtered air at 50℃. The dried polysaccharides were hydrolyzed by 2 mL of 2 M trifluoroacetic acid (TFA) containing 300 μg of *myo*-inositol as an internal standard in a screw-capped test tube for 1 h at 121℃ and then, dried by air flow at 50℃. The hydrolyzed monosaccharides were reduced by sodium borohydride and acetylated with acetic anhydride in the presence of 1-methylimidazole as a catalyst (Blakeney et al., 1983). The acetylated monosaccharides were dissolved in 100 μL of acetone and one μL was introduced into a GLC system (M600D, Young-Lin Instrument Co., LTD, Seoul) equipped with a flame ionization detector and a capillary column (SP-2380, Supelco, Park, Bellefonte, PA, USA). The oven temperature was raised from 180℃ to 230℃ at a rate of 4℃/min. The sugar content was determined by the ratio of peaked area. Data from one experiment with three determinations are given.

RESULTS AND DISCUSSION

Changes in amounts and contents of cell wall polysaccharides during dedifferentiation and proliferation

The Susp. I was directly induced from the epicotyl segments of kidney bean seedlings in liquid MS medium supplemented with 2,4-D, 1.0 and kinetin, 0.5 mg/L. And the Susp. I was subcultured in the same medium for 30 days. The total cell wall sugar amount (12.5 mg/g FW) of the epicotyl segments decreased during dedifferentiation (Susp. I, 7.5 mg) and proliferation (Susp. II, 3.3 mg). The cellulose amount (mg/g FW) more sharply decreased than that of hemicellulose during dedifferentiation and proliferation. However, the amount of pectin was maintained at the lowest level, ranging of 0.5~1.0 mg (Figure 1A).

The cellulose content of the epicotyl segments was 62%. The content decreased from Susp. I (57%) to Susp. II (44%). Hemicellulose content of the Susp. II was slightly increased to 35%. The pectin content of the Susp. II also slightly increased (Figure 1B).

The cellulose content of the Susp. I (57%) and II (44%) was lower than that (62%) of the differentiated epicotyl segments. The content of cellulose in the suspension-cultured cells varied among plant species, being reported below 20% in callus and suspension cells of barley strains (Yeo et al., 1995, 1998), at 23% in sycamore cells (Talmadge et al., 1973), 25% in *Vinca rosea* (Takeuchi and Komamine, 1987), 42% in carrot (Masuda et al., 1984), 46% in *Zinnia elegans* (Ignold et al., 1988), and 62% in *Rosa glauca* (Chambat et al., 1981). The high cellulose content was related with differentiation of tracheary elements (Ignold et al., 1988). It is generally accepted that the plant cell walls that develop during the proliferation and elongation of cells are primary ones, while those that are synthesized after the cessation of cell elongation are secondary ones. It has been reported that the secondary ones are often rich in cellulose (Heigler, 1985).

Changes in neutral sugar components of non-cellulosic fractions during dedifferentiation and proliferation

To determine whether or not there were any differences in neutral sugar components, the acetylated monosaccharides of pectin and hemicellulose fractions were analyzed by GLC (Table 1). The neutral sugar components of the pectin fraction of the epicotyl segments consisted of galactose (81.1%), arabinose (8.3%), rhamnose (5.4%), and glucose (4.5%) in a descending order. The neutral sugar components of the Susp. I consisted of arabinose (40.6%), glucose (21.3%), galactose (16.0%), rhamnose (14.2%), mannose (7.3%), and xylose (0.5%) in a descending order. These results suggested that among pectic polysaccharides, arabinogalactan I or rhamnogalacturonan I was synthesized during dedifferentiation. Susp. II consisted of arabinose (37.9%), rhamnose (24.5%), galactose (20.3%), glucose (8.6%), mannose (6.5%), and xylose (2.2%), which are similar to those of Susp. I except with low level of glucose and high level of xylose. The UA contents were 20% of the total amount of pectin. There was no difference in UA content in the Susp. I and II. The pectic polysaccharides of suspension-cultured cells consisted mainly of arabinose, xylose, and galactose (uronic acid) (Kato and Nevins, 1982). The ratio of galactose to rhamnose was about 1:1. Therefore, they are probably the components of rhamnogalacturonan I (RG I), an acidic polysaccharide. It has been reported that RG I, which contains a backbone of α1,4-linked galacturonic acid and α1,2-linked rhamnose, is a major component of primary cell wall of dicotyledonous plants, and that, in sycamore suspension-cultured cells, the ratio of galacturonic acid to rhamnose is about 1:1, probably as a regular, alternating sequence (Brett and Waldron, 1990).

In the hemicellulose fractions, the neutral sugar components from the epicotyl segments mainly consisted of galactose (43.0%), arabinose (28.7%), and glucose (19.4%) in a descending order. Susp. I and II had more arabinose and less xylose, mannose, galactose, and glucose than the epicotyl segments had (Table 1). The contents of UA were below 2.3% of the total amount of hemicellulose, much less (a tenth) than that (24%) of the pectin. These results suggested that arabinogalactan II (AG II), possesses a highly-branched galactan core, containing 1,3- and 1,6-linked galactose units, was the one most likely synthesized during dedifferentiation since many suspension-cultured angiosperm cells have been found to secrete arabinogalactan-protein in

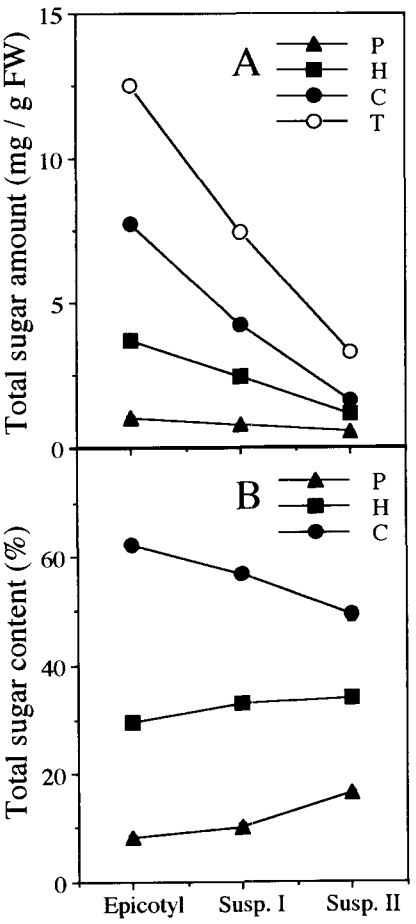

Figure 1. Changes in sugar amount and content of cell wall fraction during dedifferention and proliferation. P, pectin fraction ; H, hemicellulose fraction ; C, cellulose fraction ; T, total cell wall fraction. Standard error bars were hidden within the symbols (n=3).

Table 1. Changes in neutral sugar components of pectin and hemicellulose fractions during dedifferentiation and proliferation of kidney-bean from the epicotyl segment to the Susp. I and II

Material	Cell-wall fraction	Neutral sugar component (%)						
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc
Epicotyl segment	Pectin	5.4	-	8.3	tr	tr	81.1	4.5
Susp. I		14.2	-	40.6	0.5	7.5	16.0	21.3
Susp. II		24.5	-	37.9	2.2	6.5	20.3	8.6
Epicotyl segment	Hemicellulose	tr	-	28.7	8.8	0.1	19.4	43.0
Susp. I		tr	-	51.6	9.3	tr	8.5	30.6
Susp. II		tr	-	64.4	6.5	tr	0.2	28.9

Rha, rhamnose; Fuc, fucose; Ara, arbinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; -, not detected; tr, trace (< 0.1%). Data represent means of triplicates.

Table 2. Comparison of proliferation of Susp.I in media supplemented with growth regulators and nitrogen sources or without for 30 days

Medium	Growth regulator*	Nitrogen source **	Fresh weight (mg)/ flask (pH)	Proliferation rate (fold)***
A	-	-	879^{c} ± 18 (4.2)	2.3 ± 0.0
B	-	+	737^{b} ± 6 (5.1)	1.9 ± 0.0
C	+	-	6241^{d} ± 207 (4.7)	16.1 ± 0.5
D	+	+	658^{a} ± 41 (5.1)	1.7 ± 0.1

* 2,4-D, 1.0 mg/L and kinetin, 0.5 mg/L; **Ammonium nitrate, 16.5 g/L and potassium nitrate, 19.0 g/L; ***Fresh weight / initial fresh weight. The initial fresh weight of Susp. I was 388 ± 3 mg (n=3). Data from one experiment with triplicated samples are given. The alphabets on shoulder of the mean represent differences at 5% level of Duncan's multiple range test.

which the polysaccharide portion is very similar to AG I, in spite of that AG II is found as a component of gymnosperm cell walls (Brett and Waldron, 1990). Other high levels of arabinose, glucose, and xylose would be components of xylans, glucuromannan, xyloglucan, callose, and β1,3, β1,4-glucan among hemicellulosic polysaccharides. A quantitative analysis of hemicellulosic polysaccharide revealed active changes of galactose and xylose in the wall during *in vivo* cell extension and maturation, suggesting that xyloglucan was synthesized (Loescher and Nevin, 1972; Nishitani et al., 1979; Terry et al., 1981). The glucose component is considered to be callose (β1,3,-glucan) *in mechanically-agitated suspension-cultured* cells. And the glucose component in hemicellulose of *Gramineae* is also considered to be β-(1,3; 1,4) glucan (Sakurai, 1991). Levels of β-(1,3; 1,4) glucan in *Gramineae* have been reported to be high in young leaves, coleoptiles, and stems, and low in old tissues (Buchala and Wilkie, 1971). Therefore, the high level (29.8%) of glucose of Sus. II harvested after 30 days of subculture (at stationary phase) suggested that the cells had primary cell walls characteristic of young tissue cells.

Effects of growth regulators and nitrogen source on proliferation of the Susp. I

Table 2 shows amounts and rates of proliferation of the Susp. I cultured in the revised media A, B, C, and D for 30 days. The medium C with growth regulators and without nitrogen source showed the highest proliferation rate (16.1 folds) among the media. The decreasing order was the medium A, B, and D at the 5% significant level of Duncan's multiple range test. These results indicated that growth regulators promoted cell division, while nitrogen source inhibited it. After 30 days of culture, the pH in media

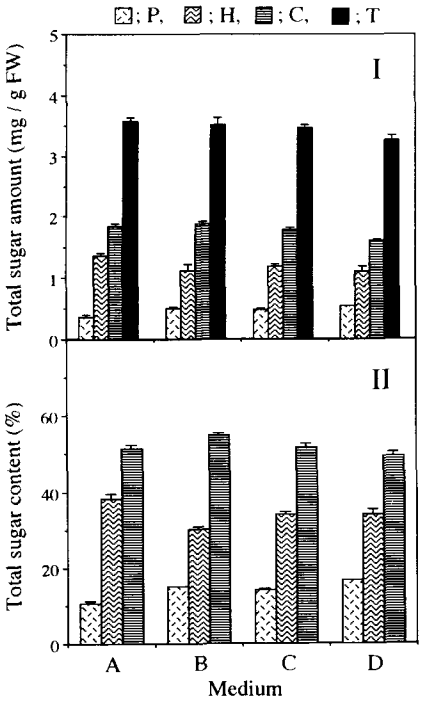

Figure 2. Effects of media on sugar content of cell wall fraction of the suspension-cultured cells. A, control ; B, nitrogen source (KNO_3, 19.0 g/L and NH_4NO_3, 16.5 g/L) ; C, growth regulator (2,4-D, 1.0 mg/L and kinetin, 0.5 mg/L) ; D, growth regulator (2,4-D, 1.0 mg/L and kinetin, 0.5 mg/L) and nitrogen source (KNO_3, 19.0 g/L and NH_4NO_3, 16.5 g/L) ; P, pectin fraction ; H, hemicellulose fraction ; C, cellulose fraction. Vertical bars represent SEs (n=3).

changed, ranging from 4.2 to 5.1 (Table 2). However, it is unlikely that the proliferation is related to pH.

Effects of growth regulators and nitrogen source on content of cell wall polysaccharide

There was no difference in the total sugar amount (mg/g FW) of the cell wall fraction among the media A, B, C, and D (Figure 2, I). However, the cellulose content in the media was 50~55%. The hemicellulosecontent (30%) in medium B was lower than that (35~40%) in other media A, C, and D. On the other hand, the pectin content (10%) in medium A was lower than those (16~18%) in other media (Figure 2, II). These results suggested that both growth regulator and nitrogen source were promotive factors for non-cellulosic polysaccharide synthesis.

Effects of growth regulator and nitrogen source on neutral sugar composition of non-cellulosic fractions

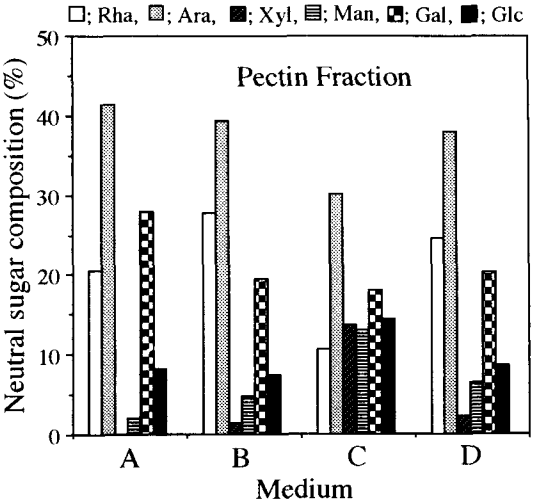

Figure 3. Effects of media on the neutral sugar composition (%) of pectin fraction of the suspension-cultured cells. A, control ; B, nitrogen source (KNO_3, 19.0 g/L and $NH4NO_3$, 16.5 g/L) ; C, growth regulator (2,4-D, 1.0 and kinetin, 0.5 mg/L) ; D, growth regulator (2,4-D, 1.0 mg/L and kinetin, 0.5 mg/L) and nitrogen source (KNO_3, 19.0 g/L and NH_4NO_3, 16.5 g/L) ; Rha, rhamnose; Ara, arabinose ; Xyl, xylose ; Man, mannose ; Gal, galactose ; Glc, glucose. Fucose was under detection limit. Data represent means with three determinations of one experiment.

To investigate the effect on the turnover of the non-cellulosic polysaccharides of the suspension-cultured cells grown in the media A, B, C, and D for 30 days, the acetylated monosaccharides of the pectin and hemicellulose fractions were analyzed by GLC (Figures 3 and 4).

In the pectin fractions, rhamnose, arabinose, and mannose contents in medium B were higher, while galactose content was lower than the content in medium A (control). On the other hand, in medium C, rhamnose and arabinose content were lower, but xylose, mannose, and glucose contents were higher than in medium D (Figure 3). These results suggested that growth regulators inhibited the synthesis of rhamnogalacturonan I or arabinogalactan I and promoted that of other pectic polysaccharides, and nitrogen source *vice versa*.

In the hemicellulose fractions, there was no difference in neutral sugar component in the media A and B. In the medium C, arabinose content was lower, but galactose and xylose contents were higher than in medium D (Figure 4). These results suggested that growth regulator inhibited synthesis of arabinogalactan II, while nitrogen source inhibited the synthesis of xyloglucan. Xyloglucan exists commonly in hemicellulosic polysaccharides in *in vivo* monocot and dicot