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Long-Term Effects of Several Biochemical and Physical Factors on Cellulose Biosynthesis in Callus and Suspension Cultures of Normal and Mutant Barley (*Hordeum vulgare* L.) Strains Producing Less Cellulose

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To clarify a low level of cellulose biosynthesis of the *in vitro* cultured cells, the effects of several biochemical factors such as carbon sources (sucrose, maltose, and UDPG), antioxidants (ascorbic acid and glutathione) and physical factors such as artificial pressure, high gravity, on the cellulose production in barley callus and suspension cultures were investigated. In the suspension culture of two barley strains, the supplement of different concentrations (0, 1.5, 3.0, and 4.5%) of sucrose or maltose into the medium for 30 days did not promote the cellulose production and 4.5% of sugar supplement was rather inhibitory in one strain. However, in the presence of sucrose at 3%, UDPG (3 or 10 mM) supplement, as a precursor for cellulose, promoted 1.2~1.3 fold of the production in two strains. A low concentration (3 mM) of ascorbic acid and glutathione promoted 1.5 and 1.2 fold of the production in two strains, respectively. These results suggest that low cellulose biosynthesis of the *in vitro* cultured cells is due to a decreased level of the UDPG in the cytosol, and that the oxidative condition of external medium impedes cellulose synthesis in some manners. Artificial pressure applied to the callus promoted 1.4 fold of the cellulose production. High gravity (5,000 or 10,000 *g*) applied to the suspension-cultured cells by centrifugation did not cause a substantial change.

Keywords: barley (*Hordeum vulgare* L.), cellulose production, carbon source, UDPG, antioxidant, artificial pressure and high gravity, callus and suspension-cultured cell.

INTRODUCTION

Cell wall, a major structural element of plant cells, plays an important role in protection of protoplasts from mechanical forces and in the mechanical support of plant tissues and organs (Taiz, 1984; Masuda, 1990; Sakurai, 1991; Hoson, 1993). The crystalline cellulose polysaccharide in the matrix is a major factor in the regulation of cell shape and rigidity. But the cellulose synthesis and its regulation remain unclear (Delmer, 1987; Delmer *et al.*, 1993) and in dispute (Okuda *et al.*, 1993; Albersheim, 1997).

Kokubo *et al.* (1991) reported that three isogenic barley mutants that developed fragile culms produced less amount of cellulose than corresponding normal strains. We determined the amount of cell wall polysaccharides of callus and suspension-cultured cells derived from the above three isogenic lines of fragile barley mutants and their corresponding normal lines (Yeo *et al.*, 1995). The results demonstrated that the cellulose contents of calli and suspension-cultured cells of normal barley strains were ca. 1/5~1/6 of those of highly differentiated culm of normal barley strains grown in the field. The cellulose contents of normal strains under *in vitro* culture condition were close to those of their corresponding mutant strains. The results also indicated that the cells oriented toward cell division, such as calli and suspension-cultured cells, produced more non-cellulosic polysaccharides and

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less cellulose than differentiated cells did. The content of cellulose in suspension-cultured cells varied among plant species, being reported at 23% in sycamore cells (Talmadge *et al.*, 1973), 26% 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). It was deduced that the walls of cultured cells of the former two species are composed of primary walls, but the latter three ones are composed of secondary walls, which comprised tracheary elements (Ignold *et al.*, 1988). The average content of cellulose is estimated as ca. 45% in the primary cell walls of monocotyledonous intact plants (Sakurai, 1991).

To clarify the low cellulose biosynthesis in the cultured cells of the normal or mutant barley strains, the effects of several biochemical and physical factors on cellulose production were investigated.

MATERIALS AND METHODS

Callus and Suspension Culture

The seeds of three isogenic normal and mutant strains of barley (*Hordeum vulgare* L. cv. Ohichi, Shiroseto, and Kobinkatagi 4) were collected on June 20, 1996 at the experiment farm of Faculty of Integrated Arts and Sciences, Hiroshima University. They were stored with calcium chloride as a desiccant in a refrigerator until use.

The seeds were sterilized with 70% EtOH for 1 min, then with 2% sodium hypochlorite for 15 min. They were allowed to germinate on two layers of filter paper (Whatmann No. 2) in 100-ml beakers containing 4 ml of deionized water that had been autoclaved for 15 min at 121°C. The beakers were placed under white fluorescent light at an intensity of about 15 $\mu E\ m^{-1}s^{-1}$ with a 16-h light/8-h dark cycle at 25°C.

For suspension culture, the middle parts of root of the 2~3 days-old seedlings were excised (5 mm-long) and inoculated into a liquid SH (Schenk and Hildebrandt, 1972) medium (pH 5.9) supplemented with 10^{-5}M 2,4-D and 3% sucrose. After one month of suspension culture (with rotation at 120 rpm), the suspension cells (ca. 200 mg FW) were subcultured in a 100-ml flask containing 30 ml of the same medium with different concentrations of sucrose and maltose (0, 1.5, 3.0, and 4.5%) and of uridine diphosphate glucose (UDPG) (0, 1, and 10 mM), and glucose (1.6%), uridine diphosphate (UDP) (10 mM) or uridine (U) (10 mM) in the presence of 3.0% sucrose. To investigate effects of antioxidants on cellulose synthesis, the suspension cells were subcultured in the same medium supplemented with different concentrations of ascorbic acid and glutathione (0, 3, and 10 mM). To investigate the effect of high artificial gravity, the suspension cells (ca. 100 mg FW) were inoculated into a Teflon centrifuge tube containing 10 ml of the same medium and cultured under the centrifugation (5,000 or 10,000 $g \times 2$ h $\times 3$ times/week) for 30 days.

After 30 days of culture, the cells were collected by a centrifugation (250 *g*, 5 min) and washed twice with deionized water. The fresh weight was determined for the proliferation rate. The suspension cells were fixed in 10 ml of methanol at 65°C for 15 min and stored at room temperature until use.

For the application of artificial pressure to callus, the scutellum tissue was selected for the callus induction. Callus from the scutellum tissue grew more than those from the other tissues (root and coleoptile) of the seedlings (Yeo and Kuraishi, 1992). The scutellum tissue was excised and inoculated into a small glass tube (id., 5.0 mm; length, 2.5 cm) vertically placed in a 100-ml flask containing 30 ml of 0.9% solid agar medium. After 30 days of culture, the degree of contact of callus with the wall of glass tube was divided into three classes, as low, medium, and high. After the fresh weight was determined, the calli were fixed in 10 ml of methanol at 65°C for 15 min and stored at room temperature until use.

Fractionation of Cell Wall Polysaccharides of Callus and Suspension-Cultured Cells

Cell wall polysaccharides of various samples were fractionated by a modified method of Sakurai *et al.* (1987). The suspension-cultured cells (200-300 mg FW) and callus (ca. 1 g FW) in methanol were centrifuged for 10 min at 1,000 *g*. The extract was designated the MeOH fraction. The residue was homogenized in deionized water with a mortar and pestle. The homogenate was boiled for 10 min to inactivate any glycanase and then centrifuged for 10 min at 1,000 *g*. The supernatant was designated the hot water (HW) fraction. The residue (crude cell-wall material) was treated with 2 ml of a solution containing 5 units ml^{-1} of porcine pancreatic α-amylase (Type I-A; Sigma, St. Louis. MO. USA) in 50 mM sodium acetate buffer (pH 6.5) for 2 h at 37°C. The crude cell-wall materials were washed

three times with deionized water by centrifugation (10 min at 1,000 *g*). The residue was hydrolyed with 1 ml of 2 M trifluoroacetic acid (TFA) for 1 h at 121°C in a screw-capped test tube. The TFA-soluble fraction was collected by centrifugation (10 min at 1,000 *g*). The supernatant was designated the non-cellulosic TFA fraction (including pectin and hemicellulose polysaccharides). The residue was washed twice with 2 ml of deionized water. The washings were combined with the TFA fraction. The TFA-insoluble fraction was washed three times with a mixture of ethanol and diethylether (1:1, v/v) and dried for one day at 25°C and for 2 days at 40°C. The dried materials were designated the cellulose fraction.

Measurement of Total Sugar Content in Fractions

Total sugar contents in each fraction were measured by a phenol-sulfuric acid method (Dubois *et al.*, 1956). The cellulose fraction was hydrolyzed with 7.5 M H_2SO_4 for 1 h at room temperature and 1 M H_2SO_4 for 1 h at 100°C, before sugar determination. The cellulose content (%) was calculated as follows,

$$\text{Cellulose content (\%)} = \frac{\text{cellulose content}}{\text{total cell wall content (TFA + cellulose fraction)}} \times 100$$

The cellulose productivity was estimated as cellulose content (%).

Analysis of Neutral Sugar Component of Non-cellulosic TFA Fraction

The neutral sugar components of the TFA fractions of calli grown under high artificial pressure and suspension cultured cells grown under artificial high gravity were determined by a gas liquid chromatography (GLC). A portion (3 ml) of the TFA fraction was placed in a screw-capped tube and dried with air flow at 50°C. One ml of 2 M TFA containing 300 μg of *myo*-inositol as an internal standard was added to the tube. The tube was autoclaved for 1 h at 121°C. The hydrolyzed monosaccharides were reduced with sodium borohydride and acetylated with acetic anhydride in the presence of 1-methylimidazole as a catalyst (Blakney *et al.*, 1983). The acetylated monosaccharides were dissolved in 200 μl of acetone and one μl was introduced into a gas-chromatograph (GC-7A, Shimazu, Kyoto) equipped with a flame ionization detector and a capillary column (SP-2380, Supelco, Park, Bellefonte, PA, USA). The column temperature was raised from 180 to 230°C at the rate of 4°C/min.

Table 1. Effects of two carbon sources (sucrose and maltose) on the sugar amounts of the symplastic and apoplastic (cell-wall) fractions of suspension-cultured cells of normal barley strains (*Hordeum vulgare* L. cv. Ohichi and Shiroseto)

Strain	Carbon source (%)	Symplastic fraction		Apoplastic (cell-wall) fraction		
		MeOH	HW	TFA	Cellulose	Total
		mg (g FW)$^{-1}$				
Ohichi	Sucrose					
	0.0	0.5±0.02	1.4±0.03	23.5±0.36	6.8±0.32	30.3±0.48
	1.5	6.7±0.48**	1.3±0.01	29.3±0.54**	6.9±0.32	36.2±0.63**
	3.0	10.2±0.53**	4.6±0.13*	35.5±0.22**	12.0±0.03**	47.5±0.23**
	4.5	21.5±1.08**	3.6±0.01	38.8±0.58*	9.6±0.10*	48.4±0.59
	Maltose					
	1.5	7.5±0.08**	2.2±0.09	28.5±0.26	10.2±0.16*	38.7±0.31**
	3.0	14.2±0.08**	4.4±0.11*	31.3±0.34*	11.4±0.28	42.7±0.44**
	4.5	11.2±0.07*	1.8±0.01	17.7±0.14**	5.5±0.06**	23.2±0.15**
Shiroseto	Sucrose					
	0.0	0.2±0.02	1.5±0.03	32.9±0.36	11.6±0.32	44.5±0.54
	1.5	5.0±0.08**	1.7±0.03	32.0±0.16	10.2±0.06	42.4±0.17
	3.0	10.2±0.40**	1.5±0.04	33.3±1.02	8.8±0.30	42.1±1.06
	4.5	10.3±0.42	1.6±0.01	26.4±0.40**	6.2±0.16	32.6±0.43**
	Maltose					
	1.5	6.6±0.09**	0.9±0.02	26.7±0.26	8.0±0.36	34.7±0.22*
	3.0	12.8±0.17**	2.6±0.02	28.7±0.12	9.2±0.22	37.9±0.13*
	4.5	18.7±0.34**	2.8±0.03	41.8±0.36**	10.1±0.16	51.9±0.37**

*Significantly smaller or larger than the control without sugar supplement at the 5% and **at 1% level.
The means and SEs of triplicates are in each case. MeOH; methanol, HW; hot water, TFA; trifluoroacetic acid.

RESULTS AND DISCUSSION

Effects of Carbon Sources (Sucrose and Maltose) on Symplastic and Apoplastic Sugar Contents

After one month of suspension culture from root segments of 2~3 days-old seedlings of the Ohichi and Shiroseto strains, the effects of sucrose and maltose added to the medium as carbon sources on sugar contents of the symplastic and appolastic fractions were investigated (Table 1). The sugar contents of the symplastic MeOH fraction of Ohichi strain increased more by sucrose supplement than by maltose supplement into the medium, while those of the Shiroseto strain did by the maltose supplement. The high supplement of maltose at 4.5% suppressed the contents of the fraction in Ohichi strain (11.2± 0.07 mg (g FW) ^{1}). The extremely low level of the fraction of both strains in the absence of carbon source accounts for almost no proliferation for both strains during 30 days. The MeOH fraction was reported to contain mono- and oligosaccharides such as glucose, fructose, and sucrose (Wakabayashi *et al.* 1991).

Exogenously supplied carbon source did not remarkably affect the sugar content of the HW fraction. The fraction contains polysaccharides of non-cell wall components such as starch and cell wall component such as arabinogalactan in primary walls (Fincher and Stone, 1983).

In the apoplastic TFA fraction with non-celluiosic polysaccharides (pectin and hemicellulose), the sugar content of the Ohichi strain increased according to the amount of exgenous sucrose, while that of the Shiroseto strain did not. Maltose supplement was rather inhibitory for the Ohichi strain, while it was promotive for the Shiroseto strain. These results suggest that the Ohichi strain favors sucrose, rather than maltose for non-cellulosic wall metabolism and the Shiroseto strain does maltose.

Effect of exogenous supply of carbon source on cellulose content was not so remarkable as that on the TFA fraction. The promotive effect was only found for the Ohichi strain supplemented with more than 3% of sucrose. For the Shiroseto strain, sucrose supplement was rather inhibitory. Maltose at 4.5% was inhibitory for the Ohichi strain, but it was slightly promotive for the Shiroseto strain. These results suggest that the activities of the invertase and synthase are strain-specific. The enzymes exist in cytosol, vacuole, and sometimes even in cell walls (Stommel and Simon, 1990).

Effect of UDPG on Cellulose Production

Glucose from UDPG is incorperated into cellulose product (Morvan *et al.*, 1991; Li and Brown, 1993; Li *et al.*. 1993; Okuda *et al.*, 1993; Kudlicka and Brown, 1997). Effect of UDPG, added as a precursor for cellulose biosynthesis, in suspension

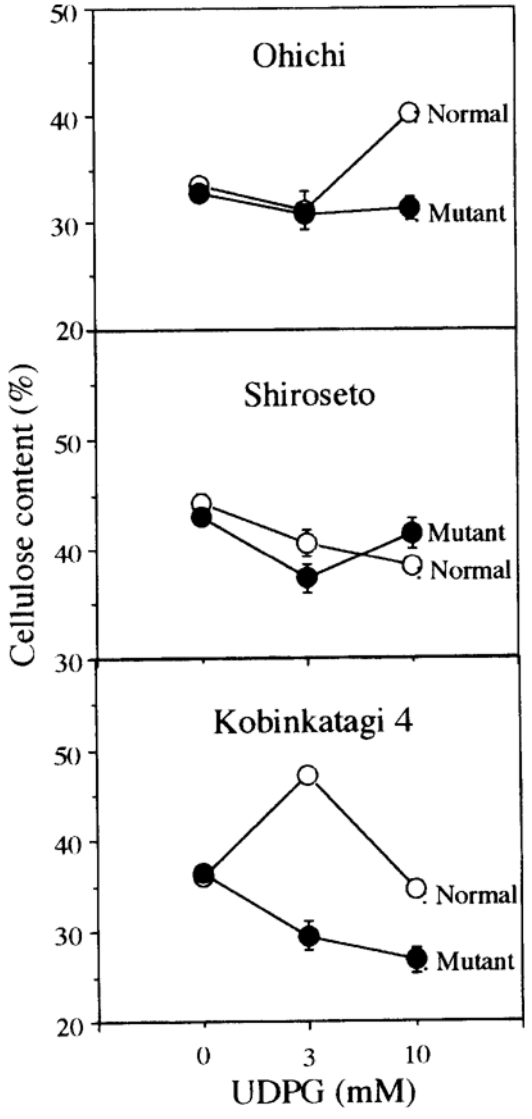

Fig. 1. Effects of UDPG on cellulose production in suspension-cultured cells of three less cellulose-prodution mutants and their isogenic normal strains. After one month of suspension culture from root segments of the seedlings in SH medium supplemented with 10^{-5} M 2,4-D and 3% sucrose, the suspension cells were cultured in the same medium supplemented with different concentrations of UDPG, a precursor for cellulose, for 30 days. UDPG; uridine diphosphate glucose. Vertical bars indicate SE of triplicates.

cultures of three less cellulose-production mutants and their isogenic normal strains was investigated by means of cellulose content experssed in % (Fig. 1). Ther was not so much differences in cellulose content between the normal and mutant of Ohichi, Shiroseto, Kobinkatagi 4 strains. The cellulose content (43%) of the Shiroseto strains without UDPG supplement was the highest. This result suggested that the Shiroseto strains had the higher cellulose productivity than other two strains had, probably due to their physiological background.

A high concentration (10 mM) of UDPG promoted 1.2 fold of the cellulose production in the normal Ohichi strain, but did not affect the production in the mutant strain. In normal and mutant strains of Shiroseto, the supplements did not promote the production. On the other hand, in the Kobinkatagi 4 normal strain, the low supplement (3 mM) promoted 1.3 fold of the production, but the supplement (3 mM and 10 mM) inhibited the production in the mutant strain. The inhibitory effect could not be explained at the moment. The promotive effects of UDPG in the above two normal strains suggested that UDPG was directly used for cellulose biosynthesis, rather than for carbon metabolism, because the enough amount (3%, 88 mM) of sucrose was already supplemented into the medium. Addition of glucose (1.6%), UDP (10 mM) or uridine (10 mM) did not affect the production in the suspension-cultured cells of the normal strains (data not shown). The promotive effect of UDPG supplement also suggests that the low cellulose biosynthesis of the cultured cells was partly due to a decreased level of UDPG precursor in the cytosol (Delmer, 1987; Lin *et al.*, 1985). There remains a further question of why the suspension cells contain fewer cellulose molecules, in terms of assembly mechanisms of cellulose-forming complexes (Delmer, 1987; Itoh *et al.*, 1984; Mizuta, 1985; Mueller and Brown, 1982). The suspension-cultured barley cells treated with 2,6-dichlorobenzonitrile reduced the levels of cellulose in their walls (Shedletzky *et al.*, 1992). Therefore, it is likely that 2,6-dichlorobenzonitrile either reduces the level of UDPG in cytosol or impedes in the assembly process of cellulose-forming complexes.

Effects of Antioxidants (Ascorbic acid and Glutathione) on Cellulose Production in Suspension Culture

In studies of cellulose synthesis by a cell-free oat coleoptile enzyme system, the cellulose synthesis was inactivated by oxidants (Ordin and Hall, 1967). The effects of two well-known antioxidants, ascorbic acid and glutathione for cell-wall on the cellulose production were investigated in suspension cultures of the normal Ohichi and Shiroseto strains (Fig. 2). Low concentrations (3 mM) of ascorbic acid and glutathione, respectively, promoted 1.5 fold of the cellulose production in the normal Ohichi strains. In the normal Shiroseto strains, the 3 mM concentration of ascorbic acid promoted 1.2 fold of the production, but that of glutathione did not promote the production, probably due to the physiological backround. These results suggest that a reduced state of apoplast (cell-wall) favors the cellulose biosynthesis, and also suggest that the apoplast under the *in vitro* culture condition is of the oxidative state. The oxidative state would concern regulation of the enzymatic activities present in the cell walls during maturation (Ricard and Noat, 1986). The mechanism of regulation will be one of the major objectives for further study.

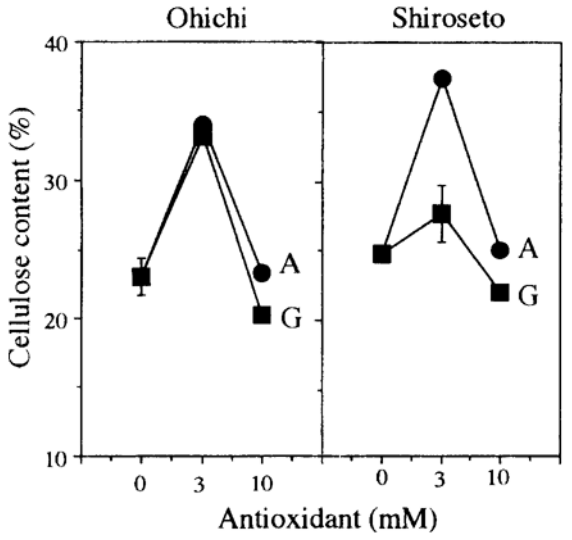

Fig. 2. Effects of antioxidants [ascobic acid (A) and glutathione (G)] for cell walls on cellulose production in suspension-cultured cells of the normal Ohichi and Shiroseto strains. After one month of suspension culture from root segments of the seedlings in SH medium supplemented with 10^{-5} M 2,4-D and 3% sucrose, the suspension cells were cultured in the same medium supplemented with different concentrations of antioxidants for 30 days. Vertical bars indicate SE of triplicates.

Effects of Artificial Pressure and High Gravity on Cellulose Production in Callus and Suspension Cultures

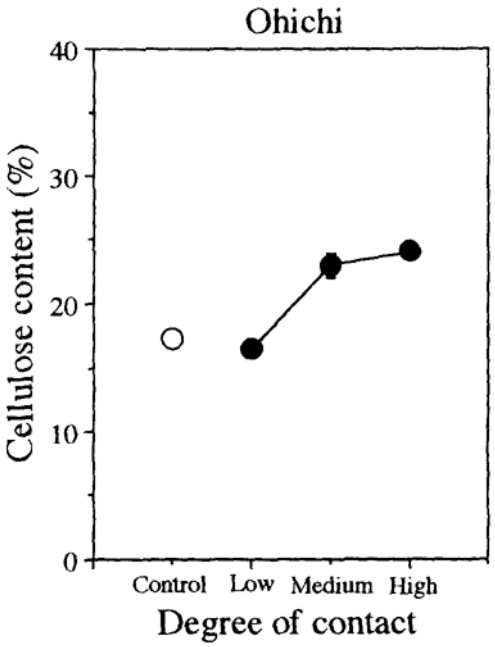

Fig. 3. Effect of artificial pressure on cellulose production in callus of the normal Ohichi strain. The callus from the scutellum tissue of the seedlings was cultured in a small glass tube (id., 5.0 mm; length, 2.5 cm) vertically placed in a 100-ml flask containing 30 ml of 0.9% agar SH medium supplemented with 10^{-5} M 2,4-D and 3% sucrose for 30 days. Three classes divided into as low, medium, and high indicate the degree of contact of callus with the wall of glass tube in 100-ml flask. Vertical bars indicate SE of triplicates.

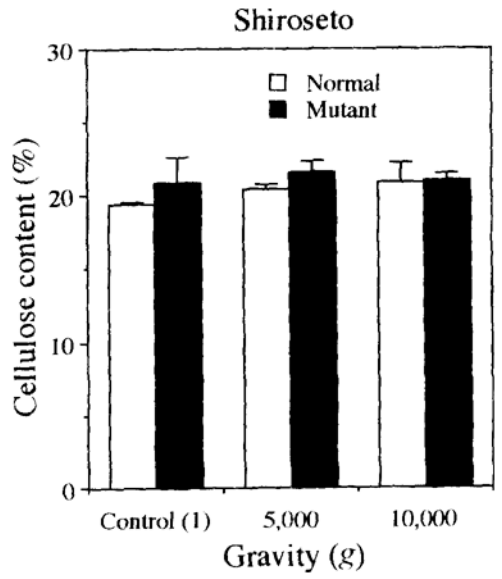

Fig. 4. Effect of artificial high gravity on cellulose production in suspension-cultured cells of the normal and mutant Shiroseto strains. After one month of suspension culture from root segments of the seedlings in SH medium supplemented with 10^{-5} M 2,4-D and 3% sucrose, the suspension cells (ca. 100 mg FW) were cultured in a Teflon centrifuge tube containing 10 ml of the medium and the high gravity (5,000 or 10,000 *g*) was applied to the suspension cells by a centrifugation (2 h×3 times/week) for 30 days. Vertical bars indicate SE of triplicates.

Artificial pressure was applied to callus culture of the normal Ohichi strain by culturing the callus within a small glass tube (Fig. 3). High gravity was applied to suspension cultures of the normal and mutant Shiroseto strains by centrifugation (Fig. 4).

The hight pressure promoted ca. 1.4 fold of the cellulose production in callus of the normal Ohichi strain, increasing at the degree of contact with glass tube wall (Fig. 3). Usually, the most of cells of intact plants are surrounded by epidermal cells with thicker cell walls. This outer peripheral tissue exerts pressure on the inner tissues. Unorganized tissue, callus or suspension-cultured cells have no epidermal cells so that the cells recieve little pressure. We attempted to produce the pressure for callus. Calli grown under pressure had a slightly higher glucose content than the control had (data not shown). This glass tube system is only a passive application of pressure to the callus, because the pressure is totally dependent on a degree of callus growth. Therefore, if the pressure had been applied more arbitrarily, the production would have been increased more consistently.

In suspension cultures of the normal and mutant strains of Shiroseto, artificial high gravity (5,000 or 10,000 *g*) did not affect the cellulose prodution, because the suspension-cultured cells was not proliferated for 30 days (Fig. 4). The little proliferation was acertained by the analysis of neutral sugar component of the non-cellulosic TFA fraction, showing that there was not any differences of the composition (%) between the cells grown under high gravities in the Teflon centrifuge tube and the control cells (data not shown). Starch grain like statolyth, perceptive for gravity, was also not confirmed in the suspension-cultured cells by the microscopic observation. Therefore, the applied high gravities would have caused a serious damage to cellular metabolism. In other graviresponses, a low gravity (20 *g*) stimulated the elongation of intact radish and cucumber hypocotyls (Kasahara *et al.*, 1995) and asymmetric distribution of β-D-glucan in the cell wall of the leaf-sheath pulvinus of oat was observed (Gibeaut *et al.*, 1990). Microgravity on a

three-dimensional clinostat also affected automorphogenesis of intact maize shoots (Hoson *et al.*, 1995). Althought the high gravity in this culture system did not affect the cell wall metabolism, unlike in the intact plants, it is likely that the high gravity affect the mode of cellulose microfibril arrangement.

The cellulose content (30~45%) promoted by UDPG, ascorbic acid, glutathione and artificial pressure in the present study was still lower than that (55~67%) of highly differentiated and mature culms of normal barley strains (Kokubo *et al.*, 1991). However, it seems that plant cell walls developed during cell proliferation are primary ones and those synthesized after cessation of cell elongation are secondary ones, often rich in cellulose (Heigler, 1985).

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