Journal of Plant Biology, June 2002, 45(2) : 90-95

Regulation of Cell-Wall Polysaccharide Components by $CaCl_2$ in Suspension Cultures of Kidney Bean (*Phaseolus vulgaris*)

Up-Dong Yeo*and Kyong-Ho Kim†

Faculty of Biological Science, College of Natural Sciences, Chonbuk National University, Chonju 561-756, Korea

We cultured the suspension cells of kidney bean in MS media supplemented with one of five concentrations of $CaCl_2$ [0, 22, 44 (control), 88, or 176 mg/L], and harvested them at the logistic (15 d) and early-stationary (30 d) phases. Cells grown at concentrations higher than 22 mg/L showed better proliferation than those at 0 mg/L. The rate of proliferation also increased with higher concentrations. We fractionated the individual sugars into symplastic (EtOH and starch) and apoplastic (low-molecular pectin, high-molecular pectin, hemicellulose, and cellulose) components. Cells treated at the highest concentration (176 mg/L) exhibited the greatest amount of sugar in the EtOH and starch fraction during the logistic phase. In contrast, cells in the early stationary phase had the highest level of sugar at treatment concentrations of less than 22 mg/L. For treatment concentrations higher than 22 mg/L on Day 15, more pectin and hemicellulose was detected at greater amounts compared with those cells treated with 0 mg/L. However, at Day 30, concentrations higher than 44 mg/L induced greater amounts of pectin and hemicellulose than from the other concentrations. Cellulose was more abundant with the 0 mg/L treatment, and contents ranged from 17.4 to 25.5% in the primary cell walls over all treatment concentrations. These results indicate that $CaCl_2$ modulates both symplastic and apoplastic sugar metabolism. Therefore, we suggest that the cell-wall structure may define the mode of polysaccharide biosynthesis during cell growth.

Keywords: $CaCl_2$, cellulose, cell wall, kidney bean, starch, suspension cell

Cell walls are a major structural element in plants, protecting the protoplasts and aiding in the mechanical support of tissues and organs (Taiz, 1984; Masuda, 1990; Sakurai, 1991, 1998; Hoson, 1993). The crystalline cellulose polysaccharide in the matrix is an important factor in regulating cell shape and rigidity. However, cellulose synthesis and its regulation remain unclear (Delmer et al., 1993; Okuda et al., 1993; Albersheim et al., 1997; Delmer, 1999).

Kokubo et al. (1991) reported that three isogenic barley mutants with fragile culms produced less cellulose than their corresponding normal strains. Furthermore, Yeo et al. (1995) determined the amount of cell-wall polysaccharides in calli and suspension-cultured cells derived from those three isogenic barley lines and the normal lines. They demonstrated that the cellulose contents from calli and suspension-cultured cells of the normal strain were only ca. 1/5 to 1/6 of those measured in the highly differentiated culms of normal, field-grown barley. Under in-vitro conditions, cellulose content in the normal strains was close to that of their corresponding mutant strains. This indicated that the cells oriented toward division, such as did the calli and suspension-cultured cells, producing more non-cellulosic polysaccharides and less cellulose than did the differentiated cells.

Cellulose contents in suspension-cultured cells vary among species, ranging from 23% in sycamore (Talmadge et al., 1973) to 26% in *Vinca rosea* (Takeuchi and Komamine, 1978), 42% in carrot (Masuda et al., 1984), 46% in *Zinnia elegans* (Ignold et al., 1988), and 62% in *Rosa glauca* (Chambat et al., 1984). In the former two species, the cultured cells are composed of primary walls, whereas the latter three consist of secondary walls that comprise tracheary elements (Ignold et al., 1988).

The low-level production of cellulose reported for in-vitro cultured barley cells is affected by several biochemical factors, such as carbon source (e.g., sucrose, maltose, and UDPG) and reduced reagents (ascorbic acid and glutathione), as well as by physical factors, such as artificial pressure and high gravity (Yeo et al., 1998). Albersheim et al. (1997) have suggested that the structure of cell-wall polysaccharides may define its mode of synthesis. Calcium is a well-known essential

*Corresponding author; fax +82-63-270-3362
e-mail y520419@moak.chonbuk.ac.kr
†Present address: National Honam Agricultural Experiment Station R.D.A., Iksan 570-080, Korea

element for growth and development. However, its effects on the structural changes of cell-wall polysaccharides are not clear.

Our objective in this study was to better understand the mode of cell-wall polysaccharide synthesis by determining the effect of $CaCl_2$ on suspension-cultured cells of the dicotyledonous kidney bean, which has a higher proliferation rate than monocotyledonous barley. We investigated polysaccharide synthesis at the logistic and the early-stationary phases of plant growth.

MATERIALS AND METHODS

Cell Culture

Seeds of the kidney bean (*Phaseolus vulgaris* L. cv. Kangnangkong 1) were soaked in running tap water for 1 d, then, germinated in a 1:1 (v:v) mixture of sand and vermiculite. After 7 d, the epicotyl segments were sterilized with 70% ethanol for 30 s and a 1% sodium hypochlorite solution for 10 min. These segments were then excised into 2- × 3- mm pieces, and transferred to a 100-mL flask containing 25 mL of a liquid MS medium (Murashige and Skoog, 1962) supplemented with 1.0 mg/L 2,4-D and 0.5 mg/L kinetin. This medium was adjusted to pH 5.8 with 1 M KOH, then, autoclaved at 121°C for 15 min. Culture conditions included white fluorescent lights (15 $\mu E\ m^{-1}s^{-1}$) and a shaking incubator (25 ± 1°C) at 120 rpm. After 30 d of culturing, the suspension cells (clusters) were sedimented in-vitro by letting them stand for 10 min. Afterward, 3 mL of the sedimented cell volume (SCV), consisting of 300 mg (fresh weight) of the suspension cells, was transferred to a 100-mL flask containing 30 mL of the same medium supplemented with 1.0 mg/L 2,4-D and 0.5 mg/L kinetin. The cells were subcultured biweekly to maintain their homogeneity.

For our experiments, these homogeneous cells were cultured for either 15 d (logistic growth phase) or 30 d (early-stationary growth phase) in various media supplemented with one of four concentrations of $CaCl_2 \cdot 2H_2O$ (MERCK) [0, 22, 88, or 176 mg/L]. The control MS medium contained 44 mg/L $CaCl_2$. Flasks were placed in a completely randomized design into the shaking incubator. At the end of each test period (15 or 30 d), the suspension cells were packed by centrifugation and washed twice with distilled water. Fresh weights were measured, and the cells were then fixed in 80% ethanol (EtOH) at 80°C for 15 min and stored in a refrigerator (4°C).

Cell-Wall Fractionation

We performed fractionation with a method modified from Sakurai et al. (1987). The samples, which had been stored in EtOH, were centrifuged for 10 min at 1,000*g*. This supernatant was then designated the EtOH fraction. Afterward, the pellet was hydrated in deionized water for 10 min. Samples were homogenized in deionized water with a mortar and pestle, and the residue was washed twice with 10 mL of 50 mM MOPS/KOH buffer (pH 7.0). The pellet was resuspended in 10 mL of 50 mM MOPS/KOH buffer (pH 7.0), and boiled for 1 min at 100°C. After cooling to room temperature, it was treated with 4 µL (20 units) of porcine pancreatic α-amylase (Type I-A, Sigma, St. Louis, MO, USA) in 50 mM MOPS/KOH buffer (pH 7.0) for 2 h at 37°C to remove the starch. After being centrifuged for 10 min at 1,000*g*, the resulting supernatant was designated the starch (S) fraction, and the pellet was washed twice with distilled water. The supernatant was combined to the S fraction. The EtOH and S fraction were called the symplastic fraction.

The homogenate was boiled three times, for 10 min each, then centrifuged for 10 min at 1,000*g*. This supernatant was then designated the hot water (HW), low-molecular pectin fraction. High-molecular pectic substances from the cell walls were extracted three times, each for 15 min, at 100°C with 50 mM EDTA in 50 mM sodium phosphate buffer (pH 6.8). The extract was designated the high-molecular pectin fraction. Afterward, the hemicellulosic substances were extracted three times with 17.5% NaOH containing 0.02% sodium borohydride, for 18 h at 25°C. This extract was designated the hemicellulose fraction. The residue was washed twice, each time with 0.03 M acetic acid, a 1:1 mixture (v/v) of ethanol and diethyl ether, and diethyl ether to remove any phenolic compounds. It was then air-dried at 40°C. The dried material was designated the cellulose fraction. The HW, pectin, hemicellulose, and cellulose fractions were called the apoplastic fraction.

Measurements of Sugar Content in Each Type of Fraction

The total amount of sugar from the symplastic fractions and the apoplastic cellulose fraction was determined according to the phenol-sulfuric acid method (Dubois et al., 1956), using the formula, total sugar (µg/mL) = 91 × A490. To prepare for this determination, the cellulose fraction was hydrolyzed with 7.5 M

sulfuric acid over an ice bath, and with 1 M sulfuric acid for 1 h at 100°C. In addition, the levels of neutral sugar (NS) and uronic acid (UA) in the pectic (HW and pectin) and the hemicellulose fractions were measured by both the phenol-sulfuric acid method of Dubois et al. (1956) and the m-hydroxydiphenyl method of Blumenkranz and Asboe-Hansen (1973). We used the formula: NS (μg/mL) = 92.2 (A490 - 0.464A520) and UA (μg/mL) = 112.4 × (A520 - 0.0272 A490), respectively. All measurements were done in triplicate. The data were then analyzed with the LSD (least significant difference) test at the 5% level.

RESULTS AND DISCUSSION

Suspension-Cell Proliferation and the Effect of $CaCl_2$

We purchased a proliferation curve to determine the necessary sampling time for investigating the effect of $CaCl_2$ on our suspension cells (Fig. 1). After 15 d (logistic phase) and 30 d (early-stationary phase), the quantity of cells in 30 mL of the liquid MS medium had increased to ca. 600 mg, twice the initial cell weight.

We used fresh-weight measurements to determine the effect of $CaCl_2$ on the rate of cell proliferation over the two test periods. At the logistic phase, the lowest level of proliferation was seen for cells treated with 0 mg/L; treatment at 22 mg/L resulted in the highest proliferation while cells treated at concentrations higher than the control level (44 mg/L) had the same,

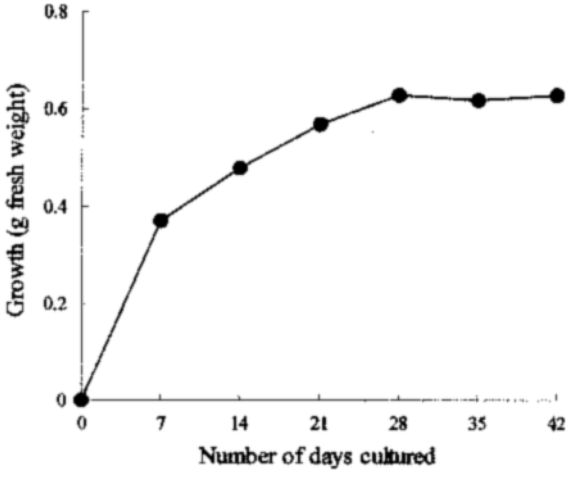

Figure 1. Growth curve of kidney bean suspension cells in liquid MS medium supplemented with 1.0 mg/L 2,4-D and 0.5 mg/L kinetin. Data are mean ± S.E. from triplicate experiments [SEs hidden in the symbol].

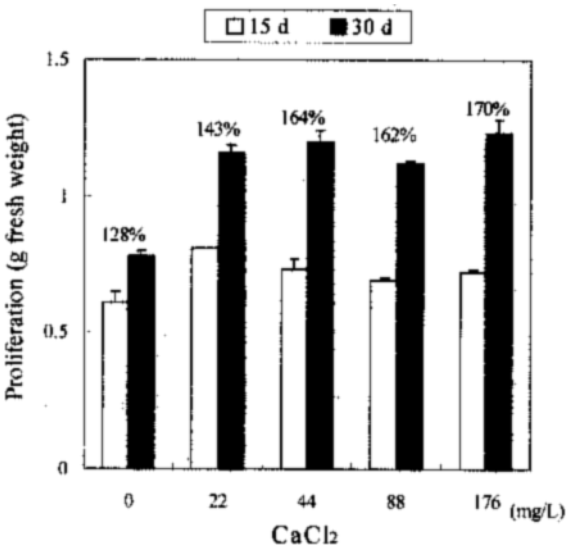

Figure 2. Effects of $CaCl_2$ on proliferation rate of kidney bean suspension cells. Figures in % on the histograms indicate the proliferation rate between logistic and early stationary phases. Data are mean ± S.E. from triplicate experiments.

intermediate amounts of proliferation. Likewise, in the early-stationary phase, cells treated with 0 mg/L exhibited the least proliferation, whereas concentrations higher than 22 mg/L induced similar amounts of proliferation (Fig. 2). The rate of proliferation (%) between the logistic and early stationary phases increased up to the control-concentration treatment, and was highest (170%) at 176 mg/L.

Effect of $CaCl_2$ on Total Sugar in the Symplastic Fractions at the Logistic and Stationary Phases

In the symplastic, EtOH and starch fractions gathered at the logistic phase (Day 15), a high concentration of $CaCl_2$ (176 mg/L) resulted in the highest amount of sugar (mg/g fresh weight; Fig. 3. upper). In contrast, at the stationary phase (Day 30), treatment with 0 mg/L produced the highest total-sugar amount in the EtOH fraction. The EtOH or MeOH fraction contained mono- and oligosaccharides, such as glucose, fructose, and sucrose (Wakabayashi et al., 1991). Another low concentration (22 mg/L) resulted in the highest amount of sugar in the starch fraction as well (Fig. 3. lower).

Effect of $CaCl_2$ on Sugar in the Apoplastic Fractions at the Logistic and Stationary Phases

We determined the total amount of sugar in the apoplastic fractions. At the logistic phase, the control

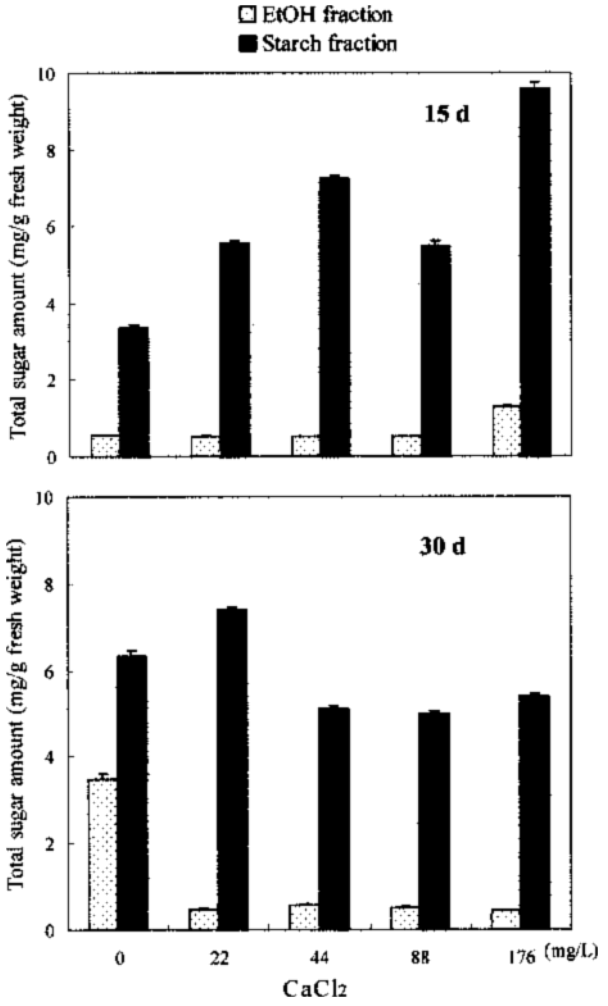

Figure 3. Effects of $CaCl_2$ on amounts of total sugar in symplastic, HW, and starch fractions at logistic (15 d) and early stationary (30 d) growth phases of kidney bean suspension cells.

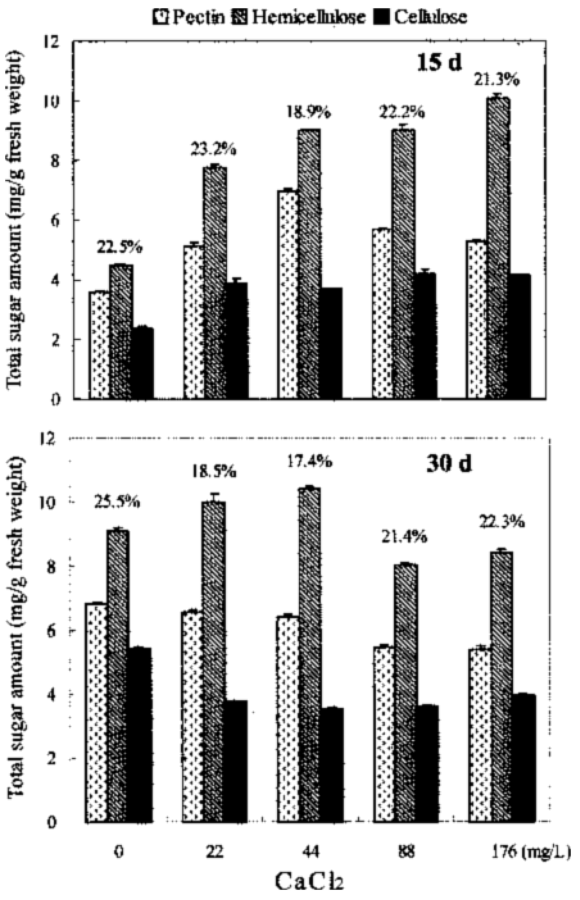

Figure 4. Effects of $CaCl_2$ on amount of total sugar in apoplastic cell-wall fractions at logistic (15 d) and early stationary (30 d) growth phases of kidney bean suspension cells. Figures in % on the histograms indicate the cellulose content as a percentage of the total for apoplastic sugars.

treatment, i.e., 44 mg/L $CaCl_2$, resulted in the highest level of NS + UA in the pectin (HW + pectin) fraction, whereas the samples from the highest concentration (176 mg/L) exhibited the greatest amount of NS + UA in the hemicellulose fraction. Those cells that had received the non-treatment (i.e., 0 mg/L) had the lowest level of cellulose. With regard to the amount of cellulose as a percentage of the total, samples from the control (44 mg/L) had the lowest proportion, 18.9%, with the remaining treatments ranging from 21.3 to 23.2% (Fig. 4. upper). At the stationary phase, $CaCl_2$ concentrations from 0 to 44 mg/L resulted in greater amounts of sugar in the pectin and hemicellulose fractions compared with cells receiving higher concentrations. The % cellulose content was highest (25.5%) in the non-treatment, while the other treatments induced levels from 17.4 to 22.3% (Fig. 4. lower). These results for % cellulose content were similar to those reported from sycamore (23%) (Talmadge et al., 1973) and *V. rosea* (26%) (Takeuchi and Komamine, 1978). However, they were higher than those found in embryogenic cells (12%) and globular embryos (10%) during somatic embryogenesis of celery (Yeo et al., 1999), which indicate that the cells also are composed of primary walls.

Based on our results, we suggest that $CaCl_2$ modulates symplastic sugar metabolism, with low concentrations promoting symplastic starch accumulation while simultaneously inhibiting apoplastic cell-wall polysaccharide metabolism. Therefore, we recommend that the optimum concentration for proliferation via symplastic metabolism of the suspension cells is 22 mg/L, and that the optimum concentration for cell-wall bio-

synthesis via apoplastic metabolism is close to the control (i.e., 44 mg/L).

Finally, in the non-cellulosic fractions, the ratios of NS to UA amounts were 10:1 for HW, 3:1 for pectin, and 10:1 for hemicellulose, all of which demonstrate incomplete fractionation. Previously, Chai et al. (1998) used GLC to analyze the NS composition of non-cellulosic (pectin and hemicellulose) fractions. They suggested that rhamnogalacturonan I in the pectin fraction and arabinogalactan II and/or xyloglucan in the hemicellulose fraction are synthesized or degraded under the influence of growth regulators (2,4-D and kinetin) and nitrogen sources (potassium nitrate and ammonium nitrate). The suspension cells in that study were cultured for 30 d (up to the stationary phase). Our results strongly indicate that $CaCl_2$ modulates the structure of cell-wall polysaccharides, a finding that agrees with that of Albersheim et al. (1997). Cells of higher plants also contain a membrane-bound glucan synthase for the synthesis of cellulose. However, almost all attempts toward its in-vitro synthesis have resulted in either the formation of only 1,3-β-glucans (callose) or very limited synthesis of 1,4-β-glucans (cellulose), so that this biosynthetic process is still poorly understood (Delmer et al., 1993; Okuda et al., 1993; Delmer, 1999). $CaCl_2$ also is involved in the stability of glucan synthase.

Vital processes for synthesis are mediated through both the specific attachment of cell walls to plasma membranes and the continuum of the cell wall-plasma membrane-endo cytoskeleton (microtubules and microfilaments; Wyatt and Carpita, 1993; Miller et al., 1997). $CaCl_2$, therefore, may modulate not only the structure of cell-wall polysaccharides, but also the arrangement of the cytoskeleton. Further investigations are needed into the relationship of the cytoskeleton with cellulose fibrils, under the influence of Ca^{2+}. Researchers must also analyze the methylation of the cellulose fraction to verify the activity of callose and/or cellulose during each growth phase.

ACKNOWLEDGEMENT

This paper was supported, in part, by research funds from Chonbuk National University.

Received March 29, 2002; accepted April 25, 2002.

LITERATURE CITED

Albersheim P, Darvill A, Roberts K, Stachelin A, Varner JE (1997) Do the structures of cell wall polysaccharides define their mode of synthesis? Plant Physiol 113: 1-3

Blumenkrantz N, Asboe-Hansen G (1973) New method for quantitative determination of uronic acids. Anal Biochem 54: 484-489

Chai Y-K, Kim K-H, Yeo U-D, Sakurai N (1998) Long-term effect of growth regulators and nitrogen sources on proliferation and turnover of cell wall polysaccharides in suspension culture of kidney bean (*Phaseolus vulgaris* L.) Kor J Plant Tiss Cult 25: 477-485

Chambat G, Baroud F, Joseleau J-P (1984) Structure of the primary cell walls of suspension cultured *Rosa glauca* cells. Plant Physiol 74: 687-693

Delmer DP (1999) Cellulose biosynthesis: Exciting times for a difficult field of study. Annu Rev Plant Physiol Plant Mol Biol 50: 245-276

Delmer DP, Ohana P, Gonen L, Benzamin M (1993) *In vitro* synthesis of cellulose in plants: Still a long way to go! Plant Physiol 103: 307-308

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28: 350-356

Hoson T (1993) Regulation of polysaccharide breakdown during auxin-induced cell wall loosening. J Plant Res 106: 369-381

Ignold E, Sugiyama M, Komamine A (1988) Secondary cell wall formation: Changes in cell wall constituents during the differentiation of isolated mesophyll cells of *Zinnia elegans* to tracheary elements. Plant Cell Physiol 29: 295-303

Kokubo A, Sakurai N, Kuraishi S, Takeda K (1991) Culm brittleness of barley (*Hordeum vulgare* L.) mutants is caused by smaller number of cellulose molecules in cell wall. Plant Physiol 97: 509-514

Masuda H, Ozeki T, Amino S, Komamine A (1984) Changes in cell wall polysaccharides during elongation in a 2,4-D free medium in a carrot suspension culture. Physiol Plant 62: 65-72

Masuda Y (1990) Auxin-induced cell elongation and cell wall changes. Bot Mag Tokyo 103: 3455-370

Miller D, Hable W, Gottward J, Ellaed-Ivey M, Demura T, Lomax T, Carpita NC (1997) Connection: The hard wiring of the plant cell for perception, signaling, and response. Plant Cell 9: 2105-2117

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol 15: 473-493

Okuda K, Li L, Kudlicka K, Kuga S, Brown RM, Jr (1993) β-Glucan synthesis in the cotton fiber. 1. Identification of β-1,4 and β-1,3-glucan synthesized *in vitro*. Plant Physiol 101: 1131-1142

Sakurai N (1991) Cell wall functions in growth and development: Physical and chemical points of view. Bot Mag Tokyo 104: 235-251

Sakurai N (1998) Dynamic function and regulation of apoplast in the plant body. J Plant Res 111: 133-148

Sakurai N, Tanaka S, Kuraishi S (1987) Changes in wall polysaccharides of squash (*Cucurbita maxima* Duch.) hypocotyls under water stress condition. I. Wall sugar composition and growth as affected by water stress.

Plant Cell Physiol 28: 1051-1058

Taiz L (1984) Plant cell expansion: Regulation of cell wall mechanical properties. Annu Rev Plant Physiol 35: 585-657

Takeuchi Y, Komamine A (1978) Changes in the composition of cell wall polysaccharides of suspension-cultured *Vinca rosea* cells during culture. Physiol Plant 42: 21-28

Talmadge K, Keegstra K, Bauer WD, Albersheim P (1973) The structure of plant cell walls. I. The macromolecular components of the walls of suspension-cultured sycamore cells with a detailed analysis of the pectic polysaccharides. Plant Physiol 51: 158-173

Wakabayashi K, Sakurai N, Kurashi S (1991) Effect of ABA on synthesis of cell-wall polysaccharides in segments of etiolated squash hypocotyl. II. Levels of UDP-neutral sugars. Plant Cell Physiol 32: 427-432

Wyatt SE, Carpita NC (1993) The plant cytoskeleton-cell wall continuum. Trends Cell Biol 3: 413-417

Yeo U-D, Han J-Y, Choi Y-E, Soh W-Y, Nakagawa N, Sakurai N (1999) Turnover of cell-wall polysaccharides during somatic embryogenesis and development of celery (*Apium graveolens* L.). J Plant Biol 42: 8-15

Yeo U-D, Soh W-Y, Nakagawa N, Sakurai N, Takeda K (1998) 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. J Plant Biol 41: 219-226

Yeo U-D, Soh W-Y, Tasaka H, Sakurai N, Kuraishi S, Takeda K (1995) Cell-wall polysaccharides of callus and suspension-cultured cells from three cellulose-less mutants of barley (*Hordeum vulgare* L.). Plant Cell Physiol 36: 931-936