Turnover of Cell-Wall Polysaccharides during Somatic Embryogenesis and Development of Celery (*Apium graveolens* L.)

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Non-embryogenic cells (NEC) and embryogenic cells (EC) were separated from cell clusters derived from the hypocotyl segments of celery seedlings, which had been suspension-cultured in MS medium supplemented with 10^{-5} M 2,4-D. The EC formed globular embryos in medium without 2,4-D. The globular embryo developed through heart-shaped, torpedo to cotyledonary embryos within 10 days. The EC and developing embryos were fractionated into symplastic [MeOH, hot water (HW), starch (S)] and apoplastic [pectin, hemicellulose, TFA (trifluoroacetic acid)-soluble and cellulose] fractions. The EC contained lower levels of sugar in the MeOH fraction and higher levels of starch than NEC. In the apoplastic fractions, there were no differences of total sugar amounts between NEC and EC. Cellulose contents were about 10% of the wall polysaccharides. During somatic embryogenesis, total sugar contents of the MeOH and HW fractions increased till the heart-shaped embryo stage, and then decreased during the torpedo and cotyledonary embryo stages. The sugar contents of the starch, pectin, TFA-soluble, and cellulose fractions did not change during the stages mentioned above. However, the hemicellulose substances remarkably increased during embryogenesis, and then decreased as the development proceeded. The neutral sugar components of the hemicellulosic fractions were analyzed. Arabinose increased markedly in EC to the globular embryo stage, but decreased as the development proceeded. Galactose increased only at the torpedo and cotyledonary embryo stages. Xylose was present at lower levels in all stages of embryogenesis than in the differentiated hypocotyl cell walls. These results suggest that there was a high turnover of arabinogalactan polysaccharides during embryogenesis, and that xylan accumulated in the cell walls of differentiated cells

Keywords: arabinogalactan, celery (*Apium graveolens* L.), cell wall, cellulose, non-embryogenic and embryogenic cell, somatic embryogenesis and development, xylan

Cell-wall polysaccharides are extensively modified during the growth and development of plant cells (Lavavitch, 1982; Taiz, 1984; Masuda, 1990; Sakurai, 1991; Hoson, 1993). Crystalline cellulose plays an important role 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). Recently, Albersheim et al. (1997) suggested that the structure of cell-wall polysaccharides may define their mode of synthesis.

Kokubo et al. (1991) reported that three isogenic barley mutants that developed fragile culms produced less cellulose than their corresponding normal strains. Yeo et al. (1995) analyzed the amount of cell-wall polysaccharides of calli and suspension-cultured cells derived from these three isogenic lines of fragile barley mutants and their corresponding normal lines. The results demonstrated that cellulose contents of calli and suspension-cultured cells of normal strains were ca. 1/5-1/6 of those of highly differentiated culm of normal field-grown strains. The cellulose contents of normal strains under the in vitro culture conditions were close to those of the corresponding mutant strains. This results indicated that the cells oriented toward cell division, such as calli and suspension-cultured cells, produced more non-cellulosic polysaccharides less cellulose than differentiated cells did. The average content of cellulose is estimated as 45% in the primary cell walls of monocotyledonous plants (Sakurai, 1991) and as 55-67% in mature culms of field-grown barley plants (Kokubo et al., 1991). In growing hypocotyls of *Pinus pinaster* seedlings, cellulose content increased from 26% to 45% (Lorences et al., 1987).

Kikuchi et al. (1995) reported that non-embryogenic callus of carrot contained higher levels of cellulose and lower levels of pectin than embryogenic callus did, suggesting that the cell-wall structure of

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embryogenic cells differs from that of non-embryogenic cells, even in the callus or suspension-cultured cells with high potential for cell division. These results imply that dynamic changes in the metabolism of cell-wall polysaccharides take place during somatic embryogenesis and differentiation. Sequential changes in the cell-wall polysaccharides during embryogenesis using a plant regeneration system have not been throughly investigated (Williams and Maheswaran, 1986). Recently the specific increase of arabinogalactan among hemicellulosic polysaccharides during embryogenesis of a monocotyledonous asparagus was first found (Yeo et al., 1998). We tried to confirm that this increase is common in other plant species.

The present study was conducted to analyze the changes in the polysaccharide structure of cell walls during somatic embryogenesis and embryo development of a dicotyledonous celery.

MATERIALS AND METHODS

Plant Materials and Cultures

Celery (*Apium graveolens* L.) seeds were sterilized with 70% ethanol for 30 s and with 1% sodium hypochlorite for 15 min. They were germinated on MS (Murashige and Skoog, 1962) medium (pH 5.8) supplemented with 3% sucrose and 0.8% agar in 100-mL beakers containing 20 mL of the medium that had been autoclaved for 15 min at 121°C. Five segments of 1 cm-long hypocotyl were excised from the 5-day-old seedlings and cultured in 30 mL of MS medium supplemented with 10^{-5} M 2,4-D and 3% sucrose in 100-mL flasks in a shaking incubator (with rotation at 120 rpm at 25°C) under white fluorescent light at an intensity of about 15 μm^{-2} s^{-1} and 16-h light / 8-h dark cycle. The cell clusters derived from the hypocotyl segments were subcultured in the same medium every two weeks for two months. After one week of subculture, the culture was centrifuged for 5 min at 20g and the supernatant (suspension cells) was washed two times with 2,4-D-free medium on nylon mesh and one gram of suspension cells was immediately weighed.

For somatic embryogenesis, one gram of cell cluster was cultured in 100 mL of 2,4-D-free medium in a 500-mL flask. The flasks of suspension culture were placed in a shaking incubator (with rotation at 120 rpm at 25°C) under the light/dark conditions described above. After 15 days of culture, the suspension culture was sedimented in a test tube for 10 min to remove non-embryogenic cells (NECs) in the upper layer. The lower layer was centrifuged for 10 min at 20g to collect embryogenic cells (ECs). ECs were subcultured in the same medium for 14 days. The homogeneous globular embryos formed were collected by sieving with stainless steel mesh (400 μm). The heart-shaped, torpedo, and cotyledonary embryos were collected under stereomicroscope (SMZ-1, Nikon).

Samples of NECs, ECs, and/or somatic embryos at four developmental stages were washed twice with deionized water and one gram was immediately weighed after brief blotting by a filter paper. They were fixed in 10 mL of methanol at 65°C for 15 min and stored at room temperature until use.

Fractionation of Symplastic and Apoplastic Sugars

Fractionation was performed by a modified method of Sakurai et al. (1987). The sample (one gram in FW) in methanol was centrifuged at 1,000g for 10 min. The supernatant was designated a symplastic, MeOH fraction. This fraction contained mono- and oligosaccharides such as glucose, fructose, and sucrose (Wakabayashi et al., 1991). The residue was hydrated with deionized water for 10 min at room temperature and homogenized with a mortar and pestle. The homogenate was boiled for 10 min to inactivate any glycanase and then centrifuged at 1,000g for 10 min. The residue was washed twice with deionized water. The supernatants were designated a symplastic, hot water (HW) fraction. This fraction contained polysaccharides of non-cell wall components such as starch and arabinogalactan-proteins (Fincher and Stone, 1983). The residue (cell-wall material) was treated with 2 mL of 5 units 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 and then centrifuged for 10 min at 1,000g. The residue was washed three times with deionized water. The supernatants were designated a symplastic, starch (S) fraction.

Pectic substance was extracted three times, for 15 min each, from cell walls with 50 mM EDTA in 50 mM sodium phosphate buffer (pH 6.8) at 95°C. Next, hemicellulosic substance was extracted at 25°C for 18 h with 17.5% NaOH containing 0.02% sodium borohydride. In a previous study (Yeo et al., 1995), the residue was found to contain appreciable amounts of neutral sugars other than glucose. Therefore, the residue was further hydrolyzed with 2 mL of 2 M trifluoroacetic acid (TFA) for 1 h at 121°C in a screw-capped test tube. The TFA-insoluble fraction was col-

lected by centrifugation (10 min at 1,000g). The supernatant was designated the TFA-soluble fraction. The residue was washed twice with deionized water, and the washings were combined with the TFA-soluble fraction. The residue (TFA-insoluble material) was washed three times, with 0.03 M acetic acid, ethanol, and then a mixture of diethyl ether and ethanol (1:1, v/v). The washed residue was dried for one day at 25°C and two days at 40°C. The dried materials were designated the cellulose fraction.

Measurements of Sugar Contents

Total sugar content of each fraction was determined 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 in ice bath and with 1 M H_2SO_4 for 1 h at 100°C, before determination. Uronic acid (UA) content in the pectin and hemicellulose fractions was determined by an *m*-hydroxydiphenyl method (Blumenkranz and Asboe-Hansen, 1973). Cell wall apoplastic sugars consisted of pectin, hemicellulose, TFA-soluble, and cellulose fractions while symplastic sugars consisted of MeOH, HW, and S fractions. Data from one experiment with three determinations are given.

Analysis of Neutral Sugar Components of Hemicellulosic Substance

The hemicellulosic substance was neutralized with glacial acetic acid over ice-cold water and dialyzed against deionized water for two days. The neutral sugar component was determined by GLC. A portion (3 mL) of the hemicellulose fraction was placed in a screw-capped tube and dried with a stream of filtered air at 50°C. One mL of 2 M TFA containing 300 μg of *myo*-inositol as an internal standard was added to the tube, and 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 (Blakeney et al., 1983). The acetylated monosaccharides were dissolved in 200 μL of acetone and one μl was introduced into a GLC system (M600D, Young-Lin Instrument Co., Seoul) 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 a rate of 4°C/min. The sugar content was determined by the ratio of peaked area. Data from one experiment with three determinations are given.

RESULTS AND DISCUSSION

Embryogenesis and Development

EC clusters (suspension cells) were induced from the hypocotyl segments of celery seedling in a liquid MS medium supplemented with 10^{-5} M 2,4-D and

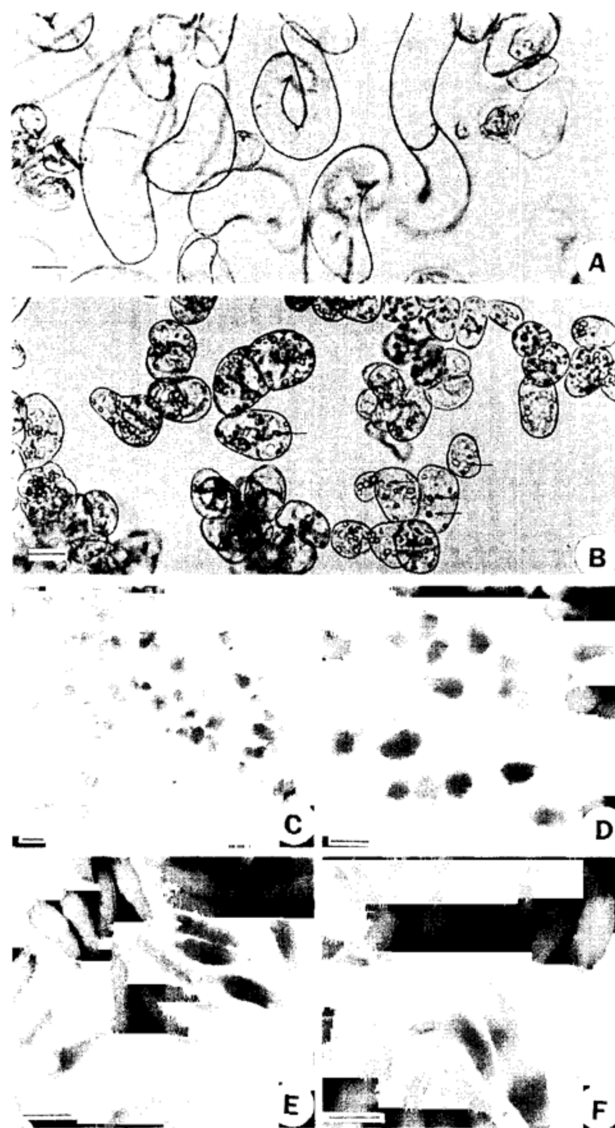

Figure 1. Non-embryogenic cells (NEC), embryogenic cells (EC), and developing somatic embryos of celery in liquid 2,4-D-free MS medium. **A**, NEC at day 0 of suspension culture (bar, 30 μm); **B**, EC at day 0 of suspension culture (bar, 30 μm), arrows indicate starch grains; **C**, globular embryos at day 14 of suspension culture (bar, 400 μm); **D**, heart-shaped embryos at day 17 of suspension culture (bar, 600 μm); **E**, torpedo embryos at day 20 of suspension culture (bar, 1 mm); **F**, cotyledonary embryos at day 25 of suspension culture (bar, 1 mm).

subcultured in the same medium. After 14 days of suspension culture in 2,4-D-free medium, large NECs and small ECs with dense cytoplasm were separated by specific gravity (Figs. 1A and B). NEC contained small amount of starch grains, while EC contained numerous starch grains. There was also a distinct difference in the cell size. The length of EC (22.4±3.7 μm) was shorter than that of NEC (100.7±11.2 μm), and the ratio of cell length to diameter of EC (0.8) was three times lower than that of elongated NEC (2.5). Globular embryos (Fig. 1C) from EC were formed on day 14 of the suspension culture and rapidly developed into heart-shaped embryos (Fig. 1D) on day 17, torpedo embryos (Fig. 1E) on day 20, and cotyledonary embryos (Fig. 1F) on day 25.

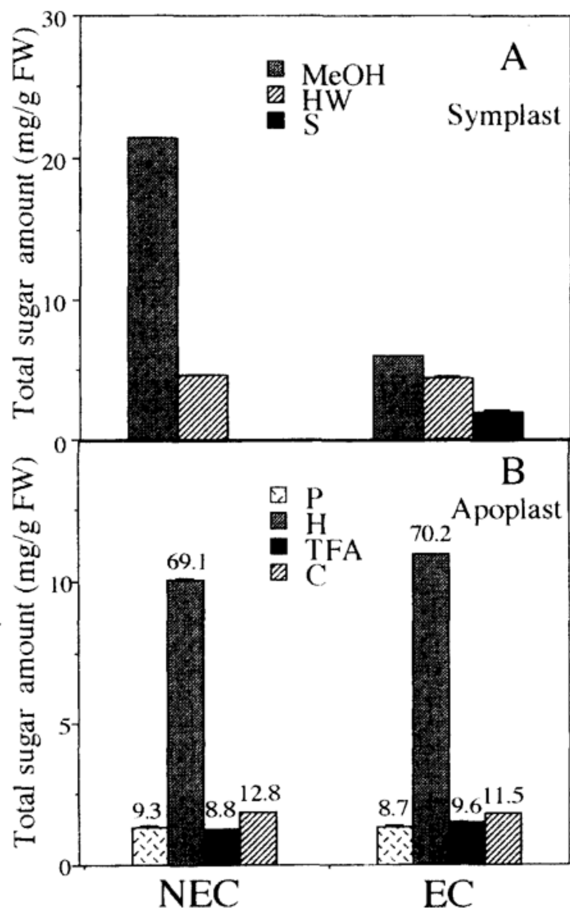

Figure 2. A comparison of sugar contents of the symplastic and apoplastic fractions between non-embryogenic (NEC) and embryogenic cells (EC) of celery. Numbers on histograms in B represent the percentage of cell wall sugars. MeOH, methanol fraction; HW, hot water fraction; S, starch fraction; P, pectin fraction; H, hemicellulose fraction; TFA, TFA-soluble fraction; C, cellulose fraction. Vertical bars indicate S.E. of triplicates.

Comparison of Total Sugar Content of Symplastic and Apoplastic Fractions between NEC and EC

To compare sugar contents between NEC and EC, the cells were fractionated into symplastic (MeOH, HW, and S) and apoplastic (pectin, hemicellulose, TFA-soluble, and cellulose) fractions.

Figure 2A shows the total sugar content of the symplastic fractions. In the MeOH fraction, the sugar content of NEC (21.4±0.1 mg / g FW) was higher (ca. 3-folds) than that of EC (6.0±0.1 mg). The low level of sugar in EC probably reflects the utilization of the sugar pool in the cytoplasm and vacuole, and/or culture medium. However, in the S fraction, the starch content of NEC (0.1±0.0 mg) was much lower than that of EC (2.1±0.1 mg). EC is characterized by having conspicuous starch grains in the cytoplasm (Williams and Maheswaran, 1986). In the HW fraction, there was no significant difference in the total sugar content in NEC and EC (4.7±0.1 and 4.4±0.1 mg, respectively).

The total sugar content of apoplastic (cell-wall) fractions was similar in both NEC and EC (Fig. 2B). The hemicellulose content (hemicellulose plus TFA-soluble fraction) was 6~10 times higher in both NEC and EC. The contents of cellulose in the cell wall were 12.8% in NEC and 11.5% in EC. It is suggested that there may be some difference in the cellulose microfibril orientation between NEC and EC to cause the substantially different cell shapes.

Changes in Total Sugar and Cellulose Contents

Figure 3 shows changes in the total sugar amounts of symplastic (MeOH, HW, and starch) fractions during somatic embryogenesis and development. The descending order of the sugar amounts was MeOH, HW, and starch fraction in all stages. The sugar content in the MeOH and HW fractions increased till the heart stage, and then decreased during the torpedo and cotyledonary stages. The sugar content of the symplastic fractions was lowest in the hypocotyl segments. The amount of starch did not change much during somatic embryogenesis and development.

Figure 4A shows changes in the total sugar amounts of the apoplastic fractions during somatic embryogenesis and development. The hemicellulose content of EC (10.1±0.1 mg / g FW) was much higher than that of the hypocotyl segments (3.3±0.1 mg). The hemicellulose content decreased to the level of the hypocotyl segments in the cotyledonary stage. It is suggested that the transient increase in hemicellulose

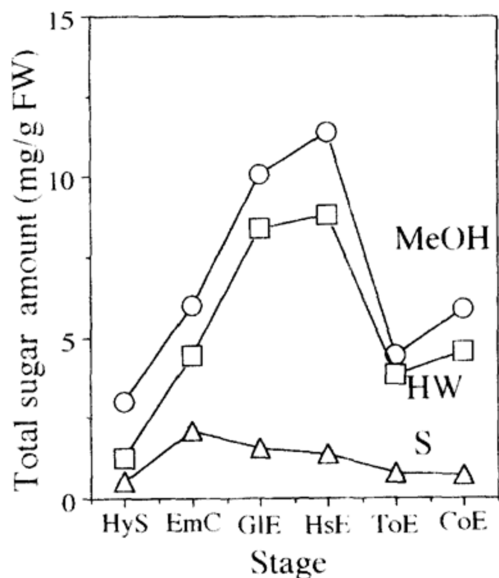

Figure 3. Changes in sugar contents of the symplastic fractions during somatic embryogenesis and development of celery. MeOH, methanol fraction; HW, hot water fraction; S, starch fraction; HyS, hypocotyl segments; EmC, embryogenic cells; GlE, globular embryos; HsE, heart-shaped embryos; ToE, torpedo embryos; CoE, cotyledonary embryos; All vertical bars indicating S.E. of triplicates are smaller than the symbols and so are not shown.

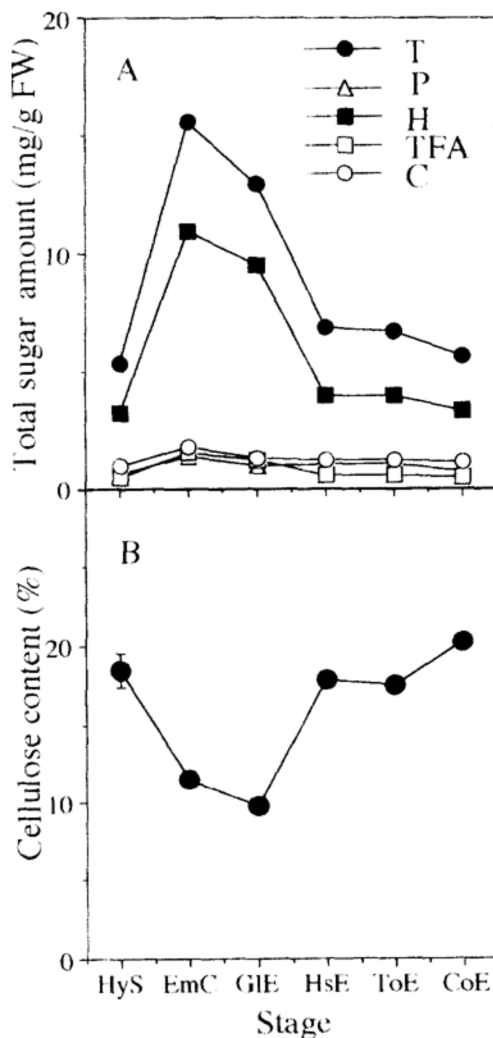

Figure 4. Changes in sugar contents and cellulose contents of the apoplastic (cell wall) fractions during somatic embryogenesis and development of celery. P, pectin fraction; H, hemicellulose fraction; TFA, TFA-soluble fraction; C, cellulose fraction; HyS, hypocotyl segments; EmC, embryogenic cells; GlE, globular embryos; HsE, heart-shaped embryos; ToE, torpedo embryos; CoE, cotyledonary embryos; Most of the vertical bars indicating S.E. of triplicates are within the symbols and so are not shown.

during embryogenesis and at the early stages of embryo development corresponds to an active turnover of hemicelluloses in the primary wall of dividing cells. The low amounts (below 2.5 mg) of other fractions (pectin, TFA-soluble, and cellulose) did not change during somatic embryogenesis and development. The richness in hemicellulose in the cell walls of EC and globular embryos suggests that the undifferentiated cells contain higher amount of hemicellulose. These results suggest that the cells actively oriented to cell division had more non-cellulosic polysaccharide, namely hemicellulose than differentiating cells did in celery.

The cellulose content of the total cell wall of EC (11.5%) was lower than that of the hypocotyl segments (18.4%) (Fig. 4B). The cellulose content of globular embyros was the lowest. The content increased again to 20% at the heart stage, then did not change as the development proceeded. The cellulose contents in the in vitro cultured cells varied among plant species: 62% in *Rosa glauca* (Chambat et al., 1981), 46% in carrot (Masuda et al., 1984), 26% in *Vinca rosea* (Takeuchi and Komamine, 1978), and 23% in sycamore cells (Talmadge et al., 1973). The cellulose content was similar to those in calli (19.6%) and suspension-cultured cells (17.9%) of barley (Yeo et al., 1995; Yeo et al., 1998c), and also similar to those in elm gall tissues (16-20%) (Yeo et al., 1997) and in sumac gall tissues (16-20%) (Yeo et al., 1998a) at early developmental stages. The plant cell walls that develop during the proliferation and elongation of cells are primary cell walls while those that are synthesized after the cessation of cell elongation are secondary cell walls. It has been reported that secondary cell walls are often rich in cellulose

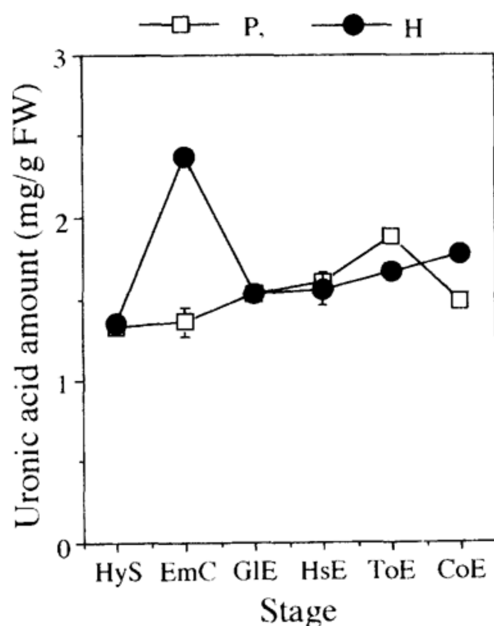

Figure 5. Changes in uronic acid (UA) contents of the pectin (P) and hemicellulose (H) fractions during somatic embryogenesis and development of celery. HyS, hypocotyl segments; EmC, embryogenic cells; GlE, globular embryos; HsE, heart-shaped embryos; ToE, torpedo embryos; CoE, cotyledonary embryos. Vertical bars indicate S.E. of triplicates.

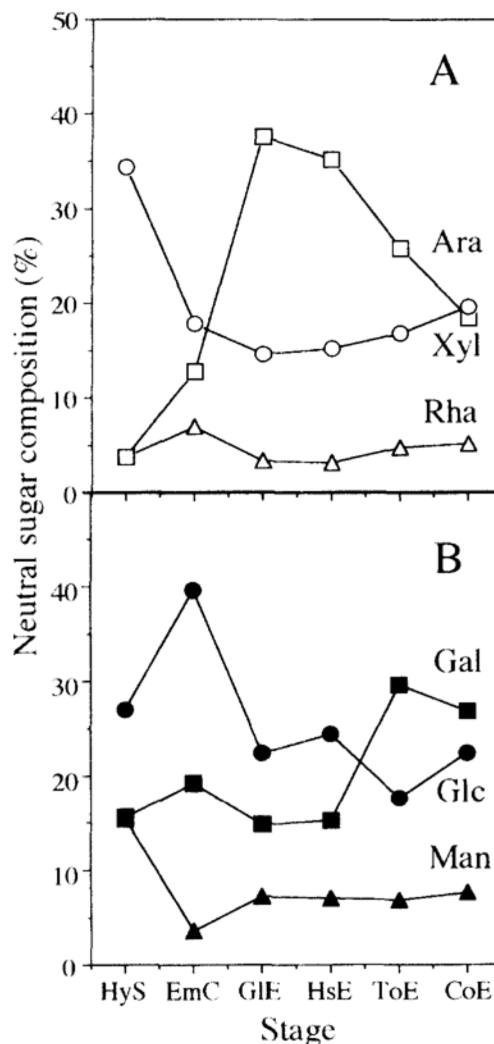

Figure 6. Changes in neutral sugar components of the hemicellulose fraction during somatic embryogenesis and development of celery. Ara, arabinose; Xyl, xylose; Rha, rhamnose; Gal, galactose; Glc, glucose; Man, mannose, HyS, hypocotyl segments; EC, embryogenic cells; GlE, globular embryos; HsE, heart-shaped embryos; ToE, torpedo embryos; CoE, cotyledonary embryos. Data represent the means of three determinations of one experiment.

(Heigler, 1985). Therefore, the high cellulose content in the cell wall of cultured cells may be associated with the loss of differentiating potential. A further comparative study of cellulose synthesis in actively growing celery plantlets, from the juvenile to the mature developmental stages, may help us to understand the regulation of cellulose synthesis and the signal for the switch from primary to secondary wall formation.

Changes in Uronic Acid (UA) Content of Pectin and Hemicellulose Fractions

The UA content in the pectin fraction did not change remarkably during somatic embryogenesis and development (Fig. 5). By contrast, the UA amount in the hemicellulose fraction markedly increased in EC, then decreased and was maintained at a consistent level as its development proceeded, similar to that of the hypocotyl segments. These results indicate that EC had more acidic side chains in its hemicellulosic polysaccharides than developing embryos or differentiating hypocotyl segments.

Changes in Neutral Sugar Components of Hemicellulose Fraction

The specific monosaccharide component of hemicellulose was analyzed by GLC. Figure 6A and 6B show changes in the neutral sugar composition (% of total neutral sugar amount) in the hemicellulose fractions during somatic embryogenesis and development. The hemicelluloses consisted of rhamnose, arabinose, xylose, mannose, galactose, and glucose.

Fucose level could not be detected using the GLC technique. Rhamnose was maintained at a low level, ranging from 3.2% to 7.0% during somatic embryogenesis and development. Arabinose content of EC (12.9%) was 4 times higher than that of the hypocotyl segments (3.8%). Arabinose level increased up to 37.7% at the globular embryo stage. Xylose content decreased from the hypocotyl segments to EC, and was maintained at a constant level during somatic embryogenesis and development (Fig. 6A). Mannose also decreased from the hypocotyl segment to EC, and was maintained to a constant level ranging from 6.7% to 7.5% after the globular embryo stage. Galactose was maintained at a constant level till the heart-shaped stage, then increased during the torpedo stage (29.5%) and cotyledonary stage (26.9%). Glucose content (39.5%) of EC was the highest of hemicelluloses. It decreased to a slightly lower level (22.3%) than that of hypocotyl segment during somatic embryogenesis and was maintained at a constant level as cell development proceeded (Fig. 6B).

Pectins are made up of a group of polysaccharides (homogalacturonan, rhamnogalacturonan I and II), rich in galacturonic acid, rhamnose, and galactose (O'Neil et al., 1990). Hemicelluloses include various species of polysaccharides, such as xylan, mamnan, xyloglucan, galactomannan, arabinogalactan, glucuromanan, callose, and 1,3; 1,4-β-glucan. In contrast to the pectins, the hemicelluloses differ greatly according to cell types and species. In most cell types, one hemicellulose predominates, with others are present in smaller amounts (Brett and Waldron, 1990). In the hemicellulose fraction, arabinose content was remarkably increased during somatic embryogenesis as well as in asparagus (Yeo et al., 1998c), but galactose content increased also in the late stage of embrygenesis. Differential increase in arabinogalactan components, abrabinose and galactose, implies the complex role of arabinogalactan molecules in embryogenesis. Kikuchi et al. (1996) reported that the embryogenic callus of carrot contained more arabinose than non-embryogenic callus did and they determined the increase in arabinose branching chains of arabinogalactan by methylation analysis. They proposed that the increased side chains of arabinose in arabinogalactan participitate in cell adhesion. In celery embryogenesis, we detected a remarkable increase in arabinose, followed by an increase in galactose, suggesting the initial elongation of arabinose side chains on the galactan backbone and increase in arabinogalactan content. These increases of arabinose and galactose did not correspond to the change in rhamnose levels, which is a key component of acid polymer of pectin, rhamnogalacturonan. Therefore, if arabinogalactan is attached to a rhamnose residue of rhamnogalacturonan as proposed by McNeil et al. (1982), only the arabinogalactan side chain elongated during embryogenesis without an increase in the amount of main backbone. This is also supported by the fact that UA content in the pectin fraction was maintained at a constant level throughout embryogenesis and development (Fig. 5). The precise role of arabinogalactan polymer in embryogenesis needs more structural analysis such as methylation.

The difference in cell wall contents between carrot and celery is that the changes in arabinogalactan was observed in pectin for carrot and hemicellulose for celery. Cell walls of carrot EC were rich in pectin (63%) (Kikuchi et al., 1995), while those of celery EC contained only a small amount of pectin (ca. 10%), even in the intact hypocotyls. The small amount of pectin in the cell walls is often regarded as a characteristic of *Poaceae* plants (Sakurai, 1991). In this sense, celery cell walls are *Poaceae*-type, even though celery belongs to the same dicot, *Apiaceae*, to which carrot does. Alternatively, celery cell walls may contain pectic polysaccharides that are resistant to EDTA-extraction.

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