Plant & Cell Physiol. 20(1): 63–74 (1979)

Growth and cell wall changes in azuki bean epicotyls I. Changes in wall polysaccharides during intact growth

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(Received July 27, 1978)

Measurement of endogenous growth rates and the mechanical property of the cell wall in various regions of light-grown azuki bean epicotyls revealed that the minimum stress-relaxation time (T_0) was the shortest in the upper region (0–30 mm below the apex) of the epicotyl, where vigorous endogenous growth took place, and became longer toward the basal region, which was mature and not growing.

In the upper region of the epicotyl, a lower percentage of α-cellulose and a higher percentage of pectic substances than in the lower region were found. The percentage of hemicellulose content was almost constant over the whole epicotyl. Major components of noncellulosic neutral sugars in the cell wall were galactose and xylose. The percentage of the galactose content to the noncellulosic polysaccharide was highest in the upper region and lowest in the basal region of the epicotyl, and a clearly negative correlation between the galactose composition and the T_0 value was obtained. On the contrary, the percentage of the xylose content was highest in the basal region and lowest in the upper region, and a clearly positive correlation between the xylose composition and the T_0 value was obtained. During the endogenous growth of the intact epicotyl, all the neutral sugars, particularly galactose, increased in the upper region, whereas in the middle and basal regions, only xylose increased. Similar changes in sugar compositions were observed during IAA-induced elongation of the segment excised from various regions of the epicotyl.

Key words: Azuki bean epicotyl — Cell wall polysaccharides — Cell wall loosening — IAA.

Rheological studies of the plant cell wall have revealed that auxin causes cell wall loosening when it induces cell elongation (*3*, *15*). Cell wall loosening, or the capacity of the cell wall to extend, has been demonstrated to be represented by the decrease in the minimum stress-relaxation time (T_0 value), which is obtained by the stress-relaxation analysis developed by Yamamoto et al. (*24–26*). As to biochemical modificaions underlying auxin-induced cell wall loosening, much attention has been paid to the metabolic turnover of noncellulosic polysaccharides of the cell wall (*11*, *12*, *16*, *23*). In monocots, such as *Avena* coleoptiles, degradation of noncellulosic β-glucans has been correlated with the auxin-induced cell wall loosening (*12*, *15*, *18*). In dicots, on the other hand, Nevins et al. have reported changes in noncellulosic polysaccharides, particularly those composed of galactose and xylose, in the epicotyl of growing bean plants (*17*). A close correlation between the T_0 value and the

Abbreviation: IAA, indole-3-acetic acid.

amount of hemicellulosic polysaccharides has been demonstrated in lettuce hypocotyls (*9*). Furthermore, Tanimoto and Igari (*22*) have reported a good correlation between the endogenous growth rate and the β-galactosidase activity in various parts of pea epicotyls, suggesting the participation of turnover of some galactans in cell wall extension.

This paper describes a relationship between the mechanical property of the cell wall represented by the T_0 value, the growth capacity, and neutral sugar compositions of the cell wall in azuki bean epicotyl, a dicotyledonous plant part. It also describes changes in the sugar compositions which took place in both the growing intact epicotyl and excised epicotyl segments growing in the presence of auxin.

Materials and methods

Plant material: Azuki (*Vigna angularis*) seeds were soaked in running tap water for 1 day at 30°C, then sown in moist vermiculite in plastic trays. After the seedlings had been allowed to grow in continuous light (ca. 2000–2500 lux) for 6 days at 25°C, they were selected for uniformity of epicotyl length (8–10 cm) (*19*).

Growth experiments: To measure endogenous growth of the epicotyl, the upper 70-mm region below the first leaves was divided into 5-mm subregions by ink marks. After the seedlings had been allowed to grow under the same conditions for 24 hr, the length of each subregion was measured. The segments excised from each region after 24-hr growth were immediately killed in boiling methanol then treated with a pronase solution of 200 ppm pronase (Kaken Kagaku Co.), 50 mM potassium phosphate buffer (pH 7.0) and 5% ethanol for 18 hr at 37°C. These pronase-treated segments were stored at −20°C until use.

For the growth experiments with excised segments, the upper 70-mm region of the epicotyl was cut into seven 10-mm subregions. From each subregion, a 7-mm segment was excised then washed in water for 2 hr. Twenty 7-mm segments were incubated for 24 hr at 25°C in light in a petri dish containing 4 ml of test solution composed of 10 mM potassium phosphate buffer solution (pH 6.0) with 50 mM sucrose with or without 0.1 mM IAA. After the incubation, the length of the segments was measured microscopically. Next, the segments were immediately killed in methanol, treated with pronase, and stored as described above.

Measurements of mechanical properties of the cell wall: The upper 70-mm region of the epicotyl was cut into seven 10-mm segments. The epidermis of the segment was peeled off with forceps into three strips. The epidermis was immediately killed in boiling methanol, treated with pronase and stored in methanol until use. Mechanical properties of the cell wall of the epidermis were measured by stress-relaxation analysis (*24*, *25*). A rehydrated strip of epidermis was fixed between the upper and lower clamps (distance: 5 mm) of an Instron tensile tester (Model TM-M), and then stretched by lowering the lower clamp (lowering rate: 20 mm/min). After the segment had received an initial stress of 5 g, the lower clamp was stopped and the relaxation process of the stress was automatically recorded for 5 sec by a HITAC-10-II minicomputer then the T_0 value was calculated according to the method of Yamamoto et al. (*26*).

Cell wall fractionation: One-cm segments excised from various regions of the epicotyl were killed in boiling methanol. After rehydration, they were homo-

genized with a mortar and pestle, washed three times each with water, acetone, and a methanol-chloroform mixture (1 : 1, v/v) then air-dried. The dried material was treated for 18 hr with pronase solution (200 ppm). The pronase-treated material was then treated for 3 hr at 37°C with a pancreatic α-amylase (Sigma Chemical Co.) solution consisting of 10 units of the enzyme and 3 mM $CaCl_2$ in 100 mM sodium acetate buffer (pH 6.5). The pancreatic α-amylase has been shown not to contain any other glycanases unlike *B. subtilis* α-amylase, which is contaminated by a β-glucanase (*8*).

The cell wall material thus obtained was extracted twice for 2 hr each with boiling water (hot water fraction) and for 2 hr with 0.5% ammonium oxalate solution at 95°C (oxalate fraction). The residue was then extracted twice for 24 hr with 10% KOH solution, followed by 24 hr extraction with 24% KOH solution containing 4% boric acid. These alkali extracts (hemicellulose fraction) were neutralized with acetic acid (*6*). The residue after the alkali extractions was washed with water, 1 mM acetic acid and ethanol, then air-dried at room temperature (α-cellulose fraction).

The total sugar content of each fraction was determined by the phenol-sulfuric acid method (*4*), and expressed as the glucose equivalent. Uronic acid contents in the hot water and oxalate fractions were determined by a modified carbazole-sulfuric acid method (*7*), and expressed as the galacturonic acid equivalent.

Analysis of noncellulosic sugar compositions of the cell wall: Pronase-treated segments were crushed between two glass plates then washed three times with water, twice with acetone and twice with a methanol-chloroform mixture (1 : 1, v/v). The dried materials were treated with pancreatic α-amylase. The noncellulosic neutral sugars in the cell wall materials were hydrolyzed for 1 hr at 121°C with 2 N trifluoroacetic acid, then the sugars liberated were reduced with an excess amount of sodium borohydride, followed by acetylation in the presence of acetic anhydride at 121°C for 3 hr. The amounts of resulting acetylated alditols were determined by gas chromatography (Hitachi Model 163) (*12, 18*). The GLC separation was performed with a liquid phase containing 0.2% of polyethyleneglycol succinate, 0.2% polyethyleneglycol adipate and 0.4% of silicone XF-1150 coated on Gas-Chrom P (100–200 mesh) (*1*).

Insoluble material which had not been hydrolyzed by trifluoroacetic acid was rinsed twice with water, twice with acetone, then air-dried at room temperature. To the dried material was added 0.2 ml of 72% sulfuric acid, and the sample was allowed to stand for 1 hr at 25°C with occasional stirring. Following dilution of the sample with 5.8 ml of water, the sugar content of the solution was determined colorimetrically by the phenol-sulfuric acid method (*4*) and defined as the α-cellulose content. α-Cellulose fraction after KOH extractions (see above) was also subjected to the phenol-sulfuric acid method.

Results

Endogenous growth rate and the mechanical property of the cell wall

Fig. 1A shows that endogenous growth occurred primarily in the upper region of the epicotyl ranging between 0–30 mm below the apex, with the apical end showing the most elongation growth.

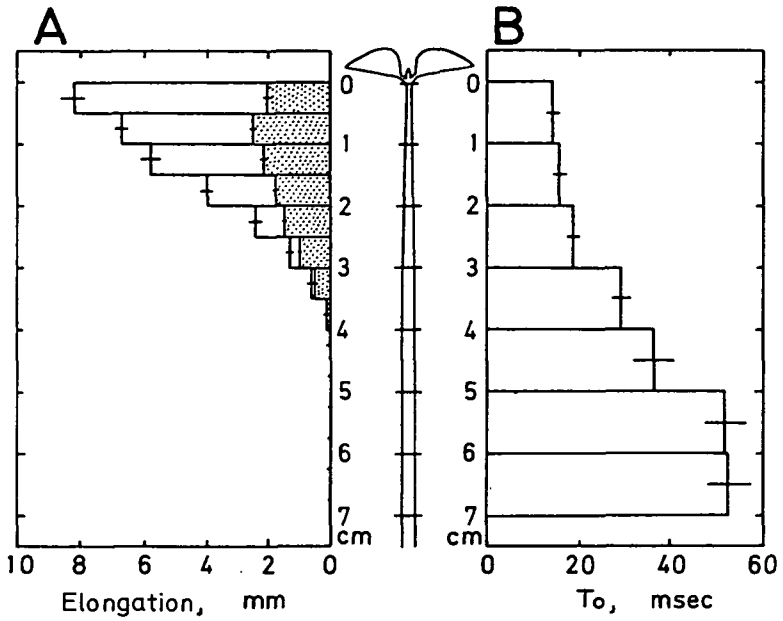


Fig. 1. *Distribution of endogenous growth rate and mechanical properties of the cell wall along the intact azuki bean epicotyl.* A. Endogenous growth in various regions of the epicotyl. The upper 70-mm region of an azuki epicotyl was divided into 5-mm subregions by ink marks. After the seedling had been allowed to grow for 8 hr (shaded pars) and 24 hr (light bars) in light (ca. 2000–2500 lux) at 25°C, the length of each subregion was measured. The means of 20 epicotyls are shown with standard errors as horizontal lines. B. Distribution of the T_0 value in the epicotyl cell wall. The upper 70-mm region of the epicotyl was cut into seven equal 10-mm segments. The epidermis of each segment was peeled off with forceps, then subjected to stress-relaxation analysis (see **Materials and methods**). The means of 20 strips of epidermis are given with standard errors.

Since the mechanical property of the epidermal cell wall has been shown to represent the capacity of the whole stem to elongate (*13*, *21*), the T_0 values of the epidermal cell wall in various regions of the epicotyl were measured. As shown in Fig. 1B, the shortest T_0 was obtained in the segments excised from the upper region of the epicotyl, where active endogenous growth took place, whereas larger T_0 values were obtained in the basal region. Comparison between T_0 values and endogenous growth rates in various regions of the epicotyl indicated that the endogenous growth occurred only in the stem region where the T_0 was shorter than about 20 msec. This agrees with the previous indication of the threshold value of T_0, which had been obtained with the epidermal cell wall of mung bean hypocotyls (*27*).

Cell wall compositions in the epicotyl

Polysaccharide compositions of cell walls in various regions of the epicotyl are shown in Fig. 2A and B. About 55% of the cell wall was composed of noncellulosic polysaccharides in the upper region of the epicotyl and about 45% in the basal region (Fig. 2B). Among the noncellulosic polysaccharides, hemicellulose was predominant throughout the epicotyl and its percentage composition was almost constant over the whole epicotyl (Fig. 2B). The cell wall in the upper region of the epicotyl

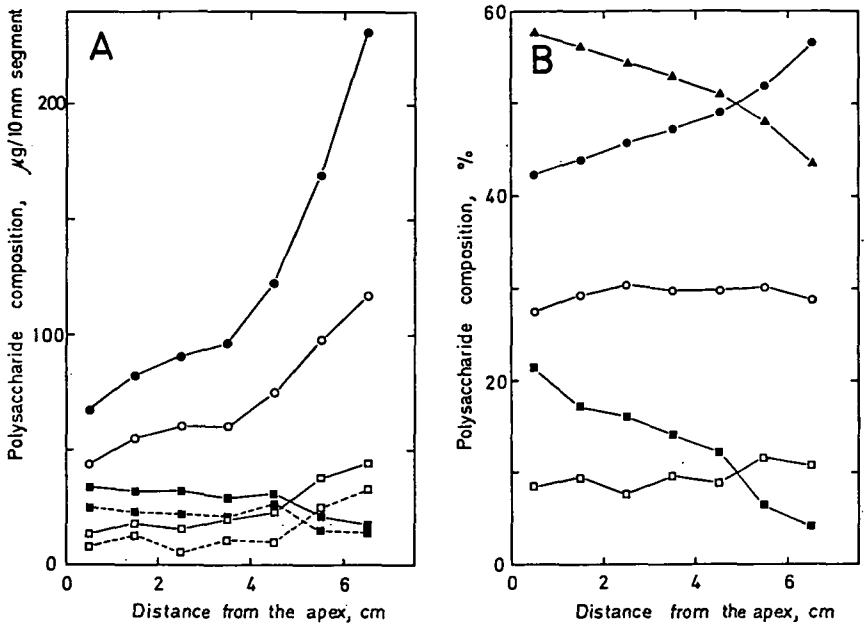


Fig. 2. *Changes in the cell wall composition in azuki bean epicotyl.* A. Cell wall composition. The upper 70-mm region of the epicotyl was cut into seven 10-mm segments, which were separated into four fractions: hot water (□), oxalate (■), hemicellulose (○), and α-cellulose (●). Total sugar contents (solid lines) and uronic acid contents (broken lines) in each fraction were determined. B. Percentage composition of each cell wall fraction. The percentage amount for each 10-mm segment of each fraction was calculated, and the same symbols as in Fig. 2A except for the total noncellulosic polysaccharides (▲) are used.

contained a higher percentage of the oxalate fraction. A large part of the hot water fraction, as well as the oxalate fraction, was composed of uronic acid. The amount of uronic acid to the total cell wall was 18% in the upper region and 11% in the lower region of the epicotyl. Further studies on the significance of uronic acids in the cell wall extension may be necessary. The neutral sugar composition of the total noncellulosic polysaccharides was investigated in the present study. Neutral sugar composition of these cell wall fractions was examined by GLC. While the hot water and oxalate fractions were composed of galactose, arabinose and rhamnose, the hemicellulose fraction contained xylose, glucose, galactose, mannose and fucose.

Fig. 3A shows the noncellulosic neutral sugar contents in the cell wall in each 5-mm region of the stem. The galactose content was almost constant over the various regions of the epicotyl, though relatively higher contents were observed in the upper-middle region about 20 mm below the apex. On the other hand, the xylose content was constant from the apex to 40 mm and remarkably increased toward the basal region.

Fig. 3B shows the percentage compositions of these neutral sugars in the total noncellulosic neutral sugars of the cell wall. The predominant sugar was galactose in the upper region, but xylose in the basal region. A relatively high

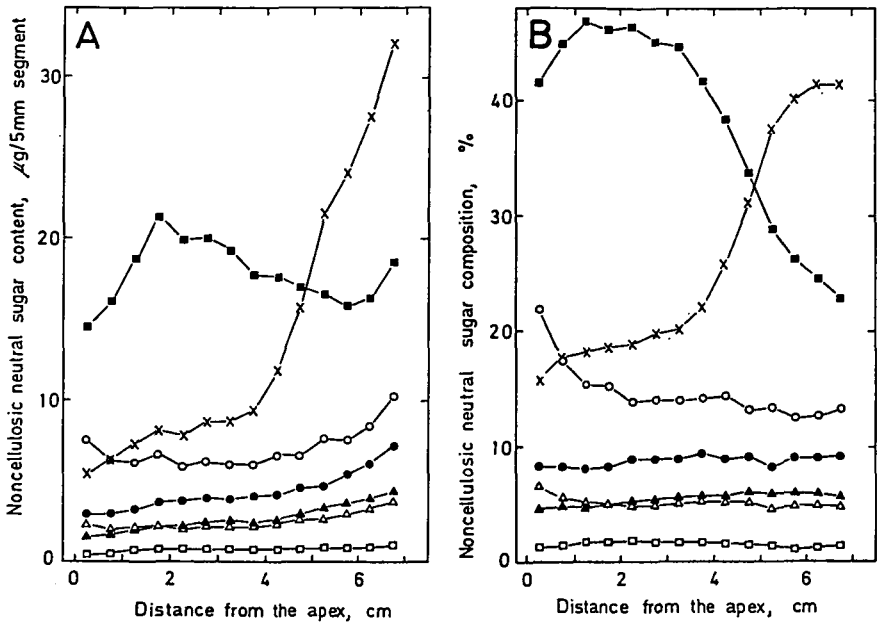


Fig. 3. *Noncellulosic sugar contents in azuki epicotyl cell wall.* A. Noncellulosic neutral sugar contents in various regions of the epicotyl. The upper 70-mm region was cut into fourteen 5-mm segments. The contents of noncellulosic neutral sugars in each stem segment were determined by GLC (see **Materials and methods**). The various neutral sugars are designated as follows: galactose (■), glucose (●), mannose (▲), xylose (×), arabinose (○), rhamnose (△), fucose (□). B. Percentage composition of noncellulosic neutral sugars in various regions of the epicotyl. The percentage composition of each component sugar in the total noncellulosic neutral sugars was calculated and shown. The symbols are the same as in Fig. 3A.

percentage of arabinose was observed in the apical end. Percentage compositions of other sugars, such as glucose, mannose, rhamnose and fucose were almost constant over the whole epicotyl.

Changes in noncellulosic neutral sugar composition during endogenous growth of the epicotyl

Fig. 4A shows changes in net contents of each sugar in each region of the epicotyl during endogenous growth for 24 hr. The xylose content increased substantially in all regions of the epicotyl. The increase was particularly conspicuous in the middle region. A remarkable increase in galactose was observed in the upper region which corresponds to the elongating zone, and a substantial decrease was observed in both the middle and basal regions. Much smaller changes were observed in the other neutral sugars. Increase in cellulose content was observed in the whole epicotyl, the largest being in the apical region.

Fig. 4B shows that in the upper region of the epicotyl, every sugar content increased up to 2–4 times the initial content of each 5-mm region. In the middle region, conspicuous increases in xylose (3.5-fold) and cellulose (2-fold) were observed. In the basal region, where the stem no longer elongated, very little increase was observed except for xylose and cellulose.

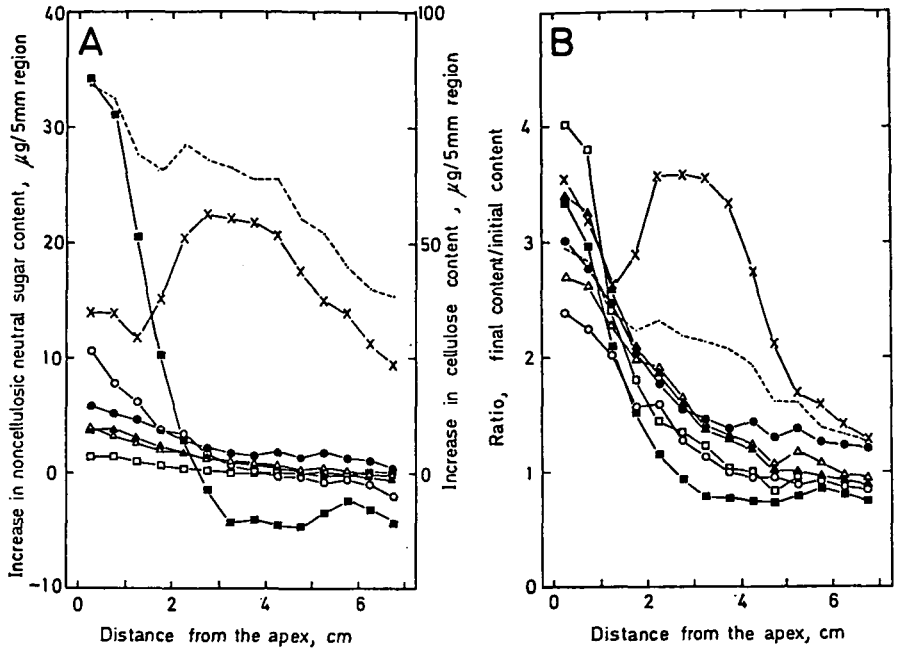


Fig. 4. *Changes in noncellulosic neutral sugars during endogenous growth in azuki bean epicotyl.* A. Changes in the net amount of neutral sugars in various regions of the epicotyl during endogenous growth. The upper 70-mm region of the epicotyl was divided into 5-mm subregions by ink marks, then allowed to grow in light at 25°C. After 24 hr, each marked subregion was excised and its cell wall composition was analyzed (see **Materials and methods**). Changes in the net amount of each neutral sugar (final amount−initial amount, μg) in each marked subregion are shown. The same symbols as in Fig. 3, except for cellulose (----), are used. B. Changes in the neutral sugar composition of polysaccharides during endogenous growth. The ratio (final amount/initial amount) of each neutral sugar in various regions of the epicotyl was calculated and shown. The same symbols as in Fig. 4A are used.

Changes in noncellulosic neutral sugar composition during auxin-induced elongation of excised epicotyl segments

In order to study the effect of exogenously applied auxin on the neutral sugar composition, 7-mm segments were excised from various regions of the epicotyl. Following the starvation of endogenous hormones and the sugar pool for 2 hr, the segment was incubated in potassium phosphate buffer solution (10 mM, pH 6.0) containing 50 mM sucrose in the presence or absence of 0.1 mM IAA. As shown in Fig. 5A, IAA-induced elongation was observed in the segments excised from the upper region of the epicotyl, above 40 mm from the apex. While maximum endogenous growth was observed in the apical end (see Fig. 1A), segments excised from 10 to 30 mm below the apex showed the largest elongation when IAA was exogenously supplied.

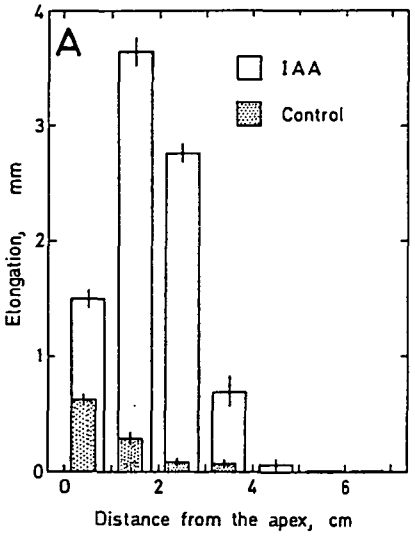
Changes in contents of the neutral sugars in segments treated with or without IAA are shown in Fig. 5B and C. IAA increased the galactose and cellulose contents particularly in the segment excised from the subapical region (10–30 mm below

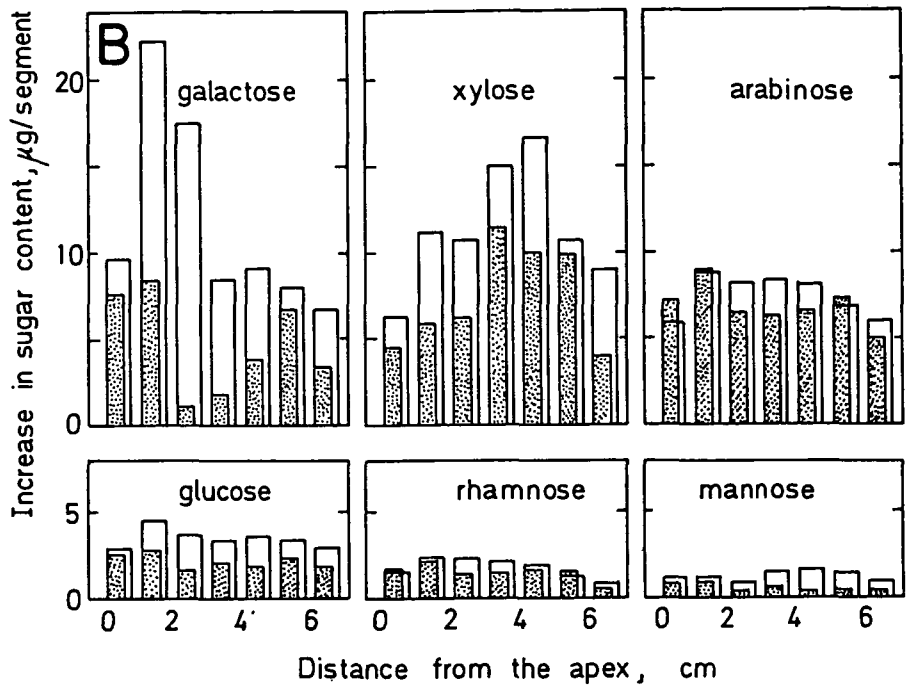
the apex) where the largest elongation of the segment was induced by IAA. Note that the pattern of auxin-induced increase in galactose content coincides well with that of auxin-induced elongation of epicotyl segments (cf. Fig. 5A and B). On the other hand, a relatively large increase in xylose and cellulose contents was caused by IAA in segments from the middle region, as well as the apical region of the epicotyl, where IAA slightly induced elongation. IAA caused a small increase in the contents of other sugars such as glucose, rhamnose, mannose and fucose.

Discussion

The results obtained in the present study demonstrate that in the young region of the upper epicotyl, all the cell wall polysaccharides, particularly those rich in galactose, increased and that in the basal region, only xylose increased. Therefore, the metabolic sequence of cell wall synthesis probably shifts from young to mature regions during the aging of the organ. As a result, the cell wall of the young elongating region is rich in galactose and that of the mature one in xylose. This is consistent with the observation of an increase in xylose and a decrease in galactose contents in whole epicotyls of growing *Phaseolus aureus* (*5, 17*) and *P. vulgaris* (*17*).

The T_0 value represents the capacity of the cell wall to extend (*24–26*). A close correlation between the T_0 value and the amount of hemicellulosic polysaccharides in the cell wall has been demonstrated, suggesting the participation of hemicellulosic polysaccharide metabolism in the process of cell wall loosening (*9*). In this study, we tried to find a possible correlation between the T_0 values and the sugar composition. A negative correlation between the T_0 values and galactose percentage in the cell wall polysaccharides, as shown in Fig. 6, implies an important role of polysaccharides composed of galactose in the growth capacity in azuki bean epicotyls. Contrary to this, a positive correlation between the T_0 values and xylose percentages (Fig. 6) suggests that polysaccharides composed of xylose play some





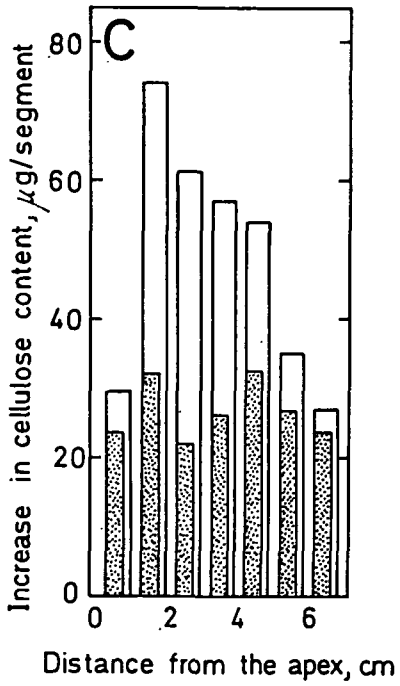


Fig. 5. *Effect of IAA on elongation and cell wall compositions of segments excised from azuki bean epicotyls.* A. IAA-induced elongation in segments excised from various regions of the epicotyl. Seven-mm segments excised from various regions of the epicotyl were incubated for 24 hr in a medium containing 10 mM K-phosphate buffer (pH 6.0) and 50 mM sucrose in the presence (light bars) or absence (shaded bars) of 0.1 mM IAA in light at 25°C. The means of 20 segments are shown with standard errors. B. Effect of IAA on changes in noncellulosic sugar content of epicotyl segments. After incubation (see Fig. 5A), the segments were killed in boiling methanol, then subjected to analysis of their cell wall compositions (see **Materials and methods**). C. Effect of IAA on changes in cellulose content of epicotyl segments. Cellulose content was determined in the segments which had been subjected to analysis of their noncellulosic neutral sugars (see Fig. 5B).

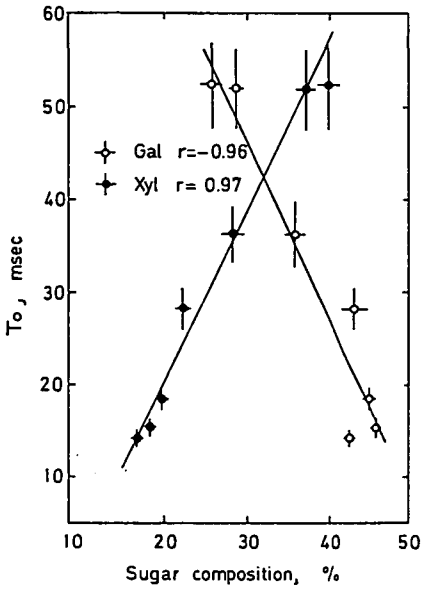


Fig. 6. *Relationship between the T_0 value and percentage compositions of galactose and xylose in azuki bean epicotyl.* T_0 values of the epidermal cell wall in various regions of the epicotyl were plotted against the corresponding sugar composition. Data from Fig. 1A and 3 were used.

part in stiffening the cell wall. The above idea is supported by the finding that considerable increase in the galactose content is coupled with elongation and the xylose content increases after completion of the elongation (Fig. 4 and 5).

According to the structural model of the sycamore cell wall (*2*, *10*, *20*), 1,4-linked galactan chains are covalently attached to both rhamnogalacturonans and xyloglucans which are in turn attached to cellulose microfibrils by hydrogen bonding. In order for a cell wall to extend, cellulose microfibrils must undergo rearrangement. Chemical modifications in the galactan thus would lead to a rearrangement of the cellulose microfibrils (*10*). The turnover of galactose residues in cell wall polysaccharides is considered to occur as dicot plants grow (*5*, *11*, *17*). A close correlation between β-galactosidase activity and endogenous growth, which was demonstrated in pea epicotyls (*21*), also suggests the occurrence of exowise degradation of galactans during growth.

In *Avena* coleoptile segments, Loescher and Nevins (*12*) and Sakurai and Masuda (*18*) demonstrated the specific degradation of noncellulosic cell wall glucan, and a close correlation between the T_0 value and the decrease in the glucan content has been found. In azuki bean epicotyl, however, neither the decrease in the glucose content nor the relationship between the glucose content and the T_0 value was observed. It is thus assumed that chemical modifications underlying cell wall loosening in azuki bean epicotyls differ from those in *Avena* coleoptiles.

In excised segments, exogenously applied IAA caused both elongation growth and an increase in the galactose content and also an increase in xylose content in the cell wall in the presence of sucrose, as observed in growing intact epicotyl. Thus, the cell wall metabolism as well as stem elongation is probably regulated, at least partly, by auxin in the intact seedling. However, interpretation of the vigorous growth and changes in cell wall compositions in the apical end as being regulated solely by IAA was difficult, as the effect of IAA was less in the apical end than in the

subapical region about 20 mm below the apex. The endogenous growth of the intact epicotyl is believed to be controlled, in addition to auxin, by some other plant hormones including gibberellins and cytokinins. In segments of azuki bean epicotyls, a synergistic effect of gibberellins on the IAA-induced elongation has been reported (*19*). Therefore, elongation growth and changes in cell wall compositions during growth, particularly those in the apical region, seem to be regulated by gibberellins as well as auxin (*14*). Further investigations of the gibberellin- and IAA-induced changes in the cell wall compositions and effects of substrate concentrations on the compositions will be reported later.

We thank Dr. Hiroh Shibaoka of Tokyo Metropolitan University and Dr. Seiichiro Kamisaka and Dr. Naoki Sakurai of our laboratory for their invaluable discussions and criticisms during this study. This work was partly supported by a grant (148013) from the Ministry of Education.

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