Control of Auxin-induced Stem Elongation by the Epidermis

By

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Abstract

Segments of the 4th and 5th internodes of light-grown pea seedlings were used for the study of control of stem elongation. With 5th internodes, at low turgor as well as at water saturation auxin primarily appeared to cause a change in cell wall properties of the epidermis but it showed little effect on expansion of the inner tissue. This was confirmed by comparison of expansion between peeled and unpeeled segments, split tests and by measurements of stress-relaxation properties of the epidermal cell wall. Segments with the central part removed elongated well in response to auxin, but the isolated epidermis showed neither auxin-induced elongation nor cell wall loosening. A fungal β-1,3-glucanase appeared, at least partly, to have a similar effect as that of auxin on elongation, by changing cell wall properties of the epidermal cell wall. Peeled segments of 4th internodes expanded very little and auxin had little effect on their epidermal cell wall properties.

Introduction

The mechanism of cell expansion in tissue segments in the majority of studies has been interpreted in analogy with that of a single cell, where the suction force is controlled by osmotic pressure of cell sap and turgor pressure (cell wall pressure). The significant role of turgor pressure in cell growth has been discussed (4) but the idea of the involvement of turgor pressure as driving force in water uptake has been questioned (2). The cell wall properties of *Nitella* have been studied using creep (8, 12) and stress-relaxation (6, 7).

When, on the other hand, coleoptile or stem tissues grow, the situation is not as simple as in the former case, owing to their structure being composed of different types of cells. van Overbeek and Went demonstrated in 1937 (16) and Thimann and Schneider in 1938 (15), that auxin primarily induced the elongation of epidermal cells but had little effect on that of parenchymatous cells when it was added to the growth solution on which segments of etiolated Avena coleoptiles and pea epicotyls were floated. They however reached different conclusions: the insensitivity of the inner tissue to added auxin was attributed to the inhibited auxin penetration through the injured surface (16); inner tissue had a different auxin concentration for growth promotion, so that the optimum auxin concentration for the stem elongation promoted elongation of epidermal cells (15). Tanimoto and Masuda (14) and Masuda *et al.* (9), using light-grown pea epicotyl segments, reported that the epidermis and the inner tissue had approximately the same optimum concentration for their growth and that auxin-induced elongation of the former was much larger than that of the latter, and was accompanied by a significant change in the cell wall properties of the former. They also reported that the auxin-induced elongation of unpeeled segments was inhibited by actinomycin D and cycloheximide which also suppressed the auxin-induced change in the cell wall properties of the epidermis but not of the inner tissue.

Using etiolated pea epicotyl segments Burström *et al.* (3) scrutinized the relationship between turgor pressure and the elasticity of the cell wall (Young's modulus, E). They found (1) that E increased nearly proportional to the turgor pressure and was at water saturation more than 50 times higher than at plasmolysis; (2) that E was higher in the epidermis than in the parenchyma; (3) that auxin caused a decrease in E; (4) that auxin induced growth both in the epidermis and in the parenchyma under a decrease in E; (5) that this was followed by an increase of E which was independent of auxin but dependent on the turgor pressure. They thus attributed the insensitivity of the parenchyma to auxin to the high tension due to water saturation.

In the present study the role of the epidermis in stem elongation caused by auxin and β-1,3-glucanase was investigated.

Material and Methods

Pea seeds, *Pisum sativum* L. cv. Alaska, were surface sterilized with sodium hypochlorite solution. They were then soaked and kept for 2 days in the dark on moistened absorbent cotton in plastic trays at 25°C. Selections for uniformity of root length were made and they were transplanted to plastic trays for water culture. Stem segments, 7 mm in length, were excised with a double-bladed cutter from the upper portion of the 4th and/or 5th internode (2–3 cm long) of pea plants grown for 8 days under continuous fluorescent light, 5000 lux, at 25±0.5°C in a growth cabinet. The segments were pooled in ice-cooled 0.01 *M* K-phosphate buffer, pH 5.5, and were then transferred to 2 or 5 ml each of test solutions buffered with 0.01 *M* K-phosphate, pH 5.5, with no sugar added, in petri dishes, 4 or 9 cm in diameter. Dishes were incubated at 25°C, shaked (60 rpm) under light at 5000 lux. The segments to be peeled were initially 15 mm long. First they were ice-cooled, then the epidermis was peeled off with forceps starting from the basal end. The epidermis could be peeled off in 2 or 3 strips.

In elongation studies with peeled segments (7 mm in initial length), segments were excised again from the upper portions of the 15 mm long peeled segments. In a set of experiment, the central part of the epicotyl segment including pith, cambium and part of vascular bundles was removed using a micro-borer (diameter: 0.5 mm) to prepare "cylinder" segments. Isolated epidermal strips, peeled from 7 mm segments, were also subjected to elongation studies. The elongation of tissue segments thus prepared, *i.e.* unpeeled, "cylinder" and peeled segments, and isolated epidermal strips, 10 segments each being floated on 2 ml of test solution, was traced at intervals by measuring segment-length with an ocular micrometer using a binocular microscope (×10).

In expansion experiments 10 each of peeled and unpeeled segments were incubated for 3–4 h in 10 ml of 0.2 *M* mannitol solution buffered with 0.01 *M* K-phosphate buffer, pH 5.5, in the presence and absence of 1 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid). The segments were then allowed to expand in 5 ml of buffer and the change in length was traced at 30, 60, 90 and 120 min of expansion period.

Split tests were performed using 2 cm long segments of the 4th and 5th internodes. The apical 1 cm was split lengthwise into 2 equal halves with a razor blade and the split segments were put into petri dishes containing 5 ml buffer solution. After 30 min the segments were removed from the solution and shadow pictures were made on bromide paper. Curvature (degree) was then measured with a celluloid protractor lined up with the straight region of each half and intact base of the segment, and the angle formed between the vertical axis of the intact base and the tangent at the point where curvatures ceased (apical tip) was read.

Cell wall extensibility was expressed in 2 different ways using a Tensilon UTM-II tensile-tester (Toyo Measuring Instruments Co., Ltd.): (1) determination of strains caused by a quick stretching producing a certain amount of stress (S_0), or that of stress produced by a certain amount of strain (5 % constant strain of the initial length); from these determinations ratios (g/mm; symbol stress/strain is conventionally used, see 18) were calculated in some cases; (2) determination of stress-relaxation parameters, T_0 and T_m, as reported previously (18). In this case a certain amount of initial stress (2×10^8 dyn/cm²), produced strain being kept thereafter, or of constant strain (5 %) was given to epidermal specimens by stretching them at the rate of 20 mm/min.

The average thickness of the epidermal cell wall was determined to 2.5 μm on electron microscopic pictures of cross-sectioned epidermal cells. The width of a strip times 2.5 μm then gave the cross-area of the epidermal cell wall in cm², and hence the stress produced was calculated.

After the segments were differently treated, the epidermis was peeled off and epidermal strips were immediately killed for 5 min in boiling methanol. A strip was fixed between 2 clamps of the tester (distance: 5 mm) and a small stretching force (ca. 3 g) was given to the specimen to take up initial slack (5). Then the specimen was given a strain, stretched by lowering the bottom clamp to produce 2×10^8 dyn/cm² of S_0 (initial stress) or 5 % strain, the decay of S being subsequently automatically recorded.

In both "non-conditioned" and "conditioned" specimens auxin elicited a similar effect on their extensibility and relaxation properties (5). When, however, the amount of stress or strain was changed, only "non-conditioned" specimens showed a linearity with regard to elastic modulus (Yamamoto and Masuda, unpublished). For this reason "non-conditioned" specimens were used in the present study.

The parameters were determined by simulating mechanical properties to a continuous viscoelastic model consisting of an infinite number of Maxwell components (19). Since the plotting of stress (S) against $\log(t + T_0)$ produced a straight line, the following equation is obtained:

$$S(t) = a - b \log(t + T_0) \quad (1)$$

where a and b are constants and t = time (s), and T_0 can be determined by extrapolating the line to S_0 (also see 1). For the system of τ spectrum (τ: relaxation time), the relaxation function is expressed as follows:

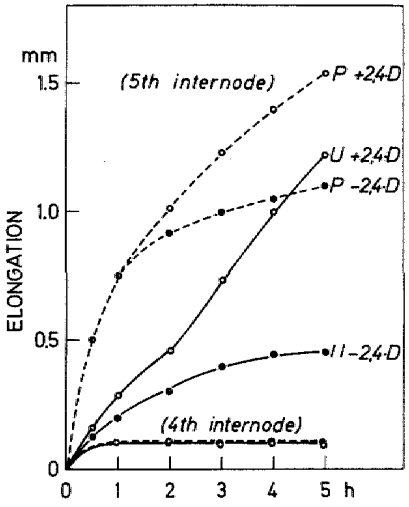

Figure 1. *Time course for elongation of peeled* (P) *and unpeeled* (U) *segments of 4th and 5th internodes in 1 mg/l 2,4-D solution.* Means of 10 segments.

$$S(t) = \int_0^\infty G(\tau)\gamma \exp(-t/\tau)d\tau \qquad (2)$$

where $G(\tau)$ is the τ spectrum of the elastic modulus and γ = strain. Finally $H(\tau)$ $(= G(\tau)\tau)$ can be obtained as

$$H(\tau) = \frac{b}{\gamma}\{\exp(-T_0/\tau) - \exp(-T_m/\tau)\} \qquad (3)$$

where $T_m = \exp(a/b)$. The $H(\tau)$ spectrum represented by Eq. (3) indicates a box-type distribution (19), T_0 and T_m being the parameters for determining the shape of the box-type distribution and representing the minimum and maximum relaxation times of the continuous model, respectively.

The enzyme preparation of exo-type β-1,3-glucanase was similar to the one used previously (18), supplied by Drs. J. Ebata and S. Oi of our University, to whom thanks are due. The enzyme preparation was isolated from the culture of a fungus *Sclerotinia libertiana* and purified through several steps. The enzyme activity was referred to as 1 unit/ml when 1 mg glucose was produced from 10 mg Sclerotan (*Sclerotinia* glucan) incubated in the enzyme solution (0.02 *M* acetate buffer, pH 4.5) for 20 min at 40°C. The enzyme activity per mg protein was 40.0 units.

Results

The time course study of the elongation of unpeeled (U) and peeled (P) segments of 5th internodes in the presence of 1 mg/l 2,4-D and in its absence (Figure 1) showed that the inner tissue had a much smaller sensitivity to auxin as compared with unpeeled segments (epidermis), although its expansion quickly occurred but seemed to level off by about 2 h. With control segments, which had a high expansion capacity of the inner tissue, it seemed likely that the rigidity of the epidermal cell wall was large enough to compress the inside against the tension due to the latter. On the other hand, with 2,4-D-treated segments, the epidermal cell wall was gradually loosened and by about 4–5 h the restraint due to the epidermal call wall and the tension due to the inner tissue seemed to be balanced. As indicated in Table 1, the cell wall parameters, particularly T_0, remarkably changed during the incubation in the presence of auxin; *i.e.* auxin-induced cell wall loosening as indicated by a decrease in T_0 was apparently found in the epidermis. T_m seemed to show a trend to increase in response to auxin as reported previously (18), suggesting that auxin changes cell wall properties by causing metabolic turnover of constituting wall polysaccharides. As previously reported (14), auxin caused no appreciable changes in the cell wall parameters of the inner tissue.

If the outward curvature of split segments is due to the removal of the restraint caused by the epidermis (epidermal cell wall loosening), it may not occur with

Table 1. *Effect of 2,4-D on cell wall parameters of the epidermis.* Segments, 15 mm in length, were floated on 0.01 *M* phosphate buffer, pH 5.5, containing or not containing 1 mg/l 2,4-D and incubated for 4 h. The epidermis was then peeled off, methanol-killed and subjected to stress-relaxation analysis, a constant strain of 5 % being given to the epidermal specimen. Means of 10 specimens with standard errors.

Segments	Treatment	T_0 (s)	T_m ($10^8 \times$ s)	$\frac{\text{Stress}}{\text{Strain}}$ (g/mm)
4th internode ...	Initial	0.0096 ± 0.0007	0.97 ± 0.21	77.2
	Buffer	0.0103 ± 0.0008	0.56 ± 0.18	74.8
	2,4-D	0.0089 ± 0.0016	2.34 ± 1.37	74.7
5th internode ...	Initial	0.0150 ± 0.0038	3.17 ± 2.71	45.9
	Buffer	0.0097 ± 0.0009	0.26 ± 0.07	37.2
	2,4-D	0.0064 ± 0.0007	5.32 ± 2.61	39.8

Table 2. *Curvatures of split segments after 4 h elongation in buffer solutions with and without 1 mg/l 2,4-D.* Apical 1 cm of segments before and after incubation was split lengthwise and floated for 30 min on 0.01 *M* phosphate buffer solution and curvatures were measured. Means of 10 segments with standard errors.

Treatment	Curvature (degree)
Initial	84 ± 4.5
Buffer, 4 h	56 ± 4.8
2,4-D, 4 h	31 ± 3.2

the segments which elongate for about 4 h in the presence of 2,4-D and then are split lengthwise. The outward curvature of elongated segments was apparently much smaller than that of control segments. The data of curvature determination indicated in Table 2 confirm the conclusion.

The segments from the 4th internodes which ceased elongating were unable to expand in response to added auxin (Figure 1). One possibility of their incapability of expansion may be that the expansion capacity of the inner tissue is almost lost, as judged from the fact that peeled segments of the 4th internodes did not elongate much (Figure 1). An alternative may be that the epidermal cell wall of the 4th internodes is incapable of loosening in response to auxin, in addition to the incapability of expansion of the inner tissue. This was really the case, as indicated in Table 1. The stress-relaxation parameters of the epidermal cell wall were similar in the 4th and 5th internodes, but stress/strain ratio (5 % strain) was much larger in the former than in the latter, indicating that the epidermal cell wall of

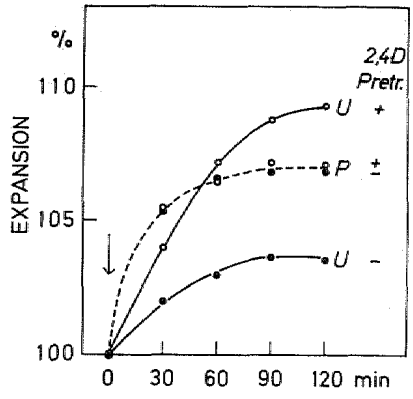

Figure 2. *Expansion of peeled* (P) *and unpeeled* (U) *segments of 5th internodes.* After pre-incubation the segments were transferred to phosphate buffer solution and change in length (%) was traced.

the former is mechanically much stronger and much less extensible under a certain tension.

Epansion experiments were then carried out to see the relationship between the expansion of the inner tissue and the cell wall extensibility of the epidermis. Peeled and unpeeled segments were pre-incubated for 4 h (balance time) in 0.2 *M* mannitol solution in the presence and absence of 2,4-D and then allowed to expand in buffer (Figure 2). It is apparent that pretreatment with auxin remarkably enhanced the subsequent expansion of unpeeled segments whereas it had practically no effect on that of the inner tissue.

In peeled segments only the epidermis was removed, leaving the adjacent cell layer (the outermost layer of

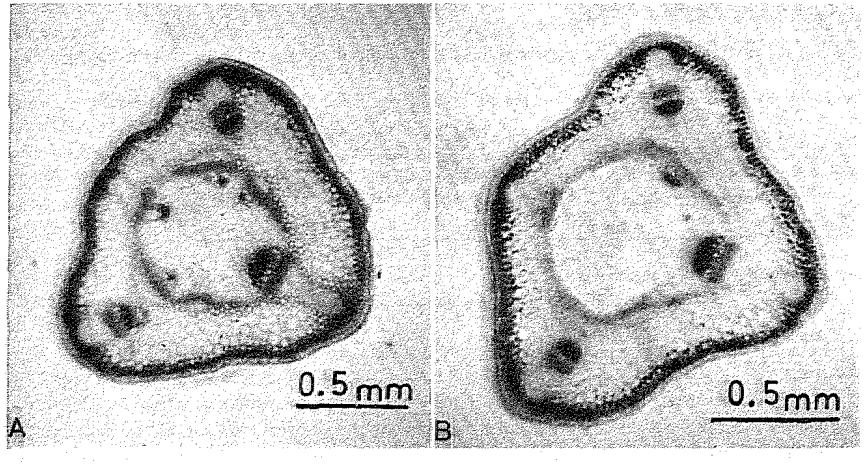

Figure 3. *Cross sections of segments with the central part intact* (A) *and removed* (B).

Table 3. *Comparison of elongation due to 2,4-D of intact and "cylinder" segments.* Initial length 7 mm.

Segments	Addition	Elongation, mm		
		1 h	3 h	5 h
Intact	Buffer	0.23 ± 0.01	0.39 ± 0.01	0.60 ± 0.04
	2,4-D	0.31 ± 0.01	0.74 ± 0.03	1.64 ± 0.03
Cylinder	Buffer	0.17 ± 0.02	0.19 ± 0.02	0.24 ± 0.03
	2,4-D	0.26 ± 0.02	0.51 ± 0.03	0.90 ± 0.04

peeled segments) intact as judged by microscopical observation. Figure 3 illustrates the cross sections of intact and "cylinder" segments. With the latter, only 10–12 cell layers were remained intact, but it well responded to auxin (Table 3), showing that the outer layer, if not the epidermis alone but several cell layers, is the major site for auxin action to induce stem elongation. A question then arises whether or not only the epidermis responds to auxin, resulting in epidermal cell wall loosening, and the inner tissue plays a major role in expanding. The epidermis was peeled off from 7 mm segments and incubated for 4 h in the presence and absence of 2,4-D. After peeling off, the length of the epidermal strip shrank about 5 % and no increase in its length and no significant change in the relaxation parameters due to auxin were observed (Table 4). Plasmolysis test indicated that epidermal cells were alive at least after 5 h incubation. It thus appears that auxin can not act on epidermal cells to induce cell wall loosening and elongation unless several layers of inner cells are attached to support auxin to cause epidermal cell wall loosening.

Induction of the elongation of unpeeled segments was caused by β-1,3-glucanase, 0.55 units/ml, just as is already reported with etiolated pea segments (17). The enzyme caused in 1–2 h a rapid elongation which thereafter went almost parallel with control level (Figure 4). Contrary to auxin the enzyme caused no elongation of the inner tissue and this could be the reason why the enzyme was unable to cause a continued elongation.

Table 4. *Effect of 2,4-D on the length and on cell wall parameters of the isolated epidermis.* The epidermis was peeled off from 7 mm segments excised from 5th internodes and then incubated for 4 h in buffer solution with and without 1 mg/l 2,4-D. After measuring the length, treated epidermal strips were methanol-killed and subjected to stress-relaxation analysis (5 % strain). Means of 10 specimens with standard errors.

Treatment	Length, mm	Cell wall parameters	
		T_0 (s)	T_m ($10^6 \times$ s)
Initial	6.60 ± 0.027	0.0144 ± 0.0018	61.4 ± 28.9
Buffer, 4 h ...	6.41 ± 0.048	0.0222 ± 0.0017	7.0 ± 1.8
2,4-D, 4 h	6.46 ± 0.034	0.0176 ± 0.0018	12.1 ± 3.8

However, the enzyme significantly changed the cell wall properties, as its effect on T_0 showed (Table 5).

If the expansion (%) of unpeeled segments (from Figure 2) was plotted against that of peeled ones at different expansion periods (Figure 5 *A*), it turned out that at a certain innner pressure (expansion of the inner tissue) the expansion of unpeeled segments pretreated with auxin was much larger than that of control segments. It is thus probable that auxin added during preincubation mainly caused a change in the mechanical properties of the epidermal cell wall. In order to confirm this, the epidermis was peeled off after pre-incubation and methanol-killed in the following experiments. Different amounts of strain, 3, 4, 6, 7 and 9 % each, were then given to the epidermal strip and the stress produced spontaneously was plotted at each strain (Figure 5 *B*). The curves (stress-strain) for auxin-treated and -untreated epidermal cell walls turned out to be similar to those (expansion of peeled segments vs. that

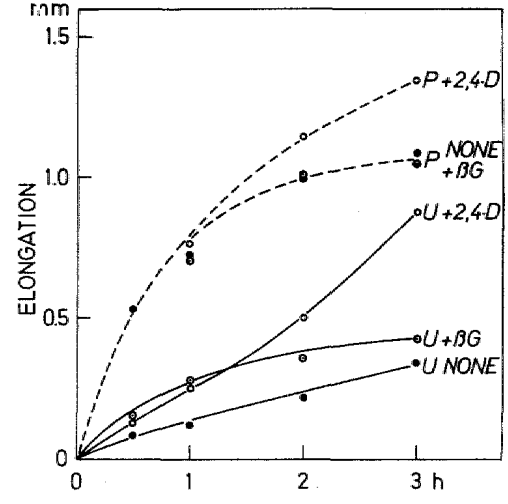

Figure 4. *Time course for elongation of peeled* (P) *and unpeeled* (U) *segments of 5th invernodes in 1 mg/l 2,4-D and 0.55 units/ml β-1,3-glucanase solutions.* Means of 10 segments.

Table 5. *Effect of 2,4-D and fungal β-1,3-glucanase on cell wall parameters of the epidermis.* Segments, 15 mm in length, were floated on 0.01 *M* phosphate buffer, pH 5.5, containing or not containing 1 mg/l 2,4-D or 0.55 units/ml fungal exotype β-1,3-glucanase and incubated for 3 h. The epidermis was then peeled off, methanol-killed and subjected to stress-relaxation analysis, a constant initial stress ($S_0 = 2 \times 10^8$ dyn/cm²) being given to the epidermal specimen. Means of 10 specimens with standard errors.

Treatment	T_0 (s)	T_m ($10^8 \times$ s)
Buffer	0.0087 ± 0.0014	2.69 ± 1.59
2,4-D	0.0062 ± 0.0009	4.23 ± 2.00
β-Glucanase	0.0057 ± 0.0017	4.51 ± 1.79

of unpeeled ones) shown in Figure 5 *A*. These results are simply accounted for if auxin changes the epidermal cell wall properties during the period of its pretreatment in 0.2 *M* mannitol solution. The data shown in Table 6 support the idea. Either at constant strain (5 %) or at constant initial stress (2×10^8 dyn/cm²) did the auxin pretreatment decrease T_0, although it had no effect on T_m.

Discussion

There have been conflicting opinions as to the quantitative importance of turgor in growth (4). Burström *et al.* (3) have elucidated, using 3rd internodes of etiolated pea seedlings, that growth occurs even at low turgor pressures. They have also reported that under normal condition at low turgor the inner tissue and the epidermis grow equally, but only the former grows at or near water saturation.

Using excised epicotyl segments growing on solutions, Burström *et al.* (3) have shown under experimental conditions that the inner tissue loses its sensitivity to auxin whereas the epidermis grows in response to the hormone. A similar conclusion has been drawn by Tanimoto and Masuda (14) and Masuda *et al.* (9) using light-grown pea stem segments. They conclude that the induction of stem elongation by auxin, at least at its initial stage, is brought about by the removal of restraint of the stem tissue by the epidermis. This conclusion has been further supported in the present study. Splitting of elongated segments under the influence of auxin produced much smaller curvature than control segments (Table 2). In growth-ceased epicotyls, 4th internodes in this case, there seemed to be no restraint of the stem by the epidermis, since peeled segments of the 4th internodes expanded very little (Figure 1).

The removal of the restraint of stems due to the epidermis when they grow is certainly caused by the cell wall loosening of the epidermis. This became apparent by the results of expansion experiments (Figures 2 and

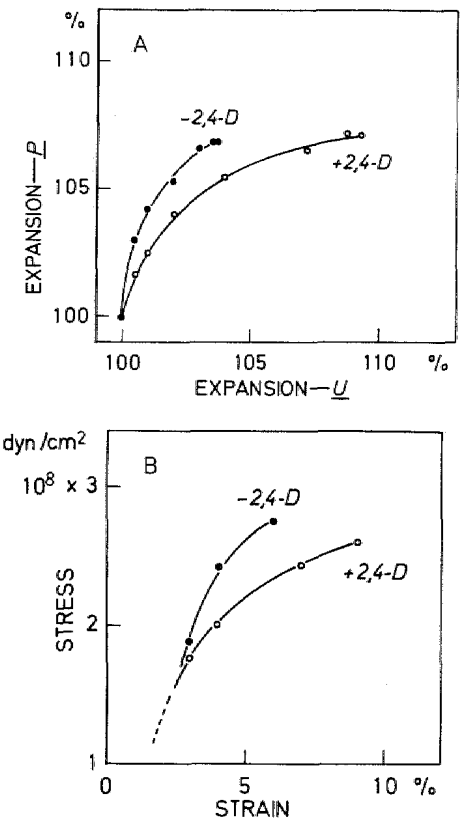

Figure 5. *A. Relationship in expansion between peeled* (ordinate) *and unpeeled* (abscissa) *segments.* Data from Figure 2 were re-plotted. *B. Stress-strain relationship of the epidermis.* A certain amount of strain (abscissa) was given to the epidermis and stress produced (ordinate) was measured. Means of 10 epidermal specimens.

Table 6. *Effect of 2,4-D added in 0.2 M mannitol solution on cell wall parameters of the epidermis.* Segments were preincubated for 4 h in 0.2 *M* mannitol solution with and without 1 mg/l 2,4-D. The epidermis was then peeled off, methanol-killed and subjected to stress-relaxation analysis. Means of 10 specimens with standard errors.

Treatment	T_0 (s)	T_m ($10^8 \times$ s)
Buffer [1]	0.0142 ± 0.0045	7.13 ± 3.38
2,4-D [1]	0.0070 ± 0.0015	2.97 ± 1.37
Buffer [2]	0.0091 ± 0.0050	17.7 ± 7.16
2,4-D [2]	0.0021 ± 0.0004	18.9 ± 8.21

[1] Constant strain of 5 %.
[2] Constant S_0 of 2×10^8 dyn/cm².

5 *A*) and of measurements of extensibility (Figure 5 *B*) and of stress-relaxation parameters (Tables 1 and 6). Not only at water saturation but also at reduced turgors, stem elongation seemed primarily dependent upon the mechanical properties of the epidermal cell wall at a certain turgor. The isolated epidermis, however, did not respond to added 2,4-D (Table 4) while segments with the central part removed did (Table 3), which suggests that several layers adjacent to epidermal cells are necessary for the latters to respond to auxin. Auxin may change the mechanical properties of the epidermal cell wall by acting on the adjacent inner cells, or directly by acting on the epidermal cells for which the adjacent inner cells are needed in some way. Plasmadesmata connecting epidermal cells and parenchymatous cells may be important but no information has so far been available. No detailed studies have been reported as to the difference in the biochemical, chemical or physical properties between epidermal cells and inner parenchymatous cells except those on fine structures by O'Brien (10, 11). Some structural differences have been reported by him but no clear indications have been given to connect the structural differences and auxin responsiveness. An autoradiographic study by Ray (13) indicated that the incorporation of labeled glucose into non-cellulosic polysaccharides of the oat and pea epidermal cell wall was stimulated by auxin. Auxin thus stimulates epidermal cell wall synthesis when it induces elongation.

Biochemical significance of changes in T_0 and T_m has not been fully understood, although they are physically established (1). However, as also reported previously (18), since β-1,3-glucanase decreased T_0 when it induced stem elongation, a decrease in T_0 may be a reflection of wall loosening. A shift in the box-type distribution of the τ spectrum is generally thought to be caused by a shortening or lengthening of the constituent molecules which appear to be metabolically controlled and to involve nucleic acid and protein metabolism (9, 14). Metabolic and biochemical studies on the constituent polysaccharides of the epidermal cell wall and their changes due to auxin are needed to explain the mechanism of auxin-induced stem elongation and auxin-induced changes in the physical parameters of the cell wall.

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