The Mechanic State of "Inner Tissue" in the Growing Zone of Sunflower Hypocotyls and the Regulation of Its Growth Rate Following Excision[1]

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Spontaneous growth of isolated inner tissue from the etiolated sunflower (*Helianthus annuus* L.) hypocotyl growing zone was investigated. A new preparation technique allowed measurements starting 3 s after excision. Elongation with respect to the turgescent and plasmolized state was quantified in terms of relative growth rates, facilitating comparison to growth in situ. Turgor and turgor-induced strain were determined. Overall longitudinal strain in inner tissues in situ was positive, indicating that compressive forces exerted by peripheral tissues are outweighed by turgor-dependent tensile stress. Inner tissue expansion following isolation depended on water uptake. Extreme plastic extension rates occurred immediately after excision, suggesting that mechanical parameters of inner tissue in situ cannot be extrapolated from the mechanics of excised sections. In the long term, excised inner tissue autonomously established values of turgor, turgor-induced strain, and relative growth rates similar to values in the living plant. These results support historic models of tissue cooperation during organ growth, in which inner tissues actively participate in the control of growth rates.

Tissue tension, i.e. mutual tensions between different cell layers in plant organs, was intensively studied by the older botanists (for review, see Peters and Tomos, 1996a). After a period of neglect, the awareness of these phenomena and their relation to growth and morphogenesis has grown again during the last 2 decades (Firn and Digby, 1977; Tomos et al., 1989; Hejnowicz, 1997). However, a largely overlooked discrepancy exists between historic and recent notions about the role of inner tissues in the growth process. To date, it appears accepted that elongating higher plant stems can be described as a two-phase composite material (Niklas, 1992) consisting of a rigid outer husk that restricts the expansion of a highly extensible inner core. To most modern researchers, this seems to imply that organ growth is controlled by the epidermal cell layers, and many recent studies focus on this tissue (Kutschera, 1992). Surprisingly, similar mechanical models had led older botanists to conclude that "the parenchymal walls are the site of the growth mechanism, and therefore they are of the highest interest" (Bonner, 1935, p 383, our translation).

The mechanical roles of different cell layers are usually inferred from the behavior of isolated tissues. Therefore, the above discrepancy might be due to contradicting interpretations of inner tissue behavior following excision. We tested this possibility by scrutinizing the nature of growth processes in isolated inner tissue from the etiolated sunflower (*Helianthus annuus* L.) hypocotyl, with particular emphasis on the relationship to growth in situ.

RESULTS AND DISCUSSION

Characterization of the Growing Zone

Time courses of relative growth rates of segmental elements of a growing organ can be characterized only if both spatial and temporal growth patterns are known. Thus, the gradient of growth intensity along that organ, i.e. its relative elemental growth rate (REGR) profile, must be determined (Erickson, 1976; Silk, 1984). The REGR profile of sunflower hypocotyls under our standard conditions of cultivation was measured in seedlings of the same age as those used in the other experiments, and exhibited a pronounced peak that was skewed toward the apex (Fig. 1).

In the present study, tissue was excised from a location near peak growth intensities (5–11 mm below the hook). During a 5-h period, the maximum duration of our experiments with excised tissues, such a segment in situ would have gained 1.25 mm in length, and its proximal margin would have moved away from the hook by some 2.4 mm (Fig. 1; for a summary of the mathematical background of such calculations, see Peters and Bernstein, 1997). The time course of segmental relative growth rate in situ (R_S) during this period is also given in Figure 1. Obviously, R_S decreases with time, because the segment moves away from the peak of growth intensity.

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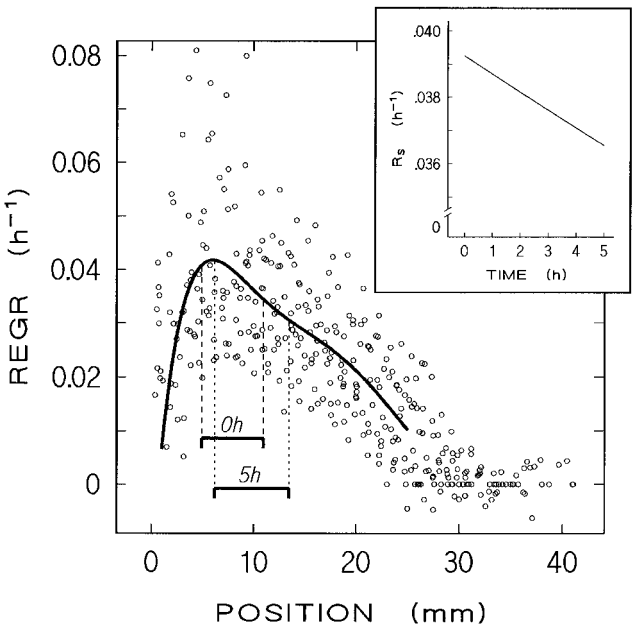

Figure 1. Spatial distribution of elongation growth along the etiolated sunflower hypocotyl, plotted as REGR versus position. Position 0 refers to the top of the hook; the shoot/root transition is located initially at position 42 mm (average length of hypocotyls at the start of the tests). The bold line represents the average REGR profile calculated from 14 individual experiments; original data from these experiments are also shown (○). The position of segments excised for further experiments (5 and 11 mm below the hook) is indicated (0h). The position to which this segment would move in a 5-h period, which is the maximum duration of our experiments with excised tissue, is also shown (5h). The time course of the R_S during the 5-h period is given in the inset.

Mechanism of Spontaneous Inner Tissue Expansion

Plant cell growth depends on turgor-induced stress in the cell wall (Taiz, 1984), and is initiated by wall stress relaxation (Cosgrove, 1993). On the molecular level, turgor-induced stress works by straining load-bearing wall components so that they irreversibly yield (Passioura and Fry, 1992; Passioura, 1994). In this context, it is crucial to understand what the "state of compression" (in which inner tissues are claimed to be; Fritsch and Salisbury, 1961; Kutschera, 1987; Niklas and Paolillo, 1998) means in physical terms.

We followed plasmolytic shrinkage of isolated inner tissue in 0.8 M mannitol (Fig. 2), which is in excess of the concentration required to completely abolish turgor-dependent expansion (not shown). Within 10 min, the tissue reached a stable length of about 84% of the initial value, equivalent to a longitudinal turgor-induced tensile strain of 0.19. Obviously, inner tissue cell walls are in a state of longitudinal tension in the intact plant. We then studied the kinetics of spontaneous extension. When isolated inner tissue was incubated in water, rapid elongation started instantaneously. It leveled off after some 20 min, when segment length had increased by about 19% (Fig. 3; see also Fig. 5). In previous studies, much smaller values for inner tissue expansion in water were found. Kutschera and Köhler (1992) reported a 4% increase in length within 5 min (11.7% in our experiments), while Hejnowicz and Sievers (1995) found a similar value (4.5%) after 10 min (14.4% in our experiments). We assume that these discrepancies are due to differences in the time that elapsed between tissue isolation and the start of measurements. Our method, which was designed to minimize this period, apparently enabled us to detect rapid early expansion that previously passed unnoticed.

We detected no length change at all in segments placed in water-saturated paraffin oil to prevent both transpiration and water uptake. When these segments were transferred to water, extension proceeded with similar kinetics as in segments without prior paraffin treatment (Fig. 3). It follows that isolation-induced inner tissue expansion is not an elastic response to a release from compressive stress, but rather is an effect of water uptake. Inner tissue expansion in water does not provide a valid measure of compressive forces exerted by outer tissues in situ, since water availability (Burström and Fransson, 1957; Boyer, 1988) or low apoplasmic osmotic potentials (Cosgrove and Cleland, 1983) might be limiting factors.

If inner tissue expansion results from water uptake rather than from compressive force release, then why do segments of longitudinally incised stems bend outwards to some extent even in dry air? The answer must be rapid water re-distribution between tissues following the destruction of organ symmetry, leading to differential tissue swelling and shrinkage (as postulated by Sachs, 1875).

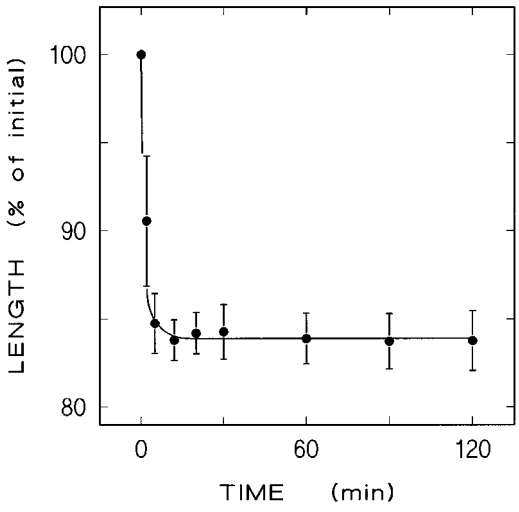

Figure 2. Time courses of shrinkage induced by plasmolysis by incubation in 0.8 M mannitol in isolated inner tissue from the etiolated sunflower hypocotyl growing zone. Length is given as a percentage of turgescent length (means ± SD, $n \geq 11$).

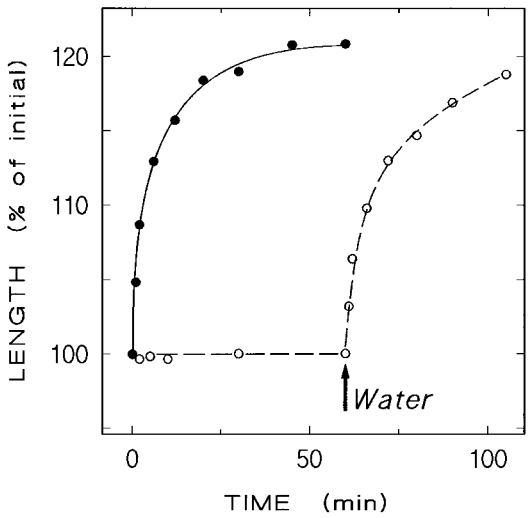

Figure 3. Time courses of spontaneous expansion of isolated inner tissue from the sunflower hypocotyl growing zone. One section (●) was transferred to water immediately upon isolation (time 0); another one (○) was first incubated in water-saturated paraffin and then transferred to water after 60 min (as indicated). No change in length occurred in the absence of water. If water was available, the kinetics of the spontaneous expansion were similar in both treatments. The graph shows a typical result from six repetitions of this experiment.

Long-Term Kinetics of Turgescent Length and Irreversible Extension

As shown above, inner tissue segments shrunk rapidly in 0.8 M mannitol to reach a stable length within a few minutes. Similar kinetics occurred if plasmolysis was induced at different times following excision and transfer to water (Fig. 4). The length of segments plasmolized at different times increased in parallel with the turgescent length, although at a different velocity. We found that plasmolized lengths differed from the control (i.e. time 0) at $P < 0.05$ at 2 min and at $P < 0.01$ (Student's t test) at 3 min after the start of the experiment.

After about 1 h, the initial phase of inner tissue expansion, characterized by exponentially declining velocity, was followed by a sustained phase of apparently constant expansion velocity (Fig. 5). The transition between the exponential and the linear growth phase was characterized by an increase in growth velocity. The time course of turgescent segment length was paralleled by a similar development of plasmolized length. A major difference between these parameters was their reaction to cyanide, an inhibitor of oxidative phosphorylation. Cyanide did not interfere with the exponential growth phase of the turgid tissue, but the subsequent linear phase was completely prevented. Conversely, the pattern of irreversible (i.e. plasmolized) length was not changed by cyanide; both the exponential and linear phase were seen, albeit at a reduced rate.

Time Courses of Turgor and Turgor-Induced Strain

Although turgescent and plasmolized length followed similar time courses, turgor-induced longitudinal strain increased drastically after tissue isolation and transfer to water (Fig. 6A). Peak values reached 0.3, i.e. turgor extended the tissue by some 30% of its irreversible length. Turgor-induced strain reapproached control values during the early parts of the linear growth phase. The development of turgor-induced strain was paralleled by the time course of turgor, which increased to more than 2-fold within 2 min before decreasing to control values again (Fig. 6B).

Inner tissue turgor before transfer to water was measured while the tissue was kept in water-saturated paraffin to prevent transpiration. It was found to be remarkably low (about 0.2 MPa, Fig. 6B) compared with values reported previously from unpeeled segments in water (Kutschera and Köhler, 1992). To resolve the discrepancy, we measured turgor in outer parenchymal cells of intact segments (i.e. without separation of tissues). Values in segments under paraffin (0.17 ± 0.037 MPa, mean ± SD, $n = 31$) were in line with results from isolated inner tissue. When segments were incubated in water, turgor (0.44 ± 0.049 MPa, mean ± SD, $n = 18$) was almost identical to the value reported previously. We also measured turgor in situ at the position from which segments were excised. The measurements we ob-

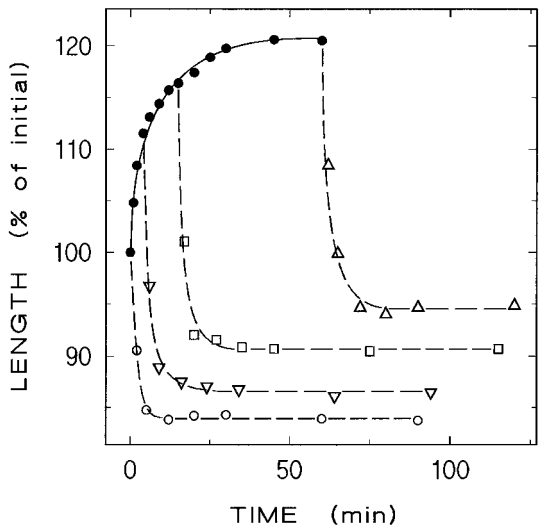

Figure 4. Time courses of spontaneous expansion of isolated inner tissue from the sunflower hypocotyl growing zone incubated in water (●), and of shrinkage induced by plasmolysis in 0.8 M mannitol immediately after tissue isolation (○), and after 5 (▽), 15 (□), and 60 (△) min (time 0 indicates excision). The length in the plasmolized state rises with time, indicating that growth (irreversible elongation) occurs during spontaneous tissue expansion. Data shown are from one representative experiment, i.e. measurements were actually made in parallel as shown (open symbols represent individuals; closed symbols represent mean values with $n \leq 4$). For averaged results from numerous repetitions, see Figure 5.

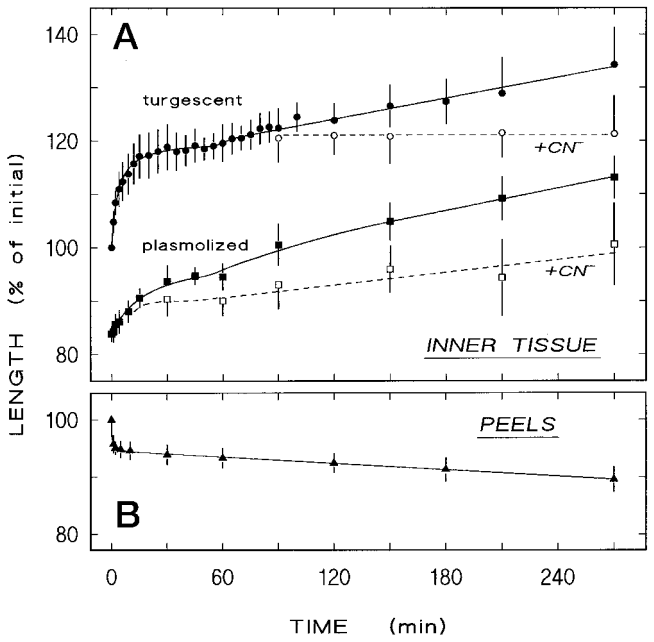

Figure 5. Time courses of spontaneous changes in length of tissue isolated from the sunflower hypocotyl growing zone at time 0. All symbols represent means ± SD. A, Time courses of turgescent length of inner tissue incubated in water (●, $n \geq 64$) and of its length after plasmolysis (■, $n \geq 24$; these data were obtained in experiments as depicted in Fig. 4). Curves shown as solid lines are segmented functions fitted to the original data (for details, see "Materials and Methods"). Both curves show an initial phase characterized by exponentially decreasing velocities of elongation and a subsequent phase of apparently stable elongation velocity. Analogous tests with 1 mM KCN in the medium are shown by open symbols (○, $n = 8$; □, $n = 6$); data from cyanide experiments not significantly different from cyanide-free tests are omitted for clarity. In the presence of cyanide, the initial phase of turgescent length increase remained unaffected, whereas subsequent steady-state growth was prevented. In contrast, the increase of plasmolized (irreversible) length was partly inhibited as a whole. B, For comparison, the development of the length of epidermal peels in water is shown ($n = 9$). Slow contraction of peels continued during the time of observation. No growth effects became evident.

tained from six individual plants ranged between 0.17 and 0.25 MPa. We conclude that turgor in water-incubated segments is no reliable measure for turgor in the living plant, where transpiration-induced turgor gradients (Meshcheryakov et al., 1992; Rygol et al., 1993), substantial apoplasmic solute concentrations (Cosgrove and Cleland, 1983), and gradients of growth-induced water potentials (Boyer, 1988) exist.

The data presented in Figures 3 to 6 suggest that the inner tissue expansion following isolation is driven by rapid water uptake, leading to a massive turgor increase. While tensile stresses induced by turgor values of about 0.5 MPa are effectively counteracted by peripheral cell walls in intact segments (Kutschera and Köhler, 1992), isolated inner tissue expands rapidly in response to such forces. Elastic cell wall extension predominates initially, as indicated by the increase in turgor-induced strain (Fig. 6A). This is in accordance with the cyanide insensitivity of the exponential expansion phase (Fig. 5).

Isolated Inner Tissue Expansion as Related to Organ Growth in Situ

Incubation of isolated inner tissue in water creates a transient situation of instability, during which cell mechanical parameters (water uptake rate, turgor, turgor-induced cell wall strain) vastly deviates from physiological values. However, after 1 to 2 h, a steady state characterized by the return of turgor and cell wall strain to values typical of intact growing zones becomes established (Fig. 6). Concomitantly, the velocity of elongation attains a constant value. How does this compare with growth in situ?

As expected from the trajectories (Fig. 5), relative growth rates of turgescent and plasmolized length

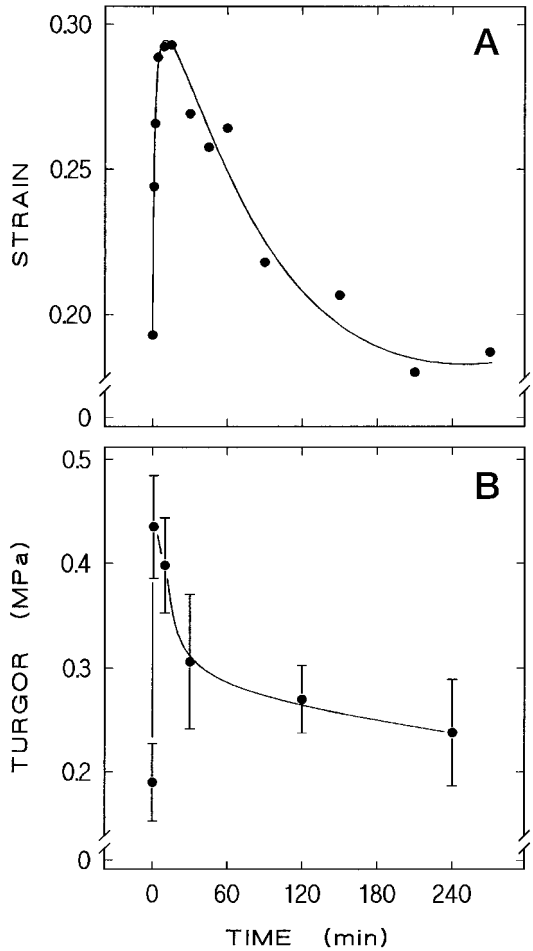

Figure 6. A, Time course of turgor-induced strain in isolated inner tissue from sunflower hypocotyl growing zones following excision at time 0 and incubation in water. Values were calculated as conventional strain from turgescent and corresponding plasmolized lengths at different times, given in Figure 5A. B, Analogous time course of turgor pressure (means ± SD, $n \geq 7$). Both parameters dramatically increase after tissue isolation, but then steadily return to control values again.

(R_{trg} and R_{pls}, respectively) rapidly decreased from maximum values at the start of the experiment (Fig. 7). Curves cross over at about 9 min, which had to be anticipated since turgor-induced strain showed a maximum at that time (Fig. 6A). Both R_{trg} and R_{pls} became stable when elongation velocities reached constant values (Fig. 7); in fact, a relative growth rate decreased over time if the corresponding growth velocity remained constant; however, the effect is too small to show in the figure. Before the establishment of apparently stable R_{trg} and R_{pls}, there were minima at about 50 min, and the transition was characterized by transient peaks. However, since relative growth rates are calculated from derivatives of functions fitted to elongation data, slight variations in the fitted curves result in substantial changes in the time courses of the relative growth rate. During the transition from the exponential to the linear growth phase, there is a considerable degree of freedom in choosing a particular function to fit. The exact shape of the relative growth rate curves between 50 and 75 min therefore is somewhat uncertain, although there is no doubt about the discontinuity that was consistently observed in the raw data. Further discussion should be postponed until continuous measurements on individual segments are available.

The in situ time course of R_S initially located at the position from which inner tissue was isolated is also shown in Figure 7 (compare Fig. 1). The correspondence with steady-state values of R_{trg} and R_{pls} is striking. Thus, isolated inner tissue is capable of establishing a stable biomechanic state in which its relative growth rate is close to values observed in intact plants. The maintenance of this state is achieved actively, as the cyanide dependency of R_{trg} and R_{pls} (Fig. 5) demonstrates.

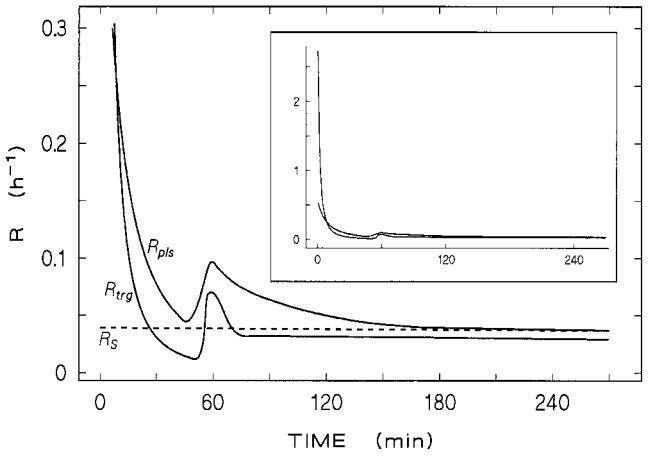

Figure 7. Time courses of R_{trg} and R_{pls} in inner tissue from the sunflower hypocotyl growing zone isolated at time 0. Curves were calculated from segmented functions fitted to elongation data (Fig. 5). The inset shows the complete time course, whereas in the main figure parts of the curves are depicted at higher resolution. Immediately after excision, relative growth rates greatly exceeded physiological values (compare Fig. 1, inset). The shape of the transients at about 60 min, representing the transition between exponentially decreasing and steady-state elongation velocities (compare Fig. 5), is subject to some uncertainty for mathematical reasons (for details, see text). The time course of relative growth rate, which segments would have experienced in situ during the equivalent period (R_S, as introduced in Fig. 1), is also given (broken line). Note the correspondence to steady-state values in excised inner tissue.

The expansion of isolated inner tissue in water initially consists of a major elastic and a minor plastic component (Fig. 5). Hejnowicz (1997) interpreted initial expansion as "fully reversible," although the original data undoubtedly show "a small plastic component in length change" (Hejnowicz and Sievers, 1996). As Figure 7 shows, the relative rate of change of plasmolized length of isolated segments (R_{pls}) during the first minute after isolation is in the range of 0.5 h^{-1}. This is 12 times the maximum rate occurring in the REGR profile of the intact organ (assuming that R_{trg} and R_{pls} are similar in situ; Fig. 1). Irreversible cell wall deformation at such an unphysiological rate must be considered relevant. Obviously, inner tissue segments that have started to expand spontaneously are in a highly artificial state, and thus are probably not valid model systems with which to study the mechanic properties of inner tissue in situ.

CONCLUSIONS

It has long been demonstrated that in growing plant stems peripheral tissues exert compressive forces on the inner ones (Sachs, 1875). More recent models emphasized this apparent "state of compression" and likened inner tissues to compressed springs (Fritsch and Salisbury, 1961) or to "giant protoplasts" (Kutschera, 1995). However, the overall stress regime in a physical body depends on all acting forces. The trivial fact of plasmolysis-induced shrinkage in inner tissue proves that compressive stresses exerted by peripheral cell layers are outweighed by turgor-dependent tensile stresses in situ. Inner tissue expansion following isolation is driven by water uptake and is characterized by further increases of both the tensile strain of the cell walls and the compression of the protoplasts (as indicated by the rise in turgor). There is no conceivable analogy between these processes on one hand and the expansion of a spring following the release from compressive forces on the other. Thus, the inner tissue "state of compression" is a figure of speech of obscure physical relevance. In the context of cellular growth regulation, where irreversible cell wall extension depends on tensile wall strain, this phrase is misleading and should be avoided.

In recent years, various approaches were used to quantify the mechanics of tissue tension. These attempts focussed on the mechanical characters of different portions of the apoplast (Vincent and Jeronimidis, 1991; Hejnowicz and Sievers, 1995, 1996; Niklas and Paolillo, 1998). To fully understand the

mechanics of non-lignified plant organs and the phenomenon of tissue tension, aspects of organ and tissue water relations will have to be integrated into the existing models. In this context it is important that excised, water-immersed segments are not generally adequate models for the undisturbed organ in situ, as we have demonstrated.

Isolated inner tissue is capable of establishing and maintaining endogenous growth rates strikingly similar to values in situ. This ability is absent from isolated peripheral tissue (Fig. 5), which had convinced older plant physiologists that inner tissue growth is "active," in contrast to the "passive" growth of epidermal layers (Peters and Tomos, 1996a). Quite obviously, this notion led to different research strategies than the modern opinion that shoot growth is controlled by the epidermis (Peters and Tomos, 1996b). Our present study supports the idea that cells of inner tissues form an active part in the control of organ growth, and that they are a promising system with which to study growth regulation on the cellular level.

MATERIALS AND METHODS

Plant Material

Seeds of sunflower (*Helianthus annuus* L. cv Frankasol) were sterilized in 150 mM NaClO for 30 min, soaked in tap water for 6 h, and sown on moist vermiculite. Seedlings were kept in the dark at 23°C. When hypocotyls had reached a length of 4 to 5 cm, straight-grown seedlings were selected for experiments.

REGR Profiles

Segments about 1 mm long were marked on hypocotyls with india ink. Initial segment lengths (L_0) were measured to the nearest 22 μm using a horizontal stereomicroscope fitted with eyepiece graticules. Segment lengths were measured again (L_t) after 7 h (Δt). Segmental relative growth rate (R_S) was calculated as (Hunt, 1982):

$$R_s = \frac{(\ln L_t - \ln L_0)}{\Delta t} \quad (1)$$

R_S was plotted versus average segment position (Peters and Bernstein, 1997). Polynomial regression functions (4th–7th order) were fitted to yield estimates of REGR profiles. A profile's integral over the growing zone equals the average organ growth velocity during Δt. Organ growth velocity was also determined directly by measuring hypocotyl length with a ruler to the nearest 0.5 mm for assessment of accuracy of REGR profiles. Plants in which both values deviated by more than 18% (the maximum error of the ruler experiments, i.e. 1 mm, expressed in percentage of the average growth increment during Δt) of the directly measured organ growth velocity were discarded. An average REGR profile was calculated from the remaining ones. The validity of the REGR analysis was tested by applying it to elongation data created by a model growing zone, as described previously (Peters and Bernstein, 1997; Peters and Felle, 1999). Idealized time courses of R_S were derived from the profile by the algebraic relationships summarized by Peters and Bernstein (1997).

Tissue Isolation

Epidermal Peels

A segment (5–11 mm below the hook) was marked with Indian ink and measured to the nearest 22 μm. A strip containing the marks was peeled off with fine forceps, and the time course of shrinkage of the peel in water was followed under a stereomicroscope.

Inner Tissue

A method was developed to allow determination of initial segment length before the separation of inner and outer tissues and to reduce the period between tissue isolation and the start of measurements to below 3 s (for details, see Fig. 8). Changes in length of isolated segments were measured to the nearest 22 μm. Distilled water augmented with 2 mM KCl served as the basic incubation medium; we refer to this solution as water throughout the text.

Growth Analysis

Functions were fitted to elongation data using standard scientific PC software. To model the complex behavior, segmented functions had to be used, obeying the continuity condition that first derivatives of neighboring functions have to be identical at their meeting point. Time courses of relative growth rate (R) were calculated from the segmented functions according to the definition of R (Hunt, 1982):

$$R = \frac{1}{L}\frac{dL}{dt} = \frac{d\ln L}{dt} \quad (2)$$

Turgor-Induced Strain

After tissue plasmolysis in 0.8 M mannitol, relative turgor-induced extension was calculated as conventional strain (Cauchy strain) ε:

$$\varepsilon = \frac{L_s}{L_0} - 1 \quad (3)$$

with L_S and L_0 denoting the stressed (turgescent) and unstressed (plasmolized) lengths, respectively.

Turgor Measurements

Turgor was measured with a cell pressure probe (Tomos and Leigh, 1999). A microcapillary filled with silicon oil and connected to a pressure transducer was inserted into a cell. Cell sap entered the capillary, and the position of the cell sap/silicon oil boundary (meniscus) was controlled by a piston modifying the volume of the system. Turgor was

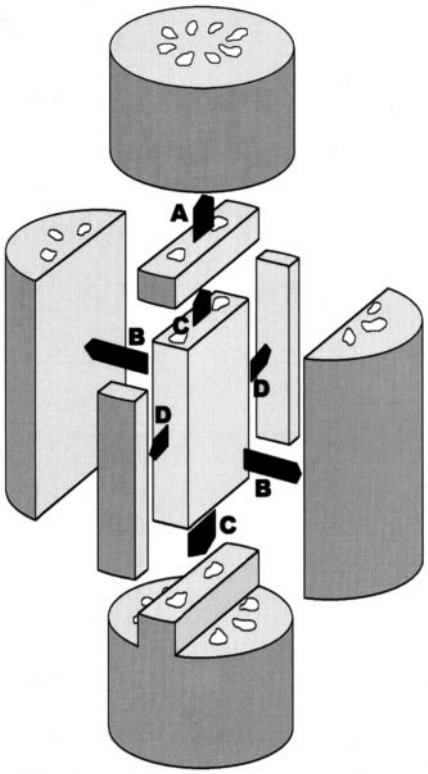

Figure 8. Diagram describing the preparation of inner tissue segments. The hypocotyl top was cut off 4 mm below the hook (step A). Two parallel longitudinal incisions were made to separate one inner and two symmetric outer segments. The outer segments, containing most of the peripheral tissue, were removed (step B). A portion was cut from 1 mm below the top of the central segment with two razor blades mounted in parallel at a fixed distance on a holder (step C). Distance between blades (i.e. length of excised tissue in situ: about 6 mm) was checked before and after each experiment to the nearest 11 μm. The preparation to this stage took less than 7 s. Segments were taken out of the cutter (time 0 for the time courses of inner tissue expansion) and were freed from peripheral tissues on both sides by longitudinal cuts through the cortical parenchyma (step D). Inner tissue excisates consisting of pith parenchyma, vascular bundles, intervascular parenchyma, and inner portions of cortical parenchyma were transferred to the test medium. Specimens in which this procedure was not finished within 3 s after removal from the cutter were discarded.

determined in isolated segments from the hypocotyl growing zone, or 5 to 7 mm below the hook in intact plants, which were kept upright with their roots in the substrate. It was often difficult to obtain reliable measurements, because the capillary tip became blocked by the viscous cytoplasm in these poorly vacuolized cells. Under these circumstances, constant pressure readings are likely to be artifacts. Therefore, we routinely performed pressure relaxation cycles, i.e. sudden increases and decreases of cell volume by some 5% to 10% evoked by movements of the meniscus, and followed the spontaneous pressure recovery. Cells in which not at least two consecutive cycles could be performed or in which the half-time of pressure recovery exceeded 3 s were discarded.

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