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**Extensibility of isolated cell walls in the giant tip-growing cells of the xanthophycean alga *Vaucheria terrestris***

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**Abstract** Apical cell wall fragments isolated from the giant-cellular xanthophycean alga *Vaucheria terrestris* sensu Götz were inflated with silicon oil by applying internal pressure ranging from 0.1 to 0.7 MPa, and the time-course of the cell wall deformation was recorded and analyzed by videomicroscopy. The cell wall extensibility in the tip-growing region was estimated by the pressure required for cell wall extension, the amount of total extension until cell wall rupture and the rate of cell wall extension. Apical cell walls exhibited gradual extension, or creep, during inflation, which was eventually followed by rupture at the apical portion, whereas no significant extension was found in the cylindrical basal portion of the cell wall fragment. Besides the largest extension observed around the tip, significant extension was also observed along the subapical region of the cell wall. The wall extensibility was dependent on buffer pH used for infiltration before inflation. The optimum pH for the extension was about 8.0, but the cell wall was much less extensible after infiltration with an acidic buffer. The cell wall extensibility was dependent on the pH of the buffer used before inflation, regardless of that used in the previous infiltration. Moreover, pretreatment of the cell wall with a protease caused considerable loosening of cell walls, but affected

the pH dependence of cell wall extensibility little. These results indicate that the extensibility of the cell walls in the giant tip-growing cells of the alga is distinct from that of plant cells that exhibit “acid growth” in its dependence on environmental pH and the role of cell wall proteins.

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**Keywords** Cell wall extensibility, pH dependence, Subtilisin, Tip growth, Turgor pressure, *Vaucheria*

**Abbreviations** (none)

## Introduction

Cell surface expansion, or cell growth, in plants involves the extension of existing cell walls and deposition of new cell wall components. Therefore, knowledge about cell wall extensibility in growing plant cells should provide fundamental information for studying the mechanisms that control plant cell growth. For example, the force required for cell wall extension and the environmental factors that affect cell wall extensibility could be determined by biomechanical experiments on the cell walls. Cell wall extensibility, in principle, has been investigated by measuring the deformation of cell walls, cells or tissues in response to force applied externally (Cosgrove 1989, 1993; Kutschera 1996).

Earlier studies of cell wall extensibility have been carried out using giant internodal cells of the green alga *Nitella* (e.g., Metraux et al. 1980). These studies were followed by studies on the growing tissues in angiosperms (reviewed in Kutschera 1991; Cosgrove 1993). In angiosperms, since growing cells are

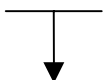
embedded in compact tissue, it is generally difficult to observe the growing cells directly and to analyze growth patterns of the cells, with few exceptions (Schnyder et al. 1990; Fricke and Flowers 1998). Nevertheless, assuming that the whole extension in a tissue represents cell wall extension in individual cells that compose the tissue, cell wall extensibility has been investigated mainly by stretching a living or killed tissue at the opposite ends with external force (Cosgrove 1993). Such investigations have revealed several factors that affect cell wall extensibility. For example, stress relaxation in cell walls was accelerated by pretreating the tissues with plant growth substances (reviewed in Masuda 1990), and a non-enzymatic cell wall protein increased the rate of long-term cell wall extension under an acidic condition (Cosgrove 1996). In addition, another cell wall protein had a function in decreasing yield stress, which is the minimum stress required for the initiation of tissue extension (Okamoto-Nakazato et al. 2000).

Tip growth is a mode of plant cell growth in which the expansion of the cell surface is largely limited to the apical region of the cell, which is typically a cylindrical cell with a dome-like apex. Growth in many kinds of tip-growing cells, e.g., pollen tubes, root hairs, protonemata, rhizoids, hyphae and algal thalli, can be observed directly, and, therefore, spatial and temporal patterns of cell expansion have been studied in detail (Chen 1973; Kataoka 1982; Bartnicki-Garcia et al. 2000; Shaw et al. 2000). In addition, physiological activities in cells that control tip growth have been intensely studied, including localized ion flux across the plasma membrane and intracellular transportation along cytoskeletal elements, (e.g., Cai et al. 1996; Malho and Trewavas 1996; Pierson et al. 1996;

Bibikova et al. 1998). However, due to difficulty in manipulating the small region of cell apices, cell wall extensibility in the growing apices has not been measured in any tip-growing cells. Therefore, in the tip-growing cells, fundamental information about mechanical properties of cell walls in the growing region is lacking, except for a few exceptions in which the artificially elevated turgor pressure required for cell wall rupture (Benkert et al. 1997; Money and Hill 1997) or force generated by growing hyphae (Johns et al. 1999) has been measured in living cells.

Thus, the mechanical properties of cell walls in the growing region remained to be investigated in tip-growing cells. We have tested the use of cell wall fragments isolated from tip-growing regions in several giant-cellular algae in order to overcome the technical problems in manipulating the limited apical regions of tip-growing cells and to analyze cell wall deformation caused by artificially applied stress. In the present study, we isolated an apical cell wall fragment from the giant-cellular xanthophycean alga *Vaucheria terrestris* and inflated it by applying internal pressure. Deformation of the cell wall during inflation was recorded using time-lapse videomicroscopy until cell wall rupture. The time course of cell wall deformation was analyzed in order to clarify the cell wall extensibility in the growing region. In addition, effects of environmental pH and protease treatment on the cell wall extensibility were examined to identify possible physiological factors that could control the cell wall extensibility.

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## **Materials and Methods**

### **Algal material**

A unialgal strain of *Vaucheria terrestris* sensu Götz was obtained from Dr.

Hironao Kataoka (Tohoku University, Sendai, Japan), and cultured in double strength YCT medium (Kataoka 1987; modified by increasing the concentration of the vitamin mixture 100-fold) under the conditions of temperature  $20 \pm 2^{\circ}\text{C}$  and illumination ca.  $4 \text{ W m}^{-2}$  in 12 h:12 h light/dark cycles provided by cool fluorescent lamps.

#### Buffers and enzyme solution

Experimental buffers of pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 were made by mixing 20 mM Tris(hydroxymethyl)aminomethane and 20 mM 2-morpholinoethanesulfonic acid in various ratios, and designated as 20 mM Tris/Mes buffer. A proteolytic enzyme (P8038; subtilisin Carlsberg; Lot No. 21K1653) was purchased from Sigma, suspended in 20 mM Tris/Mes (pH 7.0) at concentration  $1 \text{ mg ml}^{-1}$ , filtrated through a membrane filter (2012-003, Iwaki Glass Co. Ltd., Tokyo, Japan) and used for protease treatment of the cell wall fragment. In the negative control experiment, an enzyme suspension autoclaved at  $121^{\circ}\text{C}$  for 20 min was used.

#### Isolation and inflation of cell wall fragments

A cell fragment including the actively growing tip, which was determined by whether it had an apical cytoplasmic zone, was excised by scissors under a dissecting microscope. After the cell fragment was transferred into a small drop of medium placed on a transparent rubber sheet, the protoplasm was gently extruded by stripping off the cell fragment from tip to base using a glass instrument with a suitable, small rounded tip as described by Menzel and Schliwa (1986). A



**Fig. 1**

3-5 mm apical cell wall fragment was cut off from the basal portion by a razor, washed with the experimental buffer 3 times, and transferred into a small drop of the buffer on a transparent plastic plate. The plastic plate was placed on an inverted microscope (CK, Olympus Co., Tokyo) equipped with a micromanipulator (MMO-202N, Narishige Scientific Instrument Laboratory, Tokyo).

A glass needle was made by pulling a glass tube (GD-1, Narishige) using a needle puller (PC-10, Narishige). A 230 g weight was used for needle pulling and the heating voltage indicator was at 63.5. The needle tip was rounded by heat using a microforge (MF-900, Narishige) and the needle was curved about 30° at the point where the needle was 120-150 micrometers thick using the microforge. A small volume of silicon oil (KF-96L-2CS, a gift from Shin-Etsu Chemical Co. Ltd., Tokyo) was sucked into the needle through the tip. The needle was installed in a needle holder (HI-7, Narishige) attached to the micromanipulator. After installation, the needle tip was moved into the experimental buffer on the plastic plate and a small amount of the buffer, usually 0.7-1.0  $\mu$ l, was sucked into the needle. The air pressure, ranging from 0.05 to 0.8 Mpa, was provided by reducing the pressure (ca. 20 MPa) of air from a scuba diving tank using an air pressure regulator. The air pressure was measured by a semiconductor pressure sensor (MPS3320T; a gift from Mitsubishi Electric Corporation, Tokyo), which had been calibrated using an ordinary pressure gauge with Bourdon tubes. The lower air pressure was then sent into the needle holder.

The needle tip was inserted into the cut end of the cell wall fragment, and

the end of the cell wall fragment was tucked up against the needle with a bamboo stick. The junction between the cell wall fragment and the needle tip was pulled out from the buffer solution, glued with a cyanoacrylate instant adhesive (Aron Alpha, Toagosei Co. Ltd., Tokyo), and submerged into the buffer solution again. The cell wall fragment was infiltrated with the buffer by applying weak pressure (0.05 MPa) inside the needle until the cell wall fragment was filled with silicon oil. During infiltration, small dust particles occasionally found in the buffer solution moved to the apical region of the cell wall fragment, indicating that the buffer solution permeated that area.

Some cell wall fragments were infiltrated with the buffer solutions containing protease prepared as described above, separated from the glass needle by excision with a razor and incubated at 30°C for 24 h. Then, the cell wall fragments were infiltrated with an experimental buffer without protease as above.

Glass powder made by grinding coverslips with a mortar and pestle was suspended in a poly-L-lysine solution (P8920, Sigma; diluted 1:10 in distilled water), incubated for 1-5 h, washed with distilled water 3 times, and suspended in the experimental buffer of the desired pH. After infiltration of the cell wall fragment with the experimental buffer, a small volume of the glass powder suspension was added to the buffer solution in which the cell wall fragment was situated. The cell wall surface was marked with particles of glass powder by vigorously pipetting the mixture of glass powder suspended in buffer and then washed with the buffer solution without glass powder to remove excess glass powder. After starting

image acquisition by time-lapse videomicroscopy, air pressure was raised to the desired internal pressure for inflating the cell wall fragment. All experiments of cell wall inflation were carried out at  $19 \pm 1^\circ\text{C}$ . The experimental setup for infiltration and inflation of the isolated cell wall described above is diagrammatically indicated in Fig. 1.

#### Image acquisition and analysis

Cell wall deformation during inflation was observed through an objective lens (LWD x 40, Olympus). Analog video images were acquired by a CCD camera (MTV-7366, Mintron Enterprise Co. Ltd., Taipei, Taiwan) through a photographic lens (NFK 2.5 x LD, Olympus), transformed into a digital video signal using a media converter (DVMC-DA1, Sony Corp., Tokyo) and fed into a personal computer through an IEEE 1394 interface. A time-lapse digital video movie was made by the computer software MotoDV (Digital Origin Inc., Mountain View, U.S.A.) with a frame acquisition interval of 0.5, 1 or 2 s.

The extension of the cell wall was determined according to the displacement of the glass powder particles along the cell wall surface on the video frames. The earliest frame after the focus was adjusted to the midst plane of the apical dome of the cell wall fragment (usually within 5 s after internal pressure application) was regarded as the first frame, whereas the frame just before cell wall rupture was regarded as the last frame. Five to 15 frames (usually 10 to 12) at regular time intervals beginning with the first frame and ending with the last frame were selected from the movie.


Each of the selected video frames was transferred to the working area of

**Fig. 2**

the illustrating software Illustrator (version 8.0, Adobe Systems, San Jose, U.S.A.). Figure 2 diagrammatically indicates the way of measuring the meridional perimeter and diameter of the cell wall. The outline of a cell wall fragment including the whole apical dome and distal part of the cylindrical basal portion was traced by a Bézier curve with five control points. A straight line parallel with the outline along the cylindrical cell wall portion was drawn and regarded as the longitudinal axis (broken line in Fig. 2). A straight line perpendicular to the longitudinal axis was drawn from the positions of the glass powder particles. The distance between the intersection of this line and the cell wall outline was regarded as the cell wall diameter at the position where the glass particle was located ( $d$  in Fig. 2). The farthest point from the cylindrical basal portion of the cell was regarded as the tip of the cell. The total length of the outline, the segmental perimeter length of the outline between neighboring glass particles, and the cell wall diameter were measured using the plug-in software, BPT-Pro (Baby Universe Inc., Yokohama, Japan).

According to Cosgrove (1993), cell wall extensibility has been defined in several ways. From the list of various meanings of 'extensibility': (Table 1 in Cosgrove 1993), (i) stress at breaking point, (ii) strain rate/stress and (iii) strain at breaking point were regarded as parameters of cell wall extensibility in the present study. Consequently, the extent of cell wall extensibility was estimated by three measures: (i) the internal pressure required for cell wall extension followed by cell wall rupture within 15 min, (ii) the total and maximum rate of meridional cell wall extension during cell wall inflation, and (iii) the amount of the cell wall's total

meridional extension until its rupture. It is generally known that plant cell walls are made of a network of cell wall polymers bound to each other, and that during plastic extension of the wall by mechanical stress, a load-bearing binding between the polymers breaks and the polymer slips until it is rejoined with another adjacent polymer (reviewed in Cosgrove 1993, 1996). Given that this is also true for *V. terrestris* cell walls, the first and second measures of cell wall extensibility indicated above might reflect the strength of polymer bindings and slipperiness between the polymers. The third one would depend on ductility of the cell wall, or the tendency that stress concentration on the crack of the material is dissipated in the form of polymer slippage (Niklas 1992). Here, total meridional extension was the increase in the whole meridional perimeter ( $M$  in Fig. 2) relative to initial cell diameter ( $D$ ) between the first frame and the last frame of the video movie. The average rate of meridional cell wall extension (creep rate) was calculated by dividing total meridional extension by the time that passed between the first and last frame. The creep rate was not constant during inflation as shown in Fig. 4. The largest extension rate observed in the successive three selected frames during inflation was regarded as the maximum creep rate.

  
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## Results

### Meridional extension of isolated cell walls

The inflation of isolated cell walls by internal pressure with silicon oil, which had been infiltrated in a neutral buffer, caused gradual extension in the apical region of the cell wall (Fig. 3). The silicon oil did not leak through the cell wall until eventual rupture of the cell wall at the apical region. In Fig. 4, changes in whole

Fig. 3

Fig. 4

meridional perimeters along the apical region of the cell wall after internal pressure application were presented in order to indicate the time-course of cell wall extension quantitatively. The results of five experiments using internal pressure 5.0, 5.5 or 6.0 MPa, where cell wall rupture occurred after more than 5 s and less than 15 min after application of internal pressure, are summarized in Table 1. As shown in Fig. 4, the cell walls exhibited time-dependent extension, or creep, until they ruptured. The extension rate during creep, which is indicated in Table 1 and also recognized as the inclination of the graph in Fig. 4, was greater in the cell walls inflated by high pressure than in those by lower pressure. On the contrary, the total extension was generally larger in the cell walls slowly inflated by low pressure than in those quickly inflated by high pressure (Fig. 4 and Table 1). This might be because the cell wall's viscosity is a non-Newtonian fluid, which is dependent on the stress rate (Niklas 1992).

**Table.**  
**1**

#### Meridional cell wall extension at different environmental pH

The measures of cell wall extensibility, i.e., total extension of whole meridional perimeter and creep rates of cell wall fragments, after infiltration of 20 mM Tris/Mes buffer at pH ranging from 4.0 to 9.0 are shown in Table 2. The pressure required for extension and rupture of cell walls was significantly higher in acidic conditions compared with low pressure in pH 7.0 or higher. Total meridional extension in pH 4.0 buffer was only one tenth to twentieth of that in weak alkaline buffers. These results indicate that, judging from the amount of deformation and the force required for deformation of the material, the apical cell wall of *V. terrestris* is very extensible in weak alkaline conditions, whereas it exhibits poor

**Table.**  
**2**

extensibility in acidic conditions. Although the extension was not as significant in neutral or weak alkaline conditions, the cell wall extension and rupture in acidic conditions was observed in the apical region also.

Total and maximum creep rates were also quite low in acidic conditions. For a more convenient comparison of cell wall extensibility among cell walls at different environmental pH, extensibility indices were calculated for each pH as follows. Maximum creep rates obtained from five experiments using each of three internal pressures were plotted over internal pressure (Fig. 5). Linear regression was drawn in order to calculate the presumptive internal pressure that would cause a maximum creep rate, 1% cell diameter s<sup>-1</sup>. This pressure was designated as an extensibility index of the cell wall fragment at the pH, and consequently, a low index indicates that only low internal pressure is required for a certain creep rate, and thus, the extensibility of the cell wall is high. As a result, the extensibility indices plotted against various pH examined in the present study show a gentle peak at pH 8.0 and a sharp decline toward acidic pH (Fig. 6). These results indicate that the cell wall is much less extensible in acidic than in neutral and weakly alkaline environments judging from the rate of deformation of the material.

**Fig. 5**

**Fig. 6**

In order to examine the reversibility of the pH dependence of cell wall extension, buffers of two different pH, 5.0 and 8.0, were used alternately for infiltration of the cell wall before cell wall inflation. As shown in Table 3, the measures of extensibility in cell walls infiltrated with the buffer of pH 5.0 after that of pH 8.0 exhibited similar characteristics to those infiltrated with pH 5.0 only (Table 2). Similarly, the cell wall infiltrated with the buffer of pH 5.0 and then with

**Table.  
3**

pH 8.0 showed the extensibility characteristics similar to those of the cell walls infiltrated with pH 8.0 buffer only. Thus, the cell wall extensibility's pH dependence was reversible.

#### Spatial distribution of cell wall extension in living cells and isolated cell walls

In order to examine the local distribution of extension along the apical cell wall, the outline of the cell wall was divided into several segments separated by the points where the particles of glass powder adhered. Meridional extension of each segment's length and radial extension of the local cell wall diameter at each point were measured. The amount that the segment or the local cell wall diameter extended was divided by its initial length and plotted over initial meridional distance from the tip as in Fig. 7. This graph shows the segmental extension profile that indicates the local distributions of both meridional and radial extensions of the cell wall. The segmental extension profile of a living, tip-growing cell is indicated in Fig. 8. It shows only a single discrete peak in the narrow segment including the tip. No extension in the subapical regions, where the distances from the tip are longer than half of the cell's diameter (0.5 in Fig. 8), was observed, indicating that cell expansion occurs exclusively in the limited region around the living cell's tip.

**Fig. 7**

**Fig. 8**

In Fig. 9, the segmental extension profiles made from inflation experiments of isolated cell wall fragments are indicated. Data were based on the total extensions observed until cell walls ruptured. Five independent experiments were carried out under various internal pressures at pH 8.0, the optimum pH for meridional extension of isolated cell walls. In most cases, both meridional and

**Fig. 9**

radial extensions were the longest in the segment including the tip of the cell wall fragment. Large extensions indicated by additional peaks or shoulders of the graph were also found around subapical regions where the distances from the tip were around half of the cell diameter ( $\pm 0.5$  in Fig. 9). In addition, the region in which small, but significant, extension occurred extends up to the basal part of the apical dome where the distance was longer than the cell diameter ( $\pm 1$  in Fig. 9). The time-course of extension of each segment indicated no significant difference in the timing of extension among segments (not shown), indicating that there is no tendency for early extensions to occur in particular portions of the cell walls. Thus, isolated cell walls from *V. terrestris* exhibited significant extension in both meridional and radial directions, and even in the subapical and basal regions, during inflation by internal pressure.

#### Extension of isolated cell walls after protease treatment

After 24 h treatment with the protease subtilisin, the isolated cell wall became extremely loosened. Total extension of whole meridional perimeter and creep rates of protease-treated cell walls at pH 5.0 or 8.0 are shown in Table 4. Internal pressure required for extension and rupture of the cell walls at pH 8.0 was remarkably reduced to about one fourth of that for intact cell walls at the same pH. Total meridional extension in pH 8.0 buffer was reduced to about a half of that observed in intact cell walls. The extensibility index of protease-treated cell walls at pH 8.0 was 0.10, which is about one fifth of that of an intact cell wall at the same pH. Thus, the protease treatment raised the cell wall extensibility about five-fold from the viewpoint of stress required for deformation and strain rate per

<p><b>Table.</b></p> <p><b>4</b></p>
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stress of the material, but the treatment made the cell wall brittle rather than ductile judging from the amount of deformation of the material until breakage.

Even after the protease treatment, the cell wall extension remained pH-dependent. Internal pressure required for cell wall extension at pH 5.0 was significantly greater than at 8.0, and the total meridional extension in pH 5.0 buffer was about half of that in pH 8.0. The creep rates were also lower than those in pH 8.0 and the extensibility index at pH 5.0 was 0.16, about 60% higher than that at pH 8.0. Thus, the protease treatment in the present study did not alter the pH dependence of cell wall extension. In addition, changes in cell wall extensibility did not occur by treatment with autoclaved protease (see Table 4; extensibility index was 0.45 at pH 8.0).

The protease treatment also caused changes in direction and distribution of cell wall extension. The segmental extension profile obtained from triplicate experiments of protease-treated cell walls in pH 8.0 buffer is presented in Fig. 10. In many cases, the peak in the apical region, including the tip, is not as significant as in intact cell walls (Fig. 9), but large extension, especially in the radial direction, occurred along a broad region extending to the basal part of cell wall where the distance from the tip was equal to or greater than cell diameter. The tendency for a large radial extension to occur along the basal region resulted in remarkable transverse swelling of the cylindrical region of the cell wall fragment before rupture (Fig. 11). In addition, the cell wall rupture also occurred in the cylindrical basal regions during inflation of protease-treated cell walls in most cases (five in six cell wall fragments used in the experiments shown in Fig. 10).

**Fig.  
10**

**Fig.  
11**

## Discussion

In the present study, we used giant tip-growing cells because they enabled us to manipulate a limited region of the apical cell walls in order to remove protoplasm and insert a glass needle with a tip wide enough to avoid closure by cytoplasmic debris. Examination of cell walls' images during inflation obtained by time-lapse videomicroscopy provided a convenient tool for analyzing temporal and spatial patterns of cell wall extension. These enabled us to investigate the extensibility of cell walls isolated from growing regions in the tip-growing cells for the first time. Because the cell wall fragments used were isolated immediately after being excised from the actively growing cell, we believe that their extensibility should reflect the mechanical properties of cell walls in living, tip-growing cells. One could argue that the original turgor pressure in the living cells was released during wall isolation and changes in wall stress caused by such treatment may possibly alter the cell wall's mechanical properties. However, it is at least possible to compare the present data with those obtained from the previous studies on the extensibility of isolated cell walls.

### pH dependence of cell wall extension

The cell wall extensibility's dependence on pH in relation to cell growth has been investigated extensively in stems and coleoptiles of angiosperms. There have been a number of studies that show promotion of the cell wall extensibility by lowering environmental pH in such cells (reviewed in Rayle and Cleland 1992). Several physiological events that may raise the cell wall extensibility in response to cell wall acidification have been suggested. For example, protonation of

pectins in acidic environments should inhibit their solidification, or gelling, with divalent cations resulting in the cell wall loosening. The environmental pH could possibly control the activities of enzymes bound to cell walls. Some of these enzymes, e.g., pectin methylesterase and peroxidase, might be concerned with the mechanical properties of the cell wall by loosening it in an acidic condition (reviewed in Hayashi 1991). As mentioned above, two species of non-enzymatic cell wall proteins, expansin and yieldin, have been known to promote cell wall extension in acidic environments by raising creep rate and lowering yield stress for extension, respectively (Cosgrove 1996; Okamoto-Nakazato et al. 2000). In addition, acid-induced expansion has been reported in cell walls isolated from giant cells of green algae, *Valonia* (Tepfer and Cleland 1979) and *Nitella* (Metraux et al. 1980). In *Valonia*, it is suggested that the acid-induced cell wall extension is not governed by cell wall proteins because the promotion of cell wall extension was not inhibited by the denaturation of proteins by 8 M urea, whereas the same treatment diminishes acid-induced cell wall loosening in oat coleoptiles (Tepfer and Cleland 1979).

In contrast to the promoted cell wall extension in acidic conditions observed in the elongating cell as mentioned above, the optimum pH for cell wall extensibility in *V. terrestris* is around 8, and the extension is significantly suppressed when pH is acidic. Since the response of the cell wall extensibility was instantaneous, the pH dependence is not likely governed by time-dependent reactions like enzymatic reaction. Moreover, since the pH dependence was almost reversible, the alteration of the cell wall extensibility according to the environmental pH is not due to irreversible loss of some structural components

from the cell wall, in particular pH.

The mechanisms that cause extension in weak alkaline environments and/or poor extensibility at low pH are unknown. In the present study, the pH dependence of the cell wall extension remained significant even after the extensive proteolysis that considerably loosened the cell walls. Although the possibility of remnant protein(s) cannot be excluded, the above result implies that the non-proteinous component(s) may govern the pH-dependent change in the cell wall extensibility. The pH-dependent association between cellulose and xyloglucan (Hayashi 1991) would be one of the candidates for such controlling mechanisms. The cell wall composition in the tip-growing region should be investigated in order to examine the components that control pH-dependent cell wall extensibility.

The actual turgor pressure of *V. terrestris* has not been directly measured. However, one turgor pressure measurement of *V. terrestris*, calculated from the cell's osmotic pressure (0.23 Osm, personal communication from Dr. Hironao Kataoka), was about 0.56 MPa, which equals the internal pressure required for extension and rupture of the isolated cell walls at neutral pH. The turgor pressures directly measured in other freshwater giant-cellular algae ranged from 0.4 MPa to 0.5 MPa (Green et al. 1971; Zhu and Boyer 1992), in which the internal pressures used for cell wall extension were included. It is naturally presumed that in the active tip-growing living cells of *V. terrestris*, the cell wall environment should be controlled so that its extensibility is secured by neutral or weak alkaline conditions. Direct measurement of the pH of tip-growing cell walls has been carried out in root hairs (Bibikova et al. 1998), and a similar

investigation in *V. terrestris*, accompanied with the present result on pH-dependent extensibility in the isolated cell walls would provide significant information on mechanical properties of cell walls in the tip-growing living cells.

Spatial distribution of extension in isolated cell walls and living tip-growing cells

The fragmental extension profile of the cell walls in the tip-growing living cells of *V. terrestris* (Fig. 8) showed a prominent extension in the narrow region including the tip. The amounts of both meridional and radial extensions immediately decrease while moving away from the tip. This represents the local distribution of cell extension similar to a previous relative elemental growth rate profile obtained from continuous observation of living *Vaucheria* cells (Fig. 6a in Kataoka 1982). Moreover, patterns of local growth distribution with a single discrete peak around the tip similar to those in *Vaucheria* have also been found in tip-growing living cells in other organisms including algal rhizoids (Chen 1973), fungal hyphae (Bartnicki-Garcia et al. 2000) and root hairs (Shaw et al. 2000).

The fragmental extension profiles of the isolated cell wall shown in Fig. 9 also indicate that the tip of the cell wall was the area extended most by internal pressure. However, they also exhibited large meridional and radial extensions around subapical regions, where no extension was observed in living cells. Cell wall extension under lower pressures than those shown in Table 2 resulted in occasional extension, and rupture of these cell walls took a much longer time (30 min to several h) after pressure application. Even in such experiments, significant extensions were also observed in the subapical region (data not shown). In cases where the cell wall is not significantly thicker in the subapical region than in the tip,

those additional extensions can be attributed to a curvature with a longer radius in the region than in the tip (Fig. 3). Nevertheless, the presence of additional extensions in the subapical region of isolated cell walls implies the possibility of additional forces, other than turgor pressure, driving the extension of cell walls in the tip-growing *V. terrestris* cells.

In contrast to the turgor-driven growth of diffuse-growing cells (Ray et al. 1972; Cosgrove 1996), recent studies on the relationship between growth rates and turgor pressure in the tip-growing cells have revealed that the growth rate is not dependent on cell turgor pressure (Benkert et al. 1997) and considerable cell growth occurred even when turgor pressure was decreased to nearly zero (Harold et al. 1996; Pickett-Heaps and Klein 1998). It has been proposed that the stress that expands the apical cell walls in such tip-growing cells was not provided by turgor pressure, but by rheological force generated by the movement of myosin along the actin cytoskeleton (Money 1997). Studies on the relationships between artificially controlled “turgor” pressure and cell growth rate have also been conducted on living giant cells of green algae where diffuse growth occurs (Green et al. 1971; Zhu and Boyer 1992). Physiological studies similar to such ones, as well as morphological investigations of the precise cytoskeletal assembly in the apical cytoplasm, are in progress in *V. terrestris* in order to clarify the mechanism generating the impetus for expanding cell walls in tip-growing living cells.

Role of cell wall proteins

In the present study, digestion of cell wall proteins by a protease dramatically loosened the cell walls in *V. terrestris*. This is indicated by a four-fold reduction in the stress required to extend the walls. On the other hand, the amount of extension until rupture (breaking strain) was also reduced. These results suggest the existence of protease-sensitive structural proteins that restrict cell wall extension by strengthening the cell wall in the alga. A well-known example of cell wall proteins that potentially strengthen the cell wall is extensin, a major structural hydroxyproline-rich glycoprotein in angiosperm cell walls. Since the protein is particularly abundant in cell walls of mature and fully differentiated tissues (Ye and Varner 1991; Tire et al. 1994), it has been postulated that extensin improves the integrity of the cell wall by means of intermolecular cross-linking to one another and forming a network interpenetrated by other cell wall components (reviewed in Cassab 1998). Despite information about the structure, distribution and regulatory properties of the protein, there is no evidence for its functions in controlling the mechanical properties of cell walls (reviewed in Showalter 1993). Cell wall glycoproteins homologous to extensin have been found in several tip-growing cells (Rubinstein et al. 1995; Baumberger et al. 2001; Bucher et al. 2002). Therefore, it is also possible to argue that such proteins are present in the cell walls of *V. terrestris*.

In contrast to the results that cell wall extension and rupture were invariably observed in the apical region of intact cell wall fragments regardless of the environmental pH, the protease treatment induced considerable changes in the local distribution and the direction of the cell wall extension. The prominent transverse expansion and rupture were frequently observed in the cylindrical

basal region of the protease-treated cell wall fragments during inflation by internal pressure (Figs. 10, 11). This implies that the presumptive cell wall protein(s) that could strengthen the cell wall may function as a hoop that limits the radial expansion of the cylindrical portion of the cell, where the cell wall microfibrils were arranged longitudinally rather than transversely (Kataoka 1982). The necessity of such structural components is also suggested by the fact that the wall stress generated by internal pressure in the radial direction is twice as strong as that in the longitudinal direction (Pickett-Heaps and Klein 1998). In order to examine the existence of such cell wall protein(s), which might be an important component for cell morphogenesis in tip-growing cells, attempts to isolate and characterize proteins that bind to *V. terrestris* cell walls are in progress.

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## **Acknowledgements**

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## Figure legends

**Fig. 1** Experimental setup for infiltration and inflation of apical cell wall fragment isolated from *V. terrestris*. A: Scuba diving air tank, B: Air pressure regulator, C: Semi-conductor air pressure sensor, D: Solenoid-operated air valve, E: Needle holder, F: Silicon oil, G: Glass needle, H: Glue, I: Isolated cell wall, J: Experimental buffer, K: Plastic plate

**Fig. 2** Diagram of measurement of meridional distance from the tip ( $m$ ), local cell wall diameter ( $d$ ), cell diameter ( $D$ ) and whole meridional perimeter including apical region ( $M$ ). G: Particle of glass powder

**Fig. 3** Deformation and rupture of apical cell wall fragment during inflation by 0.5 MPa internal pressure. The cell wall fragments had been infiltrated with 20 mM Tris/Mes buffer pH 7.0. (a) 0 s, (b) 138 s, (c) 216 s after application of internal pressure. An arrow indicates the site of cell wall rupture where a jet of the silicon oil was emitted

**Fig. 4** Changes in whole meridional perimeter of apical cell wall fragments during inflation by various internal pressures until rupture of the fragment. Closed triangle: 0.45 MPa, open square: 0.50 MPa, open circle: 0.55 MPa. The cell wall fragment had been infiltrated with 20 mM Tris/Mes buffer pH 7.0

**Fig 5** Relationship between internal pressure and maximum creep rate during cell wall inflation in environmental pH 5.0 and 8.0. Average (closed square: pH 5.0, open square: pH 8.0), standard deviation (vertical bars) and linear regression curve are indicated. Data were obtained from five independent experiments

**Fig. 6** Extensibility indices of cell wall fragments at different environmental pH.

Calculation of the indices is explained in the Results section

**Fig. 7** Diagram of segmental extension measurements and calculations, and a graphical expression of the segmental extension profile

**Fig. 8** The segmental extension profile observed in a tip-growing living cell during 20 min incubation

**Fig. 9** The segmental extension profile observed in isolated apical cell wall fragments during inflation by various internal pressures until rupture of the fragment. The cell wall fragment had been infiltrated with 20 mM Tris/Mes buffer pH 8.0

**Fig. 10** The segmental extension profile observed in apical cell wall fragments during inflation by various internal pressures until rupture of the fragment. (**a** to **c**) 0.1 MPa, (**d** to **f**) 0.13 MPa. The cell wall fragments had been incubated in protease solution for 24 h and infiltrated with 20 mM Tris/Mes buffer pH 8.0. Cell wall rupture occurred in cylindrical basal region of the cell wall fragments, except for that shown in **e**

**Fig. 11** Deformation of apical cell wall fragment during inflation by 0.125 MPa internal pressure. The cell wall fragments had been incubated in protease solution for 24 h and infiltrated with 20 mM Tris/Mes buffer pH 8.0. (**a**) 0 s, (**b**) 79.5 s after application of internal pressure. At 80 s, the cell wall ruptured in the cylindrical basal portion out of the field of view. Note the remarkable transverse swelling along the cylindrical portion of the cell wall

Table 1. Time of rupture after beginning of inflation, total extension of whole meridional perimeter until rupture and total and maximum creep rate during inflation of apical cell wall fragments isolated from *V. terrestris* under various internal pressures. The apical cell wall fragments had been infiltrated with 20 mM Tris/Mes buffer pH 7.0. Average and standard deviation obtained from five independent experiments are shown

Internal pressure (MPa)	Time of rupture (s)	Total meridional extension (% cell diameter)	Creep rate	
			Average (% cell diameter s <sup>-1</sup> )	Maximum
0.50	340 (± 194)	46.8 (± 7.4)	0.183 (± 0.122)	0.298 (± 0.177)
0.55	168 (± 104)	46.9 (± 9.1)	0.350 (± 0.152)	0.757 (± 0.335)
0.60	35 (± 27)	26.5 (± 11.2)	1.192 (± 1.115)	1.581 (± 1.120)

Table 2. Total extension of whole meridional perimeter until rupture and total and maximum creep rate during inflation after infiltration with buffer (20 mM Tris/Mes buffer) of various pH. Average and standard deviation obtained from five independent experiments are shown except for triplicates at pH 4.0

pH	Internal pressure <sup>a</sup> (MPa)	Total meridional extension (% cell diameter)	Creep rate	
			Average (% cell diameter s <sup>-1</sup> )	Maximum
4.0	0.70	4.1 (± 2.8)	0.03 (± 0.03)	0.04 (± 0.03)
	0.75	4.3 (± 3.3)	0.03 (± 0.01)	0.05 (± 0.02)
	0.80	6.2 (± 3.9)	0.09 (± 0.04)	0.18 (± 0.10)
5.0	0.60	17.8 (± 7.2)	0.05 (± 0.03)	0.13 (± 0.07)
	0.65	15.2 (± 2.6)	0.14 (± 0.06)	0.40 (± 0.27)
	0.70	18.4 (± 8.4)	0.27 (± 0.12)	0.50 (± 0.24)
6.0	0.55	31.2 (± 4.2)	0.09 (± 0.04)	0.18 (± 0.08)
	0.60	33.4 (± 7.3)	0.30 (± 0.24)	0.50 (± 0.31)
	0.65	22.8 (± 3.1)	1.02 (± 0.60)	1.52 (± 0.60)
7.0	0.50	46.8 (± 7.4)	0.18 (± 0.12)	0.30 (± 0.18)
	0.55	46.9 (± 9.1)	0.35 (± 0.15)	0.76 (± 0.34)
	0.60	26.5 (± 11.2)	1.20 (± 1.12)	1.58 (± 1.12)

Table 2. (continued)

8.0	0.45	48.8 ( $\pm$ 3.7)	0.14 ( $\pm$ 0.03)	0.21 ( $\pm$ 0.05)
	0.50	43.1 ( $\pm$ 4.4)	0.33 ( $\pm$ 0.15)	0.64 ( $\pm$ 0.47)
	0.55	43.76 ( $\pm$ 16.5)	1.58 ( $\pm$ 0.71)	1.91 ( $\pm$ 0.76)
9.0	0.45	44.3 ( $\pm$ 6.4)	0.17 ( $\pm$ 0.18)	0.34 ( $\pm$ 0.38)
	0.50	40.6 ( $\pm$ 4.3)	0.60 ( $\pm$ 0.25)	0.95 ( $\pm$ 0.40)
	0.55	44.9 ( $\pm$ 5.2)	0.67 ( $\pm$ 0.25)	1.03 ( $\pm$ 0.40)

<sup>a</sup> Internal pressures are those where cell wall rupture occurred between 5 to 900 s (15 min) after application of internal pressure

Table 3. Total extension of whole meridional perimeter until rupture and total and maximum creep rate during inflation after alternate infiltration with two buffer solutions (20 mM Tris/Mes buffer) of different pH. Average and standard deviations obtained from five independent experiments are shown


pH		Internal pressure <sup>a</sup> (MPa)	Total meridional extension (% cell diameter)	Creep rate	
1st	2nd			Average	Maximum
				(% cell diameter s <sup>-1</sup> )	
5.0	8.0	0.45	42.2 (± 4.1)	0.29 (± 0.20)	0.43 (± 0.32)
		0.50	44.4 (± 9.4)	0.97 (± 0.96)	1.08 (± 0.73)
		0.55	35.5 (± 16.9)	1.72 (± 1.10)	2.06 (± 0.99)
8.0	5.0	0.60	16.1 (± 7.5)	0.04 (± 0.04)	0.06 (± 0.05)
		0.65	12.6 (± 5.0)	0.06 (± 0.06)	0.19 (± 0.17)
		0.70	10.8 (± 5.6)	0.16 (± 0.11)	0.4 (± 0.35)

<sup>a</sup> Internal pressures are those where cell wall rupture occurred between 5 to 900 s (15 min) after application of internal pressure

Table 4. Total extension of whole meridional perimeter until rupture and total and maximum creep rate during inflation after protease treatment and infiltration with buffer (20 mM Tris/Mes buffer) of pH 5.0 or 8.0. Average and standard deviations obtained from five independent experiments are shown

pH	Internal	Total meridional	Creep rate	
	pressure <sup>a</sup>	extension	Average	Maximum
	(MPa)	(% cell diameter)	(% cell diameter s <sup>-1</sup> )	
5.0	0.13 <sup>b</sup>	14.0 (± 6.5)	0.38 (± 0.44)	0.73 (± 0.58)
	0.15 <sup>b</sup>	17.7 (± 5.3)	0.55 (± 0.46)	0.92 (± 0.75)
8.0	0.10 <sup>b</sup>	26.7 (± 4.6)	0.76 (± 0.66)	1.45 (± 1.30)
	0.13 <sup>b</sup>	25.4 (± 13.4)	3.78 (± 1.44)	6.63 (± 1.76)
8.0 <sup>c</sup>	0.45	59.3 (± 13.0)	0.43 (± 0.71)	1.04 (± 1.48)
	0.50	60.4 (± 14.9)	0.82 (± 0.44)	1.43 (± 1.21)
	0.55	45.0 (± 14.3)	1.62 (± 1.39)	2.14 (± 1.33)

<sup>a</sup> Internal pressures are those where cell wall rupture occurred between 5 to 900 s (15 min) after application of internal pressure <sup>b</sup> protease-treated cell walls were so fragile that a narrower range of internal pressures were used <sup>c</sup> experiments using autoclaved protease as a negative control for protease treatment

  
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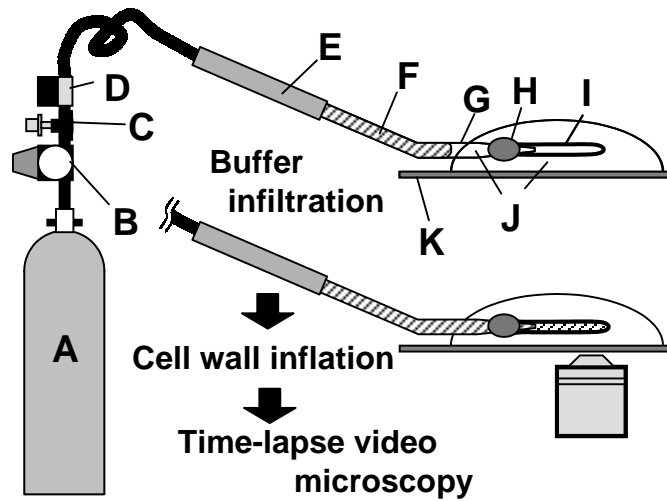
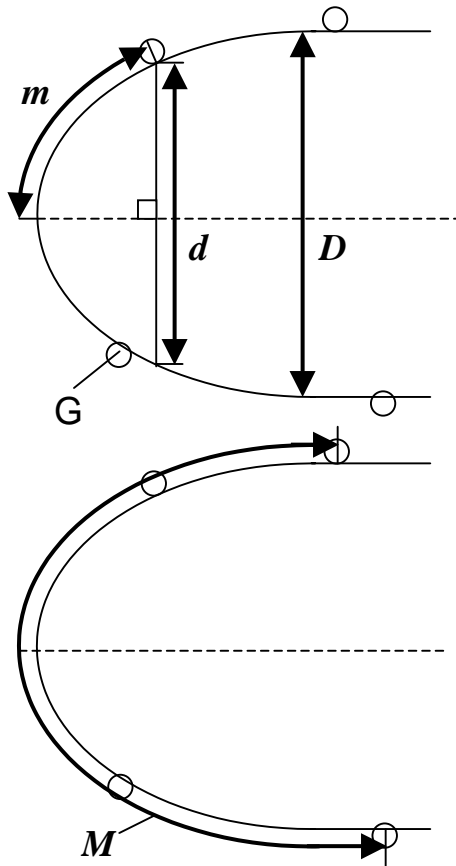
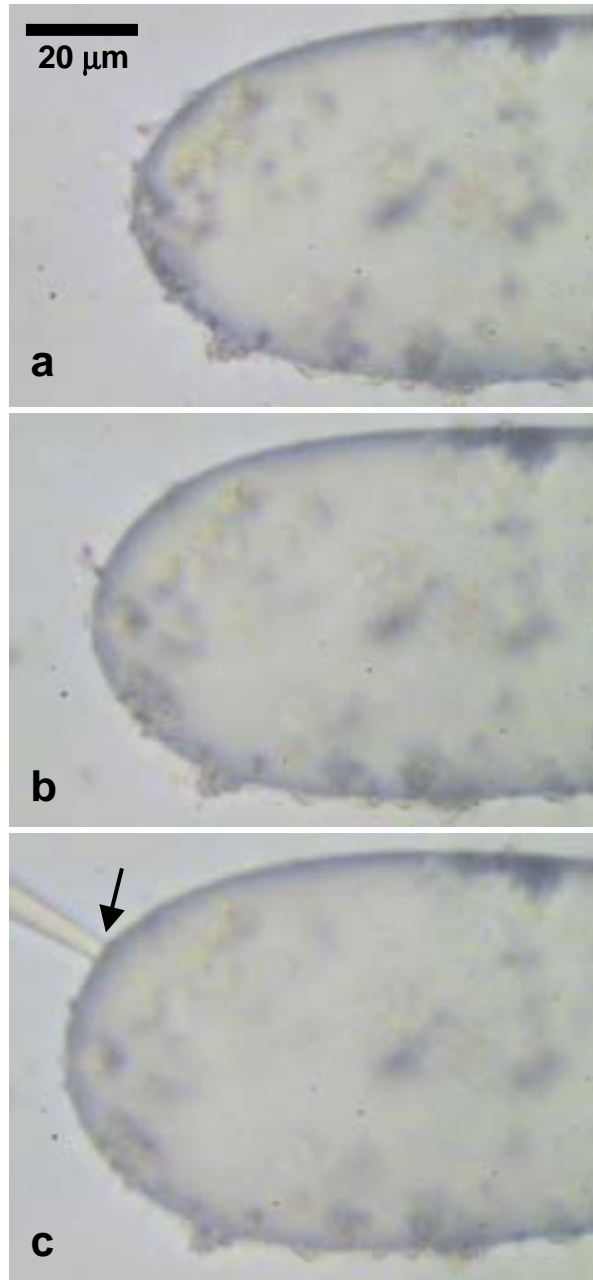


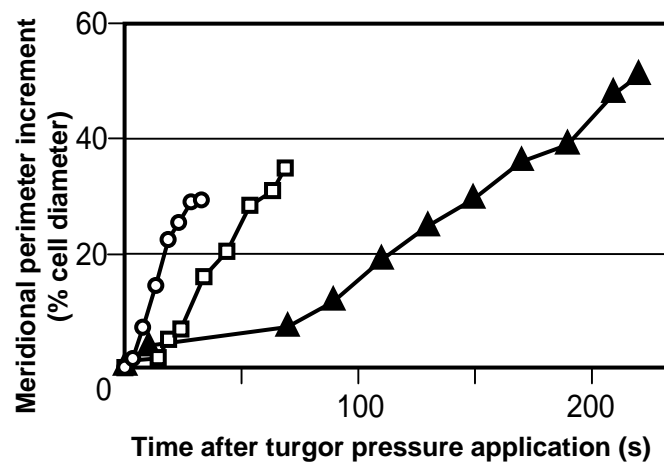
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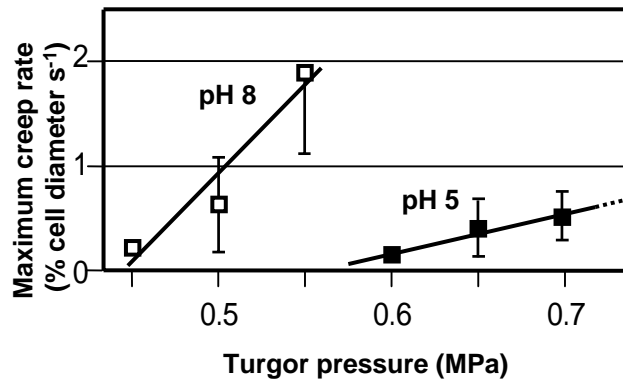
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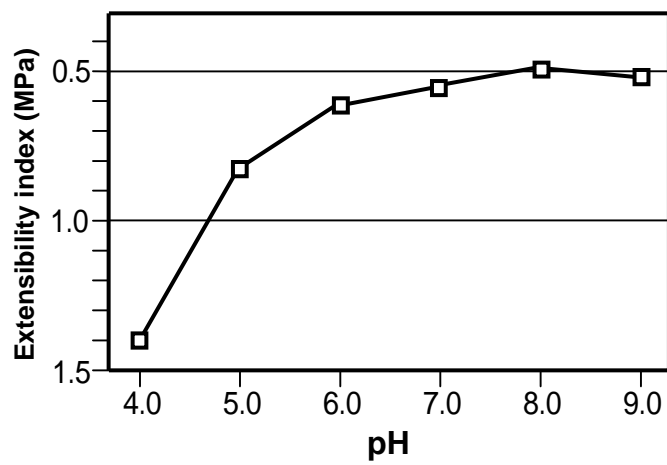
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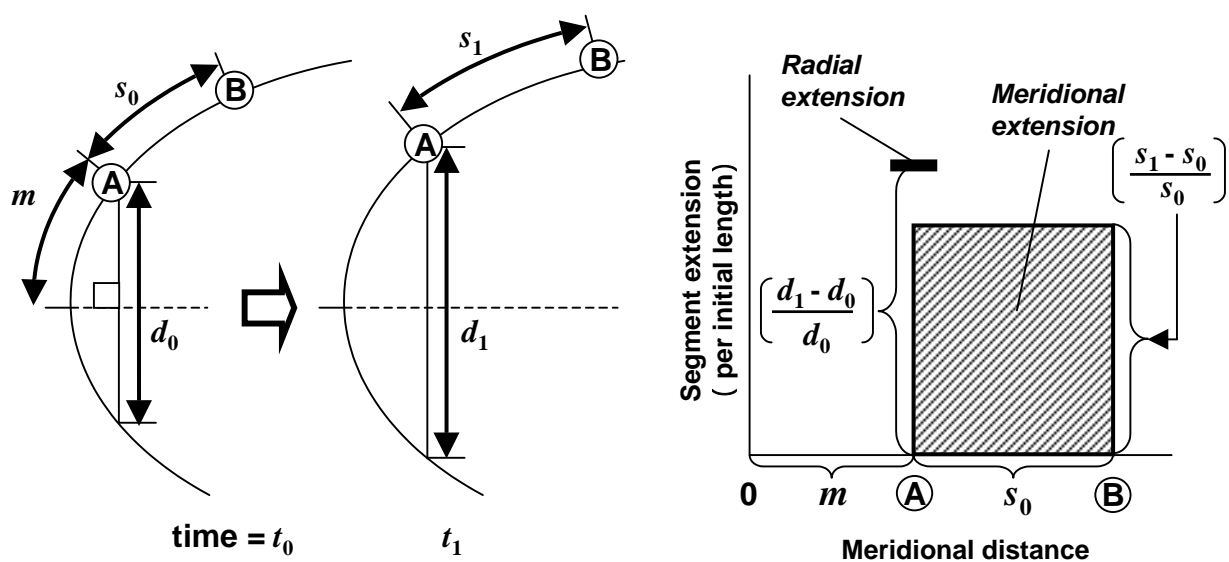
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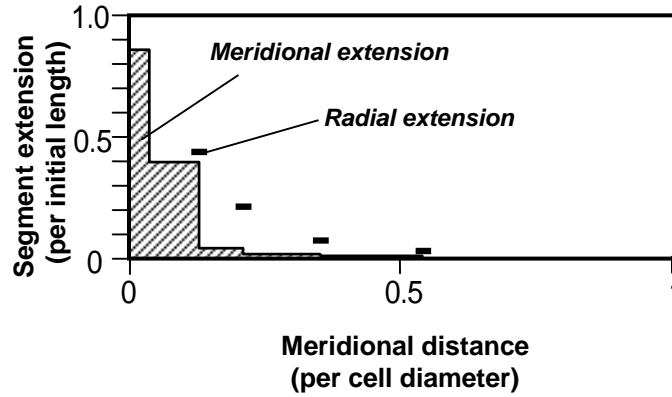
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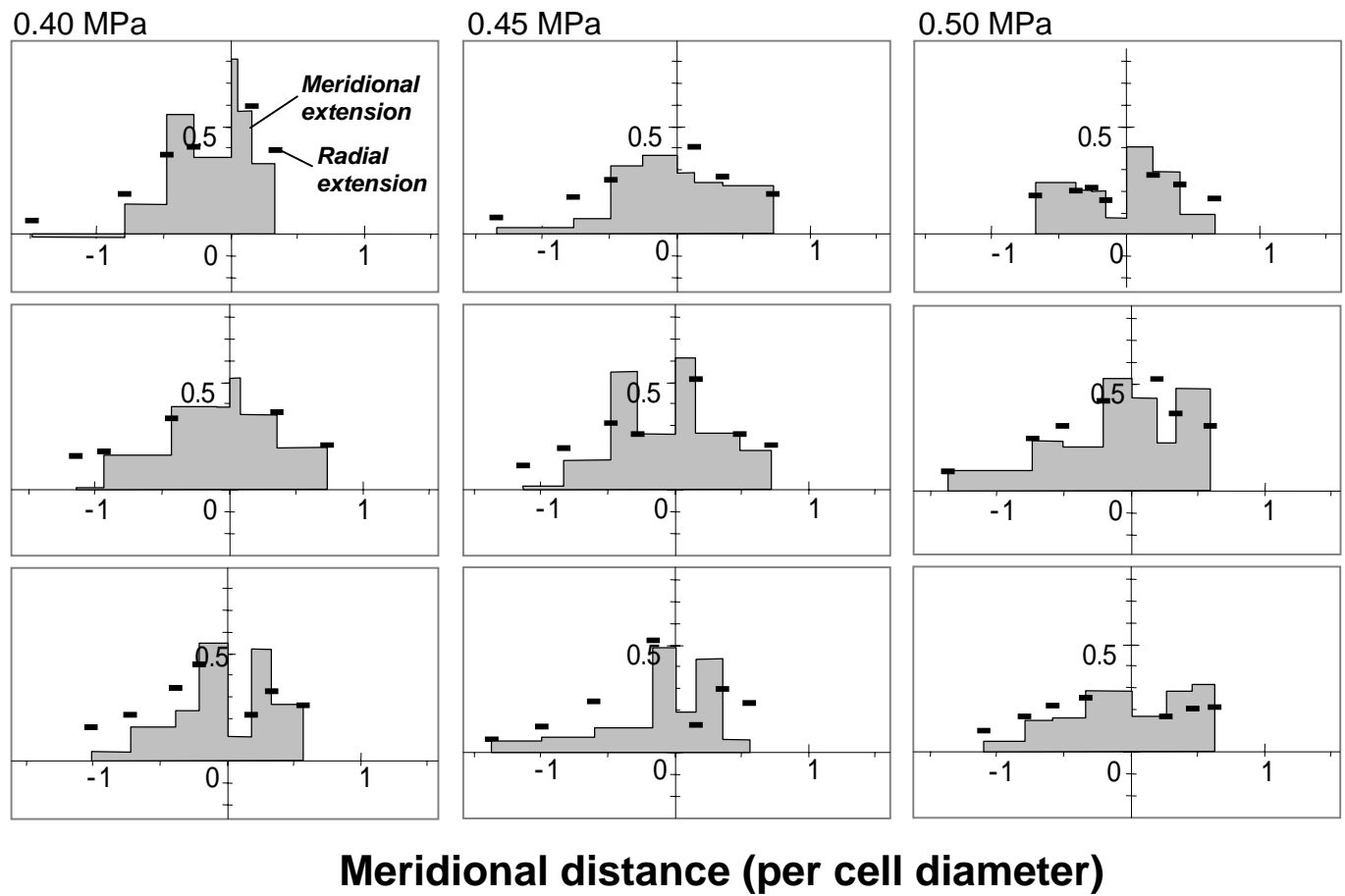
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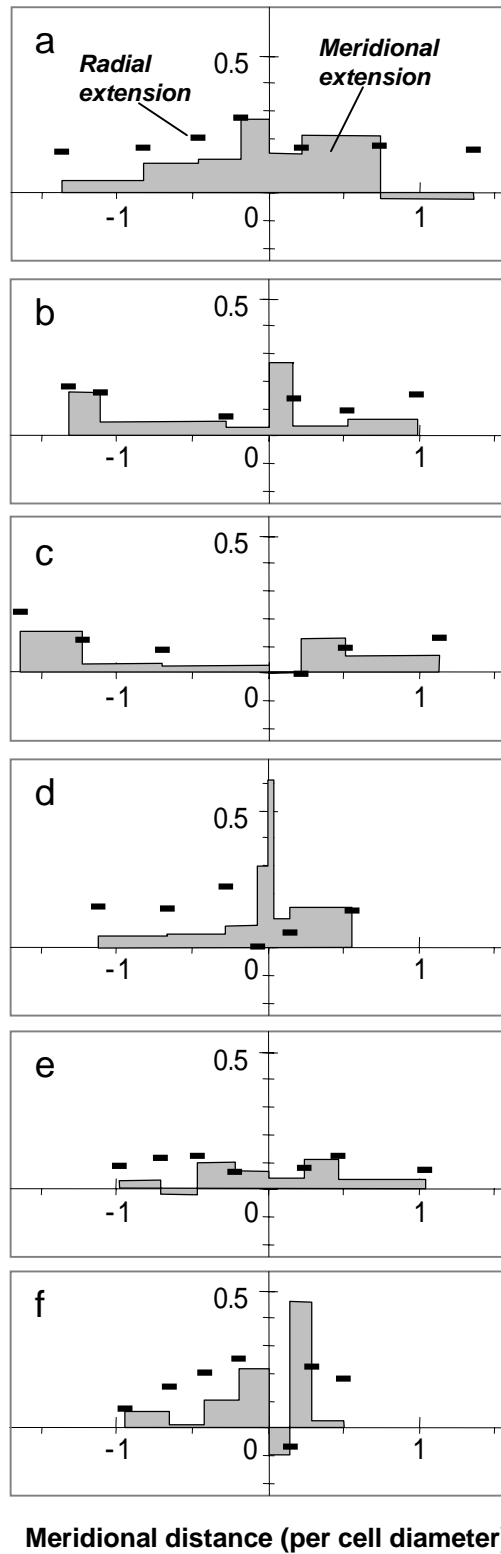
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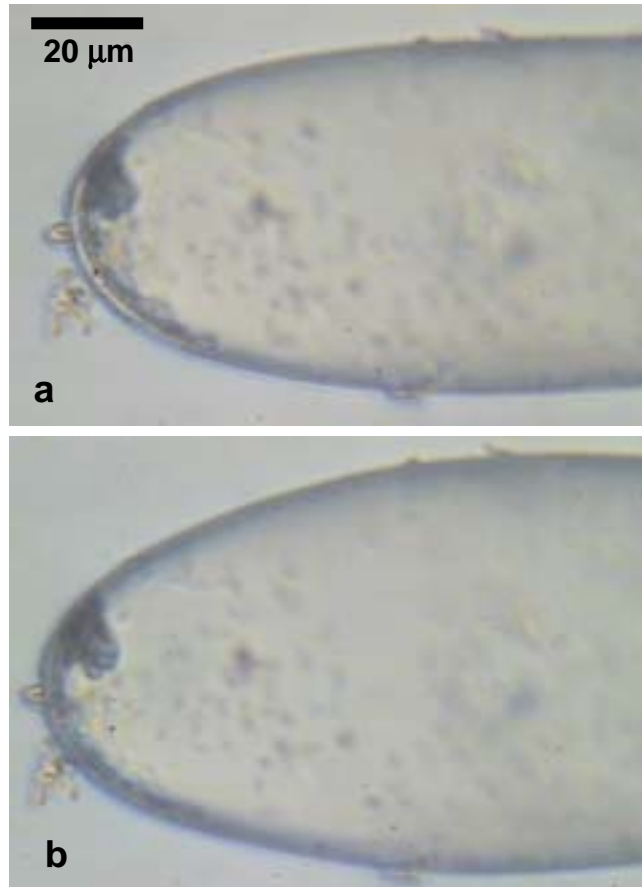
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