Turgor, temperature and the growth of plant cells: using *Chara corallina* as a model system

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Abstract

Rapid changes in turgor pressure (*P*) and temperature (*T*) are giving new information about the mechanisms of plant growth. In the present work, single internode cells of the large-celled alga *Chara corallina* were used as a model for plant growth. *P* was changed without altering the chemical environment of the wall while observing growth without elastic changes. When *P* was measured before any changes, the original growth rate bore no relationship to the original *P*. However, if *P* of growing cells was decreased, growth responded immediately without evidence for rapid changes in wall physical properties. Growth occurred only above a 0.3 MPa threshold, and increasing *P* caused small increases in growth that became progressively larger as *P* rose, resulting in a curvilinear response overall. The small changes in growth close to the threshold may explain early failures to detect these responses. When *T* was lowered, the elastic properties of the cell were unaffected, but growth was immediately inhibited. The lower *T* caused *P* to decrease, but returning *P* to its original value did not return growth to its original rate. The decreased *P* at low *T* occurred because of *T* effects on the osmotic potential of the cell. At above-normal *P*, growth partially resumed at low *T*. Therefore, growth required a *P*-sensitive process that was also *T*-sensitive. Because elastic properties were little affected by *T*, but growth was markedly affected, the process is likely to involve metabolism. The rapidity of its response to *P* and *T* probably excludes the participation of changes in gene expression.

Key words: Elastic effects, cell walls, cell enlargement, wall properties.

Introduction

In most plant cells, turgor pressure (*P*) is required for growth. The *P* must be above a minimum, and growth appears as a steady increase in size if *P* is steady (Cleland, 1971; Taiz *et al.*, 1981; Taiz, 1984; Passioura, 1994). When *P* is increased, there are early adjustments often interpreted as alterations in wall physical properties (Green *et al.*, 1971; Green and Cummins, 1974; Kuzmanoff and Evans, 1981; Matthews *et al.*, 1984; Ortega *et al.*, 1989; Serpe and Matthews, 1992, 1994). The alterations were thought to maintain the growth rate nearly constant (Green *et al.*, 1971). In support of this concept, little or no changes were observed in growth rates at several permissive *P* (Green *et al.*, 1971; Zhu and Boyer, 1992). On the other hand, at higher *P*, rates increased as *P* increased (Green *et al.*, 1971; Zhu and Boyer, 1992). Sometimes rates increased only at *P* above those normally present in the cells (Zhu and Boyer, 1992). Moreover, it has recently been reported that most of the early responses to *P* were elastic (Proseus *et al.*, 1999). When elastic extension was subtracted from the total enlargement of the cells, the growth rate changed with *P* but showed little or no evidence for alterations in wall physical properties, which raises anew the question of how growth responds to *P*.

Enlargement is one of the most fundamental activities of plants, and there are many simultaneous processes involved. Several could be *P*-dependent. For example, *P* might affect the insertion of new wall polymers (Ray, 1962; Robinson and Cummins, 1976). Metabolic processes could be involved because low temperature (*T*) is inhibitory (Ray and Ruesink, 1962; Haughton and Sellen, 1969; Proseus *et al.*, 1999). Enzymes in the wall might

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participate by affecting the bonds between the polymers (Passioura and Fry, 1992; Carpita and Gibeaut, 1993; Cosgrove, 1997), and would be inhibited by low T. However, because enzyme activities are little affected by pressures having the magnitude of P, the P is unlikely to control enzyme activity directly.

On the other hand, P stretches the wall and probably causes wall polymers to slide past each other, deforming the wall to a permanently larger size without a metabolic contribution (Lockhart, 1965a, b; Cleland, 1971; Taiz, 1984; Passioura and Fry, 1992; Cosgrove, 1993, 1997). However, in order for this mechanism to represent growth, the T response should be similar to that for growth, the stretching should be irreversible and the sliding should continue when internal cell metabolism is inhibited.

There could be an altered expression of genes whose products change wall metabolism or the mix of wall polymers. However, in order for this mechanism to explain the effect of P, the response must be as fast as the response to P.

These possibilities were explored by developing a method that rapidly and permanently changes P without altering the wall environment (Zhu and Boyer, 1992). Low T was used to remove elastic effects so that growth alone could be observed (Proseus et al., 1999). These methods were combined with digital techniques having high resolution. The objective was to change P rapidly, remove elastic effects and observe in detail how growth responded to P and T in a single plant cell.

Materials and methods

Plant material

Cultures of *Chara corallina* Klien ex. Willd., em. R.D.W. were maintained as previously described (Zhu and Boyer, 1992). Culture pH and T were sampled at several times during the course of the study to ensure a stable pH at 8–8.5 and culture T from 22–23 °C. Fluorescent fixtures and ambient sunlight in the culture room provided the cultures with continuous 10–15 µmol m^{-2} min^{-1} PAR at the medium surface. For each experiment, a single internode cell was excised by hand from the distal portion of the thallus. Growing cells were usually taken from the region near the apex of the thallus and mature, non-growing cells were taken from lower portions of the thallus. The initial length of each cell was measured before placement in the growth apparatus. All experiments were conducted in the growth medium in a controlled environment chamber with T and light conditions matching the conditions in the cultures.

Experimental apparatus

A large pressure probe was used to monitor P and perform P clamps according to methods described previously (Zhu and Boyer, 1992; Proseus et al., 1999). Basically, the experimental internode cell was excised from the plant, branches were removed, and the cell was placed in a trough in growth medium. One end was held in a scissors-like gate and the tip of the capillary for the pressure probe was inserted into the immobilized end of the cell. The capillary had been pre-loaded with solution from other internode cells (which were discarded). After measuring the initial P, the cell solution was injected to increase P, or removed to decrease P. For increasing P, repeated small injections were made until the new P remained steady without further injection. The cell was then permanently at the new P. This procedure worked because injecting cell solution caused water to move out of the cell, but the new solute to remain inside. As the solute concentration built up with the small injections, the new osmotic potential supported the new P without further injection. For decreasing P, cell solution was removed, and the new P became steady after many small removals. This worked because solute was removed from the cell and the entering water diluted the cell solution, causing a new lower P.

Cell elongation was monitored with a radial position transducer (RVIT, Lucas Control Systems, NJ, USA) attached by wire to the free end of the cell. T was monitored with a fine thermocouple in the medium alongside the cell. The apparatus was carefully designed and tested to minimize any T response from the equipment itself. The reader is referred to Proseus et al. (Proseus et al., 1999) for more detailed descriptions of T control, medium delivery, and apparatus construction.

A datalogger system (Campbell CR7, Campbell Scientific, Logan UT, USA) provided a digital record of each experiment (Proseus et al., 1999). Cell P (MPa), L (µm), growth rate (dL/dt), and T (°C) were recorded once every 5 s or once every minute as described previously (Proseus et al., 1999).

Separating elastic extension from growth

Because changes in P caused expansion or contraction of the cell that were partially elastic, the method of Proseus et al. was used to remove the elastic component and reveal growth alone (Proseus et al., 1999). For the *Chara* internode cells, enlargement was almost entirely longitudinal and was largely independent of cell length. Therefore, the elastic and growth components were expressed in simple linear form by an equation first suggested by Ortega (Ortega, 1985, 1990) and simplified to:

$$\frac{dL}{dt} = m(P - P_c) + \frac{L_o dP}{\varepsilon_L dt} \qquad (1)$$

where dL/dt is the longitudinal extension rate (m s^{-1}), m is the irreversible extensibility of the cell wall (m s^{-1} MPa^{-1}), L_o is the original cell length (m), and ε_L is the longitudinal component of the elastic modulus (MPa). The term $m(P - P_c)$ is the growth component and the term $L_o(dP/dt)/\varepsilon_L$ is the elastic component. Proseus et al. (Proseus et al., 1999) eliminated the first term by exposing the cells to cold T that prevented growth but did not alter the elastic properties of the cell. According to equation 1:

$$\frac{dL}{dt} = \frac{L_o dP}{\varepsilon_L dt} \qquad (2)$$

By subtracting equation 2 measured at cold T from equation 1 measured at warm T in the same cell, the growth component was revealed:

$$\frac{dL}{dt} = m(P - P_c) \qquad (3)$$

It should be noted that m represents all of the biological and physical factors affecting growth and not simply inert polymer effects.

Hydraulic conductivity

Cell hydraulic conductivity Lp (m s^{-1} MPa^{-1}) was measured by subjecting internode cells to P pulses (0.03 MPa). The half

time ($t_{1/2}$) of the P relaxation was recorded (Steudle and Zimmerman, 1974; Zimmerman and Steudle, 1975), and Lp was calculated according to:

$$Lp = \frac{\ln 0.5V}{t_{1/2}A(\varepsilon_v - \Psi_s)} \qquad (4)$$

where V is the cell volume (m³), A is the cell surface area (m²), ε_v is the volumetric elastic modulus (MPa), and Ψ_s is the cell osmotic potential (MPa). A and V were determined from the measurements of length and diameter of the cell prior to the experiment. Ψ_s was assumed to equal $-P$ as shown by Zhu and Boyer (Zhu and Boyer, 1992). ε_v was calculated from the volume of solution injected to accomplish the 0.03 MPa P pulse according to:

$$\varepsilon_v = 0.03 \frac{V}{dV} \qquad (5)$$

where dV is the volume of injected cell solution measured from the change in position of the cell solution/oil meniscus in the probe capillary.

Evaluation of earlier study

In order to obtain a more detailed assessment of the growth/P relationships in *Chara* internodes, computer-aided techniques were used to reanalyse a portion of the data reported in the study by Zhu and Boyer (Zhu and Boyer, 1992). Since the earlier investigation did not use a datalogger or a computer, no digital records of the data were available. Therefore, several of the original graphs of cell length and P were scanned directly into a desktop computer and saved as enlarged pictures (PICT format). The pictures of each graph were then viewed with GraphicConverter software (Lemke Software, Piene, Germany) on a Power Macintosh 6100 computer. The y-axis to x-axis proportionality of each picture was increased from 1:1 to 3:1 to exaggerate the slope of the cell length versus time trace. The growth rate of a cell at each value of P was measured from the enhanced graphs by selecting two points on the cell length trace where growth appeared to be steady. Pixel coordinates for the two points were recorded, and the slope of a straight line connecting the two points was used as a measurement of the average dL/dt for the interval between the points. The growth rates obtained for the individual measurements were plotted against the corresponding P also measured directly from the enlarged graphs.

Results

The internode cells grew at elevated rates for about 30–40 min after excision from the intact plant. The experiments were delayed until after that time, when growth was steady for several hours or was slowly decelerating (Fig. 1). Cytoplasmic streaming occurred throughout the experiments.

Response to P

The cells grew in a dilute medium whose Ψ_w was nearly zero. As a consequence, the Ψ_s essentially equalled $-P$ (Zhu and Boyer, 1992). The P clamp operated by adding or removing cell solution. This allowed P to be changed, but increasing P above the original subjected the wall to
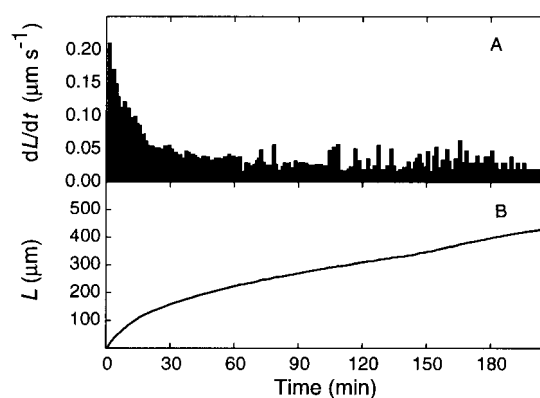

Fig. 1. Growth rates (A) and L (B) for an internode cell of *Chara* at various times after excision from the parent plant. Rates in (A) were obtained from digital data in (B). Area under the curve in (A) was darkened to emphasize small differences in rate. Original length of the cell was 12 mm. T was 23 °C.

larger forces than in the intact plant. Decreasing P below the original did not subject the wall to these large forces.

It was tested whether growth responded similarly to these forces by measuring the original P and making the steps. When a step-down P clamp was used (left side of Fig. 2A), the cell shrank immediately (left side of Fig. 2B) and small removals of cell solution held the new P nearly constant (Fig. 2A). There were slight variations in the length of the cell because of the small removals. However, the effects were small and growth resumed at a slower rate immediately after the step-down (slope of the trace became lower after the step-down, Fig. 2B). When P was returned to the original level (right side of Fig. 2A), there was a rapid elongation followed by a gradual transition to a steady growth (right side of Fig. 2B).

Exposing the same cell to cold eliminated growth but had little effect on the shrinkage or rapid elongation or gradual transition (7.3 °C, Fig. 2C). The shrinkage and rapid elongation were subtracted from the total response at 23 °C in Fig. 2B. The gradual transition was not subtracted because it was sometimes smaller in the cold. With these subtractions, growth responded immediately and smoothly to the step-down from the original P (Fig. 2D, left side). There were no gradual changes. During the step-up, elongation immediately increased and, after the gradual transition, the growth rate resumed at near the original rate before the P step (Fig. 2D, right side). The slight spikes in Fig. 2D (asterisks) can be disregarded because they were caused by an inability to start the P steps at precisely the same time in the 5 s interval between individual data, and they appeared in the subtraction.

In general, a similar pattern emerged when P was stepped up from the original (Fig. 3) instead of being stepped down as in Fig. 2. The step-up caused rapid elongation followed by a gradual transition to more rapid growth (left side, Fig. 3B). The return to the original P
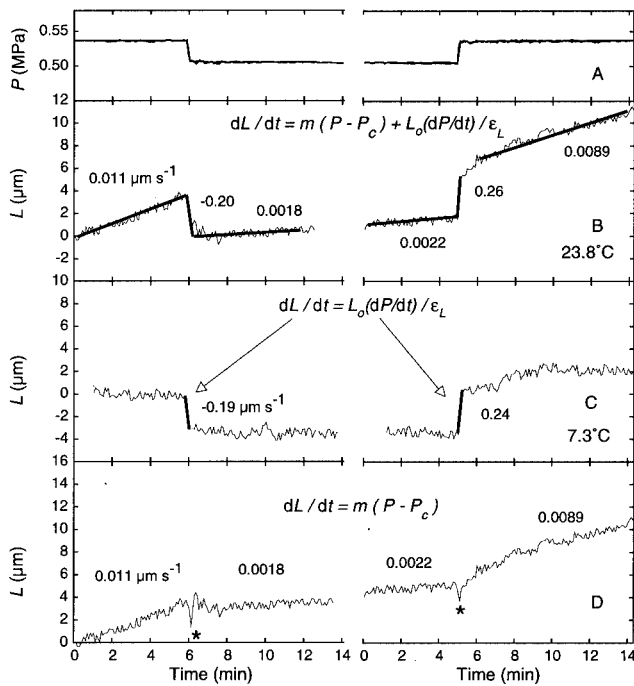
Fig. 2. Growth in response to *P* step below the original *P* in a single internode cell of *Chara*. (A) Original *P* of the cell, followed by a downward step and return to the original *P*, (B) total elongation at 23.8 °C, (C) total elongation in the same cell at 7.3 °C, and (D) growth obtained at each *P* by subtracting heavy lines in (C) from corresponding locations in (B). Original *P* was measured before any other manipulations were done, and represents the *P* existing naturally in the intact cell. *P* steps in (A) consisted of large initial removal or addition of cell solution followed by many small removals/additions that caused slight variations in length (B). After about 10 min, no further removals/additions were required and *P* remained steady. Two superimposed traces are shown, one for the elongation in (B) and one for elongation in (C). The relevant form of equation 1 is shown in (B), (C), and (D). Data for thin lines are running averages of 20 s for measurements every 5 s, and data for heavy lines are regressions for the data in the thin lines underneath. Numbers beside heavy lines are rates obtained from the slopes of the regressions. The gap between the heavy lines in (B) during the return to original *P* indicates the gradual transition to steady elongation. * In (D) show brief spikes originating from slight asynchronies in (B) and (C) that were not related to growth. The asynchrony was caused by the inability to start the *P* steps in (B) and (C) at identical times within the 5 s interval between consecutive data points. *L* is shown as the length beyond L_o at the beginning of the trace. Originally, cell length was 20 mm, growth rate was 0.011 μm s^{-1}, and *P* was 0.536 MPa.

caused rapid shrinkage followed by immediately decreased growth (left side, Fig. 3B). In the cold, the step-up showed a gradual transition to the new rate but the transition was small. During the step-down, there was no evidence for this transition. After subtracting the rapid elastic component in Fig. 3C (heavy lines) from the total elongation in Fig. 3B, there was an immediate elongation response to each *P* step (Fig. 3D). During the step-up, elongation was more rapid and gradually settled to a steady rate after about 2 min. Following the immediate response during a step-down, new steady growth occurred with no gradual transition. At the end of the experiment, the growth rate was much slower than the original one
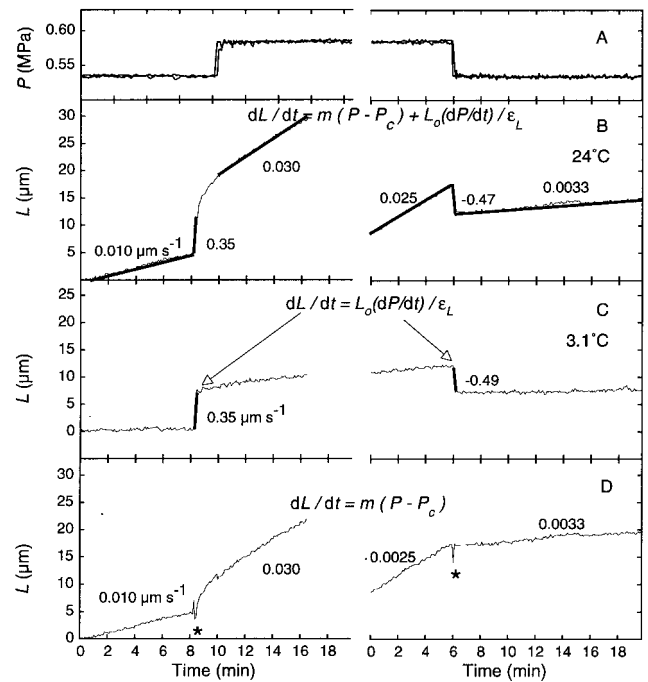

Fig. 3. Same as Fig. 2 except the *P* was stepped up above the original *P*. In (B), the gradual transition is large after the upward step (gap between heavy lines). In (D), the growth rate after the return to the original *P* was less than the original rate. Originally, cell length was 16 mm, growth rate was 0.010 μm s^{-1}, and *P* was 0.532 MPa.

(0.0033 versus the original 0.010 μm s^{-1}) despite a return to the original *P* (0.53 MPa). Therefore, the step-up above the original *P* caused an effect on subsequent growth that was different from a step-down from the original. It should be noted that the step-up exposed the cell to a higher *P* than the maximum normally present in the cell (controlled by its original Ψ_s). This is in contrast to the step-down experiment (Fig. 2) where *P* was never above the maximum.

For simplicity in the remaining experiments, the elastic and growth components will not be separated, but the initial rates will be interpreted as though there is an underlying growth (and gradual transition during step-up) that continues, as in Figs 2 and 3. The approach can be seen in Fig. 4, where a step-down causes a temporary large decrease in rate (Fig. 4B) but a steady underlying growth rate appears after elastic effects are subtracted and the small removals/additions of cell solution are completed (Fig. 4C). By not taking the time to carry out the subtraction, many steps could be used and growth rates observed over a wide range of *P* but the data interpreted in terms consistent with Figs 2 and 3.

Using this approach in Fig. 5, it is evident that growth rates decreased after each *P* step-down (steps 2–5). There were a few negative rates (cell shrinkages) as solution was removed, but otherwise the change to the new slower growth rate was immediate with no evidence for gradual changes. By step 5, *P* had decreased about 0.16 MPa
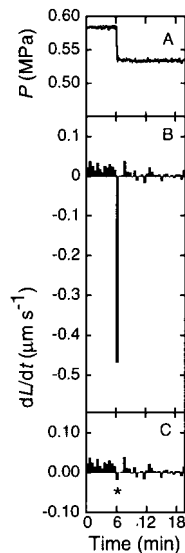
Fig. 4. Growth shown as rate (dL/dt) during a P step-down. (A) P step-down, (B) total elongation rate at 23 °C, and (C) growth rate at 23 °C. Total rate in (B) was measured directly. Growth rate in (C) was obtained after subtracting elastic component from total rate in (B), as in Figs 2 and 3. The area under the curve in (B) and (C) was darkened to emphasize small differences in rate; * shows a spike not related to growth, as in Fig. 2.

Fig. 5. Elongation of a single *Chara* internode cell subjected to a series of P steps below the original P. (A) P steps, (B) L, (C) dL/dt, and (D) growth rates at various P. In (B), numbers beside curve correspond to P steps in (A). In (C), dL/dt were determined from total elongation in (B) once per minute. For downward steps 2–5, negative dL/dt in (C) are caused by initial rapid cell shrinkage followed by subsequent small shrinkages as the cell solution was removed until P became steady in (A). For upward steps 6–10, initial rapid swelling is followed by subsequent small swellings as cell solution was added until P became steady in (A). However, in contrast to downward steps, the upward steps are also accompanied by a gradual transition to steady growth. In (D), dL/dt are taken from the slope of a regression line fit to the appropriate steady segment of L in (B) during the last few minutes of the P clamp. Originally, cell length was 16 mm, growth rate was 0.056 μm s^{-1}, and P was 0.520 MPa.

(Fig. 5A) and growth had decreased nearly to zero (Fig. 5B, C). When P was then increased in steps 6–10, the cells elongated rapidly at first followed by a gradual transition to steady growth, partly because of small additions of cell solution necessary to maintain each higher P. By step 9, P had returned to its original level shown at step 1, and steady growth had returned nearly to the original rate. Note that, in contrast to steps-down, there were gradual transitions to the new steady rate after each step-up (Fig. 5C, Steps 6–10). These represent the gradual transitions to the new rate shown in Figs 2 and 3 after a step-up. It was necessary to wait until growth was steady at each step to identify the underlying rate, and a plot of this growth at various P showed small growth responses at low P and larger ones at high P, indicating that the response was curvilinear (Fig. 5D). At P below about 0.36 MPa (70% of the original P), growth did not occur. This P is designated as P_c, the critical threshold pressure for growth.

In contrast to this experiment involving P below the original, stepping P above the original exposed the wall to forces greater than had occurred under natural conditions and caused a marked growth stimulation (Fig. 6). By step 7 (Fig. 6A), P was 0.2 MPa above the original, and the growth rate was nearly 5× the original rate (Fig. 6D). As in Fig. 3, if the high P was lowered to the original P, the growth rate was always slower than the original rate (data not shown).

When these data at high and low P were combined (Fig. 7), the growth response was curvilinear over the
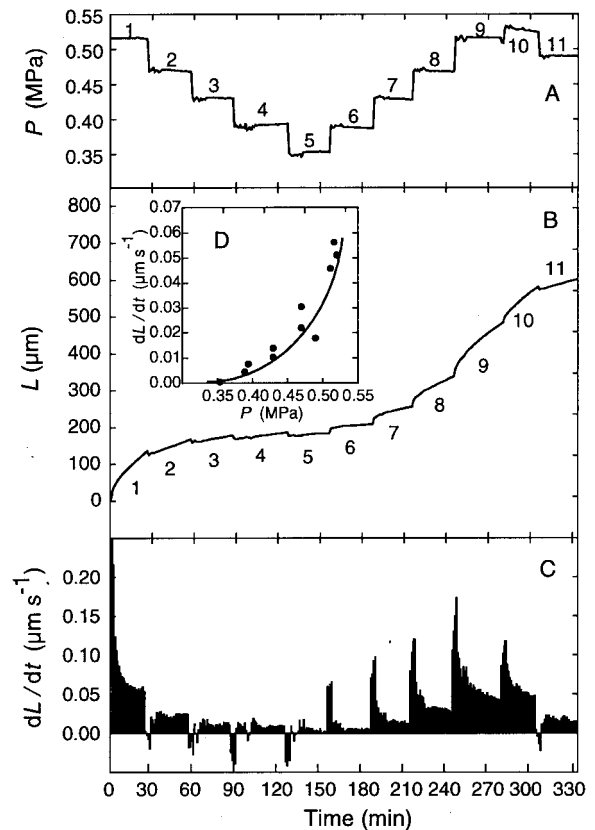
entire range of P. P_c occurred at 70–90% of the original P. Figure 8 shows that there was no correlation between the original growth rate and the original P of the cells (Fig. 8A) or the values for P_c (Fig. 8B). Therefore, $P–P_c$ was approximately the same in all cells regardless of the original growth rate. The mean $P–P_c = 0.12$ MPa ($n = 7$, Fig. 8C).

As controls, mature cells treated similarly showed rapid elastic and subsequent gradual responses to a P step up, but no growth. Figure 9A shows that rapid initial elongation was evident at each P step above the original (vertical heavy lines, Fig. 9B) followed by a slower gradual transition to zero growth (gap between heavy lines, Fig. 9B). Although the P were above the original and thus higher than would occur normally, cell lengths eventually became
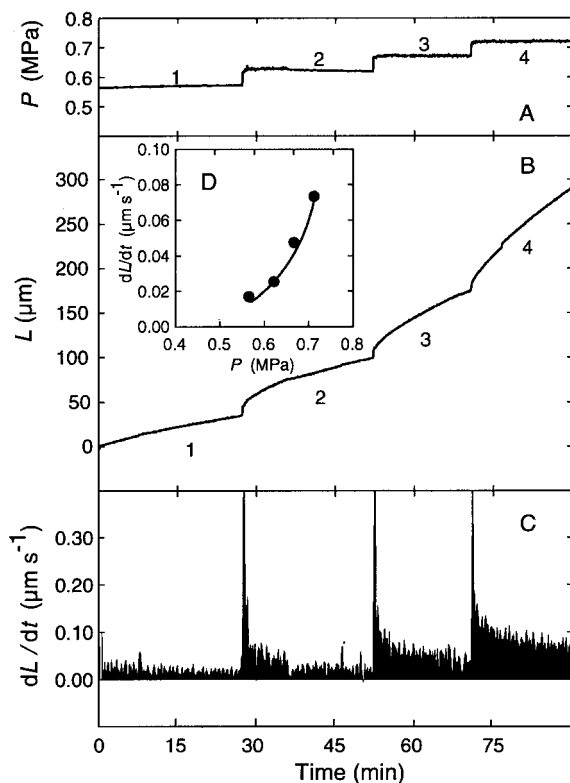
Fig. 6. Same as Fig. 5 except *P* steps were above the original *P*. Originally, cell length was 16 mm, growth rate was 0.017 μm s^{-1}, and *P* was 0.555 MPa.
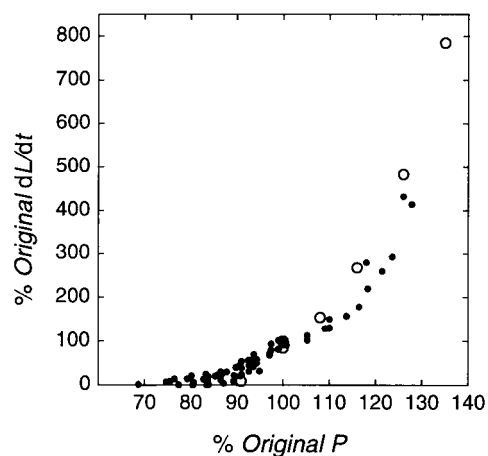

Fig. 7. Growth rates at *P* below and above the original *P* in several cells. Open circles show the response of a single cell.

constant at each new *P* (Fig. 9B). As a consequence, steady rates of elongation centred on zero (Fig. 9C) and growth was undetectable for all *P* (Fig. 9D).

Response to T

During these experiments, we found that a decrease in *T* always caused a decrease in *P*. In order to conduct the above experiments, it was necessary to adjust *P* to make
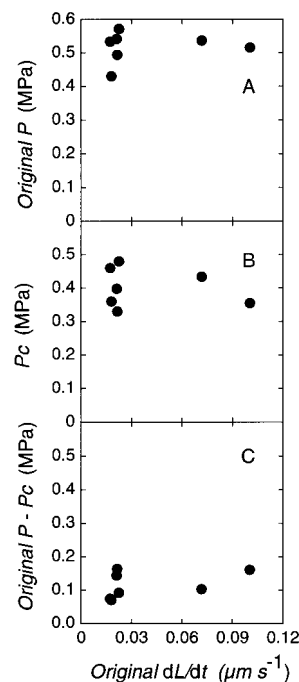

Fig. 8. Pressures in cells having various original growth rates. (A) Original *P* in the cell, (B) P_c measured as in Fig. 5, and (C) Original $P–P_c$ for each cell.

up for this effect. If *P* was not adjusted, *P* decreased about 0.03 MPa (compare *P* at 1 and 2, Fig. 10A) when *T* of 23 °C was decreased to 8 °C (Fig. 10C). Growth was prevented at the low *T* (Fig. 10B). The effect was completely reversible and *P* recovered when *T* was returned to 23 °C (compare *P* at 1 and 3, Fig. 10A). Growth also recovered (Fig. 10B). It should be noted that the gradual responses of growth to *T* in this experiment were caused by the gradual changes in *T* and not by delayed responses to *P*.

The effect of *T* on *P* was such a consistent factor in our experiments that its origin was investigated. According to the van't Hoff relation, *T* affects Ψ_s in proportion to the Kelvin *T*:

$$\Psi_s = -RT\left(\frac{n_s}{V}\right) \qquad (6)$$

where Rn_s/V is a proportionality constant consisting of the gas constant *R* (8.32×10^{-6} MPa m^3 mol^{-1} K^{-1}), the number of moles of solute n_s (also a constant), and the volume of water in the cell *V* (nearly constant, and the slight variation will be ignored). It seemed possible that *T* could have acted on Ψ_s, and *P* simply reflected this action. This theory was tested by forming the ratio of Ψ_s for any two *T*, causing the constants in equation 6 to cancel. The Ψ_s ratio was then the same as the *T* ratio, and because -*P* approximated Ψ_s in these cells:

$$\frac{\Psi_{s2}}{\Psi_{s1}} = \frac{T_2}{T_1} \approx \frac{P_2}{P_1} \qquad (7)$$
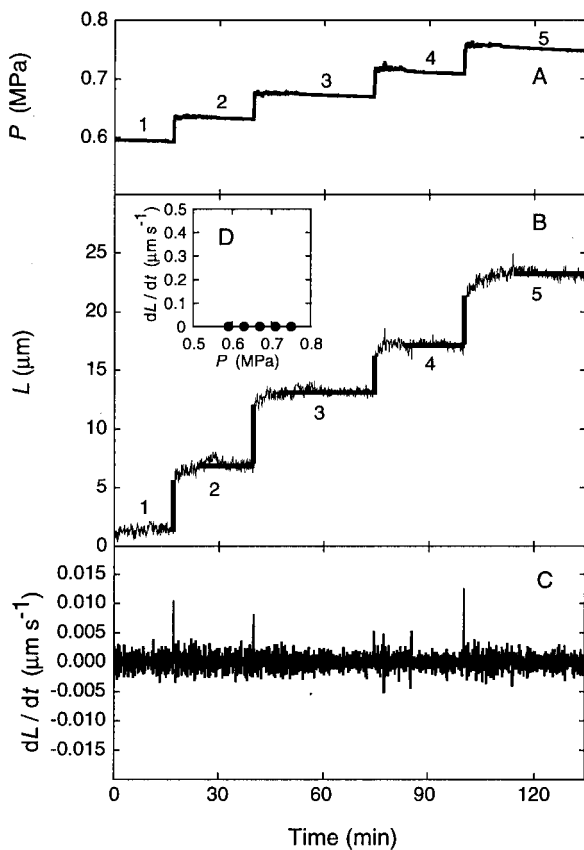
Fig. 9. Same as Fig. 5, except cell was mature (control) and *P* steps were above original *P*. Heavy lines show rates during step-up and after trace became stable. Gap between heavy lines is gradual transition to final rate. Originally, cell length was 21 mm, growth rate was 0.0 μm s^{-1}, and *P* was 0.594 MPa.

Fig. 10. Low *T* decreased original *P* in *Chara* internodes. (A) *P*, (B) *L*, (C) *T*. In (A), (1) shows original *P*, (2) shows effect of low *T*, and (3) shows the effect of a return to original *T*. All measurements were in the same cell, and *P* was allowed to reflect the effects of *T* passively. (D–F) are the same as (A–C) except *T* was not returned to the original *T* and instead *P* was returned to the original *P* using a *P* step at low *T* (D, 3). Note that in (C) and (F), *T* change required about 10 min and was thus slower than *P* change in (D, 3). *P* and *L* showed gradual responses in (B) and (E, 2) attributable to the slow changes in *T*.

Figure 11 shows that *T* affected *P* by nearly the same amount as *T* affected Ψ_s (the *P* response shown as the solid line nearly coincided with the Ψ_s response shown as the 1 : 1 dashed line). Therefore, most of the effect of *T* on *P* was through its effect on Ψ_s.

This effect raised the possibility that low *T* inhibited growth by decreasing *P* rather than by directly inhibiting chemical or metabolic reactions. This possibility was tested by lowering *T* (Fig. 10F), but returning *P* in the cold to the level it had been in the warm (Fig. 10D). Figure 10E shows that growth remained inhibited and did not return to the original rate. It exhibited only the rapid elastic and gradual responses to *P*.

On the other hand, if *P* was increased above the original level, growth resumed. Decreasing *T* to 8 °C (Fig. 12C) caused *P* to decrease (Fig. 12A, step 2) and growth to be completely inhibited (Fig. 12B, step 2), but increasing *P* above the original level caused growth to begin again (Fig. 12B, steps 3–5). At each step, elongation showed elastic and gradual components that were similar to those at 23 °C.

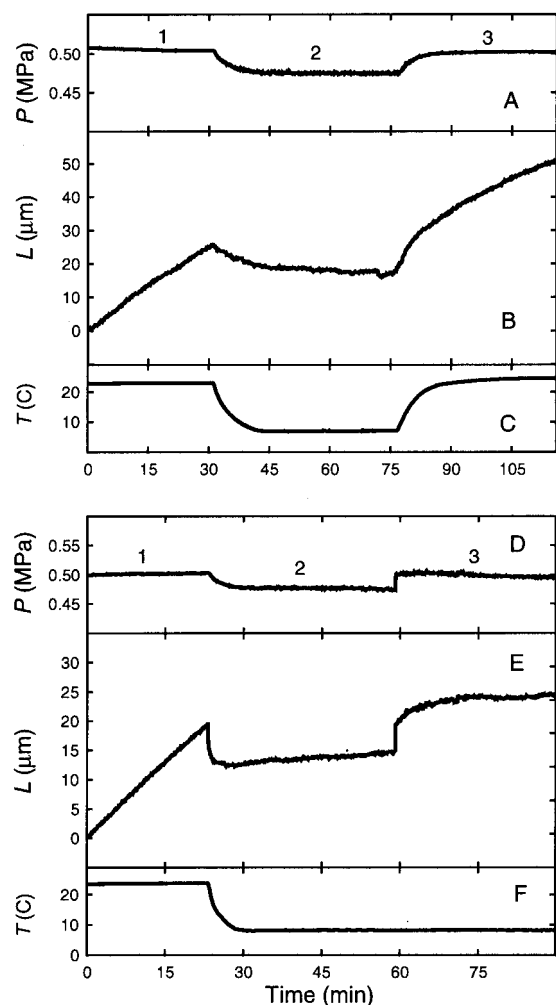The growth resumption in the cold indicated that P_c

appeared to have shifted to higher *P* (Fig. 13A). This effect was clearer at *T* of 0.1 °C (Fig. 13B) which was markedly inhibitory to growth at all *P* (Fig. 13B). However, P_c had moved above the original *P*, and growth began again slowly at very high *P*. For this experiment, the *P* was adjusted before chilling and again after chilling to keep it identical for each cell at the two *T*. Low *T* could inhibit growth only by inhibiting factors other than *P*. Growth could resume at low *T* only because of high *P*.

The cell hydraulic conductivity *Lp* (m s^{-1} MPa^{-1}) was also measured in order to determine whether the growth inhibition at low *T* could be attributed to limited water uptake. Chilling decreased cell *Lp* from a mean of 2.33 m s^{-1} MPa^{-1} at 23 °C (range: 1.45–3.01 m s^{-1} MPa^{-1},
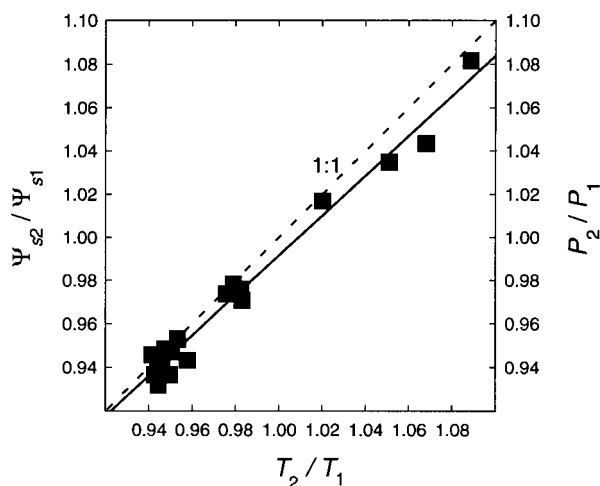
Fig. 11. Effect of T on Ψ_s (dashed line, calculated from Equation 7) and P (solid line, directly measured). Solid line is formed by regression through data points (■). T_2/T_1, Ψ_{s2}/Ψ_{s1}, and P_2/P_1 equal 1.00 at T of growth medium (296 K).

$n=5$) to 1.80 m s^{-1} MPa^{-1} at 7 °C (range: 1.27–2.47 m s^{-1} MPa^{-1}, $n=5$). Thus, the mean Lp in the cold was 77% of that at 23 °C.

Discussion

P affects growth

These data show that growth was rapidly and closely coupled to P in individual cells of *Chara corallina*. When P was low, growth did not occur. When P was higher, cells capable of growth grew according to P. The response was observed over a wide range including P higher than normally present in the cells. Clearly, at least one P-requiring process was essential for growth and the whole growth process could be prevented if P was below the required range. In a similar fashion, low T prevented growth. The effect was evident if P was kept rigorously constant, but could be reversed by increasing P. Growth thus involved reactions sensitive to both P and T.

At the same time, each cell had its own characteristic growth rate at the same P. The characteristic rate had developed over long times as the cell developed in the intact plant. It was not possible to predict how fast a cell would grow at its original P even though growing cells responded similarly to variations in P. Mature cells did not grow regardless of P. This suggests that long-term plant growth was regulated internally, as has frequently been observed in other experimental systems (Cleland, 1971; Taiz, 1984).

Does P_c change when P changes?

Green *et al.* suggested that P_c changes as P changes, thus regulating growth rates (Green *et al.*, 1971). If P_c changes are regulatory, they must occur in one direction during a step-down and in the reverse direction during a step-up,
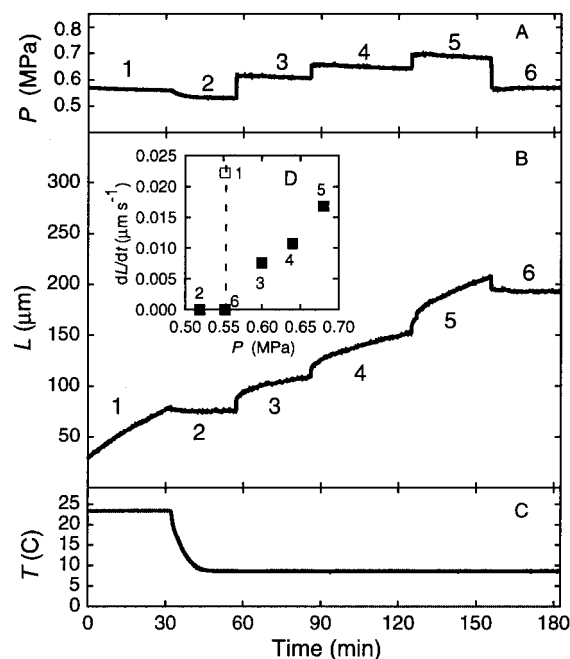

Fig. 12. Elongation at high P when a cell is cold-treated. (A) P, (B) L, (C) T, and (D) dL/dt at various P. Numbers beside curves correspond to P steps in (A). P step 1 is original P in warm conditions, and P step 6 is an identical P but in the cold. P steps 3–5 show growth in the cold at P higher than the original P. Vertical dashed line in (D) is original P. Originally, cell length was 6 mm, growth rate was 0.022 μm s^{-1}, and P was 0.568 MPa.

i.e. P_c changes would need to occur in both directions in order to generate regulatory action. If P_c changed only in one direction, a return to the original growth rate would not be possible when P returned to the original level. Figure 14 shows a diagrammatic representation of the changes observed with the P clamp. A P step-down followed by a comparable P step-up caused the growth rate to return to the original level (Growth$_3$ = Growth$_1$, Fig. 14). Rapid elastic change accounted entirely for the cell shrinkage during the step-down (Fig. 2), as also reported by Proseus *et al.* (Proseus *et al.*, 1999). It should be noted that the elasticity was measured independently, but in the same cell in which growth was measured, thus maximizing the chance of detecting a change in P_c. Because the elastic effects were distinct from growth, and growth changed immediately to a slower rate, there was no evidence for a regulation of P_c during the step-down. Without regulatory action of P_c during a step-down, it follows that there could have been no rapid regulatory action of P_c during a step-up.

P was directly changed using the P-clamp in order to maintain the normal chemical environment of the cell wall and to avoid the use of external osmotica. Osmotica expose the wall to large concentrations of solute, which can alter growth rates in a solute-specific manner, as shown by Zhu and Boyer (Zhu and Boyer, 1992). *Chara* showed a temporary growth cessation with mannitol that
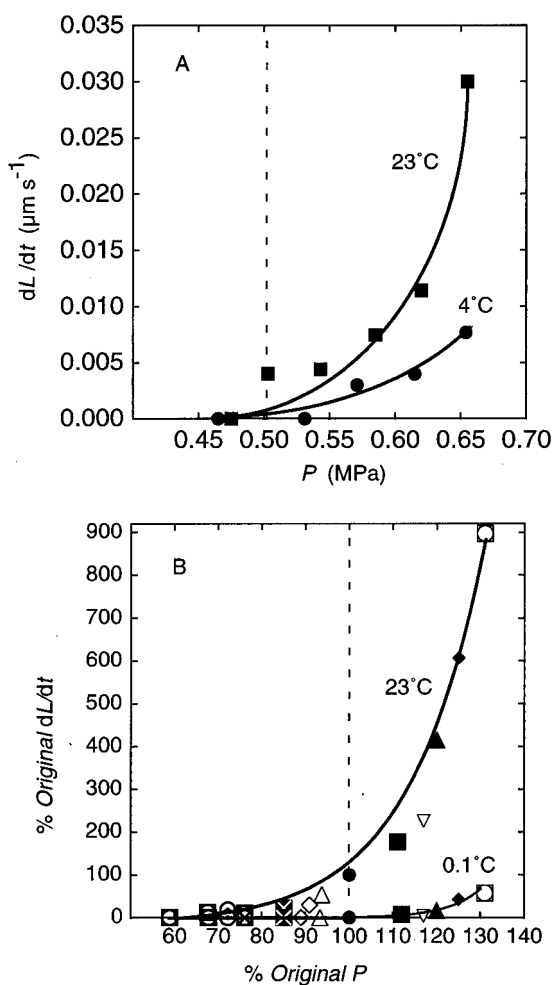
Fig. 13. Growth above and below the original *P* in the cold. (A) Single cell. *P* steps in (A) were made first at 23 °C and then at 4 °C. (B) Many cells. Each pair of symbols in (B) represents growth of a single cell at identical *P*. *P* was first adjusted above or below the original *P* at 23 °C, and growth was measured. The *T* was decreased to 0.1 °C, *P* was adjusted to the same level, and growth was again measured. Vertical dashed line represents original *P*.

Fig. 14. Schematic diagram of a *P* step-down followed by a *P* step-up using the *P* clamp. Not shown are small length variations following the rapid elastic changes, attributable to small removals or additions of cell solution to maintain the new *P*.

was absent when *P* alone was changed in the same cell (Zhu and Boyer, 1992). This indicates that the temporary growth cessation was attributable to a direct effect of the solute on the chemical environment of the wall. It is likely that some of the differences between these results and other studies based on osmotica (Green *et al.*, 1971; Hsiao and Jing, 1987; Frensch and Hsiao, 1994, 1995; Serpe and Matthews, 1992) can be attributed to these solute effects. Adverse effects of osmotica have been reported by others (Adebayo and Harris, 1971; Greenway, 1974; Hughes and Street, 1974; Zimmerman, 1978; Nagahashi *et al*, 1990; Money and Harold, 1992; Passioura and Fry, 1992; Zhu and Boyer, 1992). The solute effects are usually attributed to the plasma membrane being permeable to the solutes, pooling of the solutes in the cell wall, or inhibition of enzyme activity in the wall (Adebayo and Harris, 1971; Greenway, 1974; Hughes and Street, 1974;
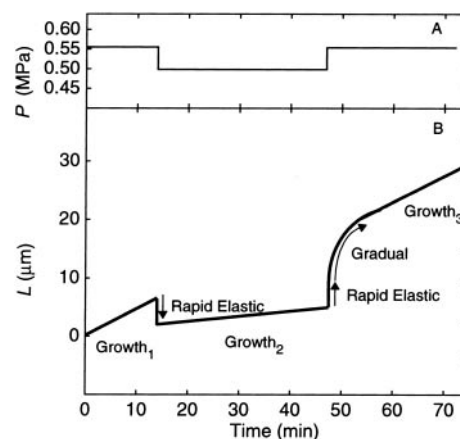
Zimmerman, 1978; Nagahashi *et al*, 1990; Money and Harold, 1992; Zhu and Boyer, 1992). It seems that osmotica give a first approximation of the growth response to *P* but the results need to be interpreted cautiously and include the possibility of direct solute effects acting on the cell.

The *P* clamp made it possible to measure P_c in single cells. Because growth was a curvilinear function of *P* and not linear, it was not possible to extrapolate from a few *P* measurements to find P_c. If it had been possible to extrapolate from the data in Fig. 5B, the first 0.05 MPa step-down would indicate a P_c of 0.44 MPa when the actual P_c was approximately 0.35 MPa. Therefore, a direct measure of P_c was necessary. With that measure, the *original* $P - P_c$ were around 0.1 MPa and similar to those in multicellular plant and fungal systems (Matthews *et al.*, 1984; Boyer *et al.*, 1985; Hsiao and Jing, 1987; Okamoto *et al.*, 1990; Ortega *et al.*, 1991).

Other authors have questioned whether P_c changed during short-term experiments (Ortega *et al.*, 1989; Cleland, 1971). In corn coleoptiles, no evidence of a change of wall mechanical properties was found when external osmotica were changed (Hohl and Schopfer, 1992), and Green and Cummins thought the immediate physical extensibility of oat coleoptiles could have remained stable under similar conditions (Green and Cummins, 1974). Adjustments of P_c over longer time scales have been reported in multicellular plants (Matthews *et al.*, 1984; Shackel *et al.*, 1987; Nakahori *et al.*, 1991; Serpe and Matthews, 1994; Maruyama and Boyer, 1994), but it seems that over short time scales they may not play the regulatory role originally envisioned by Green *et al.* (Green *et al.*, 1971).

What is P_c?

Despite the likelihood that P_c is unaffected by rapid changes in *P*, it was affected by *T*. P_c increased at low *T*,

as also reported by other authors (Pritchard *et al.*, 1990*a*, *b*; Boyer, 1993). In this work, low T moved P_c above the P normally prevailing in the cell, and growth could not occur. Increasing P reversed the effect. This recovery indicates that T and P compensate for each other in the growth process.

One possibility is that P_c is a rheological property of the wall, and low T causes the wall to become more viscous thus requiring larger forces for deformation (higher P) for growth. This theory is an extension of the rheological concept of growth proposed by Lockhart (Lockhart, 1965*a*, *b*). However, the increased viscosity for growth should show in the elastic and viscoelastic behaviour of the same polymers. Provided a polymer is above its glassy transition T, its rheological properties show little change with T until it melts (Sperling, 1992). Cross-linking like that in cell walls (Carpita and Gibeaut, 1993) extends the T range for stable rheological behaviour (Sperling, 1992). No significant effect of T (0–50 °C) was reported on the stress/strain ratio of isolated cell walls from *Nitella* (Haughton and Sellen, 1969). Proseus *et al.* found a stable elastic modulus of *Chara* cells in a T range extending from maximum growth to completely inhibited growth (Proseus *et al.*, 1999). These observations argue against the rheological mechanism.

An alternate possibility is that P_c involves a metabolic process that is inhibited by low T. Because of the metabolic involvement, its T-response likely would differ from that of elastic behavior. Because it also would require P, the process would proceed more rapidly at low T if P was raised, in agreement with the findings of this study.

Nature of rapid changes and gradual transitions

Figure 14 shows that rapid changes always were present when P changed. Proseus *et al.* found that most of them were reversible and thus elastic (Proseus *et al.*, 1999). They could be seen when growth occurred, or at low T or low P when growth was absent. They were present in mature cells that could not be induced to grow at any P. Consequently, they appeared to be purely physical responses accompanying the growth response to P. They probably involved straightening of folds or coils in the individual polymer molecules, with a return nearly to the original conformation when P was returned to its original level (Sperling, 1992). Because this elastic stretching exhibited a negligible response to T (Proseus *et al.*, 1999), the affected bonds were weak. The effects could be subtracted from the total elongation to more clearly reveal growth.

On the other hand, the gradual component of Fig. 14 begins with a small irreversible stretching in the first 10 s of a step-up (as described by Proseus *et al.*, 1999). This stretching was termed viscoelastic and was followed by a gradual stretching that lasted 10–20 min and was present whether the cells were growing or not. It tended to become larger and take longer at higher P, suggesting that strong bonds were being altered between molecules. Accordingly, the change likely involved a displacement of wall polymers relative to other polymers that did not readily reverse when the increased tension was relieved (Sperling, 1992; Proseus *et al.*, 1999). It is worth noting that viscoelastic changes were not detected during a step-down. Growth slowed at the lower P despite the absence of viscoelastic effects. Therefore, although an irreversible displacement of wall polymers probably can contribute to growth (Fry, 1989; Carpita and Gibeaut, 1993), growth is not the same as the gradual viscoelastic change observed only during a step-up.

In addition, the gradual component of Fig. 14 tended to be larger after a longer time at Growth$_2$ following a step-down. This increment was present only in growing cells and was in addition to the viscoelastic effects observed in the presence or absence of growth. The increment appeared to be the 'metabolic relaxation' described by Lockhart (1965*b*) or 'stored growth' (Okamoto *et al.*, 1990; Nakahori *et al.*, 1991). More work is needed to determine this additional, time-dependent component of the gradual transition.

Regardless of the nature of this metabolic component when the force on the wall is changed, physically-based rapid elastic and gradual viscoelastic changes are inevitable because of the polymeric nature of the wall structure (Sperling, 1992). As a result, while elastic and viscoelastic properties of the wall can be observed in all cells, their magnitudes will differ according to the cell wall composition (Probine and Preston, 1962; Probine and Barber, 1966). In these experiments, the elastic/viscoelastic changes were always smaller in mature than in growing cells, probably because of differences in wall composition (Metraux, 1982; Morrison *et al.*, 1993; Proseus *et al.*, 1999).

Nature of growth changes

Curvilinear growth rate/P curves have been reported in other plant systems and could hold clues to the mechanisms controlling plant growth (Green and Cummins, 1974; Matthews *et al.*, 1984; Pritchard *et al.*, 1990*b*; Hohl and Schopfer, 1992). For example, Green *et al.* (Green *et al.*, 1971) observed little change in growth rates around P_c, and Zhu and Boyer (Zhu and Boyer, 1992) reported a switch-like onset of growth around P_c. Because these effects were observed at P close to P_c, they occurred in the range where only small changes in rate were observed. Thus, it seems possible that small changes in growth rates went undetected in the previous work of Green *et al.* (Green *et al.*, 1971) and Zhu and Boyer (Zhu and Boyer, 1992). In order to test this possibility, the published recorder tracings of Zhu and Boyer (Zhu and Boyer,

1992) were reanalysed using the methods of this paper. Figure 15A shows that a *P* response was present and was small at *P* close to P_c, as seen in the curvilinear response of Fig. 5 in this study. Plotting the results for all of the cells (Fig. 15B) confirmed that a *P* response was always observed (curvilinearity was not obvious in this plot, but the data were limited). In the light of this finding, it is concluded that small *P* responses were beyond reliable detection with the earlier methods, and a *P* response was actually present.

Lockhart (Lockhart, 1965*a*, *b*, 1967) suggested a rheological model of growth in which the wall acts as an inert polymer deformed by the force of *P*. In this model, growth is a continuous viscoelastic slippage of wall molecules past each other, driven by the tension from *P*. Accordingly, it seems that growth should have a *T* response like that of elastic and viscoelastic deformation. However, it did not and when *T* was low, elastic deformation and a portion of viscoelastic deformation continued but growth was abolished. Lockhart (Lockhart 1965*a*, *b*) simplified his theory by excluding the biosynthesis of new wall, but the *P*-dependent delivery of molecules to the

wall may be an important feature of growth, as emphasized by Passioura and Fry (Passioura and Fry, 1992) and Roberts (Roberts, 1994). Robinson and Cummins (Robinson and Cummins, 1976) found more cellulose and matrix polysaccharides being delivered to the wall at high *P* than at low *P*. Therefore, the *P* response of growth may have involved the delivery of these molecules to the walls or the molecular assembly of the walls. Low *T* could affect this delivery or other features of the wall assembly process.

An alternate theory is that *P*-responsive changes in gene expression could account for the growth changes. *P*-responsive changes in gene expression have been reported in plants (Guerrero *et al.* 1990) and many have been observed when Ψ_w changes (Mason *et al.*, 1988*a*, *b*). They typically require several minutes to hours in plants undergoing changes in growth rates (Mason *et al.*, 1988*a*, *b*). Long-term changes such as altered wall composition or regulation of growth patterns undoubtedly involve these changes, and they probably control the original growth rates observed at the original *P* of the cells. However, they are unlikely to be involved in rapid *P* effects because new growth rates were steady within seconds. The immediate steadiness implies that a process necessary for growth responded directly to *P*.

Effect of T on P and Lp

While low *T* undoubtedly diminished metabolic activity, it also decreased *P* directly. Because the effects were predicted by the van't Hoff relation, the *T* appeared to act on Ψ_s, which became less negative. Because *P* was similar to $-\Psi_s$, the *P* became lower. The authors are unable to explain why Hertel and Steudle (Hertel and Steudle, 1997) found no effect of *T* on *P* in *Nitella* internode cells. The generality of the van't Hoff relation indicates that *P* should decrease at low *T* in all plant cells provided other factors are not overriding.

In multicellular plants, on the other hand, the generality may be obscured by growth-induced Ψ_w (Boyer, 1968; Molz and Boyer, 1978; Boyer, 1988). Growth-induced Ψ_w develop in growing tissues absorbing water from distant, sparse xylem in growing regions (Boyer, 1968; Molz and Boyer, 1978; Boyer, 1988). The *P* is below the maximum defined by Ψ_s, which forms a downward Ψ_w gradient extending from the xylem to the expanding tissues and moving water into the enlarging cells (Boyer, 1968; Nonami and Boyer, 1993; Fricke and Flowers, 1998; Martre *et al.*, 1999). The gradients are prominent in shoot tissues (Westgate and Boyer, 1985; Barlow, 1986; Nonami and Boyer, 1993; Fricke and Flowers, 1998; Martre *et al.*, 1999) but less so in root tissues because water surrounds the roots (Silk and Wagner, 1980). In single cells such as *Chara*, water surrounds the cells and growth-induced potentials are too small to detect (Zhu
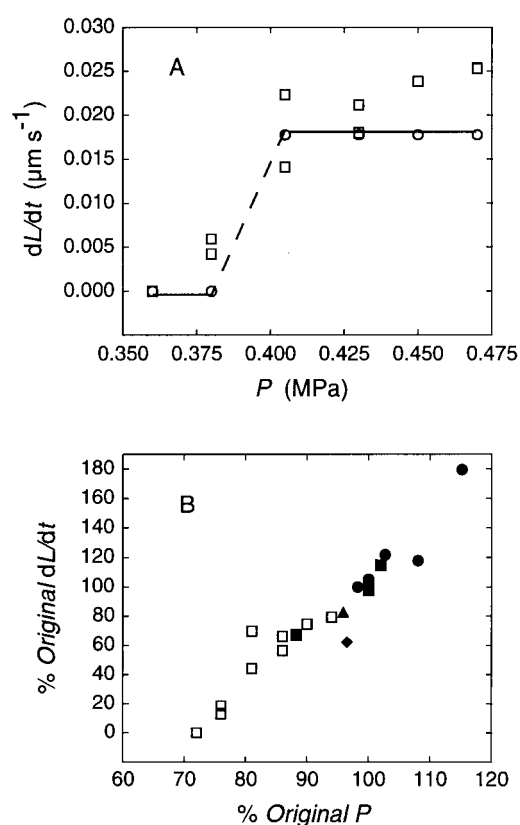

Fig. 15. Reanalysis of the data of Zhu and Boyer (Zhu and Boyer, 1992). (A) Single cell showing increasing response of growth rate to *P* as *P* increases above P_c (□) in comparison to the original analysis (○) presented in Fig. 6 of Zhu and Boyer (Zhu and Boyer, 1992). (B) composite of cell responses from Fig. 5 (□), Fig. 6 (●), Fig. 7 (●), Fig. 11 (◆) and Fig. 12 (■) of Zhu and Boyer (Zhu and Boyer, 1992).

and Boyer, 1992). In soybean stems, growth-induced Ψ_w are significant (Nonami and Boyer, 1993), and Boyer (Boyer, 1993) showed that low T increased P. This effect is the opposite of what happened in *Chara* and occurred because T inhibited growth, diminishing the gradient in growth-induced Ψ_w. The resulting increase in P overrode the decrease in P that would have occurred otherwise. Similar increases in P at low T were noted in growing leaves of *Lolium* and *Poa* spp, and in roots of maize (Woodward and Friend, 1988; Thomas *et al.*, 1989; Pritchard *et al.*, 1990*a*) and tissue cultured soybean stems (Ikeda *et al.*, 1999), but roots of wheat showed decreases like *Chara* (Pritchard *et al.*, 1990*b*).

Another complicating factor is continued solute uptake that may cause P to increase when growth is inhibited by low T, particularly in roots of multicellular plants (Prichard *et al.*, 1988, 1990*b*). Because *Chara corallina* cells do not have the ability to quickly adjust Ψ_s (Bisson and Bartholomew, 1984), they provide a unique test of P/T relationships in a living system free from the complicating factors in complex tissues.

Effects on Lp were similarly predictable in *Chara*. Low T increases the viscosity of water flowing through membranes, causing an apparent decrease in membrane Lp. In these experiments, the Lp at $7\,^{\circ}\text{C}$ was 77% of that at $23\,^{\circ}\text{C}$, and the increased viscosity of water would decrease Lp to 65% of its original value at $23\,^{\circ}\text{C}$. Therefore, the entire decrease in Lp was attributable to the change in viscosity of water. Others also observed lower Lp at cold T (Tomos *et al.*, 1981; Thomas *et al.*, 1989; Boyer, 1993; Hertel and Steudle, 1997). In this work, the decrease was insufficient to account for the complete inhibition of growth, which would require Lp to be near zero at $7\,^{\circ}\text{C}$.

Significance of m

The preceding conclusions direct our attention to m, the slope of the line described by equation 1. The m is the only remaining factor linking P to growth rate, and because growth varies with a wide range of factors, the m includes their effects. In particular, over long times it combines the possibility of large amounts of newly synthesized wall (Roberts, 1994), alterations in rate caused by growth regulators such as auxin (Cleland, 1971; Taiz, 1984) and inhibitions by treatments that disrupt cell metabolism such as cold (Ray and Ruesink, 1962; Barlow and Adam, 1989; Thomas *et al.*, 1989; Pritchard *et al.*, 1990*a, b*; Boyer, 1993; Berman and DeJong, 1997), anoxia (Carr and Ng, 1959; Ray and Ruesink, 1962), or metabolic inhibitors (Robinson and Cummins, 1976; Zhu and Boyer, 1992; Schindler *et al.*, 1994). Each of these factors involves cell metabolism, and our inability to predict growth from the *original P*, P_c, or *original* $P - P_c$ indicates that these metabolic factors controlled m and thus the long-term growth of the cell. Over short times, the

response of growth to P was large and suggests that at least one of these factors is P-responsive. The identity of the P-responsive step(s) awaits further experiment.

Conclusions

The methods used here maintain the wall environment constant while changing P over a wider range than is normally possible in the cell. The methods allow small differences in growth to be resolved without elastic effects. They show that P- and T-responsive process(es) play a central role in plant growth and have the distinctive features of a minimum P requirement that shifts with T, a curvilinear response to P, and a rapid change in rate when P changes without a requirement for changes in wall physical properties. Wall properties change over long times, however. As a result of these features and the rate-controlling nature of the P- and T-responsive process(es), the inhibitory effects of low T can be partially overcome by P. This implies that methods that increase P in growing cells might improve growth rates at low T. The marked inhibition of growth by low T suggests that the process has important metabolic features in addition to any rheological ones.

Acknowledgements

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