Identifying cytoplasmic input to the cell wall of growing Chara corallina

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Abstract

Plants enlarge mostly because the walls of certain cells enlarge, with accompanying input of wall constituents and other factors from the cytoplasm. However, the enlargement can occur without input, suggesting an uncertain relationship between cytoplasmic input and plant growth. Therefore, the role of the input was investigated by quantitatively comparing growth in isolated walls (no input) with that in living cells (input occurring). Cell walls were isolated from growing internodes of Chara corallina and filled with pressurized oil to control turgor pressure while elongation was monitored. Turgor pressure in living cells was similarly controlled and monitored by adding/removing cell solution. Temperature was varied in some experiments. At all pressures and temperatures, isolated walls displayed turgor-driven growth indistinguishable in every respect from that in living cells, except the rate decelerated in the isolated walls while the living cells grew rapidly. The growth in the isolated walls was highly responsive to temperature, in contrast to the elastic extension that has been shown to be insensitive to similar temperatures. Consequently, strong intermolecular bonds were responsible for growth and weak bonds for elastic extension. Boiling the walls gave the same results, indicating that enzyme activities were not controlling these bonds. However, pectin added to isolated walls reversed their growth deceleration and returned the rate to that in the living cells. The pectin was similar to that normally produced by the cytoplasm and deposited in the wall, suggesting that continued cytoplasmic input of pectin may play a role in sustaining turgor-driven growth in Chara.

Key words: Cell wall, Chara corallina, cytoplasmic input, growth, intermolecular bonds, pectin, turgor pressure.

Introduction

Plant enlargement is restricted to certain localized tissues where the cells rapidly extend 10–100-fold while water enters osmotically. The process is driven by hydrostatic pressure inside the cell (turgor pressure, $P$) that causes the primary wall to yield. Above a critical $P$, the yielding is irreversible and considered to be growth (Cleland, 1971; Taiz et al., 1981; Taiz, 1984). During growth, new wall is deposited that prevents the primary wall from becoming too thin and subject to rupture (Roberts, 1994).

The presence of new wall is direct evidence for input from the cytoplasm while the walls grow. However, an enlargement resembling growth can be demonstrated if growing cells are killed and their walls subsequently deformed with an external force (Cleland, 1971; Taiz et al., 1981; Taiz, 1984). Isolated strips of primary walls behave similarly (Probine and Preston, 1962). It has been proposed that wall enzymes might be a form of cytoplasmic input that would act to loosen or tighten inter-polymeric bonds between structural polysaccharides and account for the growth in the isolated walls as well as living cells (Cosgrove, 2000; Kutschera, 2001; Fry, 2004). However, growth is completely inhibited by small decreases in $P$ (0.1–0.2 MPa), which has been difficult to reconcile with continued enzymic loosening or tightening of wall polymers (Fry, 2004). Chemical inhibitors of enzyme action such as KCN can inhibit enlargement (Taiz, 1984) but they sometimes decrease $P$ (Zhu and Boyer, 1992), suggesting that membrane integrity can be compromised and growth inhibited more for physical reasons than a lack of cytoplasmic input.
Alternatively, cytoplasmic input might consist of polysaccharide destined to become part of the new wall. Proseus and Boyer (2005) reported an inability of polysaccharides to enter an existing wall unless $P$ was raised to a level causing growth. Proseus and Boyer (2006) found that pectins began to gel at these $P$, suggesting that $P$ could be involved not only in wall extension but also wall assembly. Importantly, the $P$ directly affected the rate that new wall was deposited in the living cells.

As a consequence, $P$ may be a link between the assembly and extension of cell walls, but the identity and role of cytoplasmic input remains obscure. Part of the uncertainty arises from the experimental difficulty of eliminating the input while maintaining a normal wall environment and ability to develop $P$. One way to approach the problem is to isolate walls and subject them to $P$ in the lumen previously occupied by the cytoplasm. The importance of such experiments has been suggested for some time (Metraux et al., 1980; Taiz et al., 1981; Nonami and Boyer, 1990). They became possible recently when a method to generate growth-sustaining $P$ inside isolated walls was developed (Proseus and Boyer, 2005). The following work was undertaken to use this approach to investigate the nature of cytoplasmic input for plant growth. By using a simple cellular system, growth rates could be quantitatively compared when cytoplasm was present or absent under conditions otherwise identical to those in which the plants were grown.

**Materials and methods**

**Plant materials**

Single internode cells containing primary walls were obtained from the elongating, apical portion of the alga *Chara corallina* (Klen ex. Wild., em. R.D.W.) grown in laboratory cultures at 22 °C in continuous light of 10–15 μmol photons m$^{-2}$ s$^{-1}$ of photosynthetically active radiation. The rhizoids were anchored in bottom sediment above which the thallus grew in water occasionally supplied with 1 mM chloride salts of Na, K, Mg, and Ca. The medium developed a pH of 7–8 and had an osmotic potential of $-0.01 \pm 0.01$ MPa. After excision, the cells continued to elongate for several hours at rates similar to those in the intact plant (Zhu and Boyer, 1992). Non-growing, mature cells were obtained from lower portions of the plant where the walls displayed characteristic helical bands of rigid cellulosic secondary walls in the electron microscope (Metraux, 1982; Morrison et al., 1993). No CaCO$_3$ banding was observed except in the oldest mature cells.

The experiments were conducted in a 4 ml trough or 6 ml cup in a controlled environment chamber that reproduced the growth conditions (culture medium with irradiance of 10–15 μmol m$^{-2}$ s$^{-1}$, and $P$ and $T$ varied as shown). The pH of the culture medium was stable because of a small amount of natural buffering capacity.

**Measuring and controlling $P$**

For living cells shown in Fig. 1A, one end of the cell was held in a scissors-like gate in the trough containing 4 ml of culture medium, and the microcapillary tip of a pressure probe (Steudle and Zimmermann, 1974) was inserted into the vacuole. The initial $P$ was measured (usually 0.45–0.55 MPa) by returning the cytoplasm to its original position at the cell wall. $P$ was then decreased by withdrawing cell solution (vacuolar and cytosolic). After a series of small adjustments, the new $P$ was stable and no further manipulation was necessary (Zhu and Boyer, 1992). The solution removed from the cell was inside the microcapillary and continuously in contact with the solution remaining in the cell. The $P$ became stable because enough cell solute had been removed from the cell to dilute the cell contents. Conditions in the cell were otherwise unchanged, and only $P$ had been altered inside the cell. The procedure could be reversed by returning the cell solution to the cell interior, increasing $P$ and eventually returning it to its original level. It should be mentioned that the cytoplasm streams throughout this procedure and will plug the opening in the microcapillary if the opening is small. By making the opening large (75 μm), plugging rarely occurred although small amounts of cytoplasm flowed into the microcapillary.

![Fig. 1](image-url) Methods for measuring and altering turgor pressure ($P$) inside living cells (A) or isolated walls (B) of young *Chara corallina* internodes that are growing. The tip of the microcapillary removes or returns cell solution (A) or oil (B) to the cell interior thus allowing control of $P$ in either system. Expanded view shows matrix polysaccharides (cross-hatch), but not cellulose microfibrils embedded in the matrix. Culture medium penetrates the wall. Plasma membrane (PM) and the oil/water interface (O/W) are located in similar positions in the cell (A) and isolated wall (B), respectively. Other parts of the apparatus are not shown but are described by Proseus et al. (1999).
For isolated walls shown in Fig. 1B, the experiments were conducted in a shallow plastic cup with 6 ml of culture medium. P was controlled using mineral oil from the pressure probe to fill the lumen previously occupied by the cytoplasm. The isolation, attachment of the wall to the microcapillary, and filling of the lumen was carried out in culture medium as described in detail by Proseus and Boyer (2005), except a different glue was used (‘Zap-A-Gap CA+’, Super Glue Corp., Rancho Cucamonga, CA, USA) instead of ‘Quick-Tite’ whose manufacture had been discontinued. After the microcapillary was mounted on the pressure probe, ‘artificial’ P as high as 0.45 or 0.5 MPa was generated by moving a rod into or out of the oil reservoir. The P developed because the oil did not enter the wall structure. The oil was trapped inside the lumen by the surface tension between the oil and water at the inner wall face. The same forces prevent the plasma membrane from entering the wall structure in living cells. Figure 1B (O/W) shows the oil/water meniscus in approximately the same position as the plasma membrane in a living cell (Fig. 1A; PM).

In order to preserve metabolic and enzymatic activities, the cells and isolated walls were continuously immersed in culture medium taken directly from the parent culture in all of the experimental procedures. In this way, the enzymes normally active in the isolated walls were maintained in an active state. For some experiments, the enzyme activities were eliminated in isolated walls by boiling for 10 min and cooling before gluing the walls onto the microcapillary.

Measuring and controlling T

The influence of temperature (T) on elongation rates was determined by circulating the culture medium from the trough or cup through a Peltier chiller coil as described by Proseus et al. (1999). Because P in a live cell is a function of T (Proseus et al., 2000), the P had to be adjusted by injecting or removing small amounts of cell solution as T changed. T affects P because the osmotic potential of the cell solution responds to T, and P follows. In the isolated walls, cell solution was absent and this effect was not present. Only small injections of oil were needed to maintain a stable P in the wall lumen as T varied.

Measuring elongation

While the P control was carried out through the immobilized end of the cell or wall, the other, free end of the cell or wall was connected to a tungsten wire leading to a radial position transducer (Proseus et al., 1999). The wire had a fork that could be gently inserted over the free end. For isolated walls, enough oil was injected to generate P of approximately 0.05 MPa, and the wire was attached without twisting or tearing the wall.

Elongation (ΔL) was recorded with a resolution of 1 μm, with ΔL=0 at the beginning of the experiment. During periods of stable P, steady elongation rates (dL/dt) were calculated from the ΔL data by selecting the time period of interest (approximately 10 min) and calculating the slope of a line of best fit through the ΔL values for that period. Treatments were imposed only when a reasonably stable dL/dt was observed at a stable P, usually 20–30 min after stable P was established.

Acquiring data

Instead of making cell solution or mineral oil injections manually, an automatic controller maintained P in the cells or isolated walls, as described by Proseus and Boyer (2005). A datalogger (CR7X, Campbell Scientific, Logan, UT, USA) and strip chart recorder monitored the output every 5 s from the pressure probe, position transducer, and a thermocouple immersed in the medium around the cell or isolated wall. A computer continually displayed dL/dt. The data in the datalogger were downloaded to a computer for processing.

Supplying exogenous pectin

Medium taken directly from the Chara cultures was filtered to remove cells and debris larger than 0.2 μm. Pectin (80% potassium salt of polygalacturonic acid obtained from citrus albedo, from Sigma, St Louis, MO, USA) was used to make a 6 mg ml⁻¹ solution (35 μM, pH 7) in the culture medium. Analysis of this pectin at the Complex Carbohydrate Research Center (Athens, GA, USA) indicated a mean molecular weight of 170 kDa.

Results

When oil was initially injected into the lumen of walls isolated from mature cells (Fig. 2A), the wall elongated rapidly (Fig. 2B), but soon ceased to elongate after P rose to 0.5 MPa. No further elongation was detected when viewed at high resolution (Fig. 2B, inset). On the other hand, the same conditions caused continued elongation if the walls were isolated from young cells (Fig. 2E, inset). An initial rapid rate was apparent (Fig. 2E), but decelerated and continued slowly for many hours. T was held constant and nearly the same for both kinds of cell (Fig. 2C, F).

P in live cells was usually around 0.5 MPa. It could be stepped up or down in the live cells (Fig. 3A–C) or isolated walls (Fig. 3D–F). Elongation responded immediately by extending or shrinking (Fig. 3B, E). The step-down reversed much of the initial rapid extension that occurred during the step-up. This reversible component was considered to be elastic deformation (E in Fig. 3B, E), as in Proseus et al. (1999).

After the rapid response to the step up, there was a gradual transition to a steady rate that was always faster at the higher P than at the lower P (Fig. 3B, E). The steady extension was not reversible and could be clearly seen after the step-down (G in Fig. 3B, E). This irreversible deformation was considered to be elongation ‘growth’. Lateral growth was negligible (data not shown). T was steady and comparable throughout these P changes (Fig. 3C, F).

When growth was compared over long times in live cells and isolated walls while P was held at 0.5 MPa, the rate was initially more rapid in the isolated walls than in the live cells (Fig. 4, inset). However, the rate soon decelerated in the isolated walls, and growth became much slower than in the live cells (Fig. 4). The decelerated rate continued for the 20 h duration of the measurements. The crossover from the rapid to the decelerated rate in the isolated walls provided an opportunity to compare extension in the walls and live cells while rates were similar (Fig. 4; dashed box in inset). For example, in six living cells during this time, mean elongation rates were 0.029 μm s⁻¹ (SD=0.021, range=0.015–0.071 μm s⁻¹) with mean original P of 0.518 MPa (SD=0.049, range=0.43–0.57 MPa). At the same time, nine isolated walls displayed mean rates of 0.020 μm s⁻¹ (SD=0.008, range=0.008–0.031 μm s⁻¹) when P was held at 0.5 MPa. Unless otherwise indicated, the following experiments were conducted within the 90 min shown in...
the dashed box (Fig. 4; inset) to minimize differences in rates between the live cells and isolated walls.

Figure 5 shows that growth was not observed unless $P$ was above a critical level in live cells or isolated walls. In the cells, steady growth ($dL/dt$ in Fig. 5A) began at $P$ of 0.35–0.47 MPa and accelerated rapidly as $P$ rose farther to 0.5–0.6 MPa. In the walls, growth behaved similarly (Fig. 5B). When the isolated walls were boiled for 10 min
then mounted in the apparatus and tested, growth displayed the same response to $P$ as in the live cells (Fig. 5C). Details for a single isolated wall in Fig. 5D–F revealed a transition from elastic to steady growth after each $P$-step. This type of response was essentially the same as in the living cells shown in Fig. 3.

These tests were made while $T$ was held constant (Fig. 5F), but when the converse experiment was done by varying $T$ while $P$ was held constant, growth was near zero at $T$ below 10–12 °C in living cells, isolated walls, and walls isolated and boiled before the measurements (Fig. 6A–C). When $T$ was above 10–12 °C, growth was detected and accelerated markedly as $T$ rose (Fig. 6A–C). Details for a single isolated wall in Fig. 6D–F indicate that the wall responded immediately to $T$ and displayed steady growth as soon as $T$ became steady, resembling that seen in living cells by Proseus et al. (1999, 2000).

This experiment suggested that $T$ had the same effect on the growth of isolated walls as in live cells. However, because the effects of $T$ are usually reversible in live cells (Proseus et al., 2000), a similar test was made with isolated walls by briefly exposing them to low $T$ and returning them to the original $T$. Exposure to $T$ of 10–15 °C caused growth nearly to cease in a live cell (Fig. 7A–C) and an isolated wall (Fig. 7D–F), but the growth resumed when $T$ returned to the original level. The resumption began at the cell length that was present before the exposure to low $T$. In effect, the low $T$ delayed growth until $T$ returned to its original level.

**Fig. 4.** Elongation ($\Delta L$) of live young Chara internodes having natural $P$ near 0.5 MPa (Live Cells, thick lines) compared with isolated walls from similar internodes having $P$ of 0.5 MPa (Isolated Walls, thin lines). Data begin after determining the original $P$ in the living cells or filling and pressurizing oil in the isolated walls. Inset shows magnified view of elongation during the first 130 min. Dashed box in inset indicates when most experiments were done. $P$ and $T$ (22 °C) are not shown.

**Fig. 5.** Growth rates ($dL/dt$) at various turgor pressures ($P$) but constant temperature ($T$) in (A) live cells ($n=4$), (B) isolated walls ($n=3$), or (C) isolated walls boiled before measurement ($n=3$). Each symbol type represents values from an individual cell or wall. Detailed responses for one isolated wall from (B) are shown for $P$ in (D), elongation ($\Delta L$) in (E), and temperature ($T$) in (F).
In addition to low $T$ as an inhibitor, live cells were exposed to other metabolic inhibitors (KCN, NaN$_3$, FCCP, DCMU, brefeldin A, abscisic acid, RNase) at various concentrations alone or in combination. These either had little effect or more often were growth inhibitory with a nearly concurrent decrease in $P$ (data not shown), as reported by Zhu and Boyer (1992) for DCMU+FCCP. In no instance was $P$ maintained for long times while growth was inhibited. Consequently, attempts to inhibit growth at high $P$ using chemical inhibitors instead of low $T$ were unsuccessful.

On the other hand, growth was accelerated when pectin as polygalacturonic acid was supplied to the culture medium around isolated walls (Fig. 8B, solid trace). For a time after the addition, growth in the walls was as rapid as in the live cells in normal medium (Fig. 8B; dashed traces). Even after 15 h, the rate in the wall remained slightly higher than prior to the addition of polygalacturonic acid.

**Discussion**

It is rare that *in vivo* growth can be directly compared to the enlargement of walls isolated from identically treated cells and exposed to growth-sustaining $P$. In the present work, the comparison was made at various $P$ and $T$ without altering the wall environment, which was maintained in the culture medium. For a time, growth rates were similar in the walls and cells and it was possible to compare growth in the two systems under near-identical conditions except for input from the cytoplasm.

Isolated walls displayed $P$ and $T$ responses of growth that were indistinguishable from those in living cells except for a deceleration evident after the walls had been isolated for an hour or so. Taiz and Richmond (1984) and Metraux *et al.* (1980) also saw a similar deceleration in the extension of isolated *Nitella* walls when they were pressurized with mercury. In *Chara*, the deceleration indicated that a growth-sustaining activity was lacking, but all the other hallmarks of growth were present. These were an elastic response to $P$, a critical $P$ that had to be exceeded before growth began, a $P$-dependent rate of extension, an irreversible $P$-driven extension, and a high $T$-responsiveness (Probine and Preston, 1962; Haughton *et al.*, 1968; Cleland, 1971; Preston, 1974; Richmond *et al.*, 1980; Taiz, 1984; Proseus *et al.*, 1999, 2000). In addition, the multi-directional tension generated by $P$ caused a unidirectional extension, which was another characteristic of growth in the living cells (Probine and Preston, 1962; Baskin, 2001, 2005). Therefore, the ability to sustain high growth rates was the clearest manifestation of cytoplasmic input in the living cells.
Cytoplasmic input to growth

In previous work, the cytoplasm was often considered to modify wall properties rapidly when $P$ changed (Cleland, 1971; Taiz, 1984). For example, Green et al. (1971) and Green and Cummins (1974) used osmotica with the charophyte *Nitella* and with coleoptiles to explore the response to $P$ in vivo, concluding that cytoplasmic activity maintained growth rates constant while $P$ changed. Zhu and Boyer (1992) studied $P$ changes without using osmotica in *Chara* and agreed with Green et al. (1971) that rates remained constant while $P$ changed. However, recent analyses by Proseus et al. (2000) found that the rates were not constant and, instead, were too small for differences to be detected with the measuring systems of Zhu and Boyer (1992), and probably Green et al. (1971). Nevertheless, Proseus et al. (1999, 2000) proposed that cytoplasmic input was required for growth because, in living cells, low $T$ was inhibitory and is well-known to inhibit metabolic activity.

Therefore, it was unexpected that isolated, boiled walls continued to be sensitive to $T$ during growth, where cytoplasmic activities were obviously absent. The isolation should have preserved enzymes in the wall because isolation was carried out in the culture medium where the cells had been grown, but boiling would eliminate the activity of these enzymes. Therefore, neither cytoplasmic nor wall enzymes could affect the results with boiled walls, but the walls continued to behave like the living cells at various $P$ and $T$ except for the deceleration mentioned above. This behaviour indicates that the responses were inherent features of the matrix structure rather than enzymes in the cytoplasm or walls.

In living cells, the inhibition of metabolism by low $T$ would decrease the supply of intermediates to the wall among many other effects. However, the results indicate that these decreases, while inevitable, were not so limiting as the response of the wall structure itself. Ray and Ruesink (1962) and Proseus et al. (2000) considered the $T$ response to originate from metabolic activity in the cytoplasm, but concluded that the response resided close to the final steps in the growth process. In view of the present results, these investigators probably were observing the $T$ response directly in the matrix structure of the wall.

Wall bonds

What features of matrix structure could account for the $P$ and $T$ responses of the primary wall? The wall is a composite of transversely-oriented cellulose microfibrils embedded in a matrix of cross-linked polysaccharides (McCann et al., 1990; Passioura and Fry, 1992; Carpita and Gibeaut, 1993; McCann and Roberts, 1994; Passioura, 1994; Baskin, 2001, 2005). The matrix precursors (pectins, hemicelluloses) are secreted essentially fully formed from Golgi-derived vesicles by exocytosis (Boffey and Northcote, 1975; Robinson et al., 1976; Perrin et al., 2001). They gather in a periplasmic layer and cross-link to form a gel-like wall matrix. Morrison et al. (1993) and Popper and Fry (2003) reported that pectins are the predominant matrix polysaccharide in the primary wall of the

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**Fig. 7.** Turgor pressure ($P$), elongation ($\Delta L$), and temperature ($T$) at varying times before and after exposure to low $T$ in a living *Chara* internode cell (A–C) or isolated wall (D–F). $P$ was held constant at all $T$. (A, D) $P$; (B, E) $\Delta L$; (C, F) $T$ in the live cell and isolated wall, respectively. Similar results were obtained in three replications of this experiment.
charophytes *Nitella* and *Chara*, and it follows that pectins are a predominant form of cytoplasmic input to the wall.

When tension is applied to the wall by $P$, the cellulose prevents transverse extension, and the matrix polymers extend mostly along the long axis of the cell. Proseus *et al.* (1999) reported that the tension first affects the reversible elastic bonds between matrix polymers. The intertwining, somewhat mobile or coiled polymeric segments in the wall matrix readily stretch and become more ordered under the stress. At the macroscopic level, this effect is illustrated by the rapid, reversible, elastic effects of $P$. The bonds controlling elasticity are relatively weak and nearly unaffected by $T$ (Proseus *et al.*, 1999).

However, the present results identify another type of bond affected by $T$. This second type is much stronger than the weak bonds responsible for elastic effects. Figure 9 shows an Arrhenius plot of the latter type of bond, represented by steady $dL/dt$ in isolated walls at various $T$, with $P$ above the critical level (above 0.35 MPa). For comparison, the same plot shows the $T$ response of the elastic bonds ($\varepsilon$) in the wall identified by Proseus *et al.* (1999). The response of $dL/dt$ differs from that of $\varepsilon$ despite detection of both kinds of bonds in walls grown identically. While $\varepsilon$ is not a rate and thus not strictly interpretable in Arrhenius terms, the rate of elastic deformation was essentially instantaneous in the present experiments, indicating that the bonds were indeed weak. By comparison, the steepness of the $dL/dt$ plot and gradual deformation that occurred above the critical $P$ indicates that the bonds controlling irreversible deformation were strong. The steepness indicates that high activation energy was involved in the reaction, and only a few of the potentially deformable bonds had sufficient energy. As in synthetic polymers (Billmeyer, 1971; Sperling, 1992), these bonds would slowly break and reform, slipping relative to their neighbours when under stress. The high activation energy is consistent with the presence of strong bonds having a requirement for a critical $P$ to be exceeded before irreversible deformation began. Because they were observed in the isolated walls, the two types of bonds are inherent properties of the wall polymers.

Haughton and Sellen (1969) used Arrhenius concepts to analyse the irreversible extension of isolated walls of algae in detail, but the type of bond affecting wall extension was not identified. Metraux *et al.* (1980) reported apparent dual thresholds in *Nitella* internode walls when artificial $P$ was produced by mercury injection to affect irreversible deformation. Beginning at a $P$ of 0.05 MPa, Metraux *et al.* (1980) increased $P$ for brief periods and in response the cell walls exhibited irreversible extension that was a function of the magnitude of the pulse. The high threshold of 0.45 MPa reported by Metraux *et al.* (1980) is similar to the critical $P$ observed here in *Chara* undergoing growth.
at steady $P$, and may share the same origin in the strong bonds. The lower threshold observed by Metraux et al. (1980) was not observed in the present work perhaps because the cyanoacrylate glue used in Chara was less deformable than the beeswax/rosin junction used by Metraux et al. (1980). Richmond et al. (1980) observed stretching of the latter kind of junction. It is difficult to assess with confidence, but the lack of a lower threshold in the present work might then be related to the difference in glue junction rather than wall behaviour. Also, Richmond et al. (1980) found that the capacity for irreversible deformation measured with brief pulses differed from that measured with long-term extension associated with growth.

**Polygalacturonic acid and cell wall elongation**

The ability of polygalacturonic acid to restore high growth rates in isolated walls indicates that a substance normally supplied by the cytoplasm affected the strong bonds in the wall. This activity has not been reported before (Cosgrove, 2000). In Chara, polygalacturonic acid containing small amounts of glucuronic acid constitutes most of the pectic fraction (Popper and Fry, 2003). Polygalacturonic acid thus appears to be the predominant matrix component being delivered to the walls by the cytoplasm. Generally during cell enlargement, the wall may become thinner or thicker perhaps by as much as two-fold (Taiz, 1984; Kutschera, 1990, and references therein; Bret-Harte et al., 1991). But because plant cells often enlarge by 10–100-fold, much of the wall is new at maturity (Roberts, 1994) and polygalacturonic acid delivery must be substantial.

Morrison et al. (1993) and Popper and Fry (2003) report that the carboxyl groups of charophyte polygalacturonic acid are largely unmethylated. Consequently, the groups can dissociate to the anionic form, creating a large capacity for multivalent cation binding and cross-linking in the primary wall (Morikawa and Senda, 1974a; Gillet et al., 1992, 1998). When the wall was placed under elastic extension, Morikawa and Senda (1974b) and Morikawa et al. (1974) found that some of the carboxyl groups oriented relative to the long axis of the cells, i.e. some of the pectins became oriented. Accordingly, some of the pectin may bear the load of deformation in the existing wall, although other matrix components might also contribute.

Using these concepts, the two types of bonds are shown diagrammatically in Fig. 10 for pectin in an isolated wall. The pectin has a high mobility when the wall is unstrained by $P$ (Fig. 10A). As $P$ increases, weak interpolymeric bonds break, causing the pectin to orient and stretch elastically, decreasing its mobility (Fig. 10B). The stretching causes some of the pectin cross-links to become load-bearing. As $P$ increases above a critical level, strong bonds between load-bearing cross-links begin to slip (Fig. 10C). The wall grows in length. As the isolated wall lengthens, additional cross-links come under load. As the number of load-bearing links increases, the wall tightens. The rate of slippage decelerates, decelerating the irreversible growth of the wall. The pectins may thus account for the weak and strong bonds in the wall, as well as the deceleration occurring after the wall is isolated.

It is important to note that this extension model for the isolated wall requires no input from the cytoplasm or any wall enzymes. The two types of wall bonds with their $P$ and $T$ responses are inherent properties of the wall matrix alone. The matrix polysaccharides are the extensible components, and the cellulose restrains lateral extension. However, while this model accounts for all of the growth properties of the isolated wall, its assumptions can only be tentatively offered until more definitive evidence for the identity of load-bearing polymers is in place.
Because continued rapid growth in living cells occurs while new wall is deposited, the growth activity of pectin is particularly noteworthy. Proseus and Boyer (2005, 2006) found that the wall acted as an ultrafilter to retain polysaccharides delivered by the cytoplasm. P concentrated the retained polysaccharides in a periplasm between the plasma membrane and inner wall face. At $P$ of 0.5 MPa (sufficient to support growth), some of the polysaccharides began to enter the wall. Proseus and Boyer (2006) report that the high $P$-induced concentrations in the periplasm also caused gelling of pectin. If $P$ was too low to support growth, gelling and wall deposition were inhibited (Proseus and Boyer, 2006). Consequently, $P$ high enough to support growth in the living cell appeared to promote cross-linking and wall penetration of polysaccharides destined to become part of the wall matrix, creating a seamless combination of new and old primary wall while growth occurred. It follows that these phenomena accompany the action of $P$ and $T$ on the weak and strong bonds in the isolated wall shown in Fig. 10. Therefore, a complete model of growth in living cells will need to include the action of new wall on the weak and strong bonds in the existing wall.

**Relation to enlargement in land plants**

It increasingly appears that all land plants (embryophytes) diverged from ancestral charophycean algae about 400–500 million years ago (Niklas, 1992; Scherp et al., 2001). Morphological, ultrastructural, biochemical, and molecular evidence indicates that charophytes such as *Chara* and *Nitella* are the most closely related to the ancestral algal types (Chapman, 1985; Graham, 1985; Niklas, 1992; Scherp et al., 2001).

The monosaccharide composition in *Chara* primary walls resembles that in terrestrial plants (Preston, 1974; Metraux, 1982; Morrison et al., 1993), but the polymers are somewhat different (Popper and Fry, 2003). Although the homoalacturonan or rhamnogalacturonan pectic component in the walls of actively growing *Nitella* and *Chara* consists almost exclusively of non-methyl-esterified pectin (Morrison et al., 1993; Gillet and Liners, 1996; Popper and Fry, 2003), most terrestrial plant walls have a mix of methyl- and non-methyl-esterified pectin (Knox et al., 1990; McCann and Roberts, 1994; Goubet et al., 2003). Terrestrial plants (bryophytes and vascular taxa) often contain boron in the walls generally found as borate di- or methyl-borate. In the walls of actively growing *Nitella* and *Chara* walls did not affect gelling of pectin. If $P$ is too low to support growth, gelling and wall deposition were inhibited (Proseus and Boyer, 2006). Consequently, $P$ high enough to support growth in the living cell appeared to promote cross-linking and wall penetration of polysaccharides destined to become part of the wall matrix, creating a seamless combination of new and old primary wall while growth occurred. It follows that these phenomena accompany the action of $P$ and $T$ on the weak and strong bonds in the isolated wall shown in Fig. 10. Therefore, a complete model of growth in living cells will need to include the action of new wall on the weak and strong bonds in the existing wall.

In terrestrial species, the growth-supporting roles proposed for various wall enzymes centre on cleavage of wall polysaccharides (endoglucanases) (Goldberg, 1980; Huber and Nevin, 1981; Fry, 1989), rearrangement of interpolymeric bonds (e.g., xyloglucan endotransglycosylases) (Fry et al., 1992; Nishitani and Tominaga, 1992) or other putative functions for ‘osmiophilic deposits’ in periplasm (Kutschera, 2001) and expansins (McQueen-Mason and Cosgrove, 1995; Cosgrove, 1998). Boiled oat coleoptiles (Tepler and Cleland, 1979) and maize coleoptiles exposed to metabolic inhibitors or destruction of the protoplast (Ding and Schopfer, 1997) lost their responses to acid. Boiling cucumber hypocotyls for 15 min or exposure to proteases irreversibly inhibited wall extension (Cosgrove, 1989).

The lack of enzyme involvement in *Chara* wall growth is counter to these concepts in terrestrial plants (Cosgrove, 2000; Kutschera, 2001; Fry, 2004), but expansins are apparently absent in *Chara* (unpublished observation, discussed by Cosgrove, 2000). XET activity was not detected with cells from cultures used in the current study (Yajun Wu, personal communication). Application of exogenous xyloglucanase to *Chara* or *Nitella* walls did not produce oligosaccharide fragments known to be required for XET activity (Popper and Fry, 2003). A lack of enzyme involvement in *Chara* is consistent with the findings of Metraux and Taiz (1977) and of Taiz et al. (1981) that the ability of acidic media to promote elongation in isolated *Nitella* walls was not affected by boiling. Similar results were found in boiled walls of the chlorophyte alga *Valonia* by Tepler and Cleland (1979).

Growth in *Chara* tends to be slower than in terrestrial plants. A typical *Chara* internode with a length of 15 mm grows at about 0.02 mm s$^{-1}$ to give a relative growth rate of 0.5% h$^{-1}$ averaged over the entire length of the cell. For comparison, elongating regions of stems, leaves, nodal roots, and silks measured in this laboratory in maize had relative growth rates of 6%, 8%, 15%, and 0.8% h$^{-1}$, respectively, averaged over the entire growing region (Westgate and Boyer, 1984, 1985). For seedlings, average rates of 8% h$^{-1}$ were typical for the whole elongating region of seedling pea stems (Matyssek et al., 1988), and 3.7% h$^{-1}$ for soybean hypocotyls (Meyer and Boyer, 1972). For further comparison, primary roots had relative growth rates averaged over the whole growing region that were 20% in maize according to Erickson and Sax (1956), 30% in arabidopsis, 12% in alfalfa, 26% in lettuce, 28% in tomato, and 30% in timothy according to Van der Weele et al. (2003). Thus, it seems that the more advanced walls of terrestrial species may contain special adaptations for rapid growth. Seen in this light, the similarity in wall growth properties between *Chara* and terrestrial species may indicate an underlying similarity in molecular mechanism that in terrestrial plants includes additional adaptations for enhancing growth rates.
Conclusions

Growth in Chara cells is highly responsive to P and T but does not depend on the activity of wall enzymes. Because the cell walls after isolation quantitatively exhibited all the growth behaviour of the live cells for a short time, the growth behaviour resided in the matrix structure of the wall and not altered cytoplasmic metabolism. P enlarged the wall by reversible elastic deformation superimposed on a sustained deformation that was irreversible. The two kinds of deformation were identified with weak bonds that ordered the wall polymers elastically as they came under tension from P and strong bonds that caused polymers to slip under high tensions from P. The latter bonds were associated with growth.

Because the isolated walls initially grew at least as rapidly as the live cells but soon decelerated in rate, the deceleration was the main manifestation of the missing cytoplasm. Pectin is a matrix component of charophyte primary walls and is normally supplied by the cytoplasm. It was lacking for the isolated walls, and supplying pectin rejuvenated their growth, allowing the strong bonds to resume slipping at the rate occurring in live cells. It is concluded that newly-supplied matrix polysaccharides, such as pectin, may need to be supplied to the wall by the cytoplasm in order to sustain growth.

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