

Evans Review:

Cell wall biosynthesis and the molecular mechanism of plant enlargement

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Abstract. Recently discovered reactions allow the green alga *Chara corallina* (Klien ex. Willd., em. R.D.W.) to grow well without the benefit of xyloglucan or rhamnogalactan II in its cell wall. Growth rates are controlled by polygalacturonic acid (pectate) bound with calcium in the primary wall, and the reactions remove calcium from these bonds when new pectate is supplied. The removal appears to occur preferentially in bonds distorted by wall tension produced by the turgor pressure (P). The loss of calcium accelerates irreversible wall extension if P is above a critical level. The new pectate (now calcium pectate) then binds to the wall and decelerates wall extension, depositing new wall material on and within the old wall. Together, these reactions create a non-enzymatic but stoichiometric link between wall growth and wall deposition. In green plants, pectate is one of the most conserved components of the primary wall, and it is therefore proposed that the acceleration-deceleration-wall deposition reactions are of wide occurrence likely to underlie growth in virtually all green plants. *C. corallina* is one of the closest relatives of the progenitors of terrestrial plants, and this review focuses on the pectate reactions and how they may fit existing theories of plant growth.

Additional keywords: calcium, *Chara corallina*, gel, growth, irreversible deformation, pectate, pectin, tension, turgor pressure.

Introduction

The ability to grow larger is one of the most important activities of plants. All of development depends on it because cells divide as development occurs, and growth is part of the division cycle. After division, young plant cells generally enlarge further by 10- to 1000-fold before they mature. The latter enlargement controls most of the growth and shape of organs, allowing plants to reach the light, acquire nutrients and water, and develop reproductive structures. Reproductive success thus depends on the underlying enlargement process, giving it a primary significance for evolution.

This review considers plant enlargement in the knowledge of recently discovered reactions linking the process to cell wall biosynthesis. The reactions depend on unique chemistry, and the aim is to explore ways the reactions might fit the wider contexts of plant biology. Helpful early reviews describe physiological and biochemical aspects of enlargement and cell wall structure (Northcote 1969; Cleland 1971; Preston 1974; Taiz *et al.* 1981; Taiz 1984; Ray 1992; Carpita and Gibeault 1993). Later reviews focus on gene expression, and understanding the formation of wall precursors (Carpita *et al.* 1996; Darley *et al.* 2001; Scheible and Pauly 2004; Seifert 2004; Somerville *et al.* 2004; Saxena and Brown 2005; Bacic 2006; Farrokhi *et al.* 2006; Lerouxel *et al.* 2006; Somerville 2006). Excellent overviews are given by Kutschera (2001), Fry (2004) and Cosgrove (2005). But mostly missing from these treatments are the enigmatic later steps in wall assembly and the specific chemistry controlling

rates of enlargement. How does the solid composite wall extend while more polymeric material is integrated into itself? What are the specific chemical structures controlling the extension? Answers to these questions are important, and are beginning to emerge.

Background

Cells enlarge under the control of the tough cell wall, termed the primary wall, which is extended by cell wall tension produced by the turgor pressure (P) inside the cell. Normally, P is rather high, around 0.5 MPa or more, which is equivalent to two to three times the pressure in an automobile tyre. When P is sufficiently high, the wall extends both irreversibly and reversibly, which enlarges the cell compartment. The irreversible extension is considered to be 'growth'. While the extension occurs, new material is simultaneously synthesised and deposited in the wall. Maintaining wall thickness is essential because enlargement of 10- to 1000-fold would make the wall too thin to hold P . In most plant cells, the thickness remains the same within a factor of about two (Taiz 1984; Kutschera 1990; and references therein; Bret-Harte *et al.* 1991). Consequently, in the living cell, wall enlargement and wall biosynthesis go together.

The primary wall is an intricate, largely noncovalent network of polymers. Cellulose microfibrils are cross-linked by hydrogen bonds to a matrix of hemicellulose intercalated with pectins and a small amount of structural protein (e.g. Preston 1974; Carpita and

Gibeaut 1993; McCann and Roberts 1994; Fry 2004; Cosgrove 2005). The microfibrils have a high tensile strength, and are often laid down in an orientation perpendicular to the long axis of the cell. With this orientation, lateral extension is inhibited and the wall extends mostly lengthwise (Probine and Preston 1962; Baskin 2001, 2005). The longitudinal extension stretches the matrix between the microfibrils, spreading the microfibrils apart. The orientation of the microfibrils thus controls organ shape while the matrix controls growth rate.

The later steps in this process have remained enigmatic in part because of the difficulty in controlling P . In terrestrial plants, enlarging cells are small, densely packed, and considerably distant from a vascular supply. Gradients in water potential and P are required to move water and solutes from the distant vessels to the enlarging cells to feed the growth process (Boyer and Silk 2004). Because the source of the gradients is in the enlarging cells, the gradients are considered growth-induced (Boyer 2001). If the tissues are then excised, P can change and alter the gradients, creating an uncontrolled variable affecting enlargement. P -dependent enlargement is obscured by these changes.

New approaches

Because of this situation, single algal cells have proven useful for enlargement studies. Water and solute surround the cells, causing growth-induced water potentials to be negligible (Zhu

and Boyer 1992). P can be controlled by inserting the tip of a microcapillary into the cell and injecting or withdrawing cell solution without altering any other property in or around the live cell (Zhu and Boyer 1992). An important feature is the ability to conduct manipulations in the culture medium, including isolating the wall, mounting it on a microcapillary, and supplying or injecting diverse molecules (Proseus and Boyer 2005, 2006a). All metabolic and enzymatic functions of the live cell or isolated wall are preserved with this approach.

P -dependent elongation becomes obvious with this technique in a single cell of the large-celled green alga *Chara corallina* (Klien ex. Willd., em. R.D.W.) (Fig. 1A). The elongation resembles the growth of leaves on intact plants (Fig. 1B). Elongation begins above a critical P and increases when P increases further. Growth occurs over a similar range of P in both organisms. Likewise, the temperature response of growth in a single cell of *Chara* (Fig. 1C) resembles that of terrestrial species such as soybean (*Glycine max* L. Merr.; Fig. 1D). Growth decreases similarly in both species as temperature is lowered. These similarities probably occur because charophytes are the closest relatives of the algal progenitors of terrestrial plants (Chapman 1985; Graham 1985; Niklas 1992; Scherp *et al.* 2001). Note, however, that the terrestrial plants grow 5–20 times faster than *Chara* when P and T are favourable (cf. rates on the y-axes of Fig. 1).

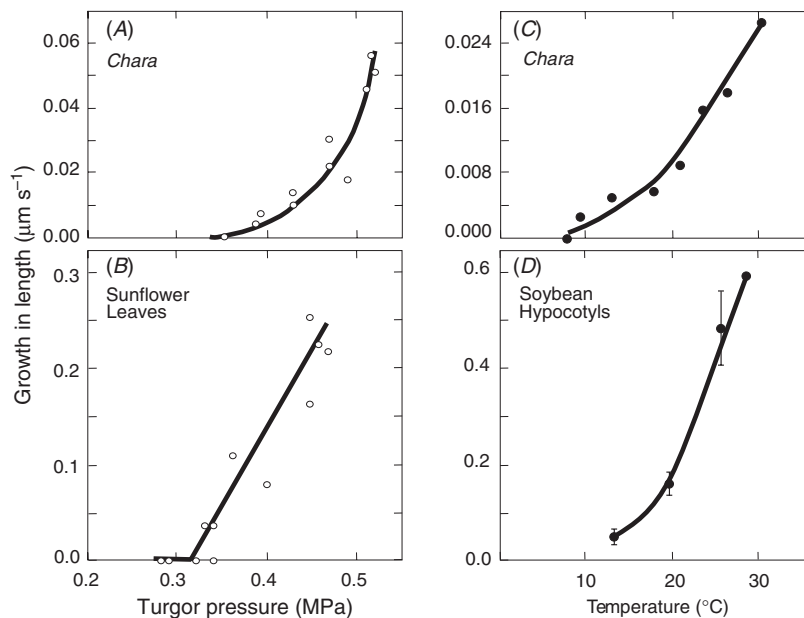


Fig. 1. (A) Growth in a *Chara* internode cell, and (B) sunflower leaves at various P , and in (C) a *Chara* internode cell, and (D) soybean hypocotyls at various T . In (A), P was changed and determined in a single live cell mounted on a microcapillary attached to a pressure probe while elongation was measured. In (B), water was withheld from the soil for various times before determining P in several intact sunflower (*Helianthus annuus* L.) leaves using isopiestic psychrometry. In (C), T was changed and controlled in a single live cell mounted on a microcapillary attached to a pressure probe while elongation was measured. P was maintained at 0.5 MPa with the probe. In (D), T was changed and controlled for 24 h in several seedlings before measuring elongation of the intact hypocotyls. P was measured with isopiestic psychrometry and was 0.45–0.65 MPa. Data in (D) are means $\pm$ 1 s.d. See Matthews *et al.* (1984), Boyer (1993), and Proseus *et al.* (2000) for additional replication, and Nonami *et al.* (1987) for the close relationship between P measured with a pressure probe and with isopiestic psychrometry.

The quantities of pectin, hemicellulose, and cellulose in primary walls of charophytes (Fig. 2A) resemble those in terrestrial plants (Anderson and King 1961; Preston 1974). The cellulose (β -1,4-glucan) is in crystalline microfibrils surrounded by a matrix of pectin and hemicellulose that is gel-like. The pectin of charophytes is composed of polygalacturonic acid (pectate, i.e. α -1,4-linked galacturonic acid with small amounts of rhamnose) and minor quantities of polyglucuronic acid (Preston 1974; Morrison *et al.* 1993; Popper and Fry 2003). Few, if any, of the carboxyl groups are methylesterified, leaving them free to be weak acids (Anderson and King 1961; Morrison *et al.* 1993). Ca^{2+} forms cross-links with these carboxyl groups, and provided there are regions of at least 10–20 consecutive unmethylated galacturonic carboxyls in neighbouring pectate molecules, clusters of two or possibly four molecules will form non-covalent interpolymer associations with divalent cations (Grant *et al.* 1973; Morris *et al.* 1982; Powell *et al.* 1982; Jarvis 1984; Jarvis and Apperley 1995; Ralet *et al.* 2001; Willats *et al.* 2001). The associations create strong junction zones if Ca^{2+} is the cross-linking ion, and a gel can result (Jarvis 1984; Jarvis and Apperley 1995; Willats *et al.* 2001). Mg^{2+} does not form a gel (Proseus and Boyer 2006c). Calcium is abundant in *Chara* walls, and magnesium is detected (Fig. 2B). In primary walls of charophytes, if calcium is removed, the wall begins to disassemble (Gillet and Liners 1996).

The pectin rhamnogalactan II that complexes boron in terrestrial walls appears to be absent in *Chara* (Matsunaga *et al.* 2004; O'Neill *et al.* 2004). Also in contrast to the primary walls of terrestrial plants, *Chara* walls lack isoprimeverose (xylosyl- α -(1 $\rightarrow$ 6)-glucose) and thus a fundamental unit of xyloglucans (β -1,4-linked glucan with α -D-xylosyl side branches) (Popper and Fry 2003). Instead,

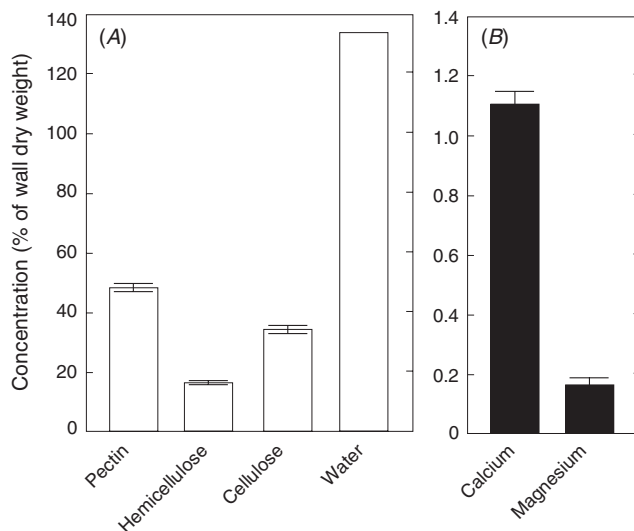


Fig. 2. The composition of the primary wall of charophytes. (A) Polysaccharides and water. (B) Inorganic constituents. Also analysed were boron, copper, iron, potassium, sodium and zinc but these elements were not detected. Polysaccharides are for *Nitella* from Morrison *et al.* (1993), water and inorganic elements are for *Chara* from Proseus and Boyer (2006c). Data are means $\pm$ s.e. ($n = 3$ –4).

the hemicellulose contains xylose-rich polymers whose structures remain to be elucidated.

The crystalline cellulose does not contain water, but there is considerable water in the interstices of the gel-like matrix (Fig. 2A). Whether in *Chara* (Shepherd and Goodwin 1989; Proseus and Boyer 2005) or terrestrial species (Read and Bacic 1996), the water-filled interstices have diameters of ~ 4 –5 nm. Small molecules like glucose (0.42 nm) or calcium (hydrated diameter of 0.88 nm) readily pass through, but most polysaccharide polymers are much larger and do not enter unless P is present (Proseus and Boyer 2005). Taken together, these observations suggest that the structure of the charophyte wall is a simplified version of the terrestrial wall (Anderson and King 1961; Preston 1974; Morrison *et al.* 1993; Popper and Fry 2003).

Nature of growth-controlling bonds

When primary cell walls are isolated from *Chara*, filled with oil in place of cytoplasm, and pressurised to the P normally found in the live cells (0.5 MPa), they grow nearly as rapidly as the live cells for 1–2 h (Proseus and Boyer 2006b). Even when the isolated walls are boiled for 10 min and cooled before pressurisation, their growth rates are similar to those in the live cells (Fig. 3). Since wall enzymes are highly unlikely to survive this treatment [except in extremophiles, see Van den Burg *et al.* (1998)], it seems safe to conclude that growth is controlled by a non-enzymatic reaction in the wall itself. The reaction depends on P (Fig. 3A) and T (Fig. 3B) in the same fashion as in the live cell (Fig. 3) and in terrestrial species (Fig. 1B, D). P and T responses of the live cell are thus controlled by the P and T responses in the wall without the activity of enzymes. This remarkable result indicates that growth is a non-enzymatic activity in *Chara*, and the cytoplasm and wall enzymes appear to play no part other than to determine wall structure (Proseus and Boyer 2006b).

Another consequence of this T -responsive growth is that it resembles the T response of metabolism or enzymes. Indeed, as recently as 2000, Proseus *et al.* (2000) used the T response of live *Chara* cells to conclude that growth was under metabolic control. It is now clear that this was likely incorrect and the T response is an inherent property of a non-enzymatic bond in the wall structure (Proseus and Boyer 2006b). The bond is weaker than covalent or electrostatic ones that require enzymes to lower their activation energy for metabolic reactions. The activation energy for the growth-controlling reaction is inherently moderate and allows reasonable rates of reaction without enzyme mediation.

Others have reported supporting results. Metraux and Taiz (1977) and Taiz *et al.* (1981) in *Nitella*, and Tepfer and Cleland (1979) in the chlorophyte alga *Valonia*, report no effect of boiling the walls on the ability of acidic media to promote elongation. But oat coleoptiles that were boiled (Tepfer and Cleland 1979) or maize coleoptiles that were 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 exposing them to proteases irreversibly inhibited wall extension (Cosgrove 1989). It seems that enzyme activity may affect the growth of terrestrial species, perhaps because there are wall bonds not present in charophytic algae.

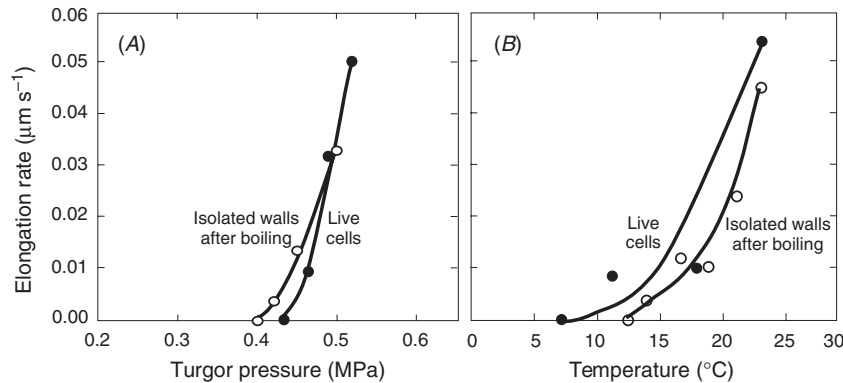


Fig. 3. Elongation rates at various P or T measured in live *Chara* cells or in walls isolated from similar cells, boiled for 10 min, then cooled and measured. Note the similarity to the P and T responses of terrestrial species in Fig. 1. For the P experiments, P was varied using a pressure probe. For the T experiments, P was maintained at 0.5 MPa using a pressure probe. Data are single measurements in a single cell or wall. For replication, see Proseus and Boyer (2006b).

What is the nature of the non-enzymatic but growth-controlling bond in *Chara*? The rapid growth of isolated *Chara* walls is not sustained beyond 1–2 h, and soon becomes slow while the live cells continue to grow rapidly (Proseus and Boyer 2006b). Evidently, the cytoplasmic contribution to wall structure is necessary for sustained wall growth. The cytoplasm supplies precursors for wall biosynthesis, and when Proseus and Boyer (2006b) added pectate to the culture medium around pressurised walls of *Chara*, it rejuvenated growth. The rejuvenating action lasted for several h and required only a low pectate concentration ($35 \mu\text{M}$ K-pectate having a molecular weight of 170 kDa, supplied to the culture medium at pH 7).

Because pectate is a prominent wall constituent, the cytoplasm supplies it to the wall for wall biosynthesis, and the rejuvenation is likely to occur *in vivo*. Consistent with this prediction, pectate supplied to live cells caused their growth to accelerate (Fig. 4A, B). Similar behaviour was seen in isolated walls (Fig. 4C, D). The effect required pectate because removing pectate and returning the original medium caused growth to revert to its original rate (Fig. 4). These results indicate that the cytoplasm sustains the growth-controlling reaction in the wall by providing a supply of precursors, specifically pectate, for wall biosynthesis.

But how could such a system work? Did the new pectate intercalate within the structure of the existing wall, taking up space between the polymers and thus expanding the matrix? This is unlikely because Proseus and Boyer (2005) report no spontaneous polysaccharide entry into the wall if the solution is at atmospheric pressure and the molecular weights are ~ 38 kDa or larger, as in this experiment. This rules out intercalation (i.e. intussusception) as a mechanism of pectate action. Also, because enzymes had no effect on wall growth, enzymatic mechanisms are ruled out. Without intussusception or enzyme activity, it seems possible that pectate might act chemically, directly on the bonds controlling the P and T responses of the cells in Fig. 3.

The latter possibility is supported by the action of newly supplied pectate on the calcium and magnesium in the wall (Proseus and Boyer 2006c). New pectate removed these

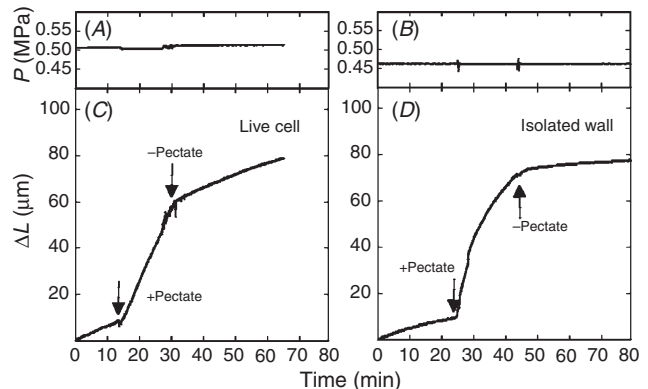


Fig. 4. Supplying pectate to the culture medium (downward arrow), then removing it from (A and C) live cells, or (B and D) isolated walls. Removal involved washing with fresh culture medium (upward arrow). Turgor pressure (P) is shown in (A and B). Change in cell length (ΔL) is shown in (C and D). Temperature was constant at 23°C . Pectate was 170 kDa, $35 \mu\text{M}$, pH 7. From Proseus and Boyer (2006c).

elements from the wall, and similar but larger losses occurred when the wall was exposed to low concentrations of EGTA (2.5 mM , pH 7). Because EGTA is a strong chelator of calcium, pectate appeared to be acting as a chelator. Indeed, growth accelerated with EGTA in the same way as with the pectate (Proseus and Boyer 2006c). Moreover, the EGTA completely removed magnesium from the wall without loss in wall integrity. Some calcium remained, suggesting that wall magnesium was inactive in wall growth while calcium was important.

If so, re-supplying Ca^{2+} without pectate should replace the lost calcium in the wall and cause growth to revert to the original rate before new pectate was supplied. As shown in the experiment in Fig. 4, the fresh culture medium washed away the pectate, and Ca^{2+} normally in the medium caused the growth to revert to its original rate. The culture medium had a concentration of 0.61 mM Ca^{2+} , and, therefore, only a low concentration of Ca^{2+} was necessary to replace that lost from the wall. Similar

results were obtained with EGTA in live cells (Proseus and Boyer 2006c). Hence, pectate newly supplied by the cytoplasm acts to remove calcium from the growth-controlling bond in the cell wall, and the culture medium acts to re-supply it.

During biosynthesis, the new pectate is in the cytoplasm and exposed only to low Ca^{2+} concentrations, typically sub-micromolar around $0.1 \mu\text{M}$ (Clarkson and Hanson 1980; Hepler and Wayne 1985). In *Chara*, the calcium pump secretes Ca^{2+} at a rate of $0.078 \text{ nmol m}^{-2} \text{ s}^{-1}$ (Reid and Smith 1992), or $\sim 1\%$ of the rate of uronic acid delivery to the periplasm as pectate ($\sim 7.2 \text{ nmol m}^{-2} \text{ s}^{-1}$, calculated from Appendix 2 by Proseus and Boyer 2006c). Consequently, pectate is delivered to the wall essentially without Ca^{2+} , perhaps as a K^+ salt.

The wall is where new pectate first meets Ca^{2+} in significant concentrations. Sentenac and Grignon (1981) calculate a high concentration of Ca^{2+} in the interstitial water of cell walls. Newly synthesised pectate would bind this Ca^{2+} moderately strongly, with a pK_a for dissociation of 3.5 (Van Cutsem and Gillet 1983; Jarvis 1984). Because of the dissociation, the new pectate would exchange calcium continually with the interstitial Ca^{2+} and the bound wall calcium. This exchange would cause the gel structure of the wall matrix to be dynamic, able to re-orient, and have a tendency to slip when placed under sufficient tension from *P*.

Tension and the molecular mechanism of cell enlargement

Given that calcium pectate seems to be the molecular site controlling the growth rate of these cells, it becomes important to consider how *P* affects the site. When *P* rises in cells, it puts wall polymers in tension. Proseus and Boyer (2006c) filled isolated walls with oil where the cytoplasm had been and created tension by pressurising the oil to *P* of 0.5 MPa. When as much as 95% of the wall calcium was removed, the walls grew rapidly but remained intact. This indicated that only 5% of the calcium pectate bonds were capable of bearing the entire tension from *P* in the wall matrix. If more than 95% of the calcium was removed, massive elongation occurred and the isolated wall soon broke, leaking the oil to the outside (Proseus and Boyer 2006c). *P* fell to zero. Without these few bonds, wall integrity was lost. Because they could bear most of the load from *P*, it follows that they also controlled irreversible wall deformation by *P* and thus growth. New pectate must have acted on them.

When the tension was varied by varying *P* in isolated *Chara* walls, newly supplied pectate was active only when *P* was above a critical value (Proseus and Boyer 2007). If *P* was below 0.2 MPa, newly supplied pectate had no growth-accelerating activity. In other words, tension appeared to make the load-bearing bonds susceptible to calcium loss. Removing their calcium would decrease the cross-bridging between neighbouring pectate molecules, weaken the load-bearing cross bridges, and allow polymer slippage that irreversibly deformed the wall.

Proseus and Boyer (2007) suggested that the ladder-like structure of pectate might explain this action (Fig. 5A). The rungs of the ladder are galacturonic acid and the single side rail is the α -(1-4) oxygenic bond connecting the rungs (Ridley *et al.* 2001). The side rail alternates from one side of the ladder to the other between rungs. The carboxyl groups also

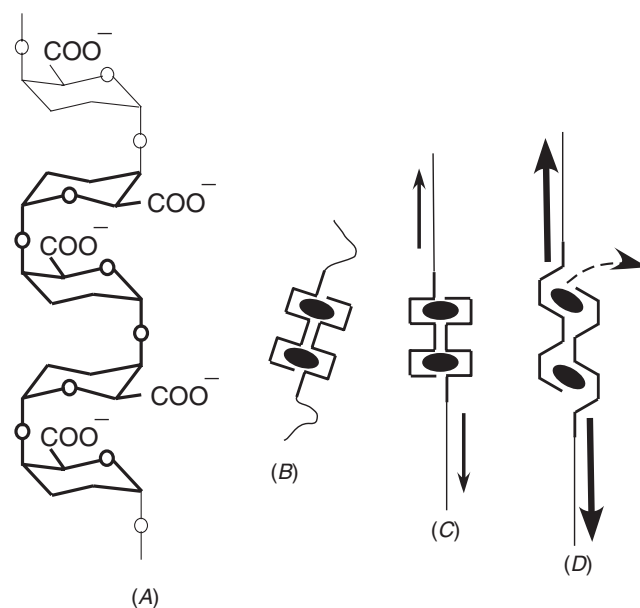


Fig. 5. Pectate structure and suggested distortion in *Chara* cell walls under tension from *P*. (A) Ladder-like structure of pectate (polygalacturonic acid). Hydrogen and hydroxyl groups are not shown. (B) Two anti-parallel, cross-linked pectate chains before *P* develops. Bold portion of pectate in (A) shows position of two calcium pectate cross-links in a junction zone in (B) (with calcium as black ovals). (C) Same as (B) but chains are in moderate tension from low *P* (small arrows). (D) Same as (B) but chains are in high tension from normal *P* of 0.5 MPa (large arrows). High tension distorts pectate, weakening calcium pectate cross-links and causing calcium to be released more readily (dashed arrow) than from undistorted links. From Proseus and Boyer (2007).

alternate. As a result, two anti-parallel ladders create an 'egg-box' structure that appears to sequester Ca^{2+} with coordination bonds as in Fig. 5B, shown for two calcium cross-links (Grant *et al.* 1973; Morris *et al.* 1982; Powell *et al.* 1982; Jarvis 1984; Jarvis and Apperley 1995; Ralet *et al.* 2001; Willats *et al.* 2001). When 10–20 of these calcium cross-links are adjacent on the ladder, a junction zone forms that is strong enough to create a gel.

Proseus and Boyer (2007) suggested that the ladder structure would be susceptible to distortion by *P*. At low *P*, the ladder would straighten (Fig. 5C). Beyond a critical *P*, distortion would increase the distance between adjacent galacturonic residues as in Fig. 5D and lengthen the coordination bonds with calcium. The lengthening would weaken them and reduce the pK_a for dissociation below 3.5. In thermodynamic terms, calcium in the wall would exist in a mixed population of undistorted (pK_a 3.5) and a few distorted calcium pectate bonds (pK_a lower than 3.5). Calcium would tend to migrate towards the undistorted bonds because of their higher affinity for calcium. Newly-supplied pectate is undistorted, so it would remove calcium most easily from the distorted bonds having low pK_a . The number of distorted bonds would decrease per unit area of wall, causing growth to accelerate.

This distortion model generates several predictions. First, there should be two kinds of calcium pectate bonds in the cell wall at normal *P*, a few bonds that are distorted (Fig. 5D) and many that are not (Fig. 5B, C). Proseus *et al.* (1999) and Proseus

and Boyer (2006b) detected two kinds of wall bonds when *P* stretched the wall: weak ones that controlled coiling and orientation of wall polymers (reversible elastic), and strong ones that controlled irreversible deformation of the wall (irreversible growth). The strong ones inevitably required a critical tension (critical *P*) before they could be broken, as in Fig. 3A.

A second prediction is that a fraction of the pectate should become oriented when charophyte walls are placed under tension. Using optical methods, Morikawa and Senda (1974) and Morikawa *et al.* (1974) found that some of the wall pectate aligned parallel to the long axis of the cell if tension was applied to isolated walls of *Nitella*. Richmond *et al.* (1980) similarly observed increasing order in *Nitella* walls when placed under tension.

A third prediction is that growth acceleration should be progressive. More acceleration should be observed when tension increases incrementally, because more of the calcium pectate bonds should become distorted. This would create a progressively steeper growth response as *P* increased, which was observed (Figs 1A, 3A) and also seen when new pectate was supplied (Proseus and Boyer 2007). Thus, higher *P* weakens more cross-links.

A fourth prediction is that wall polymers distorted by tension relax when calcium is removed. After relaxing, the polymers would remove calcium from other distorted chains. Wall calcium would migrate throughout the pectate matrix. This auto-catalytic action would cause acceleration to occur for long times, as observed by Proseus and Boyer (2006b).

Finally, Proseus and Boyer (2006c) found that auto-catalysis by new pectate could be quenched by high Ca^{2+} concentrations. This demonstrated that flooding the new pectate with externally supplied Ca^{2+} could stop removal of calcium from distorted wall pectate. Basically, the Ca^{2+} bound to the 170 kDa pectate, preventing its accelerating action but also causing it to gel in and around the wall. This 2-fold action of high Ca^{2+} concentrations revealed that newly-supplied pectate acted on distorted wall pectate and accelerated growth and, after binding Ca^{2+} and gelling, the new pectate also decelerated growth. Indeed, when saturated with Ca^{2+} , the junction zones were so numerous that they could prevent growth.

Tension-dependent pectate cycle

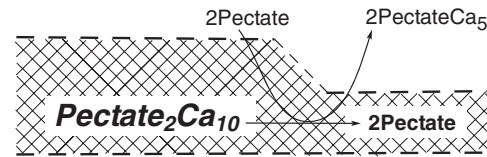
The rate of wall biosynthesis determines how rapidly new pectate is supplied to the wall. Assuming each load-bearing junction zone contains 10 Ca^{2+} ions bound to two pectate molecules, the overall biosynthesis reaction for junction zones in wall pectate would be:



where the substrates are soluble pectate from the cytoplasm (probably as acid or potassium salt) and Ca^{2+} ions from the culture medium. The product is two anti-parallel chains of pectate cross-linked by calcium in the wall to form a junction zone.

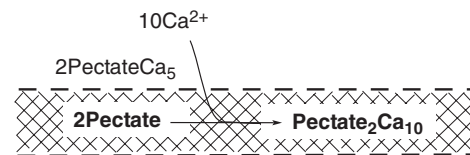
But the junction zone also is the site of the *P*-dependent removal of calcium from load-bearing bonds. The overall reaction (1) thus includes several subreactions when *P* is high enough to distort the junction zones (i.e. above 0.2–0.3 MPa in

Chara). Exposing the distorted pectate to new, undistorted pectate gives subreaction 1:



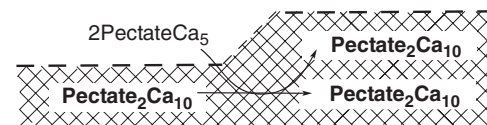
where a distorted junction zone ($\text{Pectate}_2\text{Ca}_{10}$) gives up its calcium to new pectate from the cytoplasm (2Pectate outside of the crosshatched wall). The junction zone in the wall dissociates to 2Pectate , and loses its distortion as it relaxes. Wall growth accelerates because the number of load-bearing (distorted) junction zones has decreased, weakening (loosening) the wall and allowing slippage under tension from *P*. This wall deformation is irreversible, and the wall becomes thinner.

The relaxed 2Pectate is able to bind calcium more tightly, and Ca^{2+} is absorbed from the external medium leading to subreaction 2:



which re-forms the junction zone and re-tightens the structure. Growth decelerates, but the junction zones are now spread over a larger wall area because of the previous wall deformation, and the wall remains thin.

At the same time, the newly formed 2PectateCa_5 of subreactions 1 and 2 (outside of the wall) can bind to the wall in subreaction 3:



This new wall material adds a junction zone, returning the density of junction zones to the original level, and returning the wall to its original strength and thickness.

The acceleration-deceleration-biosynthesis sequence of these subreactions sum to the overall reaction (1) and, in reality, probably occur in a continuum while the wall extends, driven by *P*. With extension, new junction zones would become distorted, and the subreactions would repeat to create a *P*-dependent pectate cycle (Fig. 6).

The cycle shows a strict stoichiometry between the supply of new pectate from the cytoplasm, wall extension, and the deposition of new wall. But the stoichiometry depends on *P*, and subreaction 1 ceases when *P* decreases below 0.2 or 0.3 MPa in *Chara*, blocking the entire cycle. For this reason, low *P* blocks not only growth, but also wall biosynthesis simultaneously, as reported by Cleland (1967) and Proseus and Boyer (2006a). But

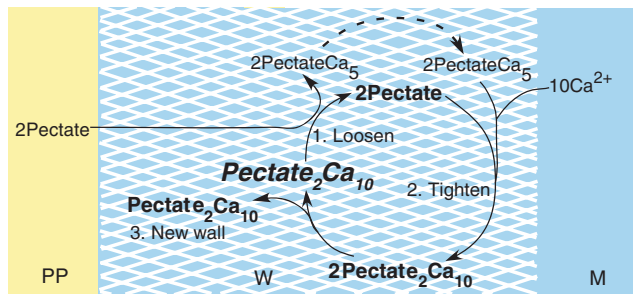


Fig. 6. *P*-dependent pectate cycle in *Chara* cell walls consisting of subreactions 1 (loosen), 2 (tighten), and 3 (new wall) described in the text. PP, layer containing newly supplied pectate next to inner wall face; W, wall containing the cycle; M, culture medium containing Ca²⁺; bold font, wall constituents; bold italic font, distorted junction zone; normal font, soluble precursors. Drawing is not to scale. From Proseus and Boyer (2007).

Proseus and Boyer (2006a) found that exocytosis continued unabated, and the pectate cycle predicts an accumulation of pectate at the inner wall face under these conditions. With a return to normal *P*, the enlarged pectate pool would remove calcium from the wall more rapidly than normal and the subreactions would speed up until the pool would be consumed. Growth would appear to have been ‘stored’ while *P* was low. Proseus and Boyer (2008) simulated this effect by supplying pectate to isolated walls at low *P* as though the cytoplasm had continued to supply it. Although the isolated walls did not normally show evidence of stored growth, they did so when *P* was returned to normal in the presence of the pectate. This provides strong evidence for the *P*-dependent pectate cycle in the wall, and also identifies wall precursors, especially new pectate, as an important cause of stored growth.

The cycle predicts that faster growth occurs with faster release of pectate by the cytoplasm, or lower molecular weight of the pectate (fewer junction zones), or more blockage of the free carboxyl groups (perhaps by methylesterification to give fewer junction zones). Also, faster growth would occur without external Ca²⁺ for a time, but this is likely to be lethal eventually. Without Ca²⁺, the wall would fail to strengthen as it was being stretched irreversibly, and would lose integrity at normal *P* or might fail to adequately support the plasma membrane.

Other factors could interrupt the stoichiometry of the cycle as well. If the molecular weight of pectate is too small to form junction zones (seen in fig. 6 by Proseus and Boyer 2006c), accelerated extension might occur without wall tightening, creating runaway extension. If the cells were starved photosynthetically, the pool of precursors might be reduced while the wall continued to extend at normal *P*, creating thin walls. If the plant was exposed to cations other than Ca²⁺, the chelation chemistry of pectate might be interrupted. Perhaps these effects account for some of the previously noted difficulties in linking wall deposition to plant growth rates (Taiz 1984; Kutschera 1990).

Cell wall biosynthesis

If pectate controls wall extension but ends up as part of the wall, the polymers must be integrated into the existing structure.

Matrix polysaccharides, including pectate, are synthesised in Golgi-derived vesicles and released to the wall by exocytosis (Northcote and Pickett-Heaps 1966; Robinson *et al.* 1976; Moore *et al.* 1991; Perrin *et al.* 2001), where they are deposited around the cellulose microfibrils at the inner wall face. But many find their way into the existing wall structure (Northcote and Pickett-Heaps 1966; Ray 1967). It is difficult to explain how such large polymers enter the wall. Talbott and Ray (1992) report that the molecular weights of matrix polysaccharides in pea (*Pisum sativum* L.) stems are in the range from 40 to 1000 kDa, which would give them diameters of ~9 nm to more than 20 nm when in solution. Interstices in the wall are only ~5 nm in diameter (Read and Bacic 1996) and too small for spontaneous entry of wall polymers in this size range.

In agreement with this observation, Proseus and Boyer (2005) found in *Chara* that polysaccharides such as dextran (α-1,6-glucan) in the 9 to 20 nm range do not enter the wall spontaneously. At low *P*, they remain in a layer adjacent to the inner wall face. But with normal *P* of 0.5 MPa, the polymers became concentrated against the inner wall, and those with diameters of 9–11 nm readily moved into and through the wall (see cover photograph). Thus, the end of the polymer appeared to enter one of the interstices, and *P* provided the energy to push the rest through while uncoiling it. The polymer moved snake-like into the matrix (reptation) where it would come into intimate contact with polymers already in the wall. For calcium pectate, this would produce a seamless integration of new wall material into the old wall structure. Supporting evidence for this mechanism comes from plasmolysed cells, which synthesise wall precursors but fail to deposit them in the wall (Robinson and Cummins 1976; Robinson *et al.* 1976; Ueda and Yoshioka 1976; Kroh and Knuiman 1985). When the cells are deplasmolysed, deposition begins (Robinson and Cummins 1976). New matrix polysaccharides thus appear to enter the wall by a *P*-driven mechanism, allowing large polymers to become part of an existing polymeric structure.

Very large polymers of ~500 kDa (diameters larger than 20 nm) failed to enter the wall even with *P* of 0.5 MPa (Proseus and Boyer 2005, 2006a). Figure 7 indicates that they remained at the inner wall where the osmotic potential of the polymer solution equilibrated with *P*. At an osmotic potential of –0.5 MPa, dextrans developed concentrations around 50% (weight of polymer/weight of water) while 170 kDa pectate reached 14% (Proseus and Boyer 2006a). These concentrations are similar to those in the gel-like matrix of the existing wall (matrix ~37% in charophytes, calculated from Fig. 2). At these concentrations, pectate begins to gel spontaneously (Proseus and Boyer 2006a). No gelling occurred if *P* decreased to 0.4 MPa, because the concentration of large dextran decreased to 45% and pectate to 11% when in equilibrium with the diminished *P* (Proseus and Boyer 2006a). Figure 8 indicates that live cells respond to this diminished *P* by depositing less wall than at 0.5 MPa. They also decreased their growth as shown in Fig. 1. These results indicate that, in addition to its effects on growth, *P* is a major contributor to wall assembly by concentrating matrix polymer solutions and pushing some of the polymers into the existing wall. Through this mechanism, new wall appears to be assembled in the pectate cycle.

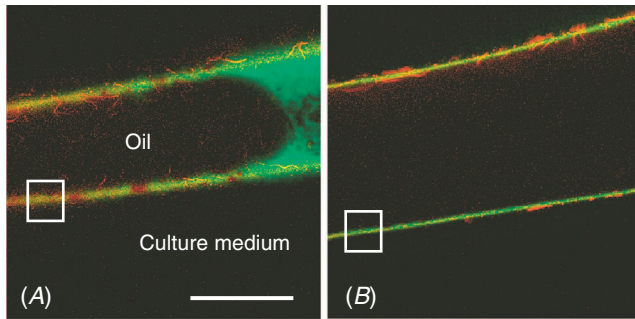


Fig. 7. Turgor pressure (P) concentrates dextran (green) that is too large (580 kDa, 27 nm diameter) to move through a cell wall isolated from a *Chara* internode. (A) Low P of 0.05 MPa creates dextran layer 45 μm thick (measured in white box). (B) Same wall a few minutes later after raising P to 0.5 MPa. Dextran thickness diminishes to 23 μm and indicates the concentration equilibrates with P . No dextran moved through the wall for 5 h. The wall is stained with Congo Red and is 5 μm thick. Scale bar = 0.5 mm. From Proseus and Boyer (2006a).

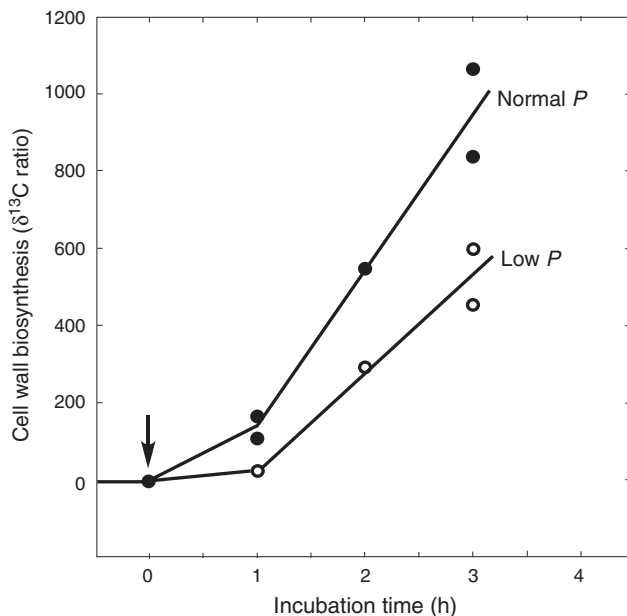


Fig. 8. Rates of wall deposition in live *Chara* internode cells at a normal turgor pressure (P) of 0.5 MPa or at a low P of 0.4 MPa. In the same cells, growth was measured simultaneously and was rapid at high P but substantially decreased by the low P . See Proseus and Boyer (2006a) for details.

Terrestrial plants

Pectate is present in the primary walls of all terrestrial plants where it embeds the other wall components and forms the middle lamella (Carpita and Gibeaut 1993; Popper and Fry 2003, 2005). It is present in most green algae, and pectate relatives such as polymannuronic acid and polyguluronic acid are often found in other algal forms (brown and red algae, Kloareg and Quatrano 1988). During the evolution of green plants, pectate, together with cellulose, was one of the most conserved wall components (Carpita and Gibeaut 1993; Popper and Fry 2003, 2005). Calcium pectate chemistry was probably similarly conserved.

This view suggests that the pectate cycle could underlie the growth control of virtually all green plants.

Because the junction zones have sufficient strength to bear the load of P or even prevent growth completely (Proseus and Boyer 2006c), *Chara* can control its growth rate by controlling the number of junction zones per unit of wall. In agreement with this concept, terrestrial species such as oat (*Avena sativa* L.) experienced a growth-accelerating action of auxin, which had increased the supply of pectin (and other matrix precursors) to the walls of the coleoptiles (Baker and Ray 1965; Ray and Baker 1965). Ca^{2+} quenched part of the acceleration. Ca^{2+} appeared to alter the biochemical wall-loosening in these structures (Cleland and Rayle 1977). Also like *Chara*, calcium chelators enhanced deformation of tomato (*Lycopersicon esculentum* Miller) epidermis when under tension, and Ca^{2+} abolished the effect (Thompson 2005).

In *Arabidopsis thaliana* (L.) Heynh, a substantial portion of the wall pectate is methylesterified. Derbyshire *et al.* (2007) used *Arabidopsis* mutants for gibberellic acid and transformants for certain pectin methylesterases to vary the degree of esterification in the walls. The cells grew faster when methylesterification was more prevalent. They conclude that esterification of the carboxyl groups blocks their ability to bind calcium, decreasing the number of junction zones and making pectate more extensible by P . In effect, the esterification loosens the walls.

It is easy to see why this might happen. P elongates the cells by pushing the end walls apart, and the force on the end walls decreases dramatically as their area decreases (P is force/area). Because the radius of an *Arabidopsis* cell (12 μm) is $\sim 3\%$ that of a *Chara* cell (375 μm), the end wall has only $\sim 0.09\%$ of the area in *Chara* and the force is correspondingly less at the same P . With so little force, *Arabidopsis* cytoplasm supplies pectate with only a few chemically reactive carboxyl groups, making it easier for P to deform the walls irreversibly. As the cells mature, pectinmethylesterase appears to reduce esterification, allowing more calcium binding and making the walls less deformable. It is worth noting that the middle lamella consists mostly of non-esterified pectate whose high strength with calcium may be necessary to hold the cells together and create a tissue. Taken together, these findings indicate that growth is likely to be controlled by calcium pectate not only in *Chara* but also in multicellular terrestrial species.

Xyloglucans in the wall matrix have also been suggested to control growth in terrestrial species. The xyloglucans readily form hydrogen bonds with cellulose, and Passioura and Fry (1992), Passioura (1994) and Fry (2004) proposed that the xyloglucans might act as tethers between microfibrils that could control wall extension. In pea stems supplied with exogenous xyloglucan, the cell walls decreased their growth rates as though the xyloglucans might have a load-bearing function (Takeda *et al.* 2002). But their absence in *Chara* excludes them for general growth control. In a terrestrial species, Cavalier *et al.* (2008) demonstrated that xyloglucan-less double mutants of *Arabidopsis* are able to grow. Without wall xyloglucan, these authors attribute growth control to other wall components such as pectins.

Xyloglucans are thought to cross-bridge between pectins and cellulose microfibrils with intertwining segments of the molecules or with hydrogen bonds (Passioura and Fry 1992;

Passioura 1994; Fry 2004) or covalent bonds (Thompson and Fry 2000). A substantial fraction of xyloglucan is covalently linked to pectin in terrestrial species (Thompson and Fry 2000; Popper and Fry 2005, 2008). This suggests that, when present in the wall, xyloglucans contribute to load-bearing by pectins but are not essential. Instead, the xyloglucan portions of the xyloglucan-pectate might bind to the microfibrils by hydrogen bonding while the pectate portions might bind to adjacent pectate by calcium cross-bridges, creating a cross-linked xyloglucan-pectin matrix with tethers between microfibrils.

Several theories of growth control involve enzymes that cleave wall polysaccharides (endoglucanases) (Goldberg, 1980; Huber and Nevins 1981; Fry 1989), or rearrange interpolymeric bonds (e.g. xyloglucan endotransglycosylases) (Fry *et al.* 1992; Nishitani and Tominaga 1992). Other putative proteins have been implicated in wall extension but are of unknown identity ('osmiophilic deposits') (Kutschera 2001) or function (expansins that might disrupt non-covalent polysaccharide bonds in the wall [McQueen-Mason and Cosgrove 1995; Cosgrove 1998; Cosgrove 2005]). In *Chara*, expansins are apparently absent (Cosgrove 2000), and although there is evidence for each of these theories, Fry (2004) points out that the presence of an enzyme of known activity is no assurance that it controls growth rate. Another difficulty is that enzymes would need to weaken wall structure at normal P but cease to function when P decreases and growth ceases. No enzyme has shown this P -dependent activity. Instead, it seems reasonable that some of the enzyme-based enlargement mechanisms could co-exist and interact with a non-enzymatic pectate cycle. An example is the pectin methylsterase studied by Derbyshire *et al.* (2007). The enzyme-based component of enlargement might then account for the rapid growth of cells of terrestrial plants compared with the slower growth of a typical *Chara* internode (Fig. 1).

A prominent enzyme-based theory is the acid growth hypothesis involving plasma membrane H^+ -ATPases that could acidify the wall and cause the wall to extend (Hope and Walker 1975; Metraux *et al.* 1980; Rayle and Cleland 1992). In live charophyte cells, the plasma membrane normally maintains a pH between 4.5 and 5.0 at the inner wall face (Hope and Walker 1975; Metraux *et al.* 1980). With the medium at pH 7 or 8, a pH gradient probably exists across these walls. Van Cutsem and Gillet (1983) found that H^+ could displace wall calcium in charophytes, giving a rationale for growth control by the acid. However, Proseus and Boyer (2006c) varied the pH of the culture medium with little effect on *Chara* enlargement perhaps because substantial Ca^{2+} was always in the medium, and wall calcium displaced by H^+ was replaced by Ca^{2+} from the medium. Pectate remained active in accelerating growth from pH 3.5 to pH 11 in the live cells. Therefore, the pH gradient appeared to play no significant role in growth under culture conditions.

Chemical inhibitors also have been used to determine whether enlargement is metabolically or enzymatically mediated (Cleland 1971; Taiz 1984). But in *Chara*, extensive tests indicated their inhibition was always accompanied by a collapse in P (Proseus and Boyer 2006b). In terrestrial species, P is rarely measured, so the interpretation of inhibitor action remains uncertain.

In terrestrial plants, gradients in growth-induced water potentials complicate these concepts. Substantial gradients

move water into the enlarging cells in multicellular tissues (Boyer and Silk 2004), and P can be diminished while P drives enlargement in the same cells. Given this situation, the osmotic potential of the cells must generate sufficient P to allow the lowered P in the gradient while sustaining sufficient P for wall extension. When the growth-induced water potential varied diurnally in the growing region of maize leaves, the osmotic potential kept pace and also varied diurnally (Westgate and Boyer 1984; Tang and Boyer 2008). P was thus maintained. In this way, P -driven enlargement appears to co-exist with the water potential gradient. The cell osmotic potential may be an important feature of growth control in multicellular tissues.

Remaining issues

Although the experimental evidence focuses on pectate for growth control, a growth accelerating action of pectate has not been reported in terrestrial species to date. The difficulty may lie with P , which was rigorously controlled in single cells of *Chara* but cannot be so easily controlled in multicellular tissues. Lacking P control, accelerated growth often is accompanied by diluting cell contents and decreasing P that would counter the growth acceleration. Also, growing tissues have numerous cell walls and plasma membranes. The entry of exogenous pectate becomes uncertain with these structures, and supplying pectate for long times might be necessary to overcome this problem. But the pectate binds Ca^{2+} , and Ca^{2+} deprivation for long periods of time can have potentially inhibitory or lethal effects for plant cells. In live cells of *Chara*, long exposure to Ca^{2+} deprivation was not necessary, and normal growth returned when normal concentrations of Ca^{2+} returned in the fresh culture medium. Brief calcium deprivation thus had no permanent effect.

The *Chara* experiments involved large amounts of exogenous pectate of low concentration while the cytoplasm usually supplies small amounts of pectate at high concentration. Professor Lincoln Taiz (personal communication) indicates that when the lumen of isolated *Nitella* walls was filled with pectate solution and pressurised, the walls extended faster than controls but soon lost wall integrity. This confirms pectate's activity but also indicates that large amounts of pectate may be problematic when concentrated by P . More work on these effects seems warranted.

Also needing work is signal transduction between the wall and the cytoplasm. According to the pectate cycle (Fig. 6), when pectate (among other wall precursors) is supplied more rapidly, growth and wall deposition become more rapid at normal P . But the supply could also take the form of changes in molecular weight or altered chemical form of the pectate (e.g. methylsterified carboxyl groups) that would require control signals between the wall and cytoplasm. Also, when P is low and pectate and other wall precursors accumulate at the inner wall, feedback signalling would be expected to control further biosynthesis. Subtle metabolic mechanisms would be required for these signals.

Conclusions

The primitive wall of *Chara* contains calcium cross-linked with pectate which, when under tension from P , appears to be the

primary growth-controlling bond in this plant. The growth-controlling feature of the bond is an immediate calcium loss to newly synthesised pectate on its way to the wall. Growth accelerates as a result, and then decelerates as the new pectate (now calcium pectate) is deposited in the wall. Despite the non-enzymatic character of these reactions, this chemistry acts in walls of live cells. The chemistry creates *P* and *T* responses like those in terrestrial plants, and it is proposed that the chemistry is the underlying growth mechanism in virtually all green plants. Xyloglucan, rhamnogalactan II, and methylesterification are often present in species other than *Chara*, and probably play modifying roles in this chemistry.

The activity of newly synthesised pectate is traced to its ability to act as a stronger calcium chelator than the load-bearing pectate in the wall. The load-bearing pectate bonds appear to be weakened by distortion of the pectate structure by tension generated from *P*. The weak bonds lose calcium, which decreases the number of load-bearing cross-links and accounts for the accelerated growth. Having removed calcium, the new calcium pectate is able to bind to pectate in the existing wall, accounting for growth deceleration and new wall deposition.

P concentrates the new pectate because the wall acts as an ultrafilter. Although most matrix polysaccharides are too large to enter the wall spontaneously, many enter with *P* and come into intimate contact with existing wall polymers. The high concentration and contact with existing polymers helps to create a tough, seamlessly-synthesised gel enclosing cellulose microfibrils and other components of the primary wall.

It is proposed that these reactions occur in sequence in a pectate cycle that links wall biosynthesis to growth in a *P*-dependent way. Although the cytoplasm supplies pectate and other wall precursors, the pectate cycle occurs entirely in and around the wall, and represents critical terminal steps in wall growth and assembly. Consequently, in plants, growth during wall biosynthesis may be determined to a large extent by the rate at which new polymers with chemically reactive carboxyl groups are supplied to the wall.

Acknowledgement

I thank Professor Lincoln Taiz for so generously sharing his unpublished experiments with pressurised pectate solutions in the lumen of isolated walls of *Nitella*. Special thanks are also due to Dr John Passioura and Professor Stephen Fry for many thoughtful discussions, and Professor JKE Ortega for helpful comments on the manuscript.

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