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Disruption of hydrogen bonding between plant cell wall polymers by proteins that induce wall extension

(cellulose/paper/plant cell enlargement)

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ABSTRACT **Plant cell enlargement is controlled by the ability of the constraining cell wall to expand. This ability has been postulated to be under the control of polysaccharide hydrolases or transferases that weaken or rearrange the load-bearing polymeric networks in the wall. We recently identified a family of wall proteins, called *expansins*, that catalyze the extension of isolated plant cell walls. Here we report that these proteins mechanically weaken pure cellulose paper in extension assays and stress relaxation assays, without detectable cellulase activity (exo- or endo- type). Because paper derives its mechanical strength from hydrogen bonding between cellulose microfibrils, we conclude that expansins can disrupt hydrogen bonding between cellulose fibers. This conclusion is further supported by experiments in which expansin-mediated wall extension (*i*) was increased by 2 M urea (which should weaken hydrogen bonding between wall polymers) and (*ii*) was decreased by replacement of water with deuterated water, which has a stronger hydrogen bond. The temperature sensitivity of expansin-mediated wall extension suggests that units of 3 or 4 hydrogen bonds are broken by the action of expansins. In the growing cell wall, expansin action is likely to catalyze slippage between cellulose microfibrils and the polysaccharide matrix, and thereby catalyze wall stress relaxation, followed by wall surface expansion and plant cell enlargement.**

Plant cells are surrounded by a tough polymeric wall that acts like a straitjacket to constrain and shape the cell. The typical wall of higher plant cells contains crystalline cellulose microfibrils embedded in a gel-like matrix of mixed-linked polysaccharides and protein (1–4) and is placed under considerable tensile stress by the internal hydrostatic pressure of the cell. Prior to maturation, plant cells enlarge by a factor of 10 to more than 100 times the original volume. This enlargement is initiated by stress relaxation of the wall, which leads secondarily to water uptake by the cell and surface expansion of the wall (5).

Despite intensive study, the molecular mechanisms underlying wall relaxation and expansion remain poorly understood. It is commonly proposed that wall "loosening" enzymes, such as wall hydrolases or transglycosylases, cleave tension-bearing polymers to initiate wall relaxation and cell enlargement (1, 6, 7). Alternative ideas invoke phase transitions in the gel-like matrix (8), perhaps associated with wall synthesis, biochemical modifications of the pectin network, or changes in the ionic environment of the wall (reviewed in ref. 1). Unfortunately, none of these mechanisms has been shown capable of causing extension of isolated walls, so they remain attractive but unproved hypotheses.

We recently identified a class of wall proteins from cucumber and oat seedlings with the ability to induce extension of isolated plant cell walls (9, 10). We have named this class of proteins *expansins*. Two expansins were purified from cucumber cell walls with molecular masses of 29 and 30 kDa, referred to here as Ex29 and Ex30, respectively. The activity of these expansins was correlated with the growing state of the tissues from which they were isolated. Furthermore, expansin activity showed similar biochemical sensitivities to pH, metal ions, and proteases as exhibited by the extension of native cell walls. Our results indicate that expansins mediate at least part of the "acid-growth" responses found in most plant species (11).

Contrary to conventional ideas, these wall extension proteins do not exhibit hydrolytic activity on cell walls (9). Neither do they possess xyloglucan endotransglycosylase activity (12), an activity which has recently attracted attention as a potential "wall loosening" mechanism (13, 14). Furthermore, we have found that these expansins do not cause time-dependent weakening of cell walls and that their effects on the mechanical properties of walls are fully reversible by heat inactivation—results that argue against a hydrolase-type mechanism of action (unpublished observations).

These observations led us to suspect that expansins catalyze wall expansion by reversibly disrupting noncovalent interactions within the cell wall. This hypothesis is difficult to investigate because such activity would leave little trace of its action. Moreover, the heterogeneous composition of the plant cell wall and its complex structure present many possibilities for noncovalent bonds. Therefore, as a simpler model system, we examined the action of expansins on filter paper. Such paper is composed of cellulose fibers held together by hydrogen bonding, which endows paper with its mechanical properties (15). We do not propose cellulose paper as a model of the plant cell wall, but use it solely to assay the ability of expansins to disrupt hydrogen bonding between cellulose fibers *in vitro*. Any effects of these proteins on paper would be easier to interpret than effects on cell walls, which contain at least three coextensive polymer networks, any of which has the potential to be load bearing and thus to influence the mechanical properties of the cell wall (1, 16). Here we report evidence that purified expansins can weaken the hydrogen bonding between paper fibers without degrading the cellulose molecule. This mechanism of action is further tested by the action of urea, deuterated water (D_2O), and temperature on extension of expansin-treated walls.

MATERIALS AND METHODS

Plant Materials. Seeds of cucumber (*Cucumis sativus* L., cv. Burpee Pickler, from A. W. Burpee, Westminster, PA) were sown on water-soaked paper (KimPak seed germination paper K-22; Seedburo Equipment, Chicago) and germinated in the dark at 27°C for 4 days. For wall extractions,

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Abbreviation: D_2O, deuterated water.

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etiolated seedlings were harvested under laboratory lighting by excising the growing region of the hypocotyl (upper 4 cm) with a razor blade and were floated on cold buffer (10 mM KH_2PO_4, pH 7.4/3 mM $NaHSO_3$) before grinding in a blender. For extensometer assays, the upper 1 cm of the hypocotyl was excised and stored at −20°C before use.

Protein Purification. Two expansin fractions were purified as described (9). Briefly, proteins were extracted with buffered 1 M NaCl from washed cell wall fragments isolated from growing hypocotyl sections as described above. Proteins were precipitated from this extract with ammonium sulfate and sedimented by centrifugation. S1 and S2 fractions (enriched in Ex29 and Ex30, respectively) were purified by HPLC (C3 column followed by sulfopropyl cation-exchange column) (9). Protein concentrations were estimated by using Coomassie protein assay reagent (Pierce) with a standard curve constructed with bovine serum albumin (Pierce).

Extension Assays. Extension measurements were made with a constant load extensometer as described (9, 17). Paper strips (Whatman no. 3, 10 mm by 2 mm) were secured between two clamps (with about 5 mm between the clamps) under a constant tension of 20 g force. Plastic cuvettes were fitted around the specimens and filled with bathing solution (generally 50 mM sodium acetate, pH 4.5). Movement of the lower clamp was detected with an electronic position transducer and recorded on a microcomputer. Extension of cucumber hypocotyl walls was measured in the same fashion, except that the apical 1-cm region of the hypocotyl was fixed between the two clamps (5 mm between clamps). We refer to wall specimens from frozen, thawed, and abraded hypocotyls as "native" walls (17). "Heat-inactivated walls" were frozen, thawed, and abraded hypocotyls treated with a 10-min incubation in water at 80°C. This treatment inactivated the endogenous extension mechanisms. "Reconstituted walls" were heat-inactivated walls that were subsequently treated with purified expansin fractions.

For the D_2O experiments, 1 part of 1 M sodium acetate in H_2O was mixed with 19 parts of pure D_2O (99.9%) and adjusted to an apparent pH of 4.5 by addition of acetic acid. The final D_2O concentration was approximately 95%.

For the temperature experiments, a special extension chamber was constructed that allowed the wall of the cuvette to be cooled or heated with flowing water. Temperature of the solution within the cuvette was measured to 0.1°C with a miniature thermistor. The temperature reached steady-state values within 5 min and was generally held for an additional 20 min to estimate extension rate.

Stress Relaxation Assays. Strips of Whatman no. 3 filter paper (10 mm by 2 mm) were held between two clamps in a custom-made tensile tester (17), with 5 mm of paper between the clamps. Samples were extended at 170 mm/min until a force of 20 g was attained and then held at constant strain. Force was detected by a force transducer attached to the lower clamp and recorded for 5 min by a microcomputer with a minimum sampling interval of 2 ms, gradually increasing to 2 s (17). The relaxation spectrum was calculated as the derivative of the force with respect to log(time).

Paper Hydrolysis. Cellulase (from *Trichoderma viride*; Boehringer Mannheim) and Ex29 (S1 fraction) were extensively washed and filtered on Centricon-30 microconcentrators (Amicon) to remove soluble sugar contaminants. Ex29 and cellulase were then incubated with 10 strips (3.5 mg) of Whatman no. 3, in 1 ml of 50 mM sodium acetate, pH 4.5 for 5 h at 25°C. After incubation, the reaction mixes were filtered through 0.2-μm Centrex filters (Schleicher & Schuell) to remove particulate matter. Solutions were assayed colorimetrically for reducing sugars by using *p*-hydroxybenzoic acid hydrazide, with glucose as a calibration standard.

Viscometry Assays. Carboxymethylcellulose, sodium salt, high viscosity (Sigma), was dissolved at 20 mg/ml in 50 mM sodium acetate, pH 4.5. An 0.8-ml sample of this solution was mixed with 0.2 ml of Ex30 (S2 protein, 10 μg/ml) or 0.2 ml of *Trichoderma* cellulase (100 μg/ml), and the viscosity was measured periodically in a rolling ball viscometer (18).

RESULTS AND DISCUSSION

Our first approach was to investigate the action of expansins on the extension of filter paper held under a constant load. Plant walls exhibit a long-term "creep" when treated with expansins in this assay (9, 17). Our results with paper are presented in Fig. 1. When filter paper was bathed in buffer and placed under tension, it showed a low initial rate of extension which declined almost to zero by 30 min. When a purified expansin fraction from cucumber hypocotyls was added to the bathing solution, the rate of extension increased until, after about an hour of extension, the paper broke. When this protein was boiled in water for 5 min prior to addition, no effect on extension was seen. When similar experiments were carried out using bovine serum albumin at 100 μg/ml, no such effects were seen, indicating that nonspecific proteins do not weaken the paper (data not shown). Treatment with 8 M urea caused the paper to break (not shown), as expected of a treatment that should disrupt hydrogen bonding between paper fibers. In contrast, ionic and nonionic detergents (1% SDS, 1% Triton X-100) and 4 M NaCl showed no effects in this assay, indicating that hydrophobic and electrostatic interactions play a minor or no role in the mechanical properties of paper.

A *Trichoderma* cellulase preparation also caused extension and breakage of the paper (Fig. 1), but it required a concentration of 100 μg/ml to produce this effect, whereas only 5 μg/ml of the expansin fraction was required for a similar effect. Cellulase and expansin differed markedly in their hydrolytic activities. Prolonged incubation of paper strips with cellulase released soluble sugars (Fig. 2*A*), whereas the expansin fraction had no effect in this exoglucanase assay. Similarly, cellulase reduced the viscosity of carboxymethylcellulose solutions, whereas the expansin fraction exhibited no effect in this sensitive assay for endoglucanase activity (Fig. 2*B*). These results indicate that the weakening of paper by cellulase may be attributed to hydrolytic activity, whereas

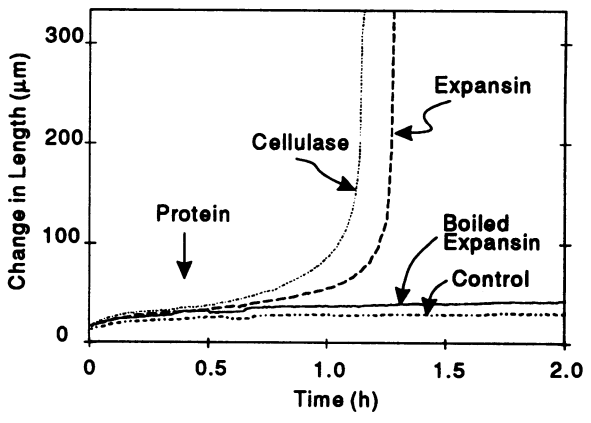

FIG. 1. Effects of expansin and cellulase on extension and breakage of filter paper, as assayed with a constant-load extensometer. Paper strips were clamped in an extensometer in 50 mM sodium acetate, pH 4.5. After 20 min the buffer was exchanged for 0.4 ml of the same buffer containing various protein additions. They were (per ml of buffer): 100 μg of cellulase from *Trichoderma viride*, 5 μg of expansin, 5 μg of expansin inactivated by boiling for 5 min in water (data shown are for Ex29; similar results were obtained with Ex30). The control contained no protein additions. The figure shows representative traces from six independent experiments, all of which showed similar results.

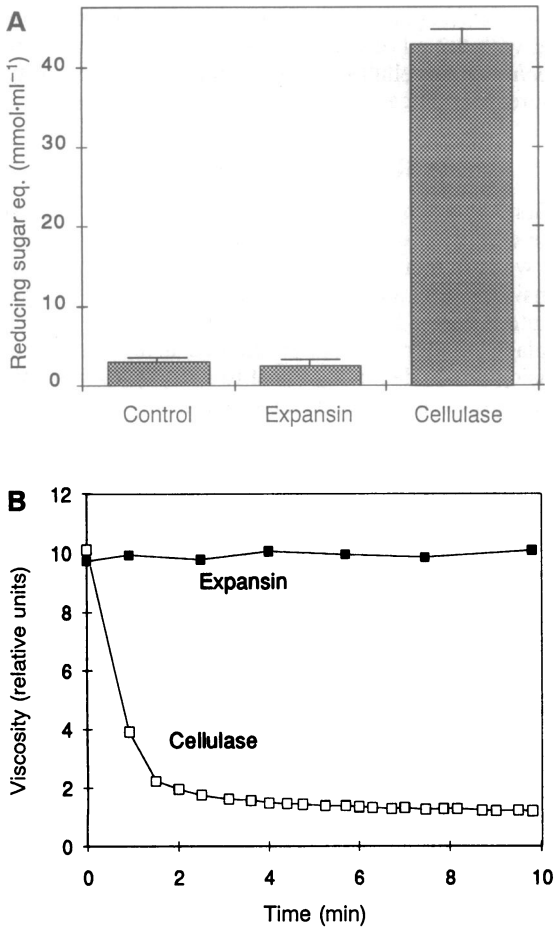

FIG. 2. Comparison of hydrolysis of paper (*A*) and reduction of carboxymethylcellulose viscosity (*B*) by expansin and cellulase. (*A*) Cellulase exhibits exoglucanase activity, whereas expansin does not. Filter paper strips were incubated with 50 mM sodium acetate, pH 4.5, containing Ex29 (S1 fraction, 5 μg/ml), *Trichoderma* cellulase (100 μg/ml), or no protein (Control). Solutions were then assayed for the release of soluble reducing sugars after 5 h of incubation (mean ± SEM of five measurements). Similar results were obtained with Ex30 (S2 fraction, data not shown). (*B*) Cellulase exhibits endoglucanase activity, whereas expansin does not. *Trichoderma* cellulase (0.2 ml, 100 μg/ml) or Ex30 (S2 fraction, 10 μg/ml) was added to 0.8 ml of carboxymethylcellulose solution (20 μg/ml in 50 mM sodium acetate, pH 4.5), and viscosity was measured in a rolling ball viscometer. Experiments were carried out twice with similar results.

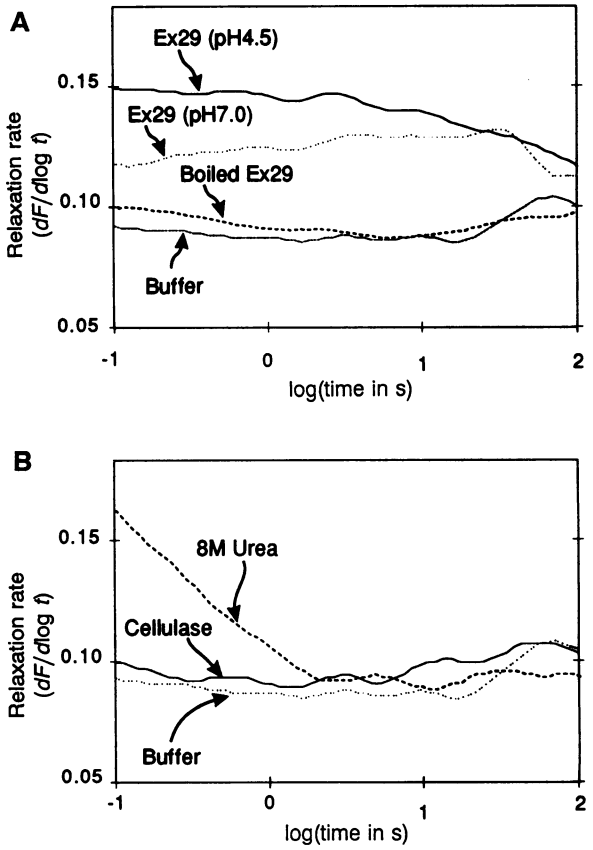

FIG. 3. Effects of expansin, cellulase, and urea on the stress relaxation spectrum of paper. (*A*) Filter paper strips were soaked in a solution containing Ex29 (5 μg/ml) in 50 mM sodium acetate, pH 4.5, or 50 mM Mes, pH 7.0, for 5 min and then stored briefly on ice until they were extended and the relaxation rate was measured with a tensile tester. *F*, force in g; *t*, time in s. Stress relaxation spectra for controls (buffer solutions only) at pH 4.5 or pH 7.0 were not significantly different from each other, and only data for pH 4.5 are included. Boiled Ex29 (5 μg/ml) had been boiled for 5 min in 50 mM sodium acetate, pH 4.5. Similar results were obtained with Ex30 (data not shown). (*B*) Methods were the same as in *A* except that *Trichoderma* cellulase (100 μg/ml) in 50 mM sodium acetate, pH 4.5, or 8 M urea (pH 7.0) was used to soak the paper strips. Data are the averages of 10 measurements. Experiments were repeated three times with similar results.

the effects of the expansin fraction were not associated with cellulose hydrolysis.

As a second approach, we measured the effects of expansins and cellulase on stress relaxation of paper. For these experiments the paper is held between two clamps and quickly stretched until a predetermined force is attained, then held at constant length. The force subsequently decays as the load-bearing polymers rearrange themselves into a low-stress condition. Active expansin fractions considerably enhanced the rate of stress relaxation in the paper when assayed at pH 4.5 (Fig. 3*A*), indicating that the protein renders the paper more compliant. The effect of expansins at pH 7.0 was less, in agreement with the reported pH optimum (pH 4.0–4.5) for expansin activity in plant cell walls (9). In contrast, cellulase had little effect on stress relaxation of paper (Fig. 3*B*), a further indication that its mode of action is quite different from that of expansin. It also follows from these results that the cellulose-binding domain of cellulase, which is postulated to disrupt hydrogen bonding between β-1,4-glucan chains within the cellulose microfibril (19, 20), does not behave in the same manner as expansins.

Urea (8 M) caused a significant enhancement of the rate of stress relaxation in paper strips (Fig. 3*B*), confirming the importance of hydrogen bonding in the mechanical properties of paper. Urea accelerated relaxation in early parts of the spectrum (less than 1 s), whereas expansin effects were apparent across the entire period of measurement (Fig. 3*A*). Probably urea weakens all the hydrogen bonds between fibers in the paper, causing most of the stress to relax very quickly. Expansin activity, in contrast, appears to be slower, perhaps involving a progressive disruption of bonds and movement of the protein as the fibers slide apart.

Our results indicate that expansins are capable of disrupting hydrogen bonding between cellulose fibers in paper. In native walls it is unlikely that expansins act exactly in this fashion because cellulose microfibrils do not make direct contact with each other; instead, microfibrils are coated with a surface layer of hydrogen-bonded matrix polysaccharides (heteroxylans, xyloglucans, mannans, or related glycans) and embedded in a gel-like polysaccharide matrix that keeps microfibrils apart. Hence, we expect that, in the native cell wall, expansins induce slippage between the microfibril and its surface coat or between the surface polysaccharide and interacting matrix polymers. In native walls, such slippage

results in a slow, steady extension of the wall (typically 30–40% extension before breakage; see ref. 17), whereas paper extends in response to expansin with only a brief extension before it breaks, presumably because of the shortness and randomness of the hydrogen-bonded overlap between fibers.

We reasoned that addition of moderate concentrations of urea might act synergistically with expansins in an extensometer assay, by weakening the hydrogen bonding between wall polymers. In agreement with this idea, addition of 2 M urea doubled the extension rate of both native walls and walls reconstituted with expansin (Fig. 4). Without active expansins, 2 M urea had only a minor effect on wall extension. Higher concentrations of urea (8 M) were inhibitory (not shown), presumably because they denatured the wall proteins and perhaps caused some rigidification of the matrix by reducing the osmotic force that keeps the gel-like matrix swollen (21, 22).

Replacement of H_2O in the buffer with D_2O inhibited extension of expansin-reconstituted walls by 36% (n = 4; SEM = 4.6%). It had a similar effect on extension of native cell walls. Because the hydrogen bond formed by deuterium is about 20% stronger than that formed by H (23) and because deuterium will exchange with accessible hydroxyl groups in the wall, we expected the deuterium treatment to strengthen the bonding between wall polymers and reduce extension, as we found. However, this result is not compelling because part of the D_2O effect may also be a direct effect on the expansin protein.

To estimate the number of hydrogen bonds broken by expansin, we examined the temperature sensitivity of extension in walls reconstituted with Ex30 (S2 fraction; approximately 10 μg/ml in 0.4 ml). The extension rate increased at higher temperatures, with a temperature coefficient of 0.0343 per degree between 13.5°C and 23°C (n = 5; SEM = 0.0032) and 0.0406 per degree between 23°C and 28.5°C (n = 5; SEM = 0.0023). These values correspond to an apparent Q_{10} of about 2.2 and 2.5 and an apparent activation energy of 59 and 71 kJ·mol^{-1}. If we assume that this activation energy arises from the need for expansins to break hydrogen bonds between wall polymers, we estimate that 3 or 4 hydrogen bonds need to be broken during the rate-limiting step in wall extension (using 14–20 kJ·mol^{-1} for the O—H ··· H hydrogen bond; see ref. 23). Assuming further that one hydrogen bond per glucose is formed between cellulose and its polysaccharide coat, this activation energy would correspond to disruption of hydrogen bonding along a stretch of 3 or 4 glucose residues on the glucan backbone.

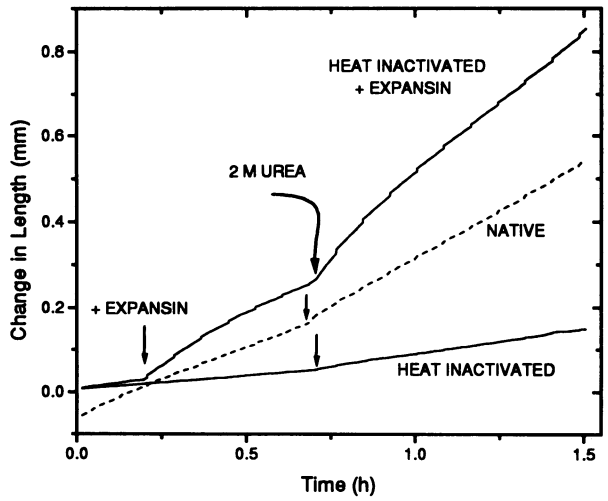

FIG. 4. Effects of 2 M urea on extension of native walls, walls reconstituted with expansin, and heat-inactivated walls. Native walls or heat-inactivated walls were clamped in the extensometer in 50 mM sodium acetate, pH 4.5. At about 20 min Ex29 (S1 fraction, 10 μg/ml) was added to one set of heat-inactivated walls. At about 45 min the incubation solutions were replaced with 2 M urea buffered with 50 mM sodium acetate, pH 4.5. These traces are representative of four trials for each treatment.

With the above facts in mind, our tentative model for expansin action in native walls goes as follows: The protein binds to a hydrogen-bonded polysaccharide complex at the surface of cellulose microfibrils (water may serve as an intermediate partner in the hydrogen bonding of the complex). Because expansins associate more tightly with cellulose coated with matrix polysaccharides than with clean cellulose or with soluble matrix polysaccharides (unpublished observation), we suggest that expansins act at the interface between the microfibril and the matrix by disrupting hydrogen bonding between a heteroduplex 3 or 4 sugar residues in length. If the polysaccharide complex bears wall stress, the two polymers will tend to pull apart when bonding is weakened in this fashion and such action, once started, will tend to go to completion because the wall stress borne by the polymers will become concentrated in the remaining bonds that bind the two polymers together. Once the hydrogen-bonded complex has pulled apart, the protein releases because its affinity for single chains is much less than for the paired complex. It is now ready to bind another complex and start the cycle again. The catalytic activity in this hypothetical cycle is unusual because it is driven not by chemical energy but by mechanical energy (the energy released by stress relaxation of the polymers). This wall loosening mechanism could account, at least partly, for the common observation that plant cell enlargement is dependent on turgor pressure, which ordinarily generates large tensile stress in plant cell walls. This process would not progressively weaken the wall during expansion because, after the wall polymers have slipped, new hydrogen-bonded associations could form between previously separated chains, thereby restoring the mechanical integrity of the wall.

Slippage between cellulose and a xyloglucan coat was previously considered a potential mechanism for the acid-induced extension of plant walls, but this idea was discarded with the discovery that binding of pure xyloglucan to pure cellulose was not sensitive to pH in the range that causes the acid-extension response of walls (24). Thus a direct effect of pH on xyloglucan binding was rejected, but the involvement of wall proteins in the acid extension response was not considered. Our model differs from previous ones in that we envision that the pH dependence resides in the catalytic activity of the expansin protein rather than a direct effect of pH on interchain hydrogen bonding.

In addition to these proteins acting as agents that catalyze plant wall extension, we also note that expansins can act on coated commercial papers. Thus these proteins may prove useful in paper processing and recycling applications.

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