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Extension growth of the water mold *Achlya*: Interplay of turgor and wall strength

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ABSTRACT **When hyphae of the water mold *Achlya* were subjected to osmotic stress, imposed with polyethylene glycol (PEG)-300 or sucrose, turgor pressure fell in proportion to the increase in external osmotic pressure. There was no evidence of turgor regulation, even over a period of days, yet the extension rate was unaffected until turgor was reduced to less than a third of the normal level of 0.6–0.8 MPa (6–8 bars). Measurements of the pressure at which the hyphae burst indicate that they respond to osmotic stress by softening their apical cell walls, sustaining extension growth despite reduced turgor pressure. The effect of osmolytes excluded by the wall was very different; superfusion of growing hyphae with PEG-6000 or dextran-6000 reduced turgor and stopped extension but did not induce wall softening. Furthermore, the hyphae did not resume growth during an hour or more of continuous exposure to these substances. Although the two classes of osmolytes have the same effect on turgor, they may induce different strains within the cell wall; this might then affect the capacity of the organism to detect the drop in turgor or to soften its cell wall. The interplay between turgor and wall strength supports the proposition that turgor supplies the driving force for extension and that production of the standard hyphal form requires a balance between hydrostatic pressure and a resistive cell wall.**

Most plant and microbial cells are highly pressurized structures whose walls withstand the tension exerted by up to 2.0 MPa (20 bars) of hydrostatic pressure (1–3). This pressure, or turgor, is generated by water influx due to the difference in osmotic pressure between the protoplasm and the external milieu. Turgor provides skeletal support and, so it is thought, the driving force for expansion. Biophysical models suggest that turgor exerts an isotropic force upon the inner surface of the cell wall, and localized compliance results in the generation of cellular form (4–7).

Many organisms, both prokaryotes and eukaryotes, respond to osmotic stress by the accumulation of compatible solutes (1, 3, 8–10), either by synthesis or by uptake from the external medium. The resumption of growth parallels solute accumulation; although few experiments have measured changes in total solute concentration, it seems likely that turgor is rebuilt under these conditions. These studies indicate that turgor is necessary for the growth of walled cells but do not identify its role in the manifold processes that underlie surface expansion.

The lower eukaryote *Achlya* is a convenient subject for investigations on the role of turgor in apical growth; it produces relatively large aseptate hyphae that lend themselves to direct measurement of turgor with a micropipet-based pressure probe (11). We report here that hyphae of *Achlya* lack a mechanism for turgor regulation but instead sustain apical growth with reduced turgor by softening their cell walls. Our observations reinforce the view that turgor supplies the mechanical force for extension and provide some insight into the genesis of hyphal form.

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MATERIALS AND METHODS

Organism and Culture Conditions. Colonies of *Achlya bisexualis* Coker, strain T5 (female; ATCC 14524) were grown on the defined medium $DMA_{3.2}$ (12), solidified with 2% agar. Cells were also cultured in liquid medium of the same composition for flow chamber experiments and amino acid analysis. The osmotic pressure of the basal medium was 0.05 MPa.

Osmometry. Osmotic pressures of culture media were measured with a 5100C vapor pressure deficit osmometer (Wescor, Logan, UT) as described (13).

Flow Chamber Experiments. Hyphae were superfused by gravity flow in a Plexiglas chamber (14). Flow rates of 1–2 ml/min were established and the volume of liquid in the chamber was regulated by vacuum aspiration to ≤1 ml. Hyphal extension was monitored over successive 5-min intervals by reference to an eyepiece reticule. Measurements of the position of the hyphal tip were made to the nearest 2.5 μm, providing an accuracy of ±0.5 μm/min for the extension rates. Experiments were repeated at least three times for each solute.

Analysis of Long-Term Growth. Colonies were grown on $DMA_{3.2}$ agar supplemented with various concentrations of sucrose or PEG-300 to supply osmotic pressures up to 1.3 MPa. Growth was assessed after 48 hr of incubation at 22°C by measuring three randomly selected colony diameters from each of three replicate plates (15). Relative growth rates were calculated with reference to colony diameters on the unsupplemented $DMA_{3.2}$ agar. In addition, extension rates of individual hyphae were measured as described above.

Measurement of Turgor and Burst Pressure. Direct measurements of hyphal turgor were made with a micropipet-based pressure probe (11), as illustrated in Fig. 1. The instrument presently in use has been modified: the control-rod micrometer screw, which is used to set the oil pressure within the probe and thereby to position the oil meniscus in the tip of the micropipet, is now motorized with a dc micromotor and joystick controller (Stoetling). Micropipets with tip diameters of 0.5 μm were pulled from filamented glass capillaries (11) and then broken against the edge of a cover glass to give outer diameters of 2–4 μm for the experiments. The opening of the pipet tip was often blocked by the deposition of vesicular material in the vacuole within 10 min of penetration, as reported earlier for work with microelectrodes (16). Continuous measurement of turgor during the 30-min duration of most experiments was therefore abandoned. Instead, the same hyphae were penetrated at different times during the experiment, and turgor was measured for 3–8 min.

The burst pressure of the hyphal tip was measured by increasing the oil pressure beyond that of the turgor, so that

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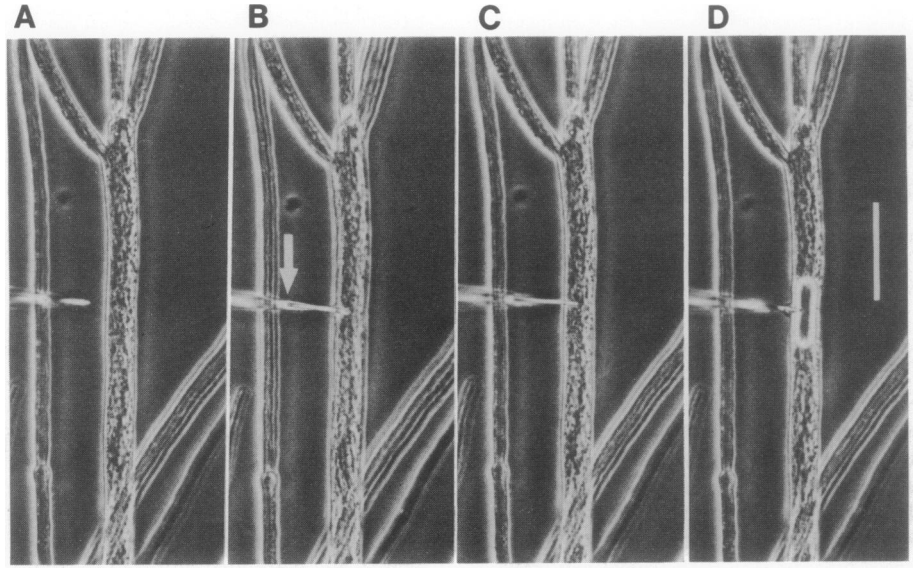

FIG. 1. Phase-contrast photomicrographs illustrating the measurement of turgor pressure from growing hyphae of *A. bisexualis*. (*A*) Oil-filled micropipet is positioned close to the hyphal surface, 400 μm behind the growing apex. (*B*) The hypha is penetrated, and pressurized cell sap displaces the silicone oil in the pipet tip; arrow indicates position of the oil/cell sap interface. (*C*) The oil is repositioned to the pipet tip by increasing the oil pressure (see *Materials and Methods*); oil pressure now matches turgor. (*D*) Following turgor measurement, oil pressure is raised further, oil is injected into the cell, and burst pressure is measured when the apical wall ruptures. (Bar = 100 μm.)

oil was injected into the central vacuole (Fig. 1*D*). Oil pressure was then increased further until the apical wall ruptured. The peak pressure coincident with bursting represents a qualitative measure of the tensile strength of the apical cell wall.

Amino Acid Analysis. Liquid-grown hyphae were macerated in 10% (wt/vol) trichloroacetic acid and filtered through Whatman 540 hardened ashless filter paper. The filtrate was extracted with an equal volume of ethyl ether, and the aqueous phase was collected for analysis. The aqueous phase was filtered through an ultrafiltration disc (cutoff, M_r 10,000; Millipore), neutralized with triethylamine, derivatized with phenylisothiocyanate, and run on a Waters Pico.Tag amino acid analysis system (Waters).

RESULTS

Hyphal extension in *A. bisexualis* is extremely sensitive to osmotic stress. In a flow chamber, superfusion with growth medium supplemented with PEG-300 or sucrose (external osmotic pressure, 0.3–0.4 MPa) quickly reduced the rate of extension (Fig. 2), and the hyphal apices became more rounded. However, within 10 min of sustained superfusion, extension rates recovered and the apices resumed their streamlined form. If the superfusate was then exchanged for the unsupplemented growth medium, the cells invariably burst and cytoplasm and vacuolar fluid were expelled. The position of the lesion was variable, but usually occurred within 100 μm of the tip. In the unsupplemented medium, recordings of hyphal turgor pressure ranged from 0.6 to 0.8 MPa (mean ± SEM = 0.69 ± 0.01 MPa, n = 12). During exposure to PEG-300 or sucrose, turgor dropped in direct proportion to the increase in external osmotic pressure (Table 1 and Fig. 3). Notably, recovery of the extension rate was not accompanied by an increase in turgor. There was also no evidence of the proline accumulation reported by Luard (17) for the related fungi *Phytophthora* and *Pythium*. During a 30-min exposure of logarithmic-phase cells of *Achlya* to 0.4 MPa of PEG-300, there was no significant change in the hyphal amino acid content (data not shown).

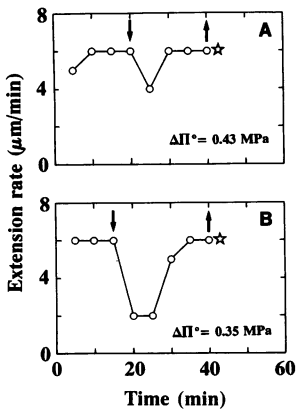

FIG. 2. Effect of wall-permeant solutes on hyphal extension rate in $DMA_{3.2}$ medium with 160 mM PEG-300 (*A*) or 150 mM sucrose (*B*). Arrows indicate influx and washout of osmolyte; star indicates hyphal rupture. $\Delta\Pi^0$ represents the increase in external osmotic pressure from the basal 0.05 MPa of the unsupplemented medium.

Both PEG-300 and sucrose diffuse freely through the hyphal cell wall of *Achlya* (18). The effects of larger solute molecules, excluded by the wall, were quite different. Superfusion of hyphae with solutions of PEG-6000 or dextran-6000 with an osmotic pressure of 0.3 MPa stopped extension

Table 1. Effect of PEG-300 on hyphal turgor, burst pressure, and extension rate

	Turgor pressure, MPa (n)	Burst pressure, MPa (n)	Extension rate, μm/min (n)
Control	0.69 ± 0.01 (12)	0.94 ± 0.04 (6)	2.8 ± 0.06 (5)
Post-PEG	0.39 ± 0.02 (11)	0.63 ± 0.04 (6)	2.6 ± 0.39 (5)

Hyphae were grown on thin films of $DMA_{3.2}$ agar (12) on 22-mm square coverglasses and were covered with a drop of liquid medium for the experiments. Control measurements were made in unsupplemented liquid $DMA_{3.2}$, which was then removed by vacuum aspiration and replaced with $DMA_{3.2}$ supplemented with PEG-300 (160 mM; increase in external osmotic pressure, 0.43 MPa). The second set of measurements was made after at least 5 min exposure to PEG. On average, rates of extension were 50% lower than those measured in the flow chamber (see Fig. 2), which supplied the cells with a continuous stream of fresh medium. Sample size is indicated in parentheses.

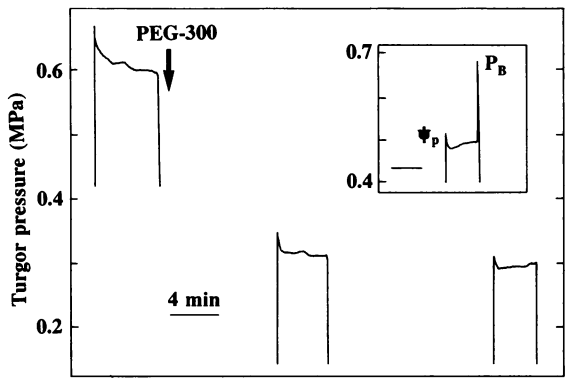

FIG. 3. Recordings of hyphal turgor pressure before and during exposure to 160 mM PEG-300. Hyphae were cultured as described in Table 1. The horizontal portion of each trace represents the turgor. Turgor was first measured in unsupplemented $DMA_{3.2}$, followed by two measurements from the same hypha after replacement of the medium with medium containing PEG (arrow). (*Inset*) Measurement of burst pressure (P_B) from a separate hypha with reduced turgor (Ψ_p).

(Fig. 4), and no recovery occurred during sustained exposure for >60 min. Turgor pressure was reduced to between 0.4 and 0.5 MPa in response to the imposed stress, and remained at this lower level as long as exposure to PEG-6000 or dextran continued. When the superfusate was exchanged for the unsupplemented medium, turgor pressure returned to the control value of about 0.7 MPa and the hyphae resumed extension without bursting.

Wall Softening. Evidently, *Achlya* does not regulate turgor in response to osmotic stress; instead, the resumption of growth during superfusion with PEG-300 or sucrose was accompanied by softening of the apical cell wall. A measure of this wall modification is the "burst pressure" determined with the pressure probe, which records the critical pressure at which the apical wall ruptures after oil injection into the hypha (Table 1 and Fig. 3). In the unsupplemented medium, growing hyphal apices burst at hydrostatic pressures about 0.2 MPa in excess of turgor. The burst pressure declined in parallel with turgor as the external osmotic pressure was raised. We interpret the phenomenon of sub-apical bursting after the return to the unsupplemented medium as a reflection of the response to osmotic stress: the softened cell wall ruptured when turgor returned to the control level. Turgor reduction imposed with the nonpenetrating solutes was not accompanied by wall softening.

No Turgor Regulation During Prolonged Osmotic Stress. When *Achlya* was cultured on agar plates supplemented with PEG-300 or sucrose, the hyphae behaved in much the same way as described above for the response to hyperosmotic shock (Fig. 5). There was no adjustment of turgor for at least 24 hr, and the cells continued to grow at the reduced turgor pressures imposed by osmotic stress. The relationship between burst pressure and external osmotic pressure indicates that the wall-softening response evident after <10 min of exposure to the osmolytes persisted for at least 24 hr, so that the burst pressure always exceeded turgor by about 0.2 MPa (Fig. 5). Control rates of extension (measured either by colony diameter or by the observation of individual hyphae) were maintained for 3 days or more, despite the severe turgor reduction produced by external osmotic pressures of 0.2–0.6 MPa. At still higher osmolyte concentrations, the growth rate diminished markedly, but hyphal morphology was normal at turgor pressures as low as 0.07 MPa. The only visible indication of growth with reduced turgor was a slight rounding of the hyphal apices and an increase in diameter. For example, at an external osmotic pressure of 0.50 MPa, when turgor was reduced to 0.2 MPa, mean hyphal diameter increased from 29 ± 1.2 μm (control; n = 10) to 33 ± 1.8 μm (n = 10).

Aberrant Growth Continues in the Absence of Turgor. When turgor was reduced below 0.07 MPa apical extension ceased, but slow, poorly polarized growth continued even at external osmotic pressures above 1.2 MPa. In the absence of turgor regulation, turgor should be abolished in all hyphae at these osmotic pressures (Fig. 5). Indeed, once the external osmotic pressure exceeded 1.0 MPa, no turgor could be detected with the pressure probe, whose resolution was

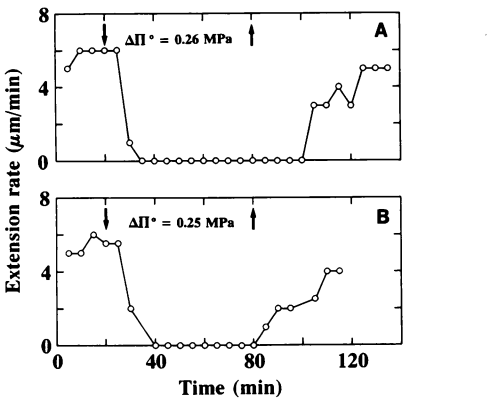

FIG. 4. Effect of wall-impermeant solutes on hyphal extension rate in $DMA_{3.2}$ with PEG-6000 (140 g/kg) (*A*) or dextran-6000 (240 g/kg) (*B*). Arrows indicate influx and washout of osmolyte.

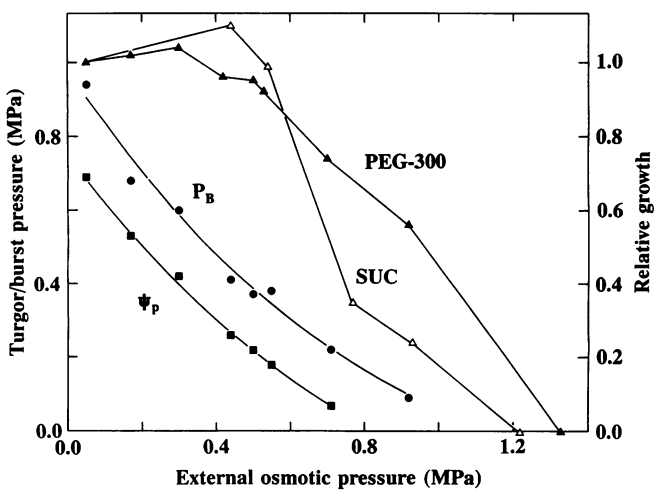

FIG. 5. Long-term effects of PEG-300 and sucrose on turgor pressure (Ψ_p), burst pressure (P_B), and relative growth. Turgor and burst pressure: *Achlya* was grown on thin films of agar supplemented with various concentrations of PEG-300 or sucrose; turgor and burst pressure were measured on 14- to 24-hr cultures as described in *Materials and Methods*. Quadratic curves were fitted by regression analysis (r^2 = 0.97 for turgor and 0.94 for burst pressure). The graph shows mean values for both parameters, based on 3–17 measurements at each osmotic pressure; the maximum standard error was 0.04 MPa for both turgor and burst pressure. Growth: *Achlya* was grown on agar plates supplemented with osmolytes as above [PEG-300 or sucrose (SUC)]. Relative colony diameters at 48 hr were plotted as a measure of the growth rate; maximum standard error was 0.03 (dimensionless). The pattern of inhibition was confirmed by measurements of the rate of hyphal extension from individual cells (data not plotted). These remained between 7 and 9 μm/min over the range 0.05–0.44 MPa of external osmotic pressure, but decreased to 4 μm/min at 0.70 MPa and 3 μm/min at 0.92 MPa. Note that since the extension rate varied during the development of the colonies, the measurements made after 48 hr of incubation do not correspond to the *cumulative* growth expressed in terms of relative colony diameter.

about 0.02 MPa. Therefore, slow growth continued in the apparent absence of turgor.

At these high osmotic pressures PEG-300, sucrose, and sorbitol had identical effects on morphology. The cells that developed ranged in shape from ovoid to spherical and sometimes gave rise to misshapen outgrowths. Growth was largely limited to the fluid on the agar surface; the inability of these forms to penetrate the solid medium may be an independent indication that they lack turgor. When the misshapen cells were transferred to unsupplemented medium, outgrowths with normal hyphal morphology developed within 2 hr.

DISCUSSION

When microorganisms are subjected to hyperosmotic shock, cellular expansion is at first curtailed and then resumes at close to the normal rate. The familiar response to osmotic stress is the restoration of turgor by the uptake or synthesis of compatible solutes (1–3, 8–10). Likewise, organisms subjected to sustained osmotic stress generally exhibit elevated cellular solute concentrations. Within the fungi there are numerous reports of compatible solute synthesis (17, 19–21); *Phytophthora* and *Pythium*, oomycetes like *Achlya*, accumulate proline when the external osmotic pressure is raised with sucrose or KCl (17). *Achlya* displays an alternative strategy: it does not regulate turgor in the short or the long term but extends despite the reduced pressure. Measurements of the burst pressure suggest that the hyphae compensate for the reduced turgor by softening their apical cell wall (Table 1 and Fig. 5).

A precedent for our observations comes from the work of Green *et al.* (22), who demonstrated that the giant-celled alga *Nitella* does not regulate turgor but responds to osmotic stress by metabolic adjustment of the strength of its wall. The related freshwater alga *Chara corallina* also fails to maintain constant turgor during osmotic stress (23). Modulation of the strength of the cell wall dominates growth-rate control among higher plants (24–26) and in the giant sporangiophore of the fungus *Phycomyces* (27). *Achlya* appears to be the first instance of this mechanism among tip-growing microorganisms. Some years ago, Thomas and Mullins (28) observed that branching in *Achlya* is correlated with increased secretion of endocellulase and suggested that this enzyme effects wall softening. Whether cellulases are responsible for the phenomenon reported here remains to be investigated.

Lockhart (29) and Ray *et al.* (30) developed a model for pressure-dependent plant cell growth which predicts that the rate of expansion is a function of the difference in osmotic pressure between the cell and its surroundings (equal to the turgor pressure), the extensibility of the cell wall, the permeability to water, and the size of the cell. In addition, turgor must exceed a critical value, termed the "yield threshold," to drive the expansion of the wall (24, 25). In *Nitella* there appears to be a lower limit for the adjustable yield threshold, since growth did not resume once turgor fell below 0.2 MPa (22). In the case of *Achlya* this limit must be much lower, possibly negligible, since the cells continue to grow in the absence of any measurable turgor, albeit with aberrant morphology. There is also no evidence that the reduction in turgor must exceed some threshold value to elicit the wall-softening response. Instead, the data shown in Fig. 5 suggest that the strength of the apical wall is a continuously variable function of turgor pressure.

When *Achlya* is grown on medium supplemented with PEG-300, sucrose, or sorbitol (osmotic pressure above 1.0 MPa), abnormal forms are produced. It is possible that these shapes report toxic effects of the high concentrations of osmolytes used in these experiments, or contaminants therein. However, the simpler interpretation, that they result from the lack of turgor, is supported by both the unitary effect of the different solutes used and the reversibility of the effect on form. We suggest that as hyphal turgor falls below 0.07 MPa (10% of normal) and the cell envelope becomes increasingly compliant, normal morphogenesis is disrupted. We do not know whether perturbation of secretion or loss of wall rigidity is responsible, nor what drives the slow, formless enlargement under these conditions. However, production of the standard hyphal shape evidently requires a balance between positive hydrostatic pressure and a resistive cell wall.

In the course of these experiments, we made the surprising observation that cells whose turgor was reduced by osmolytes large enough to be excluded by the wall did not resume growth, nor did they exhibit wall softening. The reason for this effect is not known. In both wall-penetrating and nonpenetrating osmolytes, the water potential of the protoplasm and the cell wall should approach equilibrium with the external milieu. High concentrations of PEG-300 and sucrose induce plasmolysis by diffusing through the cell wall and withdrawing water from the protoplast. By contrast, during dehydration with PEG-6000 or dextran-6000, the osmolyte cannot enter the wall; in this case, the water potential of the wall is thought to be diminished by development of a negative hydrostatic pressure within its water-filled pores (31). Under these conditions the cell contracts as a whole, without separation of the plasma membrane from the wall, a phenomenon termed cytorrhysis (18). The osmolyte concentrations used in our experiments were too low to elicit either plasmolysis or cytorrhysis. Instead, in both cases the elastic wall would be expected to contract along with the shrinking protoplast (18), yet the physiological consequences are quite different. A possible explanation may be that the strain developed within the wall is not the same when it is dehydrated by permeant or impermeant solutes. Such a difference may affect the capacity of the organism either to detect the drop in turgor or to soften its cell wall.

Although our findings are consistent with the hypothesis that hydrostatic pressure provides the mechanical force for cellular expansion, we have not quite excluded the possibility that turgor is required merely to appress the plasma membrane to the wall, perhaps to maintain the proximity of wall synthases and nascent wall. This interpretation of the role of turgor was put forward by the botanist Reinhardt (32), among others, in relation to hyphal growth and remains current (33). The inhibition of growth by nonpenetrating osmolytes argues against this view; turgor is reduced and extension ceases under these conditions, even though contact between wall and membrane is unaffected. The most plausible interpretation of our findings is that turgor drives hyphal extension and that the interplay between pressure and wall strength enables *Achlya* to sustain growth and normal morphology over a wide range of environmental circumstances. For a freshwater organism, nutrient deprivation is a more likely contingency than osmotic stress. Indeed, when *Achlya* hyphae are transferred to a non-nutrient medium, extension continues for hours despite a progressive decline in turgor. We propose that the wall-softening response is primarily an adaptation, not to fluctuations in the external osmotic pressure, but to episodes of nutrient deficiency.

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