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Original research article

***In vitro* cell wall extensibility controls age-related changes in the growth rate of etiolated *Arabidopsis* hypocotyls**

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Running title: Age-related changes in growth and extensibility

Abstract

Plant cell growth is controlled by cell wall extensibility, which is currently estimated indirectly by various microtensile and nano/microindentation techniques. Their outputs differ in the accuracy of growth rate and *in vivo* extensibility prediction. Using the creep method we critically tested several metrics (creep rate, creep rate·stress⁻¹, *in vitro* cell wall extensibility (ϕ) and *in vitro* cell wall yield threshold (γ)) for their ability to predict growth rates of etiolated *Arabidopsis thaliana* (L.) Heyn. hypocotyls. We developed novel approaches for ϕ and γ determination and statistical analysis based on creep measurements under single loads coupled with wall stress calculation. The best indicator of growth rate was ϕ because the three-fold developmental decrease in the growth rate of 4- versus 3-day-old hypocotyls was accompanied by a three-fold decrease in ϕ determined at pH 5. Although the acid-induced expansin-mediated creep of cell walls resulted exclusively from increasing ϕ values, the decrease in ϕ between 3- and 4-day-old hypocotyls was not mediated by a decrease in expansin abundance. We give practical recommendations on the most efficient use of creep rate, creep rate·stress⁻¹, ϕ and γ in different experimental situations and provide scripts for their automated calculations and statistical comparisons.

Additional keywords: expansion growth, biomechanics, creep test, cell wall stress, yieldins

1 Introduction

2
3 Plant growth, considered as irreversible cell expansion, is primarily controlled by cell wall
4 extensibility at the biophysical level, which is described by the Lockhart equation
5 (Lockhart 1965):

$$6 \quad (1/V)(dV/dt) = \Phi(P - Y),$$

7 where $(1/V)(dV/dt)$ is the relative growth rate expressed as a change in cell volume (V), Φ
8 is the wall yielding coefficient, P is the turgor pressure, and Y is the yield threshold, the
9 minimum turgor required for growth. The above equation states that the growth rate is a
10 linear function of turgor in excess of yield threshold. Φ corresponds to the slope of the line
11 describing the linear dependence of growth rate on turgor. This characteristic shows the
12 sensitivity of the growth rate to changes in turgor. The terms Φ and Y characterize cell wall
13 extensibility, which can be defined as the wall ability to extend irreversibly during growth
14 (Cosgrove 1993). This ability depends on mechanical properties of cell wall polymers and
15 their continuous modification *in vivo* by numerous wall-localized enzymes.

16 Several methods for *in vivo* measurements of Φ and Y values in relation to growth
17 have been developed (Cosgrove 1993). Unfortunately, these methods are not applied at
18 present, possibly because they only allow a very limited spectrum of experimental
19 manipulations with plants. Instead, microtensile tests along with nano- and
20 microindentation techniques are currently used to study the wall biomechanics (Burgert
21 and Keplinger 2013; Routier-Kierzkowska and Smith 2013). These approaches do not
22 measure Φ and Y values, but derive different biomechanical cell wall characteristics, which
23 may or may not have a link with the growth rate and the wall extensibility. It is, therefore,
24 critical to choose an appropriate method in the studies of a cell wall basis of plant cell
25 expansion and find optimal biomechanical characteristics giving growth-relevant
26 information.

27 The most commonly used microtensile tests include the stress/strain method, the *in*
28 *vitro* stress-relaxation, and the creep method. In the stress/strain method a cell wall sample
29 is extended at constant rate while stress (the force acting on the wall divided by its cross-
30 sectional area) and strain (the ratio of the current wall sample length to its original length)
31 are simultaneously recorded. Then the tensile modulus is calculated as the ratio of stress to
32 strain (Ryden *et al.* 2003). The *in vitro* stress-relaxation involves very rapid stretching of a
33 wall sample to a predetermined length, after which the decay in wall stress over time is
34 recorded. Several biomechanical characteristics are derived from the kinetics of wall stress

1 decay (Hansen *et al.* 2011). The creep method involves the application of a constant load
2 to a wall sample and subsequent measurement of its deformation against time (McQueen-
3 Mason *et al.* 1992). As turgor does not change appreciably in the course of plant cell
4 expansion (Cosgrove 1986), the constant stress generated in the cell wall during creep
5 measurements mimics the action of turgor on the growing wall better than the rapidly
6 changing stress in the stress/strain and *in vitro* stress-relaxation techniques. This defines
7 the advantage of the creep method over the other two techniques.

8 The creep method has much lower spatial resolution compared with the modern
9 micro- and nanoindentation techniques. However, the results of the former are easier to
10 link with growth rate than the results of the latter, because of several reasons:

11 (i) The direction of wall strain in the creep test coincides with the main direction of
12 organ growth, while that in the micro(nano)indentation is usually perpendicular to the
13 growth vector (Milani *et al.* 2013);

14 (ii) Growth is associated with irreversible cell wall deformation, which is the main
15 component of wall creep (Suslov and Verbelen 2006). On the contrary,
16 micro(nano)indentation techniques measure mostly reversible cell wall deformations,
17 which may have no direct link with growth (Peaucelle *et al.* 2011);

18 (iii) Micro(nano)indentations are usually performed on turgid tissues. So it is not
19 easy to distinguish the physical properties of plant tissues defined by cell walls from those
20 conferred by turgor (Radotić *et al.* 2012; Routier-Kierzkowska *et al.* 2012). The use of
21 plasmolysed tissues does not solve this problem, because osmotica are known to cause
22 adaptive changes in wall biomechanics (Frensch and Hsiao 1995). On the other hand, the
23 results of creep measurements are not affected by turgor pressure because they are
24 traditionally performed with frozen/thawed plant organs. This treatment is specially used to
25 disrupt protoplasts and eliminate turgor (Cosgrove 2011).

26 Thus, the creep method has many advantages over classical, as well as recently
27 introduced techniques. However, only some results of the creep test obtained under
28 carefully optimized measurement conditions provide growth-relevant cell wall
29 biomechanical characteristics. Among others, it is very important to select an appropriate
30 constant load that causes a wall stress comparable to that existing *in vivo*. *In vivo* wall
31 stress is difficult to estimate because it depends on many factors including turgor, cell
32 geometry and stress anisotropy within multicellular plant organs (Baskin 2005; Baskin and
33 Jensen 2013). In view of this problem, creep rates have often been compared under a broad
34 range of loads with the implication that at least one of them would induce a wall stress

1 corresponding to that *in vivo* (Thompson 2001; Suslov and Verbelen 2006; Vandenbussche
2 *et al.* 2011).

3 In a more advanced approach, it is not the creep rates themselves that are measured
4 under different loads, but their dependencies on the applied load were compared. This
5 approach was first used by Okamoto and Okamoto (1994), who found a linear dependence
6 of creep rate on the load and characterized it by ϕ (*in vitro* cell wall extensibility) and y (*in*
7 *vitro* cell wall yield threshold), *in vitro* analogs of the parameters Φ and Y from the
8 Lockhart equation. The value of ϕ corresponds to the slope of the line characterizing the
9 linear dependence of creep rate on the load, while the value of y reflects the minimal load
10 at which creep starts (Okamoto and Okamoto 1994). Originally, the values of ϕ and y were
11 determined on the basis of serial loading. Equal loads were added to the wall sample
12 sequentially at similar time intervals. The creep rate was measured for each loading step
13 and plotted against the respective total load extending the wall sample. This approach
14 allows the values of ϕ and y to be determined for each individual wall sample. Thus, with
15 several samples the two parameters can be easily compared using routine statistical tests
16 (Taguchi *et al.* 1999; Ezaki *et al.* 2005). The main problem with ϕ and y determined as
17 above is that their values can vary depending on the protocol of serial loading (Takahashi
18 *et al.* 2006). In this case the creep rate at each step is affected by the previous history of
19 wall extension. For example, the creep rates of identical wall samples under a 12 g total
20 load could be different if this load was achieved in two (6 g + 6 g), three (4 g + 4 g + 4 g)
21 or four (3 g + 3 g + 3 g + 3 g) steps of serial loading.

22 To address the problem of protocol-imposed variability of ϕ and y determined by
23 serial loading, an alternative approach based on creep measurements under single loads
24 was developed (Miedes *et al.* 2013). First, the wall stress generated by different loads in
25 the creep test was determined. Then, different cell wall samples were extended under one
26 of several loads, generating a biologically-relevant wall stress. The resulting creep rates
27 were averaged for each load used. Their averaged values depended on wall stress in a
28 linear fashion, allowing the calculation of ϕ and y values. In this approach, the creep rate
29 under each load does not depend on creep rate measurements under different loads. As a
30 result, the ϕ and y values thus obtained are much less affected by the protocol of study than
31 those determined by serial loading. However, the *in vitro* wall yielding parameters
32 calculated by the new approach cannot be determined for individual wall samples and are
33 based on the whole population of cell walls analyzed. This creates a challenging problem

for statistical comparisons of ϕ and y values, which was not addressed in Miedes *et al.* (2013).

In the present work a novel method for statistical comparisons of the *in vitro* wall yielding parameters was developed, and their role in developmental changes of *Arabidopsis thaliana* (L.) Heyn. hypocotyl growth rate was studied. Besides ϕ and y , creep rate and creep rate·stress⁻¹ were critically assessed for their ability to predict *in vivo* growth rates. Scripts written in R software provided here allow easy calculation and statistical analysis of these biomechanical characteristics.

Materials and methods

Plant material and growth conditions

A. thaliana wild type Col-0 plants were grown on ES medium (Estelle and Somerville 1987). The seeds were stratified for 3 days at 4°C and synchronous germination was induced by exposure to fluorescent white light (150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ True Light; Philips, Eindhoven, The Netherlands) for 4 h at 21°C. The moment of transfer to light is considered as time zero for all the experiments. After the induction period the Petri dishes were wrapped in four layers of aluminium foil, placed vertically in an environmentally controlled growth cabinet (cooled incubator BRC120, Bioconcept-FirLabo, Beun De Ronde, Drogenbos, Belgium), and the plants were grown in darkness for up to 5 days at 21°C.

Measurement of hypocotyl growth

To measure the length of hypocotyls, the foil was removed from the Petri dishes under green safe light, they were photographed using a Canon 50D digital camera, wrapped in foil again, and placed back into the growth cabinet until the next measurement. All these manipulations had no effect on hypocotyl growth, as revealed by comparison with control seedlings grown in continuous darkness. Hypocotyl length was measured as the distance between the uppermost root hairs around the collet and the base of the ‘V’ made by the petioles of the cotyledons (Scheres *et al.* 1994) using ImageJ software (Abramoff *et al.* 2004). The relative growth rate of hypocotyls was calculated with the formula: $(\ln L_n - \ln L_{n-1})/T$, in which L_n is the hypocotyl length at t_n , L_{n-1} is the hypocotyl length at t_{n-1} , and T is the time between t_n and t_{n-1} (Hunt 1990). To calculate the relative growth rate of 3(4)-day-old hypocotyls more accurately, their length was measured at two additional time

points: one before and another after the respective age (Fig. 1), and these values were used in the above formula.

Extensiometry

To study developmental changes in the biomechanics of hypocotyls, the constant-load (creep) method was used. Etiolated wild type arabidopsis seedlings were transferred individually into 1.5 ml Eppendorf test tubes, frozen by plunging the closed tubes into liquid nitrogen, stored at -20°C and used for extensiometry within two weeks after freezing. Heat-inactivation was carried out as described in Boron *et al.* (2015). For this purpose distilled water (1.5 ml, 22°C) was added to Eppendorf test tubes with frozen samples, the tubes were transferred to a preheated (90°C) thermomixer (Eppendorf Thermomixer® comfort) and incubated with 300 rpm shaking for 3 min. Then, the heat-inactivation was rapidly stopped by transferring the tubes to a 1000 ml beaker with water at 22°C. Finally, the frozen/thawed heat-inactivated arabidopsis seedlings were transferred to small Petri dishes filled with distilled water and kept in a refrigerator at 4°C for 1–2 h before being used in creep tests.

In vitro extension of frozen/thawed arabidopsis hypocotyls was measured with a custom-built constant-load extensometer described in Suslov and Verbelen (2006) using the same procedure as in Miedes *et al.* (2013). A 5 mm long hypocotyl segment (starting from 1.5 mm below the cotyledons) was secured in the extensometer and preincubated in a buffer (20 mM MES-KOH, pH 6.0 or 20 mM Na-acetate, pH 5.0) in the relaxed state for two minutes, after which it was extended in the same buffer under a constant load (400 mg, 500 mg, 600 mg, or 700 mg) for 15 min. The relative creep rate (for brevity referred to as ‘creep rate’ below) was calculated with the above-mentioned formula for relative growth rate as detailed in Miedes *et al.* (2013).

Calculation of cell wall cross-sectional area and tensile stress generated in arabidopsis hypocotyls under a constant load

The creep rate of hypocotyls is proportional to the stress (a force divided by the cross-sectional area across which it acts) in their cell walls resulting from the action of a constant load. To calculate this stress, the cross-sectional area of the hypocotyl cell walls was determined by measuring their dry weight per unit length (Cleland 1967) assuming that the wall density (ρ) is 1.5 g cm⁻³ (Gibson 2012). By definition, $\rho = m/V$, where m is mass and V is volume. For a segment of a cylindrical organ like a hypocotyl $V = l \cdot A$, where l is the

length of the segment (5 mm), and A is its cross-sectional area. From the above equations $A = m/(\rho \cdot l)$ and can easily be calculated using the known length of the hypocotyl segments (5 mm), their measured mass and assuming that $\rho = 1.5 \text{ g cm}^{-3}$.

To prepare samples for cell wall cross-sectional area determination, 5 mm long segments (corresponding to those used in the creep test) were excised from fresh arabidopsis hypocotyls with a custom-made double-bladed cutter. Between 60 and 80 hypocotyl segments were transferred to a container for critical point drying (Microporous Specimen Capsules and Caps; 120-200 μm pores, Electron Microscopy Sciences (EMS); AURION, Wageningen, The Netherlands). This cylindrical container with a lid is made from highly porous inert material easily permeable for organic solvents. Each container with hypocotyl segments was transferred to a separate borosilicate glass Petri plate, where the segments were extracted and dehydrated by four sequential 1 h washes in chloroform:methanol (1:1, v/v) followed by a 1 h wash in diethyl ether with subsequent air drying in a fume hood. This protocol of dehydration is analogous to routine cell wall processing for FTIR analysis (Miedes *et al.* 2011). The weight of the dry wall material prepared from 60-80 5 mm long segments of arabidopsis hypocotyls was determined using a balance (SARTORIUS 2405, Germany) with a resolution of 1 μg .

The wall stress (MPa) during the uniaxial extension of arabidopsis hypocotyls *in vitro* under a constant load was calculated as the ratio F/A (F : tensile force (N), A : hypocotyl cross-sectional area (m^2)). The loads 400, 500, 600 and 700 mg used in the creep test generate tensile forces of 0.00392 N, 0.00490 N, 0.00588 N and 0.00687 N, respectively.

Analysis of statistical data

If not stated otherwise, comparisons between two groups (differences between 3- and 4-day-old hypocotyls) were performed using a Student's two-tailed t -test; multiple comparisons (differences between pH 6.0; pH 5.0; and pH 5.0 heat-inactivated) were made with ANOVA followed by a Games-Howell's post-hoc test.

Non-parametric estimates of the mean and variance of the ratio creep rate-stress⁻¹ were obtained by determining the ratio in 10000 bootstrap samples of size 4 obtained by sampling with replacement from the available measurements of creep rate and wall stress (Methods S1, Script S1 in Supplementary Material). Comparisons of the ratio estimates between groups were then performed using a Student's two-tailed t -test and, when needed,

corrected for multiple comparisons by maintaining the false discovery rate at 5% (Benjamini and Hochberg 1995) (Methods S1, Script S1 in Supplementary Material).

The dependence of creep rate on wall stress was estimated by fitting Model II linear regression models with errors in both variables using the Maximum Likelihood Functional Relationship implementation from Ripley and Thompson (1987), a variant of Deming regression, assuming the residual standard error is proportional to the standard error of the sample (Methods S1, Script S2 in Supplementary Material). The code of the method is available at <https://stat.ethz.ch/pipermail/r-help/2010-February/227865.html>. *In vitro* cell wall extensibility was estimated as the slope of the regression line with stress as the independent and with creep rate as the dependent variable. The *in vitro* yield threshold was estimated by regressing stress as a dependent variable on creep rate as the independent variable and taking the intercept of this line. This is a valid approach because the Maximum Likelihood Functional Relationship method is symmetric and gives the same result, regardless whether x is regressed on y or y on x , as pointed out in Ripley and Thompson (1987). Non-parametric standard errors for the slope and the intercept were derived using leave-one-out jackknife.

Regression significance was determined by testing the significance of the slope coefficient, using the slope and standard error estimates to calculate a t -ratio and testing on a t -distribution with $N-p-1$ degrees of freedom where N is the number of loads, p is the number of predictors in the regression (1 for linear regression) (Methods S1, Script S2 in Supplementary Material).

Differences between slopes and intercepts of regression models were tested by computing a test statistic (t -ratio) for the difference between the parameters of two models using the formula:

$$t = \frac{coef_1 - coef_2}{\sqrt{0.5(\sigma_1^2 + \sigma_2^2)}}$$

where $coef_1$ and $coef_2$ are the parameter estimates of two regression models and σ_1 and σ_2 are the respective standard errors of the estimates, and testing on a two-tailed t -distribution with df_1+df_2 degrees of freedom, where df_1 and df_2 are the degrees of freedom of the two regression models (2 in all cases) (Methods S1, Script S2 in Supplementary Material).

Results

Hypocotyls of wild type Columbia-0 arabidopsis plants elongate through cell extension without accompanying cell divisions (Gendreau *et al.* 1997). In etiolated arabidopsis seedlings grown on Petri plates under sterile conditions hypocotyls demonstrated developmental changes in their growth rate (Fig. 1). Three-day-old hypocotyls grew almost three-fold as fast as four-day-old hypocotyls [$4.45 \pm 2.24 \text{ \%} \cdot \text{h}^{-1}$ versus $1.70 \pm 1.17 \text{ \%} \cdot \text{h}^{-1}$ (mean $\pm$ s.d., $n=123$; the difference is significant at $P < 0.001$, Student's two-tailed t -test)], which makes them a convenient model to study the wall physical properties controlling the rate of plant cell extension. Five mm long subapical segments extended in the creep test incorporated essentially complete growing zones of 3- and 4-day-old hypocotyls (Gendreau *et al.* 1997), validating comparison of their growth and biomechanics.

The correct interpretation of biomechanical data with isolated cell walls is difficult in some cases when their cross-sectional areas are unknown. The wall deformation *in vitro* in the creep test is proportional to the stress (= force divided by the cross-sectional area across which it acts) generated by an external load. This means that the walls with absolutely identical composition and architecture could demonstrate different extension behavior simply because their cross-sectional areas are not the same. To take this factor into account, the wall cross-sectional areas of 3- and 4-day-old hypocotyls were estimated by measuring their dry weight per unit length (Table 1). The more mature hypocotyls were found to have thinner cell walls. Thus, each constant load used in the creep test will generate significantly higher wall stress in 4-day-old than in 3-day-old hypocotyls (Table 1).

The experimental setting for the creep test was chosen such as to realistically mimic different conditions that could exist in cell walls *in vivo*. The loads used (400, 500, 600 and 700 mg) represented the broadest possible range for arabidopsis hypocotyls. Higher loads (≥ 800 mg) were inappropriate, because they led to rupture in the majority of 4-day-old hypocotyls. Under the minimal load used (400 mg) the creep rate was well below the *in vivo* growth rate, while a further decrement in load did not allow accurate creep measurement. The pH used (pH 5 and 6) is within the physiological range for the plant apoplast (Felle 2001). A pH 5 buffer was chosen because it activates expansins and yieldins, wall loosening proteins which mediate the acid growth response (McQueen-Mason *et al.* 1992; Okamoto-Nakazato *et al.* 2000). On the other hand, a pH 6 buffer switches off the activities of these two protein classes, while being optimal for other potential cell wall loosening factors such as xyloglucan endotransglucosylase/hydrolases (XTHs) (Maris *et al.* 2011). Finally, a heat-treatment of hypocotyls was used to eliminate

activities of the majority of cell wall loosening proteins, thus demonstrating their contribution to the wall creep when results were compared with no heat-treated samples. The standard protocol for cell wall protein heat-inactivation (boiling in water for 15 s) (McQueen-Mason *et al.* 1992) was found to be too harsh for arabidopsis hypocotyls. They started drying and twisting immediately after having been taken out from the boiling water, which precluded their further use. An alternative heat-inactivation procedure was developed for arabidopsis hypocotyls involving incubation in water at 90°C for 3 min, followed by an incubation at 4°C for 1-1.5 h. Shorter incubation periods at the high temperature were insufficient to eliminate the protein-mediated cell wall loosening. On the other hand, longer incubations at 90°C increased wall creep compared with the heat-inactivation for 3 min, possibly due to thermal breakage of some bonds within the wall. Omission of the hypocotyl incubation at 4°C for 1-1.5 h occasionally resulted in their rapid drying and twisting.

Arabidopsis hypocotyls demonstrated a strong acid-induced extension *in vitro*. Their creep rate at pH 5 was three to five times higher than under the two different conditions (Fig. 2a, c, e). The increase in creep rate at pH 5 versus pH 5 heat-inactivated shows that the acid-induced cell wall extension is protein-mediated (Fig. 2a, c). Interestingly, comparison of creep rates at pH 6 and pH 5 heat-inactivated revealed different age-related trends. In 3-day-old hypocotyls the creep rates were statistically indistinguishable (Fig. 2c, e) indicating that the wall proteins active at pH 6 might not contribute to the rapid growth occurring at this developmental stage (Fig. 1). On the other hand, in 4-day-old hypocotyls the creep rate at pH 6 was significantly higher than that at pH 5 heat-inactivated under 600 mg and 700 mg loads (Fig. 2c, e), still remaining much lower compared with creep rates at pH 5 (Fig. 2a, e). This means that the wall proteins active at pH 6 could start playing a role in the growth control at later developmental stages working in concert with proteins active at pH 5. Their relative contribution to growth would be dependent on the actual pH in the apoplastic space of 4-day-old hypocotyls.

Hypocotyls of the same age used for comparison of the effects of pH and heat-inactivation were randomly selected from a common pool of plants. Consequently, they had statistically equal cell wall cross-sectional areas, and comparison of their biomechanics gave similar results whether the *in vitro* wall stress (Table 1) was taken into account by determining creep rate·stress⁻¹ values (Fig. 2b, d, f) or not (Fig. 2a, c, e). On the other hand, 3- and 4-day-old hypocotyls had significantly different cell wall cross-sectional areas (Table 1). Due to this reason, the results of comparison of their biomechanics were not

identical when expressed as creep rates (Fig. 2a, c, e) or creep rate·stress⁻¹ values (Fig. 2b, d, f). In agreement with the above-mentioned changes in growth, the creep rate was found to be significantly lower in 4-day-old than in 3-day-old hypocotyls at pH 5 and pH 5 heat-inactivated under 600 mg and 700 mg loads (Fig. 2a, c). No significant differences were observed when comparing their creep rate at pH 6 (Fig. 2e). The age-related changes in the wall properties were more prominent, when they were expressed as creep rate·stress⁻¹ (Fig. 2b, d, f). Its values were lower in 4-day-old versus 3-day-old hypocotyls at pH 5 and pH 5 heat-inactivated under all loads tested (Fig. 2b, d), while at pH 6 the difference was significant only under a 600 mg load (Fig. 2f). This example shows that creep rate used as a metric underestimates age-related changes in wall material properties due to developmental thinning of arabidopsis hypocotyl cell walls. Interestingly, creep rate (Table 2) and creep rate·stress⁻¹ values (Fig. 2b, d, f) decreased by about the same extent in 4-day-old versus 3-day-old hypocotyls at pH 5 and pH 5 heat-inactivated, while the relative changes were clearly less pronounced at pH 6.

In addition to determining creep rate·stress⁻¹ values, there is an alternative method that allows wall stress to be considered when interpreting biomechanical data. Miedes *et al.* (2013) plotted creep rates against respective wall stress values and found a linear relationship between them using a Deming regression. Unlike ordinary least squares regression, Deming regression takes into account variation in both creep rate and wall stress to fit an optimal straight line describing their relationship. This method was used for the present data (Fig. 3). Preliminary analysis (Methods S1, Script S2 in Supplementary Material) has shown that Deming regression was significant in five cases out of six. The only exception was the data for 4-day-old hypocotyls pH 5 heat-inactivated. In the other five cases the linear regression lines obtained revealed two essential characteristics describing wall mechanics: *in vitro* cell wall extensibility (ϕ) and *in vitro* cell wall yield threshold (γ). The values of ϕ correspond to the slopes of the respective regression lines and characterize the sensitivity of creep rate to changes in wall stress. The values of γ correspond to intercepts of the regression lines with the x axis and show the minimal wall stress at which creep starts. Non-parametric estimates of the standard errors of the ϕ and γ values allowed their statistical comparisons between different treatments (Methods S1, Script S2 in Supplementary Material), (Table 3). The values of ϕ at pH 5 were significantly higher than at pH 5 heat-inactivated and pH 6 both in 3- and 4-day-old hypocotyls. *In vitro* cell wall extensibility at pH 5 was also the only characteristic demonstrating significant

age-dependent changes. In 3-day-old hypocotyls it was three-fold higher than in 4-day-old hypocotyls (Table 3), closely matching the difference in their *in vivo* relative growth rates (see above). Although no significant differences in y were observed, the values of y at pH 5 were numerically higher (less optimal for cell wall creep) than at pH 6 both in 3- and 4-day-old hypocotyls (Fig. 3, Table 3). Overall, these data suggest that the strong protein-mediated acid-induced cell wall extension of arabidopsis hypocotyls results entirely from their higher ϕ values. *In vitro* cell wall extensibility also demonstrates a good correlation with developmental changes in hypocotyl growth rate (Fig. 1, 3; Table 3) implying its role in the control of plant cell extension.

To sum up, several cell wall mechanical characteristics were obtained from the analysis of creep curves of arabidopsis hypocotyls and their cell wall cross-sectional areas: creep rate, creep rate·stress⁻¹ values, *in vitro* cell wall extensibility (ϕ) and *in vitro* cell wall yield threshold (y). They differed in their ability to predict developmental changes in growth rate of arabidopsis hypocotyls with ϕ being the most accurate growth-relevant characteristic.

Discussion

In vitro cell wall yielding parameters (ϕ and y) determined using frozen/thawed plant organs have two principal differences from Φ and Y . Firstly, Φ and Y reflect not only intrinsic mechanics of wall polymers, but also all dynamic processes by which cellular metabolism controls cell wall extensibility *in vivo*, while ϕ and y give a static picture of cell walls at the moment when they were frozen, characterizing their extension not supported by cellular metabolism. Some labile factors involved in growth regulation do not survive the freezing/thawing procedure (Okamoto-Nakazato 2002), and others are rapidly metabolized during *in vitro* extension (Maris *et al.* 2009). As a result, ϕ and y may underestimate some aspects of cell wall extensibility regulation (Cosgrove 2011). Secondly, Φ and Y characterize cell wall extension resulting from biaxial turgor-induced wall stress, while ϕ and y are derived from creep caused by uniaxial stress. Uniaxial stress is known to result in larger cell wall extension than equivalent biaxial stress (Richmond *et al.* 1980). Due to this reason ϕ values may exaggerate *in vivo* cell wall extensibility. Despite these limitations *in vitro* cell wall yielding parameters are able to predict growth rate (Fig. 1, Table 3) and they proved to be efficient for the identification of novel cell wall loosening proteins (Okamoto-Nakazato *et al.* 2000). Thus, ϕ and y can be considered as

1 useful correlates of Φ and Y from the Lockhart equation. It should also be noted that Φ and
2 Y on one hand, and their respective *in vitro* correlates on the other hand are approximately
3 100-fold different numerically (Table 3; Boyer *et al.* 1985; Cosgrove 1985, 1987), which
4 has a simple explanation. Φ and Y are derived from the dependence of growth rate on
5 turgor, while ϕ and γ link creep rate and wall stress. Relative rates of growth and creep are
6 close to each other (Fig. 1, 3), while turgor and turgor-induced wall stress are
7 approximately 100-fold different (Cosgrove 1993). As a result, γ is two orders of
8 magnitude larger than Y , while ϕ (derived from division of creep rate by the difference of
9 wall stress and γ) is about 100 times less than Φ (obtained from division of growth rate by
10 the difference of turgor and Y).

11 Heat-inactivation has been routinely used to eliminate activities of cell wall
12 proteins (McQueen-Mason *et al.* 1992; Maris *et al.* 2009). We found that this treatment
13 could have side effects beyond protein inactivation. First, cell wall weakening was
14 observed with heat-inactivation longer than 3 min. This phenomenon can be explained by
15 heat-induced breakage of some non-covalent bonds between wall polymers (Suslov *et al.*
16 2010). Second, wall twisting often occurred as a result of heat-inactivation, which was
17 prevented by prolonged incubation in water. Apparently, this effect was induced by a rapid
18 water loss from the cell wall, which could decrease its extensibility (Thompson 2008). The
19 complex interaction between these independent processes having opposite effects on cell
20 wall extension can explain why a linear dependence of creep rate on wall stress was not
21 found in 4-day-old heat-inactivated hypocotyls (Fig. 3b). All these factors should be taken
22 into account when interpreting the results of experiments with heat-inactivation.

23 In addition to methodological findings, the present research has revealed several
24 important biological aspects of growth regulation at the cell wall level. The strong acid-
25 induced creep of etiolated arabidopsis hypocotyls observed at pH 5.0 was protein-
26 mediated, as evidenced by its high sensitivity to the heat-treatment which inactivates cell
27 wall proteins (Fig. 2, 3). Two protein classes were implicated in the acid-induced cell wall
28 extension, expansins (McQueen-Mason *et al.* 1992) and yieldins (Okamoto-Nakazato *et al.*
29 2000). In the case of arabidopsis hypocotyls yieldins would hardly contribute to the acid
30 creep because of their low expression in this organ (Jamet *et al.* 2009) and their poor ability
31 to resist freezing/thawing used here for wall sample preparation (Okamoto-Nakazato
32 2002). On the other hand, expansins are abundantly expressed in arabidopsis hypocotyls
33 (Jamet *et al.* 2009), thus being the main candidate players behind the acid-induced cell wall
34 extension. This response was found to be caused exclusively by higher ϕ values both in 3-

1 and 4-day-old hypocotyls (Fig. 3, Table 3), implying that expansins stimulate only ϕ and
 2 have no effect on γ . Interestingly, an exogenously applied α -expansin increased ϕ and
 3 decreased γ in reconstitution experiments with cucumber hypocotyls (Takahashi *et al.*
 4 2006). The discrepancy between these results and our data most probably has a
 5 methodological basis. Takahashi and coauthors used the ramped sweep analysis, a variant
 6 of serial loading, to determine ϕ and γ . In our research they were estimated by independent
 7 creep rate measurements under one of several loads. In the former case the creep rate at
 8 each step of loading depends on the previous history of the wall sample extension, which
 9 could lead to protocol-related artifacts. The expansin-mediated decrease in γ observed by
 10 Takahashi *et al.* (2006) might be one of them.

11 The three-fold decrease in the growth rate between 3- and 4-day-old hypocotyls
 12 (Fig. 1) was accompanied by the practically equal decrease in *in vitro* cell wall
 13 extensibility (Fig. 3, Table 3). Thus, ϕ is a good predictor for *in vivo* growth rate. Despite
 14 the fact that expansins seemingly exert their effect via ϕ , changes in their protein
 15 abundance could not be the mechanism of the age-dependent decrease in the wall
 16 extension (Fig. 2, 3), which dropped to the same extent at pH 5 with and without heat-
 17 inactivation (Table 2). So this developmental change in wall mechanics was independent
 18 of expansins, because it was also observed when their activity was eliminated by the heat-
 19 treatment. A similar conclusion can be made from recent research on developmental
 20 changes in the growth rate of arabidopsis leaves (Goh *et al.* 2014). Their absolute
 21 expansion rate depended on the age in a bell-shaped fashion, peaking in 15-day-old leaves.
 22 Different levels of expansin overexpression had opposite effects on the rate of leaf
 23 expansion, with no changes in its temporal pattern with the maximum in 15-day-old leaves.
 24 Apparently, some heat-resistant modifications of the wall structure account for the age-
 25 dependent decrease in the wall extension observed in etiolated arabidopsis hypocotyls (Fig.
 26 2, 3). These could include cellulose microfibril realignment in the direction of hypocotyl
 27 elongation (Refrégier *et al.* 2004), resulting in cell wall strain-hardening (Suslov *et al.*
 28 2009), deesterification of pectins (Derbyshire *et al.* 2007) with their subsequent calcium-
 29 mediated cross-linking (Jarvis 1984), and a tyrosine-based cross-linking of cell wall
 30 structural proteins (De Cnodder *et al.* 2005).

31 Interestingly, the age-related decrease in wall extension at pH 6 (Fig. 2f, Table 2)
 32 was clearly less prominent than at pH 5 with and without heat-inactivation (Fig. 2b, d,
 33 Table 2). In 3-day-old hypocotyls the creep rate was similar at pH 6 and pH 5 heat-
 34 inactivated, while in 4-day-old hypocotyls it became significantly higher in the former case

1 than in the latter (Fig. 2c, e). This change could be explained by increased expression of
2 some cell wall loosening proteins active at pH 6 in 4-day-old compared with 3-day-old
3 hypocotyls, which partially compensates for the drop in the wall extension caused by the
4 heat-resistant changes in the wall structure (see above). The candidate proteins active at pH
5 6 could be xyloglucan endotransglucosylase/hydrolases (XTHs), the activity of which was
6 shown to increase in the course of arabidopsis hypocotyl development (Miedes *et al.*
7 2013). Thus, it follows that wall proteins active at pH 5, presumably expansins, play a
8 crucial role in the control of cell wall extension in 3-day-old hypocotyls. In 4-day-old
9 hypocotyls a trade off between them and the proteins active at pH 6 could regulate the rate
10 of extension. Their actual contribution will be dependent on the apoplastic pH in 4-day-old
11 hypocotyls. In this regard it would be very interesting to estimate developmental changes
12 in apoplastic pH within arabidopsis hypocotyls using a novel genetically-encoded
13 biosensor (Gjetting *et al.* 2012).

14 Cell wall characteristics based on creep and cell wall cross-sectional area analysis
15 (creep rate, creep rate·stress⁻¹, ϕ and γ) have different advantages and limits of use.

16 Creep rate is the characteristic of choice whenever the wall samples under analysis
17 are known to have equal cross-sectional areas. This condition is normally met if control
18 and test samples are randomly selected from a common pool of cell wall material, as, for
19 example, in studies where purified proteins are tested for their ability to induce cell wall
20 extension (McQueen-Mason *et al.* 1992; Van Sandt *et al.* 2007). Creep rate is also
21 preferred as long as there is experimental evidence that wall samples of different origin
22 have statistically-indistinguishable cross-sectional areas (Park and Cosgrove 2012). The
23 main advantage of this characteristic is that creep rates of different samples can be
24 compared using well-known parametric statistical tests without additional manipulations.

25 Creep rate·stress⁻¹ is the most universal characteristic whenever the cross-sectional
26 areas of the wall samples under comparison are significantly different. It can be
27 successfully used even in the situation when creep rate does not depend on wall stress in a
28 linear fashion. However, creep rate·stress⁻¹ does not allow the data to be reported concisely
29 if the wall samples are compared under many different loads. In this case all significant
30 differences, which could comprise a long list, should be specifically mentioned.

31 *In vitro* cell wall extensibility (ϕ) and *in vitro* cell wall yield threshold (γ) can be
32 considered as a more advanced alternative to creep rate·stress⁻¹, which are also used in the
33 situation of different cell wall cross-sectional areas. Unlike creep rate·stress⁻¹, they allow a

much more concise description of the data: cell wall extension measured under many different loads is characterized by only two parameters. More importantly, ϕ and γ can give an insight into the mechanism of changes in wall mechanical properties. For example, acidic buffer was shown to modify wall biomechanics via ϕ (this study), while XTH overexpression exerted its effect on the walls by changing γ (Miedes *et al.* 2013). However, *in vitro* cell wall yielding parameters can only be used if the regression of creep rate against wall stress is statistically significant, which, unfortunately, is not always the case (Fig. 3b). The disadvantage of both creep rate·stress⁻¹ and *in vitro* cell wall yielding parameters is that they depend on both creep rates and stress values, with their associated errors, making it difficult to calculate their means and standard deviations and conduct statistical testing. These issues have been solved here 1) through the use of bootstrap resampling to estimate the means and standard errors of creep rate·stress⁻¹ and 2) by Deming regression combined with jackknife to estimate ϕ and γ with their errors. The values are then compared using standard statistical tests. The methods are implemented in a set of R scripts, which are made freely available (Methods S1, Script S1, Script S2 in Supplementary Material).

Figure 4 summarizes different experimental situations discussed above and gives practical recommendations on the most efficient use of creep data in each of them. Overall, the present study has shown that the creep test is a very powerful tool to characterize growth-relevant cell wall biomechanical properties.

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Table 1. Tensile stresses generated in cell walls of hypocotyls *in vitro* under constant loads

Asterisks (*) mark significant differences between 4-day-old and 3-day-old hypocotyls ($P < 0.05$, Student's two-tailed t -test, $n = 4$).

	3-day-old hypocotyls	4-day-old hypocotyls
Dry weight (μg) ^A	2.06 ± 0.23	$1.56 \pm 0.20^*$
Cross-sectional area (μm^2) ^B	275 ± 31	$208 \pm 27^*$
Tensile stress from 400 mg load (MPa) ^C	14.4 ± 1.6	$19.1 \pm 2.5^*$
Tensile stress from 500 mg load (MPa)	18.0 ± 1.9	$23.8 \pm 3.1^*$
Tensile stress from 600 mg load (MPa)	21.6 ± 2.3	$28.6 \pm 3.7^*$
Tensile stress from 700 mg load (MPa)	25.2 ± 2.7	$33.4 \pm 4.4^*$

^A Dry weight of cell walls from one 5 mm long hypocotyl segment (mean $\pm$ s.d.) was calculated from the measured dry weights of four samples each containing 60-80 5 mm long hypocotyl segments.

^B Cell wall cross-sectional area (A) of one hypocotyl segment (mean $\pm$ s.d., $n = 4$) calculated from the equation $A = m/(\rho \cdot l)$, where m is the dry weight of cell walls from one 5 mm long hypocotyl segment (g), ρ is the cell wall density (assumed to be 1.5 g cm^{-3}) and l is the length of a hypocotyl segment (5 mm).

^C Tensile stress generated in the cell wall of a hypocotyl segment *in vitro* is calculated as the ratio F/A , where F is tensile force (N, Newton), A is cell wall cross-sectional area (m^2). The loads 400 mg, 500 mg, 600 mg and 700 mg used to extend hypocotyl segments generate tensile forces of 0.00392 N, 0.00490 N, 0.00588 N and 0.00687 N, respectively.

Table 2. Age-related changes in creep rate in arabidopsis hypocotyls

The age-related changes were expressed as a ratio for creep rate calculated by dividing the creep rate of 4-day-old hypocotyls by the creep rate of 3-day-old hypocotyls measured under the same load and pH. These ratios of creep rate, their s.d. and statistical comparisons were obtained using the same approach as for creep rate·stress⁻¹ (Methods S1, Script S1 in Supplementary Material). The creep rate data were taken from Fig. 2*a, c, e*. Groups compared under the same load are marked with different letters ^a and ^b when they are significantly different from each other ($P < 0.05$, Student's two-tailed *t*-test with FDR).

	Load			
	400 mg	500 mg	600 mg	700 mg
Ratio for creep rate				
pH 5	0.79 ± 0.09	0.67 ± 0.11	0.59 ± 0.10 ^a	0.53 ± 0.07 ^a
pH 5 heat-inactivated	0.74 ± 0.11	0.69 ± 0.11	0.46 ± 0.08 ^a	0.62 ± 0.12 ^{ab}
pH 6	0.96 ± 0.12	1.04 ± 0.23	0.93 ± 0.12 ^b	0.84 ± 0.14 ^b

Table 3. *In vitro* cell wall extensibility (ϕ) and *in vitro* cell wall yield threshold (y) of arabidopsis hypocotyl cell walls

The values of ϕ ($\pm$ s.e.) correspond to slopes of the fitted straight lines in Fig. 3. The values of y ($\pm$ s.e.) correspond to intercepts of the fitted straight lines with the x axis in Fig. 3. ND – not determined because Deming regression was not significant in this case (Methods S1, Script S2 in Supplementary Material). * Significantly different in 4-day-old hypocotyls compared with 3-day-old hypocotyls ($P < 0.05$, Student's two-tailed t -test). † Significantly different at pH 5 compared with pH 5 heat-inactivated and pH 6 ($P < 0.05$, Student's two-tailed t -test with FDR).

	3-day-old hypocotyls		4-day-old hypocotyls	
	ϕ (% h ⁻¹ MPa ⁻¹)	y (MPa)	ϕ (% h ⁻¹ MPa ⁻¹)	y (MPa)
pH 5	1.23 $\pm$ 0.35 [†]	12.66 $\pm$ 1.99	0.44 $\pm$ 0.10 ^{*,†}	14.90 $\pm$ 2.53
pH 5 heat-inactivated	0.16 $\pm$ 0.04	7.05 $\pm$ 2.57	ND	ND
pH 6	0.24 $\pm$ 0.12	10.47 $\pm$ 3.79	0.15 $\pm$ 0.04	12.93 $\pm$ 3.46

Fig. 1. Growth of etiolated Columbia-0 arabidopsis hypocotyls. Data are means $\pm$ s.d. ($n=123$).

Fig. 2. Age-related changes in the wall biomechanics of etiolated arabidopsis hypocotyls. Creep rate of frozen/thawed arabidopsis hypocotyls at pH 5 (*a*), pH 5 after heat-inactivation (*c*), and pH 6 (*e*). Data are means $\pm$ s.d. ($n=8-10$). Creep rate $\cdot$ stress $^{-1}$ values for hypocotyls determined at pH 5 (*b*), pH 5 after heat-inactivation (*d*), and pH 6 (*f*). Data are means $\pm$ s.d. obtained using random bootstrap re-sampling with 10000 iterations. Letters ‘a’ indicate significantly lower creep rates (creep rate $\cdot$ stress $^{-1}$ values) in 4-day-old compared with 3-day-old hypocotyls under otherwise identical conditions ($P < 0.05$, Student’s two-tailed t -test). Letters ‘b’ indicate a significantly higher creep rate (creep rate $\cdot$ stress $^{-1}$ values) at pH 6 than at pH 5 after heat-inactivation ($P < 0.05$, for creep rate: Games-Howell’s post-hoc test performed after ANOVA; for creep rate $\cdot$ stress $^{-1}$: Student’s two-tailed t -test with FDR). Creep rate (creep rate $\cdot$ stress $^{-1}$) at pH 5 was significantly higher than at pH 6 and pH 5 heat-inactivated for the respective age under all loads tested (not shown on the plots; $P < 0.01$, for creep rate: Games-Howell’s post-hoc test performed after ANOVA; for creep rate $\cdot$ stress $^{-1}$: Student’s two-tailed t -test with FDR). Note that the data for pH 5 (*a*, *b*) are differently scaled than those for pH 5 heat-inactivated (*c*, *d*) and pH 6 (*e*, *f*).

Fig. 3. Dependence of creep rate of frozen/thawed arabidopsis hypocotyls on the applied stress. Hypocotyls of (a) 3-day-old and (b) 4-day-old plants were extended at pH 6.0; pH 5.0; and pH 5.0 after heat-inactivation. Creep rate and stress values were taken from Fig. 2 and Table 1, respectively, and are reported with their s.d. The straight lines were fitted to the data using the Deming regression taking into account the variance in both creep rate and wall stress. In the case of 4-day-old hypocotyls extended at pH 5 after heat-inactivation the regression was not significant, and its result is shown as a dotted line. The slopes of the fitted straight lines correspond to *in vitro* cell wall extensibility (ϕ) values, and their intercepts with the x axis correspond to *in vitro* cell wall yield threshold (γ) values.

Fig. 4. Recommended procedures for analyzing cell wall extension using the creep method.