Mapping nano-scale mechanical heterogeneity of primary plant cell walls

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Abstract

Nanoindentation experiments are performed using an atomic force microscope (AFM) to quantify the spatial distribution of mechanical properties of plant cell walls at nanometre length scales. At any specific location on the cell wall, a complex (non-linear) force-indentation response occurs that can be deconvoluted using a unique multiregime analysis (MRA). This allows an unambiguous evaluation of the local transverse elastic modulus of the wall. Nanomechanical measurements on suspension-cultured cells (SCCs), derived from Italian ryegrass (Lolium multiflorum) starchy endosperm, show three characteristic modes of deformation and a spatial distribution of elastic moduli across the surface. ‘Soft’ and ‘hard’ domains are found across length scales between 0.1 µm and 3 µm, which is well above a typical pore size of the polysaccharide mesh. The generality and wider applicability of this mechanical heterogeneity is verified through in planta characterization on leaf epidermal cells of Arabidopsis thaliana and L. multiflorum. The outcomes of this research provide a basis for uncovering and quantifying the relationships between local wall composition, architecture, cell growth, and/or morphogenesis.

Key words: Atomic force microscopy, cell mechanics, Lolium multiflorum, nanoindentation, primary cell walls, suspended culture.

Introduction

Measuring mechanical properties of cell walls presents a significant but important challenge. One of the key factors contributing to the complexity of plant cell walls is the non-uniform spatial distribution of mechanical properties such as the elastic modulus. This effect has recently been demonstrated at the tissue level for shoot apical meristems (SAMs) of Arabidopsis thaliana. Kierzkowski et al. (2012) found that hypo-osmotic treatment of the meristems causes 45–80% surface expansion of peripheral cells but only 25% for cells in the central area. These results are supported by direct
nano-mechanical measurements on plasmolysed SAMs using atomic force microscopy (AFM), where the elastic modulus of cells in the apex is measured to be $5 \pm 2\,\text{MPa}$ compared with only $1.5 \pm 0.7\,\text{MPa}$ at the periphery (Milani et al., 2011). The significance of such local wall mechanical heterogeneities has become increasingly relevant in a number of biological processes and was again highlighted upon discovering that the auxin transporter PIN1 responds to mechanical cues, indicating that the mechanical map of the meristem may coordinate polarity (Braybrook and Peaucelle, 2013).

Mechanical properties of walls are determined by the composition and organization of both the macromolecular components of the wall and the water content. These components provide structural support as well as a signalling function via a feedback mechanism that informs the interior biochemical machinery of the cell of its mechanical environment, as well as placing constraints on possible realizations of plant cell wall microstructure. The latter stems from the fact that not all microstructures that can be assembled for a given cell wall composition provide an adequate mechanical barrier that (i) counter-balances the turgor pressure; and (ii) has tuneable properties that enable cell growth and deformation. Little, however, is known about mechanical heterogeneity within walls at the cellular level.

We hypothesize that mechanical heterogeneity and the resultant local gradients of mechanical stress are important factors driving the assembly of polysaccharides at the cell wall level. Such local mechanical stresses acting in concert with intermolecular interactions such as hydrogen bonding provide a physical mechanism of formation of different types of microstructures with a wide range of mechanical properties. During their development, cells may ‘utilize’ this mechanism to adopt a certain mechanical phenotype, ‘stiff’ or ‘soft’, which is essential for the formation of growth zones and morphogenetic specialization.

While there are reports suggesting mechanical heterogeneities at the level of a single cell, the magnitude of the variations remains uncharacterized. Using AFM indentation on Arabidopsis suspension-cultured cells (SCCs), Rodotić et al. (2012) observed ‘stiffness’ to range from ~20 kPa to 800 kPa. Although nano-scale mechanical heterogeneities have not been widely reported for higher plants, they are seen in yeast cells in the form of raft-like structures; the microstructure of the chitin wall is readily revealed using AFM imaging of the cell surface (Touhami et al., 2003). The structural basis for mechanical heterogeneities is suggested to be associated with the microstructural arrangement of cellulose fibrils in the wall, including their density and degree of association/aggregation. Recently, the presence of cellulose fibril bundles that inform such structural heterogeneity was documented in onion (Allium cepa) epidermal cells (Kafle et al., 2014; Zhang et al., 2014) and maize (Zea mays) parenchyma (Ding et al., 2014). These bundles, in addition to the preferred in-plane orientation of cellulose fibrils in the wall, that is, the mechanical response to applied deformation in the longitudinal direction (along the length of fibrils) is different from that in the transverse direction (perpendicular to the fibrils’ preferred orientation) (Cosgrove, 2000, 2005; Cosgrove and Jarvis, 2012; Park and Cosgrove, 2012; Ding et al., 2014; Kafle et al., 2014; Zhang et al., 2014). Differences in stiffness may also arise from the heterogeneous composition of the wall, which is extensively documented (McCann et al., 1997; Burton et al., 2010; Lee et al., 2011, 2013), with nearly all constituent cell wall polymers displaying at least some level of chemical heterogeneity. We do not yet have the measurement capability to provide direct links between cell wall composition and local micromechanical properties, but a step towards achieving this goal is to provide a robust methodology for mapping nano-scale mechanical properties.

Currently, the effect of microstructure-based heterogeneities on the overall mechanical properties of tissues and plant organs is not well understood. The models applicable for direct experimental analysis are generally based on continuum mechanics (Gibson, 2005; Boudaoud, 2010; Dyson et al., 2012) and do not incorporate consideration of the underlying microstructure. Microstructure is a term used to describe the appearance or arrangement of structural components (or phases) in a material across a broad range of length scales (from nanometres to centimetres). Although continuum models shed some light on static mechanical properties (Huang et al., 2012), they are inadequate for developing the structure–property–function relationships that are necessary to describe the complex dynamic and transient mechanical properties of plant cell walls, plant cells, and tissues (Altartouri and Geitmann, 2015). Recently, Milani et al. (2014) showed that the expression patterns of some genes correlates with the elasticity of the cell walls. Observations of such correlations provide key evidence of a connection between the mechanics of the wall and its biosynthesis.

In this study, we examine the mechanical properties of plant cell walls using SCCs derived from Italian ryegrass (Lolium multiflorum) starchy endosperm. The use of Lolium SCCs enables us to probe mechanical heterogeneity in a commelinoid monocot, which, in contrast to eudicots, is rich in mixed-linkage glucan (MLG) and heteroxylans (HXs), and with relatively low levels of cellulose, xylol glucan, and pectin (Table 1). We use novel microfabricated microwell arrays to entrap cells physically without the need for clamps, sticky tape, or adhesive layers that can disturb plant material and produce artefacts associated with adhesion and uncontrolled deformation. A detailed characterization of micromechanical properties using AFM nanoindentation and our advanced multiregime analysis (MRA) routine (Bonilla et al., 2015) reveals heterogeneous distribution of elastic moduli on the outer wall surface of Lolium SCCs, including ‘soft’ and ‘hard’ domains. We also quantify micromechanical heterogeneity in planta using leaf epidermal cells of Arabidopsis thaliana and L. multiflorum seedlings as a representative dicot and commelinoid monocot, respectively. The results suggest that the domain structure of mechanical heterogeneity at the micrometre level is an inherent property of plant cells and tissues, and may have significant repercussions for our understanding of cell growth and morphogenesis.
Materials and methods

Plant materials

Lolium multiflorum SCCs: The Lolium SCCs were derived from the starchy endosperm of L. multiflorum grains 9–10 d post-anthesis (Smith and Stone, 1973b). Briefly, endosperms were placed onto solid modified White’s medium (Smith and Stone, 1973b) for 7 d and the resulting calli were placed into liquid medium for bulking at 27 °C in the dark in a shaking incubator at 130 rpm. After establishment, the cell culture was grown in 250 ml Erlenmeyer flasks containing 150 ml of modified White’s medium (Smith and Stone, 1973b); ionic strength 0.435 mol l−1, osmotic pressure 1.17 MPa. The cultures were maintained in the dark at 27 °C with constant shaking at 130 rpm. Subculturing was conducted every 10 d by weighing 30 g (fresh weight) of cells and transferring the cells to 150 ml of fresh medium. The polysaccharide composition of the primary cell walls of these cells was determined to be 30–50% (1,3;1,4)-β-glucan (MLG), 20–30% HX, and 5–15% cellulose.

Arabidopsis thaliana and Lolium multiflorum plant growth conditions: Arabidopsis thaliana seeds (Columbia-0 ecotype) were surface sterilized with 70% (v/v) ethanol and 0.01% (v/v) Tween-20 for 5 min, rinsed in absolute ethanol, air-dried, and individual seeds plated on half-strength Murashige and Skoog (MS) medium (Sigma) with 2% (w/v) sucrose and 0.8% (w/v) agarose (Sigma) in Nunclon Petri dishes (35 × 10 mm, Thermo Scientific). Plates were incubated at 4 °C for 3 d in the dark then grown for 3 weeks in a growth chamber (120 μmol m−2 s−1) under a 16 h day (20 °C)/8 h night (17 °C) regime. Lolium multiflorum seeds were imbibed in water overnight then placed on filter paper (Whatman) in a Nunclon Petri dish and grown for 7 d in natural light (12 h light, 12 h dark, 22 °C).

Cell preparation for AFM imaging of untreated walls: To image the surface of the cell walls, the L. multiflorum cells were washed with a 10× volume of White’s medium and then the medium was exchanged to de-ionized water. A copious amount of water (24 °C) was used to remove all loosely bound components of the wall. After washing, the cell suspension was frozen overnight at −18 °C. Before milling, samples were pre-cooled for 5 min in liquid nitrogen. Cryomilling was done in the Freezer/Mill 6850 SPEX (Metuchen, NJ, USA) for two cycles with 2 min of cooling time in between the cycles; each milling cycle was performed at 10 strokes s−1 for 5 min. The thawed suspensions of the cell wall fragments were sieved through a 90 μm mesh sieve, and the filtrate was collected. Then the filtrate was passed through a 40 μm nylon mesh cell strainer (Falcon™ Cell Strainer, Fisher Scientific), and the retentate was washed with copious amounts of water. After washing, the wet cake of the cell wall fragments with rough sizes between 40 μm and 90 μm was re-suspended in 0.01% sodium azide solution in de-ionized water. The samples were kept in the fridge and used for AFM microscopy analysis within 3 d.

Treatment of the cells with the Updegraff reagent: To elucidate the underlying microstructure of the cellulose network, L. multiflorum cells were treated using Updegraff reagent by the procedure outlined in Updegraff (1969). The reagent was prepared using 30 ml of de-ionized water mixed with 120 ml of glacial acetic acid and 15 ml of concentrated nitric acid (both from Sigma). A suspension of cells was washed with a 10× volume of White’s medium and then the medium was exchanged to de-ionized water. Cells were then separated on a 40 μm nylon mesh cell strainer (Falcon™ Cell Strainer, Fisher Scientific) and drained under vacuum to form a moist cake. The Updegraff reagent was slowly added to the pellet; the final ratio of the reagent was 100 mg ml−1 of cell cake. The suspension was then placed on a boiling water bath for 60 min. Upon cooling, the cells were centrifuged and Updegraff reagent was replaced with water until pH 7 was reached. The samples were kept in the fridge and used for AFM analysis within 3 d.

Microscopic characterization of Lolium SCCs

Microscopic characterization of Lolium SCCs using CLSM: Lolium SCCs were assessed by CLSM (Zeiss LSM 710, Germany) with a three-dye staining procedure. Calciffluor white staining solution (1 g l−1 in de-ionized H2O) with pre-added Evans blue (0.5 g l−1; Sigma Aldrich) was used in 1:10 dilution to visualize glucans of the cell wall. Nile red (Sigma Aldrich) was dissolved in absolute ethanol at

| Cell materials | Loliun SCC<sup>a</sup> (Smith and Stone, 1973a) | Lolium leaf epidermis<sup>b</sup> (Chesson et al., 1985) | Arabidopsis leaf<sup>c</sup> (Pettolino et al., 2012) |
|---------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Cellulose     | 15.7                                          | 38.8                                          | 29.7                                          |
| Xyloglucan    | –                                             | 10.9                                          | 10.5                                          |
| Mixed-linkage glucan | 47.6                                        | 6.3                                           | –                                             |
| Pectin HG     | –                                             | –                                             | –                                             |
| Pectin RG I   | { 4.7                                          | }−14                                          | 25.5                                          |
| Xylan (includiing arabinoxylan and glucurono-arabinoxylan) | 30.0                                          | 26.7                                          | 6.4                                           |
| Mannan        | –                                             | + (Tr)                                        | 3.2                                           |
| Arabinogalactans (AGI and AGII) | 1                                             | + (Tr)                                        | 6                                             |
| Arabinans     | –                                             | −1                                            | 2.3                                           |
| Others        | −5                                            | 3.3 (including galactans)                      | 7.2                                           |

Notations used: (~) approximate figures, (–) not detectable, +(Tr) trace amounts.

<sup>a</sup> Cell walls isolated from L. multiflorum endosperm grown in liquid suspension culture.

<sup>b</sup> Intact primary epidermis cell walls prepared from early cut leaves of L. multiflorum.

<sup>c</sup> De-starched A. thaliana leaf cell walls, alcohol-insoluble residue preparation.
a concentration of 200 µg ml⁻¹ to form a 100× stock solution, and was used to visualize lipid inclusions and membranes. Fluorescein diacetate (FDA) (Sigma Aldrich) was dissolved in acetone at a concentration of 2 mg ml⁻¹ to form a 100× stock solution, and was used to visualize/assess viable protoplasts. All dyes were used without further purification. If the fraction of viable cells after sieving was >80%, the sample was accepted for further analysis.

Additionally, we performed staining using Aniline Blue Fluorochrome (Biosupplies Australia Pty Ltd, Cat. No. 100-1) and Decoloured Aniline Blue (Mori et al., 2006) for callose, but this showed no appreciable fluorescence, indicating low levels of callose in L. multiflorum SCC walls.

**Microscopic characterization of Lolium SCCs using transmission electron microscopy (TEM):** To prepare L. multiflorum SCCs for TEM, small drops of liquid suspension containing cells were placed in freezer hats and high pressure frozen in a Leica PACT for TEM, small drops of liquid suspension containing cells were recorded over the PDMS substrate. This was done to cross-check the cantilever spring constant in addition to the standard calibration performed using the thermal noise method (Higgins et al., 2006). It was also important to control the position of the AFM tip over the cell’s apex to ensure that the indentation is normal to the cell surface (Dufrene et al., 2001). The optical image was also a way to control for any motion/rotation of the cell inside the well. The FICs were only recorded on the cells that were stable and seated firmly within the microwell. To avoid cell damage and minimize plastic deformation due to prolonged contact, the maximum indentations were limited to 500 nm, and maximal forces to 500 nN, which is considered sufficient for plant cells (Fernandes et al., 2012). Measurements were done in modified White's medium filtered twice through a 0.2 µm pore size membrane filter (MilliQXS MCE, Millipore, Ireland). After adding the buffer, the system was thermostated for 10–30 min to ensure minimal cantilever drift.

**AFM force curve spectroscopy**

**FCS on Lolium SCCs:** AFM measurements were performed using a Nanowizard II (JPK Instruments, Berlin, Germany) mounted on an inverted fluorescent optical microscope. The Nanowizard II machine was equipped with the CellHesion module that enabled extension of the cantilever movement in the Z direction up to 100 µm. All measurements were performed in the closed loop mode. Indentation curves were recorded using driving speeds ranging from 200 nm s⁻¹ to 1000 nm s⁻¹ depending on the cantilever stiffness to minimize the impact of hydrodynamic drag that at all times was <5 Å in the deflection equivalent (Vinogradova et al., 2001).

The probes used were pre-manufactured AFM tips; PNP-TR Si3N4 (R <10nm) (NanoWorld AG, Germany), DNP Si3N4 (R ~20nm) (Bruker AFM Probes, CA, USA), and Mikromasch NSC/ CSC Si tips (R <10nm) (NanoWorld AG). Immediately before use, probes were cleaned in oxygen plasma for 5 min and then were mounted and immersed in the experimental cuvette with buffer. The spring constant (k) of the sensors ranged from 0.05 N m⁻¹ to 1.0 N m⁻¹, and was determined using the Asylum Research GetReal™ routine that utilizes a combination of the thermal noise and the Sader methods (Higgins et al., 2006).

A poly(dimethylsiloxane) (PDMS) microwell substrate measuring 16 × 23 × 1 mm was designed to retain the individual cells in situ during the AFM indentation. The array contains 21 000 microwells, the diameter of which ranges from 55 µm to 75 µm in 5 µm steps to accommodate variability in cell size. The microwell array was fabricated by a standard soft lithography technique outlined in Bonilla et al. (2015) and Chen et al. (2011). The Petri dish with a microwell array was also equipped with in- and outflow tubing connected to a syringe pump. The system allows solvent exchange and perfusion of hypertonic solutions.

In a typical experiment, the microwell substrates were positioned in the instrument and a 1 ml aliquot of filtered Lolium SCCs was added. After ~60 s, the substrate was examined using a low magnification objective (4×4/10) to identify microwells with trapped cells of matching size as illustrated in Fig. 1. The cantilever was then positioned in close proximity to the cell, and a set of force-indentation curves (FICs) were then recorded over the PDMS substrate. This was done to cross-check the cantilever spring constant in addition to the standard calibration performed using the thermal noise method (Higgins et al., 2006). It was also important to control the position of the AFM tip over the cell’s apex to ensure that the indentation is normal to the cell surface (Dufrene et al., 2001). The optical image was also a way to control for any motion/rotation of the cell inside the well. The FICs were only recorded on the cells that were stable and seated firmly within the microwell. To avoid cell damage and minimize plastic deformation due to prolonged contact, the maximum indentations were limited to 500 nm, and maximal forces to 500 nN, which is considered sufficient for plant cells (Fernandes et al., 2012). Measurements were done in modified White’s medium filtered twice through a 0.2 µm pore size membrane filter (MilliQXS MCE, Millipore, Ireland). After adding the buffer, the system was thermostated for 10–30 min to ensure minimal cantilever drift.

**FCS data processing:** Raw force versus distance curves were recorded as a function of the voltage output from the position-sensitive device versus the calibrated z-position of the piezotranslator. The output voltage of the position-sensitive device was converted into a deflection in metres by calculating the slope of the constant compliance measured against a glass/Si wafer substrate in the same buffer/solvent used to record the FIC in cells. The force was calculated by multiplying deflection by the cantilever spring constant. The zero position was determined as the cross-section point of the baseline and the tangent line corresponding to the onset of the indentation curve, where cantilever deflection started to deviate from the baseline. Positive values were attributed to the indentation section of the curve. The apparent separation was calculated by subtracting cantilever deflection from the z-position of the piezotranslator.

For experiments with modulation of the osmotic pressure, a mannitol–trehalose solution was used to induce plasmolysis of the cells. Mannitol (Sigma Aldrich, 0.779 M) and trehalose (LabChem, 1.191 M) were dissolved in de-ionized water to prepare a 6.6 MPa osmotic pressure solution. This solution was added using a syringe pump to elevate the osmotic pressure up to 3.5 MPa. Injection speed was 50 µl min⁻¹. The injection was carried out by a stepwise addition of 200 µl aliquots of osmolyte solution.
further assumptions were made as to whether the initial parts of
the response corresponded to surface forces or actual indentation.
Further analysis was performed using a dedicated custom-written
MATLAB code that is described in detail elsewhere (Bonilla
et al., 2015).

Results and Discussion

Interpretation of force indentation profiles for small-
scale localized cell deformations using an MRA

In Fig. 2A (and Supplementary Fig. S1 at JXB online), a typi-
cal individual cell of L. multiflorum SCCs is shown under fully
turgid conditions. The size of the cells ranges from 20 µm to
100 µm. The thickness of the cell wall, as indicated by the
Calcofluor white staining, is between 0.5 µm and 2 µm; this
result is further verified by TEM imaging (Supplementary
Fig. S2). In Fig. 2B, the representative images of cell ‘ghosts’
after treatment with Updegraff reagent show that the cellu-
lose subnetwork of the wall is continuous, and its thickness
is comparable with the thickness of unmodified walls. These
observations led us to a mechanical model of the SCC as a
thick-walled pressurized shell.

The presence of the wall makes deformation of plant cells
complex; a number of mechanical responses occur simultane-
ously and often with indistinguishable contributions towards
the force–indentation profiles (for a conceptualized overview
of mechanical parameters used in interpreting complex defor-
mations, see Supplementary Fig. S3; Supplementary Table
S1). All types of deformations can be broadly divided into
two categories: reversible (or elastic) and non-reversible (or
dissipative). The former correspond to a spring-like action;
that is, the substrate deforms under the applied force, but
reverts back to its original shape once the force is released.
The latter category is when the deformation profiles during
compression and decompression differ. Typical examples of
non-reversible deformations are viscous, plastic, and viscoe-
lastic deformations.

The elastic (reversible) deformations in plant cell systems are
non-trivial to interpret. During the indentation cycle (Fig. 3A),
the cell wall deforms, as does the turgid protoplast. The total
indentation in this example comprises two contributions; one
is from the cell wall compression, and another from its bend-
ing, as illustrated in Fig. 3B for the case of a blunt AFM tip
indenting a small segment of the wall. Although both defor-
mations are assumed to be elastic, the complexity stems from
the anisotropy of the wall (properties in transverse and lon-
gitudinal directions are different), and the presence of a tur-
gid protoplast. These factors result in a scenario where the
observed deformation profile reflects different cell wall proper-
ties. The compression component is primarily associated with
the transverse Young’s modulus of the wall and its transverse
Poisson ratio. The bending can be characterized using an effec-
tive membrane spring constant, which is a function of the geo-
metric curvature of the wall, turgor pressure, the longitudinal
Young’s modulus, as well as a number of other parameters
such as the cell wall shear modulus. (Supplementary Table S1).

We interpret this complex mechanical response using an
MRA that incorporates multiple mechanical models (Bonilla

Fig. 1. A bright-field image of a microwell array showing the distribution of cells and cell/clusters that sedimented into the well. The chance occurrence
of a single cell sinking into the microwell of the matching size was utilized for AFM nanoindentation measurements. In the inset, a dual illumination
(bright-field and reflected light) optical micrograph of a L. multiflorum SCC (a) confined within a PDMS microwell (b). The cell wall (c) is visible as a shell
surrounding the cell. An AFM cantilever (d) is positioned above the cell so that the tip (e) is positioned approximately above the apex of the cell. (This
figure is available in colour at JXB online.)
et al., 2015), whereby the scenario in Fig. 3B is represented as a system of ‘springs’ or ‘mechanical resistors’ that are connected in a series (Li and Bhushan, 2002).

The MRA and the corresponding automated routine allow interpretation of multiple deformation regimes that may be convoluted within a single force–indentation test. The method also ensures that force analysis complies with assumptions and applicability limits of mechanical models.

In Fig. 4 we present typical examples of indentation curves collected from L. multiflorum SCCs plotted in linear (Fig. 4A, C) and logarithmic (Fig. 4B, D) co-ordinates. The latter enables a better visualization of low force deformations, which otherwise may be obscured by other mechanical responses that dominate the force–distance profile (Bonilla et al., 2015).

A typical FIC is a sequence of compression and decompression tests (Fig. 3A). The compression is recorded first when the AFM cantilever tip indents the sample, while the decompression is recorded when a tip is retracted from the surface. The low force regime is dominated by a weak repulsive interaction which spans the indentation depth anywhere between 50 nm and 500 nm. Further indentation results in a high force region dominated by the mechanical deformation of both the cell and cell wall (Fig. 3B). Figure 4 also illustrates the magnitude of the hysteresis between the approach and retraction curves that is suggestive of contributions from both viscoelastic and plastic deformations. The moment the tip detaches from the surface, an adhesive interaction may manifest itself by producing a pull-off peak. Typically little or no adhesion is observed, although at certain locations on the cell surface multiple detachment peaks are recorded, consistent with stretching of surface-bound polymers (Supplementary Fig. S4). Due to the rarity (~1 in 20 curves) of such events, the detailed analysis was not performed; however, a qualitative evaluation suggests that the most likely mechanism of these interactions is through unspecific physical entrapment of loosely attached polysaccharides by the AFM tip during its exit from the wall. Another feature of the indentation profiles is that a significant number of curves have discontinuities, as shown in Fig. 4C and D. These discontinuities are likely to be associated with the penetration of the tip into the voids of the polysaccharide mesh of the wall.

In addition to the MRA of the force–indentation data, the dissipative parameters such as the area of hysteresis (proportional to the dissipated energy) and the plastic deformation have been recorded. The latter is measured as a distance between the approach and retract branches of the FIC at the point of zero force (for reference, see Supplementary Fig. S3B). These measures provide important insights into possible dissipative mechanisms within the system, and therefore have to be considered in interpretation of the results from MRA analysis.

The majority of data sets recorded for Lolium SCCs using an AFM tip display a behaviour characterized by a three-resistor model that describes three types of deformation
Nano-scale mechanical heterogeneity outlined in Fig. 3A and B: (i) polymer steric repulsion model, \( n_1 < 1 \) (Balastre et al., 2002; Iyer et al., 2009; Bremmell et al., 2013); (ii) elastic membrane model, \( n_2 \sim 1 \) (Begley and Mackin, 2004; Vella et al., 2012); and (iii) thin film elastic model, \( n_3 \geq 2 \) (Dimitriadis et al., 2002; Gavara and Chadwick, 2012). The latter is an extension of a well-established Hertzian–Sneddon model (Hertz, 1882; Sneddon, 1965) that accounts for a finite thickness of the deformed material, as is the case with plant cell walls (see Supplementary Model S1 for details of each deformation model and the equations used).

First, the low force regime with \( n < 1 \) is attributed to the interaction of the tip with loosely adsorbed or protruding polymers, which are evident from TEM images (Supplementary Fig. S2). Such dependency is consistent with a mechanism whereby ‘grafting’ density of polymer chains changes with separation. This can be realized either for a polydisperse brush where density increases as the tip ventures deeper into the polymer layer, and starts to probe shorter chains, or through the lateral displacement of polymer molecules originating from the conical shape of an AFM tip that effectively wedges in between the chains (Balastre et al., 2002; Bremmell et al., 2013; Cuellar et al., 2013). The presence of such a loose polymer layer is consistent with the fact that walls of \( L. \) multiflorum SCCs have a relatively low content of cellulose (<15%/dry weight), but are rich in MLG and arabinoxylan (Table 1).

The second regime with \( 0.9 < n < 1.4 \) is identified as a combination of two resistors, \( n \sim 1.5 \) and \( n \sim 1 \); note, if these occur concomitantly, an intermediate value of exponent is observed. A power law response with \( n \sim 1.5 \) is predicted for a Hertzian contact deformation exerted by a spherical punch, which is satisfied for small deformation when \( \delta < 2 \times R_{\text{Tip}} \). A linear response (\( n \sim 1 \)) is predicted by theories for deflection of membranes and localized (central) deformations of spherical shells, provided that the relative deformation \( \varepsilon = \frac{\delta}{R_{\text{cell}}} \ll 1 \) which is easily satisfied in the small deformation AFM experiments. The values of the spring constant in the MRA model do, however, depend on the scenario that is at play (Begley and Mackin, 2004; Vella et al., 2012). For small deformations of a pressurized spherical shell, \( K_M \) is estimated to be 50 N m\(^{-1}\) when \( P_{\text{turgor}} = 3 \) MPa (Arnoldi et al., 2000). However, the measured values of the effective membrane spring constant are found in the range between 0.01 and 2, which is consistent with the case of an unpressurized spherical shell in the bending-dominated regime (Vella et al., 2012) or, alternatively, with the indentation of a flat membrane (Begley and Mackin, 2004). In the latter
case, one has to assume a membrane cut-off area with radius smaller than $\sqrt{R_{cel}h}$, where $h$ is cell wall thickness. We always observe this regime to precede the transverse elastic deformation of the wall, which suggests that the characteristic bending length is limited to very narrow areas on the cell surface ($l_B << h$) (Dumais, 2007; Audoly and Boudaoud, 2008; Sultan and Boudaoud, 2008). Such a scenario is consistent with the tip indenting the areas in between polysaccharide fibres, and where the cellulose fibre mesh would act as a boundary for local deformations due to its intrinsic stiffness.

The third deformation regime observed is consistent with the indentation of the cell wall in the transverse direction, for which $1.5 < n < 4$. The lower boundary ($n=1.5$) corresponds to the Hertzian deformation exerted by a spherical indenter, which is consistent for an AFM tip at small indentations that is truncated as a cone or pyramid. For larger indentations, a conventional Sneddon’s solution for a cone predicts $n=2$. For a majority of FIC curves, however, larger values of the exponent are observed that we attribute to a non-linear deformation of the thin wall, where deformation is comparable with wall thickness ($\delta \perp h$).

We assume that for $n > 4$ the system is at the limit of the linear (or quasi-linear) elastic approximation; thus, these regions were excluded from the analysis.

**Mechanical mapping of individual cells of Lolium SCCs**

In order to map the mechanical properties of the Lolium SCC walls reliably, numerous 1D tracks are recorded with 2–3 curves per point and ~100–300 curves per track. By doing so, the distance between spatial points is less than ~30 nm, which enables probing of whether the change in the modulus is a continuous function of the position.
or random. Figure 5 shows typical traces of the effective Young’s modulus as a function of the position on the cell surface recorded on two different cells along a 2.6 µm path. The observed variations in the elastic parameters greatly exceed the accuracy of the measurements at each point, and the measured distribution of heterogeneity is consistent with the hypothesis of micromechanical domains. As shown previously (Bonilla et al., 2015), the high level of accuracy is also ensured by extracting elastic parameters from both approach and retraction parts of the indentation curve, from which the MRA routine isolates Hertzian components from other types of force response.

To illustrate further the presence of mechanical heterogeneities, we have collected 2D force volume plots over 5 × 5 µm areas. Figure 6 presents a typical map of the effective Young’s modulus and membrane spring constant. The maps allow measurement of the variation in elastic modulus (Fig. 6A) which is found to span at least three orders of magnitude, between 0.01 MPa and 10 MPa. The median and mean are 120 kPa and 700 kPa, respectively, which are similar to those previously reported for AFM measurements on single plant cell systems (Milani et al., 2011; Radotic et al., 2012). The maps of the membrane spring constant, $k_M$, presented in Fig. 6B reveal similar variations in the magnitude and the spatial distribution. The membrane spring constant is related to the longitudinal elastic modulus of the wall in a localized area around the AFM tip, hence both maps provide complementary data characterizing mechanical heterogeneity of the anisotropic wall.

![Elastic Modulus](image1.png)

**Fig. 6.** 2D force–volume plots of the elastic modulus (A) and the membrane spring constant (B) of *Lolium* SCCs. All plots were recorded over a 5 × 5 µm area. The white areas on the spring constant map (B) correspond to the areas where determining such a type of deformation was not experimentally possible. (This figure is available in colour at JXB online.)
The key finding is that the spatial distribution of heterogeneities of both the Young’s modulus and membrane spring constant has a clear domain structure, covering length scales from several hundreds of nanometres up to a few micrometres. This is because the presence of large-scale domains of mechanical heterogeneities, with the Young’s modulus varying spatially by at least two orders of magnitude, is suggestive of heterogeneity in the microstructure of the deposited cell wall polysaccharides. Figure 7 shows a typical AFM image of the surface of a cryo-milled Lolium SCC wall fragment washed with de-ionized water, which reveals an apparently homogenous and amorphous surface. We note that the deposition of cell wall fragments on the mica surface is random and hence some of the surfaces are representative of the outer surface of the wall and others of the inner surface of the wall, which is in contact with the plasma membrane. From imaging alone we are unable to distinguish between these surfaces. These results suggest that the surfaces of Lolium SCCs are probably covered by loosely associated non-cellulosic polysaccharides, masking any underlying heterogeneity.

To obtain more detailed representation of the underlying cellulose network, we have treated SCCs with Updegraff reagent. The results presented in Fig. 8 show a heterogeneous distribution of the acid-resistant fragments of cellulose fibrils. The key features of this microstructure are that heterogeneity covers a number of length scales, from tens of micrometres to a few hundred nanometres, as well as containing domains with varying density and orientation of cellulose fibrils. The smallest length scale of such microstructural features corresponds well to that found on the mechanical maps, as can be gauged by comparing images in Figs 8C and 6 (5 × 5 µm area), and Fig. 8D with a 1D track in Fig. 5 (2.6 µm).

The imaging results (Fig. 8A, B) are also suggestive of the presence of even larger scale heterogeneities that may span a few tens of micrometres. In order to test this hypothesis, we performed indentation experiments to generate 1D tracks over a larger area by recording along a spiral trajectory. Figure 9 shows a typical result obtained using a fully turgid cell. The size of the circles in Fig. 9A is proportional to the average Young’s modulus obtained using MRA at a specific point, suggesting the presence of well differentiated soft and stiff areas in the wall. These are shown in more detail in Fig. 9B, where the modulus data are plotted versus the curvilinear position, with \( x = 0 \) representing the origin of Fig. 9A. One can see a clear minimum in modulus between 100 µm and 150 µm. The location of this minimum also corresponds to a minimum in the elastic membrane spring constant \( k_M \) (Fig. 9C) and a maximum in plastic deformation (Fig. 9D). We note that for the majority of samples tested, no correlation between plastic deformation and the elastic modulus is observed, which indicates that the observed correlation in a specific location is indeed indicative of an underlying physical mechanism rather than an artefact of analysis. The results clearly show the presence of distinct ‘soft’ regions of the cell wall.

The findings reported here are in a good agreement with recent results by Zhang et al. (2016), where reticulated deposition of cellulose fibrils as well as heterogeneous spatial distribution of soft and rigid matrix polymers (i.e. pectins and cellulose) in the walls of onion epidermis walls have been extensively documented. We note that measurements by Zhang et al. (2016) are performed on a recently deposited layer of the wall; that is, the images are taken of the inner layers adjacent to the plasma membrane. In that respect, such a pattern of deposition may share similarity with the outer regions of the Lolium SCC walls probed in our experiments. It is possible to suggest that in the walls of Lolium SCCs formed without restrictions of neighbouring cells, the bundled and reticulated structure of cellulose deposits may persist through to the outer layers. The heterogeneous deposition of the wall, as well as some irregular deposits in the outer surface of the wall, are also reported in the work by Digiuni et al. (2015), who investigated callus-derived single cells of A. thaliana.

It is therefore possible to conclude that observed domains of mechanical heterogeneity do correspond to the irregular
and bundled deposition of cellulose and to that of the corresponding subnetwork of hemicelluloses and/or pectins.

In planta mechanical mapping of leaf epidermal cells of A. thaliana and L. multiflorum

In order to test the nano-scale distribution of the elastic modulus on mature cell walls within a tissue/organ, we tested abaxial and adaxial epidermal pavement cells of A. thaliana and L. multiflorum leaves. Figure 10A shows a typical AFM height image of the abaxial surface of an A. thaliana epidermal pavement cell. The mechanical tests were performed in the 2D force volume regime (32 × 32 pixels) across a 25 µm² area. The map of the Young’s modulus (Fig. 10B) shows spatial variability qualitatively similar to that observed in L. multiflorum SCCs. The variation in the modulus for the epidermal cells (both adaxial and abaxial) is markedly smaller than for Lolium SCCs; we suspect this reflects how the more mature epidermal cells have a denser distribution of polysaccharides. Also since the walls have a polylamellate structure of cellulose deposition, we expect the outer lamella to bear less resemblance to the meso-scale structure of the freshly deposited lamellae (Zhang et al., 2016).

The MRA analysis of 2D force–volume maps from indentation of L. multiflorum leaf epidermal cells (abaxial) show a marked difference compared with A. thaliana. Most of the data sets are well described by a single elastic membrane resistor, as shown in Fig. 11. This feature is easily detected in the histogram analysis of the power law exponents, which led us to run the MRA with membrane and Hertzian resistors; the latter in anticipation of a small portion of the data with n >1. Interestingly, areas of high K_M correspond well to areas of low plasticity, and vice versa. The fact that two parameters estimated through independent methods agree with each other suggests that the soft areas are not only less stiff but also display non-linear yielding behaviour characteristic of viscoelastic deformation.

We interpret the experimental findings on the three systems studied based on the cell wall composition of each cell type as summarized in Table 1. The Lolium leaf epidermis has the highest cellulose content, which coincides with the minimal transverse deformation of its walls, and the force response is dominated by the membrane-like deformation, namely the ‘thin-walled balloon’ model. It should also be noted that the xyloglucan content that may mediate the links between cellulose fibrils is similar in Arabidopsis and Lolium leaves, and hence one has to be careful with attributing differences in the mechanical response to the difference in the level of xyloglucan-mediated tethers or to the effect of xyloglucan on cellulose–cellulose interfibre links (Park and Cosgrove, 2012). This is further supported by the fact that Lolium SCCs have only a very low level of xyloglucans, if any, yet the wall is characterized by an appreciable elastic modulus in the order
of megapascals, which is higher than that of *A. thaliana* leaf epidermis cells. In part, this can be attributed to the postulated layered structure of the *A. thaliana* walls, with the outer layer being softer than the inner layer (Radotic *et al.*, 2012; Digiuni *et al.*, 2015). Another possible explanation may be the lubricating effect of pectin on cellulose–cellulose interfibre junctions to effect lower resistance of the wall to deformation.

The substantial presence of MLG and pectin in *Lolium* SCCs and *A. thaliana* epidermal walls, respectively (Table 1), results in softer walls, compression of which can be measured even at small indentations. This is in contrast to the *L. multiflorum* epidermis cells that exhibit undetectable cell wall compression, with the mechanical response being dominated by the wall deflection. We note that the average values of the membrane spring constants, \( k_M \), for *Lolium* SCCs and *L. multiflorum* leaf epidermis are of the same order of magnitude, which indicates that elastic properties in the longitudinal direction (in the plane of the wall) are not too dissimilar. It is possible, therefore, to deduce that the absence of the wall compression modes in *Lolium* leaf does not stem from the fact that its wall is ‘softer’ in the longitudinal direction, and the apparent absence of wall compression is likely to be associated with the lower levels of MLGs and pectins. This conclusion is consistent with the force curves for *Lolium* SCCs and *A. thaliana* leaf epidermis cells which feature well-pronounced deformations associated with a fluid-like layer which precedes the elastic deformation of the wall, and which is consistent with a layer of gel-like polysaccharides such as MLG or pectin.

Based on the different composition of the walls in the plant systems studied, it is also possible to propose a hypothesis about the role of xylans in the mechanics of the wall, as both *Lolium* systems feature similar and rather substantial amounts of xylan (~30 molar %). One possible mechanism is via poroelastic control of water permeability that creates a condition where fluid resistance results in the elastic-like response. Such pressurization may also contribute to the non-linear wall deformation that was observed for *Lolium* SCCs, whereby the effective elastic modulus increases with indentation. The role of xylans can also be inferred from the observed values of the plastic deformation characteristic for both *Lolium* systems. Such irreversible deformation would be consistent with the viscous drainage of water-solubilized xylan during poroelastic deformation (Lopez-Sanchez *et al.*, 2014, 2015; Bonilla *et al.*, 2016). We also note that although the values of maximum indentations used in this study are of the order of a few micrometres, they are still insufficient to induce a buckling transition within the area around the indenter which also could manifest itself as an irreversible deformation (Nasto *et al.*, 2013). The buckling zone is expected for indentations of the order of \( \sqrt{R_{cell} \cdot h_{wall}} \), which for a typical cell with the radius of 30 µm and wall thickness of 3 µm are of the order of 8 µm (Paulose and Nelson, 2013).
Non-turgid cells of Lolium SCCs

In Fig. 12A and B, representative examples of plasmolysed (non-turgid) cells are presented. One can see that plasmolysis at its extreme causes either partial (Fig. 12A) or complete detachment of the protoplast (Fig. 12B). This serves as an important validation that, at least at the highest value of the differential osmotic pressure, the plasma membrane is not in contact with the wall, and hence the mechanical properties of the wall would be close to those of an unpressurized elastic shell. We note that even under turgor, the shape of the cells often deviates from perfectly spherical (see Fig. 2A). This non-spherical shape of the majority of cells suggests that the magnitude of the turgor pressure is not sufficient to put the wall under a significant level of pre-stress. In other words, the SCCs deposit a wall that is tougher than required to counter-balance the turgor pressure. Hence results for plasmolysed SCCs should be treated with a degree of caution, as they may not fully reflect the behaviour of cells in tissues.

Figure 12C shows the same cells before and after plasmolysis. One can clearly see patterns where deformations around the circumference of the cells are non-homogeneous. Some regions (indicated by the arrows on the figure) appear to have undergone buckling transition, which provides indirect, although visually clear evidence for the presence of mechanical heterogeneities. The analysis by Paulose and Nelson (2013) shows that mechanical and geometrical heterogeneities of the shells greatly reduce the threshold of buckling, making buckled shapes less sensitive to imperfections. This predicts, in a

![Image](attachment:fig10.png)

**Fig. 10.** AFM height image (A) of the abaxial epidermal layer of an Arabidopsis thaliana leaf epidermal cell. The apex area on one of the cells (boxed area) was also tested using the 2D force–volume mapping technique. The mechanics data are well described by the Hertzian model, with maps of the Young’s modulus (B) showing strong evidence for the presence of heterogeneities. Pixel size 156 nm, indenter radius 20 nm. (This figure is available in colour at JXB online.)
rather counter-intuitive fashion, that introducing heterogeneity could make the buckling transition more reliable than for a uniform shell.

The 1D track method was used to map the mechanical properties of *Lolium* SCCs before and after exposure to high osmotic pressure solutions. Figure 13 presents an example where selected mechanical properties are recorded along a 1D track over an ~30 µm arc on the cell surface, thus covering a distance comparable with the cell's diameter. One can see two microscopic domains, a stiffer one (between ~20 µm and 30 µm) and a softer one (between 0 µm and ~20 µm). Upon changes in osmotic pressure, the elastic modulus of the softer domain did not exhibit much change, while the opposite is observed for the stiffer domain, where the elastic modulus dropped. These results suggest that these two locations differ in the level of pre-stress they exhibit: the higher modulus should be attributed to the areas where wall properties are influenced by strain stiffening. Upon a reduction in differential pressure and subsequent relaxation, the changes in modulus correspond to the reduction in strain stiffening. This interpretation is also supported by the fact that the non-linear elastic modulus (which effectively probes stiffening properties) shows a much more modest reduction compared with the Hertzian modulus.

The membrane spring constant showed little change in response to variation in osmotic pressure, which supports the hypothesis that this quantity is associated with the localized deformation of the domains of gel-like cell wall material confined between cellulosic scaffolds. Indeed, the bending deformations are limited by the longitudinal extensional elastic modulus of the wall (Arnoldi *et al.*, 2000; Boulbitch, 1998, 2000; Boulbitch *et al.*, 2000), which, due to the predominantly longitudinal orientation of cellulose fibrils, is expected to be much higher than the transverse one (Gutierrez *et al.*, 2009; Yi and Puri, 2012).

In comparison with other nanoindentation studies on plant cells, where no effect of osmotic pressure is found, we emphasize that these were restricted to much smaller deformations than in our current study. This includes the study of Milani *et al.* (2011), where the amplitude of applied deformations was negligible compared with cell size and much smaller than wall thickness. Similarly, Fernandes *et al.* (2012) measured mechanical properties on live Arabidopsis roots before and after plasmolysis and found no statistically significant effect of turgor pressure. This was attributed to the small radius of the tip used to probe cell elasticity. Our results are, however, in agreement with more recent studies in which larger indentations were employed (Beauzamy *et al.*, 2015; Weber *et al.*, 2015). It appears that in order to probe the effect of turgor pressure, one needs to apply deformations comparable with wall thickness (i.e. δ~h_wall). This conclusion is based on knowledge from the mechanics of elastic shells that the component of stress in the transverse direction is changing from –P_T at the boundary with the turgid membrane to that of the atmospheric pressure in the outer layers (Landau and Lifshits, 1959). Meanwhile, the longitudinal components of

![AFM height image](https://example.com/afm.png)  
**Fig. 11.** AFM height image (A) of the abaxial epidermal layer of a *L. multiflorum* leaf epidermal cell. The area on one of the cells (boxed area) was also tested using the 2D force–volume mapping technique. The mechanics data are well described by the elastic membrane bending model (B) that shows the presence of heterogeneities. The map of the plastic deformation (C) recorded over the same area showed significant correlation with the elastic spring constant. Pixel size 156 nm, indenter radius 20 nm. (This figure is available in colour at JXB online.)
the stress are only weakly changing with the position along the thickness of the wall, and hence can be safely assumed as constant. This implies that the force response towards small indentations in the transverse direction is identical irrespective of cell turgidity.

**Concluding remarks: implications of heterogeneous mechanical properties on plant cell growth**

Mechanical forces in cell walls are a key feedback control mechanism in plant growth, morphogenesis, and development (Mirabet et al., 2011; Peaucelle et al., 2011; Kierzkowski et al., 2012; Lucas et al., 2013; Routier-Kierzkowska and Smith, 2013). In addition to a direct effect on signalling, mechanical stress is a part of the intercellular communication mechanism that determines a cell’s response to environmental stresses and interactions with pathogens (Santiago et al., 2013). However, our understanding of the effect of mechanical forces on biological signalling is limited due to the inherent complexity and anisotropy of the plant cell wall microstructure, as well as its heterogeneous composition and distribution of polysaccharides (Cosgrove, 2000, 2005; Cosgrove and Jarvis, 2012; Park and Cosgrove, 2012; Wolf et al., 2012; Ding et al., 2014; Doblin et al., 2014; Kafle et al., 2014; Zhang et al., 2014).

Our results strongly suggest that variations in microstructure and composition have a major influence on mechanics, with values of Young’s modulus of the cell wall spanning several orders of magnitude. Our results contribute to the existing evidence for the heterogeneous distribution and variability of plant cell mechanical properties (Radotic et al., 2012; Routier-Kierzkowska et al., 2012), and for the first time provide mapping data and size characterization of such heterogeneities.

The biological implications of nano-scale mechanical heterogeneities remain unknown; we hypothesize that they

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**Fig. 12.** Typical examples of *Lolium* SCCs after plasmolysis (mannitol–trehalose solution) in which either partial detachment (arrows show residual attachment points) (A) or full detachment (B) of the protoplast from the cell wall was observed. In (B), staining with Calcofluor white (upper left), FDA (upper right), and Nile red (bottom left) shows cell wall, cell cytoplasm, and lipid membrane and lipid inclusions, respectively. The overlay of all three channels is given in the bottom right panel. (C) *Lolium* SCC walls before and after plasmolysis. The inset shows additional Nile red and FDA staining to visualize changes in the cytoplasm following the osmotic treatment. The arrows point to the areas of buckling and regions of irregular deformation (both in the top cell). Deformation of the bottom cell is mostly homogenous and can be used as a reference. (This figure is available in colour at JXB online.)
may serve as an important prerequisite for development of mechanical zones and patterns which have a strong association with plant cell expansion; so-called reaction–diffusion patterns (Nagata et al., 2013). In addition, the domain structure of mechanical properties may also be related to the distribution and clustering of microtubules (Xiao et al., 2016). It is possible that the non-homogeneous distribution of microtubules impacts the wall mechanics via non-uniform deposition of cellulose. However, the opposite is also possible, whereby mechanical feedback favours certain clustering of cytoskeletal elements in a way analogous to how mechanical heterogeneity induces fragmentation of actin filaments in animal cells (De la Cruz et al., 2015).

In conclusion, we hypothesize that multi-scale and microstructural factors may act alongside the variability in wall polymer composition, degree of cross-linking, and molecular architecture of constituent polysaccharides to affect the mechanical properties of the cell walls (Burton et al., 2010; Milani et al., 2011; Vogler et al., 2013; Cornault et al., 2014). This work demonstrates that the presence of inherent heterogeneities in primary plant cell walls contributes to their mechanical properties. Future developments should be aimed at correlating the different inherent levels of microheterogeneities, derived from mechanical, compositional, and structural properties, into a model of primary plant cell walls that explicitly reflects its function as a ‘mechano-sensor’ regulating plant form and function.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Typical examples of Lolium SCCs imaged in modified White’s medium under fully turgid conditions.

Figure S2. A representative TEM image of the Lolium SCC wall.

Figure S3. Schematic examples of force–deformation curves for different types of mechanical deformations, such as elastic, plastic, viscoelastic, and viscous.

Figure S4. Typical examples of FICs observed on Lolium SCCs.

Table S1. A conceptualized summary of mechanical parameters expected to be involved in mechanical responses of the cell wall during AFM nanoindentation.

Model S1. Mechanical resistor models.

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