*Plant Biotechnology* 37, 417–422 (2020)
DOI: 10.5511/plantbiotechnology.20.1016a

***Original Paper***

# In situ measurement of cell stiffness of Arabidopsis roots growing on a glass micropillar support by atomic force microscopy

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Received September 8, 2020; accepted October 16, 2020 (Edited by S. Sawa)

**Abstract** Atomic force microscopy (AFM) can measure the mechanical properties of plant tissue at the cellular level, but for in situ observations, the sample must be held in place on a rigid support and it is difficult to obtain accurate data for living plants without inhibiting their growth. To investigate the dynamics of root cell stiffness during seedling growth, we circumvented these problems by using an array of glass micropillars as a support to hold an *Arabidopsis thaliana* root for AFM measurements without inhibiting root growth. The root elongated in the gaps between the pillars and was supported by the pillars. The AFM cantilever could contact the root for repeated measurements over the course of root growth. The elasticity of the root epidermal cells was used as an index of the stiffness. By contrast, we were not able to reliably observe roots on a smooth glass substrate because it was difficult to retain contact between the root and the cantilever without the support of the pillars. Using adhesive to fix the root on the smooth glass plane overcame this issue, but prevented root growth. The glass micropillar support allowed reproducible measurement of the spatial and temporal changes in root cell elasticity, making it possible to perform detailed AFM observations of the dynamics of root cell stiffness.

**Key words:** atomic force microscopy, cell elasticity, force measurement, micro glass device.

## Introduction

Plants dynamically change their morphology in response to mechanical stresses such as gravity, obstructions, wind, soil density, soil compaction, and grazing (Scippa et al. 2008). To better understand the mechanisms underlying these morphological adaptations, it is essential to elucidate the static and dynamic forces in plant cells (Vogler et al. 2015). *Arabidopsis thaliana* is an important model plant for research in basic plant physiology and morphology (The Arabidopsis Genome Initiative 2000). Many Arabidopsis genes related to morphological changes have been investigated by a combination of microscopy and molecular genetics (The Arabidopsis Genome Initiative 2000), but our understanding of the forces affecting Arabidopsis root cells remains limited.

Atomic force microscopy (AFM) uses a probe to contact the sample and therefore is a promising tool for direct observation of forces on Arabidopsis cells (Beauzamy et al. 2015; Braybrook 2015; Fernandes et al. 2012; Milani et al. 2011; Peaucelle 2014) and the root is an especially important target for these analyses. For the probe to contact the sample, the specimen must not move; therefore, a major challenge of AFM is the lack of a suitable method to anchor growing tissues during observation. For example, if a root is not held in place as it grows, the AFM cantilever cannot respond to the elasticity of the root. In conventional preparation procedures, the sample is cut and fixed to the glass substrate with adhesive. However, for in situ observation of living plant cells, the sample must be intact, unaltered, and able to grow normally.

In this study, we used a platform containing arrays of glass micropillars to hold intact Arabidopsis roots for AFM observations. Glass materials are appropriate for these applications because of their high physical and chemical stability and mechanical hardness. The downside is that these properties prevent its microfabrication. Nevertheless, our fabrication techniques for

Abbreviations: AFM, atomic force microscopy; NA, numerical aperture; MS medium, Murashige and Skoog medium.
This article can be found at http://www.jspcmb.jp/
Published online December 11, 2020

micro electro mechanical systems (MEMS) allowed us to fabricate glass micropillar arrays in micrometer scale. Indeed, in our previous work, we developed several kinds of micro glass devices and assessed their advantages for cell manipulation and detection (Yalikun and Tanaka 2017; Yalikun et al. 2016).

In this study, we cultured Arabidopsis seedlings on a glass substrate with micropillars and measured the root cell elasticity directly on the substrate. We compared these cell elasticity measurements with those of roots placed on a smooth glass surface and roots fixed with an adhesive on the glass. The AFM cantilever was able to contact the root elongating into the gaps between the micropillars, but it was difficult to maintain contact with roots on the smooth glass substrate. The reliability of our measurements was confirmed from the relationship between the force applied by the cantilever and indentation of the cell. Finally, the effectiveness of this method was demonstrated by measuring cell elasticity along the root in the direction of elongation and quantifying the spatial and temporal variation throughout root growth.

## Materials and methods

### *Fabrication of glass micropillar support*

The glass substrate with micropillars was fabricated by standard photolithography coupled with a conventional glass wet-etching method (Yalikun and Tanaka 2016, 2017). Figure 1A illustrates the fabrication processes as follows: (i) A positive photoresist (OFPR800-23cp, Tokyo Ohka Kogyo) with a thickness of 1 $\mu$m was spin-coated on a Cr (75 nm)/Au (50 nm) thin film-covered glass slide (TEMPAX, Matsunami Glass, thickness: 0.7 mm, size: 70×30 mm). (ii) The glass slide was exposed to UV light through a photomask, in which a circular array pattern (diameter: 0.1 mm, interval: 0.3 mm) was engraved, and the OFPR was developed with an organic alkali solution (NMD-3, Tokyo Ohka Kogyo Co., Ltd.). (iii) Au/Cr film without the OFPR protection were removed by etching solutions AURUM-100 (Kanto Chemical) and Hicretch-s1 (Wako Pure Chemical Industries, Ltd.), respectively, to form a mask pattern. (iv) The glass surface without the mask was etched by 50% hydrofluoric acid for a few minutes. (v) The mask pattern was then removed. The fabricated glass substrate (Figure 1B, C) was cut into 15 ×15 mm squares for the following experiments.

### *Culture on the glass substrate with micropillars*

*Arabidopsis thaliana* ecotype Columbia (Col-0) was cultured as shown in Figure 2A. The seeds were sterilized in 70% ethanol, and then placed on the edge of the glass substrate on a 35-mm glass-bottom dish (Glass $\phi$27 mm, No. 0, IWAKI brand ASAHI GLASS CO., LTD.). The glass micropillars with seeds were covered with 2.0% (w/v) low-melting point agarose (PrimeGel Agarose LMT, TaKaRa). The seeds on the glass substrate were

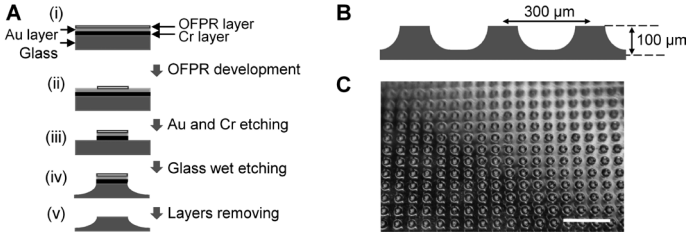


Figure 1. Glass micropillar array for holding *Arabidopsis thaliana* roots. A) Fabrication processes. (i) A positive photoresist (thickness: 1 $\mu$m) was spin-coated on a Cr (75 nm)/Au (50 nm) thin film on a glass slide (thickness: 0.7 mm, size: 70×30 mm). (ii) The glass slide was exposed to UV light through a photomask. (iii) Au/Cr film without the OFPR protection were removed by wet etching. (iv) The glass surface without the mask was etched by a hydrofluoric acid. (v) The mask pattern was removed. B) Design and C) micrograph of the glass micropillar array.

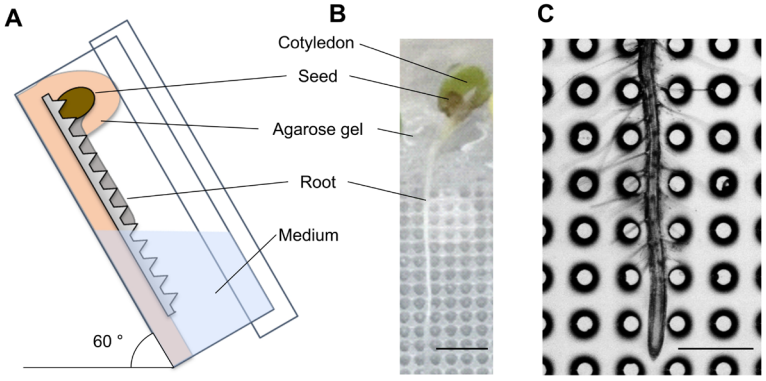


Figure 2. Culture of *Arabidopsis thaliana* on the glass substrate with micropillars. A) Schematic of the culture. B) Photograph of 4-day-old roots on the substrate. Bar is 1 mm. C) Micrograph of root hairs entangled with micropillars. Bar is 500 $\mu$m.

put in a glass-bottom dish and incubated at 4°C for 2 days, and then placed in a plant incubator (LPH-240/410SP, NK system) at a 60° angle with the seeds at the top of the substrate. The bottom end of the dish was filled with a liquid medium supplemented with 4.4 g $l^{-1}$ Murashige and Skoog (MS) medium (Duchefa Biochemie), 2 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 5.7, adjusted with KOH), and 10 g $l^{-1}$ sucrose to provide humidity and to feed the seedlings. The seedlings were cultured under long-day conditions (16-h light/8-h dark) at 22°C in 60% humidity. In the AFM measurement, the seedlings in the glass-bottom dish were gently covered with liquid MS medium and set on an inverted microscope (IX 71, Olympus). After the measurement, the dish was returned to the plant incubator. The AFM measurement was performed in a few days interval.

### *AFM measurement*

Seedlings on the glass substrate was observed by an inverted microscope (IX 71, Olympus) through 4× objective lens (UPlanFl NA: 0.18, Olympus) and measured by an AFM system (Nanowizard 4, JPK Instruments) equipped on the microscope. An AFM cantilever (force constant: 42 N $m^{-1}$, SD-Sphere-NCH, NANOSENSORS) was attached to the head of the AFM system and placed on the top of the seedling. The cantilever tip is shaped into a hemisphere (400 nm radius) to avoid damaging the contact point on the cell. The space between the cantilever and the sample was filled with the liquid MS medium. The positions of interest were chosen by adjusting the mechanical position control of the cantilever on the root cells guided by observation under the inverted microscope. AFM detection was performed point-to-point in 10×10 points onto an area of 10×10 $\mu$m. The accumulated data were analyzed by the Hertz model (Johnson 1987), in which the apparent elastic modulus of the cell was estimated by Young's modulus. The average and standard distribution of the elastic modulus in the detection area were estimated. As a reference experiment, the measurement was performed for roots cultured for 4 days and fixed on a glass-bottom dish with an adhesive (Bio-compatible glue, JPK Instruments) immediately before the measurement.

## Results

### *Root growth on the glass micropillar support*

Arabidopsis seedlings were grown on the glass substrate with micropillars (Figure 1) as shown in Figure 2A (for the details, see Materials and methods). The main root elongated into the gaps between the micropillars for 30% of seedlings, and once the root elongated into the gaps, it remained within the micropillars (Figure 2B). Root hairs appeared after 3 days of culture and grew entangled with the micropillars while the main root was held in the micropillars (Figure 2C).

### *AFM measurement of the root cell on the glass micropillar support*

The force measurement of the main root was performed by contacting the root with the AFM cantilever as shown in Figure 3A. When the surface of the root was pushed by the cantilever, the cantilever was bent by a repulsion force and indentation of the root cell. The relationship between the repulsion force and height of the cantilever, resulting in the bending of the cantilever, is shown as a force–height curve (right-hand graph in Figure 3A). The solid curve indicates force–height dependence in the push-down of the cantilever. The measurement is performed from left to right of the curve. The cantilever contacts the cell at the origin of the horizontal axis and induces indentation with the force loading of the cantilever. When the applied force reaches a certain value (preset maximum value of the loading force, called the set point), the cantilever is pulled up from the cell. The force–height dependence in the pull-up is the dashed curve in the graph of Figure 3A, which is measured from right to left. In the normal AFM measurements, the push-down and pull-up curves differ slightly because the pull-up process involves attractive interactions between the cell and cantilever.

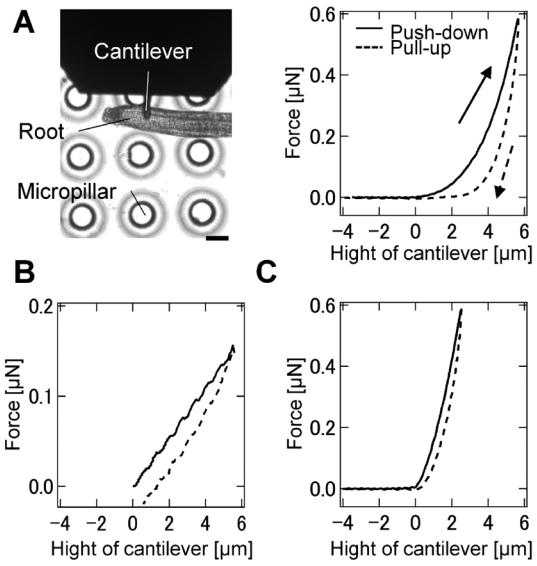


Figure 3. Measurement of force–height curves of an *Arabidopsis thaliana* root. A) Micrograph of the root in the experiment (left) and typical force–height curve (right). Bar is 100 $\mu$m. B) Force–height curve obtained from a root placed on a smooth glass substrate in water. C) Force–height curve from a root immobilized on a glass substrate by an adhesive.

For comparison, we first tested roots on a smooth glass substrate (Figure 3B). However, the push-down and pull-up curves had large differences and were unstable, i.e., their positions changed measurement-by-measurement. This indicated that reliable measurement is not possible without proper mounting because force loading by the cantilever causes the root to slip on the smooth glass substrate. Next, the root was fixed on the smooth glass substrate with adhesive (Figure 3C). The force curves had good reproducibility and showed a similar tendency with

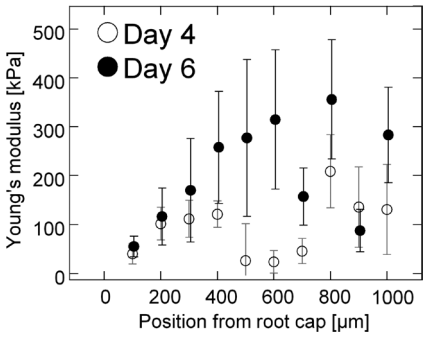


Figure 4. Changes in the cell elasticity of an *Arabidopsis thaliana* root on the glass micropillar support during seedling growth. The Young's modulus was estimated by the AFM measurement and Hertz model analysis. The measurement was performed from the root cap (origin of the horizontal axis) to root base (positive direction of the horizontal axis) with 100 $\mu$m interval. In each measurement position, force–height curves were acquired in 10×10 points onto an area of 10×10 $\mu$m. The average and standard deviation of modulus estimated from the 100 data points are plotted as circles and error bars, respectively. The white circles are modulus of a seedling cultured for 4 days. The seeding was returned to the plant incubator, cultured for 2 days, and measured again. Black circles are modulus of the seedling cultured for 6 days in total.

curves measured on the glass substrate with micropillars. Although the curve of the sample fixed by the adhesive has a greater slope than the curve of the sample in the micropillar support in comparison of Figure 3A and 3C, the difference is within the experimental errors due to individual differences of samples and the spatial inhomogeneity of the root cell surface.

### *Age-dependent elasticity of root cells in young Arabidopsis seedlings*

AFM measurement with the micropillar support allowed us to evaluate changes in cell elasticity throughout the course of seedling development without inhibiting root growth. In contrast, for the measurement using adhesive, we could not evaluate the same sample over time because the adhesive inhibited root growth. As shown in Figure 3A, the AFM cantilever can contact the root growing in the micropillar support. We measured force–height curves along the direction of root elongation, then estimated the cell elasticity based on the Hertz model (Johnson 1987). We repeated the measurements for over 5 samples and successfully obtained data for a ~1,000-$\mu$m region starting from the root tips as the course of root growth force–height curves were acquired 100 points (10×10 points) onto an area of 10×10 $\mu$m. Since the area includes not only the center of the cell but also the edge, the estimated Young's modulus is an index of the average stiffness and its standard deviation of the root surface.

The modulus tended to increase from the root tip to the root base, though an extremely low modulus was observed at some points. The possible reason for the irregularity is that, rather than measuring the indentation of the cell, the cantilever detected bending of a root hair. Despite such measurement artifacts, we observed reliable spatial and temporal variance in the cell elasticity of the same sample using the glass micropillar support.

The 6-day-old root showed a larger elasticity than the 4-day-old root in the region more than 400 $\mu$m away from the root tip (Figure 4). The region around 400 $\mu$m from the root tip in the 6-day-old root corresponds to the transition from the meristematic zone to the elongation zone (Somssich et al. 2016); therefore, the increase in the measured values from the root tip to the root base should be related to cell elongation. Interestingly, the 4-day-old root cells showed lower module with narrower ranges compared to the 6-day-old root (Figure 4). These results suggest the cell elasticity in Arabidopsis roots is regulated in an age-dependent manner.

## Discussion

In this study, we used the glass micropillar support to hold the roots of Arabidopsis seedlings during AFM measurements. The roots did not avoid the micropillars but elongated between gaps (Figure 2B). This suggests that toxic chemicals used in the micropillar fabrication process do not remain in the culturing and boron trioxide in the glass substrate has also no negative effect on the root growth. However, the root elongation in the micropillar support seemed to be a little slower than that on the smooth glass substrate. The micropillars would exert mechanical stress on the root during elongation. We must take mechanical stress due to micropillars on the root during elongation into consideration. The stress could be affected by the micropillar design including the pillar diameter, height, and interval between pillars.

The root is held firmly in the glass micropillar support (Figure 3A). This is because the root hairs are entangled with the micropillars and support contact of the root with the rigid substrate (Figure 2C). There is possibility that the AFM detects point elasticity of all cell layers or whole root sample, if the root sample is not fixed on the glass substrate. On the present experimental condition, as the sample is firmly held by the micropillar support, these artifacts would be neglected. Previously, we measured Arabidopsis roots on a smooth glass substrate (Figure 3B), on hard agarose gel, and a micropillar made of polydimethylsiloxane (PDMS), which is a common material used to fabricate microfluidic devices (Hida et al. 2014, 2015). We were not able to obtain reliable data with these substrates because AFM senses not only the bending and indentation of the root but also indentation of the substrate material, which was softer than the root or glass. Reliable data could be obtained when the root was fixed with double-sided tape, or when both sides of the root were sealed by a thin silicone seat, but these methods also probably prevent root growth. Our AFM

detection method using the micropillar support provides an alternative to these previous methods for evaluating cell elasticity while minimizing negative effects on root growth.

Previously, the cell stiffness of the Arabidopsis has been investigated by several measurement techniques. Dyson et al. has been applied a pressure probe method to measure the turgor pressure of the root epidermal cells in the developmental zone. The pressure was estimated to be ~400 kPa (Dyson et al. 2014), corresponding to several times of the atmospheric pressure (101 kPa). Apparent stiffness of the meristem measured by AFM has been reported to be around ~3 MPa (Long et al. 2020). The stiffness of the leaf has been respectively estimated to be ~100 MPa by nanoindentation measurement (Forouzesh et al. 2013). The apparent stiffness of the plant cell is not only due to the elasticity of the cell wall but also due to turgor pressure, which also induces tension in the cell wall. Since the cell wall stiffness would increase in the order of root, stem, and leaf, the apparent stiffness would also increase in this order. Taking these knowledge into account, the Young's modulus estimated by the present AFM measurement is reliable as the apparent stiffness, resulting in both the cell wall stiffness and the turgor pressure. Now we are further analyzing the AFM force curve date in theory and numerical simulations.

The Young's modulus increased from the root tip to the root base in the 6-day-old root (Figure 4). The measured root region is within the meristematic zone, elongation zone, and the beginning of the differentiation zone (Figure 4; Benfey et al. 2010; Somssich et al. 2016). Thus, the changes in the Young's modulus should be mostly related to the transition from the meristematic zone to the elongation zone. A similar observation was reported by Fernandes et al. (2012). The cell growth rate and cell wall extensibility change along the root axis, and the cell growth rate is correlated with turgor pressure (Cosgrove 2016; Pritchard 1994). Therefore, the changes in Young's modulus of roots observed here would reflect the increase in turgor pressure and cell wall dynamics to regulate wall extensibility (Cosgrove 2005, 2016). The standard deviation of the Young's modulus increased with the increase of the average. This suggests that the cell growth enhances the spatial inhomogeneity of the elasticity from the center to the edge of the root cell.

Root development involves substantial regulation of cell wall biosynthesis and modification (Cosgrove 2005, 2016; Somssich et al. 2016). For example, the pectin (1→4)-$\beta$-D-galactan accumulated in the cell walls at the transition zone between the meristematic zone and the elongation zone (McCartney et al. 2003). Moreover, in the elongation zone, cortical microtubule re-orientation is actively controlled along with cellulose synthesis, and cell wall loosening due to specific enzyme reactions was observed during rapid cell growth (Cosgrove 2005, 2016; Somssich et al. 2016). We have not yet directly linked the observed changes in cell elasticity to specific cell wall components/enzymatic activities; however, future analysis with Arabidopsis mutants and specific enzymes will uncover the molecular mechanisms that control cell elasticity during root development.

For the accurate investigation of the root development, the evaluation should be performed for the same cells in the root growth. However, we cannot measure the same cells by the present system. In addition, root hair elongations in the progress of the root growth make the difficulty of the measurement. For further accurate evaluation, it is required to improve the detection system.

Taking our results together, we were able to reliably quantify spatial and temporal variations in cell elasticity of Arabidopsis roots by AFM detection using the glass micropillar support. This method makes it possible to perform detailed observations of the dynamics of root cell elasticity and can be used on the diverse mutants of Arabidopsis, as well as with chemical screening strategies. This method could be used to provide new insight into the mechanical properties of plant cells and their regulation, which contribute to morphological adaptation in plants.

## Acknowledgements

This work was supported in part by the MEXT KAKENHI Grant-in-Aid for Scientific Research on Innovative Areas "Plant-Structure Optimization Strategy" Grant Number JP18H05493 to E.A., K.O., and Y.H. and JP18H05484 and JP18H05489 to M.O. and T.D. Furthermore, support from the Amada Foundation to Y.Y. is gratefully acknowledged.

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