Cell wall extensibility during branch formation in the xanthophycean alga Vaucheria terrestris

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Cell wall extensibility during branch formation in the xanthophycean alga *Vaucheria terrestris*

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Abstract

In the tip-growing filamentous cell of the xanthophycean alga *Vaucheria terrestris* sensu Götz, a new growing tip develops in the non-growing, cylindrical region of the cell that was exposed by local illumination. The present study examined changes in the strength and extensibility of the cell wall of the new growing tip and in the matrix components of the inner surface of the cell wall. The internal pressure required to rupture the cell walls decreased remarkably during the early to middle stages of growing tip development, but the cell wall hardly extended before rupture. In contrast, during the middle and late stages of development, cell walls were extended by internal pressure. Atomic force microscopy revealed that protease-resistant, fine granular matrix components were present only at the apical portion of a normal growing tip, and were absent in the non-growing cylindrical region. In the early and middle stages of new growing tip development, these matrix components appeared in the cell walls in patches. These results suggest that first cell wall strength decreases and then cell wall extensibility increases in the development of new growing tips, and that protease-resistant, fine granular matrix components may be involved in rendering a cell wall extensible.

Keywords: Atomic force microscopy, Cell wall, Cellulose microfibril, Extensibility, Strength, Morphogenesis

Abbreviations: AFM Atomic force microscopy

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Introduction

Growth and morphogenesis of plant cells involve the expansion of existing cell walls and the deposition of new cell wall material. Therefore, the mechanical properties of existing cell walls, such as extensibility and strength, are fundamental to controlling plant cell growth and morphogenesis. In fact, many biomechanical studies on the multicellular tissues of angiosperms have shown that growth rates of plant cells are correlated with cell wall extensibility (Masuda 1990; Kutschera 1991; Cosgrove 1993). It has been generally believed that the mechanical properties of cell walls in such plants are controlled by the modification of matrix components of the cell wall and changes in their interactions with the cellulose microfibril skeletal components (Carpita and Gibeaut 1993; Cosgrove 1997, 1999).

In tip-growing cells, where growth of the cylindrical cell is limited in the domed end of the cell, there have also been a number of reports concerning the mechanical properties of the cell wall in a variety of organisms (e.g., Haughton and Sellen 1969; Money and Hill 1997; Dumais et al. 2004). Mine and Okuda (2003) have studied cell wall extensibility in tip-growing cells of the giant-cellular xanthophycean alga *Vaucheria terrestris*. Their experimental technique applied pressure to the isolated cell wall from tip-growing regions by applying internal pressure to silicone oil filling in the cell wall sac. They found that the cell wall in the tip-growing region was extended most, whereas the cell wall in the mature, cylindrical region was hardly extended (Mine and Okuda 2003). Extensibility of the tip-growing region was highest at pH 8 but very poor in acidic environments. Treatment of the isolated cell wall with the protease subtilisin caused significant weakening of the cell wall, but the pH dependency of the cell wall

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extensibility remained. These results imply the existence of two distinct factors controlling the mechanical properties of the cell wall: one factor promoting cell wall extensibility in a pH-dependent manner in the tip-growing region (extensibility factor), and the other protease-sensitive factor maintaining cell wall strength (strength factor).

Parker et al. (1963) reported that the cell wall of *Vaucheria* consisted of layers of randomly oriented cellulose microfibrils and hot water-extractable matrix polysaccharide consisting mainly of glucose and some uronic acids. Recently, we reported the surface structures of intact and protease-treated cell walls of *V. terrestris* using atomic force microscopy (AFM) (Mine and Okuda 2006). Protease treatment of the cell wall caused loss of matrix components that contained embedded cellulose microfibrils in the inner surface and that covered microfibrils in the outer surface throughout the cell. However, even after the enzymatic removal of a significant amount of matrix components, fine granular components remained among microfibrils in the apical portion of the tip-growing region of the cell. Based on these observations, we proposed that the protease-degradable matrix components that embed and cover microfibrils throughout the cell relate to the strength factor of the cell wall, and protease-resistant fine granular matrix components in apical portion relate to the extensibility factor of the cell wall (Mine and Okuda 2006).

On the other hand, the *V. terrestris* cell spontaneously forms branches by producing new growing tips in mature, non-growing, cylindrical regions of the cell. In this branch formation process, an area of cell wall in the cylindrical region mechanically strong enough to bear high tensile stress generated by turgor pressure should be transformed into the extensible cell wall that may develop into the growing tip. During this transition of the cell wall from strong to extensible, alternation of the

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above-mentioned factors controlling the cell wall extensibility and strength might occur. Moreover, it has been reported that branch formation in this alga can be artificially induced by irradiation with blue light on a limited region of the cell (Kataoka 1975; Takahashi et al. 2001). This enables us to obtain cell wall samples from distinct stages of branch formation and extensibility.

Here we examined the changes in the extensibility and strength of the cell wall during branch formation in this alga, in order to clarify the role of the cell wall's mechanical properties in the morphogenetic process. We measured the change in the internal pressure required for the extension and rupture of cell walls isolated from the region where the branch formation had been induced by blue light irradiation, as well as the total extension and extension rate of the pressurized cell wall until rupture. In addition, using AFM, we examined the fine structure of the inner surface of protease-treated cell walls from the area of branch formation. This was to explore cell wall components related to the restoration of the extensibility in the mature, non-growing cell wall.

Materials and methods

Algal material and solutions

A unialgal strain of *V. terrestris* sensu Götz was obtained from Dr. Hironao Kataoka (Tohoku University, Sendai, Japan) and cultured as described previously (Mine and Okuda 2003). The experimental buffer was a mixture of 20 mM Tris(hydroxymethyl)aminomethane and 20 mM 2-morpholinoethanesulfonic acid (pH 7.0 or 8.0). The pH 7.0 buffer was used in protease treatment, whereas the pH 8.0 buffer

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was used for extension experiments. The proteolytic enzyme (P8038, Sigma; subtilisin Carlsberg; Lot No. 21K1653) was suspended in the buffer (pH 7.0) at a concentration of 10 units mL^{-1} for protease treatment of cell wall fragments.

Induction of branch formation

Branch formation was induced by irradiation of microscopic illuminating a limited region on the cylindrical part of the cell using a similar method to that described by Takahashi et al. (2001). Briefly, apical cell fragments 1.5 to 3 mm long were excised with scissors from cultured thalli, transferred to fresh culture medium and cultured for two days in the same conditions as the stock culture. Actively growing, non-branched regenerated cells were selected and transferred into the illumination vessel filled with medium using a Pasteur pipette. The illumination vessel was a rectangular container constructed of plastic plates, with inner dimensions 50 mm wide, 40 mm long and 3 mm deep (Fig. 1). A glass plate (25 mm wide, 40 mm long, and 2 mm in height) was placed on the bottom of the chamber so that cells were raised to the focal range of microscopic observation. On the top of the plate, two parallel streaks of silicon grease had previously been drawn at a separation about 1 mm. Cells were laid parallel to each other between the grease streaks and immobilized by gently pressing a small piece of cover slip over the grease streaks. The illumination vessel was then covered with a transparent plastic lid, sealed with silicon grease and placed on the specimen stage of an Olympus BHA light microscope. Cells were observed through the 20x objective.

A daylight filter LBD (Olympus) was inserted just above the microscope illuminator on the microscope base and a slit formed by the edges of two razor blades was

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positioned about 40 mm above the filter. The height of the condenser was adjusted so that the slit image was in the focal plane of the cells. The width of the slit was adjusted so that it was 0.2 mm in the microscopic image. The opening of the condenser diaphragm was set at 2.5 as indicated on the adjustment ring. The tungsten lamp in the microscope was set at 7 V, as measured by the built-in voltmeter. The position of the illumination vessel was adjusted so that the edge of the slit was perpendicular to the axis of the cells and at 2-4 mm from the cell apex. Except for the light path, the vessel was covered by a cardboard enclosure to minimize irradiation from ambient light.

Takahashi et al. (2001) reported sequential changes in cell structure in the irradiated region during branch formation. In the present study, branch formation was divided into the following four stages (Fig. 2): (i) aggregation of protoplasm—chloroplasts accumulate in the irradiated region resulting in thickening of protoplasm; (ii) formation of a new growing tip with a transparent patch and slight bulging of the cell surface at the patch; (iii) dome-like protrusion—the localized expansion at the new growing tip which makes the protrusion of the cell surface almost hemispherical; and (iv) extension of a new branch with a dome-like growing tip and subsequent cylindrical basal region. Takahashi et al. found that the irradiation time required for branch formation was variable. We irradiated cells continuously for 5 to 8 h and then examined them under the microscope to determine their stage of branch formation.

Extension experiment on isolated cell wall

After irradiation, cells were transferred into a plastic Petri dish containing fresh medium

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and excised at 1 mm proximal to the irradiated region. The excised cell was tightly knotted with a nylon thread (0.08 mm in diameter; gift of Toray Industries Inc., Tokyo, Japan) 1 mm distal to the irradiated region and the apical cell wall portion distal to the knot was removed. The knot was lifted out of the medium and the cell wall around the knot was glued with a cyanoacrylate adhesive (Aron Alpha, Toagosei Co. Ltd., Tokyo, Japan), then submerged back into the medium in order to harden the glue and seal that end of the cell. The cell wall was isolated by squeezing out the protoplasm as previously described (Mine and Okuda 2003). Removal of the protoplasm was sometimes incomplete due to the complicated cell wall shape at the branch protrusion (as seen in Fig 4d), but such remnant protoplasm did not affect the mechanical properties of the isolated cell wall. Isolated cell walls were further prepared for the extension experiment as in Mine and Okuda (2003), except that bending of the glass needle was omitted. Briefly, a glass needle filled with the experimental buffer (pH 8.0) and silicone oil (KF-96L-2CS, a gift from Shin-Etsu Chemical Co. Ltd., Tokyo, Japan) was inserted into the other end of the isolated cell wall and glued, and the inside of the glass needle was pressurized at 100 kPa to infiltrate the cell wall with the experimental buffer from inside to outside of the cell wall and to fill the cell wall with the oil. Tensile stress was further applied by increasing the internal pressure by 4 kPa s^{-1} until the cell wall ruptured. We stopped increasing the internal pressure at 800 kPa even when the cell wall did not rupture, because of the upper limit of the pressure regulator.

In cell walls at branch formation stages (ii), (iii) and (iv), protrusion of the cell surface at the new growing tip maintained its shape even after removal of protoplasm. The cell wall was rotated so that a side view of the protrusion was obtained, using the 10x objective under an inverted microscope (Zeiss Axiovert S 100). During the above

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extension experiment, changes in the shape of the cell wall around the new growing tip were recorded with time-lapse images at 1 s intervals with a CCD camera mounted on the microscope controlled by a computer, as previously described (Mine and Okuda 2003). Each video frame was transferred to a file of the software PowerPoint (version 97, Microsoft Corporation, Tokyo, Japan) and the cell wall outline was traced. The length of the trace was calculated using computer software (Scion Image, Scion Corporation, Frederick, MD, USA). The total extension (% cell diameter) of the cell wall until rupture was calculated as the difference between the perimeter length at the initial pressure (100 kPa) and that at the video frame just before cell wall rupture, divided by the diameter of the cell at the initial pressure (Fig. 5). The extension rate (% cell diameter s^{-1}) was calculated by subtracting the perimeter length in a video frame from that in the video frame 2 s later, which was also divided by the diameter of the cell at the initial pressure, and further dividing by two.

Observation of cell wall surface fine structure

After irradiation, cells that had reached any stage of branch formation were transferred into fresh medium and cut at the proximal boarder of the irradiated region. Protoplasm was squeezed out at the cut and the cell wall portion about 0.2 mm from the cut was excised. The excised cell wall portion was turned inside out using a pair of heat-extended glass tubes installed on a micromanipulator, as shown in Fig. 3. The cell wall portion was then incubated in a protease solution prepared as above at 30ºC for 24 h. The protease treatment has been shown to partially remove the matrix components of the cell wall (Mine and Okuda 2006). It was carried out in the present study to test for the presence of

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protease-resistant, fine granular matrix components of the cell wall, which have been previously found specifically in the apical portion of tip-growing region (Mine and Okuda 2006). After enzymatic treatment, the cell wall fragments were washed with buffer, immobilized on a glass cover slip pretreated with aqueous polyethyleneimine P-70 solution (Wako Pure Chemicals, Osaka, Japan; original 30% solution diluted 1:300 with distilled water), and air-dried. The cover slips were mounted on the specimen holder of an atomic force microscope (JEOL JSPM-4200, at the Industrial Technology Center of Kochi Prefecture, Japan) equipped with a probe (OMCL-AC160TS, Olympus). The inner surface of the cell walls in the irradiated region was observed with the microscope using AC mode. Topographic images (512 x 512 pixels) were obtained by scanning a 5 x 5 μm or 1 x 1 μm specimen area at a scanning speed 0.33 or 0.67 ms per pixel, respectively. Other scanning conditions were unchanged from the preset conditions. For comparison, the inner surface of protease-treated cell walls isolated from apical and cylindrical portions of normal tip-growing regions of the cell were also examined as in the previous study (Mine and Okuda 2006).

Results

Changes in cell wall extensibility during branch formation process

In cells at stage (i) of branch formation, rupture did not occur at 800 kPa in three of the eight cell wall fragments isolated. The remaining five cell walls ruptured at an average pressure of 760 kPa (Table 1). From stages (ii), (iii) and (iv), five cell walls for each stage were tested. All cell walls ruptured, with the required pressure decreased gradually with

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progression of the branch formation stage. The average pressure required for cell wall rupture was 532.4 kPa at stage (ii), 460.8 kPa at stage (iii), and 455.6 kPa at stage (iv) (Table 1). The differences between the average pressures at stages (i) and (ii) and between the average pressures at stages (ii) and (iii) were statistically significant (Student's *t*-test; $P < 0.01$).

Cell walls isolated from cells at stage (i) ruptured suddenly, and cell wall extension was too small to measure at this stage. Cell wall extension notably increased as the branch formation process proceeded. At stage (ii) (Table 1, Fig. 4a-c), the cell wall extended 4.2% of the cell's diameter before rupture in average; the average extension at stage (iii) was 15.5% (Table 1, Fig. 4d-f); and finally the cell wall at stage (iv) showed 25.4% extension in average (Table 1, Fig. 4g-i). In addition to the amount of extension, the rate of extension also increased with further branch formation (Table 1).

Fine structure of cell wall surface in branch-induced cell region

The observations were carried out on cell wall specimens isolated from three different cells at stage (i) of branch formation. Microfibrils densely crossed with each other and arranged in random directions were observed (Fig. 7a, b), as was seen in the cell wall from the basal, cylindrical portion of the tip-growing region of the cell (Fig. 6c, d). However, in all areas examined (six areas in total) at stage (i), no matrix components, including fine granular ones, were detected.

We further observed the fine structure of the inner surface of cell walls isolated from two different cells for each of stages (ii) and (iii). Topographic images of the inner surface of the cell walls at stage (ii) in the area of the bulge showed bright patches

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indicating that the specimen surface are locally raised in these patches (Fig. 7c). Fine granular matrix components were observed among cellulose microfibrils in some of these patches (Fig. 7d). In the cell wall at stage (iii), fine granular matrix components were also observed in some patches (Fig. 8a, b), but no matrix components were observed in other areas (Fig. 8c, d). We were not successful in turning the cell wall in the elongated bulge at stage (iv) inside-out, so the fine structure of the cell wall inner surface at this stage remains to be observed.

Discussion

Using cell walls isolated from the normal tip-growing region of cells of *V. terrestirs*, we have previously demonstrated that mechanical properties such as extensibility and strength of the cell wall are related to cell growth pattern (Mine and Okuda 2003). In the present study, changes in the mechanical properties of the cell wall during the branch formation process were examined. Mature, non-extensible cell wall in the cylindrical region of the cell was converted into young, extensible cell wall in the tip-growing region of a newly formed branch. The pressure at which cell wall rupture occurred was initially much higher than the pressure obtained from previous extension experiments (400 to 500 kPa) for cell walls isolated from the tip-growing region (Mine and Okuda 2003). The rupture pressure significantly decreased until stage (iii) (Table 1, Fig. 4), which is similar to the pressure (463 kPa) required for extension of the isolated cell walls from the tip-growing region at a comparable rate (Mine and Okuda 2003).

As pointed out in Dumais et al. (2004), the tensile stress exerted on the cell wall inflated by internal pressure is proportional to the internal pressure, inversely

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proportional to the thickness, and roughly in inverse relation to the curvature of the wall. It is probable that the wall becomes thinner in actively expanding regions than in non-growing regions. Although we did not collect data on the local wall thickness, cell wall thinning would increase the tensile stress remarkably in the cell-surface protrusion. Moreover, cell wall rupture often occurred in the subapical region of the new growing apex (Fig. 4f, 4j), where the meridional curvature was smaller than in other regions. Similar subapical extension was also observed in the cell wall isolated from the growing tip region (Mine and Okuda 2003). Measurement of local thickness and curvature of the isolated cell wall, and of the local wall extension observed in the extension experiments, will provide detailed information on the cell wall's mechanical properties during branch formation.

On the other hand, cell wall extension until rupture significantly increased as the branch formation proceeded and finally reached to about half of the amount measured in the normal tip-growing region under a lower, stable pressure (48.8% cell diameter at 450 kPa; Mine and Okuda 2003). Moreover, the maximum extension rate also increased and, in stage (iv), reached more than 2% of cell diameter s^{-1}, as also observed in the previous study on the cell wall isolated from normal tip-growing region under stable pressure (Mine and Okuda 2003). Thus, cell wall extensibility increased during the branch formation process. This implies that the mechanical properties of the cell wall are related to the rather complicated cell morphogenetic process in which the growing region is newly produced in a previously non-growing region of the cell.

A number of cellular morphogenetic events where a new tip-growing region is generated in previously non-growing cell surfaces are known. For example, in branch formation in fungal hyphae and giant cellular algae, arrangement of cytoskeletons and

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other intracellular structures specifically found in the branch forming regions have been reported (Okuda et al. 1993; Riquelme and Bartnicki-Garcia 2004, Mouriño-Pérez 2006). Root hair formation is another extensively investigated example (Carol and Dolan 2002). In this case, localized cell protrusion occurs on the cell surface along the side of a trichoblast that has been fully extended by diffuse growth in an earlier developmental stage of the cell. Although the molecular mechanisms of root hair initiation are not fully understood, physiological factors such as protein phosphatase and calcium ion (Biblikova and Gilroy 2000), and the arrangement of the cytoskeleton (Baluška et al. 2000) that could be related to the localization and initiation of root hair formation have been investigated. There have also been studies relating root hair initiation and factors affecting the cell wall's mechanical properties. Localized cell wall acidification, which probably promotes wall extensibility, was reported to be necessary for root hair bulging (Bibikova et al. 1998). Recently, an expansin (see below) expressed specifically in roots was identified and shown to be required for root hair initiation (Kwasniewski and Szarejko 2006). In *V. terrestris*, Takahashi et al. (2001) reported protoplasmic aggregation accompanied by changes in the arrangement of cytoskeletons during stage (i) of branch formation. The present study provides the first evidence of changes in cell wall strength and extensibility following cytoskeletal changes in the branch formation.

The pressure at which the cell wall ruptured significantly decreased from stage (i) to stage (ii) and from stage (ii) to stage (iii), but the decrease was not statistically significant between (iii) and (iv). The average pressure required for cell wall rupture at stage (i) (759.6 kPa) is still far greater than the turgor pressure in this alga (560 kPa) calculated from the cell's osmotic pressure (0.23 Osm, personal communication from Dr. Hironao Kataoka). But, the pressure became lower than this turgor pressure at stage (ii)

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and subsequent stages (Table 1). In contrast, the amount of extension and maximum extension rate increased from stage (ii) to stage (iv). This suggests that during early stages of branch formation the cell wall is weakened without remarkable promotion of the extensibility of the cell wall, probably resulting in the decrease in the threshold (yield) stress for initial cell wall extension in the early stages. The present results also evoke the question of why the turgor pressure does not rupture the cell wall in the tip-growing region of the living cells. If the actual turgor pressure measured by more reliable methods is also significantly lower than the rupture pressure of the isolated cell wall, the existence of cell structures that protect the cell wall from rupture by excessive turgor should be considered; these might resemble, for example, the helmet-like, rigid structure postulated to exist in tip-growing hyphae (Money, 1997).

In our previous study on cell walls isolated from tip-growing regions of this alga (Mine and Okuda 2003), the cell wall was weakened by a protease treatment throughout the cell and radial expansion of the enzymatically weakened cell wall in the region near the cylindrical region often occurred. These results suggest that such cell wall weakening also occurs during the early stages of branch formation, enabling cell wall extension for initial branch protrusion by turgor pressure. It is also known that a major part of the cell wall matrix components is removed by protease treatment (Mine and Okuda 2006), suggesting a close relationship between the protease-degradable matrix components and the protease-sensitive strength factor of the cell wall. Therefore, observations of the fine structure of the cell wall at these stages will provide useful information about the occurrence of such structural modification of matrix components of the cell wall.

Both the total amount of extension and the maximum extension rate continuously increased until stage (iv), implying that improvement of cell wall

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extensibility continues even after stage (iii), where the decrease in yield stress has apparently been completed. We have previously reported that the protease treatment used in the present study weakened the cell wall without affecting pH-dependency of the cell wall extensibility (Mine and Okuda 2003). We have also reported that the fine granular matrix components remained among the microfibrils after protease treatment of the cell wall inner surface of the apical portion of the tip-growing region in this alga (Mine and Okuda 2006). Therefore, we conclude that these protease-resistant fine granular matrix components are concerned with the factor controlling the pH-dependent extensibility of the cell wall. The observation in the present study that the fine granular matrix components were found in patches among microfibrils of the inner cell wall surface at stages (ii) and (iii), where the cell wall extensibility was significantly promoted (Figs. 5, 7) supports this conclusion. Although technical problems precluded our ability to make observations at later stages, these matrix components should come to occupy a larger area in the tip of the protrusion in stage (iv) and later, since the protrusion finally forms a new normal growing tip of the cell where the matrix components were present throughout its apical portion (Mine and Okuda 2006; Fig. 6a, b in the present study).

In angiosperms, it has been reported that the extensibility of cell walls is controlled by proteinous cell wall components, such as expansins (Cosgrove 1996), xyloglucan endotransglycosylase (Nishitani 1997; Rose et al. 2002), and specific cellulases (Park et al. 2003). Many of these molecules are believed to control cell wall extensibility by affecting intermolecular binding of cellulose microfibrils with other matrix components, such as xyloglucans. Therefore, it is reasonable to presume that such cell wall components accumulate in spaces between microfibrils in growing cell regions. To clarify the relationship between the matrix components and cell wall extensibility,

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further morphological investigations are in progress by us on the matrix components, including their distribution along cell wall sections and possible secretion processes from the protoplasm into the cell wall, as well as histochemical identification of the components.

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References

Baluška F, Salaj J, Mathur J, Braun M, Jasper F, Samaj J, Chua N-H, Barlow PW, Volkman D (2000) Root hair formation: F-actin-dependent tip growth is initiated by local assembly of profilin-supported F-actin meshworks accumulated within expansin-enriched bulges. Dev Biol 227: 618–632

Bibikova TN, Jacob T, Dahse I, Gilroy S (1998) Localized changes in apoplastic and cytoplasmic pH are associated with hair development in *Arabidopsis thaliana*. Development 125: 2925–2934

Biblikova T, Gilroy S (2000) Calcium in root hair growth. In: Ridge RW, Emons AMC (eds) Root Hairs. Springer Verlag, Tokyo, pp 141-163.

Carol RJ, Dolan L (2002) Building a hair: tip growth in *Arabidopsis thaliana* root hairs. Philos Trans R Soc Lond B Biol Sci 357: 815–821

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Deleted: 24

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J 3: 1–30

Cosgrove DJ (1993) How do plant cell walls extend? Plant Physiol 102: 1–6

Cosgrove DJ (1996) Plant cell enlargement and the action of expansins. BioEssays 18: 533–540

Cosgrove DJ (1997) Relaxation in a high-stress environment: the molecular bases of extensible cell walls and cell enlargement. Plant Cell 9: 1031–1041

Cosgrove DJ (1999) Enzymes and other agents that enhance cell wall extensibility. Annu Rev Plant Physiol Plant Mol Biol 50: 3971–3417

Dumais J, Long SR, Shaw SL (2004) The mechanics of surface expansion anisotropy in *Medicago truncatula* root hairs. Plant Physiol 136: 3266–3275

Haughton PM, Sellen DB (1969) Dynamic mechanical properties of the cell walls of some green algae. J Exp Bot 20: 516–535

Kataoka H (1975) Phototropism in *Vaucheria geminata* II. The mechanism of bending and branching. Plant Cell Physiol 16: 439–448

Kutschera U (1991) Regulation of cell expansion. In: Lloyd CW (ed) The cytoskeletal basis of plant growth and form. Academic Press, London, pp 149–158

Kwasniewski M, Szarejko I (2006) Molecular cloning and characterization of beta-expansin gene related to root hair formation in barley. Plant Physiol 141: 1149-1158

Masuda Y (1990) Auxin-induced cell wall loosening. Bot Mag Tokyo 103: 345-370

Mine I, Okuda K (2003) Extensibility of isolated cell walls in the giant tip-growing cells of the xanthophycean alga *Vaucheria terrestris*. Planta 217: 425–435

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Mine I, Okuda K (2006) Fine structure of cell wall surfaces in the giant-cellular xanthophycean alga *Vaucheria terrestris*. Planta DOI 10.1007/s00425-006-0424-8

Money NP (1997) Wishful thinking of turgor revisited: the mechanics of fungal growth. Fungal Genet Biol 21: 173-187

Money NP, Hill TW (1997) Correlation between endoglucanase secretion and cell wall strength in oomycete hyphae: implications for growth and morphogenesis. Mycologia 89: 777–785

Mouriño-Pérez RR, Roberson RW, Bartnicki-Garcia S (2006) Microtubule dynamics and organization during hyphal growth and branching in *Neurospora crassa*. Fungal Genet Biol 43: 389–400

Nishitani K (1997) The role of endoxyloglucan transferase in the organization of plant cell walls. Int Rev Cytol 173: 157–206

Okuda K, Matsuo K, Mizuta S (1993) The meridional arrangement of cortical microtubules defines the site of tip growth in the coenocytic green alga, *Chamaedoris orientalis*. Bot Mar 36: 53–62

Park YW, Tominaga R, Sugiyama J, Furuta Y, Tanimoto E, Samejima M, Sakai F, Hayashi T. (2003) Enhancement of growth by expressin of poplar cellulase in *Arabidopsis thaliana*. Plant J 33: 1099–1106

Parker BC, Preston RD, Fogg GE (1963) Studies of the structure and chemical composition of the cell walls of Vaucheriaceae and Saprolegniaceae. Proc Roy Soc B 158: 435-444

Riquelme M, Bartnicki-Garcia S (2004) Key differences between lateral and apical branching in hyphae of *Neurospora crassa*. Fungal Genet Biol 41: 842–851

Rose JKC, Braam J, Fry SC, Nishitani K (2002) The XTH family of enzymes involved in

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xyloglucan endotransglucosylation and endohydorolysis: Current perspectives and a new unifying nomenclature. Plant Cell Physiol 43: 1421–1435

Takahashi F, Hishinuma T, Kataoka H (2001) Blue light-induced branching in *Vaucheria*. requirement of nuclear accumulation in the irradiated region. Plant Cell Physiol 42: 274–285

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Figure legends

Fig. 1.

Schematic drawing of illumination vessel used for induction of branch formation. The rectangular container made of a plastic base plate (B) and a frame (F) was filled with medium. *Vaucheria* cells (V) were laid parallel between two streaks of silicon grease (G) drawn on a glass plate placed on the bottom of the container and covered with a small cover slip (S). After covering with a transparent lid (T), light from the microscope lamp (L) that passed through a narrow slit made of two razor blades (R) irradiated the cells through the microscope's condenser (C). The opening of the slit and the position of the condenser were adjusted to illuminate a 200 µm-wide region in the plane of the cell. During branch formation, cells were observed through an objective lens (O)

Fig. 2.

Fig. 2a-d Schematic drawing of outlines of irradiated cell regions in the four stages of branch formation in *Vaucheria terrestris* recognized in the present study. **a** Stage (i). Protoplasm has accumulated in the irradiated region but there is no change in the shape of the cell surface. **b** Stage (ii). A new growing tip is formed, with slight bulging of the cell surface. **c** Stage (iii). A dome-like protrusion of the cell surface resulted from the localized expansion at the new growing tip. **d** Stage (iv). The protrusion grew further, becoming a new branch with a cylindrical basal region

Fig. 3

Fig. 3a, b Schematic drawing of how cell wall fragments were turned inside-out. Outside

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of cell wall is shaded and inside is clear. **a** A glass needle with a larger tip diameter (NL) was inserted into the cell wall fragment (CW) through the initial cut end at the boarder of the irradiated region. The other end of cell wall was folded to close this end temporarily and immobilized by lightly pressing the tip of a second glass needle with a thinner tip (NS) from the opposite direction. **b** Unfolded portion of the cell wall fragment was inverted over the thinner-tipped needle by tucking up to the opposite side using a pig hair bristle from a paint brush. This resulted in the cell wall portion that included the irradiated region being turned completely inside-out, allowing direct access to the inner surface of the cell wall

Fig. 4

Fig. 4a-i Series of images of cell wall fragments isolated from cells at various stages of branch formation. The fragments were tensed by increasing internal pressure applied to the silicone oil filling the cell wall. **a-c** Cell wall fragment at stage (ii). **a** At 100 kPa. **b** 508 kPa. The cell wall perimeter had been extended by 2.7% of the initial cell diameter. **c** Cell wall rupture 1 s after Fig. 4b. The protrusion of the cell wall surface was slightly enlarged before rupture. **d-f** Cell wall fragment at stage (iii). **d** At 100 kPa. **e** 476 kPa. Extension was 9.0%. **f** Cell wall rupture 1 s after Fig. 4e. Cell wall was markedly extended before rupture. **g-i** Cell wall fragment at stage (iv). **g** At 100 kPa. **h** 476 kPa. Extension was 22.0%. **i** Cell wall rupture 1 s after Fig. 4h. Cell wall was extended more than in Fig. 4d, e. Scale bar (40 μm) in Fig. 4a also applies to Fig. 4b-i

Fig. 5.

Fig. 5a, b Schematic drawing of the measurement of cell wall extension on still images of

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time-lapse video of an inflated cell wall specimen. **a** A cell wall specimen lightly inflated at low initial pressure (100 kPa). The cell diameter (D) was measured at this time. Two objects (R1 and R2) (protoplasmic or other debris) along the cell wall perimeter on both sides of the branch protrusion were selected as the starting and the ending points for measurement. The length of the cell wall outline between R1 and R2 was measured as the initial cell perimeter (P0). Pressure was then increased at a rate of 4 kPa s^{-1}. **b** The same cell wall after swelling of the cell wall, especially at the branch protrusion. The cell perimeter at this time (Pt), measured as in **a,** was significantly longer than P0. The difference between P0 and Pt, divided by D, was regarded as the cell wall extension (% cell diameter) at time t

Fig. 6.

Fig. 6a-d Topographic images obtained by atomic force microscopy of inner surface of cell wall isolated from tip-growing region of the cell. Matrix components had been partially removed by protease treatment. **a, b** Apical region. **a** 5 x 5 µm area. **b** a 1 x 1 µm area of Fig. 6a. Note fine granular matrix components present among cellulose microfibrils throughout scanned area. **c, d** Basal, cylindrical region. **c** 5 x 5 µm area. **d** a 1 x 1 µm area of Fig. 6c. Note matrix components cannot be detected among cellulose microfibrils. Image heights of Fig. 6a-d are 250 nm, 45 nm, 122 nm and 103 nm, respectively

Fig. 7.

Fig. 7a-d Topographic images of inner surface of cell wall isolated from cell at stage (i) or (ii) of branch formation process. Protease treated. **a, b** 5 x 5 µm areas of cell wall

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surface isolated from two individual cells at stage (i). Note matrix components are not seen among cellulose microfibrils. **c, d** Stage (ii). **c** 5 x 5 µm area. Square area in brighter patch represents scanned area for Fig. 7d. **d** a 1 x 1 µm area of Fig. 7c. Note in stage (ii) fine granular matrix components present in the brighter patch; the patch is higher than other area where no matrix components are detected. Image heights of Fig. 7a-d are 262 nm, 303 nm, 342 nm, and 131 nm, respectively

Fig. 8.

Fig. 8a-d Topographic images of inner surface of cell wall isolated from cell at stage (iii) of branch formation process. Protease treated. **a** (inset) Photograph of the cell wall sample taken by a built-in light microscope. *Arrows* point to the positions of the probe tip at which scanning was carried out to obtain topographic images. *Arrows 1-3* indicate the scanned positions for Fig. 8a, 8c and 8d, respectively. Unnumbered arrow points to the position of a 5 x 5 µm topographic image (not shown) that did not show any matrix components. Scale bar = 50 µm. **a** 5 x 5 µm area. Square area in brighter patch represents scanned area for Fig. 8b. **b** a 1 x 1 µm area of Fig. 8a. Note fine granular matrix components present in the brighter patch. **c, d** Other areas of the same cell wall fragment as Fig. 8a. Note the bright patches of different sizes without fine granular matrix components. Image heights of Fig. 8a-d are 207 nm, 68 nm, 183 nm, and 142 nm, respectively

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Tables

Table 1

Changes in the extensibility of the cell walls isolated from *V. terrestris* cells during branch formation obtained from five independent extension experiments for each stage.

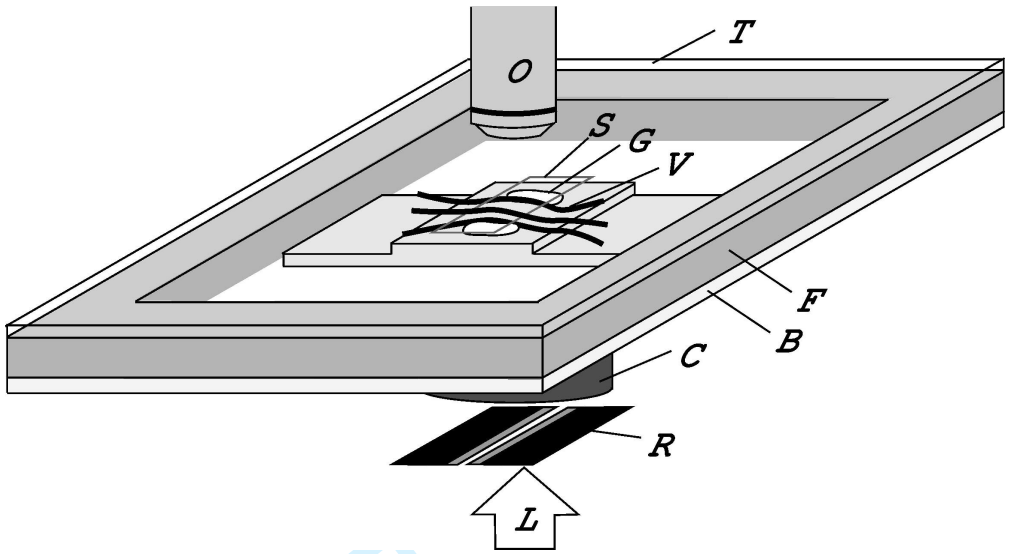
Stage	Pressure[a] (kPa)	Total extension[b] (% cell diameter)	Maximum extension rate[c] (% cell diameter sec^{-1})
(i)	759.6 ± 34.0	-	-
(ii)	532.4 ± 28.5 *	4.2 ± 2.9	0.97 ± 0.49
(iii)	460.8 ± 26.3 *	15.5 ± 8.0 *	1.75 ± 0.56 *
(iv)	455.6 ± 23.0	25.4 ± 3.9	2.62 ± 0.49

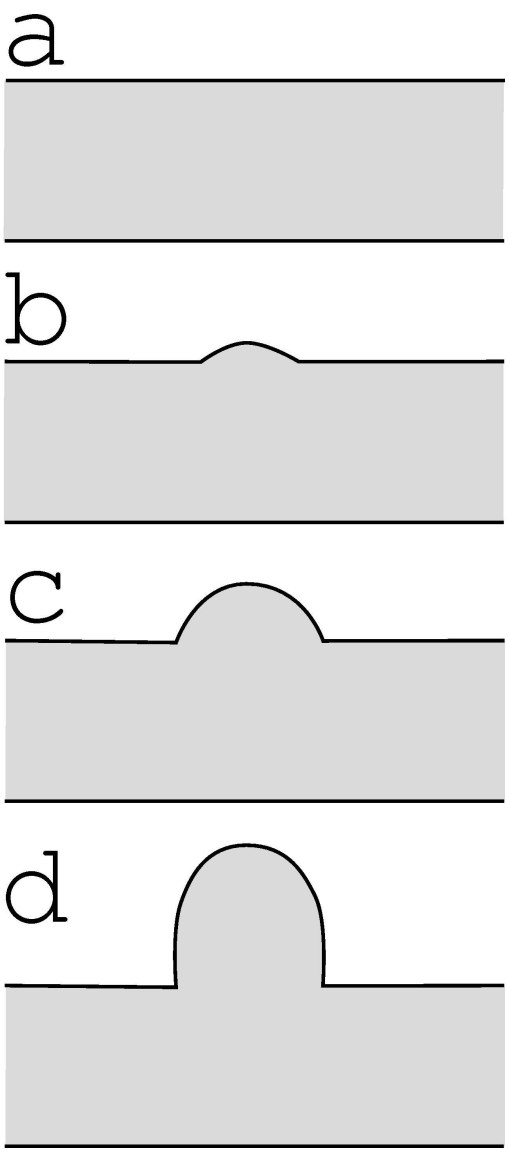
* Statistically significant difference between averages by Student's *t*-test ($P < 0.01$)

[a] Pressure at which cell wall ruptured.

[b] Total extension of cell wall perimeter until rupture.

[c] The maximum extension rate of cell wall perimeter until rupture.

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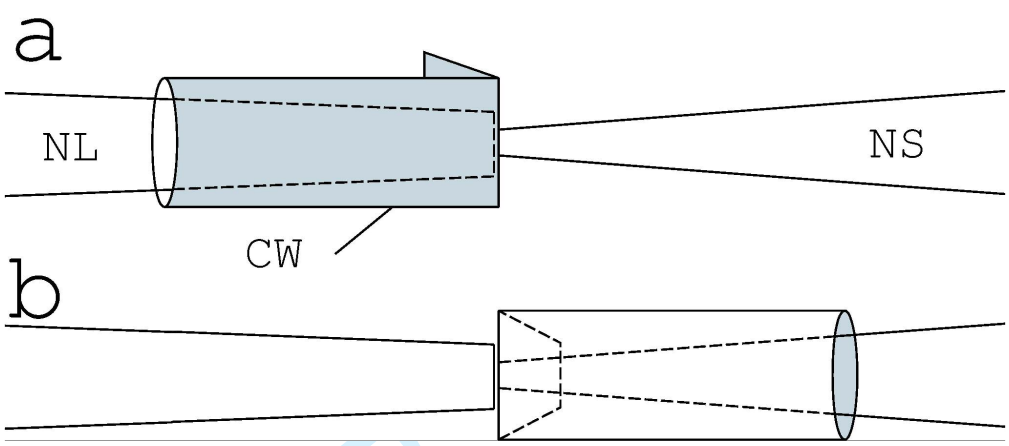

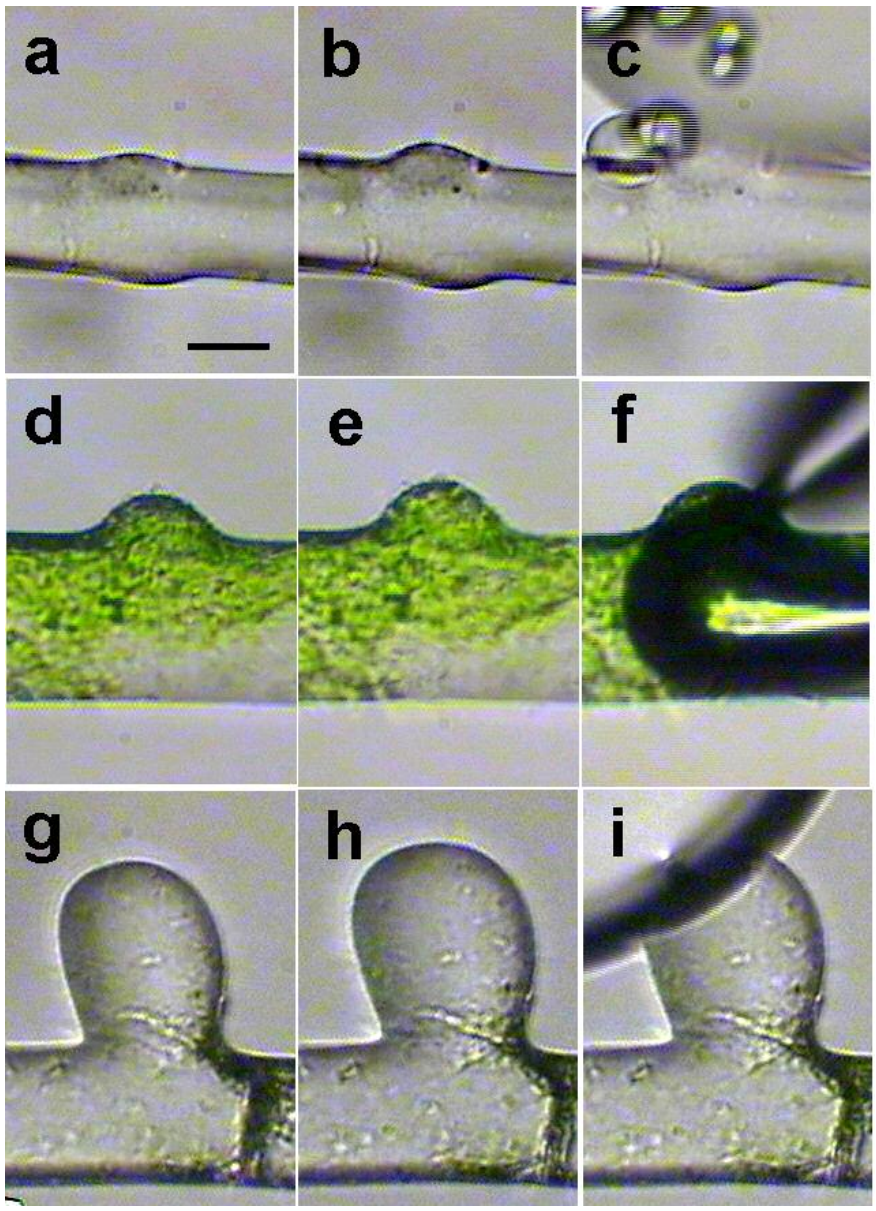
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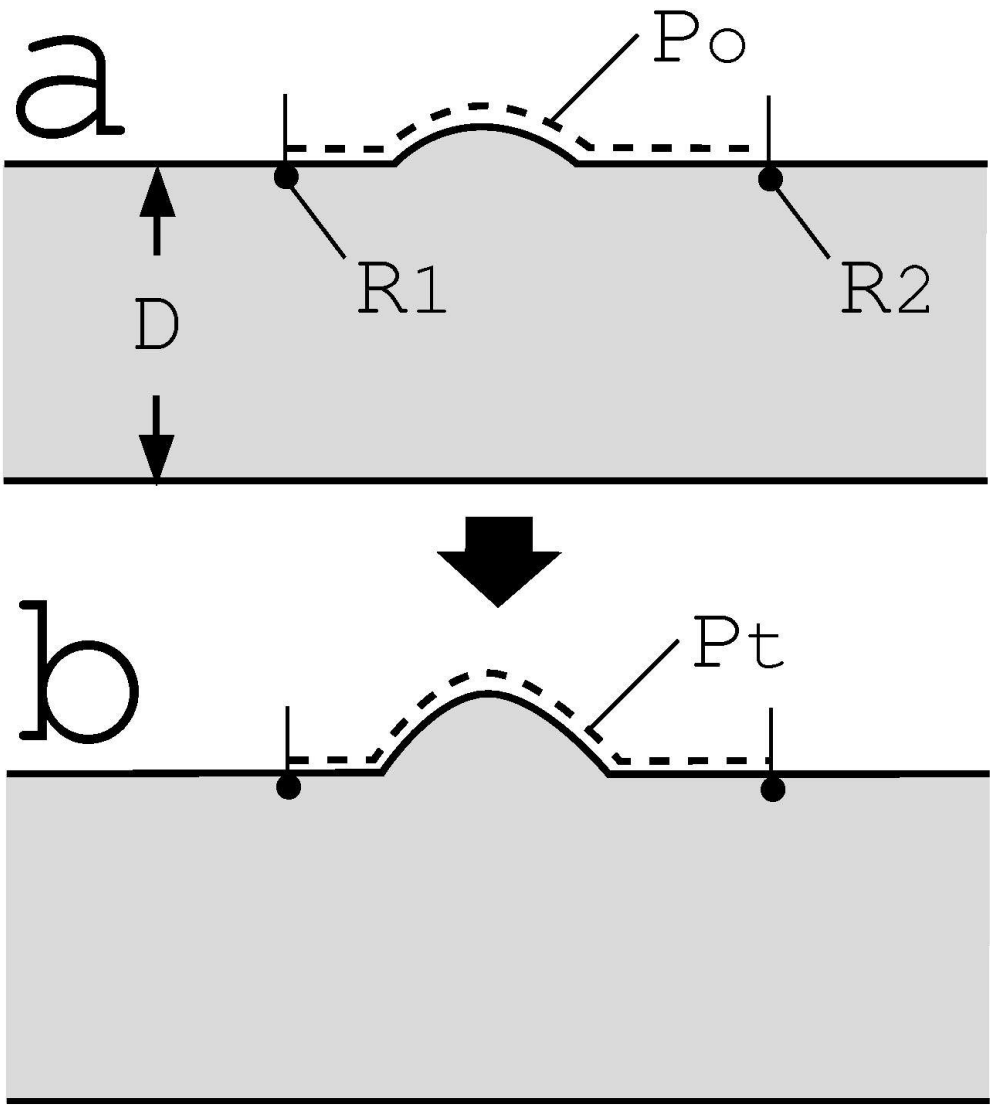
a
Po
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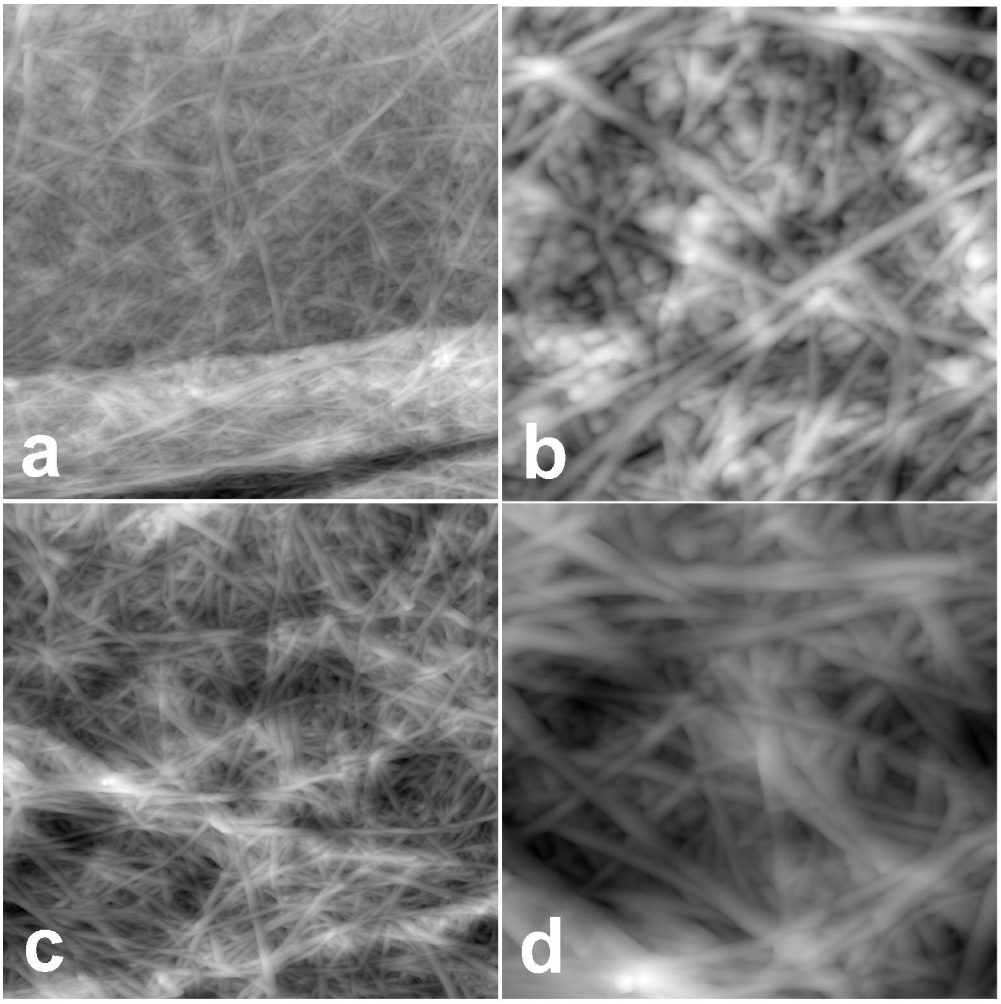
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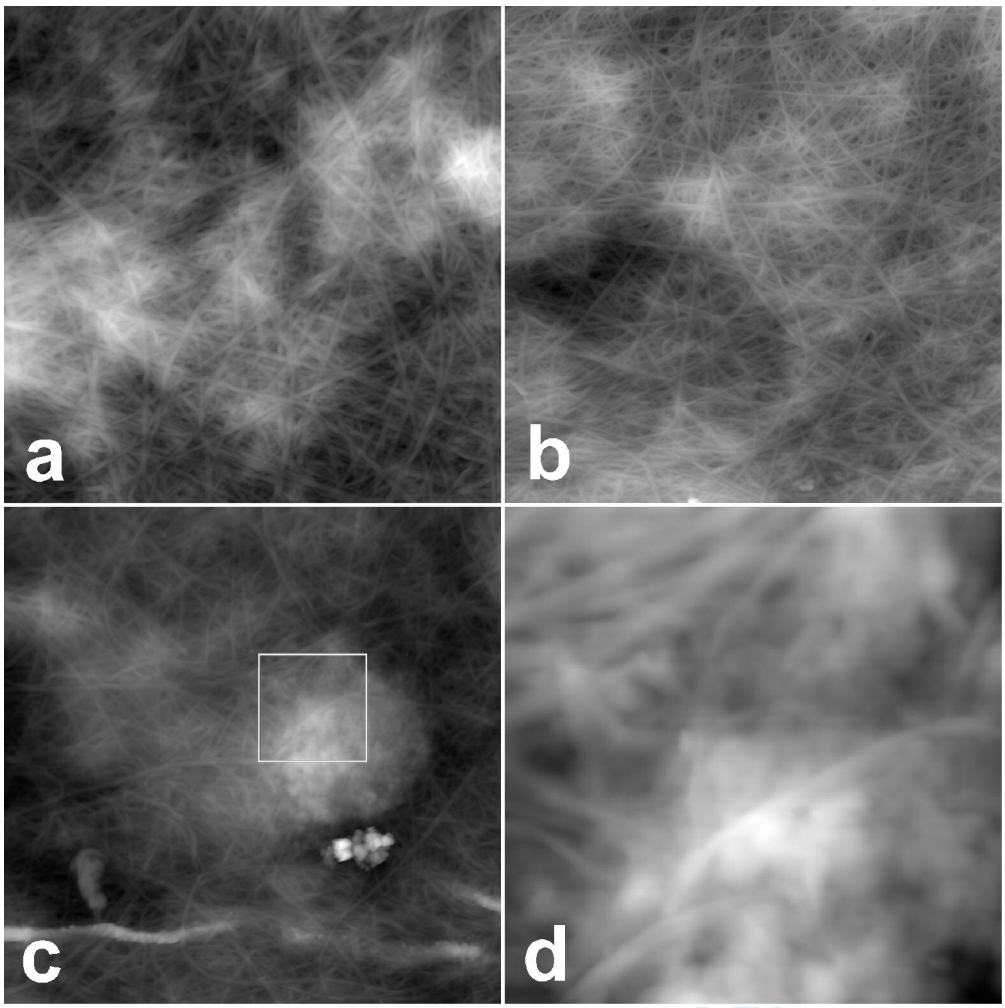
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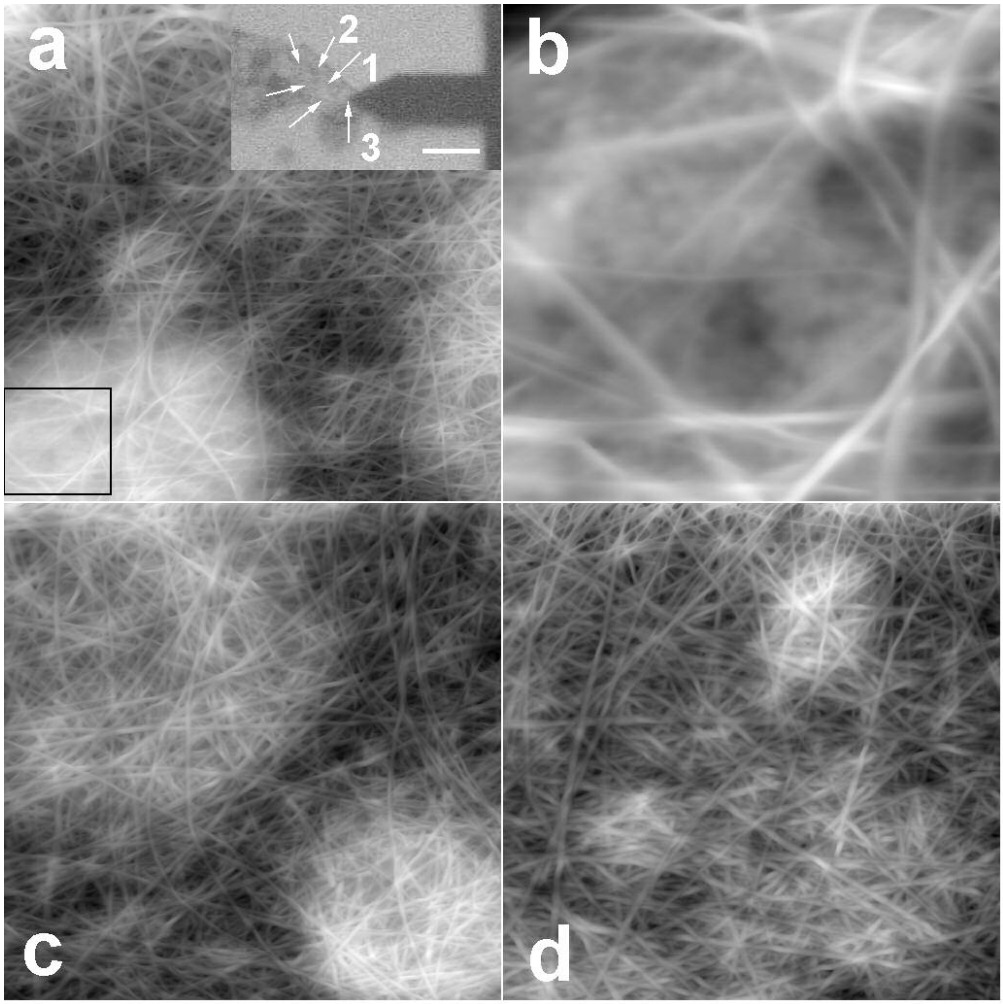
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