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Development of a Method to Measure Mechanical Properties of Single Elongated Callose Fibers in Protoplast Cultures of *Larix Leptolepis* and *Betula Platyphylla*

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Abstract

We developed a method to measure mechanical properties of single fibers of callose in liquid protoplast cultures of *Larix leptolepis* and *Betula platyphylla*, which were formed in media containing 50 mM of $MgCl_2$ or 100 mM of $CaCl_2$, respectively. Tensile test was performed using two micromanipulators loading micropipettes under an inverted microscope. Spring constant of the pipette used was first calibrated and calculated from using a microbalance. The callose fiber was wired between the two micropipettes. The Young's modulus of single fibers for *Larix* and *Betula* was 7-9 kPa (1.4-1.9 x 10^4 N/m^2) though the diameters of the fiber varied from 10 μm for *Larix* and 22-26 μm for *Betula*. No big difference was found between experiments with and without medium containing high concentrations of salts. Tensile strength at break was 1.1-1.8 kPa (2.3-3.6 x 10^3 N/m^2). The values are compared to other materials including cellulose containing plant cell wall, cell membranes, and amorphous callose. The value of the Young's modulus observed was discussed.

Keywords: callose, fiber, mechanical property, protoplast cultures, Young's modulus

1. Introduction

We reported novel long and large spiral callose fibers developed in plant protoplast cultures of a fast-growing coniferous tree, *Larix leptolepis* and a fast-growing broadleaved tree, *Betula platyphylla* by treatment with high concentrations of $MgCl_2$ or $CaCl_2$ in the liquid media (Sasamoto et al., 2003). Spiral structures with diameters of 10 to 30 μm and up to 2 mm in length were observed under an inverted microscope. The fibers were proven to be composed of callose by using specific degrading enzymes for beta-1,3-glucan and beta-1,4-glucan. And sub-structures of a *Larix* fiber, bundles of fibrils (0.7 μm diameter) and sub-fibrils (0.17 μm diameter), were also clarified by atomic force microscopy (Fukumoto et al., 2005). Callose is a natural component of plant cell wall and it is abundant in pollen tubes (Cresti & van Went, 1976). In some situation, its formation can be regarded as a stress response (Chen & Kim, 2009). Our reported fiber structure of callose is unique. It elongates from a single point on the surface of a protoplast, and it is easily separated from a protoplast (Sasamoto et al., 2003; Fukumoto et al., 2005).

Measurement of mechanical properties of unique callose fiber is an interesting topic, which might contribute to the development of an alkali-labile nano-machine at a cellular level (Oyanagi et al., 2014). Mechanical properties of single tomato suspension cell (Blewett et al., 2000) and large sized (several centimeters) discs of cell wall components including cellulose, were measured by compression test (Chanliaud et al., 2002). Parre and Geitmann (2005) found that the amorphous polymer, callose, is related to the tension stress in pollen tubes, in which cell wall is composed of not only callose but also pectin and cellulose. The mechanical properties of plasma membrane of plant protoplasts were measured by aspiration with micropipettes (Wolf & Steponkus, 1983). By improvement of the holding of single cells, Onishi et al. (2006) reported measurement of Young's modulus of an animal cell adhered on glass plate by micropipette aspiration using micromanipulators. Simple tensile testing of a whole protoplast fiber is important to our understanding of the fundamental mechanical

properties of the callose fibers. However, since the size of protoplast fibers is of the order of μm diameter and mm in length and they also float in a liquid medium, the reported methods in the literature could not be applied directly. In this report, we developed a novel method to measure mechanical properties of single fibers of callose using micropipettes mounted on micromanipulators under an inverted microscope.

2. Method

2.1 Culture of Protoplasts

Fiber formation from protoplast cultures of *Larix leptolepis* and *Betula platyphylla* was performed as described previously (Sasamoto et al., 2003). Briefly, *Larix* protoplasts were isolated by 1% Cellulase RS (Yakult) and 0.25% Pectolyase Y-23 (Seishin) in 0.4 M mannitol solution from embryogenic cells, which were sub-cultured in mCD medium containing 7 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 3 μM of benzyladenine (BA), and 3% sucrose. Protoplasts were cultured in NH_4NO_3-free Murashige and Skoog's (MS, Murashige and Skoog 1962) basal medium containing 6% sucrose, 10 μM each of 2,4-D and BA and 50 mM $MgCl_2$ in a 96-well (No.3075, Falcon) or a 24-well (No. 3047, Falcon) culture plate. *Betula* protoplasts were isolated from leaves of shoot culture, by 1% each of Cellulase R-10 (Yakult) and Driselase 20 (Kyowa Hakko Kogyo) in 0.6 M mannitol solution for 20 hrs. The leaves were floated on enzyme solution without cutting. Protoplasts were cultured in 1/2 strength MS basal medium containing 3% sucrose, 1 μM each of naphthaleneacetic acid and *N*-(2-chloro-4-pyridyl)-*N'*-phenylurea (CPPU, Sigma) and 100 mM $CaCl_2$. Fibers were used after 1-2 week of culture for *Larix* or 2 month of culture for *Betula*. They were incubated in a humid incubator (CO_2-incubator without the supply of CO_2-gas, APC-30D, CL-30, ASTEC Co. Ltd.) at 28°C.

2.2 Fabrication of Micropipettes

Micropipettes were prepared by a pipette puller (PB-7, Narishige) from 10 μL calibrated pipettes (Drummond). Picking up-pipettes were made as described previously (Sasamoto et al., 2000). Briefly, after one-step heating procedure (dial 600), the tips were removed by using a sandpaper (fine, No. 2000) to make inner diameter of 50 to 200 μm and washed with water. For tensile testing, tip of 10 μm diameter and 7 mm long was made by a two-step heating procedure (dial No. 1: 70, No. 2: 600). And their tips were cut into different lengths by using a microforge (MF-900, Narishige), or were smoothened by burning. Shape of micropipettes was modified by a micro-burner (Prince) as previously described (Ogita et al., 1999).

2.3 Calibration of Micropipettes

Calibration of micropipettes was performed before tensile testing of fibers for measuring the load of the order of μN (Onishi et al., 2006). The wide end of a micropipette was connected to a microstage for fine motion in the vertical direction. The micropipette was moved downwards and pressed against a microbalance (AB104, Mettler Toledo). The pressing load caused the deflection of the tip of the micropipette like a cantilever beam. The load and deflection of the tip were measured by the microbalance and a microscope, respectively.

The spring constant of the micropipette was obtained from the slope of the load versus deflection plot. In the linear range, its spring constant k_p is given by Equation (1), where δ_p is the deflection of a micropipette, F_p is the load applied to the micropipette.

$$k_p = \frac{F_p}{\delta_p} \tag{1}$$

After calibrating micropipette *A* with a tip length $l_{p,A}$, micropipette *B*, with a different tip length $l_{p,B}$ was calibrated using the relationship between the spring constant and the tip length of micropipettes as cantilever beams (Timoshenko, 1955) by Equation (2) where $k_{p,A}$ and $k_{p,B}$ are the spring constants of micropipettes *A* and *B*, respectively.

$$k_{p,B} = k_{p,A} \frac{{l_{p,A}}^3}{{l_{p,B}}^3} \tag{2}$$

2.4 Tensile Testing of Fibers

Protoplast fibers of about 10 μm diameter for *Larix* and of about 30 μm diameter for *Betula* were selected under an inverted microscope. They were picked up and transferred to a medium or pure water in a well of a 4-well plastic dish (Nunc) or a 60 mm center-well organ culture dish (BD Falcon) by using a micromanipulator as previously described (Oyanagi et al., 2014; Sasamoto et al., 2003). The medium for *Larix* contained 50 mM

$MgCl_2$, while that for *Betula* contained 100 mM $CaCl_2$ or pure water to study the effect of a medium containing high concentrations of Ca^{2+} ions. The dish was set on the microscope stage of the micro-tensile testing system which we have designed (Figure 1a). A single fiber in the dish was held with the fine ends of two micropipettes prepared as in sections *2.1* and *2.2*. The wider ends of two micropipettes were connected to two manual-type micromanipulators (MM188, Narishige-Nikon; MO202, Narishige) and microinjectors (IM-188, Narishige) set to an inverted microscope (IX-71, Olympus). One deflectable micropipette was slowly moved in the horizontal direction to stretch the fiber. The fiber stretching process was observed and recorded with a CMOS camera combined with a video recorder (ivis HF M52, Canon), which was connected to a microscope with an attachment (NY-VS (811276), Microscope Network).

Two methods to hold a single fiber by two micropipettes have been newly developed. In one method for *Larix* fibers, a single fiber was wired between two micropipettes to make a loop of the fiber and stretched (Figure 1b). In the other method for *Betula* fibers, both ends of a single fiber was pushed down onto the bottom of the dish by one rigid micropipette of which the tip was smoothened by burning and was wider than the other deflectable micropipette to make a loop of the fiber (Figure 1c). The fiber loop was hooked on the deflectable micropipette and stretched.

2.5 Evaluation of Mechanical Properties of Fibers

The load of a single fiber F_f is half the load of a micropipette F_p because a loop of the fiber was made between micropipettes during tensile testing (Figure 1b, c). The fiber load F_f was obtained using Equation (1) with the deflection δ_p and spring constant k_p of the calibrated micropipette as in the Equation (3).

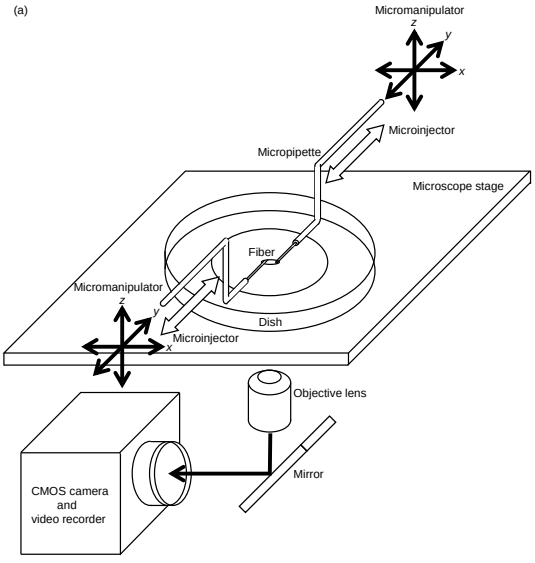
$$F_f = \frac{1}{2}F_p = \frac{1}{2}k_p\delta_p \tag{3}$$

Young's modulus of the fiber E_f was obtained by dividing the stress by the strain as in the Equation (4),

$$E_f = \frac{\dfrac{F_f}{A_f}}{\dfrac{\Delta l_f}{l_f}} \tag{4}$$

where A_f is the cross-sectional area of the fiber obtained from its diameter d_f by assuming the cross section as circular, l_f and Δl_f are the initial fiber lengths and its elongation between the points holding the fiber by two micropipettes, respectively. δ_p, d_f, l_f, and Δl_f were measured under the microscope of the tensile test system. When F_f was maximum, the tensile strength of the fiber σ_B was given by Equation (5).

$$\sigma_B = \frac{F_f}{A_f} \tag{5}$$



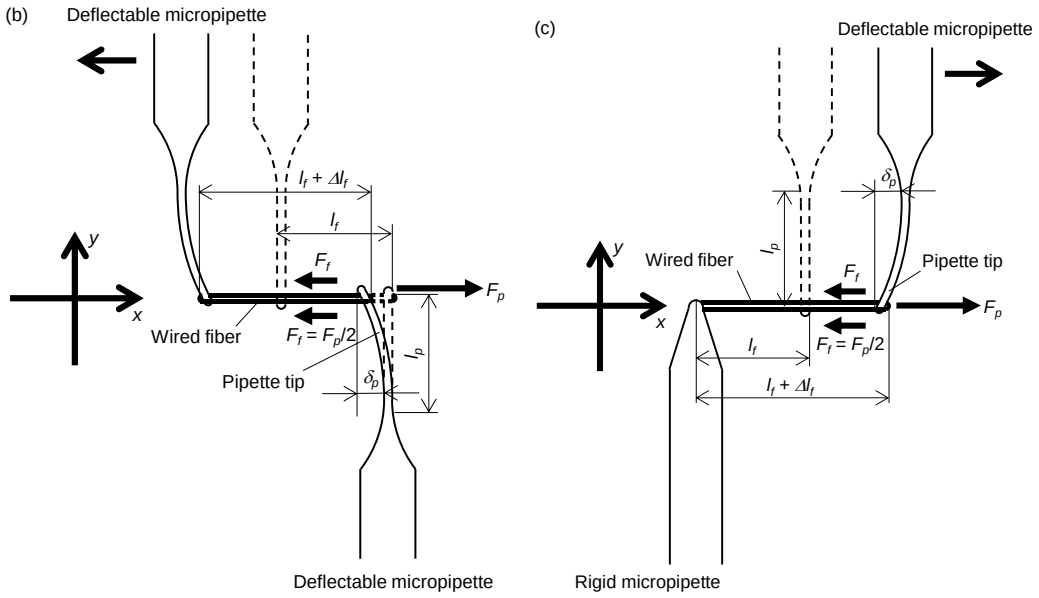


Figure 1. Schematic diagram of the micro tensile test system (a) and the detail of its test section for a *Larix* fiber (b) and a *Betula* fiber (c)

3. Results

3.1 Calibration of Micropipettes

The calibration of a micropipette (tip length 3.97 mm) was performed. The relationship between the load and deflection was almost linear (Figure 2). The spring constant of the micropipette was calculated as 7.28 mN/m from Equation (1). The spring constant of another micropipette (tip length 3.45 mm) was determined as 11.1 mN/m from Equation (2). During tensile testing, the latter micropipette was used for measuring the load of fibers.

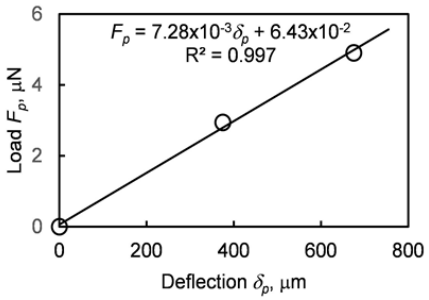


Figure 2. Load versus deflection of a micropipette for calibration

3.2 Tensile Testing of Fibers

3.2.1 *Larix Fiber*

An example of the time-lapse micrographs of the deformation of a *Larix* fiber in a medium containing 50 mM $MgCl_2$ during tensile testing is shown in Figure 3. When the fiber was firstly twisted, it was successfully wired between two deflectable micropipettes to make a loop of the fiber (Figure 3a). When the left micropipette was slowly moved to the left by a micromanipulator, the fiber was untwisted and made straight without the deflection of the right micropipette, the fiber length was 230 μm and defined as the initial fiber length l_f (Figure 3b). As the left micropipette continued to move, the fiber was stretched (Figure 3c) and finally fractured (Figure 3d).

Just before fiber fracture, the deflection of the fixed right micropipette reached a maximum and its deflection δ_p was measured as 21.6 μm using the captured image of the micropipette deformed from its initial shape (Figure 4, dotted line). The maximum load applied to the *Larix* fiber F_f was determined as 0.120 μN using Equation (3) and at that time, the maximum fiber elongation Δl_f was measured as 38.5 μm. Since the diameter of the fiber d_f was 10.2 μm from the captured image, the cross-sectional area A_f was 81.7 μm^2. Using Equation (4), Young's modulus of the *Larix* fiber E_f was determined as 8.75 kPa (1.75 x 10^4 N/m^2). Using Equation (5), the tensile

strength of the fiber σ_B was obtained as 1.47 kPa at the strain $\Delta l_f / l_f$ of 0.170 at the breaking point.

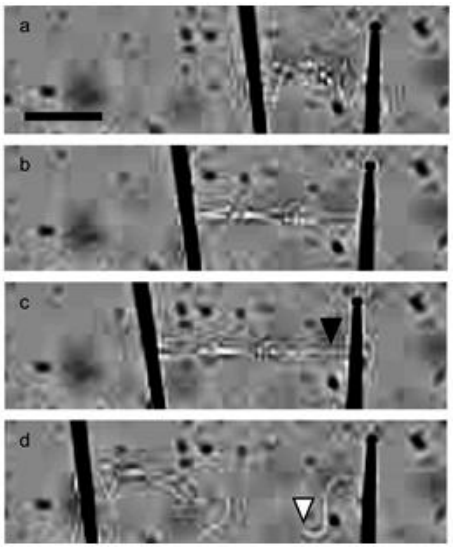


Figure 3. Time-lapse micrographs of deformation of a *Larix* fiber during tensile testing. The twisted fiber was wired between micropipettes in initial state to make a loop of the fiber (a). While the left micropipette was moved to left, the fiber was made straight (b) and elongated (c). The fiber fracture finally occurred (d). Black and white arrowheads indicate the fiber in elongated and fractured state, respectively. Scale bar, 100 μm

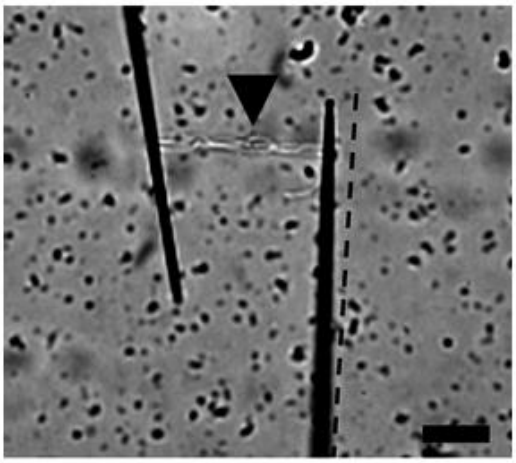


Figure 4. Micrograph of deflection of micropipettes in tensile testing for a *Larix* fiber. The arrowhead indicates the fiber wired between two micropipettes. While the left micropipette was slowly moved to left, the fixed right micropipette was deflected from its initial state (dotted line) and the fiber was elongated. The shape of the right micropipette shows the maximum deflection just before the fiber failure. Scale bar, 100 μm

3.2 Betula Fibers

In the tensile testing of a *Betula* fiber in a medium containing 100 mM $CaCl_2$, it was also successfully held with rigid and deflectable micropipettes (Figure 5). In the same manner as the *Larix* fiber, the diameter d_f, Young's modulus E_f, tensile strength σ_B, and strain at the breaking point $\Delta l_f / l_f$ of the *Betula* fiber were obtained as 25.5 μm, 9.30 kPa (1.86 x 10^4 N/m^2), 1.13 kPa, and 0.122 using the initial fiber length l_f of 1123 μm, the maximum micropipette deflection δ_p of 104 μm, the maximum fiber load F_f of 0.579 μN, and the maximum fiber elongation Δl_f of 137 μm, respectively.

In the tensile testing of a *Betula* fiber in pure water, the diameter d_f, Young's modulus E_f, tensile strength σ_B, and strain at the breaking point $\Delta l_f / l_f$ of the *Betula* fiber were obtained as 21.5 μm, 6.95 kPa (1.39 x 10^4 N/m^2), 1.78 kPa, and 0.256 using the initial fiber length l_f of 404 μm, the maximum micropipette deflection δ_p of 116 μm, the maximum fiber load F_f of 0.643 μN, and the maximum fiber elongation Δl_f of 103 μm, respectively.

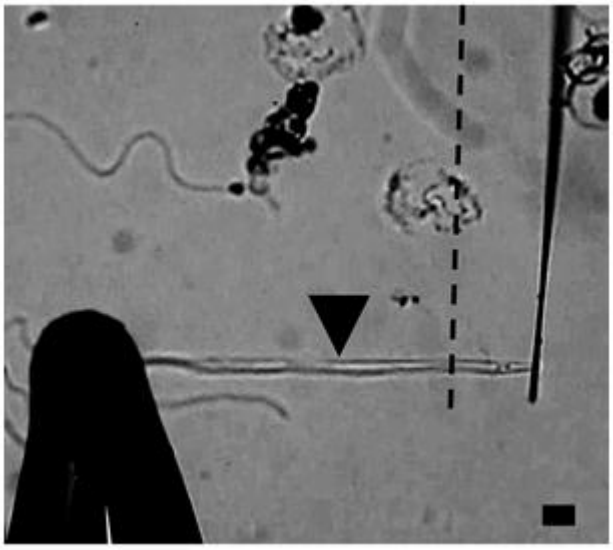


Figure 5. Micrograph of deflection of micropipettes in tensile testing for a *Betula* fiber. The arrowhead indicates the fiber wired between two micropipettes. While the right micropipette was slowly moved from the initial position (dotted line) to right and the rigid left one was fixed, the fiber was elongated and the right micropipette was deflected from its straight position. The shape of the right micropipette shows the maximum deflection just before the fiber failure. Scale bar, 100 μm

The diameter d_f, Young's modulus E_f, tensile strength σ_B, and strain at the breaking point $\Delta l_f / l_f$ in each tensile testing are summarized in Table 1.

Table 1. Mechanical properties of single fibers of callose developed in plant protoplast cultures.

Fiber type	Diameter d_f, μm	Young's modulus E_f, kPa	Tensile strength σ_B, kPa	Strain at the breaking point $\Delta l_f / l_f$
Larix in medium	10.2	8.75	1.47	0.17
Betula in medium	25.5	9.3	1.13	0.122
Betula in pure water	21.5	6.95	1.78	0.256

4. Discussion

When mechanical properties of single suspension-cultured tomato cells were investigated by micromanipulation probe on glass surface, the Young's modulus was 2.3 GPa in an uni-axial compression testing (Blewett et al., 2000; Wang et al., 2004). Chanliaud et al. (2002) reported Young's modulus (0.2-0.5 GPa) of pure cellulose by compression testing using cm-sized sheet materials with ~1 mm thickness and reduction of values by incorporation of pectin and xyloglucan, which are the components of plant cell wall. They also reviewed the Young's modulus (0.1-4 GPa) of plant cell walls and cells of different species. Cellulose is a natural fiber-forming glucan with beta-1,4-linkage composed of microfibril, which size is much smaller (1/ 1000) than the callose sub-fibrils (Fukumoto et al., 2005). Compression testing for sheet type materials of plant cell wall composites might result in an overestimation of the Young's modulus. Therefore, it is difficult to apply such a method on small protoplast fiber composed of callose. A value of 1.25 MPa was obtained in an uni-axial tensile testing, in which the size of material was 30x3x1 mm (Whitney et al., 1999). A low value, 0.25 MPa was obtained when xyloglucan was added to cellulose. Parre and Geitmann (2005) found that amorphous polymer, callose, is related to the tension stress in pollen tubes, which are composed of callose, pectin and cellulose. The modulus value, 4-5 μN / μm was reported using microindentation test. This value corresponds to 3.4 MPa. Mechanical properties of the plasma membrane of plant protoplast (Wolf & Steponkus, 1983) and animal cells (Onishi et al., 2006) were measured by aspiration using micromanipulator. The former value was 230 mN /m and the latter was 100 μN / m. These values correspond to 0.3 kPa and 4.0 kPa, respectively. As the protoplast fibers are thin and elongated, aspiration method could not be applied. It is needed to measure the mechanical property of whole fiber, but not of localized portion.

In this report, unique tensile test was developed using micromanipulators-loading micropipettes. New methods

for wiring of a *Larix* fiber between two micropipette and the pushing down of *Betula* fibers for testing were successful. Improvements of holding methods of fibers was a prerequisite for the measurement of mechanical property of unique callose fiber. Before the development of the method of wiring of a fiber between two micropipettes by friction force between a fiber and glass surface, several glues were tried in vain to attach fibers to the tip of micropipettes (data not shown). The Young's modulus for the callose fiber was of the order of 10^4 [N/m^2]. This is the first report that the mechanical properties of a single callose fiber were determined. The value is two-fold larger than that of animal cells, and much smaller than those of plant cell, protoplast membrane and cell wall components. Tensile strain at break ($\Delta l_f / l_f$, 0.12-0.26) was similar in value as cellulose (Chanliaud, 2002).

Though, high concentration of Ca^{2+} (100-200 mM) in the protoplast culture medium is a prerequisite for *Betula* fiber formation, no big difference was obtained for Young's modulus from the tensile test in the culture medium and in pure water. Under tensile testing, high concentrations of Ca^{2+} ions are not necessary at the surface of the fiber structures. Furthermore, the value of Young's modulus of *Larix* fibers was similar to those of *Betula* fibers, which was described as area base. The differences between *Larix* and *Betula* are that large *Betula* fibers (30 μm diameter) take a long period (2 months), while *Larix* fibers (10-20 μm diameter), take 1-2 weeks to form (Sasamoto et al., 2003; Fukumoto et al., 2005). As similar Young's modulus values were obtained for both *Larix* and *Betula* fibers, similar substructures of callose fibers can be considered.

Recent reports indicate that protoplast cultures of salt-tolerant mangrove plants can serve as excellent callose fiber materials as fiber formation (4 μm diameter) takes only a few days of culture and without the need of additional divalent cations (Kurita et al., 2008, 2009; Oyanagi et al., 2012). In our laboratory, similar spiral fiber structures were also observed in a protoplast culture of *Arabidopsis* recently (Sasamoto et al., unpublished data) which might offer information on molecular biology in callose fiber formation. The method developed in this paper for measuring mechanical properties of callose fibers could be applied to different protoplast fibers. The callose fibers, which are developed in liquid protoplast cultures of several plant species, are easy to separate from the original protoplasts, when they were transferred to pure water or in medium containing fixation chemical, *e. g.* glutaraldehyde (Oyanagi et al., 2014). There is no deposition of callose on membrane of fiber-forming protoplast, as aniline blue dye stains only the fiber itself but not the surface of protoplast (Fukumoto et al., 2005). Difference in mechanical properties between callose fibers and the protoplast surface might be considered.

Callose is soluble in alkaline conditions which is a unique characteristic among cell wall polymer components. Clarifying the mechanical properties of callose fiber might help for possible use as spiral microfilament material in micro-world for specific medicinal purpose (Oyanagi et al., 2014).

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References

Blewett, J., Burrows, K., & Thomas, C. (2000). A micromanipulation method to measure the mechanical properties of single tomato suspension cells. *Biotechnology Letters, 22*, 1877-1883. http://dx.doi.org/10.1023/A:1005635125829

Chen, X. Y., & Kim, J. Y. (2009). Callose synthesis in higher plants. *Plant Signaling & Behavior, 4*, 489-492. http://dx.doi.org/10.4161/psb.4.6.8359

Chanliaud, E., Burrows, K. M., Jeronimidis, G., & Gidley, M. J. (2002). Mechanical properties of primary plant cell wall analogues. *Planta, 215*, 989-996. http://dx.doi.org/10.1007/s00425-002-0783-8

Cresti, M., & van Went, J. L. (1976). Callose deposition and plug formation in Petunia pollen tubes in situ. *Planta, 133*, 35-40. http://dx.doi.org/10.1007/BF00386003

Fukumoto, T., Hayashi, N., & Sasamoto, H. (2005). Atomic force microscopy and laser confocal scanning microscopy analysis of callose fibers developed from protoplasts of embryogenic cells of a conifer. *Planta, 223*, 40-45. http://dx.doi.org/10.1007/s00425-005-0065-3

Kurita, A., Hasegawa, A., Hayashi, N., & Sasamoto, H. (2008). Novel Callose fiber formation developed in protoplast cultures of a mangrove plant. *Proceedings of the 72th Annual Meeting of the Botanical Society of Japan*, P. 233.

Kurita, A., Fukumoto, T., Hayashi, N., Shiraishi, T., & Sasamoto, H. (2009). Characteristics of novel elongated

callose fiber and regulatory mechanisms of the fiber formation in tree protoplast cultures: Mechanical properties; AFM; LCSM; TEM; GFP gene transformation. *Proceedings of the 59th Annual Meeting of the Japan Wood Research Society*, p. 96.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum, 15*, 473-497. http://dx.doi.org/10.1111/j.1399-3054.1962.tb08052.x

Ogita, S., Sasamoto, H., & Kubo, T. (1999). Selection and microculture of single embryogenic cell clusters in Japanese conifers: *Picea jezoensis*, *Larix leptolepis* and *Cryptomeria japonica*. *In Vitro Cellular and Developmental Biology Plant, 35,* 428-431. http://dx.doi.org/10.1007/s11627-999-0061-6

Onishi, T., Shiraishi, T., & Morishita, S. (2006). Mechanical Properties of a Cultured Osteoblast. *Journal of advanced Science, 18,* 95-98. http://dx.doi.org/10.2978/jsas.18.95

Oyanagi, T., Hasegawa, A., Sasamoto, H., Hayashi, N., & Fukumoto, T. (2012). Cell divisions and callose fiber formation in cultures of protoplasts isolated from suspension cells of a mangrove plant, *Sonneratia caseolaris*. *Proceedings of the 62nd Annual Meetings of the Japan Wood Research Society*, p. 116.

Oyanagi, T., Kurita, A., Fukumoto, T., Hayashi, N., & Sasamoto, H. (2014). Laser Confocal Scanning Microscopy and Transmission Electron Microscopy to Visualize the Site of Callose Fiber Elongation on a Single Conifer Protoplast Selected With a Micromanipulator. *Journal of Plant Studies, 3,* 23-29. http://dx.doi.org/10.5539/jps.v3n2p23

Parre, E., & Geitmann, A. (2005). More than a leak sealant. The mechanical properties of callose in pollen tubes. *Plant Physiology, 137,* 274-286. http://dx.doi.org/10.1104/pp.104.050773

Sasamoto, H., Wakita, Y., Yokota, S., & Yoshizawa, N. (2000). Large electro-fused protoplasts of *Populus alba* selected by a micromanipulator: Techniques and some characteristics of cells and their regenerants. *Journal of Forest Research*, 5, 265-270. http://dx.doi.org/10.1007/BF02767120

Sasamoto, H., Ogita, S., Hayashi, N., Wakita, Y., Yokota, S., & Yoshizawa, N. (2003). Development of novel elongated fiber-structure in protoplast cultures of *Betula platyphylla* and *Larix leptolepis*. *In Vitro Cellular and Developmental Biology Plant, 39,* 223-228. http://dx.doi.org/10.1079/IVP2002388

Timoshenko, S. (1955). *Strength of Materials*. Van Nostrand, New York.

Wang, C. X., Wang, L., & Thomas, C. R. (2004). Modelling the mechanical properties of single suspension-cultured tomato cells. *Annals of Botany, 93,* 443-453. http://dx.doi.org/10.1093/aob/mch062

Wolfe, J., & Steponkus, P. L. (1983). Mechanical properties of the plasma membrane of isolated plant protoplasts. Mechanism of hyperosmotic and extracellular freezing injury. *Plant Physiology, 71,* 276-285. http://dx.doi.org/10.1104/pp.71.2.276