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Effects of Salts on Helical Protoplast-Callose-Fiber Formation and Cell Division in Leaf Protoplast Culture of *Arabidopsis thaliana*: Ultrastructure of PCF Using Transmission Electron Microscopy

Manabu Hayatsu[1, 2], Suechika Suzuki[1], Futoshi Ishiguri[3], Shinso Yokota[3] & Hamako Sasamoto[1, 3, 4]

[1] Research Institute for Integrated Science, Kanagawa University, Hiratsuka, Kanagawa 259-1293, Japan

[2] Division of Microscopic Anatomy, Graduate School of Medical and Dental Sciences, Niigata University, Niigata, 951-8510, Japan

[3] School of Agriculture, Utsunomiya University, Utsunomiya 321-8505, Japan

[4] Faculty of Environment and Information Sciences, Yokohama National University, Yokohama 240-8501, Japan

Correspondence: Hamako Sasamoto (and Shinso Yokota), Utsunomiya University, Utsunomiya 321-8505, Japan. Tel: 81-28-649-5539. E-mail: 20smh213235@gmail.com; yokotas@cc.utsunomiya-u.ac.jp

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Abstract

Effects of four salts addition (10-200 mM), NaCl, KCl, $MgCl_2$, and $CaCl_2$ were examined on the growth of protoplasts and formation of helical 1.5 mm long, protoplast-callose-fibers (PCF) in *Arabidopsis thaliana* liquid leaf protoplast cultures. The basal medium was Murashige and Skoog's medium containing 1 μM 2,4-dichlorophenoxyacetic acid and 0.1 μM benzyladenine, 3% sucrose, and 0.4 M mannitol. The protoplast division was highly stimulated by the addition of 50 mM Ca^{2+} ion but was totally inhibited by 50 mM Mg^{2+} ion. Inhibition by K^+ and Na^+ ions was in-between. By contrast, PCF formation, whose numbers were counted under a fluorescence inverted microscope after Aniline Blue staining for β-1,3-glucan (callose), was stimulated by both K^+ and Ca^{2+} ions but inhibited by Mg^{2+} ion. After selecting *Arabidopsis* PCF using a micromanipulator, the sub-fibril ultrastructure was examined by transmission electron microscopy (TEM). The PCFs of *Betula platyphylla* and *Larix leptolepis*, cultured with 200 mM Ca^{2+} and 50 mM Mg^{2+} ions, respectively, after long-term storage and rehydration, were examined by TEM. The effects of different factors were discussed on PCF formation and sub-structures in different herbaceous and tree plant species.

Keywords: β-1,3-glucan, protoplast culture, protoplast fiber, salts, TEM

1. Introduction

Novel helical (or spiral) fiber structures were repeatedly found in the protoplast cultures of two clones of *Betula platyphylla* and of conifer *Larix leptolepis.* These structures were first observed in liquid protoplast cultures from the leaves of *Betula platyphylla* (Wakita *et al*., 1993) and from the embryogenic suspension-cultured cells of *Larix leptolepis* and *Cryptomeria japonica* (Sasamoto & Ogita, 2000; Sasamoto *et al*., 2001). Recent progress in the study of protoplast is summarized below.

1.1 Protoplast-Callose-Fiber (PCF) Formation in Betula and Larix

The protoplast-callose-fiber (PCF) was efficiently observed under an inverted microscope in the liquid protoplast cultures of suspension cultured cells from *Larix leptolepis* (with addition of 50 mM $MgCl_2$ after two weeks of culture), and of leaves from *Betula platyphylla* (with addition of 100 mM $CaCl_2$ after one month or more of culture at low pH). Blue-green UV fluorescence, specific for the β-1,3-glucan (callose) component, was observed after Aniline Blue staining, indicating that callose is the main component of the fibers. The PCF elongated (*e.g.*, 2 mm long) from large spherically enlarged protoplasts but not from divided protoplasts. *Larix* PCFs with a helical structure were also observed by transmission electron microscopy (TEM) (Sasamoto *et al*., 2003).

The substructure of *Larix* PCFs was further examined by atomic force microscopy (AFM), laser confocal scanning microscopy (LCSM), and the callose component of the PCF was determined enzymatically (Fukumoto *et al*., 2005). LCSM using Alexafluor 488 phalloidin, which binds to actin protein, after electric treatment,

visualized the site of callose fiber elongation from a single *Larix* protoplast selected with a micromanipulator. Thin sub-fibrils and a twisted structure were determined by TEM (Oyanagi *et al*., 2014). A unique tensile test using micromanipulators under an inverted microscope investigated the mechanical properties of single PCFs from *Larix* (10 μm diameter), and from *Betula* (20-30 μm diameter) with and without high concentrations of Ca^{2+} ions in the test medium. A similar Young's modulus (value/area, $1.4\text{-}1.9 \times 10^4$ N m^{-2}) was obtained for both PCFs, and use of such alkali labile PCFs in medicinal research was discussed (Oyanagi *et al.*, 2017).

1.2 Effects of Salt Ions on PCF Formation and Protoplast Growth in Different Plant Species

Since the findings of *Larix* and *Betula* PCFs, the stimulation factors for such helical PCF formation have been investigated in protoplast cultures of both tree species (Fukumoto, 2006; Fukumoto *et al*., 2005; Kurita-Tashiro *et al*., 2020; Oyanagi *et al*., 2012a,b, 2024; Sasamoto *et al*., 2020b; and Seyama *et al*., 2008 though the culture conditions were wrongly described), and herbaceous plant species (Fukumoto, 2006; Sasamoto *et al*., 2020a; Tagawa & Kondo, 2018).

We first predicted an inverse relationship between the inhibition of protoplast growth and stimulation of PCF formation by divalent cations at high concentrations. However, a single salt condition as a sole factor for PCF formation has not been found. In suspension cultured cells of herbaceous *Vinca rosea*, 50-200 mM Ca^{2+} ion stimulated PCF formation but inhibited protoplast growth (Fukumoto, 2006). In *Larix* PCF formation, Ca^{2+} ion was less stimulatory than Mg^{2+} ion (Fukumoto 2006; Sasamoto *et al*., 2003).

The effects of not only divalent cations but also monovalent cations, Na^+ and K^+, were also investigated on protoplast growth (non-spherical enlargement with cell wall formation and protoplast division), and on PCF formation of herbaceous plants and trees have been investigated as follows.

All four salts (NaCl, KCl, $MgCl_2$, and $CaCl_2$) inhibited the growth of protoplasts obtained from poplar leaves and herbaceous tobacco BY2 suspension cultured cells (Fukumoto *et al*., 2004). No formation of PCF was observed in the former, while in the latter, PCF was formed with only 75 mM Mg^{2+} (Fukumoto 2006).

In protoplast culture of suspension cells obtained from the Na^+ salt-tolerant mangrove tree, *Bruguiera sexangula*, Ca^{2+} and Mg^{2+} ions stimulated protoplast growth at low concentrations, but inhibited it at high concentrations (Fukumoto *et al*., 2004). Although no stimulation of PCF formation was observed with high concentrations of cationic ions, electric treatment for cell fusion (Sasamoto *et al*., 2000) caused rapid (within one week) PCF formation (Kurita-Tashiro *et al*., 2020).

In suspension cultured cells of the Na^+ halophilic mangrove tree, *Avicennia alba,* Na^+ and Mg^{2+} ions stimulated protoplast growth (Hasegawa *et al*., 2013), and Na^+ ion, at only low concentrations, stimulated slow PCF formation (> 0.5 mm long with 20 μm diameter), though Ca^{2+} ion inhibited both protoplast growth and PCF formation (Kurita-Tashiro *et al*., 2020).

In suspension cultured cells of another mangrove tree, *Sonneratia caseolaris*, Mg^{2+} ion, but not Ca^{2+} ion, stimulated both protoplast growth (Sasamoto & Hasegawa, 2021) and rapid PCF formation in sucrose medium (Oyanagi *et al*., 2012a,b; 2024).

1.3 Sub-structures of PCF

Fine sub-structures (*e.g*., fibril and sub-fibril) of helical PCF were investigated using TEM, atomic force microscopy (AFM) and laser confocal scanning microscopy (LCSM), and single cell TEM which was performed to clarify the cellular elongation site, in *Larix* (Fukumoto, 2006; Fukumoto *et al*., 2005; Oyanagi *et al*., 2014) and mangrove trees, *e.g*., *Avicennia alba, Bruguiera sexangula* (Kurita-Tashiro *et al*., 2020), and *Sonneratia caseolaris* (Oyanagi *et al*., 2012a,b, 2024).

The hollow fibril sub-structure of *Betula* PCF was observed by TEM using the antibody of callose (Seyama *et al*., 2008). Sub-fibrils and tubes-like structures of *Bruguiera sexangula* PCF were observed by single cell TEM (Kurita-Tashiro *et al*., 2020).

1.4 Leaf Protoplast Cultures of Arabidopsis thaliana (Arabidopsis) and Sub-structure of the PCF

Leaf protoplast cultures of *Arabidopsis thaliana* have been established for research on allelopathy using protoplast co-cultures with recipient lettuce (Sasamoto *et al*., 2013; 2017). In lettuce, low concentrations of Ca^{2+} and Mg^{2+} ions stimulated early protoplast growth (Sasamoto *et al*., 2020b), while the formation of PCFs has not been observed.

In this study, we investigated uniqueness of the effects of four salt ions (Na^+, K^+, Mg^{2+} and Ca^{2+}) on the protoplast growth and rapid PCF formation in leaf protoplast cultures of *Arabidopsis.*

TEM characterized the fine sub-structures of PCFs from leaf protoplasts of *Arabidopsis* after selection using a micromanipulator.

1.5 TEM of Long-Period-Stored and Rehydrated PCFs of Betula and Larix

For the efficient accumulation of the PCFs, effects of the long-term storage at dried condition and rehydration were also investigated.

Protoplast culture plates of PCFs obtained from *Betula* leaves and *Larix* suspension-cultured cells (Fukumoto *et al*., 2005; Oyanagi *et al*., 2014, 2017; Sasamoto *et al*., 2003) were naturally dried, and stored under a sterile condition for an extended period (*ca.* ~10 years).

In this study, we rehydrated the PCFs and selected them using a micromanipulator. Their sub-structure was observed by TEM analysis and compared with those of fresh PCFs obtained from *Betula* and *Larix,* which were previously reported (Oyanagi *et al*., 2014; Sasamoto *et al*., 2003; Seyama *et al*., 2008). They were also compared with the sub-structures of PCFs obtained from freshly prepared *Arabidopsis* (*1.4*) and from other plant species previously reported (*1.2, 1.3*).

2. Method

2.1 Protoplast Culture of Arabidopsis thaliana

Aseptically grown seedlings of *Arabidopsis thaliana* ecotype Columbia (Col) or Landsberg *erecta* (L*er*), and leaf protoplasts were cultured as reported (Sasamoto *et al*., 2017, 2020a). Briefly, seeds in a bag of Miracloth^R were sterilized with 0.2-0.5% NaClO solution for 5 min, washed with autoclaved water three times, planted on 1/2 salts MS (Murashige & Skoog, 1962), 1% sucrose, and 1-1.5% agar in a Plant Box (7.5×7.5×10 cm), and cultured under 14 to 16 hr light condition (60-140 µ*E*) for 2 to 3 weeks at 24-25ºC. Protoplasts were isolated in 0.4 M mannitol solution containing 1% Cellulase R10 plus 0.15% Pectolyase Y23 in a 24-well culture plate for 2 to 3 hrs at room temperature. Protoplasts were filtered through 80 µm pore size nylon mesh and washed three times with 0.4 M mannitol solution by centrifugation at 100 *g* (900 rpm) for 5 min.

Medium was 50 µL of liquid MS basal medium containing 1 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 µM benzyladenine (BA), 3% sucrose, and 0.4 M mannitol in a 96 well culture plate (Falcon No.3072). NaCl, KCl, $MgCl_2$, and $CaCl_2$ were added at a final concentration of 0, 10, 25, 50, 100, and 200 mM. The medium was adjusted to pH 5.8 with KOH before autoclaving at 120ºC for 20 min. Sterile water (100 µL) was supplied in-between wells. Plates were sealed with two layers of Parafilm^R. Final protoplast densities were $5\text{-}100 \times 10^3$ mL^{-1}. They were cultured at 28ºC in a humid incubator (CO_2- incubator without the supply of CO_2 gas, APC-30DR, ASTEC Co., Ltd.), or in a Peltier-type incubator (MEE CN-25C, Mitsubishi) with a humid box with wet papers.

Protoplast growth and PCF formation were observed periodically under an inverted microscope (Olympus CK40, IX71). Numbers of non-spherically enlarged protoplasts (E), divided protoplasts (D), and PCFs were counted. Protoplast growth (E+D) was described as the % of control without additional salts.

PCFs were also stained with Aniline Blue solution (0.5%, pH8.5), which was added to the culture medium at a final concentration of 0.05%. PCFs were counted using a fluorescence inverted microscope (Olympus IX71, IX70, UV filter U-MWU2, WU).

2.2 PCF of Betula and Larix after Long-term Storage and Rehydration

After culturing the PCFs of *Betula* (2.2.1) or *Larix* (2.2.2) for several months, the 24-well culture plates or 96-well culture plates containing PCFs of *Betula* or *Larix* were naturally dried and stored in a sterile condition for about 10 years. After rehydrated with the autoclaved H_2O of the same volume of the old culture medium in wells, PCF was selected using a micromanipulator (*2.3*) and analyzed by TEM *(2.4).*

2.2.1 *Betula* PCF

PCFs of *Betula platyphylla* were prepared from the leaf of shoot culture as previously reported (Sasamoto *et al*., 2003). Leaf protoplast was isolated by overnight floating on 0.6 M mannitol solution, containing 1% each of Cellulase R10 and Driselase, passed through a 42 µm pore size mesh. Washed with osmoticum solution three times by centrifugation at 100 *g* (800 rpm) for 3 min. Protoplasts were cultured in 1/2 MS salt medium containing 1 µM each of naphthalene acetic acid (NAA) and *N*-(2-chloro-4-pyridyl)-*N'*-phenylurea (CPPU), 3% sucrose, 0.6 M mannitol, and 100 or 200 mM $CaCl_2$. pH was adjusted at 3.5 before autoclaving at 121ºC for 20 min. Protoplast densities were 5×10^4 mL^{-1}, or 10^5 mL^{-1} in 300-500 µL medium in a 24-well culture plate. About 1 mL H_2O was supplied between wells. They were cultured at 28ºC in a humid incubator.

2.2.2 *Larix* PCF

PCFs of *Larix leptolepis* of suspension cultured cells were prepared as previously reported (Fukumoto *et al*., 2005; Sasamoto *et al*., 2003), except for selected strains of the suspension cultured cells cultured in high glutamine medium (2000 mg mL^{-1}) (Fukumoto 2006; Oyanagi *et al*., 2014). Protoplasts were isolated using 1% Cellulase RS and 0.05% Pectolyase Y-23 in 0.4 M mannitol solution for1-2 hrs. Protoplasts were purified by passage through a 42 μm sized nylon mesh and washed by centrifugation at 100 *g* for 4 min with mannitol solution 3 times. Then the protoplasts were cultured in NH_4NO_3-free MS medium containing 10 μM each of 2,4-D and BA, 6% sucrose, 0.4M mannitol, and 50 mM $MgCl_2$, pH 5.8 in a 96-well or a 24-well culture plate. Protoplast density was 2×10^4 mL^{-1}. 100 μL or about 1 mL H_2O was supplied in-between wells.

2.3 Micromanipulation of PCF

Fresh PCF of *Arabidopsis* (*2.1*), and rehydrated PCFs of *Betula* (2.2.1) and *Larix* (2.2.2) were picked up and transferred to H_2O in a 4-well culture plate (Nunc or SPL-30004, SPL Life Sciences Co. Ltd.) using a micromanipulator (M-152 or MM-89, Narishige) with a microinjector (Pneumatic Microinjector IM-11-2, Narishige) under an inverted microscope (Olympus CK40 or IX-71). The micropipettes for selecting the PCFs were made from a glass capillary (10 μL, Drummond) using a micropipette puller (PC-10, Narishige). They were bent with a micro-burner (Pen Burner, Prince) as described by Ogita *et al.* (1999). PCFs were washed with H_2O in a microtube by centrifugation at 2000 rpm and cold stored in *ca*.2% glutaraldehyde solution before processing for TEM (*2.4*). PCFs of *Larix* were also centrifuged at 10,000 rpm.

2.4 TEM of PCF

Samples for TEM observation were prepared as previously described (Hayatsu *et al*., 2012, 2014, 2017). Samples were fixed in 2% glutaraldehyde with PB (pH7.2) at 4ºC for more than 24 hrs and fixed in 1% osmium tetroxide with PB (pH7.2) at 4ºC for 12 hrs. The fixed samples were then dehydrated through a series of graded acetone and were embedded in 100% Epoxy resin (100 g Epon was composed of 54 g Epon812, 26.5 g MNA, 19.5 g DDSA, and 1.5 g DMP-30, all from Nisshin EM Co. Ltd., Tokyo, Japan) at 4ºC for 48 hrs, and were polymerized with pure Epoxy resin at 60ºC for 48 hrs. Ultrathin sections (60–80 nm thickness) were cut with an ultramicrotome (EM UC7, Leica Microsystems Inc., Wetzlar, Germany) with a diamond knife (Ultra, DiATOME, Switzerland). These sections were stained with uranyl acetate for 10 min and lead citrate for 5 min, mounted on 150-mesh copper grids, and were imaged by using TEM (H-7650, Hitachi, Tokyo, Japan) at an accelerating voltage of 80 kV, and TEM (JEM-2100, JEOL, Tokyo, Japan) at that of 100 kV.

3. Results & Discussion

3.1 Effects of Salts on the Leaf Protoplast Growth of Arabidopsis thaliana

Figure 1 shows the growth of leaf protoplast culture from *Arabidopsis* (L*er*) in the control medium without additional salts. Spherical protoplasts after one day (Figure 1a), non-spherically enlarged after 8 days (Figure 1b), and divided colonies after 12 days (Figure 1c) of culture were observed, respectively. Protoplast growth was similar to *Arabidopsis* (Col), which was previously reported (Sasamoto *et al*., 2017).

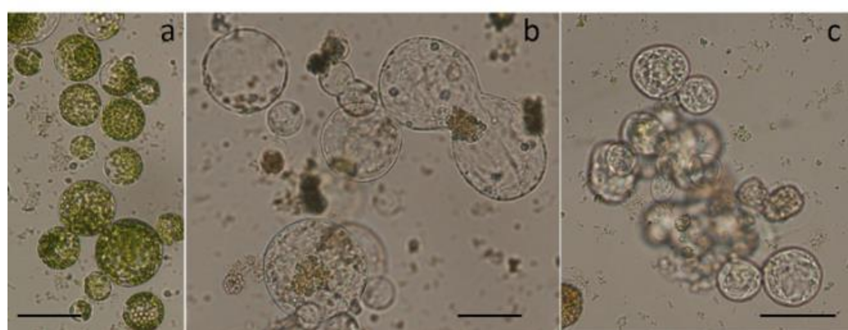

Figure 1. Protoplast culture of *Arabidopsis thaliana* without additional salts after one day (a), 8 days (b), and 12 days (c). Protoplast densities were 8×10^4 mL^{-1} (a, c) and 4×10^4 mL^{-1} (b). The basal medium was MS with 2,4-D 1 μM, BA 0.1 μM, 3% sucrose, and 0.4 M mannitol. Bar = 50 μm

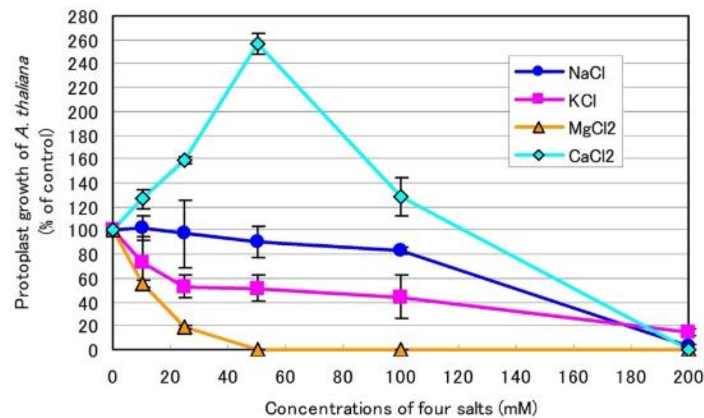

Figure 2. Effects of additional salts on the protoplast growth (E+D, % of control) of *Arabidopsis thaliana* after 8 days of culture. The basal medium was MS with 2,4-D 1 μM, BA 0.1 μM, 3% sucrose, and 0.4 M mannitol. Protoplast densities were 4-8 × 10 ^{4} mL^{-1}. At zero control, numbers (E+D) were 29/well (76% was E)

As shown in Figure 2, 25-50 mM $CaCl_2$ had a prominent stimulatory effect on leaf protoplast growth in *Arabidopsis*. Other salts were inhibitory, and 50 mM $MgCl_2$ was totally inhibitory.

Such stimulatory effect of Ca^{2+} ion (10-25 mM) on protoplast growth was also observed in the protoplast culture of lettuce, which showed no PCF formation (Sasamoto *et al*., 2020b).

For the leaf protoplast cultures of *Arabidopsis*, we used MS basal medium containing 2,4-D 1 μM, BA 0.1 μM, 3% sucrose, and osmoticum, which has been used for the protoplast co-culture method for allelopathy, and measured the effect of test plant (*Arabidopsis*) protoplast on the protoplast growth of recipient lettuce, *i.e.,* on the numbers of non-spherically enlarged (cell wall formation stage) and divided protoplasts (division stage) (Sasamoto *et al*., 2017).

MS basal medium, which is widely used for culturing plant cells, contains 0.2 mM Na^+, 21.25mM K^+, 1.5mM Mg^{2+}, and 3 mM Ca^{2+}. The ion concentrations were the same as those of NH_4NO_3-free MS medium and modified amino acid medium, which were used for protoplast cultures of *Larix* suspension cultured cells (Fukumoto *et al.*, 2005) and of *Avicennia alba* callus (Hasegawa *et al*., 2013; Sasamoto *et al*., 2020b). NH_4NO_3-free MS basal medium was better (in which growth occur at wide range of concentrations of hormonal conditions) than MS basal medium for protoplast growth of leaves from *Arabidopsis* (Sasamoto *et al*., 2020a), lettuce (Sasamoto *et al*., 2013), and poplar (Fukumoto *et al*., 2004). For *Betula* leaf protoplast growth, 1/2 MS salts and acidic pHs were also used (Sasamoto *et al*., 2003; Wakita *et al*., 1993).

3.2 Effects of Salts on PCF Formation in Arabidopsis thaliana Leaf

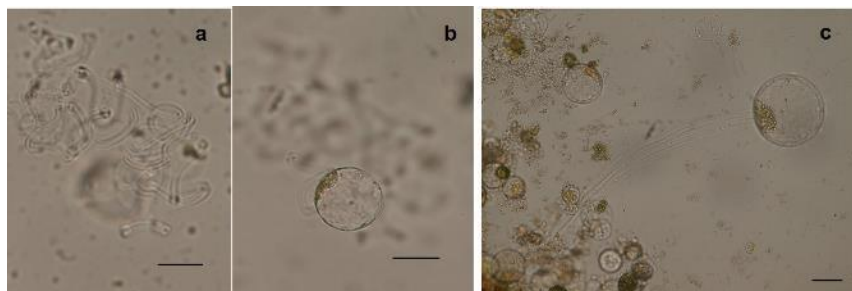

Figure 3. Helical structure of PCF from *Arabidopsis thaliana* after 5 days of culture. Photos of different focus (a,b), without the addition of salts at a density of 5 × 10 ^{4} mL^{-1}. With 10 mM $CaCl_2$ at 1 × 10^4 mL^{-1} (c). The basal medium was MS with 2,4-D 1 μM, BA 0.1 μM, 3% sucrose, and 0.4 M mannitol. Bar = 50 μm

As shown in Figure 3, helical PCFs of *Arabidopsis* were observed under an inverted microscope after several days of culture. Helical fiber elongated from a floating spherically enlarged protoplast (Figure 3b). PCFs had diameters of *ca*.10 μm (Figure 3a) to *ca*. 35 μm (Figure 3c). They were similar to the PCFs of *Larix* and *Betula,* which elongated from a specific site on spherically enlarged protoplast without cell division (Sasamoto *et al*., 2003; Fukumoto *et al*., 2005). Observation of PCF after less than a week of culture revealed speedy PCF

formation, which was similar to that in *Bruguiera sexangula* (Kurita-Tashiro *et al*., 2020) and in *Sonneratia caseolaris* (Oyanagi *et al*., 2012a,b; 2024), compared with those after 2 weeks in *Larix,* one to two months in *Betula* (Sasamoto *et al*., 2003), and in *Avicennia* mangrove protoplasts (Kurita-Tashiro *et al*., 2020).

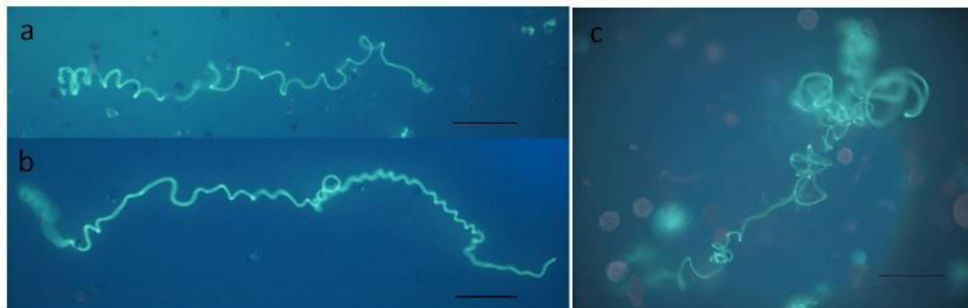

Figure 4. Fluorescent protoplast-callose-fibers of *Arabidopsis thaliana* stained with Aniline Blue after 30-40 days of culture. With the addition of 25 mM KCl at 4×10^{4} mL^{-1} (a), 10 mM $CaCl_2$ at 8×10^{4} mL^{-1} (b), and 50 mM $CaCl_2$ at 9×10^{3} mL^{-1}(c). The basal medium was MS with 2,4-D 1 µM, BA 0.1 µM, 3% sucrose, and 0.4 M mannitol. Bar =100 µm

Figure 4 shows *ca*. 1-1.5 mm long PCFs stained with Aniline Blue, observed under a fluorescent microscope. Figure 4a shows an elongated helical PCF in the medium containing an additional 25 mM KCl, and Figure 4b and c show PCFs in the medium containing an additional 10 and 50 mM $CaCl_2$, respectively.

The callose component of PCFs was determined by using specific degrading enzymes for callose and cellulose in *Larix* (Fukumoto *et al*., 2005), mangrove trees, *Bruguiera sexangula* (Kurita-Tashiro *et al*., 2020), and *Sonneratia caseolaris* (Oyanagi *et al*., 2012a,b; 2024). Observation of specific blue-green fluorescence after Aniline Blue staining of helical PCFs under a fluorescence inverted microscope can be used for a criterion of PCFs (Sasamoto *et al*., 2003).

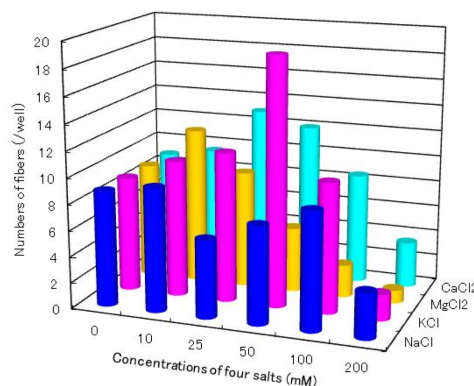

Figure 5. Effects of additional salts on the protoplast-callose-fiber formation after 40 days of culture. PCFs were counted after staining with Aniline Blue. Protoplast density was 4 to 8×10^4 ml^{-1}. The basal medium was MS, with 2,4-D 1 µM, BA 0.1 µM, 3% sucrose, and 0.4 M mannitol

Figure 5 shows the effects of four salts on the number of *Arabidopsis* PCF per well cultured for 40 days. The PCFs were counted under a fluorescent microscope after Aniline Blue staining, as in *Sonneratia caseolaris* PCFs (Oyanagi *et al*., 2024). There were fewer PCFs after 27 days of culture under an inverted microscope without staining, though the tendency of the salts effect was similar (data not shown). The highest stimulation was observed by addition of 50 mM KCl. As the MS basal medium contains *ca*.20 mM K^+ ion, the highest number of PCFs was obtained in the medium containing *ca*.70 mM K^+ ion. The addition of 25-50 mM $CaCl_2$ also stimulated PCF formation in leaf protoplasts of *Arabidopsis.* The addition of 50 mM $MgCl_2$ was inhibitory.

3.3 Different Effects of Salt Ions on Protoplast Growth and PCF Formation in Different Plants

We initially predicted that a high concentration of divalent cation is the stimulatory factor for PCF formation and inhibitory factor for protoplast growth as in *Betula* leaf and *Larix* suspension-cultured cells (Sasamoto *et al*.,

2003; Fukumoto *et al*., 2005). However, the presence of divalent cation in a high concentration (100 mM or 50 mM) was not the only factor stimulating PCF formation in leaf protoplasts of *Arabidopsis*. In *Arabidopsis* (Figures 2 and 5), the addition of 50 mM K^+ ion inhibited growth, but highly stimulated PCF formation; Ca^{2+} ion stimulated both protoplast growth and PCF formation; Mg^{2+} ion inhibited both growth and PCF formation.

Stimulation of leaf protoplast growth by Ca^{2+} ion was observed in *Arabidopsis* (Figure 2), lettuce (Kurita-Tashiro *et al.,* 2020), and suspension cells of salt-tolerant mangrove tree, *Brugiera sexangula* (Fukumoto *et al*., 2004). Inhibition of protoplast growth by Ca^{2+} ion has been repeatedly reported in Na^+ halophilic mangrove trees, *e.g*., *Avicennia alba* and *Sonneratia alba* (Hasegawa *et al*., 2013). In these halophilic mangrove cells, specific Na-Ca elemental distribution was found using quantitative X-ray microanalysis of elements with cryo-TEM (Hayatsu *et al*., 2014; 2017).

In the protoplasts of the halophilic mangrove, *Avicennia alba*, all salts tested inhibited PCF formation except for 10 mM NaCl (Kurita-Tashiro *et al*., 2020). In another mangrove, *Sonneratia caseolaris,* only Mg^{2+} ion strongly stimulated both protoplast growth and PCF formation. PCF elongated from early divided protoplasts with a division plate (Oyanagi *et al*., 2012a,b; 2024).

The rate of stimulation and inhibition by the addition of salt ions on protoplast growth (Figure 2) and PCF formation (Figure 5) in *Arabidopsis* was not high. Ten times stimulation of PCF formation by electric treatment was reported in a mangrove tree, *Bruguiera sexangula* (Kurita-Tashiro *et al*., 2020). In *Sonneratia caseolaris,* 50 mM Mg^{2+} ion stimulated PCF formation highly (20 times), but protoplast growth only 2-3 times (Oyanagi *et al*., 2024).

A simple inverse relation between protoplast growth and PCF formation cannot be widely applied to different plant species. Therefore, the ions stimulating PCF formation are not just divalent cations, and the effects of four salt ions in each plant protoplast culture should be examined further.

3.4 TEM Analysis of PCF

3.4.1 TEM of *Arabidopsis* PCF

Figures 6 and 7 show different TEM images of PCFs from *Arabidopsis thaliana.*

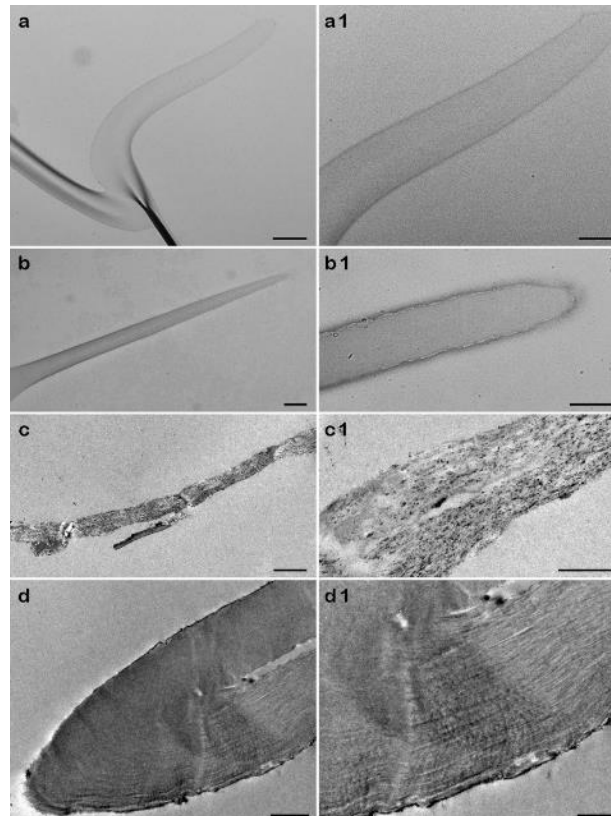

Figure 6. TEM images of branched or curved PCF of *Arabidpsis thaliana* (a-d). a1, b1, c1, and d1 are magnified images from a part of a, b, c, and d. Bars =1 μm (a, d1), 2 μm (b, c, d), and 0.5 μm (a1, b1, c1)

Figures 6a and 6b show the branched PCF of *Arabidopsis.* The curved or twisted PCF in *Arabidopsis* was ~1.3 µm in diameter (Figure 6a). The *Arabidopsis* PCF shown in Figure 6b was ~2.0 µm in diameter. The tip part (Figures 6a1, 6b1) was ~0.9 µm in diameter. They might be similar to the thin fibril structure of *Larix* PCF (Oyanagi *et al*., 2014) or the tube-like structure of *Bruguiera sexangula* PCF, elongated from a protoplast in single-cell TEM (Kurita-Tashiro *et al*., 2020).

The PCF shown in Figure 6c (0.8-1.4 µm in short-axis diameter) showed some branching, with two sub-fibrils 0.3-0.5 µm in short-axis diameter, and a gap of 0.1 µm between two sub-fibrils (Figure 6c1). Fine-fibrils (less than 0.01 µm in short-axis diameter) were observed. Figure 6d shows a curved or twisted PCF (3.7-4.3 µm in short-axis diameter), which was larger than the other PCFs in Figures 6a, 6b, and 6c. The fine-fibrils (less than 0.01 µm in short-axis diameter) were densely assembled (Figure 6d1). TEM images of *Arabidopsis* PCFs in Figures 6c and 6d were similar to those of *Larix* PCFs reported (helical filamentous structure, Sasamoto *et al*., 2003; twisted fibril structure, Oyanagi *et al*. 2014).

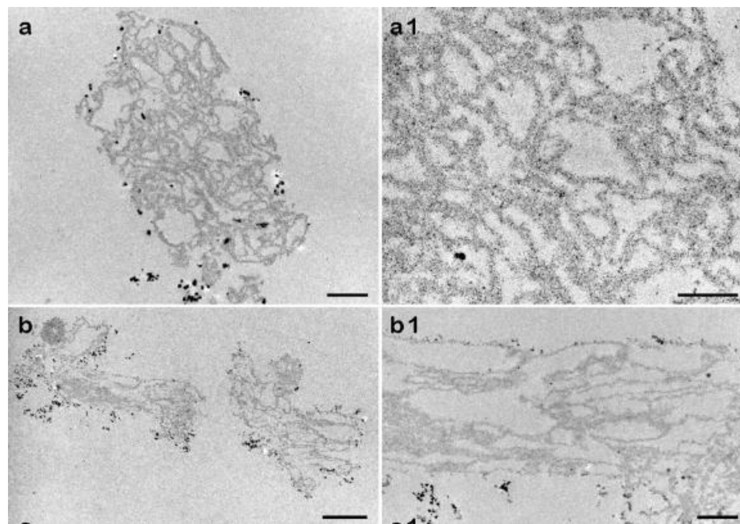

Figure 7. TEM images of PCF with hollow sub-structures of *Arabidopsis thaliana*. a1 and b1 are magnified images from a part of a and b. Bars =2 µm (a), 0.5 µm (a1), 5 µm (b), and 1 µm (b1)

In Figure 7a, large *Arabidopsis* PCFs (7.7 µm and 16.1 µm in short- and long-axis diameter, respectively) had densely packed hollow sub-structures. The PCF was composed of hollow sub-fibrils in various sizes and forms (short-axis diameters of ~0.6 µm in Figure 7a1).

TEM images of the densely packed hollow sub-structure of *Arabidopsi*s PCF (Figure 7a) were similar to those of *Betula* PCF previously reported (Seyama *et al*., 2008).

Both longitudinally and transversely cut hollow structures were observed in *Arabidopsis* PCF. In Figure 7b, a gap of 4.2-5.7 µm was observed between the two PCFs (~6.7 µm and 33.3 µm in short- and long-axis diameter of the left PCF, and ~9.6 µm and 26.7 µm in short- and long-axis diameter of the right PCF). The short-axis diameter of hollow sub-fibrils was ~1.0 µm, and the long diameter of hollow sub-fibrils was ~3.7 µm (Figure 7b1). The presence of a gap between PCFs or fibrils and the longer diameter of hollow sub-fibrils might indicate that the PCFs or fibrils are curved or twisted, similar to the twisted sub-fibril structure of *Larix* PCFs previously reported (Oyanagi *et al*., 2014).

The formation mechanism of large spiral elongated fibers has been explained by the twisting force of the fibrils caused by the molecular structure of β-1,3-bond in callose (Oyanagi *et al*. 2014).

3.4.2 TEM of PCF of *Betula* and *Larix* after Long-term Storage

Figure 8 shows TEM images of the stored *Betula* PCF, which had been cultured in the medium with an additional 200 mM $CaCl_2$. Figure 9 shows TEM images of the stored *Larix* PCF, which had been cultured in the medium with an additional 50 mM $MgCl_2$. They were stored for about 10 years under a dried condition and rehydrated before micromanipulation.

As in *Arabidopsis* PCF, both fibers with thin fibrillar sub-structures (Figure 6) and with densely packed hollow sub-structures (Figure 7) were also observed in stored and rehydrated *Betula* PCF (Figure 8). A similar hollow

sub-structure of fresh *Betula* PCF without storing for an extended period has been observed using TEM (Seyama *et al*., 2008).

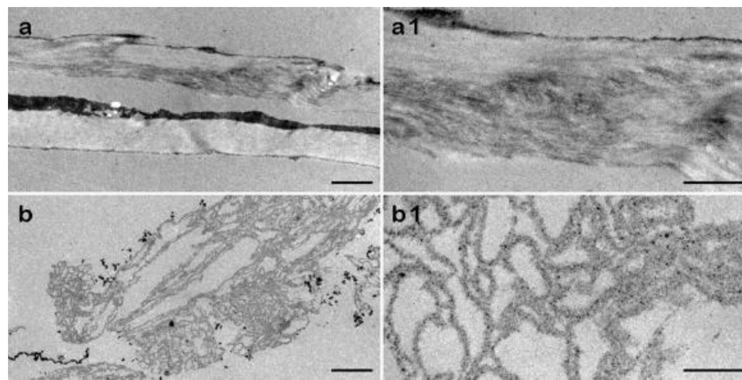

Figure 8. TEM images of PCFs with fibrillar sub-structures (a), and densely packed hollow sub-structures (b) of PCF from *Betula platyphylla* after long-term storage. a1 and b1 are magnified images from a part of a and b, respectively. Bars =1 μm (a), 0.5 μm (a1, b1), and 5 μm (b)

Betula PCFs in Figure 8a (2-2.8 μm in short-axis diameter) comprised two sub-fibrils (0.8-1.3 μm in short-axis diameter). The distance between sub-fibrils (gap) was 0.3-0.8 μm. A part of the sub-fibrils comprised a fine-fibrillar structure (Figure 8a1). The sub-fibrils of stored *Betula* PCFs shown in Figure 8a had a diameter similar to that of *Arabidopsis* PCFs (Figures 6c and 6d).

On the other hand, the stored *Betula* PCFs with densely packed hollow sub-structures were 11.5-19.5 μm in short-axis diameter (Figure 8b), similar to the long-axis diameter of *Arabidopsis* PCFs (16.1 μm, Figure 7a). Similar to *Arabidopsis* PCFs, the longitudinally or transversely cut hollow structures were observed in *Betula* PCFs. Hollow sub-structures with different short-axis diameters of ~2.8 μm were observed (Figures 8b and 8b1). Most of the hollow structures were the same in size as those of *Arabidopsis* (Figure 7). The hollow sub-structures in various sizes and forms and the gap between PCFs (~3.3 μm) in Figure 8b suggested that the stored *Betula* PCFs were partially curved or twisted.

In the TEM images of stored *Larix* PCFs, curved or twisted PCFs, and branched and curved sub-fibril structures were observed (Figures 9a and 9b). TEM has reported similar images in fresh *Larix* PCFs (helical filamentous structure, Sasamoto *et al*., 2003; twisted fibril structure, Oyanagi *et al*. 2014). *Arabidopsis* PCFs had a similar structure (Figure 6, in 3.4.1). The curved or twisted PCFs were 3.6-4.3 μm in diameter (length of the straight part) (Figures 9a, a1, and a2). Short-axis diameter values of the largest part of the PCFs and sub-fibrils were 7.9 μm and 2.8 μm, respectively (Figure 9b). Furthermore, this largest sub-fibril repeatedly branched, gradually decreasing in size from sub-fibrils with a short-axis diameter of about 1.3 μm (Figure 9b1) to sub-fibrils with a short-axis diameter of ~0.8 μm (Figure 9b2). The fine-fibrils were densely assembled.

A hollow sub-structure was also observed in stored *Larix* PCFs (7.2-11.8 μm in short-axis diameter, Figure 9c). Short-axis diameter of hollow sub-structure was 0.5-4.1 μm (Figure 9c1). However, the densely packed hollow sub-structures were not observed in transversely cut sections of *Larix* PCFs.

TEM observation of PCFs from *Betula* and *Larix* stored under a dry condition for long periods revealed that their sub-structures were similar to those of fresh *Arabidopsis* PCFs. This indicated that PCFs of *Betula* and *Larix* could be stored under a dried condition for a long period.

Fresh PCFs of *Larix* (10 μm diameter) and *Betula* (20-30 μm diameter) had been reported to have a similar Young's modulus (value/area, 1.4-1.9 × 10^4 N m^{-2}) determined by a tensile test using two micromanipulators under an inverted microscope (Oyanagi *et al*., 2017). Differences in fine structures of PCFs might not be directly related to the mechanical property of PCFs. Though, the tensile test of the fresh or dried and rehydrated PCFs must be performed depending on the use of the PCFs.

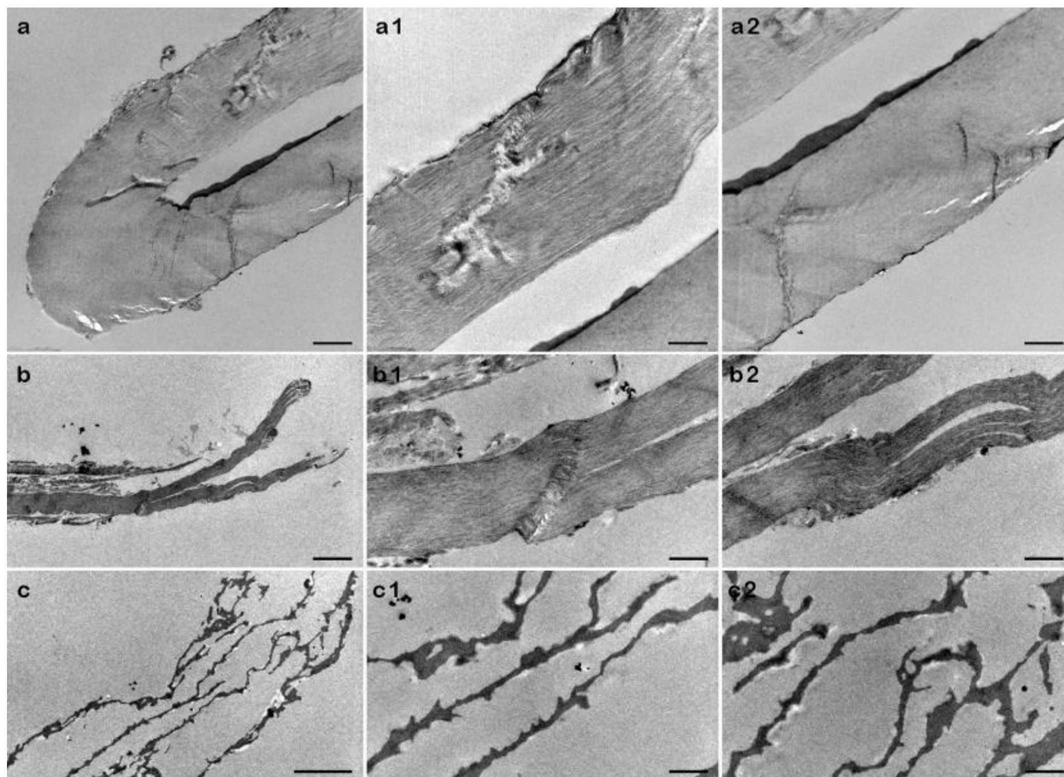

Figure 9. TEM of long-period-stored PCF from *Larix leptolepis*. PCF with non-hollow sub-structures (a, a1, a2, b, b1, and b2). PCF with the hollow sub-structures (c, c1 and c2). Bars =2 µm (a), 5 µm (b and c), 1 µm (a1, a2, b1, b2, c1 and c2)

3.4.3 Application of *Arabidopsis* PCFs

In this study, *Arabidopsis* PCFs with large diameters were observed (10~35 µm, *3.2* and *3.4*). The *Arabidopsis* PCFs had a hollow structure and branched, twisted fibril structure. *Arabidopsis* PCFs had sub-structures similar to those of *Betula* and *Larix.* Furthermore, the sub-structure of PCFs stored for a long period was similar to that of the fresh PCFs reported.

The callose is soluble in alkaline solutions which is a unique characteristic among cell wall polymer components. PCFs can be used as spiral microfilament material for specific medicinal purpose (Oyanagi *et al*., 2014, 2017).

Formation of PCFs is rapid (less than one week) in *Arabidopsis*, but slow (one month or longer) in *Betula* and *Avicennia alba* which have large diameters. An increase in diameter was not observed during culture. PCF formation in *Bruguiera sexangula* is rapid due to the electric treatment of protoplasts, but the PCFs that formed had a smaller diameter (Kurita-Tashiro *et al*., 2020).

Arabidopsis PCFs are unique materials for molecular biological studies related to callose synthesizing enzymes complex and for further research on the PCF elongation mechanism. GFP-gene transformation of *Larix* and *Bruguiera sexangula* protoplasts has been successfully performed by electric treatment using pUC18-EL2Ω -sGFP(S65T) promoter (Kurita *et al*., 2009).

The *Arabidopsis* PCFs elongated from a single spot of spherically enlarged protoplast surface, similar to *Larix* PCF, in which distribution of actin protein was detected at the PCF elongation site by LCSM using Alexafluor 488 phalloidin. A thin sub-fibril structure was observed by single cell TEM of *Larix* PCF (Oyanagi *et al*., 2014). PCFs of the *Sonneratia caseolaris* elongated rapidly from the division plate in a small divided protoplast (Oyanagi *et al*., 2024).

Application of single cell TEM using a micromanipulator on *Arabidopsis* PCF might be a method for clarifying the cellular mechanism of early PCF elongation.

4. Conclusion

The addition of 50 mM Ca^{2+} ion stimulated the leaf protoplast growth and protoplast-callose-fiber (PCF)

formation in *Arabidopsis thaliana*, while the addition of K^+ ion stimulated only PCF formation, and Mg^{2+} ion inhibited both.

Effects of salt ions on protoplast growth and PCF formation in *Arabidopsis* were compared with those of different plant species. A simple inverse relationship was not observed between optimal salt conditions for protoplast division and PCF formation.

TEM observed sub-fibril structures of *Arabidopsis* PCFs after selection using a micromanipulator. Both branched, curved, or twisted fibrillar sub-structures and densely packed hollow sub-structures were observed.

TEM analyzed the sub-fibril structure of *Betula* PCFs and *Larix* PCFs stored for a long period. The sub-structure was similar to that of fresh *Arabidopsis* PCFs. We found that PCFs stored for a long period under a dried condition could be rehydrated for micromanipulation.

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Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Informed consent

Obtained.

Ethics approval

The Publication Ethics Committee of the Canadian Center of Science and Education.

The journal's policies adhere to the Core Practices established by the Committee on Publication Ethics (COPE).

Provenance and peer review

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The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Data sharing statement

No additional data are available.

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References

Fukumoto, T. (2006). *Analysis of novel callose fiber structures and control of their development in plant protoplast cultures.* Ph.D. thesis of Yokohama National University. pp. 115

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. https://doi.org/10.1007/s00425-005-0065-3

Fukumoto, T., Nakamura, T., Suzuki, M., Ogita, S., Mimura, T., & Sasamoto, H. (2004). Different effects of four salts and pHs on protoplast cultures of a mangrove, *Bruguiera sexangula* suspension cells, *Populus alba* leaves and tobacco BY-2 cells. *Plant Biotechnology, 21*, 177-182. https://doi.org/10.5511/plantbiotechnology.21.177

Hasegawa, A., Kurita, A., Hayashi, S., Fukumoto, T., & Sasamoto, H. (2013). Halophilic and salts tolerant protoplast cultures of mangrove plants, *Sonneratia alba* and *Avicennia alba*. *Plant Biotechnology Reports, 7*, 205-209. https://doi.org/10.1007/s11816-012-0251-2

Hayatsu, M., Ono, O., Hamamoto, H., & Suzuki, S. (2012). Cytochemical and electron probe X-ray microanalysis studies on the distribution change of intracellular calcium in columella cells of soybean roots under simulated microgravity. *Journal of Electron Microscopy, 61*, 57-69. https://doi.org/10.1093/jmicro/dfr095

Hayatsu, M., Suzuki, S., Hasegawa, A., Tsuchiya, S., & Sasamoto, H. (2014). Effect of NaCl on ionic content and distribution in suspension-cultured cells of the halophyte *Sonneratia alba* versus the glycophyte *Oryza sativa*. *Journal of Plant Physiology, 171*, 1385-1391. https://doi.org/10.1016/j.jplph.2014.06.008

Hayatsu, M., Suzuki, S., Tsuchiya, S., & Sasamoto, H. (2017). Cellular calcium distribution modulates the growth of callus and protoplasts of halophyte mangrove plant, *Avicennia alba*- an X-ray microanalysis. *Journal of Plant Studies, 6*, 18-27. https://doi.org/10.5539/jps.v6n2p18

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, PA-003. pp. 96.

Kurita-Tashiro, A., Hayashi, N., Oyanagi, T., & Sasamoto, H. (2020). New Factors for Protoplast-Callose-Fiber Formation in Salt-Tolerant Mangrove Plants, *Avicennia alba* and *Bruguiera sexangula* and Analysis of Fiber Substructures. *Journal of Plant Studies, 9*(2), 1-10. https://doi.org/10.5539/jps.v9n2p1

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum, 15*, 473-497. https://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. https://doi.org/10.1007/s11627-999-0061-6

Oyanagi, T., Hasegawa, A., Fukumoto, T., & Sasamoto, H. (2012b). *Protoplast divisions and protoplast callose fiber formation in Sonneratia caseolaris*. Abstract of 18th Annual Meeting of Japan Mangrove Society, pp. 16.

Oyanagi, T., Hasegawa, A., Sasamoto, H., Hayashi, N., & Fukumoto, T. (2012a). *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 Meeting of the Japan Wood Research Society, pp. 116.

Oyanagi, T., Hayashi, N., Hasegawa, A., & Sasamoto, H. (2024). Mg^{2+} ions stimulate both helical protoplast-callose-fiber formation and protoplast division in a mangrove tree, *Sonneratia caseolaris*: Analysis of sub-fibril structures of PCF by atomic force microscopy. *Journal of Plant Studies, 13*, 1-10. https://doi.org/10.5539/jps.v13n1p1

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. https://doi.org/10.5539/jps.v3n2p23

Oyanagi, T., Kurita, A., Shiraishi, T., & Sasamoto, H. (2017). Development of a method to measure mechanical properties of single elongated callose fibers in protoplast cultures of *Larix leptolepis* and *Betula platyphylla*. *Journal of Plant Studies, 6*, 31-38. https://doi.org/10.5539/jps.v6n1p31

Sasamoto, H., & Hasegawa, A. (2021). *Effects of sucrose and galactose on halophilic nature of Sonneratia mangrove cell culture. P-105.* Proceedings of the 85th Annual Meeting of the Botanical Society of Japan. pp. 205.

Sasamoto, H., Azumi, Y., Shimizu, M., Hachinohe, Y., & Suzuki, S. (2017). In vitro bioassay of allelopathy of *Arabidopsis thaliana* by sandwich method and protoplast co-culture method with digital image analysis. *Plant Biotechnology, 34*, 199-202. https://doi.org/10.5511/plantbiotechnology.17.1204a

Sasamoto, H., Hayatsu, M., & Suzuki, S., (2020a). *Effects of Ca^{2+} and K^{+} ions on cell division and callose-fiber elongation from leaf protoplasts of Arabidopsis thaliana.* Proceedings of the 84th Annual Meeting of the Botanical Society of Japan. pp. 126.

Sasamoto, H., Hayatsu, M., & Suzuki, S. (2020b). High Allelopathic Activity of Carotenoid-accumulating Callus of a Halophilic Mangrove Plant, *Avicennia alba*: Protoplast Co-culture Method with Digital Image Analysis. *Journal of Plant Studies, 9*(1), 1-12. https://doi.org/10.5539/jps.v9n1p1

Sasamoto, H., Murashige-Baba, T., Inoue, A., Sato, T., Hayashi, S., & Hasegawa, A. (2013). Development of a new method for bioassay of allelopathy using protoplasts of a leguminous plant *Mucuna pruriens* with a high content of the allelochemical L-DOPA. *Journal of Plant Studies, 2*(2), 71-80. https://doi.org/10.5539/jps.v2n2p71

Sasamoto, H., & Ogita, S. (2000). *Formation of fiber ribbon structure in protoplast culture of Larix embryogenic suspension cultured cells.* Abstract of 18th annual meeting of Japanese association for plant tissue culture. pp. 153.

Sasamoto, H., Ogita, S., Fukui, M., Yeung, E. C., & Thorpe, T. A. (2001). *Protoplast cultures of Larix leptolepis and Cryptomeria japonica: Factors for fiber ribbon structure formation and for early somatic embryogenesis.* Abstract of 112nd annual meetings of the Japan Forest Research. pp. 660.

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. https://doi.org/10.1079/IVP2002388

Sasamoto, H., Ogita, S., & Mimura, T. (2000). *Protoplast culture and cell fusion in a mangrove, Bruguiera sexangula*. Abstract of 111st annual meeting of the Japan Forest Research. pp. 603.

Seyama, T., Kimura, S., Sasamoto, H., Abe, H., & Kondo, T. (2008). Spinning of a gigantic bundle of hollow fibrils by a spirally moving higher plant protoplast. *Planta, 227*, 1187-1197. https://doi.org/10.1007/s00425-008-0689-1

Tagawa, S., & Kondo, T. (2018). Secretion of a callose hollow fiber from herbaceous plant protoplasts induced by inhibition of cell wall formation. *Journal of Wood Science, 64*, 467-476. https://doi.org/10.1007//s10086-018-1726-8

Wakita, Y., Yokota, S., Sasamoto, H., Hosoi, Y., & Yoshizawa, N. (1993). *Comparison of colony proliferation conditions in leaf protoplast cultures between Betula platyphylla and Populus alba. P2122.* Proceedings of the 43rd annual meeting of the Japan Wood Research Society. pp. 473.