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ORIGINAL ARTICLE

Secretion of a callose hollow fiber from herbaceous plant protoplasts induced by inhibition of cell wall formation

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Abstract

The current study attempts to verify secretion of a callose hollow fiber from plant protoplasts, which was previously reported in woody plants, as a common stressed culture-induced phenomenon among plants, by re-examining herbaceous plant cultured cells of tobacco BY-2 (*Nicotiana tabacum* L. cv. Bright Yellow 2) and *Arabidopsis thaliana* allowing inhibition of cell wall formation. Then, inhibition of cell wall formation was found to induce the secretion of callose fibers by adding isoxaben, an inhibitor of cellulose synthesis, into the stressed culture medium. The inducing period for the secretion from both tobacco BY-2 and *A. thaliana* was shorter than that previously reported in *Betula platyphylla* (white birch). The secreted fibers were observed by confocal laser scanning microscopy and atomic force microscopy. The microscopic images indicated that the callose fibers from herbaceous plants did not necessarily possess a similar structure to hollow fibers from white birch. Eventually, it has been demonstrated that the secretion of callose fibers from protoplasts can be a stress-induced phenomenon commonly occurring in plants, but their hierarchical 3D-fiber structures are likely to depend on species.

Keywords Plant protoplasts · β-1,3-glucan · Callose hollow fiber · Cell wall formation · Stress response

Introduction

Previously, it was found that wood protoplasts secrete a micro-sized fiber consisting of β-1,3-glucan (callose) in the acidic culture media containing high concentration of Ca^{2+} [1–6]. The produced callose fiber from *Betula platyphylla* (white birch) had a hierarchical bundle structure composed of callose hollow sub-fibrils ranging from a few hundred nanometers to a few micrometers in width [4, 6]. In woody plants, callose occurrence, which is usually disordered deposition, is limited to cell plates, pollen tubes, plasmodesmata and protecting wounded cells [7]. In in vitro synthesis of callose using isolated membrane fraction including the callose synthase, a short fibrillar structure with a few 10 nm in width appeared [8–12]. However, the characteristic hierarchical bundle structure in the secreted micro-sized hollow fibers reported by Seyama et al. [4] and Matsuo et al. [6] has not been so far observed except for the cases of woody protoplasts.

To date, this unique phenomenon has been reported only in the protoplasts isolated from woody plant cells such as white birch leaves [1, 2, 4, 5], their callus [6], and embryogenic cells of *Larix leptolepis* (conifer) [2, 3]. Yet, we have not discovered the similar phenomenon in the herbaceous plants. The question remains whether the callose fiber production is a common phenomenon in plants or not. Therefore, the main objective of this study is to examine generality of this phenomenon among plants.

Seyama and Kondo [5] indicated that inhibition of cell wall formation could be one of the key factors for such a production of callose hollow fibers from woody plant protoplasts. They examined possibility of regeneration of cell walls in protoplasts under a stress condition by counting burst ratio of the cultured protoplast cells under a low osmotic pressure depending on the culture time. The 100% burst ratio for the stress-cultured cells was the result all through the culture period. Thus, the authors have assumed that herbaceous plant cells, which can easily and fast regenerate cell walls, could also exhibit the phenomenon when the cell wall formation is restricted.

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In the present article, isoxaben [13, 14], an extremely powerful and specific inhibitor for cellulose biosynthesis, was added to the already investigated culture media to allow herbaceous plants to produce a callose hollow fiber. Suspended cultured cells of tobacco BY-2 (*Nicotiana tabacum* L. cv. Bright Yellow 2) and *Arabidopsis thaliana*, which are representatives of herbaceous plants and model organisms of plants, were used as starting materials. Then, confocal laser scanning microscopy (CLSM) monitored their response to the stress due to the culture media to compare with the cases in protoplasts derived from woody plants reported previously [4–6]. In addition, the hierarchical 3D-fiber structures and their surface morphology were revealed using CLSM with 3D imaging technique and atomic force microscopy.

Materials and methods

Materials

Suspension cultured cells of tobacco BY-2 cell and *A. thaliana* (At wt [15], RIKEN BioResource Center. Tsukuba, Japan) were employed. Tobacco BY-2 and *A. thaliana* cultured cells were cultured in Linsmaier and Skoog (LS) [16] liquid medium containing 30 g/l sucrose, 0.2 g/l KH_2PO_4, 1 mg/l thiamine-HCl, 0.1 g/l myo-inositol and 0.2 mg/l 2,4-dichlorophenoxyacetic acid, at 25 °C under a continuous dark condition. They were sub-cultured in a fresh LS medium every 7 days.

Preparation of protoplasts

Suspension cells of tobacco BY-2 and *A. thaliana* cultured under the above condition for 4–5 days were used for preparation of protoplasts. A modified procedure of the previous one [6] was carried out as follows: The protoplasts were isolated by a treatment with 0.6 M mannitol solution containing 1% cellulase Onozuka R-S (Yakult, Co., Ltd., Tokyo, Japan) and 1% Macerozyme R-10 (Yakult). Following the enzymatic treatment, the suspension passed through a sheet of nylon mesh with a square pore size of 40 μm² to obtain the protoplasts. After washed with 0.4 M mannitol, the protoplast precipitates were collected by centrifugation (800 rpm, 150*g*, 5 min). Then, an appropriate amount of fresh 0.4 M mannitol solution was added again for further centrifugation, and this treatment was repeated twice.

Protoplast culture

Table 1 lists the culture conditions used in this study. The normal culture condition (Condition 1) was the LS medium described above including 0.4 M mannitol with pH adjusted to 5.8. Culture conditions of 2–7 indicate the culture media including additional 100, 150 and 200 mM $CaCl_2$ with a pH adjusted to 3.5 without mannitol, respectively. For the culture conditions 5–7, 3 μM of isoxaben (Sigma-Aldrich Co. LLC, Tokyo, Japan) were added. The culture solutions from isoxaben stock in ethanol were freshly diluted by the culture medium before use. Then, the cell densities were adjusted to approximately 5×10^4 cells/ml in the culture well (equivalent to 2500 cells per well), and the incubation was performed at 28 °C in the dark. All protoplasts were cultured in 96 well microplate (Corning, Inc., NY, USA), so that they were directly observed under an inversed light microscope (CK40, Olympus Co., Tokyo, Japan).

Table 1 Culture conditions employed for tobacco BY-2 and *A. thaliana* protoplasts

	Culture medium	Additive
Condition 1	LS, 0.4 M mannitol, pH 5.8	–
Condition 2	LS, 100 mM $CaCl_2$, pH 3.5	–
Condition 3	LS, 150 mM $CaCl_2$, pH 3.5	–
Condition 4	LS, 200 mM $CaCl_2$, pH 3.5	–
Condition 5	LS, 100 mM $CaCl_2$, pH 3.5	3 μM isoxaben
Condition 6	LS, 150 mM $CaCl_2$, pH 3.5	3 μM isoxaben
Condition 7	LS, 200 mM $CaCl_2$, pH 3.5	3 μM isoxaben

Confocal laser scanning microscopy

Protoplasts cultured under several conditions described above were transferred into the glass bottom 96 well dish (AGC Asahi Glass, Tokyo, Japan) for confocal laser scanning microscopy (CLSM) (Leica SP8; Leica Microsystems, Wetzlar, Germany). Then, both 10 μg/ml of calcofluor white M2R (CW: Sigma-Aldrich) and 10 μg/l of aniline blue fluorochrome (ABF: Biosupplies Australia, Pty. Ltd., Victoria, Australia) were added prior to the incubation for over 10 min. CW stains both β-1,4-glucan (cellulose) and β-1,3-glucan (callose), while ABF stains callose specifically. The pH in the medium with CW was adjusted to approximately 6.0 [17] by 1% KOH solution before CLSM observation. CW and ABF were excited at a wavelength of 405 nm in the irradiated laser beam. Fluorescence emission of CW or ABF was detected in the range of 430–470 or 430–600 nm in wave length, respectively.

Atomic force microscopy

Fibers secreted from protoplasts were observed by atomic force microscopy (AFM). Samples for AFM were prepared by washing with 50 mM methylenediaminetetraacetic acid (pH 7.0) and distilled water in three times, respectively. For observation, they were put on a microscope slide and then air-dried.

Atomic force microscopy images for surfaces of the dried sample were acquired with a MFP-3D-SA (Oxford Instruments Asylum Research, Inc., California, USA) operated at room temperature, being controlled in the non-contact mode with a scan rate 1.0 Hz to observe $15 \times 15\ \mu m^2$ areas. The AFM tip employed was an etched-silicon AFM tip (OMCL-AC240TS-C3, Olympus, Tokyo, Japan) with a nominal radius from to 7 nm, and a spring (force) constant of $2\ N\ m^{-1}$.

Image analyses

Confocal laser scanning microscopy images were processed using LAS X (Leica Microsystems, Wetzlar, Germany) and an Image J/Fiji (W. Rasband, National Institutes of Helth, Bethesda, MD) software. 3D and sectioning images were provided by integrating of sequence images of z stack in CLSM using the LAS X. Projection images were provided by integration of sequence images of z stack in CLSM using the Image J/Fiji. Cell wall formation ratio (%) was calculated based on fluorescent CLSM images, which corresponds to a ratio of cells having cell walls to the number of all cells present in the image.

Results and discussion

Secretion of callose fibers from protoplasts of tobacco BY-2 and *A. thaliana*

Protoplasts isolated from white birch leaves can proceed with cell divisions via cell wall formation under the normal condition medium; namely Murashige and Skoog liquid medium with half the strength of the standard including hormones [4]. In contrast, addition of 100–300 mM of Ca^{2+} into the medium under an acidic condition was found to induce production of a callose hollow fiber, instead of cell division for the *Betula* protoplasts [4]. Matsuo et al. [6] used the protoplasts isolated from cultured cells of callus of white birch, instead of the native cells. The protoplasts also produced callose hollow fibers in the media including Ca^{2+} in the concentration range of 100–200 mM. In general, isolated plant protoplasts are supposed to undergo cell division soon after formation of cell walls [18]. Therefore, the higher concentration of Ca^{2+} in the culture medium appears to be a critical factor to inhibit cell wall formation, but to induce secretion of the callose hollow fibers. Moreover, the acidic condition at pH 3.5 strongly influenced the phenomenon [4, 6]. Thus, the current study employed a series of media containing various concentrations of Ca^{2+} at pH 3.5 (see Table 1). Protoplasts in this study were isolated from cultured cells of herbaceous plants of tobacco BY-2 and *A. thaliana*, and cultured under the same concentration range of 100–200 mM used previously [6]. Then, isoxaben, an inhibitor of cellulose biosynthesis, was added into the culture media to allow suppression of cell wall formation to promote the fiber production as seen in woody plants [4–6].

As the result, production of micro-sized fiber appeared in both of species under the listed conditions except condition 1 as a normal culture condition for cell division (Fig. 1A, B). The fibers exhibited fluorescent emission at 430–600 nm when stained with ABF, which specifically stains β-1,3-glucan. This indicated that the secreted micro-sized fiber was composed of β-1,3-glucan (callose) (also see Fig. 6).

In protoplasts of tobacco BY-2, the fiber production was found after 3 weeks of the culture. Under pH 3.5 with 100 mM $CaCl_2$ (Cond. 2 in Fig. 1C), an efficient fiber formation occurred. Furthermore, adding isoxaben showed more efficiency in the fiber formation (Cond. 5–7 in the same image). In contrast, protoplasts of *A. thaliana* secreted a fiber under Cond. 2–4 in Fig. 1D without isoxaben after 2 weeks of the culture. With isoxaben addition (Cond. 5–7), the efficiency in the fiber secretion relatively increased.

These results in both cases suggest that the addition of isoxaben promoted the callose fiber production. It is noted that the tendency of fiber secretion in *A. thaliana* was similar to that for protoplasts of white birch callus [6]. Namely, the optimal concentration of $CaCl_2$ for producing the fiber was assumed as 100–200 mM as seen in protoplasts of white birch callus [6] (Fig. 1D).

Concerning inducing period for the fiber production, protoplasts of white birch leaves required 2 months [4], which was longer than 1 month for the protoplasts of white birch callus [6]. On the other hand, the corresponding period for tobacco BY-2 and *A. thaliana* was much shorter. The above information indicated that degree of response of the ion stress might depend on plant species. There may be two possibilities to cause the difference; one is difference in woody and herbaceous plants and the second is due to either native or cultured cells. In any event, the life cycle for such herbaceous plants may be a key factor to induce an earlier secretion of the fiber. In fact, protoplasts of *A. thaliana* were dead earlier than tobacco BY-2 and white birch (data was not shown).

Cell wall formation on herbaceous plant protoplasts

To investigate dependence of inhibition of cell walls upon occurrence of callose fibers, CLSM observation was carried out for cells stained with a fluorescent brightener, CW M2R. The fluorescent image on the cells indicated presence of cell wall components, e.g., β-1,4-glucan and/or β-1,3-glucan. Typical CLSM images are shown in Fig. 2 for both tobacco BY-2 and *A. thaliana* cultured cells, respectively. In addition, CLSM observation at a low magnification was performed to calculate the proportion of cells forming cell

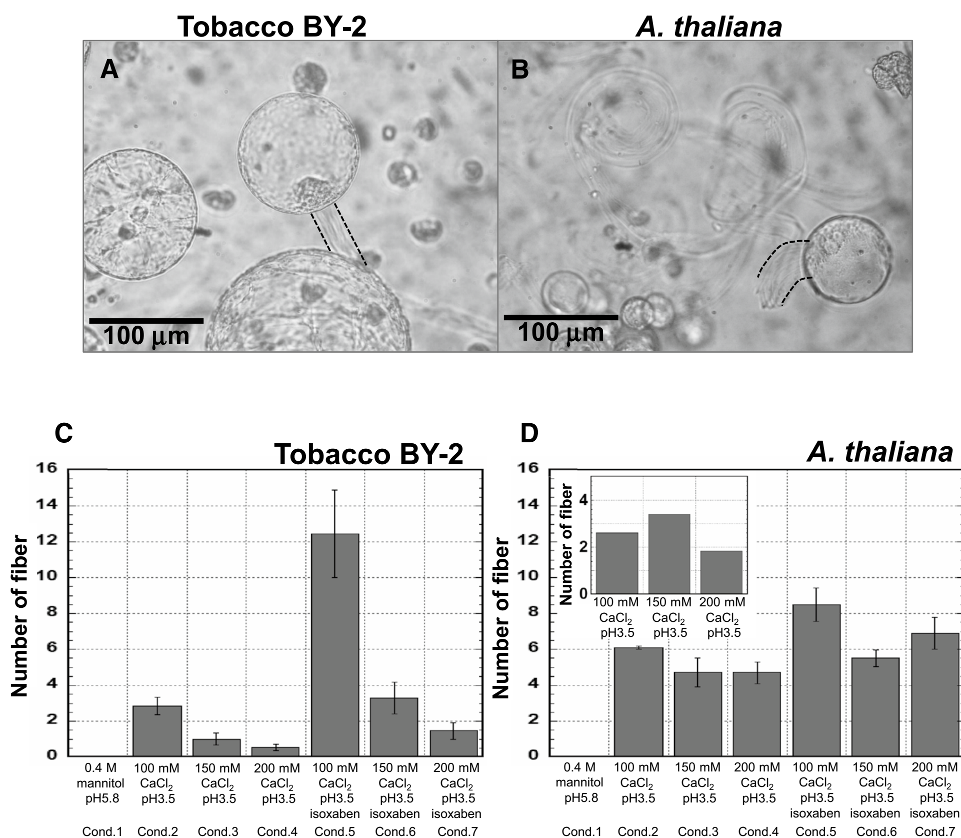

Fig. 1 Optical microscopic images and number of callose fibers secreted from protoplasts of tobacco BY-2 at 3 weeks' culture (**A, C**) and *A. thaliana* at 2 weeks' culture (**B, D**) under several conditions. Dashed lines in **A** and **B** indicate the secreted fibers. The inset in **D** represents numbers of callose fibers secreted from protoplasts of white birch (*Betula platyphylla*) reported previously [6]. The number indicates the average number of fibers per well. Cond. 1–Cond. 7 indicate from condition 1 to condition 7 in Table 1, respectively

walls based on the images for tobacco BY-2 at 3 weeks' culture as well as *A. thaliana* at 2 weeks' culture, respectively (Fig. 3). As a reference, cells at the first day of the culture in the condition 1 were also observed by CLSM as shown in Fig. 2A, E, respectively. Protoplasts of both tobacco BY-2 and *A. thaliana* did not exhibit cell wall formation in the first day of culture under the normal condition.

At 3 weeks' culture of tobacco BY-2 cells, cell elongation, cell division and formation of colonies of cells occurred. Moreover, cells were entirely covered with CW fluorescent emission (Fig. 2B), indicating that almost all cells allowed cell wall formation during the 3 weeks under the normal condition, which corresponds to the column of Cond. 1 in Fig. 3A. In the medium containing a high concentration of $CaCl_2$ (Fig. 2C), protoplasts expanded, and some protoplasts formed cell walls, but in fact mixture of both cell-walled (Fig. 2C) and non-cell walled cells (Fig. 2D) appeared. The similar mixture also appeared under the culture conditions 2–4. It is noted that elongation of cells was not observed under these conditions. When isoxaben was added into the medium, most cells were similar to the non-cell-walled cells in Fig. 2D as a representative. The frequency of cell wall formation was less than 20% (Cond. 5–7 of Fig. 3A).

At 2 weeks' culture under the normal condition (Cond. 1) of *A. thaliana*, cell division and colony formation accompanied with cell wall formation appeared (Fig. 2F). Similarly, mixture of cell-walled (Fig. 2G) and non-cell walled cells (Fig. 2H) appeared in the culture medium containing 100 mM $CaCl_2$ (Cond. 2). In the condition containing 150 and 200 mM $CaCl_2$ (Cond. 3 and 4), cell walls hardly appeared on the cultured cells (Fig. 3B). As shown in Fig. 2H, when isoxaben was added into the medium containing Ca^{2+}, most cells exhibited a similar cell image without cell wall formation.

Isoxaben is generally believed to inhibit cellulose synthesis by causing clearance of cellulose synthase A proteins (CesA) from plasma membrane [19, 20]. Since cellulose synthase isoform CES6 is supposed to discourage the activity due to presence of isoxaben in *A. thaliana* [21], the similar effect worked possibly for tobacco BY-2. Incidentally, when cultured under isoxaben added normal condition without $CaCl_2$, cell wall occurrence and secretion of the callose fibers were hardly observed in the protoplasts of tobacco BY-2 and *A. thaliana* (data was not shown). Therefore, addition of either $CaCl_2$ or isoxaben is insufficient to effective inhibition of cell wall formation in both cultured cells. Namely, the simultaneous addition of $CaCl_2$ and isoxaben is effective for inhibiting cell wall formation, which conversely induces the formation of the callose fibers. In the case of protoplasts of white birch leaves [5], cell wall formation was inhibited under a

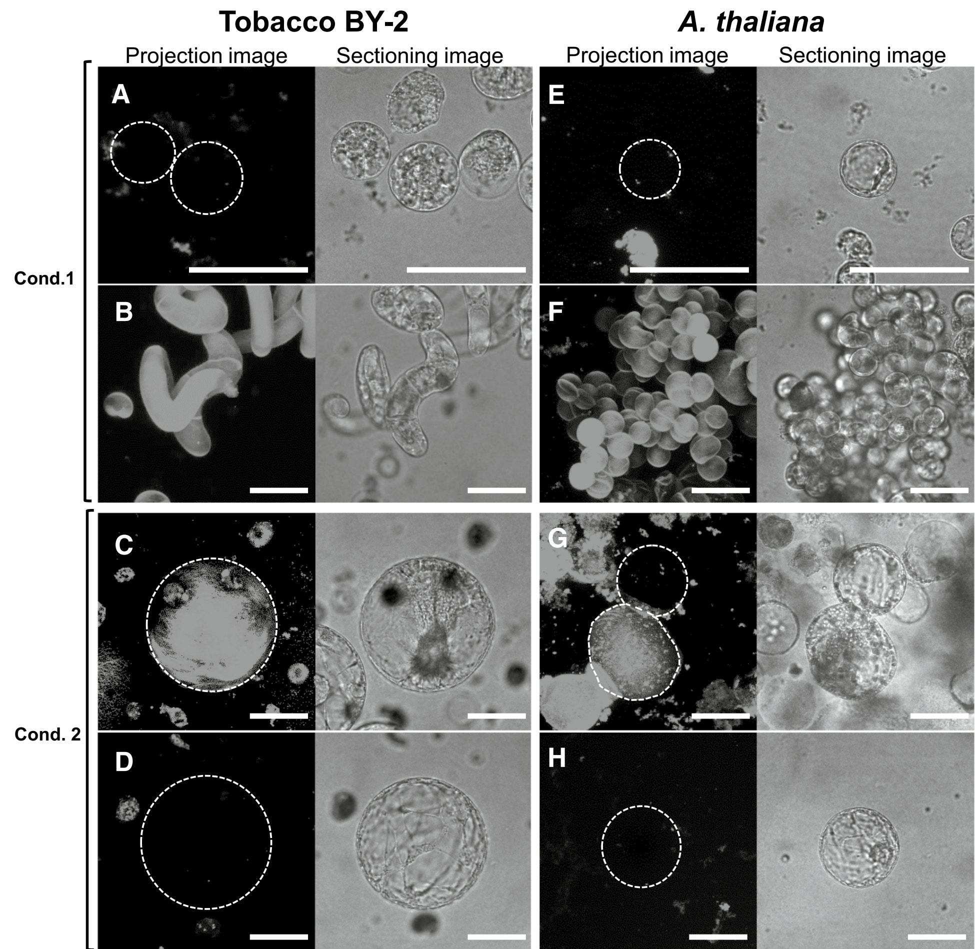

Fig. 2 CLSM projection and sectioning images of protoplasts of tobacco BY-2 (**A**–**D**) and *A. thaliana* (**E**–**H**) under condition 1 (**A, B** and **E, F**) and condition 2 (**C, D** and **G, H**), respectively. A and E are the first days' culture. Tobacco BY-2 cells (**B**–**D**) were cultured for 3 weeks, while *A. thaliana* cells (**F**–**H**) were cultured for 2 weeks. Cell walls were stained with CW. All scale bars indicate 50 µm, respectively. Cond. 1 and Cond. 2 indicate condition 1 and condition 2 in Table 1, respectively

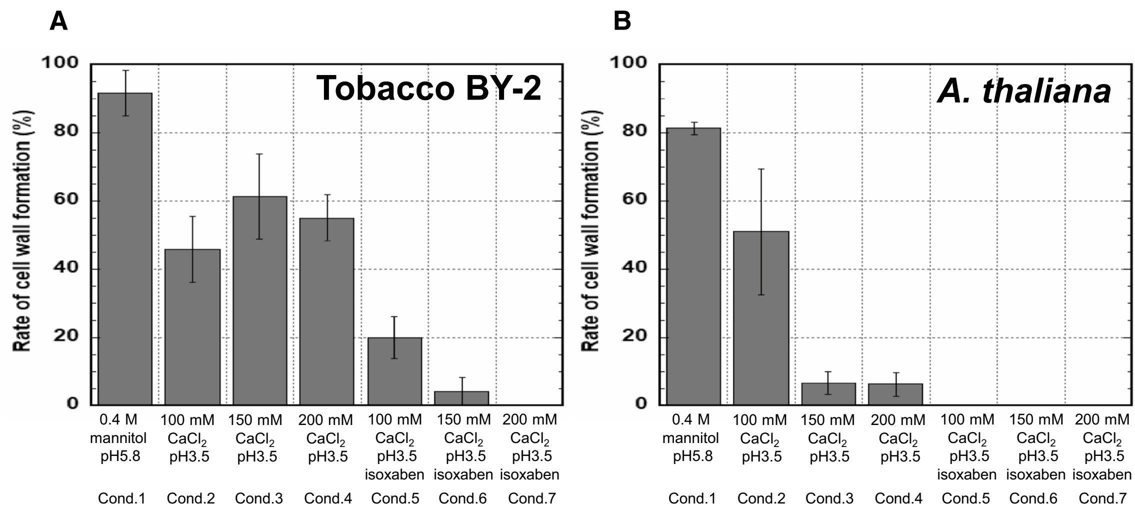

Fig. 3 Cell wall formation ratio calculated based on CLSM images of protoplasts of tobacco BY-2 in 3 weeks' culture (**A**) and *A. thaliana* in 2 weeks' culture (**B**) under conditions of Cond. 1–Cond. 7 corresponding to condition 1 to condition 7 in Table 1, respectively

medium containing 200 mM $CaCl_2$, which was similar to the protoplasts *A. thaliana* in the present study.

In both cases with tobacco BY-2 and *A. thaliana*, adding $CaCl_2$ was likely to reduce cell wall formation, but did not completely inhibit as shown in Cond. 2–4 of Fig. 3. This result indicated that a large excess addition of $CaCl_2$ to the medium resulted in gentle inhibition of cell wall formation of tobacco BY-2 and *A. thaliana*, respectively. Furthermore, addition of isoxaben assisted further suppression of cell wall formation. Under the culture conditions to restrict cell wall formation as described above, the secretion of callose fibers occurred. Therefore, it appears to be some correlation between inhibition of cell wall and producing the callose fiber in tobacco BY-2 and *A. thaliana*, similarly to woody plants [5]. In other words, suppression of cell wall formation is likely to induce the secretion of the callose fibers.

Concerning *A. thaliana*, the results of stress response showed in Figs. 1D and 3B leads to the hypothesis that relationship between *A. thaliana* and white birch is closer than that between tobacco BY-2 and white birch in systematic taxonomy by taking into account of Angiosperm Phylogeny Group classification [22].

Expansion of herbaceous plant protoplasts by culture under stressed conditions

In previous papers [5, 6], diameter size in enlarged protoplasts was supposed to correlate with the initiation of the fiber secretion. Namely, as soon as protoplasts reached 100 µm in diameter, they started to secrete a fiber in white birch's protoplasts. In the current study, changes of the cell diameter were also monitored in the protoplasts of tobacco BY-2 and *A. thaliana*, respectively. Figure 2A, E in the first day of culture showed that the cells of tobacco BY-2 and *A. thaliana* became approximately 30 and 20 µm in diameter, respectively. As protoplasts cultured in the normal condition generated colony, their individual diameters were hardly measured.

Following the cell expansion, fiber production was observed in tobacco BY-2 at 3 weeks' culture. The cell diameter reached approximately 100 µm in the optimal condition for production of the fiber, which was the condition including 100 mM $CaCl_2$ and isoxaben (Cond. 5 of Fig. 4A). Meanwhile, the diameter of cells in the conditions including 150 and 200 mM $CaCl_2$ (Cond. 6 and 7 in the same figure), which exhibited a lower induction ratio of the fiber secretion, was smaller than that in the optimal condition. A higher induction ratio is likely to correspond to higher expansion degrees of cells, which may also relate to a high activity of cells.

In *A. thaliana*, the fiber production occurred at 2 weeks' culture. The cell diameter reached approximately 50 µm all through the conditions employed (Cond. 2–7 of Fig. 4B).

The above results suggested that the cell expansion at higher concentrations of $CaCl_2$ was a common phenomenon in plants. However, the size and the induction period for production of fibers depended on the plant species. As an example of higher plants, callose fiber production of protoplasts isolated from white birch leaves started after 2 months of culture and the cell diameter became 100–200 µm [5]. In contrast, the tobacco BY-2 protoplasts required 3 weeks to reach 100 µm in diameter, which grew much faster than the case for white birch protoplasts. Furthermore, *A. thaliana* was found to produce fibers with the cell diameters less than 100 µm.

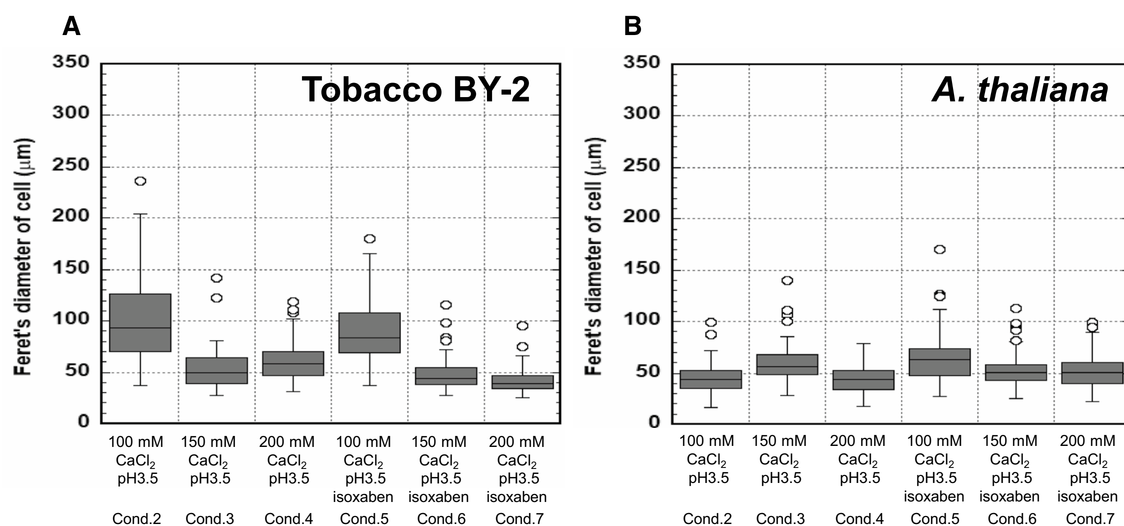

Fig. 4 Box plots of Feret's diameter of cells of tobacco BY-2 in 3 weeks' culture (**A**) and *A. thaliana* in 2 weeks' culture (**B**) under conditions of Cond. 2–Cond. 7 corresponding to condition 2 to condition 7 in Table 1, respectively

Morphology of callose fibers secreted from protoplasts of BY-2 and *A. thaliana*

The hierarchical 3D-fiber structures and the surface morphology of the secreted callose fibers were investigated using optical microscopy, CLSM and AFM. Figure 5 shows averaged width of the callose fibers of ca. 12 and 8 µm produced from protoplasts of tobacco BY-2 and A. t*haliana*, respectively. In the previous reports [5, 6], the fiber width of protoplasts derived from native birch leaves was about 30 µm [5], whereas that derived from white birch callus was about 14 µm [6]. Considering the above, herbaceous plants are likely to secrete a thinner fiber than the fiber from woody plants.

Previously protoplasts of native white birch leaves and callus found to secrete fibers having hierarchical structures composed of a bundle of hollow sub-fibrils [4, 6]. The current study using CLSM with 3D imaging and AFM surface imaging techniques has revealed difference of the 3D-fiber structures. Namely, the CLSM and AFM observations were capable of providing information on the non-destructive fibers secreted from protoplasts. In particular, it is possible to observe the in situ fibers in the culture medium using CLSM. In the case of white birch, secreted fibers were composed of a bundle of hollow sub-fibrils (Fig. 6A) as previously reported [4, 6]. On the other hand, in the case of tobacco BY-2 (Fig. 6B), most secreted fibers exhibited a single hollow structure having 10 µm in width, which was slightly thinner than a bundle of hollow sub-fibrils from white birch, and which had thicker fiber walls than those appeared in other conditions. Some fibers appeared to comprise a bundle of hollow sub-fibrils like the fiber secretion case form white birch leaves, but the number or probability of the sub-fibrils was much smaller (data was not shown). In the case of *A. thaliana* as shown in Fig. 6C, it was hard to judge presence of the hollow situation as it was densely packed as a fiber. The width of the secreted fiber was almost the half size (5 µm in width) when compared with the other cases (see Fig. 6C).

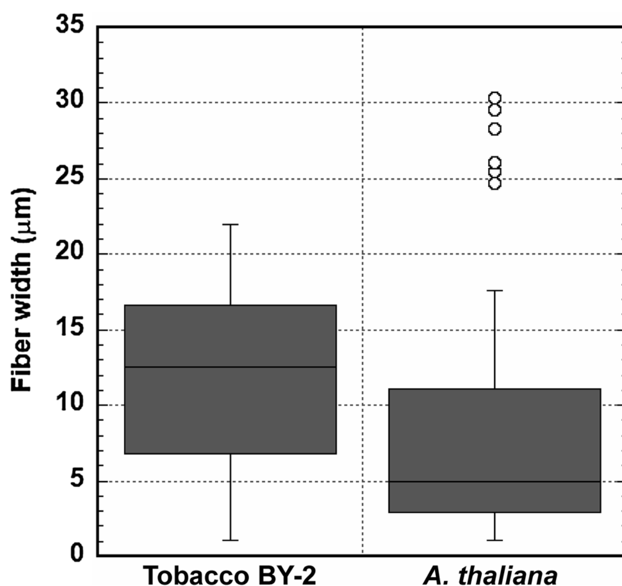

Fig. 5 Box plots of width of callose fibers secreted from tobacco BY-2 in 3 weeks' culture and *A. thaliana* in 2 weeks' culture, respectively

In addition, AFM observation indicated difference of fiber surfaces among the species. Root mean square roughness (RMS) of air-dried fiber specimens of white birch, tobacco BY-2 and *A. thaliana* were 31.549 nm (Fig. 7A), 130.747 nm (Fig. 7B) and 54.563 nm (Fig. 7C), respectively. This result suggested that fibers secreted from tobacco BY-2 and *A. thaliana* exhibited rougher surfaces than that of white birch's fiber. Thicker wall of fiber in tobacco BY-2 and denser packing of sub-fibrils in *A. thaliana* as shown in the CLSM images of Fig. 6B, C may cause the surface differences in the structural roughness.

Seyama et al. [4] reported that some granules clearly appeared on the secretion site of a bundle of callose hollow fibrils on the protoplast surface. The individual hollow fibril seemed to be secreted from a single sphere granule in the assembled state located on a spot of the cell surface. The granules connected to the end of secreted fibers are most likely to be complexes of some callose synthases that are a kind of membrane proteins. Considering this, there were two hypotheses in reason for the difference of fiber structure: (1) arrangement of callose synthases in plasma membrane were different between species, (2) fibers were composed of not only callose but also other component such as cellulose. More detailed characterization will be required in the future.

Conclusion

In our previous studies [1, 4–6], woody plants secreted callose hollow fibers under the stress condition with excess Ca^{2+} at the acidic condition of pH 3.5, and this phenomenon has been limited to woody plants. The current study attempts to confirm that the callose fiber secretion also occurs in herbaceous plant protoplasts when the cell wall formation is inhibited. Therefore, the appearance of fibers in tobacco BY2 and *A. thaliana* indicates it to be a common phenomenon among plant protoplasts due to the stressed culture condition. However, the hierarchical 3D-fiber structures and their surface morphology were not necessarily similar among species. This also indicates that arrangements of callose synthase in the plasma membrane or components of fibers may be different among species.

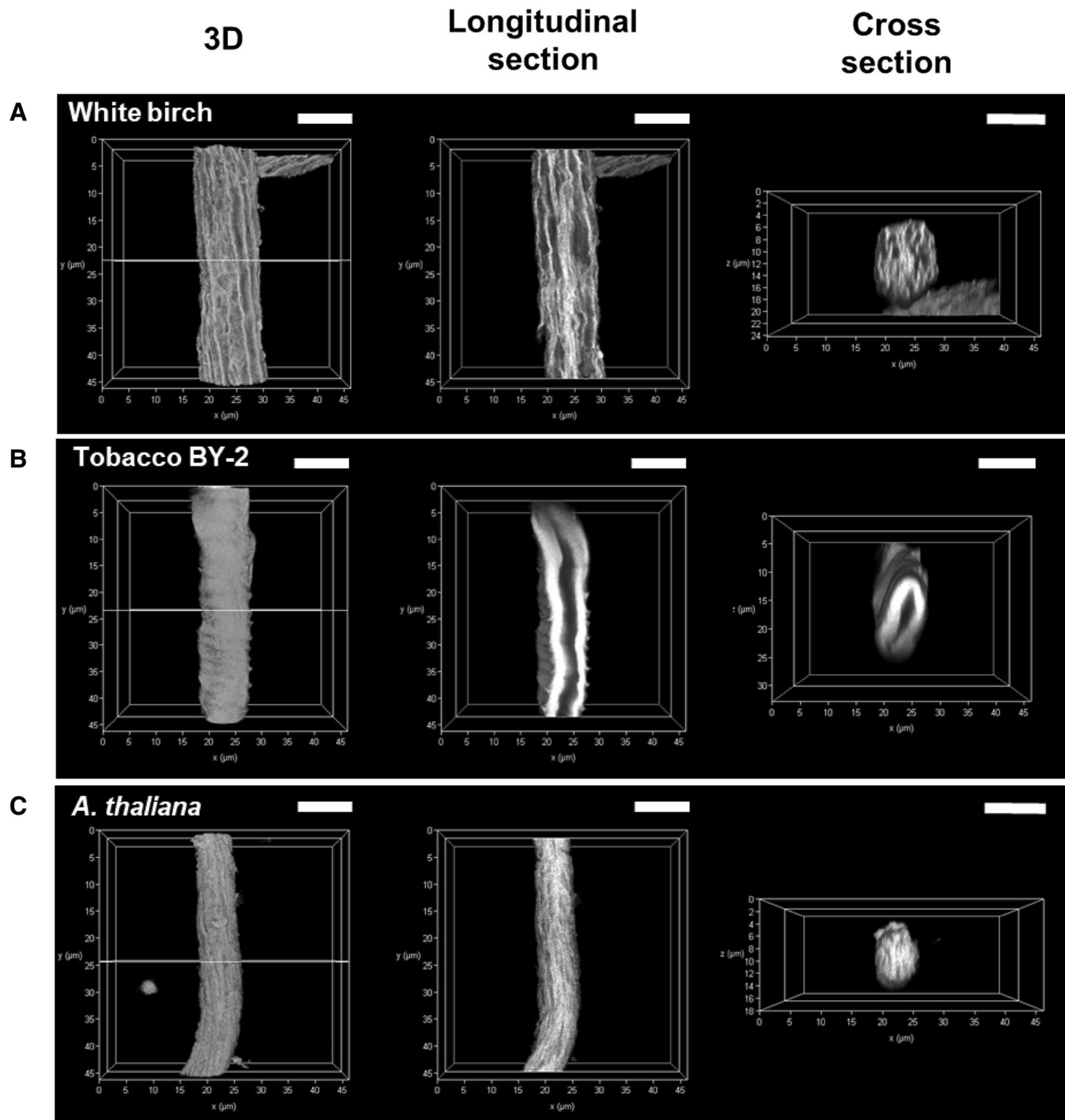

Fig. 6 CLSM 3D and sectioning images of fibers secreted from protoplasts of white birch (**A**), tobacco BY-2 (**B**) and *A. thaliana* (**C**). Fibers were stained with ABF. The individual lines in the images indicate sectioning planes. All scale bars indicate 10 μm

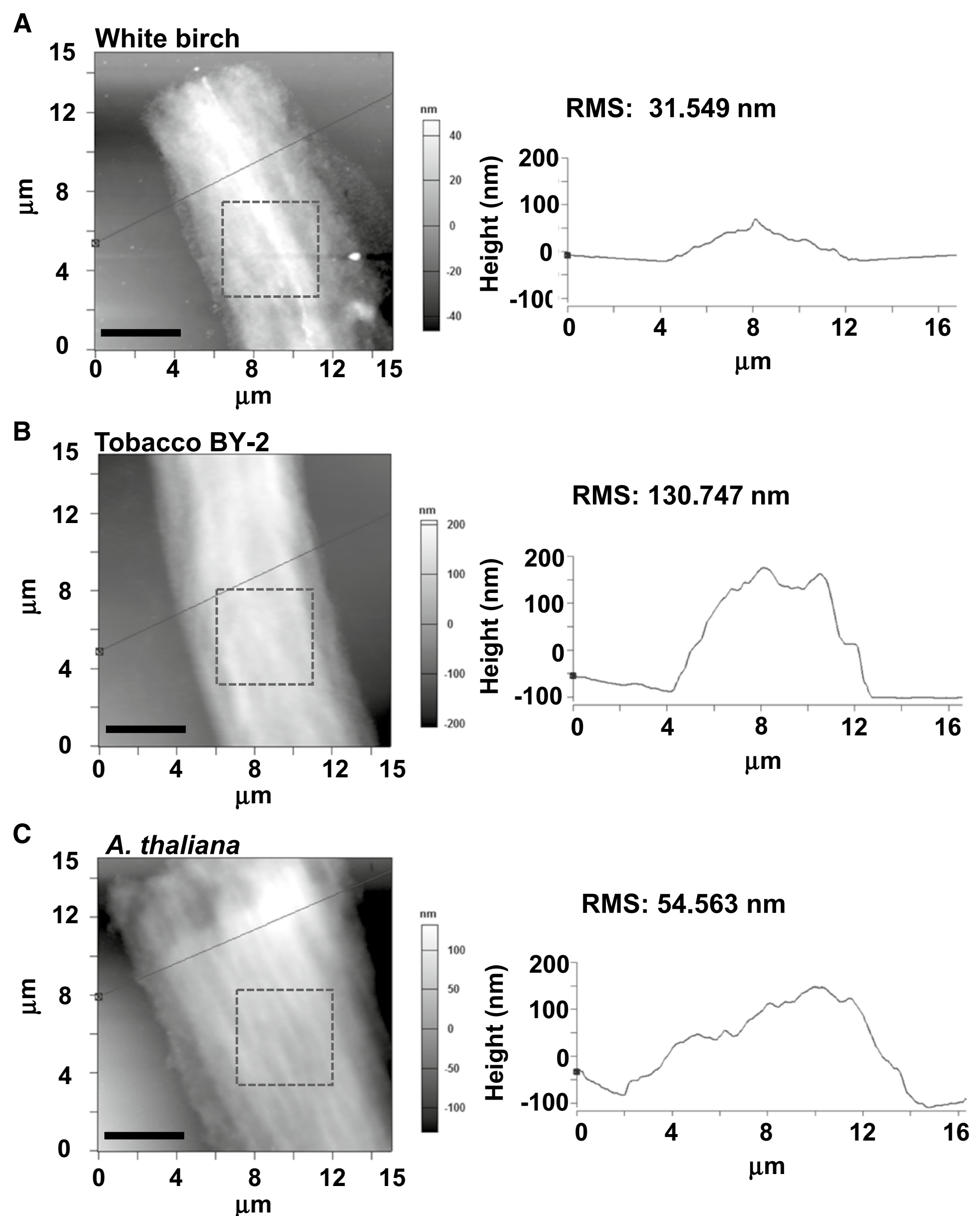

Fig. 7 AFM images of surfaces on fibers secreted from protoplasts of white birch (**A**), tobacco BY-2 (**B**) and *A. thaliana* (**C**). Left images correspond to AFM tapping mode images together with the right graphs indicating the cross-sectioning height information along the lines in the left images. RMS indicates root mean square roughness of the areas at the broken squares in the left images. All scale bars indicate 4 µm

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References

1. Kondo T, Magoshi J, Abe H, Sasamoto H (2000) Method for producing of non cellulosic callose fibers from protoplasts and the callose fiber. Japan Patent No. 3936522 (Application 2000-220419)
2. Sasamoto H, Ogita S, Hayashi N, Wakita Y, Yokota S, Yoshizawa N (2003) Development of novel elongated fiber-structure in protoplast cultures of *Betula platyphylia* and *Larix leptolepis*. In Vitro Cell Dev Biol Plant 39:223–228
3. 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
4. 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
5. Seyama T, Kondo T (2012) Morphological responses of *Betula* protoplasts in fiber spinning. Holzforschung 66:407–411
6. Matsuo S, Takenaga A, Seyama T, Kondo T (2013) Secretion of a bundle of (1→3)-*β*-glucan hollow fibrils from protoplasts of

callus suspension under a Ca^{2+}-rich and acidic stressed condition. Holzforschung 68:69–73
7. Bacic A, Fincher GB, Stone BA (2009) Chemistry, biochemistry, and biology of (1→3)-*β*-glucans and related polysaccharides. Elsevier, Saint Louis
8. Hayashi T, Read MS, Bussell M, Lin CF, Brown MR Jr, Delmmer P (1987) UDP-glucose: (1→3)-*β*-glucan synthases from mung bean and cotton differential effects of Ca^{2+} and Mg^{2+} on enzyme properties and on macromolecular structure of the glucan product. Plant Physiol 83:1054–1062
9. Bulone V, Geoffrey FB, Bruce SA (1995) In vitro synthesis of a microfibrillar (1–3)-*β*-glucan by a ryegrass (*Lolium multiflorum*) endosperm (1–3)-*β*-glucan synthase enriched by product entrapment. Plant J 8:213–225
10. Pelosi L, Imai T, Chanzy H, Heux L, Buhler E, Bulone V (2003) Structural and morphological diversity of (1→3)-*β*-D-glucans synthesized in vitro by enzymes from *Saprolegnia monoïca*. Comparison with a correspondingin vitro product from blackberry (*Rubus fruticosus*). Biochemistry 42:6264–6274
11. Him J, Pelosi L, Chanzy H, Putaux J, Bulone V (2001) Biosynthesis of (1→3)-*β*-D-glucan (callose) by detergent extracts of a microsomal fraction from *Arabidopsis thaliana*. Eur J Biochem 268:4628–4638
12. Cifuentes C, Bulone V, Emons A (2010) Biosynthesis of callose and cellulose by detergent extracts of tobacco cell membranes and quantification of the polymers synthesized in vitro. J Integr Plant Biol 52:221–233
13. Heim DR, Skomp JR, Tschabold EE, Larrinua IM (1990) Isoxaben inhibits the synthesis of acid insoluble cell wall materials in *Arabidopsis thaliana*. Plant Physiol 93:695–700
14. Fisher DD, Cyr JR (1998) Extending the microtubule/microfibril paradigm. Plant Physiol 116:1043–1051
15. Nishikiori M, Mori M, Dohi K, Okamura H, Katoh E, Naito S, Meshi T, Ishikawa M (2011) A host small GTP-binding protein ARL8 plays crucial roles in tobamovirus RNA replication. PLoS Pathog 7:e1002409
16. Linsmaier E, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol Plant 18:100–112
17. Roncero C, Durán A (1985) Effect of calcofluor white and congo red on fungal cell wall morphogenesis: in vivo activation of chitin polymerization. J Bacteriol 163:1180–1185
18. Nagata T, Takebe I (1970) Cell wall regeneration and cell division in isolated tobacco mesophyll protoplasts. Planta 92:301–308
19. Paredez A, Somerville C, Ehrhardt D (2006) Visualization of cellulose synthase demonstrates functional association with microtubules. Science 312:1491–1495
20. Gutierrez R, Lindeboom J, Paredez A, Emons A, Ehrhardt D (2009) *Arabidopsis* cortical microtubules position cellulose synthase delivery to the plasma membrane and interact with cellulose synthase trafficking compartments. Nat Cell Biol 11:797–806
21. Desprez T, Vernhettes S, Fagard M, Refrégier G, Desnos T, Aletti E, Py N, Pelletier S, Höfte H (2002) Resistance against herbicide isoxaben and cellulose deficiency caused by distinct mutations in same cellulose synthase isoform CESA6. Plant Physiol 128:482–490
22. The Angiosperm Phylogeny Group (2016) An update of the angiosperm phylogeny group classification for the orders and families of flowering plants: APG IV. Bot J Linn Soc 181: 1–20