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# Morphological responses of *Betula* protoplasts in fiber spinning

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## Abstract

In a previous article, it was reported that, under stress conditions caused by $Ca^{2+}$ ions, isolated protoplasts of *Betula platyphylla* leaves release a bundle of hollow fibrils as a stress-induced response. In the present article, details of this phenomenon have been investigated. As a special method, light microscopy combined with a 'through focus variation' technique was applied. In a regular culture medium, protoplasts could regenerate the cell walls, whereas protoplasts submitted to $Ca^{2+}$ stress could not. Under stress conditions, the protoplasts inflated and formed a huge vacuole and, after 28 days, secreted the peculiar gigantic fibril with a diameter of 150 μm. The giant fibril is composed of (1→3)-β-glucan chains, i.e., the material of callose tissue.

**Keywords:** inhibited cell wall formation; macrofibril formation; morphological responses to stress; protoplast of *Betula platyphylla*; stressed culture condition.

## Introduction

A stressed culture condition was found to induce fiber spinning in a higher plant protoplast (Kondo et al. 2000; Sasamoto et al. 2003; Fukumoto et al. 2004, 2005; Seyama et al. 2008). The stress was triggered by a high concentration of $Ca^{2+}$ ions added to a modified culture medium, MS, as described by Murashige and Skoog (1962) at pH 3.5 instead of pH 5.8 of normal culture conditions. Protoplasts isolated from leaves of white birch (*Betula platyphylla* Sukatchev var. *japonica*) under stress conditions exhibited cell expansion and secreted a gigantic bundle of hollow fibrils directly into the medium (Seyama et al. 2008). Under these circumstances, cell division was inhibited. The macrofibrils consisted of (1→3)-β-glucan chains – the raw material of callose, which is well known as a response to wounding, pathogen infection and mechanical damage (Nedukha 1998; Lherminier et al. 2003; Majewska-Sawka and Munster 2003). Callose synthesis does not involve the formation of a long fiber-like structure, as seen in the present study; short fibrillar structures are more typical (Colombani et al. 2004). The observation of the long, large fibrils can be interpreted as a type of adaptation process of plant cells under stressed culture conditions.

The development of protoplasts in plantlets is usually initiated by cell wall formation, starting from the synthesis of cellulose microfibrils under regular MS culture conditions. Cell walls in plants are not only for mechanical protection, but also for growth regulation (Martin et al. 2001; Kaczkowski 2003). This essential first step towards plant development is inhibited in the case of the formation of giant fibrils.

In the present study, we attempted to clarify the diameter of a protoplast before giant fibril synthesis. The expectation was that images created by the method 'through focus variation' would reveal new details of the process.

## Materials and methods

The plantlet of *B. platyphylla* Sukatchev var. *japonica* Kan No. 8 was cultivated under sterilized conditions in an agar medium (MS) described by Murashige and Skoog (1962), including hormones, with 2.5 μM indol-3-butyric acid (IBA) and 0.1 μM 1-naphthaleneacetic acid (NAA) for the isolation of protoplasts from the leaf tissue. Irradiation of light was in the visible wavelength range with 3000–4000 lux in strength. Irradiation intervals were alternately: 16 h irradiation and 8 h dark period at 25°C.

### Protoplast isolation

Protoplasts were isolated from young *B. platyphylla* leaves by the treatment of 3–4 leaves with 20 ml of 0.6 M mannitol solution containing 1% of cellulase ONOZUKA R10 (Yakult HONSHA Co., Ltd, Tokyo, Japan) and 1% of driselase (Kyowa Hakko Kogyo Co., Ltd, Tokyo, Japan). The leaves were immersed in the solution for 18 h without shaking. After the treatment, the leaves were transferred into a 0.6 M mannitol solution free of enzymes. The leaves were twitched gently with tweezers in the mannitol solution to liberate the protoplasts. The suspension containing protoplasts was filtered on a nylon sieve with a pore size of 40 μm. Following the second and third washing, the isolated protoplasts were collected as precipitates by centrifugation with a constant rotating rate of 800 r.p.m. for 3 min. Then, a fresh mannitol solution was added and the centrifugation was repeated twice. Finally, 1 ml of mannitol solution was added to pick up the remaining protoplasts from the solution. An aliquot part was transferred to a counter slide glass to count the cells and to observe the viability by staining with fluorescein diacetate (FDA).

### Culture condition

The protoplasts were placed in the MS liquid medium with half the strength of the standard, including hormones, 1 μM of NAA and *N*-(2-chloro-4-pyridyl)-*N*′-phenylurea (4PU). Then, 200 mM $CaCl_2$ was added to the culture medium under acidic conditions adjusted to pH 3.5 as a stressed condition (Kondo et al. 2000; Seyama et al. 2008). The cell density was adjusted to ca. $5\times10^4$ cells $ml^{-1}$ in the culture medium (2500 cells per dish) before the protoplasts were cultured at 28°C in the dark without shaking. The protoplasts in the culture plate were observed with an inversed light microscope (CK40, Olympus Co. Ltd, Tokyo, Japan). It should be noted that all data were obtained by observation of a few hundred samples and the data were averaged.

### Observation of cell expanding

Protoplasts cultured in 96 culture dishes under stressed conditions were observed with an inversed light microscope. The diameter of the protoplasts was measured after 2, 3, 4, 5 and 8 weeks of cultivation. For this purpose, protoplasts were selected at random. Observation of the protoplasts secreting a macrofibril was performed after 8 weeks of cultivation. The diameter of the protoplasts and the width of the secreted fibrils were measured simultaneously by means of an Image Pro-Plus software (Media Cybernetics, Bethesda, MD, USA).

### Fluorescent microscopy

Fibril-secreting protoplasts were stained with 0.01% aniline blue fluorochrome to detect (1→3)-β-glucan and, alternatively, with 0.1% calcofluor for clarification of cell wall regeneration. The marker solution was put into the culture medium before protoplasts were observed by fluorescent microscopy by Hg lamp and ultraviolet (UV) emission.

### Cell burst ratio

The culture periods were under normal conditions for 0, 7, 15, 20, 30 and 35 days, and only 90 days for the stressed condition. The protoplasts were then submitted to mild osmotic conditions without mannitol (Suzuki et al. 1998). The burst behavior of individual protoplasts was observed with an inversed light microscope (see above) to calculate the burst ratio related to the total number of observed cells (a few hundred).

### Observation of cytoplasm by 'through focus variation'

Cultured protoplasts under stressed conditions were observed by a Leica light microscope equipped with a cooled charge-coupled device (i.e., CCD camera; C-5810, Hamamatsu Photonics Co. Ltd, Hamamatsu, Japan) following the 'through focus variation' method, in the course of which an image is produced by digital processing of a focal image series. Protoplasts were placed on the slide glass under the microscope. A focus phase of the cross-over image was moved successively by a 2-μm step from the top to the bottom (surface-inside-surface) of the protoplasts. From the image series, a 'through focus image' or a video movie was obtained by means of Image Pro-Plus software (Media Cybernetics).

## Results and discussion

### Expansion of protoplasts

Protoplasts cultured under the stressed conditions kept expanding for the first 8 weeks, while maintaining a spherical shape (Seyama et al. 2008), but without cell division (Figures 1 and 2). Figure 1 shows the average diameter of the expanding protoplasts. About 2 weeks after the beginning of the culture, the size of the protoplasts began to increase, but the protoplasts were not significantly different to each other, as shown in a narrow standard deviation of the plot in Figures 1 and 2(a). After 3 weeks in culture, the size of individual protoplasts began to differentiate. The size deviations gradually increased and, finally, 8 weeks later, they showed larger individual differences (see change of error bars in Figure 1). This means that some protoplasts grew, while some kept their original size. With a longer culture period, the observed differences became more obvious.

Usually, protoplasts isolated from leaves are able to regenerate the cell wall in normal (stress-free) MS medium with hormones; however, under the presented stress conditions, the regeneration process was inhibited, presumably because of the presence of $Ca^{2+}$ in excess in the acidic media. Following the expansion of protoplasts for 8 weeks without cell division (Figure 2a and b), a gigantic callose fibril aggregate began to be secreted from the individual protoplast directly into the medium (Figure 2c).

The relationship between the size of the protoplasts and the width of the produced fibril is presented in Figure 3. The data for the plots presented are from protoplasts producing a callose fibril. When the diameter of the protoplasts reached the range of 100–200 μm (ellipse with broken line in Figure 3), the protoplasts began to produce the gigantic fibrils of 10-40 μm in width. Protoplasts that did not reach the indicated size range were not able to secrete gigantic fibrils. In particular, the same Figure indicates that the size of 150 μm ∅ was the most likely to initiate gigantic fibril production. Altogether, less than 10% of the total protoplasts secreted the fibril; thus, the fibril secretion is not a usual response to the culture conditions. One explanation could be that the

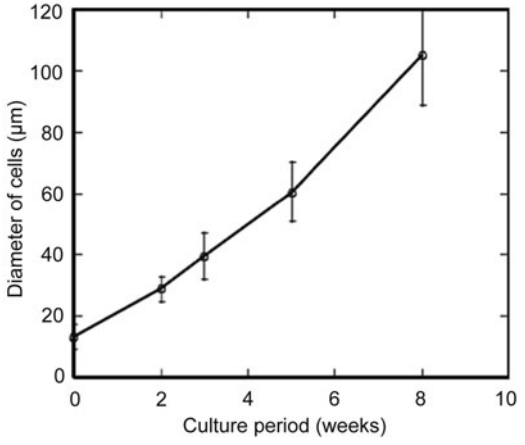


**Figure 1** Expansion of protoplasts depending on the culture period.

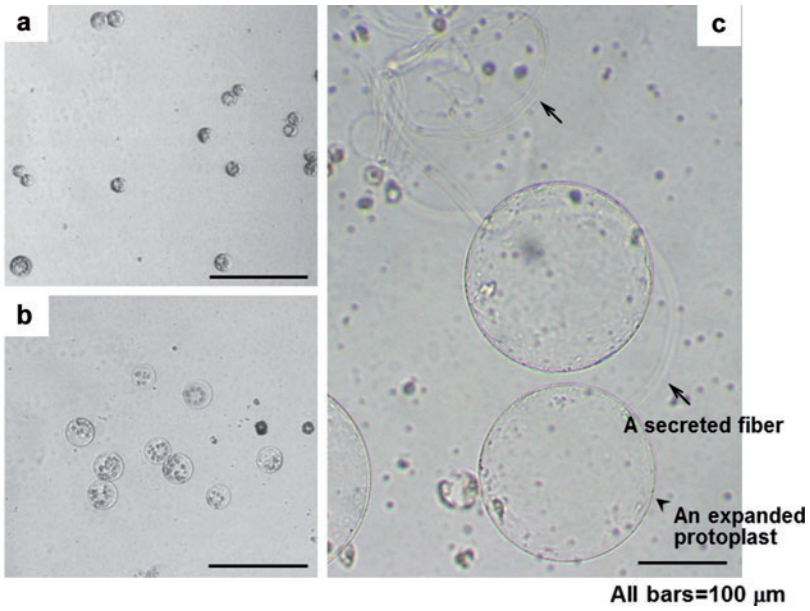


**Figure 2** Light microscopic images of the expansion of protoplasts (a) and (b). Secretion of a callose macrofibril (c). An arrowhead and an arrow indicate a secreted fiber and an expanded protoplast, respectively.

observed protoplast had different origins from various tissues. The effect may also be attributable to the possibility that there are individual differences in the expansion of protoplasts and secretion of the callose fibril.

### Protoplasts and regenerated cells under low osmotic conditions

Regeneration of protoplasts was examined under normal MS and stress conditions. To be more precise: cell wall formation was considered as an indicator for the responses of protoplasts. A plot was created 'burst ratio vs. culture period' (Figure 4). The curve with dashed line in Figure 4 shows the change of the burst ratio of protoplasts to the total examined samples cultured in the 'normal' half of the MS medium after the protoplasts were situated in the low osmotic solution. According to Suzuki et al. (1998), the burst ratio could correspond reversely to the achievement ratio of cell wall formation. In Figure 4, all protoplasts at the initial stage of 0 day culture were burst, and over 80% of cells were burst during the first 15 days in the low osmotic solution. Thereafter, the burst ratio drastically decreased, for example the 20-day-old and 35-day-old cells were burst at the ratios of less than 50% and 20%, respectively. The results can be interpreted as the strength of plasma membranes of the cells was not enough to resist the osmotic pressure at the initial stages of regeneration of the cell wall formation. With an increasing culture period, the burst ratio of cells decreased as a result of the strength of the newly formed cell walls. After 30 days under stress-free culture conditions, the cell

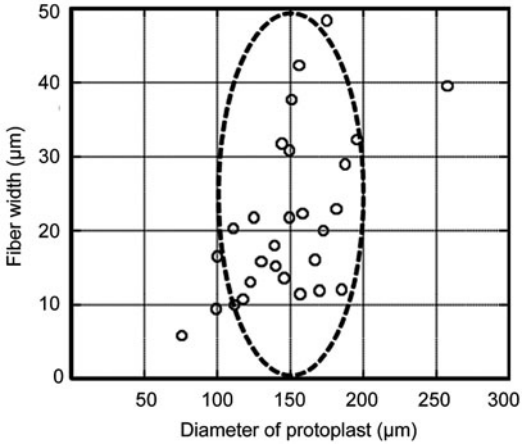


**Figure 3** Distribution of callose macrofibril width as a function of the expanded protoplast diameter.

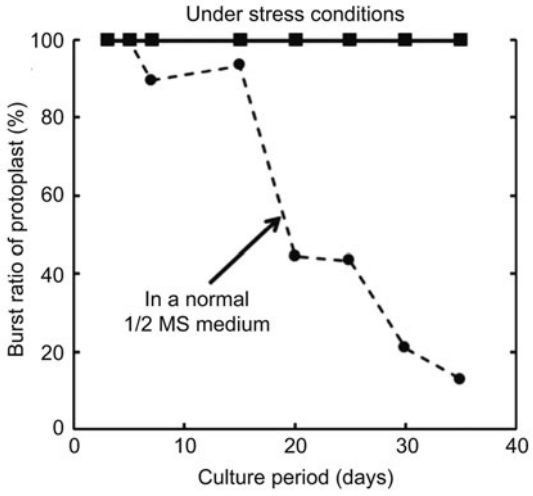


**Figure 4** Burst ratio of the protoplast under a low osmotic pressure depending on the time in culture medium.

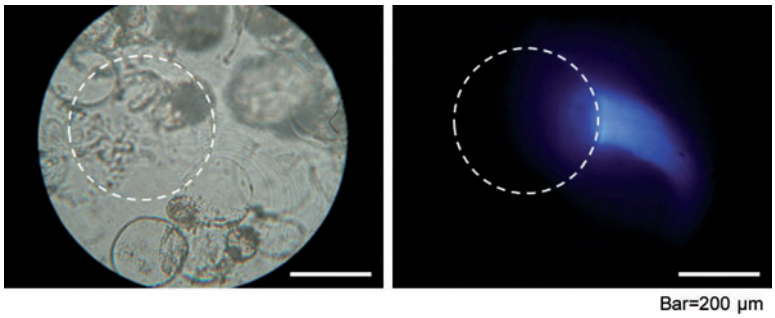


**Figure 5** Light (left) and fluorescent (right) microscopic images of a fibril-secreting protoplast stained by calcofluor white. The broken circle in the left indicates the cell at the same position of the right image.

walls appeared to be completely regenerated from cellulose microfibrils.

On the other hand, most protoplasts cultured under stress conditions were burst in the low osmotic solution after 30 days' culture, as shown by the solid line in Figure 4, and were burst even after 90 days of culture (data not shown). This indicate that cellulosic cell wall was not formed on the surface of the plasma membrane. It should be noted that the protoplasts producing a callose fibril after more than 8 weeks of culture were still easily burst. Accordingly, the stressed culture condition inhibited the regeneration of the protoplasts, as well as the cell wall formation. The expansion of protoplasts with the subsequent secretion of a callose fibril is a type of adaptation for the altered, stress environment.

The fibril secreted from protoplasts was only stained by calcofluor (in Figure 5 a blue tail), whereas the protoplast

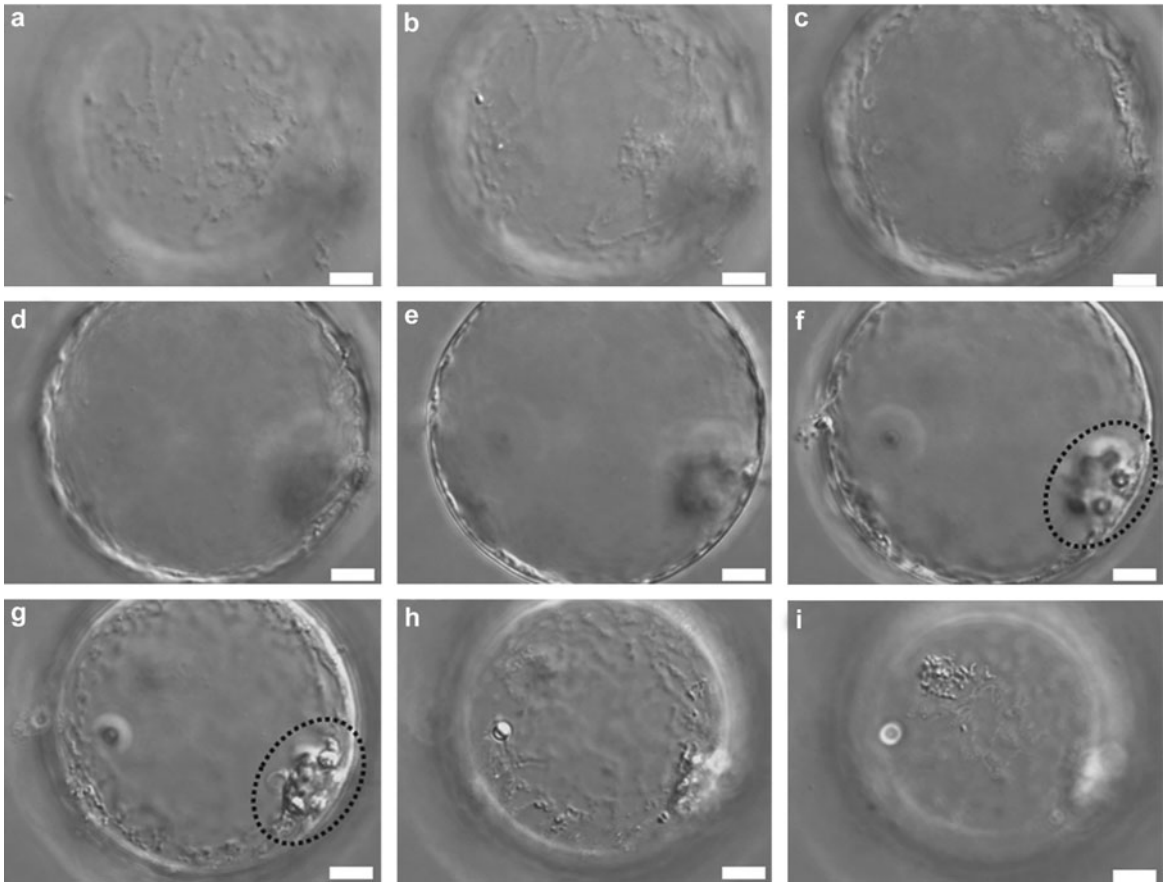


**Figure 6** A successive series of 'through focus images' from the top to the bottom of an expanded protoplast (a–i).
Note: The protoplasts were observed by a differential interference microscope. Each image step of the focus was about 10 μm. As the vacuole develops largely across the entire cytoplasm of the individual cell, the other organelles are supposed to be located aside. Thus, the organelles, except the vacuole, were located close to plasma membrane (broken circle areas). All scale bars = 10 μm.

itself was not stained by this reagent (white circle of the broken line in Figure 5). Accordingly, the protoplast secreting a callose fiber did not regenerate the cell wall by regular formation of cellulose microfibrils.

### Images by 'through focus variation'

The protoplasts cultured under the stressed conditions were observed by differential interference light microscopy combined with a 'through focus technique'. Figure 6 shows the corresponding images from the top to bottom of an expanded protoplast. The size of the protoplast is about 100 μm ∅, which is about 10 times larger than that of the initially isolated cell. There are some random lines across the surface (Figure 6a, b, h, and i). The expanded protoplasts do not appear to have a cell wall because they were not stained by the calcofluor that is supposed to recognize β-glucan molecules. This is the reason why they burst easily under low osmotic pressure conditions, as described above. Thus, the random-patterned lines are not cellulose microfibrils as building blocks for cell walls under construction. Instead, they can be considered as cytoskeleton. The protoplast also exhibits a large vacuole (Figure 6d and e, covering the entire protoplasm), while the cytoplasm, visible at deeper focal layers, is just located at a limited area inside of the plasma membrane (Figure 6f and g, marked by a broken circle) as proposed in a previous report (Suzuki et al. 1998). Probably, a cell with a large vacuole survives for a longer time because a stable vacuole may control the osmotic pressure for growth and metabolism. It seems that the cells develop a vacuole as part of the protective mechanism against a stress factor. The part within the broken line circle corresponds to the area that secretes the macrofibril, as shown in a previous report (Seyama et al. 2008).

## Conclusions

In previous reports, individual protoplasts from higher plants were found to spin a gigantic callose fibril composed of linear (1→3)-β-glucan under stressed culture conditions containing an excess amount of $Ca^{2+}$. The focus of the present study was on the secretion of the macrofibril and its characterization by means of light microscopy combined with a 'through focus variation' method. The unique response of the cells to the stressed conditions was the inhibition of cell division and then their expansion without regeneration of cell walls. On the other hand, the intracellular response was the expansion of the vacuoles so that they occupied nearly the whole cytoplasm. Following this, the production of a giant callose fibril was initiated. It is suggested that the callose fibril is secreted by a callose synthase located on the plasma membrane and not by a vacuole membrane as proposed previously.

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