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Analysis of the Formation of Protoplasts and Regeneration of Cells in *Phycomyces blakesleeanus*

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Abstract It is possible to prepare protoplasts of the zygomycete fungus, *Phycomyces blakesleeanus*, by digesting the cell wall of spore germlings with commercially available chitinase and chitosanase. However, the cells without any cell walls immediately form large aggregates, and thus, it is difficult to isolate the individually separated protoplasts. Inherent problem with the formation of aggregates in preparing protoplasts could be solved by the use of bovine serum albumin (BSA). As a result, we were able to prepare a large number of single protoplasts quickly and easily. We took time-lapse photomicrographs of the formation of protoplasts, and found that there were certain regions of the cell wall of spore germlings that were sensitive to chitinase and chitosanase, although the cell wall of the original spores is known to be insensitive to these enzymes. There are two kinds of cell walls on a spore germling; one with a bound wheat germ agglutinin (WGA), and the other a bound concanavalin A (ConA). Furthermore, only cells with walls which had bound WGA were able to regenerate, while those with walls with bound ConA were not able to regenerate.

Key words: Bovine serum albumin, concanavalin A, chitinase, chitosanase, *Phycomyces*, protoplast, spore germling, wheat germ agglutinin, Zygomycetes

In studies which were conducted the molecular genetics of fungi protoplasts have been used as potential cell type that can be used for transformation [6, 9, 15, 36]. Furthermore, hybrid cells or heterokaryons generated by fusion of protoplasts have been used in the genetic analysis of complementation and in studies of parasexual cycles [3, 35, 36]. Protoplasts have also been used as experimental materials in cell biology, for example, in studies of the regeneration of fungal cell wall and of the morphological distribution of components of the cytoskeleton, such as microtubules and actin filaments [12, 17, 37].

Phycomyces blakesleeanus is a filamentous fungus that belongs to the class Zygomycetes. It has been an important model organism in analyzing the stimulation-response systems for investigating responses to blue light, to gravity, to mechanical stretch, and to the presence of nearby barriers [7]. The main components of the cell wall are chitin and chitosan [4, 24, 43, 46]. Miyazaki *et al.* [25, 26] cloned genes from *P. blakesleeanus* for proteins of the chitin synthase family that are involved in the synthesis of cell walls and also demonstrated a multigene family consists of ten homologous genes for the chitin synthase. Recently, chitosan and its practically degraded oligosaccharides have been becoming important because of the potential applications as medical and agricultural agents. Kim *et al.* [22] reported the purification and characteristics of two types of chitosanases from *Aspergillus fumigatus* KH-94, and Yoon *et al.* [49, 50, 51] also reported the purification and characterization of a new thermostable chitosanase from *Bacillus* sp. and the cloning of a chitinase gene from *Thermoactinomyces vulgaris* KFB-C100.

In this study, we examined the formation of protoplast and its regeneration in *P. blakesleeanus*. The present findings will facilitate further studies of the molecular biology and biotechnology of *P. blakesleeanus*.

Materials and Methods

Strain and Culture Condition

In this study, sporangiospores from a wild-type strain of *P. blakesleeanus*, NRRL1555 (-), was used. Initially, 2.0×10^7 sporangiospores activated by heating at 48°C for

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10 min [42] were inoculated into a liquid SIVYC medium [45], and cultured on a "figure-eight" shaker (60 strokes/min) at 20°C under white fluorescent light (20 mW/cm^2/sec).

Enzymes and Chemicals

For digestion of cell walls, chitinase RS (Seikagaku-Kogyo, Tokyo, Japan), chitosanase RD (Seikagaku-Kogyo), and chitinase T-1 (TaKaRa-Shuzo, Kyoto, Japan) were used. Bovine serum albumin (BSA) F-V (Sigma Chemicals, St. Louis, MO, U.S.A.) was purchased from a domestic distributor, while other chemicals were obtained from Nacalai-Tesque, Kyoto, Japan.

Preparation of Protoplasts

Spore germlings which were recovered from sporangiospores cultured at 20°C for 15 h in liquid SIVYC medium were washed twice with a 0.55 M solution of sorbitol with 0.02% BSA (BSA-sorbitol). The washed germlings were suspended in 5 ml of 25 mM 2-[(4-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) buffer (pH 6.8) that contained 2.5 mg/ml chitinase RS, 2.5 mg/ml chitosanase RD, 0.55 M sorbitol (as osmotic stabilizer [44]) and 0.02% BSA in a 15-ml centrifuge tube. The centrifuge tube was then shaken gently at 30°C for 2 h on a reciprocal shaker. The formation of protoplasts was confirmed under a phase-contrast microscope (IMT-2; OLYMPUS, Tokyo, Japan), and the protoplasts were collected by centrifugation (800 ×g) at room temperature for 2 min, suspended in 5 ml of BSA-sorbitol in a 15 ml centrifuge tube, and washed three times in BSA-sorbitol by same centrifugation. Finally, the pelleted protoplasts were resuspended in 1 ml of BSA-sorbitol in a microcentrifuge tube.

Detection of Saccharide Moieties with Specific Lectins

For detecting saccharide moieties specific for lectins, we used a variety of the lectins, such as tetramethylrhodamine isothiocyanate (RITC) conjugated succinylated lectin from *Triticum vulgaris* [27, 28]. This modified wheat germ agglutinin (sWGA) was obtained from VECTOR Laboratories (Burlingham, CA, U.S.A.) and is specific for chitin-like saccharide moieties [1, 13, 48]. Fluorescein isothiocyanate (FITC) conjugated lectin from *Canavalia ensiformis*, namely, ConA was obtained from VECTOR Laboratories and is specific for α-D-mannose or α-D-glucose-like saccharide moieties [14, 38]. FITC-conjugated lectin from *Lycopersicon esculentum* (LEA) that is specific for chitin-like saccharide moieties was obtained from VECTOR Laboratories [21, 30, 31], as well as the FITC-conjugated lectin from *Pisum sativum* (PSA). It is also specific for α-D-mannose or α-D-glucose-like saccharide moieties [47]. Each modified lectin was diluted to a final concentration of 1.0 μg/ml with 10 mM Tris-HCl buffer (pH 7.5) containing 1% NaCl and 0.02% BSA (BSA-TBS).

For detection of saccharide moieties specific for each lectin, the germlings and the regenerating protoplasts were immersed in isotonic solution of the lectin in BSA-TBS solution at room temperature for 10 min. After three washes with the isotonic BSA-TBS, the samples were examined under a fluorescence microscope (Axioplan2, Carl-Zeiss, Jena, Germany) with appropriate wavelengths for excitation and emission.

Time-Lapse Recording of the Formation of Protoplasts

To record morphological changes of spore germlings, we used a time-lapse recording system that included both a Nomarski optics microscope (model BH; OLYMPUS) and a time-lapse control unit (PM-IVM; OLYMPUS).

Regeneration of Cells from Protoplasts

Protoplasts, prepared as described above, were transferred to regeneration medium, namely, SIVYC that contained 0.55 M sorbitol as an osmotic stabilizer [44]. They were then cultivated at 20°C under white fluorescent light (20 mW/cm^2/sec).

RESULTS AND DISCUSSION

The Effects of BSA on the Preparation of Protoplasts

Binding and Weber [8] and Suarez *et al.* [44] reported a method for preparing protoplasts or spheroplasts of *P. blakesleeanus*, that involved a treatment of cells with chitinase and chitosanase. In a preliminary stage, when we prepared protoplasts by their method [44], the protoplasts or spheroplasts immediately formed large cell-cell aggregates (Fig. 1D). Moreover, these aggregates immediately adhered to the walls of microcentrifuge tubes during washing or digesting with enzymes. Thus, we failed to isolate single protoplasts, but this problem was overcome by adding BSA to the washing solutions and to the solution of enzymes. Similarly, the use of BSA has been reported to prevent the adhesion of mammalian oocytes and embryos to glassware [20, 32].

In Table 1 and Fig. 1, the results obtained with BSA are shown, and it is quite clear that there is almost no adhesion and aggregation of protoplasts at concentrations of BSA above 0.01%.

The Relationship Between the Cultivation Time of Spore Germlings and the Efficient Formation of Protoplasts

The use of BSA allowed us to generate single protoplasts readily. Next, we tried to determine the optimum duration for cultivation of spore germlings to prepare protoplasts. These results are shown in Fig. 2. Germlings cultivated for 9 h or less after heat activation did not yield any protoplasts, but when germlings were cultivated for 11 to 17 h, the number of protoplasts gradually increased.

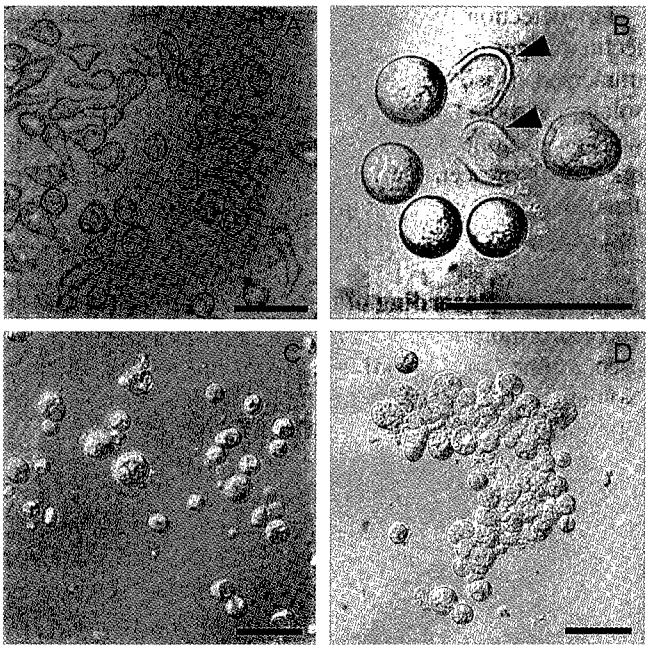

Fig. 1. Effects of BSA on the preparation of protoplasts.
A: Spore germlings 14 h after the start of cultivation. B: Protoplasts in a solution of enzymes that contained 0.02% BSA after incubation at 30°C for 120 min. Arrowheads indicate undigested cell walls derived from spores. C: Protoplasts in washing solution that contained 0.02% BSA. D: An aggregate of protoplasts that had been treated with washing solutions and enzyme solution without BSA. (See text for further details.) Bars in this figure represent 50 μm.

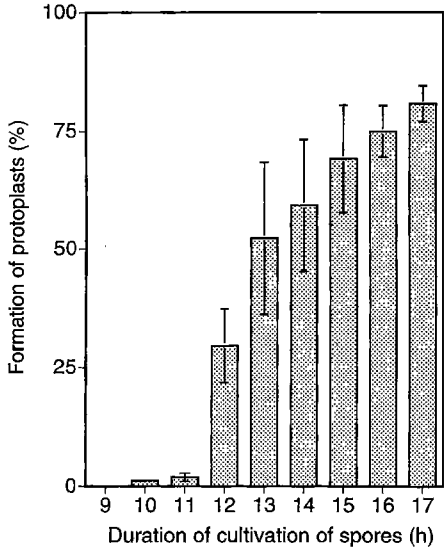

Fig. 2. Relationship between the duration of cultivation of spore germlings and the formation of protoplasts.
Values are means±S.E. of results obtained in 5 replicate experiments with 1.0×10^7 germlings in each case.

Cultivation for 18 h yielded hyphae with very long and complicated branches from which it was very difficult to isolate protoplasts.

Suarez *et al.* [44] reported that a seven hour period was an optimum time for strain NRRL1555 in SIVYC medium. However, in our study, the optimum cultivation time for spore germlings was 15–16 h, even though we used the same strain and the same medium. However, the enzymes used in these two strains were not the same. The chitinase RS and chitosanase RD used in this study were derived from *Bacillus* sp. and they contained low levels of contaminants, such as other glycosidases and/or proteases. The chitosanase used by Suarez *et al.* was isolated from the conditioned medium of *Streptomyces* no. 6 strain [18, 39] and the preparation contained other glycosidases and proteases [19, 41, 44]. In addition, we cultured spore

Table 1. Effects of BSA on the adhesion of protoplasts to microcentrifuge tubes (A) and on the aggregation of protoplasts (B).

A

Treatment	Adhesion of protoplasts to microcentrifuge tube	Aggregation of protoplasts
None	+	+
0.005% (0.05 mg/ml) in WS and SE	+	±
0.01% (0.1 mg/ml) "	±	-
0.02% (0.2 mg/ml) "	±	-
0.05% (0.5 mg/ml) "	±	-
0.1% (1.0 mg/ml) "	±	-

B

Treatment (0.2% BSA)	Adhesion of protoplasts to microcentrifuge tube	Aggregation of protoplasts
None	+	+
SE	+	±
WS and SE	±	-
CM, WS and SE	±	-

Extent of adhesion or aggregation: +, strong; ±, weak; -, none or very weak.
Abbreviations: SE, solution of enzymes; WS, washing solution (0.55 M sorbitol); CM, culture medium, namely SIVYC.

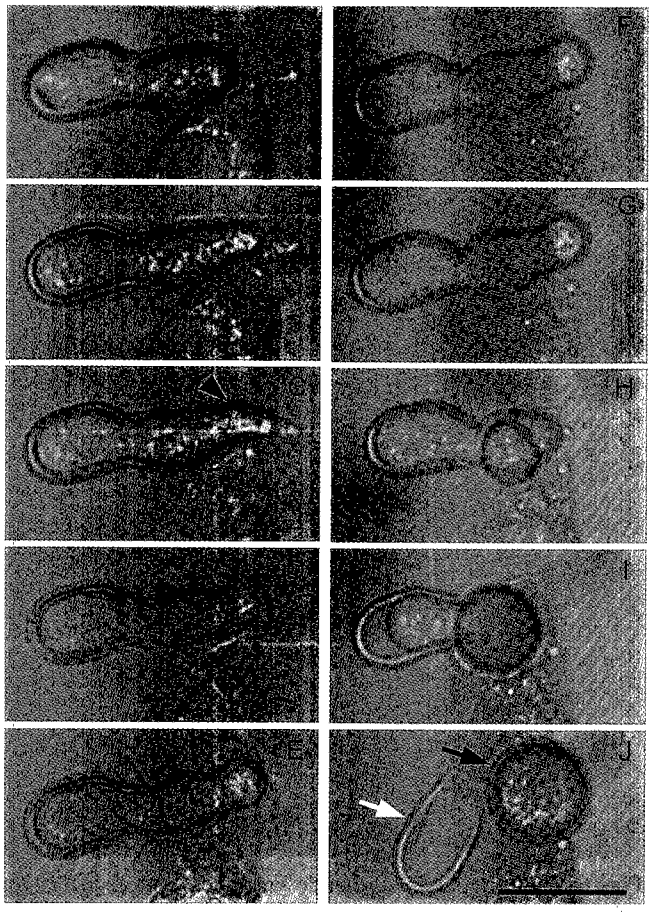

Fig. 3. Time-lapse photomicrographs showing the formation of protoplasts (Nomarski differential interference microscopy).
A, 0 min; B, 50 min; C, 70 min (arrowhead indicates a region of digested cell wall); D, 90 min; E, 110 min; F, 130 min; G, 135 min; H, 140 min; I, 145 min; J, 150 min (the black arrow indicates a protoplast, and the white arrow indicates the undigested wall of the original spore). The bar in this figure represents 25 μm.

germlings at 20°C while Suarez *et al.* cultured germlings at 22°C. The lower temperature might have retarded the growth of spore germlings.

Time-Lapse Analysis of the Formation of Protoplasts

We examined the formation of protoplast by using time-lapse photography, as shown in Fig. 3. Spore germlings were suspended in a solution of enzymes and the germlings were monitored at room temperature. Seventy-minutes after the start of digestion, we repeatedly observed a small pore or break in the sub-apical region of the cell wall of spore germlings (arrowhead in Fig. 3C). With time, the small pore increased in size and a naked cell gradually emerged from the expanded pore (Figs. 3D to 3I). An intact protoplast was visible at 150 min after the start of digestion (black arrow in the Fig. 3J). This process resembles the formation of protoplasts of *Aspergillus niger* [29] and *Schizosaccharomyces pombe* (personal communication from Dr. M. Osumi). The photomicrographs in Fig. 3 show that digestion of the cell wall of spore germlings had begins in the sub-apical region. The cell enlarged until the naked cell (or intact protoplast) emerged from the spore shell (white arrow in Fig. 3J).

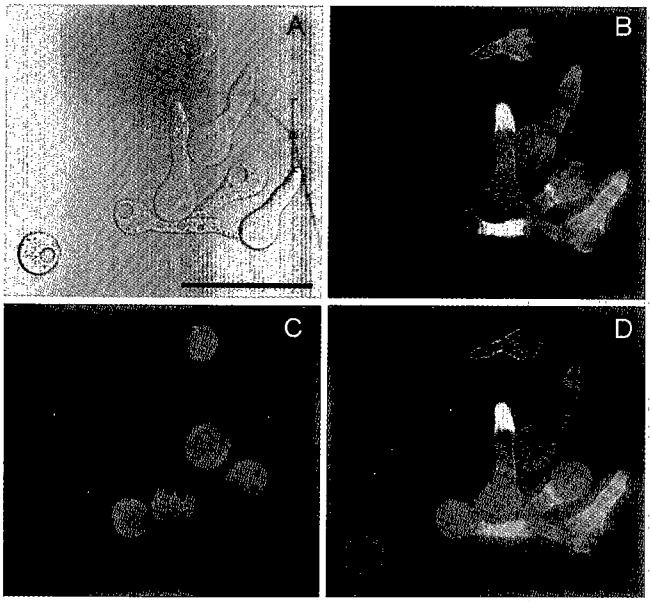

Fig. 4. Detection of saccharide moieties on the cell walls of spore germlings.
A: Photomicrograph under normal illumination. The bar in this figure represents 50 μm. B: Fluorescence photomicrograph of spore germlings, showing fluorescence due to RITC-sWGA, in the same visual field as in A. C: Fluorescence photomicrograph of spore germlings, showing fluorescence due to FITC-ConA, in the same visual field as in A. D: Fluorescence photomicrograph of spore germlings, showing fluorescence due to RITC-sWGA (red) and to FITC-ConA (green) in the same visual field as in A.

Analysis of Saccharide Moieties on the Cell Wall and Cytoplasmic Membrane of Spore Germlings using Specific Lectins

Figure 3J shows that the cell wall of the germtube was digested by chitinase RS and chitosanase RD, but the original cell wall of the spore germling was not digested by these enzymes. This observation suggests that there are at least two different kinds of cell walls on spore germling. To examine this possibility, we performed a series of experiments with lectins specific for various saccharide moieties. These results are shown in Fig. 4. Figure 4B shows that sWGA, which specifically recognizes and binds to the chitin-like saccharide moieties, was able to bind to the cell walls of the germtube. Figure 4C shows that ConA, which specifically recognized and bound to α-mannan-like or α-glucan-like saccharide moieties, was able to bind only to the original spore wall. Furthermore, Fig. 4D, in which the fluorescent image of ConA has been superimposed with sWGA, shows the relative distributions and localizations of these saccharide moieties on the spore germling. The image

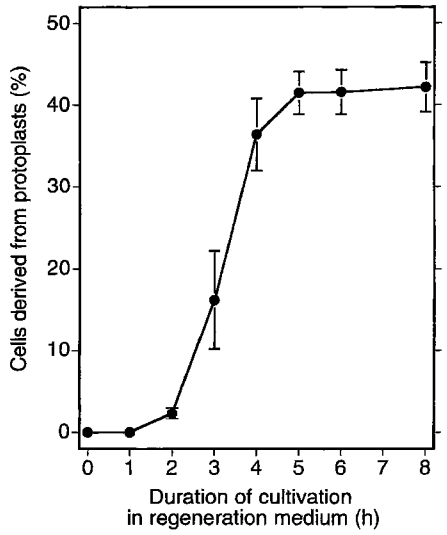

Fig. 5. Time-course of the morphological reversion of protoplasts to hyphae.
Values are means±S.E. of results obtained in 5 replicate experiments with 2.0×10^6 protoplasts in each case.

clearly revealed that the ConA-binding sites overlapped with the sWGA-binding sites in the original spore wall.

A multi-stratified organization of the cell walls of spore germlings has been observed by TEM and SEM in *Mucor rouxii* [5] and *P. blakesleeanus* [10, 11]. Furch [10] proposed a model that included a four-fold structure (mucus, perispore, epispore, and endospore layers, in turn, from the outside) for the cell wall of spore germlings. When we compare our data with Furch's models [10], we may analogize that ConA binds to the perispore and/or the epispore layers, and the sWGA binds specifically to the corresponding endospore layer. Thus, we may assume the putative perispore and epispore layers includes α-mannose or α-glucose and the putative endospore layer consists of a polymer of β(1,4) *N*-acetyl-D-glucosamine.

Regeneration from Protoplasts

We examined the time-course of the morphological changes involved in reversion of protoplasts to hyphae by taking photomicrographs at hourly intervals under a light microscope. The newly formed cells on the photomicrographs were calculated to represent the percentage (reverted cells/total protoplasts × 100). From Fig. 5, it is clear that the reversion started as early as 2 h after the transfer of protoplasts to the regeneration medium, and it was complete within 5 h. However, even when the process was completed, the percentage of reverted cells showed only 42%. This percentage was clearly twice more than that of reports made by Suarez *et al.* [44], which indicates that our method was more effective than theirs. Our improved percentage was probably due to the protective effects of BSA on the plasma membrane of protoplast, and a protective effect of

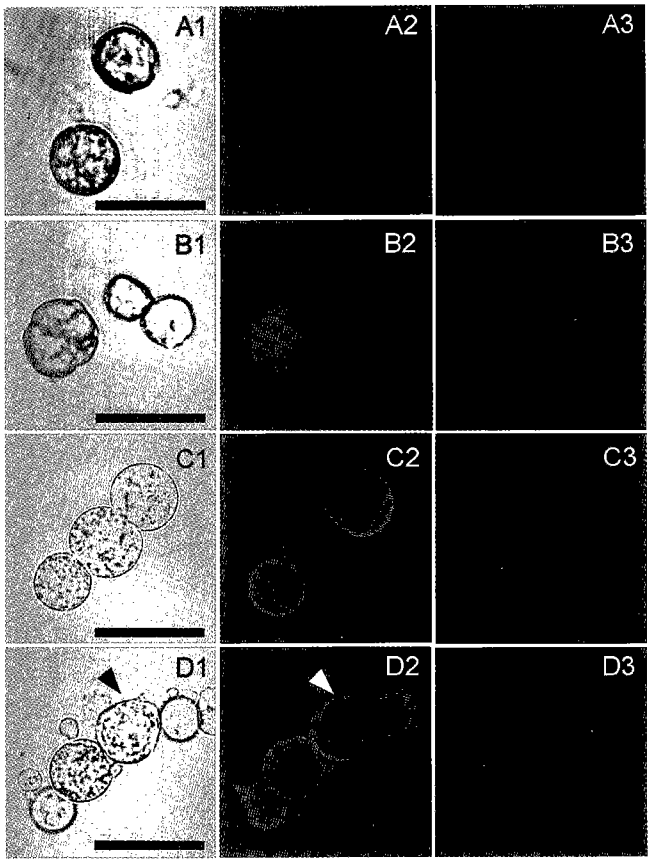

Fig. 6. Detection of saccharide moieties on regenerating protoplasts.
A1, Protoplasts before regeneration; A2, protoplasts treated with RITC-sWGA before regeneration; A3, protoplasts treated with FITC-ConA before regeneration. B1, cells 30 min after the start of regeneration; B2, cells treated with RITC-sWGA 30 min after the start of regeneration; B3, cells treated with FITC-ConA 30 min after the start of regeneration. C1, cells 60 min after the start of regeneration; C2, cells treated with RITC-sWGA 60 min after the start of regeneration; C3, cells treated with FITC-ConA 60 min after the start of regeneration. D1, cells 90 min after the start of regeneration; D2, cells treated with RITC-sWGA 90 min after the start of regeneration; D3, cells treated with FITC-ConA 90 min after the start of regeneration. Bars in this figure represent 25 μm.

BSA on the regeneration of protoplasts has also been reported by others [16, 40].

Next, we examined the possibility of the presence of saccharide moieties on the regenerating protoplasts by using lectins, and the results are shown in Fig. 6. In this investigation, there were no sWGA- and ConA-binding on the surface of protoplasts before regeneration (Figs. 6-A2 and 6-A3). However, 30 min after the start of regeneration, we confirmed sWGA-binding sites on the surface of the regenerating cells (Fig. 6-B2), while there were no ConA-binding sites (Fig. 6-B3). From 60 to 90 min, only the fluorescence that was caused by the binding of sWGA increased (Figs. 6-C2 and 6-D2) and no binding of ConA was detected (Figs. 6-C3 and 6-D4). These results suggest that, when cells are regenerating from protoplasts, only

chitin-like saccharide moieties are regenerated, while α-mannan-like or α-glucan-like saccharide moieties are not.

The patterns of distribution and localization of the fluorescence due to binding of sWGA definitely changed with time, and this clearly indicates that a cytodifferentiation occurred during the process of regeneration. There are numerous reports on the relationships between cytodifferentiation and changes in the distribution and localization of lectin-binding sites [2, 23, 33, 34, 52].

In fact, when cells were regenerated from protoplasts, chitin-like saccharide moieties were exclusively detected, and therefore, a digestion experiment was performed to confirm this possibility. As shown in Fig. 7, it was clear that, when cells were treated 120 min after the start of regeneration with RITC-conjugated sWGA, sWGA bound to the surface of the regenerated cells (Fig. 7-A2). However, 120 min after the start of regeneration, when cells were re-exposed to chitinase T-1 and then treated with RITC-conjugated sWGA, no sWGA-binding sites were detected on the surface of the redigested cells (Fig. 7-B2). Moreover, the cells which had been redigested with chitinase were reconverted to protoplasts (Fig. 7-B1). Thus, our results confirmed that the regenerated cell wall contained chitin-like saccharide moieties, but no α-mannan-like or α-glucan-like saccharide moieties were detected.

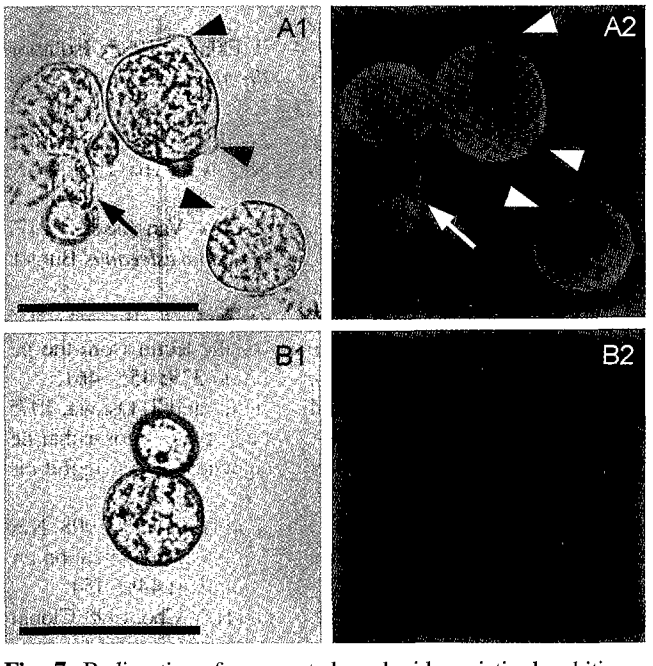

Fig. 7. Redigestion of regenerated saccharide moieties by chitinase T-1.

A1, cells 120 min after the start of regeneration (arrowheads indicate budding on regenerated cells and the arrow indicates a hypha on a regenerated cell); A2, cells treated with RITC-sWGA 120 min after the start of regeneration. B1, Protoplasts derived from cells 120 min after regeneration that had been re-treated with chitinase T-1; B2, protoplasts treated with RITC-sWGA after redigestion. See text for full details. Bars in this figure represent 25 μm.

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