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Effects of IBP on cell wall regeneration and hyphal reversion of protoplasts isolated from spores of *Pyricularia oryzae* Cav.*

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Abstract

The effects of IBP (*S*-benzyl *O,O*-diisopropyl phosphorothioate) on cell wall regeneration and hyphal reversion of protoplasts obtained from spores of *Pyricularia oryzae* Cav. isolates P2 (IBP-sensitive) and PT83 (tolerant) were examined. The pretreatment of protoplasts of P2 with 50 ppm IBP suppressed cell wall regeneration, but the treatment of previously regenerated cells of P2 did not remarkably interfere with the hyphal reversion. These results suggested that the cell wall regeneration step was more sensitive to IBP than the hyphal reversion step. The 30 min pretreatment of protoplasts of both isolates with 20 and 50 ppm also suggested that *de novo* cell wall synthesis might occur in protoplasts within 30 min after released from spores and that the IBP tolerance of PT83 might be attributable to mutation of some step(s) which is associated with cell wall synthesis. The IBP tolerance and sensitivity of protoplasts of both isolates were almost same as those of spores and mycelia which had been previously reported.

Key words: *Pyricularia oryzae*, protoplast, cell wall regeneration, hyphal reversion, IBP.

Introduction

Recently, protoplasts have been utilized successfully to examine effects of various chemicals on plasma membranes and/or synthesis of cell walls of a variety of fungi[3,6–8], since direct effects of chemicals on plasma membranes and also synthesis of cell walls are readily observed in the protoplast systm. KOBAYASHI[9] investigated the cytological effects of IBP (*S*-benzyl *O,O*-diisopropyl phosphorothiaote) on hyphae, spores and protoplasts released from hyphae of *Pyriularia oryzae* Cav. (rice blast fungus), and found that spore germination was more sensitive to IBP than mycelial growth, and in addition, that IBP interfered with cell wall regeneration, hyphal reversion or both of protoplasts. Although he did not determine whether IBP suppressed regeneration or reversion, his conclusion led us to assume that protoplasts of spore origin might be more sensitive to IBP than those of hypha origin and that the former might be more adequate to examine the IBP effects on morphogenesis of protoplasts. In this study, therefore, protoplasts released from spores of *P. oryzae* were used to examine effects of IBP on the steps from cell wall regeneration through hyphal reversion.

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Materials and Methods

Test fungus: *Pyricularia oryzae* Cav. isolates P2 (sensitive to IBP) and PT83 (tolerant)[4] were used throughout the experiments. The test isolates were grown on potato-glucose-agar (PDA) at 25C.

Test chemical: IBP (Kumiai Chemicals Co. Ltd., 96% technical grade) was used throughout the experiments. IBP, acetone and Tween 20 were mixed at the volume ratio of 6+5+1 to provide the 48% IBP stock solution[5].

Preparation of protoplasts: Protoplasts were prepared by a modification of the method of Kunoh *et al*[12]. Both test isolates were grown on V-8 juice agar medium at 25C for 7 days. To prepare spore suspension, 15 ml of sterilized water was added to the medium plates and the medium surface was rubbed with a sterilized brush. Spore suspension was filtered through 3 sheets of sterilized cheese cloth to remove mycelial residue. Spores were collected by centrifugation at 500×*g*, for 5 min, and washed three times with 0.6 M KCl and 2 mM $MgCl_2$ in 0.02 M Na-phosphate buffer, pH 7.0 (stabilizing solution). The washed spores were transferred aseptically into 20 ml of lytic enzyme solution [zymolyase 20000 (Seikagaku Kogyo Co. Ltd., Tokyo), 200 mg; β-glucuronidase (Sigma Co., USA), 200 mg] and gently shaken at 30C for 6 hr. Protoplasts were collected by two successive filtrations through glass filters (Yamato Co. Ltd., Type Nos. 2 and 3), followed by centrifugation at 400×*g* for 5 min. Protoplasts were twice washed with the stabilizing solution.

Treatment of protoplasts with IBP: Protoplasts were treated with various concentrations of IBP by the thin-layer agar-plate (TLA) method devised by KOBAYASHI *et al*[10]. Initially PDA media containing IBP at a final concentration of 10, 20 or 50 ppm were prepared and maintained at 41.5C. Two ml of the respective IBP-containing PDA was added to petri dishes placed on a stirrer controlled at 41.5C, and then 0.2 ml of the protoplast suspension was added to each petri dish, followed by gentle mixing with a stirrer. Small pieces of plastic tape (0.15 mm thick) were attached at both ends of a sterilized glass slide. Immediately after the protoplast suspension was prepared as above, it was poured onto the center of the glass slide, and pressed with another sterilized glass slide before the medium was hardened. After the medium was hardened, the top glass slide was detached, leaving a uniform (0.15 mm thick) layer of agar on the remaining glass slide. The glass slides with protoplasts were incubated in a moist environment at 25C. Following incubation for 12 and 24 hr, a coverslip was placed on the thin layer of agar and observed to evaluate cell wall regeneration and hyphal reversion, respectively.

Effects of pretreatment of protoplasts with IBP on their cell wall regeneration: The stabilizing solution containing IBP at a concentration of either 20 or 50 ppm was prepared. Five ml of this solution was added to 0.2 ml of the protoplast suspension. After incubation at 25C for 30 or 120 min, protoplasts were collected by centrifugation at 400×*g* for 5 min and were twice washed with the stabilizing solution containing no IBP. Subsequently, protoplasts were collected by centrifugation, embedded and incubated in thin-layer agar-medium of PDA with no IBP for 12 hr.

Effects of treatment of previously-regenerated cells with IBP on their reversion: Protoplast suspension was dropped onto PDA containing 0.6 M KCl and incubated at 25C for 8 hr to allow protoplasts to regenerate their cell wall. The regenerated cells were released from the medium surface by rubbing gently with a sterilized platinum loop to prepare the regenerated cell suspension. The suspension was filtered through a glass filter (No. G2) to remove partially reverted cells. The filtrate was suspended in the stabilizing solution diluted with sterilized water at the dilution rate of 2/3 to allow unregenerated protoplasts to burst. The

regenerated cells remaining in the solution were collected by centrifugation at $400 \times g$ for 5 min, and then incubated in PDA containing IBP at a concentration of 20 or 50 ppm by the TLA method as described above.

Numerical evaluation of regeneration and reversion: After incubation of TLA's as above, specimens were examined with a microscope and numbers of protoplasts, regenerated cells, and reverted hyphae were counted. Cell walls regenerated around protoplasts were stained with calcofluor white by the method of KOBAYASHI *et al*[10]. Stained specimens were observed with a Zeiss inverted interference microscope equipped with an epifluorescence attachment of a filter block with a BP365 excitation filter, FT395 dichroic mirror, and an LP395 barrier filter. The frequency of regeneration was estimated as percentage of the total number of regenerated cells per the total number (120–140 for each replicate) of protoplasts and regenerated cells observed, and that of reversion as percentage of the number of reverted hyphae per the total number (120–140) of regenerated cells with or without the hyphae. The effects of IBP were evaluated by the relative percentage of regeneration or reversion at the designated IBP concentration divided by the rate of control (no IBP).

Results

Protoplasts were all spherical when observed immediately after isolation, although they were of varying diameters. Within 0.5–6.0 hr of incubation, protoplasts responded with bluish white fluorescence, indicating cell wall regeneration, when treated with calcofluor white and observed with a fluorescent microscope. Subsequent development was categorized into the following two types: type 1- several yeast-like buds occurred from a regenerated cell but a hypha arose from an apical cell of the cell chain; and type 2- a hypha developed directly from a regenerated cell. The incidence of type 2 was much higher than that of type 1 regardless of the presence or absence of IBP in TLA.

When protoplasts of an IBP-sensitive isolate P2 was treated with IBP for 12 hr from the onset of incubation, cell wall regeneration was largely suppressed. As indicated in Fig. 1A, 39%, 23% and 8% of protoplasts regenerated cell walls at the presence of 10, 20 and 50 ppm IBP, respectively. In contrast, 85%, 73% and 31%

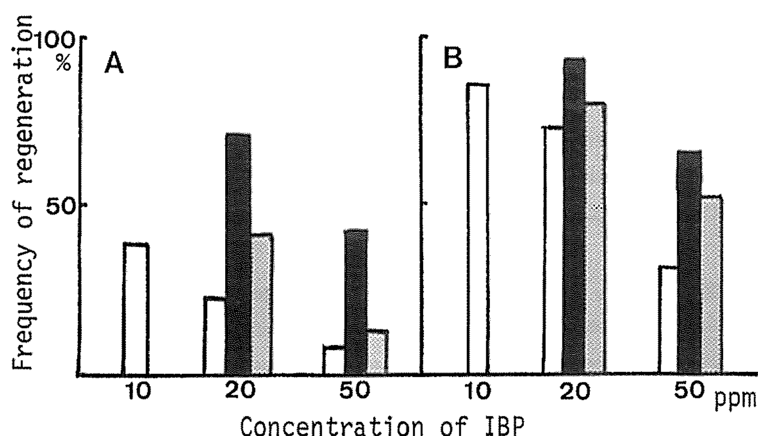

Fig. 1. Effects of various concentrations of IBP on cell wall regeneration of isolates P2 (A) and PT83 (B).
□ : Protoplasts treated with IBP from the onset to the end of incubation.
■ : Protoplasts treated with IBP for 30 min prior to incubation without IBP.
▒ : Protoplasts treated with IBP for 120 min prior to incubation without IBP.

of protoplasts of an IBP tolerant isolate PT83 regenerated cell walls at the presence of 10, 20 and 50 ppm IBP, respectively.

Reversion of a hypha from regenerated cells was similarly affected by the presence of IBP. When 10 and 20 ppm IBP were present in the medium, a hypha arose from 27 and 10% of regenerated cells of P2, respectively (Fig. 2A). However, no hyphae emerged at the presence of 50 ppm IBP. On the other hand, 73 and 65% of regenerated cells of PT83 produced a hypha even at the presence of 10 and 20 ppm IBP, respectively, and 15% of the cells did so at the presence of 50 ppm IBP.

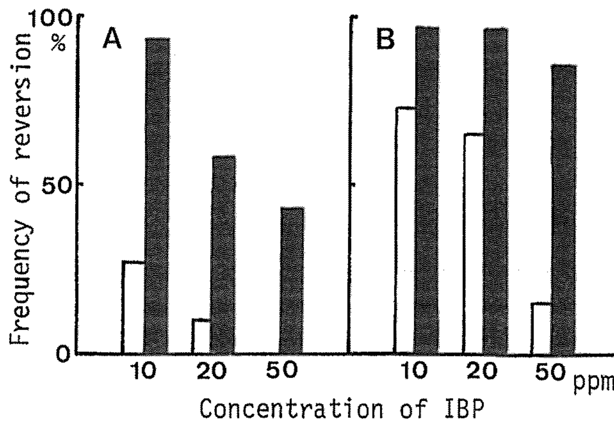

Fig. 2. Effects of various concentrations of IBP on hyphal reversion of isolates P2 (A) and PT83 (B).
□ : Protoplasts treated with IBP from the onset to the end of incubation.
■ : Previously-regenerated cells treated with IBP.

More number of protoplasts of both isolates regenerated cell walls when they were treated with IBP prior to incubation in TLA containing no IBP than when they were treated with IBP from the onset until the end of incubation. When protoplasts of P2 were treated with 20 ppm IBP for 30 and 120 min before incubation, 71% and 41% of protoplasts regenerated cell walls during the 12 hr incubation, respectively. However, the pretreatment with 50 ppm for 30 min allowed 42% of protoplasts to regenerate and that for 120 min did only 13% (Fig. 1A). PT83 was more insensitive to such pretreatments than P2. As illustrated in Fig. 1B, 93% and 80% of protoplasts of PT83 regenerated after the pretreatment with 20 ppm for 30 and 120 min, respectively. Moreover, 65% and 52% of protoplasts succeeded in regeneration when pretreated with 50 ppm for 30 and 120 min, respectively.

As indicated in Fig. 2A, 93%, 58% and 43% of previously-regenerated cells of P2 successfully produced hyphae within 24 hr, when they were treated with 10, 20 and 50 ppm IBP, respectively. Furthermore, more than 85% of previously-regenerated cells of PT83 produced a hypha regardless of the IBP concentrations.

Discussion

All of the present results indicated that protoplasts released from spores of PT83 were more tolerant to IBP than those of P2. The IBP tolerance and sensitivity of protoplasts of both isolates were consistent with those of

spores and mycelia[2,4]. Protoplasts of hypha origin are usually heterogeneous in physiology and morphology, because they are inevitably released from hyphal cells of diverse ages simultaneously[13]. In contrast, synchronization of spores is much easier than that of hyphae. Thus, protoplasts of spore origin seem to be a more adequate material to examine the tolerance of the present fungus to IBP than protoplasts of hypha origin.

When protoplasts of P2 were treated with 10–50 ppm of IBP from the onset of incubation, cell wall regeneration and hyphal reversion were suppressed remarkably (Figs. 1A and 2A). However, under such an incubation condition it is impossible to determine whether the regeneration step, the reversion step or both were suppressed by the IBP treatment, because if the regeneration step was first suppressed, then the subsequent reversion step must have been inevitably suppressed. Thus, we examined the effects of IBP on both steps separately. As illustrated in Fig. 1, the 120 min pretreatment of protoplasts with 50 ppm IBP followed by incubation without IBP greatly suppressed the regeneration step as similarly as the case of the IBP treatment for 120 hr from the onset to the end of incubation, suggesting that the 120 min pretreatment of protoplasts was enough to suppress the cell wall regeneration. On the other hand, when protoplasts of P2 were treated with 50 ppm IBP for 24 hr from the onset of incubation, no hyphae were reverted from regenerated cells (Fig. 2A). However, when previously-regenerated cells were treated with 50 ppm IBP, hyphae arose from 43% of these cells (Fig. 2A). Thus, it is most likely that the regeneration step is more sensitive to IBP than the reversion step, although we have to consider the possibility that the presence of cell walls around previously-regenerated cells might disturb the permeability of IBP into the cells.

The 30 min pretreatments with 20 and 50 ppm IBP allowed 71% and only 42% of protoplasts of P2 to regenerate cell walls, respectively (Fig. 1A), suggesting that *de novo* cell wall synthesis might occur in protoplasts within 30 min after released from spores. The data of Fig. 1B suggest that the IBP tolerance of PT83 might be attributable to mutation of some step(s) which is associated with the cell wall synthesis, since even the 120 min pretreatments with 20 and 50 ppm slightly interfered with the cell wall regeneration of this isolate. According to the biochemical data by MEDA *et al.*[14], IBP seems to inhibit cell wall synthesis of *P. oryzae* hyphae. On the other hand, AKATSUKA *et al.*[1] and KODAMA *et al.*[11] demonstrated that IBP primarily interfered with the plasma membrane through inhibition of phosphatidylcholin biosynthesis, and suggested that the apparent suppression of cell wall synthesis caused by IBP might be ascribed to the disturbance of transportation of cell wall materials from cytoplasm which was resulted from abnormal permeability of the plasma membrane. Thus, it is likely the interference of IBP with regeneration of protoplasts reflects suppression of cell wall synthesis of the present fungus through the disturbance of membrane permeability.

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イネいもち病菌 (*Pyricularia oryzae* Cav.) の胞子から分離したプロトプラストの細胞壁再生および菌糸復帰に及ぼす IBP の影響

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IBP (*S*-benzyl *O,O*-diisopropyl phosophorothioate) 感受性 (P2) 及び耐性 (PT83) のイネいもち病菌胞子から分離したプロトプラストの細胞壁再生および菌糸復帰に及ぼす同剤の影響を検討した。P2 のプロトプラストを 50 ppm IBP で30－120分前処理して培養したところ細胞壁再生は抑制されたが，プロトプラストの細胞壁を再生させた後に IBP 処理をしても菌糸復帰は顕著な阻害を受けなかった。これらの結果は，IBP 処理は菌糸復帰よりも細胞壁再生に強い抑制効果を持っていることを示している。両菌株のプロトプラストを培養前に30分間 IBP で処理すると P2 の細胞壁再生は抑制されたが，PT83 のそれは抑制されなかった。この結果は，プロトプラスト分離後30分以内に *de nove* の細胞壁再生が開始されること，及び PT83 の IBP 耐性は細胞壁再生に関わる生合成過程の変異に依存していることを示唆している。両菌株のプロトプラストの IBP 感受性および耐性は，既に明らかにされている胞子や菌糸のそれらとほぼ同じであった。