Effect of Adenosine 3′,5′-cyclic Monophosphate on Induction of Sclerotia in Rhizoctonia solani

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ABSTRACT

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Adenosine 3′,5′-cyclic monophosphate (cyclic AMP) and dibutyryl cyclic AMP induced formation of sclerotia in the isolate U-17 of *Rhizoctonia solani* which is normally unable to form sclerotia. Six isolates, including P-17, formed no sclerotia even when supplied with cyclic AMP, and five isolates (including isolate 0-0) formed sclerotia spontaneously without cyclic AMP. Endogenous cyclic AMP level was higher in isolate 0-0 than in isolate U-17. High activity of phosphodiesterase, which degrades cyclic AMP, was detected in mycelia of isolates U-17 and P-17. Isolate U-17 incorporated a significant amount of cyclic AMP, but isolates 0-0 and P-17 incorporated only small amounts. The possible participation of cyclic AMP in the induction of sclerotia is discussed.

Additional key words: anastomosis group 1, rice sheath blight fungus.

Morphological changes and macromolecular synthesis associated with differentiation of sclerotia of *Rhizoctonia solani* Kühn have been examined (5–7). Attempts to reveal the regulating events which trigger sclerotial induction in fungi have been made by many authors, and several reports have indicated that chemical substances can initiate the process. Recently factors affecting formation of sclerotia were reviewed by Chet and Henis (2), but the nature of triggering "factors" that initiate and regulate the process of sclerotial initiation of the rice sheath blight fungus is still obscure.

Adenosine 3′,5′-cyclic monophosphate (cyclic AMP) has been reported to control morphological changes in a wide variety of organisms including bacteria and fungi (15). The present report describes the effect of cyclic AMP on the induction of sclerotia in the anastomosis group 1 of *Rhizoctonia solani* Kühn.

MATERIALS AND METHODS

Organisms.—Various isolates of anastomosis group 1 of *Rhizoctonia solani* Kühn listed in Table 1 were used in this study. Sixty isolates except 0-0 to 0-4 were kindly supplied by Akira Ogoshi, University of Hokkaido, Sapporo, Japan (12,13). Of 60 isolates of anastomosis group 1, seven isolates produced no sclerotia on potato sucrose agar medium. Isolates 0-0 to 0-4 were obtained from infected rice plants in 1970 and 1971 as described previously (5,6). The fungus is synonymous with the *Rhizoctonia solani* anastomosis group 1 (AG-1) of Ogoshi (12,13) and Parmeter et al (14).

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Media and culture.—Plates containing basal medium were seeded centrally with 0.5 cm disks cut from the margin of a colony of the fungus grown on potato sucrose agar medium for 2 days. All plates were incubated at 25 C. In experiments involving the induction of sclerotia, cultures were grown on a modified Murashige and Skoog's (11) nutrient liquid medium without any organic substances except sucrose. The induction of sclerotia was not affected substantially between pH 4 and pH 6. Standard 9-cm diameter petri dishes each containing 20 ml medium were used to test sclerotial induction. After incubation for 10 days at 25 C, the percentage of plates containing sclerotia was determined.

Sclerotia formation began 3 days after seeding cultures of isolate 0-0 and after 4 days in those of isolate U-17. Although isolate P-17 formed no sclerotia, day 4 after seeding was considered to be the zero point of induction because the growth rate of this isolate was approximately the same as that of isolate U-17.

Cyclic AMP assay.—The intracellular concentration of cyclic AMP was measured in mycelia cultured in potato sucrose liquid medium. Twenty grams of mycelium were triturated for 5 min in 50 ml of cold 5% (v/v) trichloroacetic acid in a Waring Blendor. The resulting brei was centrifuged at 15,000 *g* for 20 min, and the supernatant solution was shaken with ethyl ether to remove trichloroacetic acid. The aqueous phase, concentrated to one-half under reduced pressure, was used as the crude sample.

Cyclic AMP was assayed by the protein binding method of Gilman (3). To each tube, components of the reaction mixture were added in the following order: 50 μliters of 200 mM-sodium acetate buffer, pH 4.0; 20 μliters of 40 nM-^{3}H-cyclic AMP (25 Ci/mM); 100 μliters

of sample; 20 μliters of binding protein; enough water to make the final volume 0.2 ml after addition of binding protein. Each tube was placed in ice-water for 100 min, then the contents were diluted to 1 ml with 20 mM-KH_2PO_4 buffer, pH 6.0. The contents were filtered through a Millipore cellulose ester filter disk. Each tube and filter disk were washed with 8 ml of 20 mM-KH_2PO_4 buffer, pH 6.0. The radioactivity of disks was counted by liquid scintillation techniques.

Incorporation of ^{3}H-cyclic AMP into mycelium.—To measure the amount of ^{3}H-cyclic AMP which was incorporated by mycelium, 1 ml of ^{3}H-cyclic AMP (5 μCi) was added to each petri dish. The culture was further incubated for 1 hr. The mycelium was harvested on a filter paper, washed with cold distilled water, blotted dry, and triturated for 3 min with 20 ml cold 5% (v/v) trichloroacetic acid. The resulting brei was centrifuged at 15,000 *g* for 20 min, and the radioactivity of the supernatant solution and of the trichloroacetic acid insoluble fraction was counted by liquid scintillation techniques.

Cyclic nucleotide phosphodiesterase assay.—Mycelia and sclerotia were harvested on a filter paper 1 to 6 days after seeding and were macerated for 5 min in 0.05 M tris (hydroxymethyl) aminomethane-hydrochloride (Tris-HCl) buffer, pH 7.4, in a Waring Blendor. After centrifugation, the supernatant solution was used as the crude enzyme preparation.

Reaction mixtures contained 25 pM ^{3}H-cyclic AMP, 0.1 mM cyclic AMP, 50 mM Tris-HCl buffer, pH 8.0, 5 mM $MgCl_2$ and enzyme the total volume being 0.4 ml. The mixtures were incubated for 10 min at 30 C and the reaction was then stopped by heating at 100 C for 2 min. The reaction mixture was applied to cellulose thin-layer sheets and developed with the following solvents: 1 M ammonium acetate-99% ethanol (30:75, v/v). The radioactivity of each spot was counted by liquid scintillation techniques.

Protein measurement.—Protein concentration was determined by the method of Lowry et al (9).

Sources of chemicals.—The sources of chemicals were as follows: cyclic AMP, dibutyryl cyclic AMP and other nucleotides (Sigma Chemical Co., St. Louis, MO 63178 USA), cyclic AMP assay kit and ^{3}H-cyclic AMP (Radiochemical Centre, Amersham, England).

RESULTS

Effect of cyclic AMP on the induction of sclerotia.—The twelve isolates of anastomosis group 1 of *Rhizoctonia solani* were inoculated on media with or without 10^{-5}M cyclic AMP. All cultures of isolates 0-0 to 0-4 isolated from different rice plants produced sclerotia both in the presence and absence of cyclic AMP. Isolate U-17 produced no sclerotia in the absence of cyclic AMP, but produced sclerotia in 50% of cultures supplied with cyclic AMP, when 4-day-old mycelia were used (Table 1, Fig. 1). Isolate U-17 produced sclerotia in 3% of cultures in the absence of cyclic AMP and in 56% of cultures in the presence of cyclic AMP, when 5-day-old mycelia were used. The other six isolates produced no sclerotia at all, even when supplied with cyclic AMP. Sclerotia formed in cultures of isolate U-17 were morphologically similar to those in isolates 0-0 to 0-4 (Fig. 1).

Isolates 0-0 to 0-4 grown on a modified Murashige and Skoog's nutrient liquid medium produced approximately 15 sclerotia per plate (Fig. 1). However, these isolates produced more than 50 sclerotia per plate when grown on potato sucrose agar. Formation of sclerotia was observed routinely 10 days after seeding, but no additional sclerotia were formed after prolonged incubation.

Effect of substances related to cyclic AMP on induction of sclerotia.—Various concentrations of 3′-nucleotides, 5′-nucleotides, cyclic AMP, and dibutyryl cyclic AMP were added to the medium to test for sclerotial induction in isolate U-17. Of the nucleotides tested only two of them, cyclic AMP and dibutyryl cyclic AMP, were effective in inducing sclerotia of U-17 at concentrations higher than 0.5 μM and 5 μM, respectively, when 5-day-old inocula were used (Table 2). The minimum concentrations of cyclic nucleotides that induced sclerotia were dependent on the age of inoculum; cultures seeded with 5-day-old mycelia were more sensitive to the chemicals than those seeded with 4-day-old mycelia. However, when 5-day-old mycelia were used, sclerotial induction was not observed at concentrations higher than 0.1 mM of cyclic nucleotides.

Cyclic AMP levels in different R. solani isolates.—Cyclic AMP levels in mycelium and sclerotium of isolates 0-0, U-17, and P-17 were measured at various

TABLE 1. Induction of sclerotia in various isolates of *Rhizoctonia solani* in the presence or absence of cyclic AMP

Isolate	Mycelium age (days)	Cultures[a] that produced sclerotia: Absence (%)	Presence (%)
B-60	4	0 ± 0	0 ± 0
S-3	4	0 ± 0	0 ± 0
L-7	4	0 ± 0	0 ± 0
U-17	4	0 ± 0	50 ± 3
	5	3 ± 1	56 ± 4
B-71	4	0 ± 0	0 ± 0
P-17	4	0 ± 0	0 ± 0
CS-4	4	0 ± 0	0 ± 0
0-0	4	100 ± 0	100 ± 0
0-1	4	100 ± 0	100 ± 0
0-2	4	100 ± 0	100 ± 0
0-3	4	100 ± 0	100 ± 0
0-4	4	100 ± 0	100 ± 0

[a]One hundred cultures of each isolate were incubated in the presence or absence of 1×10^{-5}M cyclic AMP at 25 C for 10 days.

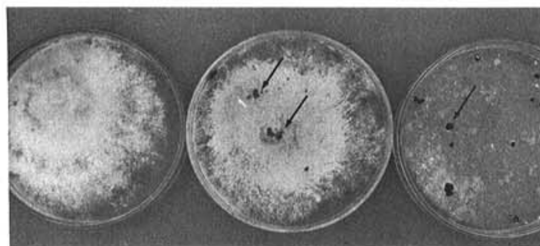

Fig. 1. Isolate U-17 of *Rhizoctonia solani* grown for 10 days with (center) or without (left) 1×10^{-4}M adenosine 3′,5′-cyclic monophosphate (cyclic AMP), and isolate 0-0 (right) grown for 10 days without cyclic AMP. Arrows indicate sclerotia.

intervals during growth (Fig. 2). Cyclic AMP level was high prior to sclerotial formation in isolate 0-0, reached a maximum level of approximately 23 pg per mg protein 2 days before sclerotial formation, and rapidly declined to about 50% of the maximum level at sclerotial formation. The accumulation of cyclic AMP in isolate U-17 was relatively low compared with isolate 0-0. In particular, the cyclic AMP level of isolate U-17 at the time of sclerotial induction was half of that observed in isolate 0-0 at the same stage. Isolate P-17 produced smaller amounts of cyclic AMP throughout the culture period tested. Cyclic AMP levels observed in isolate P-17 at and after sclerotial

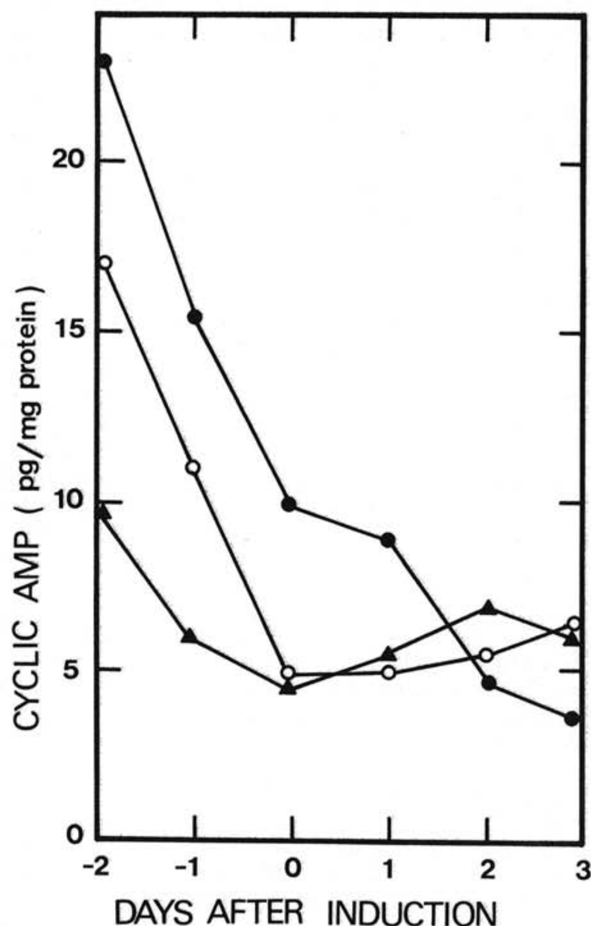

Fig. 2. Cyclic AMP levels in crude extracts obtained from mycelia (including sclerotia) of various ages in three isolates of *Rhizoctonia solani*. The time when recognizable sclerotial initials appeared in isolates 0-0 and U-17 is indicated as 0 day, and P-17 cuiltures grown for 4 days are expressed as 0 day. See Materials and Methods for further explanation. Legend: ●, isolate 0-0; ○, isolate U-17; and ▲, isolate P-17.

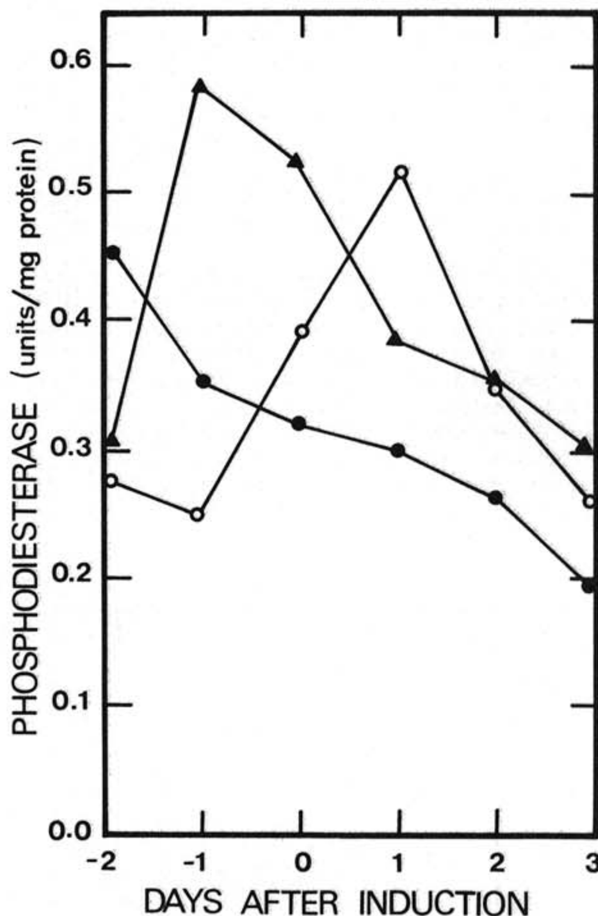

Fig. 3. Activity of cyclic nucleotide phosphodiesterase in crude extracts obtained from mycelia (including sclerotia) of various ages of three isolates of *Rhizoctonia solani*. Legend: ●, isolate 0-0; ○, isolate U-17; and ▲, isolate P-17.

TABLE 2. Effect of substances related to cyclic AMP on induction of sclerotia

	Mycelium age (days)	Percent of cultures[a] that produced sclerotia at molar concentrations:								
		1×10^{-3}	5×10^{-4}	1×10^{-4}	5×10^{-5}	1×10^{-5}	5×10^{-6}	1×10^{-6}	5×10^{-7}	1×10^{-7}
Adenosine-5′-monophosphate		0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Adenosine-5′-diphosphate		0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Adenosine-5′-triphosphate		0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Adenosine-3′-monophosphate		0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Cyclic AMP	4	59 ± 4	50 ± 4	45 ± 3	40 ± 3	40 ± 4	30 ± 2	0 ± 0	0 ± 0	0 ± 0
	5	0 ± 0	0 ± 0	0 ± 0	20 ± 1	50 ± 5	40 ± 2	80 ± 2	40 ± 3	0 ± 0
Dibutyryl cyclic AMP	4	50 ± 1	40 ± 1	40 ± 2	10 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	5	0 ± 0	0 ± 0	0 ± 0	80 ± 3	30 ± 1	40 ± 1	0 ± 0	0 ± 0	0 ± 0

[a]Fifty cultures of isolate U-17 were incubated with the chemical at the concentration indicated at 25 C for 10 days.

induction were approximately the same as those observed in isolate U-17. The level of cyclic AMP found in mycelia of isolate 0-0 corresponded well with that of cyclic AMP effective in inducing sclerotia in isolate U-17.

Regulation of cyclic AMP levels by cyclic nucleotide phosphodiesterase activities.—Crude mycelial extracts of isolates 0-0, U-17, and P-17 at various stages of growth were assayed for phosphodiesterase activity. Enzyme activity was always detected in the mycelia, whether or not they were able to form sclerotia, but the levels of enzyme activity varied during the course of sclerotial formation (Fig. 3). Enzyme activity of isolate 0-0 was relatively high in early growth stages but decreased during the course of sclerotial formation. The enzyme activity of isolate U-17 increased at the stage of sclerotial induction, reached a maximum level 1 day after induction and then declined. The enzyme activity of isolate P-17, which produced no sclerotia, was high during the early stages of growth.

Incorporation of ^{3}H-cyclic AMP into mycelial cells.—Cyclic AMP added to the culture of isolate U-17 induced sclerotia. Therefore, mycelia which had been grown in the presence of ^{3}H-cyclic AMP were fractionated to examine their ability to incorporate cyclic AMP. The radioactivity of trichloroacetic acid-soluble and -insoluble fractions was measured. The results (Fig. 4) clearly indicate that mycelia of isolate U-17 incorporated a significant amount of ^{3}H-cyclic AMP, whereas those of isolates 0-0 and U-17 incorporated relatively little. Only very small amounts of ^{3}H-cyclic AMP were taken up by mycelia of isolates 0-0 and P-17 during early stages of growth.

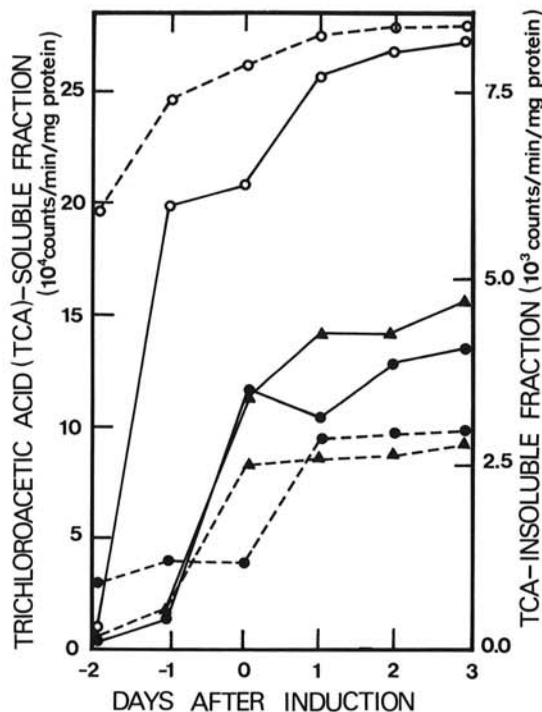

Fig. 4. Incorporation of ^{3}H-cyclic AMP into mycelia of *Rhizoctonia solani*. Legend: ——, trichloroacetic acid (TCA)-soluble fraction; – – –, trichloroacetic acid-insoluble fraction; ●, isolate 0-0; ○, isolate U-17; and ▲, isolate P-17.

DISCUSSION

The control mechanisms regulating the process of sclerotial induction in fungi are unknown, although attempts have been made by many authors to reveal the early events that trigger this process in filamentous fungi. Henis and Chet (8) suggested that unknown factors are supplied to the developing sclerotia in *Sclerotium rolfsii* by the surrounding mycelium. The existence of a morphogenetic factor(s) that triggers the induction of sclerotia in *S. rolfsii* and microsclerotia in *Verticillium albo-atrum* has been suggested by both Goujon (4) and Brandt (1).

The present results offer evidence which may help to elucidate the mechanism of sclerotial induction in *R. solani*. Sclerotial induction appears to be regulated by genetic and environmental factors and by sclerotia-inducing substance(s). Three types of *R. solani* isolates were used in the present study; five isolates (0-0 to 0-4) produced sclerotia constitutively, one (U-17) formed them only in the presence of cyclic AMP, and other six isolates produced no sclerotia in the presence or absence of cyclic AMP.

Both the spontaneous production of sclerotia and the minimum concentrations of cyclic nucleotides effective in inducing sclerotia in isolate U-17 depended on the age of the mycelium. Although it may be speculated that aged mycelium contains higher concentrations of substances that induce sclerotia, such as cyclic AMP, more research is required to elucidate the manner in which aged mycelium formed sclerotia.

Recently, Marukawa and Satomura (10) reported that cyclic AMP alone stimulated the production of melanine pigments in the medium of *Sclerotium rolfsii*. Also, a combination of cyclic AMP and sclerin further increased melanogenesis and induced the formation of sclerotium-like hyphal aggregates.

Our results show that cyclic AMP and dibutyryl cyclic AMP effectively induced sclerotia in isolate U-17. Mycelia of isolate 0-0 of *R. solani*, which are constitutively able to form sclerotia, contained a high level of cyclic AMP. Mycelia of isolate U-17 in which sclerotial induction was stimulated in the presence of cyclic AMP, contained relatively low levels of cyclic AMP, particularly at the time of sclerotial induction. This observation, together with the fact that the level of cyclic AMP increased immediately prior to sclerotial formation in isolate 0-0, indicates that a higher level of cyclic AMP probably is associated with sclerotial induction. On the other hand, phosphodiesterase activity was found in the three isolates of *R. solani*. The time when isolate U-17 showed increased specific activity of the enzyme coincided with the time of sclerotial formation in cultures supplied with cyclic AMP. Moreover, isolate U-17 was able to incorporate a significant amount of ^{3}H-cyclic AMP into its mycelial cells. Perhaps mycelium of isolate U-17, which contains a large amount of phosphodiesterase, rapidly degrades endogenous cyclic AMP, and therefore this isolate requires an external

supply of cyclic AMP to form sclerotia. On the other hand, mycelium of isolate P-17 showed a very high activity of phosphodiesterase at an earlier stage of growth, contained a low level of cyclic AMP, and had little ability to incorporate ^{3}H-cyclic AMP. This difference in incorporation ability may explain why cyclic AMP added to the culture medium was ineffective in inducing sclerotia in isolate P-17, although it was effective in isolate U-17. Thus, we conclude that cyclic AMP may play an important role in induction of sclerotia. The manner in which sclerotia are induced in the presence of cyclic AMP remains to be elucidated.

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