リボソームタンパク質のポリアクリルアミドゲル電気泳動法によるコムギからの各種雪腐病菌の検出

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Detection of Snow Mold Fungi in Wheat Plants by Means of Polyacrylamide Gel Electrophoresis of Ribosomal Proteins

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Abstract

Ribosomes were isolated from four snow mold fungi (*Pythium paddicum, P. iwayamai, Typhula incarnata* and *Fusarium nivale*) and wheat seedlings, and their proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). No difference was found in electrophoretic patterns between *P. paddicum* and *P. iwayamai*. However, there were several differences among two *Pythium* species, *T. incarnata, F. nivale* and wheat. The ribosomes were isolated also from wheat seedlings infected with *P. paddicum, T. incarnata* and *F. nivale* individually, and their proteins were analyzed by SDS-PAGE. The distinctive fungal ribosomal protein bands were detected in all patterns obtained from the infected wheat plants. The possibility of the ribosomal protein-based assay to detect snow mold fungi in wheat plants is suggested.

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Key words: snow mold fungi, electrophoresis, fungal ribosomal proteins, diagnosis.

Introduction

In Hokuriku area in Japan, three snow mold diseases have occurred on winter wheat plants (*Triticum aestivum* L.). The first is Pythium snow rot caused by *P. paddicum, P. iwayamai* and other *Pythium* spp.[2], the second is gray snow mold caused by *Typhula incarnata*, and the third is pink snow mold caused by *Fusarium nivale*. These diseases have occurred coexistently rather than singly in the fields. At present, diseased plants are diagnosed by observation of symptoms or by isolation and identification of the pathogen. However, the symptoms of these diseases are often similar and the culture method can be unreliable because a fast growing fungus may overgrow other fungi within a few days. Therefore, a new method has been required for the detection of the causual fungi in infected plants.

Marshall and Partridge[6,7] utilized ribosomal protein differences between a parasite and a host plant to detect *F. moniliforme* in stalk rotted corn. Eukaryotic ribosomes contain 70-80 different proteins[12], and in general there is no qualitative difference in ribosomal proteins from diverse tissues or organs and from various stages of develop-

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ment of the same species[13]. Furthermore, previous studies[1,4,10,12,13] have shown that there were electrophoretic and serological differences among the ribosomal proteins of different organisms. These studies suggest that the ribosomal protein-based assay has the potential to detect the snow mold fungi in host plants.

In this paper, we describe the comparison of ribosomal proteins of four snow mold fungi and a wheat plant, and the detection of fungal ribosomal proteins in infected plants by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Materials and Methods

Materials. Ten isolates of snow mold fungi shown in Table 1 were used for ribosome extraction. Isolates of *P. paddicum*, *P. iwayamai* and *F. nivale* were grown in potato sucrose broth at 15 C for 1-3 weeks, and *T. incarnata* isolates were grown at 10 C for 4-8 weeks. The mycelial mats were harvested by centrifugation or filtration, washed repeatedly with distilled water and stored at −80 C. Two cultivars (Nōrin No. 61, Yukichabo) and one line (Tōsan No. 16) of wheat plants were grown in a glasshouse until 1-1.5 leaf stage and leaves were harvested for ribosome extraction. To obtain infected plants, wheat seedlings at 3-3.5 leaf stage were inoculated with *P. paddicum*, *T. incarnata* and *F. nivale* individually by artificial inoculation method[11].

Ribosome extraction. The frozen materials of fungal and plant tissues were ground and homogenized with a mortar and pestle in two volumes of buffer A[6] (50 mM Tris-HCl, pH 8.5, 60 mM KCl, 30 mM $MgCl_2$, 0.2 M sucrose) containing 5 mM 2-mercaptoethanol. The homogenate was filtered through a double-layer of cheesecloth (the filtration was omitted for fungal homogenate) and centrifuged at $21,500\times g$ for 30 min at 4 C. The supernatant, ca. 8 ml per tube, was layered on a 1.5 ml pad of 1.5 M sucrose in buffer A containing 5 mM 2-mercaptoethanol, and centrifuged at $189,400\times g$ for 4 hr at 4 C. The resulting ribosomal pellet was resuspended in buffer B[6] (20 mM Tris-HCl, pH 7.5, 20 mM KCl, 10 mM $MgCl_2$) and the suspension was centrifuged at $23,000\times g$ for 5 min at 4 C. For further purification, the crude ribosomal suspension, less than

Table 1. Fungal isolates used

Isolate	Species	Geographic origin
H-82-73[a]	*Pythium paddicum*	Imajō-Chō, Fukui
P-7[a]	*P. paddicum*	Sabae-Shi, Fukui
W-82-15[a]	*P. paddicum*	Asahi-Chō, Fukui
W-82-50[a]	*P. iwayamai*	Takefu-Shi, Fukui
HT8301	*Typhula incarnata*	Jōetsu-Shi, Niigata
HT8302	*T. incarnata*	Ishikawa
Wh63D[b]	*T. incarnata*	Kuroisi-Shi, Aomori
HS-50[a]	*Fusarium nivale*	Ōno-Shi, Fukui
HF8001	*F. nivale*	—
HF8301	*F. nivale*	Jōetsu-Shi, Niigata

a) Supplied by Mr. S. Takamatsu, Fukui Agricultural Experiment Station.
b) Supplied by Mr. N. Matsumoto, Hokkaido National Agricultural Experiment Station.

72 A_{260} units per tube, was layered onto 10-35% (w/v) linear sucrose density gradient in buffer B, centrifuged in a Hitachi RPS 25 rotor at 52,000×*g* for 5 hr at 4 C, and fractionated into 30 fractions. Two ml of buffer B was added to each fraction and absorbance at 260 nm was measured with a Shimazu UV-240 spectrophotometer. The monosome and polysome regions of these fractions were collected, and centrifuged at 189,400×*g* for 2 hr at 4 C after adding 5 mM 2-mercaptoethanol. The resulting pellets were suspended in a small volume of 50 mM Tris-HCl, pH 8.8, and the suspension was centrifuged at 10,000 rpm for 2 min in a Hitachi RPR 18-3 rotor. The resulting supernatant was stored at −80 C for use as the purified ribosomal suspension.

SDS-PAGE. After adding equal volumes of dissociation buffer (50 mM Tris-HCl, pH 8.8, 4% SDS, 2% 2-mercaptoethanol), the purified ribosomal suspension was heated at 100 C for 5 min. The dissociated samples (1.0 A_{260} unit per well) were electrophoresed in a 10% polyacrylamide vertical slab gel, 14×10×0.2 cm, by using the discontinuous buffer system described by Laemmli[5]. For the molecular weight (mol. wt.) determinations, phosphorylase *a* (mol. wt. 92.5×10^3 daltons), bovine serum albumin (66×10^3), aldolase (39×10^3), carbonic anhydrase (28.8×10^3), tobacco mosaic virus coat protein (17.5×10^3) and cytochrome *c* (12.3×10^3) were used as marker proteins.

Results

Yield and quality of extracted ribosomes

The extraction procedure yielded an average of 14.6 A_{260} units/g of crude ribosomal suspensions from *P. paddicum* and *P. iwayamai*, 11.0A_{260} units/g from *T. incarnata*, 8.0 A_{260} units/g from *F. nivale* and 7.7 A_{260} units/g from wheat in fresh weight. When these crude ribosomal suspensions were centrifuged through a sucrose density gradient, monosome and smaller polysome peaks were detected. In particular, the monosome was predominant in *P. paddicum* and *P. iwayamai* preparations, which was probably due to polysome degradation by RNase or divalent cations in tissue[3]. The average A_{260}/A_{235} and A_{260}/A_{280} ratios of ribosomes taken after the sucrose density gradient centrifugation were 1.84, 2.04 for two *Pythium* species ribosomes, 1.79, 2.00 for *T. incarnata*, 1.67, 2.00 for *F. nivale* and 1.73, 1.99 for wheat. The less the extraneous proteins are present in a ribosomal preparation, the higher the A_{260}/A_{235} ratio is obtained[8]. Petermann and Pavlovec[9] reported that the A_{260}/A_{235} ratio of the ribosomal fraction was 1.67, which showed no extraneous proteins on electrophoretic analysis. This suggests that our ribosomal preparations were relatively free of extraneous proteins.

SDS-PAGE patterns of ribosomal proteins of snow mold fungi and wheat

Ribosomal preparations from snow mold fungal isolates and wheat plants were examined (Fig. 1). Within each species, there was no obvious difference in the electrophoretic patterns. To compare the ribosomal proteins of four different snow mold fungi and wheat, each preparation was run on the same gel (Fig. 2). No difference was found in the electrophoretic patterns between *P. paddicum* and *P. iwayamai*. However, there were several differences among two *Pythium* species, *T. incarnata*, *F. nivale* and wheat. Qualitative differences were recongnized below mol. wt. of 33×10^3 daltons (33K) among

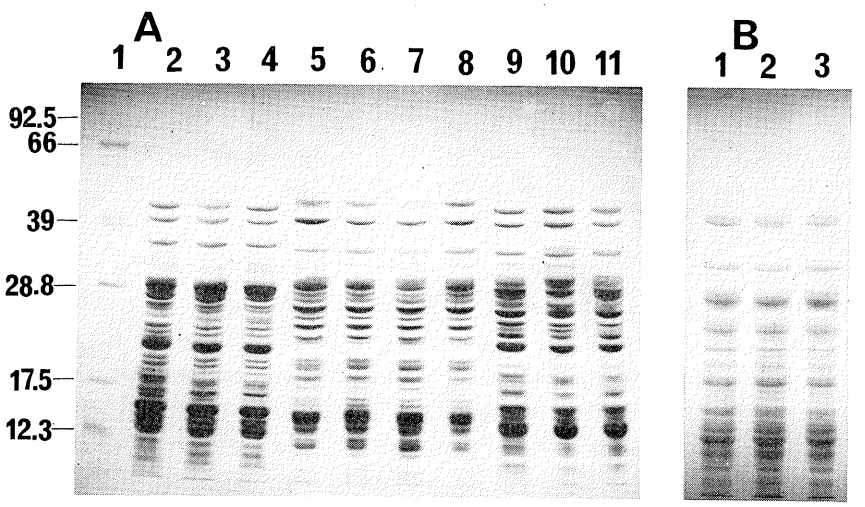

Fig. 1. SDS-PAGE patterns of ribosomal proteins of snow mold fungal isolates (A) and wheat plants (B). A: (lane 1) marker proteins ($\times 10^3$ daltons, see Materials and Methods). (lane 2) HS-50. (lane 3) HF8001. (lane 4) HF8301. (lane 5) W-82-50. (lane 6) H-82-73. (lane 7) P-7. (lane 8) W-82-15. (lane 9) HT8302. (lane 10) Wh63D. (lane 11) HT8301. B: (lane 1) Nōrin No. 61. (lane 2) Yukichabo. (lane 3) Tōsan No. 16.

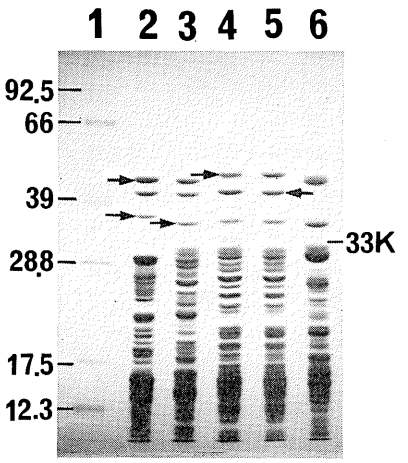

Fig. 2. SDS-PAGE patterns of ribosomal proteins of four snow mold fungi and wheat. (lane 1) marker proteins ($\times 10^3$ daltons, see Materials and Methods). (lane 2) *Fusarium nivale*. (lane 3) *Typhula incarnata*. (lane 4) *Pythium iwayamai*. (lane 5) *P. paddicum*. (lane 6) wheat. The arrows indicate the distinctive bands above mol. wt. of 33 $\times 10^3$ daltons (33K).

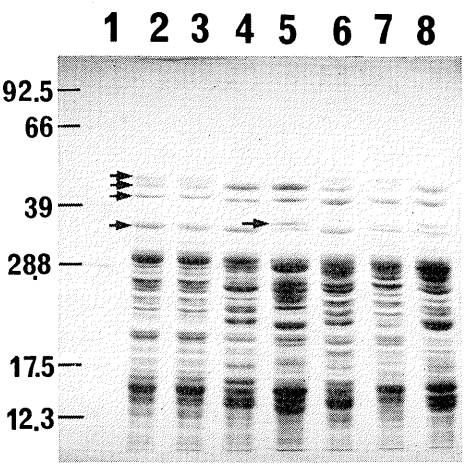

Fig. 3. SDS-PAGE patterns of ribosomal protein mixtures. Two ribosomal preparations were mixed (1:1) and co-electrophoresed. (lane 1) marker proteins ($\times 10^3$ daltons, see Materials and Methods). (lane 2) wheat + *Pythium paddicum*. (lane 3) wheat + *P. iwayamai*. (lane 4) wheat + *Typhula incarnata*. (lane 5) wheat + *Fusarium nivale*. (lane 6) *P. paddicum* + *T. incarnata*. (lane 7) *P. paddicum* + *F. nivale*. (lane 8) *T. incarnata* + *F. nivale*. The arrows indicate the distinctive bands of fungal and wheat ribosomes.

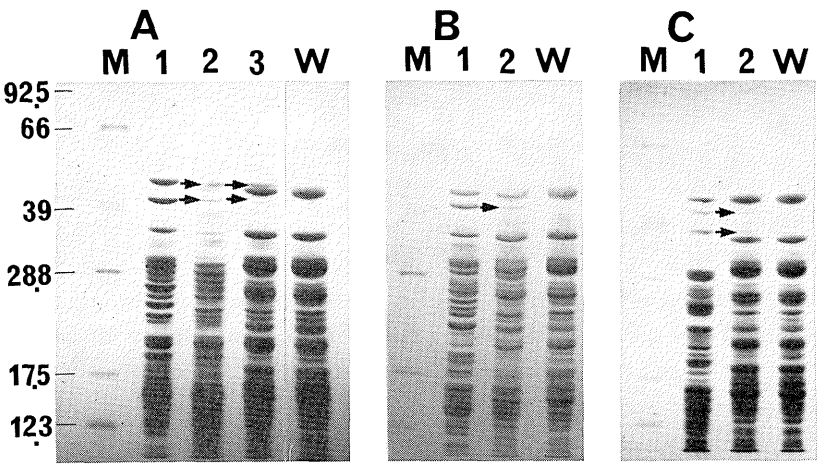

Fig. 4. Detection of fungal ribosomal proteins in the electrophoretic patterns obtained from wheat seedlings inoculated with *Pythium paddicum* (A), *Typhula incarnata* (B) and *Fusarium nivale* (C). M and W represent marker proteins ($\times 10^3$ daltons, see Materials and Methods) and the healthy wheat ribosomal pattern, respectively. A: (lane 1) *P. paddicum*. (lane 2) wheat seedlings 14 days after inoculation. (lane 3) wheat seedlings 11 days after inoculation. B: (lane 1) *T. incarnata*. (lane 2) wheat seedlings 14 days after inoculation. C: (lane 1) *F. nivale*. (lane 2) wheat seedlings 14 days after inoculation. The arrows indicate the distinctive fungal bands.

these ribosomal proteins. Qualitative differences were also found in ribosomal proteins above 33 K. From *P. paddicum* and *P. iwayamai* ribosomes, three kinds of polypeptides with 47 K, 41 K and 35 K were detected. *T. incarnata* ribosome contained three kinds of polypeptides with 45 K, 41 K and 35 K. *F. nivale* ribosome revealed three kinds of polypeptides with 45 K, 41 K and 37 K. From wheat ribosome, only two kinds of polypeptides with 45 K and 35 K were detected. These findings indicate that the 41 K band is characteristic of fungal ribosomes. When two ribosomal preparations were mixed in equal quantities and co-electrophoresed, the distinctive bands of respective fungal ribosomes could be detected in the composite pattern (Fig. 3). For example, the 47 K and 35 K bands, characteristic of *P. paddicum*, and the 45 K and 37 K bands, characteristic of *F. nivale*, were found in the composite pattern of these fungi (Fig. 3, lane 7).

Detection of fungal ribosomal proteins in infected plants

The ribosomes were extracted from wheat seedlings infected with *P. paddicum*, *T. incarnata* and *F. nivale* individually, and were electrophoresed along with those of each pathogen and wheat (Fig. 4). The distinctive fungal bands were detected in all preparations of the infected wheat plants. The 47 K and 41 K bands, which were characteristic of *P. paddicum* ribosome, were detected in two electrophoretic patterns of the plants infected with this pathogen (Fig. 4 A, lane 2 and 3). The electrophoretic pattern obtained from the plants 11 days after inoculation was similar to that of the healthy plants, whereas the pattern obtained from the plants 14 days after inoculation was sim-

ilar to that of *P. paddicum*. The 41 K band, characteristic of *T. incarnata*, was detected in the infected plants (Fig. 4 B). Similarily, the 41 K and 37 K bands of *F. nivale* were also detected in the infected plants (Fig. 4 C).

Discussion

Marshall and Partridge[6,7] characterized the ribosomal proteins as specific fungal proteins in *Fusarium* stalk rot of corn, and demonstrated that the ribosomal protein differences could be utilized for the detection of a parasite in host plants. We also studied the comparison of the ribosomal proteins of four snow mold fungi and wheat by SDS-PAGE. These ribosomal protein patterns were reproducible in different electrophoretic runs, and were uniform within each species (Fig. 1). There were several differences among two *Pythium* species, *T. incarnata*, *F. nivale* and wheat, but no difference was found between the two *Pythium* species (Fig. 2). This suggests that at least the analysis of ribosomal proteins by SDS-PAGE can find the differences among these organisms belonging to different genera.

The distinctive fungal bands could be detected in wheat plants infected with *P. paddicum*, *T. incarnata* and *F. nivale* individually (Fig. 4). The electrophoretic pattern of ribosomal preparation from wheat seedlings 11 days after inoculation with *P. paddicum* was similar to that of healthy plants, while the pattern of wheat seedings 14 days after inoculation was similar to that of *P. paddicum* (Fig. 4 A, lane 2 and 3), indicating that the detection ratio of fungal ribosomes to wheat ones increased with progress of the infection. These findings suggest that the ribosomal protein-based assay can at least distinguish among two *Pythium* species, *T. incarnata* and *F. nivale*, in infected wheat tissues. On the basis of these results, we are attempting to develop the ribosomal protein-based assay by employing serological techniques for the diagnosis of snow mold diseases.

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和 文 摘 要

竹中重仁・吉野嶺一：リボソームタンパク質のポリアクリルアミドゲル電気泳動法によるコムギからの各種雪腐病菌の検出

4種の雪腐病菌（*Pythium paddicum, P. iwayamai, Typhula incarnata* および *Fusarium nivale*）とコムギからリボソームを抽出し，SDS-ポリアクリルアミドゲル電気泳動法によって，それらのリボソームタンパク質の泳動パターンを比較した。その結果，*P. paddicum* と *P. iwayamai* との間では泳動パターンに差異が認められなかったが，2種の *Pythium* 菌，*T. incarnata, F. nivale* およびコムギの間では明らかな差異が認められた。また，*P. paddicum, T. incarnata* および *F. nivale* を各々接種したコムギからリボソームを抽出し，それらの泳動パターンを比較した結果，各種罹病コムギから病原菌のリボソームタンパク質のバンドが検出された。これらの結果から，各菌のリボソームタンパク質による本病の診断の可能性が示唆された。