Protein Synthesis during Germination and Appressorium Formation of *Colletotrichum lagenarium* Spores

By K. SUZUKI,* I. FURUSAWA, N. ISHIDA AND M. YAMAMOTO
Laboratory of Plant Pathology, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan

(Received 7 August 1980; revised 6 October 1980)

Protein synthesis during germination and appressorium formation of *Colletotrichum lagenarium* spores was investigated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and fluorography. Synthesis of polypeptides with molecular masses of 72, 43 and 38 kilodaltons was detected only in the 20000 g supernatant fraction of spore homogenates at early stages (5–25 min) of incubation. These polypeptides are probably associated with the protein synthesis essential for spore germination that occurs within 40 min from the start of incubation. A polypeptide with a molecular mass of 95 kilodaltons was specifically synthesized only when appressoria were formed. When synthesis of proteins, including the 95 kilodalton polypeptide, was completely inhibited by cycloheximide added after 1 h incubation, appressoria matured in structure but not in function; they seemed to have no ability to penetrate artificial membranes.

INTRODUCTION

It is generally accepted that protein synthesis is essential for fungal spore germination (Lovett, 1976). Qualitative and quantitative differences in proteins synthesized at the early and later stages of spore germination have been reported for *Blastocladiella emersonii* (Silverman *et al.*, 1974) and *Botryodiplodia theobromae* (Van Etten *et al.*, 1972; Wenzler & Brambl, 1978), but the proteins synthesized during spore germination have not been identified and their function has not been sufficiently discussed.

In some plant pathogenic fungi, the formation of appressoria is a prerequisite of invasion of host plants (Emmett & Parbery, 1975). A relation between formation of infection structures and protein synthesis has been reported for a few plant pathogenic fungi (Staples & Yaniv, 1976) and the formation of infection structures in rust uredospores was blocked by inhibitors of protein and RNA synthesis (Dunkle *et al.*, 1969; Ramakrishnan & Staples, 1970). Protein synthesis was also required for appressorium formation by anthracnose fungi such as *Colletotrichum trifolii* (Miehle & Lukezic, 1972) and *C. truncatum* (Staples *et al.*, 1976).

Spores of *Colletotrichum lagenarium* can germinate even if protein synthesis is inhibited by cycloheximide from 40 min after the start of incubation, but before this time protein synthesis is indispensable for spore germination (Furusawa *et al.*, 1977). Appressorium formation of this fungus is temperature-sensitive: appressoria formed when spores were incubated at 24 °C, but did not form at 32 °C (Ishida & Akai, 1969). Moreover, appressorium formation could also be influenced by antibiotics (Tani *et al.*, 1977).

In the work reported here, proteins synthesized during spore germination and appressorium formation of *C. lagenarium* were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis; the function of these proteins in the morphological differentiation of spores is discussed.

METHODS

Spores. *Colletotrichum lagenarium* (Pass.) Ellis and Halsted was cultured on potato sucrose agar medium at 24 °C for 7 d. Spores on the mycelial mat were collected with a brush and washed three times by centrifugation with ice-cold distilled water (Tani *et al.*, 1977).

Preparation of crude protein for electrophoresis. To investigate qualitative changes of proteins synthesized in *C. lagenarium* spores, 5 ml of spore suspension (5×10^5 spores ml⁻¹) containing 0.3 mM-chloramphenicol and 0.01% (v/v) Tween 20 was poured into a Petri dish (9 cm diam.) and the spores were pulse labelled with [³H]leucine [5 µCi ml⁻¹, specific activity 53 mCi mmol⁻¹ (1.96 GBq mmol⁻¹); The Radiochemical Center, Japan] for 20–30 min. The Petri dish was then placed on ice and 1 ml 355 µM-cycloheximide was added to the spore suspension to suppress further protein synthesis completely. The spores were collected by centrifugation at 1500 g for 10 min and homogenized in a Potter glass homogenizer with 100–150 µl 0.1 M-Tris/HCl buffer, pH 8.0, containing 0.01 M-Na₂EDTA and 0.01% (v/v) 2-mercaptoethanol. The homogenate was centrifuged at 500 g for 1 min to remove undisrupted spores. To the supernatant was added an equal volume of 0.0625 M-Tris/HCl buffer, pH 6.8, containing 2% (w/v) sodium dodecyl sulphate (SDS), 2% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.01% (w/v) bromophenol blue (Laemmli, 1970). The mixture was boiled for 3–5 min, then centrifuged at 3000 g for 20 min and the resulting supernatant was subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) on 10% (w/v) gels. To count radioactivity, 20 µl of each sample was placed on a Whatman 3MM filter paper (24 mm diam.). Each filter paper was washed three times with 30 ml 5% (w/v) cold trichloroacetic acid (TCA) for 15 min and then washed three times with ethanol. The radioactivity was counted in a liquid scintillation spectrometer (Packard, model 2425) as described previously (Furusawa *et al.*, 1977).

Electrophoresis and fluorography. Polyacrylamide gels were prepared according to Studier (1973). Samples of 20–60 µl were loaded on to 4.5% (w/v) acrylamide gel (about 1 cm depth) stacked on 10% acrylamide gel (10 × 15 cm, 2 mm thick). Electrophoresis was carried out using the buffer system of Laemmli (1970) at 40 V for 16 h at room temperature. In order to estimate the molecular masses of the synthesized polypeptides, human gamma globulin (mol. mass 160000 daltons), rinderserum albumin (68000), ovalbumin (45000) and myoglobin (17800) (all from Mann Research Laboratories) were co-electrophoresed. After electrophoresis, the gel was treated with 50% TCA for 4 h at 4 °C and stained with 0.1% (w/v) Coomassie brilliant blue at 35 °C for 1 h and then destained in a solution of 30% (v/v) methanol/10% (v/v) acetic acid. The gel was processed for fluorography (Bonner & Laskey, 1974) and then dried under vacuum at 65 °C for 1 h on Whatman 3MM paper. Kodak X-O-Mat X-ray film pre-exposed to a brief flash of light (Laskey & Mills, 1975) was placed in contact with the dried gel, exposed at –70 °C for 10–14 d, and developed.

Observation of the penetration process on cellulose membranes. A nitrocellulose membrane (2 × 2 cm), prepared from Visking cellulose tubing, with a uniform coating of spores was soaked in 2 ml sterile distilled water in a Petri dish (4.5 cm diam.). After 1 h incubation, the water was exchanged for 2 ml cycloheximide solution (3.55 µM). After various incubation times, the membrane was washed with sterile water, replaced in distilled water, and incubated for a further 48 h. The membrane was stained with lactophenol cotton blue or ZnCl₂/KI solution (Araki & Miyagi, 1976). After washing with water, penetration hyphae originating from appressoria and halo zones around the penetration sites were observed by light microscopy.

RESULTS

Colletotrichum lagenarium spores began to germinate within 3 h at 24 °C. The tip of the germ tube began to swell within 5 h at this temperature, and pigmented appressoria were formed by 8 h; at 32 °C, the germ tube elongated continuously without forming appressoria (Tani *et al.*, 1977). Germinating spores were pulse-labelled with [³H]leucine (5 µCi ml⁻¹) at 24 °C for a 20 min period: 5–25 min, 60–80 min or 160–180 min after the start of incubation. Many polypeptides with different molecular masses were detected on the fluorograms for each of the pulse periods (Fig. 1a). Polypeptides with molecular masses of 155, 93, 76, 72, 60, 43 and 38 kilodaltons (K) were synthesized specifically in the 5–25 min pulse period, whereas 210K and 90K polypeptides were only detected in the 160–180 min pulse period. Many of the polypeptides detected were common to the 5–25 and 60–80 min pulse periods and some were common to all three pulse periods.

To investigate the distribution of polypeptides synthesized during the three pulse periods, the crude protein fractions were separated by centrifugation into 1000 g pellet, 20000 g pellet and 20000 g supernatant fractions. The 20000 g pellet fraction contained 5–7% of the total radioactivity incorporated into acid-insoluble material. The 1000 g pellet and 20000 g supernatant fractions contained 44–60% and 35–50% of the total radioactivity, respectively.

Fig. 1. Fluorograms after SDS-PAGE of polypeptides synthesized by spores pulse-labelled with [^3H]leucine for 5–25 min (track 1), 60–80 min (track 2) and 160–180 min (track 3) after the start of incubation at 24 °C: (a) total crude protein; (b) 20 000 g supernatant fraction; (c) 1000 g pellet fraction; (d) 20 000 g pellet fraction. The radioactivity of samples in (a), (b) and (c) was adjusted to 10 000 c.p.m. and those in (d) to 1500 c.p.m. The numbers indicate the molecular masses of the polypeptides in kilodaltons.

Fig. 2. Fluorograms after SDS-PAGE of polypeptides synthesized by spores pulse-labelled with [^3H]leucine for each 30 min period until 7.5 h after the start of incubation at 24 °C (*a*) or 32 °C (*b*). The radioactivity of each sample was adjusted to 10000 c.p.m. The numbers indicate the molecular masses of the polypeptides in kilodaltons.

Fig. 3. Scanning patterns of fluorograms after SDS-PAGE of polypeptides synthesized by spores incubated under various conditions and pulse-labelled with [^3H]leucine for 1 h, starting 6 h after the start of incubation. The arrows indicate the location of the 95K polypeptide. The radioactivity of each sample was adjusted to 10000 c.p.m. Incubation conditions: (a) 24 °C; (b) 32 °C; (c) 24 °C for 4 h followed by 32 °C; (d) 24 °C in the presence of 0.5 M-sucrose; (e) 32 °C for 6 h followed by 24 °C; (f) 32 °C for 4 h followed by 24 °C.

The fluorograms of polypeptides in the three fractions are shown in Fig. 1 (b, c, d). The 72K, 43K and 38K polypeptides were found to be specifically synthesized in the 20000 g supernatant fraction only during the 5–25 min pulse period. The 155K, 93K and 60K polypeptides, however, appeared common to the 20000 g supernatant fraction for the three pulse periods. The 76K polypeptide was detected only in the 1000 g pellet fraction for the 5–25 min and 60–80 min pulse periods. Polypeptides which appeared in the 20000 g pellet fraction were detected in the other two fractions for all pulse periods.

To investigate the synthesis of polypeptides involved in appressorium formation, spores were incubated at 24 °C (appressoria formed) and at 32 °C (appressoria not formed) and pulse-labelled with [^3H]leucine (5 $\mu\text{Ci ml}^{-1}$) for each 30 min period from the start of incubation. Synthesized proteins were analysed by SDS-PAGE and fluorography (Fig. 2). In the early stage, from 0 to 3.5 h incubation, no specific polypeptide was synthesized in spores incubated at 24 °C. However, in the later stages after 3.5 h, a 95K polypeptide was newly synthesized specifically in spores incubated at 24 °C. This polypeptide was the most radioactive of those detected from the 24 °C incubation. Synthesis of this polypeptide preceded the initiation of appressorium formation by 1 h and continued during appressorium formation. At 32 °C, the 80K and 25K polypeptides were more radioactive than those at 24 °C from the start of incubation.

Fig. 4. Spore germination and appressorium formation on nitrocellulose membranes: (a) incubated at 24 °C for 48 h; (b) incubated at 24 °C in the presence of 3.55 μ M-cycloheximide for 32 h, starting 1 h after the start of incubation, followed by incubation in water for a further 48 h; (c) incubated at 32 °C for 48 h; (d) incubated at 24 °C in the presence of 0.5 M-sucrose for 48 h. After incubation the membranes were stained with ZnCl_2/KI solution (a, b) or lactophenol cotton blue (c, d). The bar markers represent 30 μ m.

The association of the 95K polypeptide with appressorium formation was then investigated. Spores of *C. lagenarium* did not form appressoria if (1) they were incubated at 32 °C from the start of incubation, (2) they were pretreated at 32 °C for more than 6 h before incubation at 24 °C, (3) they were incubated at 24 °C for the first 4 h, then subsequently at 32 °C, or (4) they were treated with 0.5 M-sucrose at 24 °C from the start of incubation (spore germination was not influenced at this concentration of sucrose – see Fig. 4d). Spores were incubated under one of these conditions and pulse-labelled with [^3H]leucine for 1 h, starting 6 h after the start of incubation. The scanning patterns of fluorograms of polypeptides synthesized under the various conditions that suppressed appressorium formation are shown in Fig. 3 (b–e): the 95K polypeptide was not detected under these conditions. On the other hand, when spores were incubated at 32 °C for 4 h after the start of incubation and then transferred to 24 °C, they could form appressoria and the 95K polypeptide was present (Fig. 3f). These results suggest that the 95K polypeptide is closely related to appressorium formation.

Table 1. *Relation between appressorium formation in the presence of cycloheximide and the ability of spore germlings to penetrate nitrocellulose membranes*

Membranes coated with spores were incubated in water for 1 h at 24 °C. The water was then replaced by 3.55 µM-cycloheximide. After the periods indicated, the membranes were washed with sterile water, scored for the presence of appressoria, and then incubated in water for a further 48 h before scoring for the presence of penetration hyphae.

Duration of cycloheximide treatment (h)	Percentage of spores forming appressoria	Percentage of appressoria with penetration hyphae
8	4.0	59.1
16	41.1	38.5
24	70.3	26.3
32	96.7	8.7
control*	97.2	75.2

* Incubated in water for 48 h.

As reported previously (Tani *et al.*, 1977), similar appressoria were formed both when protein synthesis from the first 1 h after incubation was inhibited by cycloheximide and when it was not (Fig. 4*a, b*). (In the presence of cycloheximide, spore germination and appressorium formation were, however, delayed relative to the control incubations.) The ability of appressoria formed with and without cycloheximide treatment to penetrate nitrocellulose membranes was compared. The membranes around the penetration sites did not stain with ZnCl₂/KI solution, so forming clear haloes around the appressoria. Hyphae growing in the membranes were readily observed in the control incubations (Fig. 4*a*). When spores were treated with cycloheximide for 32 h after the initial 1 h incubation (i.e. until the appressoria had matured), haloes were rarely observed around the appressoria, even after the removal of cycloheximide and a further 48 h incubation in water (Table 1; Fig. 4*b*). However, if cycloheximide was removed after only 8 or 16 h, before the appressoria had matured, penetration hyphae from the appressoria were observed after incubation in water for a further 48 h (Table 1). At 32 °C or in the presence of 0.5 M-sucrose, the spore germ tubes could elongate but they did not form appressoria and did not penetrate the nitrocellulose membranes (Fig. 4*c, d*).

DISCUSSION

Quantitative and qualitative differences in proteins synthesized during spore germination have been found in experiments both *in vivo* and *in vitro* (Van Etten *et al.*, 1972; Wenzler & Brambl, 1978), but the significance of these proteins has not been sufficiently discussed. Our previous work revealed that protein synthesis during the first 40 min was essential for the germination of *C. lagenarium* spores and suggested that this protein synthesis was qualitatively and quantitatively different from that of the subsequent incubation period (Furusawa *et al.*, 1977). In the present study, three different pulse-labelling periods were used: 5–25 min (non-germinated, protein synthesis essential for spore germination), 60–80 min (non-germinated, protein synthesis not necessary for spore germination) and 160–180 min (initiation of germ tube emergence, protein synthesis not necessary for spore germination). Of the polypeptides detected in the pulse-labelled preparations, the 72K, 43K and 38K polypeptides specific to the 5–25 min pulse period are the most likely candidates for polypeptides essential for spore germination, because spores could germinate even if protein synthesis after 40 min incubation was completely inhibited by cycloheximide.

Although protein synthesis in the early stage of incubation (0–40 min) is indispensable for the initiation of spore germination, our results suggest that morphogenesis of the appressorium does not require *de novo* protein synthesis after 40 min incubation. However,

the appearance of the 95K polypeptide was closely related to appressorium formation. It is suggested that this polypeptide might be closely connected with the function of appressoria, because appressoria formed in the presence of cycloheximide seemed to have little ability to penetrate artificial membranes. Perhaps the mechanisms involved in the morphogenesis and the function of appressoria are separated at the level of protein synthesis in *C. lagenarium*. Proteins necessary for the morphogenesis of appressoria probably already exist in dormant spores. Protein synthesis necessary for the capacity to penetrate, however, might occur during the morphogenesis of appressoria but not after their structural maturation.

From studies with inhibitors of protein synthesis, Miehle & Lukezic (1972) suggested that protein synthesis was required for spore germination and appressorium formation of *C. trifolii*. In addition, Staples *et al.* (1976) have investigated the relation between appressorium formation and nuclear division during spore germination of *C. truncatum* and showed that blocking of nuclear division inhibited appressorium formation. They have also reported that cycloheximide inhibited both appressorium formation and nuclear division. In our preliminary experiments, we found both spore germination and appressorium formation were completely inhibited if cycloheximide at a concentration higher than 35.5 μM was added to the spore suspension at any stage of germination. This suggests that high concentrations of cycloheximide not only inhibit protein synthesis but also affect other metabolic processes. The concentration of cycloheximide used in our experiments on appressorium formation (3.55 μM) was the minimum sufficient to suppress only protein synthesis in spores; no protein band was detected on fluorograms with this concentration. It is clearly important to use a dose of cycloheximide which inhibits only protein synthesis in spores if the necessity of protein synthesis for morphological differentiation of spores is to be correctly evaluated.

The authors wish to acknowledge the helpful advice of Dr H. Kunoh, Mie University.

REFERENCES

- ARAKI, F. & MIYAGI, Y. (1976). Effect of isoprothiolane on the infection process of *Pyricularia oryzae*. *Annals of the Phytopathological Society of Japan* **42**, 401–406.
- BONNER, W. M. & LASKEY, R. A. (1974). A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *European Journal of Biochemistry* **46**, 83–88.
- DUNKLE, L. D., MAHESHWARI, R. & ALLEN, P. J. (1969). Infection structures from bean rust uredospores: effect of RNA and protein synthesis inhibitors. *Science* **163**, 481–482.
- EMMETT, R. W. & PARBERY, D. G. (1975). Appressoria. *Annual Review of Phytopathology* **13**, 147–167.
- FURUSAWA, I., NISHIGUCHI, M., TANI, M. & ISHIDA, N. (1977). Evidence of early protein synthesis essential to the spore germination of *Colletotrichum lagenarium*. *Journal of General Microbiology* **101**, 307–310.
- ISHIDA, N. & AKAI, S. (1969). Relation of temperature to germination of conidia and appressorium formation in *Colletotrichum lagenarium*. *Mycologia* **61**, 382–386.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LASKEY, R. A. & MILLS, A. D. (1975). Quantitative film detection of ^3H and ^{14}C in polyacrylamide gels by fluorography. *European Journal of Biochemistry* **56**, 335–341.
- LOVETT, J. S. (1976). Regulation of protein metabolism during fungal spore germination. In *The Fungal Spore*, pp. 189–242. Edited by D. J. Weber & W. H. Hess. New York: John Wiley.
- MIEHLE, B. R. & LUKEZIC, F. L. (1972). Studies on conidial germination and appressorium formation by *Colletotrichum trifolii* Bain & Essary. *Canadian Journal of Microbiology* **18**, 1263–1269.
- RAMAKRISHNAN, L. & STAPLES, R. C. (1970). Changes in ribonucleic acids during uredospore differentiation. *Phytopathology* **60**, 1087–1091.
- SILVERMAN, P. M., HUH, M. M. & SUN, L. (1974). Protein synthesis during zoospore germination in the aquatic phycomycete *Blastocladiella emersonii*. *Developmental Biology* **40**, 59–70.
- STAPLES, R. C. & YANIV, Z. (1976). Protein and nucleic acid metabolism during germination. In *Encyclopedia of Plant Physiology New Series 4, Physiological Plant Pathology*, pp. 86–103. Edited by R. Heitefuss & P. H. Williams. Berlin: Springer-Verlag.
- STAPLES, R. C., LACCETTI, L. & YANIV, Z. (1976). Appressorium formation and nuclear division in *Colletotrichum truncatum*. *Archives of Microbiology* **109**, 75–84.

- STUDIER, F. W. (1973). Analysis of bacteriophage T7 early RNAs and proteins on slab gels. *Journal of Molecular Biology* **79**, 237–248.
- TANI, M., ISHIDA, N. & FURUSAWA, I. (1977). Effect of temperature and antibiotics on appressorium formation in spores of *Colletotrichum lagenarium*. *Canadian Journal of Microbiology* **23**, 626–629.
- VAN ETEN, J. L., ROKER, H. R. & DAVIES, E. (1972). Protein synthesis during fungal spore germination: differential protein synthesis during germination of *Botryodiplodia theobromae* spores. *Journal of Bacteriology* **112**, 1029–1031.
- WENZLER, H. & BRAMBL, R. (1978). In vitro translation of polyadenylate-containing RNAs from dormant and germinating spores of the fungus *Botryodiplodia theobromae*. *Journal of Bacteriology* **135**, 1–9.