BRIEF NOTE

Synthesis of Differentiation-Specific Proteins in Germlings of the Wheat Stem Rust Fungus after Heat Shock

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WANNER, R., FÖRSTER, H., MENDGEN, K., AND STAPLES, R. C. 1985. Synthesis of differentiation-specific proteins in germlings of the wheat stem rust fungus after heat shock. *Experimental Mycology* 9, 279-283. When uredospore germlings of the wheat stem rust fungus (*Puccinia graminis tritici*) were heat-shocked to induce differentiation, changes in the pattern of proteins synthesized were observed when the substomatal vesicles began to emerge. At this time (1.5 h after end of heat shock) two differentiation-specific proteins of approximately 34.7 and 21.9 kDa were synthesized. The synthesis of many other proteins was shut off or very nearly ceased. © 1985 Academic Press, Inc.

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Germlings of the stomatal-penetrating rust fungi develop specialized infection structures required for host penetration. When a uredospore germ tube of the bean rust fungus (*Uromyces appendiculatus*) contacts the lip of a stomatal guard cell (Wynn, 1976) or elongates on the surface of an inductive membrane (Dickinson, 1949), differentiation occurs and three specialized cells are produced—the appressorium, which occupies the stomatal opening; the substomatal vesicle; and the infection hyphae, which ramify within the mesophyll tissue. Appearance of the infection structures is accompanied by the synthesis of at least three differentiation-specific proteins (Huang and Staples, 1982).

Heat shock induces the differentiation of *Puccinia graminis tritici* germlings efficiently in the absence of contact stimuli. Heat shock thus represents another type of stimulus, and studies on it would allow examination of the differentiation process from an alternative perspective. The phys-

iological consequences of heat shock have not been studied yet. Here we report on the differentiation-specific proteins synthesized by germlings of *P. graminis tritici* in response to heat shock. The studies show for the first time that synthesis of the differentiation-specific proteins occurs about the same time as the development of the substomatal vesicle.

Our studies were pursued using collodion membranes that were prepared following the procedure described by Maheshwari *et al.* (1967); however, paraffin oil was omitted, and the membranes were poured directly onto a surface of 1.5% agar. In addition, the ratio of collodion:diethyl ether:ethanol was adjusted to 3:10:1 to improve spreading on the agar. After hardening for 30 min, both sides of the membrane were washed with sterile distilled water. The membranes were seeded with uredospores (0.1 mg/cm²) of the wheat stem rust fungus [*P. graminis* (Pers.) f. sp. *tritici* Eriks. and E. Henn.], race 32, using a set-

ting tower, and then moistened with an atomizer.

Differentiation was induced by a modification of the heat-shock procedure of Maheshwari *et al.* (1967). After the uredospores had been incubated for 1.5 h at 20°C, they were incubated for an additional 4 h at 30°C to heat shock the cells. Further development was continued at 20°C. Fungal differentiation was stopped by freezing the membranes at -20°C after removal from the agar surface. Nondifferentiated germ-lings were obtained in the same way except that incubation was continuous at 20°C. Spore germination and differentiation were monitored microscopically using differential interference contrast microscopy and Coomassie blue staining to observe the infection structures. Spores were stained with DAPI (4,6-diamidino-2-phenylindole) to observe the nuclei (Hooley *et al.*, 1982).

Proteins in germ-lings were labeled with [³⁵S]methionine (sp act 295 mCi/mmol). For this purpose, 250 cm² of membrane containing 31 mg of spores was misted with 1 ml of isotope solution that contained 30 µCi [³⁵S]methionine 30 min before harvest. The membranes were removed from the agar surface and frozen at -20°C for storage.

The membranes with the germ-lings were ground in liquid nitrogen using a mortar and pestle. Upon thawing, 5 ml of the sodium dodecyl sulfate extraction buffer of Gabriel and Ellingboe (1982) was added together with enough 0.5-mm glass beads to fill a 15-ml volume. The spores were pulverized in a vibratory cell mill at 5°C for 10 min, and the homogenate was recovered by centrifugation.

The proteins were precipitated from the supernatant solution using ice-cold (-20°C) 60% methanol containing 0.1 M ammonium acetate. The precipitate was washed once with cold methanol and twice with cold 80% acetone in water. The final pellet was dried under vacuum for 25 h and resuspended in sample buffer (Laemmli, 1970) for electrophoresis. Protein concen-

tration in the extracts was determined using a modification of the Lowry procedure (Hartree, 1972). Radioactivity of the protein extracts in the sample buffer was counted using a liquid scintillation technique. Electrophoresis was carried out at 8°C in polyacrylamide slab gels prepared according to Laemmli (1970). Protein extract containing about 50,000 cpm was pipetted into each lane of the gel.

Uredospores were germinated for 1.5 h on noninductive collodion membranes placed over water agar to ensure high germinability and a satisfactory environment for both the heat-shock step and for labeling the proteins with undiluted radiotracer. At this time, $92.5 \pm 4.8\%$ of the spores had germinated (Fig. 1). Then the heat shock was applied by a shift to 30°C for 4 h. At the end of the heat-shock period (5.5 h total), nearly 75% of the germ tubes had developed appressoria as shown in Fig. 1. A young appressorium with a developing vesicle is shown in Fig. 2a, while the nuclear condition in the appressoria is shown in Fig. 2b. Vesicles began to appear by 7 h (Fig. 1). A second round of nuclear division began as the vesicles developed. A complete set of infection structures is shown in Fig. 2c.

An example of the patterns obtained when proteins were separated by electrophoresis on polyacrylamide gels and detected by staining with Coomassie blue is shown in Fig. 3a. The electrophoretic study was carried out at gel concentrations of 12.5, 15, and 17.5% with essentially the same results. No differences were seen in the gross Coomassie blue-stained protein pattern as a result of the heat-shock treatment.

An autoradiogram of the polyacrylamide gel in Fig. 3a is shown in Fig. 3b. The autoradiogram of radioactive protein extracts from uredospores germinated for 5 h showed no differences when compared with controls. After 7 h, however, a number of protein bands could no longer be de-

FIG. 1. Timing of infection structure formation by germings of *P. graminis tritici*. Heat shock was imposed between 1.5 and 5.5 h after the start of germination. The counts were based on 200 spores for each time period.

tected, and two new protein bands appeared at 34.7 kDa and 21.9 kDa. These changes occurred during construction of the vesicles.

Differentiation induced by heat shock leads to the development of physiologically normal infection structures as shown, for example, by the recent studies of Mendgen and Dressler (1983), who demonstrated that

infection structures developed by heat-shocked germings of *P. coronata* vigorously produced haustoria upon contact with host cells. It is true that appressoria induced by heat shock do not properly localize over stomata (Staples *et al.*, 1983); however, cytological details such as mitosis and cell shape were otherwise regular.

Typically, the appearance of "heat-

FIG. 2. Photomicrographs of *P. graminis tritici* germings on collodion membranes showing the differentiation process beginning during the heat-shock period. (a) Young appressorium visualized by interference contrast microscopy ($\times 450$); (b) the four nuclei can be seen by fluorescence microscopy after staining with DAPI ($\times 450$). (c) Interference contrast photomicrograph ($\times 500$) showing a complete set of infection structures after 20 h. Ap, appressorium; Gt, germ tube; If, infection hyphae; Sp, uredospore; Ve, vesicle.

FIG. 3. Comparison of proteins synthesized by differentiated and nondifferentiated germlings of *P. graminis tritici* uredospores. Differentiated (+) and nondifferentiated (–) spores were pulse-labeled with [35 S]methionine for 30 min at the end of 5.0 and 6.5 h, respectively. Proteins were then extracted and separated on 17.5% SDS–PAGE gels. The gel was stained with Coomassie blue (a) and then autoradiographed for 10 d (b). Molecular weights were obtained by coelectrophoresis with a mixture of protein standards. In b, the same amount of radioactivity was used for each track (50,000 cpm). After 5.5 h, there were $75.0 \pm 8.9\%$ appressoria and no vesicles, while at 7.0 h, there were $76.0 \pm 8.9\%$ appressoria and $3.5 \pm 2.6\%$ vesicles.

shock” proteins and the depression of general translation activity occur immediately after a temperature shift, and the normal pattern of synthesis returns soon after the cells are returned to normal temperatures (Plesofsky-Vig and Brambl, 1985). The spectrum of proteins synthesized by the uredospores appeared not to be directly affected by the heat shock since the pattern was observed well after the cells had been returned to a lower temperature, and the pattern of synthesis was correlated with the appearance of the vesicle rather than the appressorium. The synthesis of differentiation-specific proteins was observed to occur at 7 h when construction of the vesicle began, and not immediately after the heat shock (5.5 h). Thus, both the synthesis

of the two differentiation-specific proteins (34.7 and 21.9 kDa), and the significant reduction in the synthesis of most other proteins (Fig. 3b) was correlated with the development of the vesicles (Fig. 1).

The synthesis of differentiation-specific proteins appears to begin earlier in germlings of the rust fungus (*U. appendiculatus*) induced thigmotropically on an inductive membrane of collodion and oil. In that fungus, three differentiation-specific proteins were observed (Huang and Staples, 1982). Two of the differentiation-specific proteins (18.5 and 24 kDa) were synthesized beginning with appearance of the appressorium, while that of the third (23 kDa) began with development of the vesicle. Further studies are in progress.

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