

Puccinia coronata f. sp. avenae 親和性レースの感染によるエンバク核の in vitro 転写活性増高

誌名	日本植物病理學會報 = Annals of the Phytopathological Society of Japan
ISSN	00319473
著者名	山本,弘幸 谷,利一
発行元	日本植物病理學會
巻/号	53巻1号
巻号補足	
掲載ページ	p. 21-27
発行年月	1987年1月

農林水産省 農林水産技術会議事務局筑波産学連携支援センター
Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council
Secretariat



Enhancement of *in vitro* Transcription in Nuclei of Oat Leaves Following Infection with a Compatible Race of *Puccinia coronata* f. sp. *avenae**

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Abstract

A rapid and efficient procedure for the isolation of intact nuclei was established with the primary leaves of oat seedlings infected with a compatible race of *Puccinia coronata* f. sp. *avenae*. The time required for the isolation was about 90 min and the yield of nuclei was estimated to be about 50% of total nuclei in all leaf samples. The content of DNA, RNA and protein in isolated nuclei was unchanged by the rust infection. However, time course study of ³H-UTP incorporation indicated that transcriptional activity increased to about 1.5-times of corresponding control at the early stage of uredosorus differentiation. The blockage experiments with α -amanitin suggested that qualitative changes in the transcription would most likely occur at this stage of infection.

(Received August 14, 1986)

Key words: oat, crown rust, isolation of nuclei, nuclear transcription, susceptibility.

Introduction

One of the promissive subjects in the study of obligate parasitisms is a possible contribution of nucleic acid metabolisms of the host to the establishment of susceptibility^{9,19}. Recently, the alteration in host nuclear transcription due to the change of RNA polymerase II was suggested for wheat stem rust^{8,16} and wheat powdery mildew⁷.

We previously reported²¹ that infection of oat leaves with a compatible race of *Puccinia coronata* f. sp. *avenae* is accompanied by an enhanced synthesis of mRNA (TB-RNA), rRNA and tRNA at the reproductive stage of the invaded fungus. We also demonstrated²⁴ that a bulk of rRNA increased at the initial stage of fungal differentiation towards uredosorus formation was of fungal origin. The assay of *in vitro* template activity and the blockage experiments with a RNA synthesis inhibitor, cordycepin, strongly suggested that the enhanced synthesis of mRNA at this stage of infection could be attributed to the fungus^{20,22}. Drastic decrease in rRNA content in chloroplasts was also demonstrated²³. All these results suggest that the increased synthesis of RNA species in oat leaves infected by a compatible race of the crown rust fungus is mainly due to the metabolic activation of the fungus rather than the host. These findings, however, do not rule out the possible alteration of the transcription activity in host nuclei.

* This study was supported in part by a Grant from the Ministry of Education, Science and Culture of Japan (No. 57360004).

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The objectives of the present study was to determine whether the crown rust infection would affect the RNA synthesis of oat nuclei during the establishment process of compatibility. For this purpose, we first devised a rapid and efficient method for isolation of intact nuclei from mesophyll tissues. In the subsequent experiments using isolated nuclei, we determined changes in their chemical contents and transcriptional activity during the initial stage of uredosorus differentiation.

Materials and Methods

Plants, fungus and inoculation. *Avena sativa* L. cultivar *Pc 38* was grown at 20C under continuous fluorescent light illumination (6,000–8,000 lux). The lower surface of primary leaves of 5- or 7-day-old seedlings was inoculated with compatible race 226 of *Puccinia coronata* Cda. f. sp. *avenae* Fraser and Led. as described previously^{20,27}.

Isolation of protoplasts and nuclei. Inoculated middle portions of primary leaves (about 3 cm long) were taken 3 to 5 days after inoculation. After the lower epidermis was removed by peeling, the leaf segments (1 g fresh weight) were immediately floated on 20 ml of an enzyme mixture consisting of 2% Cellulase Onozuka R 10 (Yakuruto Biochemicals Co.), 0.1% Pectolyase Y 23 (Seishin Pharmaceutical Co.) and 0.6 M mannitol and adjusted to pH 5.5 with 0.2 M KOH. After vacuum infiltration for 2 min, the segments were incubated at 25 C for 60 min with gentle shaking. Protoplasts released were filtrated through a nylon screen (50- μ m mesh) and collected by centrifugation at 100 $\times g$ for 2 min.

Nuclei were isolated by a modified method of Cress *et al.*⁵⁾. Protoplasts from 1 g fresh leaves were washed with ice chilled buffer A containing 0.26 M sucrose, 0.5 mM (2(N-morpholino) ethane sulfonic acid) (MES), 5 μ g/ml dithiothreitol (DTT), 0.15% 2-ethylhexyl alcohol (EHA), 0.6 mM Mg(Ac)₂, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), pH 6.4, and resuspended in 4 ml of cold buffer A. After 1 min, Triton X-100 was added to a final concentration of 0.5% and mixed with a Vortex. Two to 3 min later, an equal volume of buffer B containing 0.4 M sucrose, 5 mM MES, 50 μ g/ml DTT, 0.15% EHA, 5 mM CaCl₂, 6 mM Mg(Ac)₂, 25 mM KCl, 0.5 mM PMSF, pH 6.4, was added and then layered onto discontinuous gradient consisting of 1 ml of 2 M sucrose, 2 ml of 1.6 M sucrose and 2 ml of 15% Acacia dissolved in buffer B minus sucrose. The gradient was centrifuged at 724 $\times g$ for 10 min at 5 C. The pellet was resuspended and washed 3 times with buffer B.

Assay of DNA, RNA and protein contents. DNA and RNA were extracted by the method of Schneider¹⁷⁾ and determined by the diphenylamine method of Burton³⁾ and orcinol method of Schneider¹⁷⁾, respectively. Calf thymus DNA (Sigma Chemical Co.) and yeast RNA (Sigma Chemical Co.) were employed as respective standard. Protein content was determined by the method of Lowry *et al.*¹³⁾ using bovine serum albumin (Wako Pure Chemical Ind.) as a standard.

Assay of in vitro transcription. Isolated nuclei (2 $\times 10^5$) suspended in 0.3 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose were added to 50 μ l of a reaction mixture consisting of 0.5 mM each of ATP, GTP and CTP, 75 mM (NH₄)₂SO₄, 2 mM MnCl₂, 5 mM MgCl₂, 3.3 μ g of Creatine kinase (Sigma Chemical Co.), 1.2 mM

Creatine phosphate (Sigma Chemical Co.), 2 μ Ci of ^3H -UTP (Amersham; sp. act. 46 Ci/m mol), 50 mM Tris-HCl (pH 7.5). When necessary, α -amanitin (Boehringer Mannheim Yamanouchi Co.) was added to the reaction mixture to give a final concentration of 200 $\mu\text{g}/\text{ml}$.

After incubation at 30 C, 10 μl aliquots of the reaction mixture were placed on a Whatman 3MM paper (2.5 cm diam.) and added with 100 μl of 1% sodium dodecylsulphate and 10 mM ethylenediaminetetraacetic acid solution as described by David *et al.*⁶⁾. The paper was dried and then washed 3 times with a mixture of cold 5% trichloroacetic acid and 20 mM sodium phosphate and then dehydrated by washing twice with acetone. Radioactivity in the paper was counted in a toluene scintillant as described previously²⁷⁾.

Results and Discussion

Preparation of protoplasts

Primary leaves of 5- and 7-day-old seedlings were densely inoculated with uredospores. The inoculated portions were harvested 3 to 5 days later and incubated with the enzyme solution. Protoplasts liberated from these leaf pieces were mostly of mesophyll cells and cells of epidermal layers and vascular systems remained in undigested debris. Thus the protoplasts used in this study was characterized as those of mesophyll cells.

Table 1. Isolation of protoplasts from healthy and crown rust-infected primary leaves of oat cv. *Pc 38*

Sample	Number of protoplasts/g fresh wt. ($\times 10^6$)		
	3 ^{a)}	4	5
5-day-old seedling			
Uninoculated	6.99	7.75	7.50
Inoculated ^{b)}	6.55	6.68	6.89
7-day-old seedling			
Uninoculated	7.01	5.35	5.36
Inoculated ^{b)}	5.71	0.61	0.01

a) Days after inoculation.

b) Leaves were inoculated with compatible race 226 of *Puccinia coronata* f.sp.*avenae*.

Number of protoplasts from inoculated leaves was compared with that of corresponding uninoculated controls (Table 1). When 5-day-old seedlings were employed, the yields of protoplasts were similar in all experiments, regardless of healthy or infected, or of the period of infection, and more importantly the debris was free from mesophyll cells. On the other hand, when the leaves of 7-day-old seedlings were inoculated and harvested 4 and 5 days later, number of protoplasts liberated was extremely small and a mass of mesophyll cells remained in the debris. Based on these results, 5-day-old seedlings were used in the subsequent experiments.

The yield of protoplasts was determined by analyzing DNA contents leaf segments and protoplasts prepared from equal weight of leaf tissues (Table 2). The yield was

Table 2. Yields of protoplasts and nuclei from healthy primary leaves of oat cv. *Pc 38* and those inoculated with *Puccinia coronata* f. sp. *avenae* compatible race 226

Sample	DNA content ($\mu\text{g/g}$ fresh wt. of leaves)		
	Leaf	Protoplast	Nucleus
3 days after inoculation			
Uninoculated	307 (100)	196 (63.8)	152 (49.5)
Inoculated	314 (100)	188 (59.9)	147 (46.8)
4 days after inoculation			
Uninoculated	312 (100)	186 (59.6)	145 (46.5)
Inoculated	327 (100)	186 (56.9)	149 (45.6)
5 days after inoculation			
Uninoculated	307 (100)	188 (61.2)	148 (48.2)
Inoculated	309 (100)	184 (59.5)	142 (46.0)

Figures in the parenthesis are the percentage of the leaf.

about 60% of whole cells of the leaf segments and there was no essential change in the yield even after the inoculation. Since protoplasts obtained by this method were mostly of mesophyll cells, as mentioned above, and cells of epidermal layers were about 16% of leaf segments, the yield could be 70% or more of whole mesophyll cells.

In these experiments, incubation time with the enzyme solution was shortened to 60 min. Thus the time required for liberation with high yield is quite rapid and satisfactory as compared with the conventional procedure described by Verhoek *et al.*²⁵⁾ for oat primary leaves, which required 18 hr of the incubation.

Isolation of nuclei

Nuclei isolated by the method described above appeared essentially intact in shape, not agglutinated, and free from contamination with cell organella of both the host and parasite, so far as observed with a light microscope and a scanning electron microscope. The yield of nuclei estimated by the amount of DNA was 46-50 and 77-80% of original leaf tissues and protoplasts, respectively, in all experiments (Table 2). A haematometric measurement indicated that number of nuclei obtained was $5 \times 10^6/\text{g}$ fresh weight of leaves. This value is much higher than that previously reported for oat seedlings^{14,15)} and other plants^{4,11,28)}.

RNA and protein contents in isolated nuclei

The content of RNA and protein was estimated for nuclei isolated from uninoculated and inoculated leaves and expressed as ratios to DNA content (Table 3). There was

Table 3. Changes in RNA and protein contents of isolated oat nuclei of primary leaves of cv. *Pc 38* following infection with *Puccinia coronata* f. sp. *avenae* compatible race 226

Days after inoculation	Ratio to DNA ($\times 100$)			
	RNA		Protein	
	Uninoculated	Inoculated	Uninoculated	Inoculated
3	27	26	281	280
4	26	28	280	283
5	27	28	281	282

no essential changes in content at both the vegetative (day 3) and reproductive (days 4 and 5) stages of fungal development. These findings are quite contrary to those of classical works of Shaw and his co-workers^{1,2)}, who demonstrated by a cytophotometry that the contents of both RNA and protein in nuclei of wheat leaves increased at the early stage (days 2 and 3) of infection with a compatible race of *Puccinia graminis* f. sp. *tritici*. Discrepancy between these studies may probably be due to difference of the methods employed, but we assume that the results of the present study would be more accurate, because the assays were made directly of the isolated nuclei and the reactions were much more specific.

Characteristics of *in vitro* transcription

Transcriptional activity of nuclei isolated from oat leaves 3 to 5 days after inoculation was assayed by the incorporation of ³H-UTP into acid insoluble fraction and compared with that of corresponding uninoculated controls (Table 4). No substantial

Table 4. Changes in *in vitro* transcriptional activity of oat nuclei of primary leaves of cv. *Pc 38* following infection with *Puccinia coronata* f. sp. *avenae* compatible race 226

Days after inoculation	³ H-UTP incorporated (dpm) ^{a)}		
	Uninoculated (U)	Inoculated (I)	I/U (%)
3	1,318	1,314	99
4	1,359	1,910	141
5	1,473	2,236	152

a) Nuclei (2×10^5) were incubated in the transcription mixture at 30 C for 30 min.

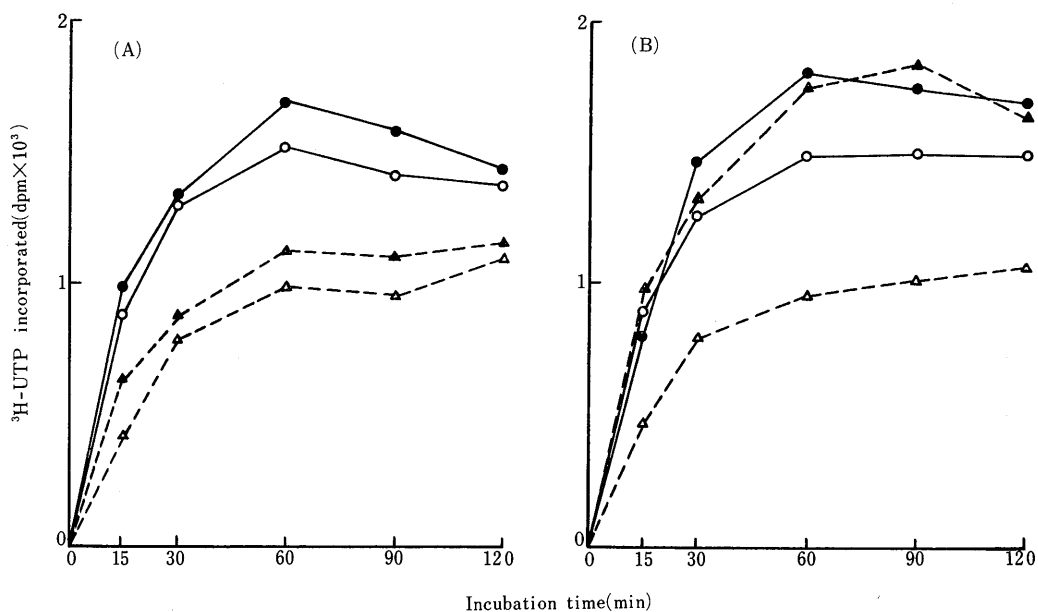


Fig. 1. Time course of ³H-UTP incorporation by oat nuclei isolated from primary leaves of cv. *Pc 38* uninoculated (open symbols) and inoculated (closed symbols) with *Puccinia coronata* f. sp. *avenae*. The nuclei (2×10^5) isolated 3 days (A) or 5 days (B) after inoculation were incubated in the reaction mixture with (broken line) or without (solid line) α -amanitin.

change in incorporation occurred in nuclei of uninoculated leaves throughout the experimental periods. In nuclei isolated from inoculated leaves, however, incorporation increased to about 1.5-fold of the uninoculated controls 4 and 5 days after inoculation, when the fungus differentiated towards uredosorus formation.

Fig. 1 gives typical patterns of the incorporation by isolated nuclei in the presence or absence of α -amanitin. The incorporation of ^3H -UTP was inhibited to about 70% by α -amanitin in both nuclei isolated from uninoculated leaves and leaves 3 days after inoculation (Fig. 1-A). On the other hand, α -amanitin had no effect on the incorporation by nuclei 5 days after inoculation, while it reduced the incorporation to about 70% of nuclei from corresponding uninoculated control leaves (Fig. 1-B).

It has been reported that α -amanitin completely inhibits the activity of RNA polymerases II and III of cauliflower at 200 $\mu\text{g}/\text{ml}$, the concentration we used in this experiment. It is also reported that there are two types of polymerases II (A and B) in several plants²⁶⁾, and recent studies have demonstrated that a certain type of polymerase II is insensitive to α -amanitin^{10,12,18)}. At present, we have no experimental datum to specify which kind of RNA polymerases might be responsible for the alteration of transcriptional behaviors in nuclei of infected leaves. The findings in this study may lead to an idea that insensitivity of oat nuclei to α -amanitin is a reflection of qualitative changes of polymerase species.

We thank Mr. T. Arawaki, Nakatado Agricultural Extension Station of Kagawa Prefecture, for his technical assistance. We are grateful to Dr. T. Onoe of Osaka Dental University, for help with the electron microscope and to Prof. S. Ouchi of Kinki University and Dr. S. Mayama of Kagawa University, for discussions.

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和 文 摘 要

山本弘幸・谷 利一 : *Puccinia coronata* f. sp. *avenae* 親和性レースの感染によるエンバク核の *in vitro* 転写活性増高

エンバク冠さび菌 親和性レース夏孢子堆の分化時における エンバク葉肉 細胞核の変化について調べた。まず、葉肉細胞核の分離方法を検討し、90分以内に約50%の収量で宿主核を得る方法を確立した。分離核のDNA, RNA および蛋白含量は測定期間中健・病間に差異はなかった。しかし、³H-UTP の酸不溶部への取込みは夏孢子堆分化初期(接種4および5日後)に健全核の約1.5倍に増高した。同時期の転写活性は、 α -アミニチンにより阻害されないことより、質的变化の生じることを示唆した。