Changes in ^{32}P -Ribonucleic Acids in Oat Leaves Associated with Susceptible and Resistant Reactions to *Puccinia coronata**

Toshikazu TANI**, Masaaki YOSHIKAWA** and Nakato NAITO**

谷 利一**・吉川正明**・内藤中人** : *Puccinia coronata* に感染したエンパク葉の
罹病ならびに抵抗反応にともなう ^{32}P -リボ核酸の変化*

Abstract

Primary leaves of two oat varieties were inoculated with *Puccinia coronata* Corda and the changes in ^{32}P incorporation into nucleic acid fractions were studied by MAK column chromatography.

In the susceptible variety, Victoria, the inoculated/uninoculated ratio of radioactivity in the rRNA fraction increased slightly at the initiation period of reproductive growth of the rust fungus (3 days after inoculation), then largely during the uredosorus development (4 to 5 days). The ratio in the sRNA fraction increased only at the beginning of sporulation (5 days). The change in the ratio for the TB-RNA as well as rapidly labeled RNA fractions showed a similar trend to that for the rRNA fraction.

In the resistant variety, Shokan No. 1, the increases in the ratio were noted to be of similar extent among the sRNA, rRNA, and TB-RNA fractions: being slight at the prehaustorial stage of infection (20 hr after inoculation), and thereafter being large until 48 hr. The rapidly labeled RNA was also increased by infection.

The base analysis of labeled RNA's indicated that the increased synthesis of rRNA may be originated from the rust fungus in the susceptible leaves, and from the host in the resistant leaves.

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Introduction

Substantial amounts of evidence have accumulated implicating nucleic acid metabolism in rust-infected plants⁶⁾. Biochemical investigations have demonstrated an increase in the total amount of ribonucleic acid (RNA) and alteration of the base composition as influenced by rust infection^{1,7,12,19,20)}. With regard to qualitative changes in the level of the functional RNA's, Wolf^{28,29)} reported an increased incorporation of ^{32}P into ribosomal RNA (rRNA) in susceptible wheat leaves affected by *Puccinia graminis*. Heitefuss and Bauer⁸⁾ found a stimulated synthesis of soluble RNA (sRNA), rRNA, and deoxyribonucleic acid (DNA) in bean leaves infected with *Uromyces phaseoli*. However, so far no available evidence has been presented for the qualitative difference of RNA's among various rust reactions.

The authors reported in a previous paper²⁵⁾ that the oat leaves inoculated with *Puccinia coronata* exhibited an increase of ^{32}P incorporation into the RNA fraction for the susceptible and resistant rust

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** Faculty of Agriculture, Kagawa University, Miki, Kagawa, Japan. 香川大学農学部

reactions. The present study was designed to compare the periodical changes in ^{32}P incorporation into different types of RNA between the susceptible and resistant reactions. The outline of this work was presented at the Annual Meeting of the Phytopathological Society of Japan held in 1970.

Materials and methods

Inoculation and ^{32}P -feeding Seedlings of the susceptible (Victoria) and resistant (Shokan No. 1) varieties of oat to the crown rust were grown at 25°C under 6,000–8,000 lux as previously described²⁵⁾. Uredospores of *Puccinia coronata* Corda were freshly harvested by the usual method¹⁶⁾ from the same culture as in the previous study²⁵⁾. Primary leaves of 7-day-old seedlings were heavily inoculated by brushing the uredospores over the whole under-surface. The inoculated and uninoculated plants were kept under moist condition for the following 20 hr. Leaf samples (70–80 leaves) with the stems of 1 cm long were detached and the cut ends were put into deionized water for 20 min and then into ^{32}P -labeled orthophosphate (100–300 $\mu\text{Ci}/\text{ml}$; carrier free) for 1 to 4 hr as in the previous study²⁵⁾. After ^{32}P -feeding, the samples were cut into pieces of 4–5 mm length and frozen at -22°C until use. The experiments with inoculated leaves were always made together with healthy leaves which were harvested from the same seedling batch and treated with the same ^{32}P -solution. The total activity of ^{32}P per fresh weight taken up into the inoculated leaves was about 70 to 90% to that of healthy leaves throughout the experiments, in both susceptible and resistant reactions.

Extraction of nucleic acid The detergent-phenol method has been extensively used for the preparation of nucleic acid from various plant tissues. The use of phenol, however, is not always effective for obtaining good RNA preparation¹⁰⁾. Recently a new method using diethylpyrocarbonate (DEP) as a deproteinizing agent has been reported with considerably good results. Therefore, in this study, the DEP-SLS method of Solymosy *et al.*²⁴⁾ was employed, with a slight modification as below.

Frozen leaf samples of 3 g (fresh weight at harvest) were homogenized under ice chilled condition in 6 ml of 0.05 M Tris-HCl buffer (pH 7.6) containing 1% sodium lauryl sulphate (SLS), 5 mM MgCl_2 , and 0.2 ml DEP, for 3 min at 20,000 rev. using a homogenizer (Nihon-Seiki, Model HB). The homogenate was incubated at 37°C for 5 min, homogenized again for 2 min at 10,000 rev. as above, and filtered through double layers of gauze to remove fibrous material. To the filtrate, crystals of NaCl was added to give a final concentration of 2.0 M and was dissolved by shaking. It was then incubated again at 37°C for 5 min, chilled in ice, and centrifuged at $10,000 \times g$ for 20 min at 0°C . The supernatant fluid was poured into 2 volumes of 90% ethanol precooled at -22°C , allowed to stand for 3–5 hr at -22°C , and centrifuged at $4,000 \times g$ for 5 min. The precipitate obtained was washed with cold 80% ethanol, suspended in 5 ml of 0.05 M phosphate buffer (pH 6.7) containing 0.4 M NaCl, and kept at $0-4^\circ\text{C}$ for 15 hr. To the supernatant obtained by centrifugation at $20,000 \times g$ for 20 min at 0°C , 0.05 M phosphate buffer (pH 6.7) was added to give NaCl concentration of 0.25 M.

Fractionation of nucleic acid Methylated albumin kieselguhr (MAK) column was prepared according to the method of Burdett and Wareing²⁾. The DEP-extracted nucleic acid was layered onto the column and was eluted with a linear gradient of 0.3 to 1.4 M NaCl buffered at pH 6.7 with 0.05 M phosphate (400 ml) giving the flow rate 40 ml/hr. Six ml fractions were collected. The remainder of ^{32}P was then eluted with 1% SLS (80 ml) as described by Ellem⁴⁾.

Determination of base composition of labeled RNA The RNA solutions obtained by MAK column chromatography were concentrated to one tenth in volume in the visking tube with polyethylene glycol 6,000 and dialyzed against deionized water at $0-4^\circ\text{C}$ for 24 hr to remove NaCl.

The visking tube was boiled before use with 1% SLS solution for 5 min. Hydrolysis of RNA was done in 0.3 N KOH for 20 hr at 37°C. The hydrolyzate was fractionated on the Dowex 1-X8 formate form column as described by Johnson *et al.*¹²⁾

The radioactivity was counted in a thin window gas flow counter (Aloka, Model PC-10 E).

Results

In the susceptible leaves, the massing of hyphae for sporulation begins 3 days, and the pseudo-parenchymatous layers appear 4 days after inoculation, developing extensive light-green flecking as a visible symptom. The development of uredosori is visible 5 days and the emergence of uredospores begins 6 days after inoculation. The resistant leaves exhibited a hypersensitive reaction. Retardation of the hyphal growth is observed at prehaustorial stage within 20–48 hr. Cessation of fungal growth occurs between 48 and 72 hr after inoculation. At this time brown watery spots are slightly visible. Detailed explanation for fungal development has been reported in the separate paper¹⁷⁾.

1. Incorporation of ^{32}P into RNA fractions

sRNA, rRNA, and TB-RNA fractions: Elution profiles of nucleic acids and ^{32}P activity for two oat varieties appeared by 4 hr-labeling are shown in Fig. 1. The major 2 peaks of the radioactivity coincide with UV absorbing peaks of sRNA and rRNA, respectively. The counts in the tenaciously bound RNA (TB-RNA) are also demonstrated in the fraction eluting with SLS. Another

Fig. 1. MAK column separation of nucleic acids in inoculated and uninoculated leaves after ^{32}P -feeding for 4 hr.

A: Victoria-inoculated (4 days). B: Victoria-uninoculated (4 days).

C: Shokan No. 1-inoculated (48 hr). D: Shokan No. 1-uninoculated (48 hr).

fraction just ahead of the DNA peak corresponds to DNA-bound RNA³⁾. This fraction was consistently observed, but the radioactivity was not high enough to determine difference between the rusted and healthy leaves. The DNA fraction in both varieties in this study was relatively high when compared with that reported by Masuda¹⁴⁾, but the purity of DNA in the tubes 39–50 in Fig. 1 was more than 92% as measured by diphenylamine method. The difference in the concentration between both may be caused by the use of different tissues²²⁾.

The counts of the tubes 17–28 and 63–80 in Fig. 1 were summed for the sRNA and rRNA fractions, respectively. The ratio of inoculated to uninoculated control for both fractions was evaluated as given in Table 1. Since the DNA content in the separation curves was very close between inoculated and uninoculated leaves, it was possible to make comparison of the radioactivity on the basis of leaf weight without any correlation. Lack of change in DNA content in the susceptible and resistant reactions during the experimental periods has been reported in the previous paper²⁵⁾.

In the susceptible variety, the inoculated/uninoculated ratio in the sRNA fraction did not increase within 2 to 4 days after inoculation. Thereafter, it increased largely to 185% at 5 days. The ratio in the rRNA fraction was noted to increase slightly at 2 and 3 days and then largely to 170–190% at 4 and 5 days. The labeled TB-RNA began to increase at 3 days and showed about 2 times high value on the following 2 days. Similar trends were also found by calculation based on dry weight. However, when it was evaluated by the specific activity (cpm/OD₂₆₀), no obvious increase was shown for sRNA and rRNA at any stages of rust development.

Table 1. Inoculated/uninoculated ratio of ³²P incorporation into sRNA, rRNA, and TB-RNA fractions after 4 hr-labeling

Period after inoculation	Fungal development	Inoculated/uninoculated ratio of cpm/fresh leaf wt. in indicated RNA fractions			
		sRNA	rRNA	TB-RNA	
Victoria					
2 days	Vegetative growth	1.15 (1.05) ^{a)}	1.24 (1.27)	1.18	
3	Initiation of hyphal massing	1.16 (1.03)	1.24 (1.08)	1.36	
4	Formation of pseudoparenchymatous layer	0.90 (0.83)	1.70 (0.81)	2.42	
5	Beginning of sporulation	1.85 (0.92)	1.93 (0.96)	1.73	
Shokan No. 1					
20 hr	Prehaustorial growth	1.21 (1.05)	1.35 (1.13)	1.20	
28	Middle stage of growth	1.58 (1.37)	2.08 (1.61)	2.14	
35	Late stage of growth	1.67 (1.53)	1.71 (1.48)	1.76	
48	Cessation of growth	1.70 (1.54)	1.88 (1.66)	1.64	

a) Figures in a bracket indicate the ratio of specific activity in cpm/OD₂₆₀.

In the resistant variety, the ratio based on fresh weight increased to similar extent among the sRNA, rRNA, and TB-RNA fractions. A slight increase was already noted 20 hr after inoculation. The large increase to 150–210% was observed within 28–48 hr. Changes in the specific activity paralleled the counts per leaf weight.

Rapidly labeled RNA: The leaves of Victoria 3 and 4 days after inoculation and Shokan No. 1 35 hr after inoculation were mainly used. Fig. 2 gives representative MAK profiles with 1 hr-labeled

Fig. 2. MAK column separation of nucleic acids in inoculated and uninoculated leaves after ^{32}P -feeding for 1 hr.

A: Victoria-inoculated (3 days). B: Victoria-uninoculated (3 days).

C: Shokan No. 1-inoculated (35 hr). D: Shokan No. 1-uninoculated (35 hr).

leaves. With both susceptible and resistant reactions, the radioactivity peak did not coincide with the OD peak. The ^{32}P incorporation into this fraction was inhibited to 70–80% relative to the water control by the pretreatment with actinomycin D ($10\text{ }\mu\text{g/ml}$) for 3 hr. When the ^{32}P was fed for 2 hr, the peak moved toward the heavy rRNA peak, and at 4 hr, both peaks coincided almost completely. Similar results were obtained by a “chase” in $0.01\text{ M NaH}_2\text{PO}_4$ for 2 and 3 hr. Thus ^{32}P -RNA in this fraction is different from rRNA. A possible contamination by bacteria, and thereby increased uptake of ^{32}P into this fraction, seemed to be improbable because the growth of bacterial colony was quite seldom on the mesophyll cells which were randomly picked out of the inoculated and uninoculated leaves and placed onto peptone-dextrose agar.

The effect of rust reaction to ^{32}P incorporation into the rapidly labeled RNA fraction is evaluated in Table 2. To eliminate contamination of labeled rRNA the counts in the tubes 81–85 in Fig. 2 were summed. The rechromatographic pattern of this region using shallower gradient ($0.7\text{--}1.1\text{ M NaCl}$)

Table 2. Inoculated/uninoculated ratio of ^{32}P incorporation into rapidly labeled RNA fraction after 1 hr-labeling

Period after inoculation	Inoculated/uninoculated ratio indicated by	
	cpm/fresh leaf wt.	cpm/OD ₂₆₀
Victoria		
3 days	1.69	1.32
4	1.81	1.28
Shokan No. 1		
35 hr	1.51	1.38

indicated that the majority of the labeled materials differed from rRNA. The results indicated that the susceptible reaction increased the ^{32}P incorporation per fresh leaf weight to 170–180%, and the resistant one to 150%. The ratio based on the specific activity also increased to about 130% for both rust reactions.

2. Base composition of labeled RNA's

Base composition of the labeled RNA of the tubes 69–72 and 74–78 in Fig. 1 and 2 was analyzed separately; the former was referred to as rRNA fraction and the latter as rapidly labeled RNA fraction. As shown in Table 3, the susceptible reaction exhibited higher contents in AMP and UMP for rRNA at 3 days, with the consequence that the GC % decreased about 6.7% in the host-parasite complex. At 4 days, AMP increased and GMP decreased in the inoculated leaves resulting in the GC % decrease of 8.8%. In the resistant reaction, on the other hand, the difference in the base ratio of rRNA between the inoculated and uninoculated leaves was slight or negligible.

Table 3. Base composition of ^{32}P -rRNA after 4 hr-labeling

Sample condition			Composition (mole %)				GC %
			AMP	GMP	CMP	UMP	
Victoria							
3 days ^{a)}	Inoculated		28.0	24.3	19.2	28.5	43.5
	Uninoculated		24.4	25.9	24.3	25.4	50.2
4	Inoculated		32.0	21.3	19.3	27.4	40.6
	Uninoculated		27.2	27.2	22.2	23.4	49.4
Shokan No. 1							
28 hr	Inoculated		26.1	28.1	20.8	25.0	48.9
	Uninoculated		25.1	27.5	22.3	25.1	49.8
48	Inoculated		26.2	28.3	19.4	26.1	47.7
	Uninoculated		25.4	28.8	20.7	25.1	49.5

a) Period after inoculation.

Table 4. Base composition of ^{32}P -RNA in rapidly labeled RNA fraction at various periods of labeling

Sample condition			Composition (mole %)				GC %
			AMP	GMP	CMP	UMP	
Victoria							
3 days ^{a)} –1 hr ^{b)}	Inoculated		27.6	25.0	22.0	25.4	47.1
	Uninoculated		25.9	25.2	23.5	25.6	48.7
4 –1	Inoculated		28.4	24.4	22.1	25.1	46.5
	Uninoculated		27.3	25.0	22.4	25.3	47.4
4 –2	Inoculated		29.2	23.0	19.7	27.6	42.7
	Uninoculated		28.7	24.8	22.2	24.3	47.0
4 –4	Inoculated		31.5	22.4	19.6	26.5	42.0
	Uninoculated		27.0	25.0	24.5	23.5	49.5
Shokan No. 1							
35 hr ^{a)} –1 hr ^{b)}	Inoculated		27.0	25.3	20.2	27.5	45.5
35 –4	Inoculated		26.0	26.8	21.2	26.0	48.0

a) Period after inoculation. b) ^{32}P -labeling period.

Table 4 shows the base compositions of rapidly labeled RNA fractions from the susceptible as well as resistant leaves at various intervals of labeling. In Victoria, the distribution of radioactivity of 1 hr-labeled RNA was similar between the inoculated and uninoculated leaves. The composition in the inoculated Shokan No. 1 was relatively higher in UMP than that in the Victoria. In the inoculated leaves of both oat varieties, the GC% similarly approached to that of ^{32}P -rRNA (Table 3) as the period of ^{32}P -feeding was prolonged, probably due to contamination with a newly synthesized rRNA.

Discussion

In the susceptible variety, Victoria, the increase of radioactivity in the rRNA fraction per fresh leaf weight was noted by 4 hr-labeling. The increased ratio of inoculated to uninoculated leaves was slight until the initiation of reproductive growth of the fungus and then large during the uredosorus development. Base analysis of the labeled RNA for these two stages indicates that the ^{32}P -rRNA in rust-infected tissues is different from the host rRNA exhibiting similar change in the ratio to the results reported with total nucleic acid in other rust diseases^{7,12}. A high ribosome concentration in the rust haustoria was suggested by electronmicroscopy⁹. The hyphae and uredospores were also shown to be rich in RNA^{12,27}. Therefore, it is quite probable that the stimulated synthesis of rRNA is originated by the parasite metabolism, especially by the reproductive activity. This view is essentially consistent with the consideration of Shaw²⁸.

When the radioactivity for Victoria is expressed on the optical density basis instead of fresh leaf weight, change in the specific activity was not found by 4 hr-labeling in any RNA fractions (Table 1). This is attributable to an abnormal rise of rRNA content which is induced by "cutting" the stems and following incubation for 4 hr. Recent investigation by the authors has indicated that rRNA content increases by cutting, as measured by optical density, in 4-day-old inoculated leaves with a lag period for 1–2 hr. More detailed experiments are now under way.

A large increase of the inoculated/uninoculated ratio of the radioactivity per fresh leaf weight was noted in the sRNA fraction only at the initiation period of sporulation. Whether it accounts for an increase of the breakdown products of high molecular RNA by the increased RNase activity in the rust-infected tissues²¹ was not determined in the present study. Therefore, further experiments are required for this fraction.

The increase in ^{32}P -rRNA in rust-infected susceptible tissues has been reported by Wolf^{28,29} with wheat leaves following infection by *P. graminis* f. sp. *tritici*. However, Heitefuss and Bauer⁸ reported that bean leaves infected by *Uromyces phaseoli* increased in the specific ^{32}P activity of sRNA, rRNA, and DNA at the sporulation stage of the fungus. The different results in the bean from the oat and wheat may be attributed to different responses of the host tissues. In the former tissues, a parasite-induced hypertrophy associated with an obvious rise of DNA concentration was shown¹³; whereas in the latter two, neither cell division around the rust pustules nor substantial change in DNA concentration was observed^{19,25}.

In the resistant variety, Shokan No. 1, not only the rRNA fraction but also the sRNA fraction exhibited a large increase of ^{32}P incorporation within 28–48 hr, before appearance of the hypersensitive necrosis. As reported in the previous paper¹⁷, the fungal development in the resistant reaction is very slight and more than 70% of the hyphae appear plasmolyzed 40 hr after inoculation. Therefore, the increase of ^{32}P incorporation can be attributed to an increased biosynthesis of RNA's by the host itself. This view would be supported by the base analysis for labeled rRNA, which indicated no difference in the base ratios between the inoculated and uninoculated leaves.

The TB-RNA eluted by SLS from MAK column after NaCl elution has been characterized in a number of studies as messenger RNA (mRNA)^{5,13,18,26}. The increase of the radioactivity in the TB-RNA fraction in this study may suggest that mRNA in the inoculated *Victoria* rises at reproductive stage of the fungus, whereas in Shokan No. 1 it rises during the period of the resistant reaction. Another species of mRNA has been recognized as rapidly labeled RNA eluting behind the heavy rRNA region^{11,15}. Short term labeled RNA at that region in the present study apparently differed from rRNA as determined by a "chase", actinomycin-treatment, and base analysis, and may be characterized as mRNA. Therefore, the increased ratio in rapidly labeled RNA may also indicate a stimulated synthesis of mRNA by the susceptible and resistant reactions.

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和文摘要

Puccinia coronata に感染したエンバク葉の罹病ならびに抵抗反応に ともなう ^{32}P -リボ核酸の変化

谷 利一・吉川正明・内藤中人

エンバク子苗第1葉に *Puccinia coronata* を接種し、各種リボ核酸区分への ^{32}P -とり込みの変化を MAK カラム法で調べた。罹病性品種のビクトリアでは、 ^{32}P の罹病/健全比は rRNA 区分において胞子堆形成開始の初期（接種3日後）から増加し、胞子堆形成期間（同4-5日後）にはいちじるしく増加した。sRNA 区分へのとり込み比は胞子形成開始期（同5日後）にだけ増加した。TB-RNA ならびに rapidly labeled RNA 区分における同比の変化は rRNA とほぼ同じ傾向を示した。抵抗性品種の勝冠1号では、罹病/健全比は sRNA, rRNA, TB-RNA 区分においてほぼ等しく増加した。増加は吸器形成期以前（接種20時間後）にすでにわずかに認められ、接種28-48時間後にいちじるしかった。Rapidly labeled RNA も同じく感染によって増加した。標識 RNA の塩基組成比を分析した結果から、 ^{32}P -rRNA の増加は罹病性品種ではさび菌に由来するが、抵抗性品種では宿主自身の合成促進に基づくかと推定した。