Presence of poly(A)-containing messenger RNA in soybean and *Phytophthora* spp.

By MASAAKI YOSHIKAWA and HAJIME MASAGO

Summary : ^{3}H-Uridine pulse-labeled RNA isolated from soybean hypocotyl and the two plant pathogenic fungi, *Phytophthora megasperma* var. *sojae* and *P. capsici* was analysed by an affinity chromatography on Sepharose 4B-poly U column and by Millipore binding assay for poly(A)-containing messenger RNA. Poly(A)-containing messenger RNA amounted to as much as 1% of total pulse-labeled RNA from either soybean or the two fungi. Sucrose density gradient centrifugation revealed that poly(A)-containing messenger RNA from the three sources was heterogenous in size except one which possessed a prominent peak at the bottom of the gradient. A culture filtrate of *P. megasperma* var. *sojae* contained substance(s) that stimulated the synthesis of poly(A)-containing messenger RNA by soybean hypocotyl to approximately 2-folds of the non-treated control.

Introduction

Considerable evidence has recently accumulated which indicates that messenger RNA and heterogenous nuclear RNA of eukaryotic cells[5], as well as viral[8] and viral directed RNAs[18], contain polyriboadenylate (poly A) sequences 50-200 nucleotides long covalently linked to the 3'-OH terminus. Addition of poly(A) appears to occur post-transcriptionally[2]. However, the informations on the presence of poly(A) in plant and fungal messenger RNA are relatively limited[5].

After the wide acceptance of that plant resistance to various parasites is genetically controlled in terms of the gene-for-gene relationship[4] and that the resistance is induced *de novo* following infection, changes in transcriptional level after infection have been receiving the prime importance as a key metabolism leading to subsequent resistance expression. Heitefuss and Bauer[6], Wolf[19], and the senior author[13][14][16] with Tani and Naito have reported increases of ribosomal RNA synthesis after infection in obligate parasitisms. However, direct evaluation of changes in messenger RNA has not been made except one[15] in which the authors found a slight increase in the *in vitro* template activity of RNA extracted from rust-infected oat leaves.

It is now possible to directly isolate messenger RNA by relatively simple methods[5] such as affinity chromatography on Sepharose-poly U and oligo(dT) cellulose columns, and binding assay on Millipore and poly U filters. This stimulated us to make comprehensive analysis

Laboratory of Plant Pathology, Faculty of Agriculture, Kyoto Prefectural University, Kyoto, Japan.
Received on July 30, 1976.
The authors are grateful to Dr. F. ibuki for technical assistance in conducting sucrose density gradient centrifugation.

on messenger RNA changes in plants after infection. The present report describes a preliminary result that demonstrates the presence of poly(A)-containing messenger RNA in a plant host, soybean, as well as its parasites, *Phytophthora* spp. and also the presence of substance(s) in fungal culture filtrate that stimulate(s) poly(A)-containing messenger RNA synthesis of the host, The effects of infection on host poly(A)-containing messenger RNA synthesis will be published elsewhere[20].

Materials and methods

Soybean (*Glycine max* (L.) Merr.) seeds were planted in plastic pans containing vermiculite : Sumirin Yuki Derma (Sumitomo Forest.) (3 : 1). Plants were grown in a growth chamber at 25°C. Twenty hypocotyls of 7- to 8-day-old plants were wounded by blades[7] and fed for 2 hr at 25°C with 10 μCi of ^{3}H-uridine (Amersham, generally labeled, 6.5 Ci/mmol, 50 μCi/ml) by placing 10 μl of the isotope solution on each wound. *Phytophthora capsici* Leonian and *P. megasperma* Drechs. var. *sojae* A. A. Hildb. was grown for 2 to 3 days in a pea broth medium (25 g frozen peas autoclaved in 1 liter water, then filtered). Emerged young hyphae (approximately 20 mg fresh weight) were placed into 0.2 ml of the ^{3}H-uridine solution and fed with the isotope for 2 hr at 25°C.

^{3}H-Uridine labeled RNA of the plant and fungi was extracted by a modification of the procedure described by Aviv and Leder[1]. Twenty wounded portions (1 cm long) of labeled soybean hypocotyls or 20 mg of labeled hyphae with 0.5 g unlabeled hyphae were pulverized in a mortor under liquid N_2 and the powder suspended in 5 ml of 100 mM Tris-HCl pH 9.0 containing 0.4 M NaCl, 1% sodium lauryl sulfate (SLS), 1 mM $MgCl_2$, and 0.1 ml diethylpyrocarbonate at 4°C. The suspension was vigorously stirred after addition of 3 ml phenol : chloroform : isoamyl alcohol (50 : 50 : 2) for 30 min at room temperature, chilled to 4°C, and the phases were separated by centrifugation at 5,000 g for 10 min. The aqueous phase was removed, poured into two volumes of ice-cold ethanol, and allowed to stand at -20°C overnight. The RNA was collected by centrifugation at 12,000 g at 0°C for 5 min. The RNA pellet was washed twice with ethanol-0.2 M NaCl (2 : 1), dissolved in 1 ml of NETS buffer (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 10 mM EDTA, 0.2 % SLS), and centrifuged at 20,000 g to remove any insoluble material.

Binding of poly(A)-containing messenger RNA to Millipore filter was performed according to Lee *et al*[9] : 0.1 ml of total RNA preparation was diluted in 20 volumes of ice-cold buffer (10 mM Tris-HCl pH 7.6, 0.5 M KCl, 1 mM $MgCl_2$). After 10 min in the cold, the solution was filtered through a Millipore filter (HA 0.45 mμ) previously soaked in the same buffer and the filter washed with 10 ml of the buffer. After drying, the filter was counted in toluene scintillation mixture consisting of 5 g PPO and 0.2 g POPOP per liter of toluene.

Poly(A)-containing messenger RNA was also separated from total RNA by affinity chromatography on Sepharose 4B-poly U (Pharmacia) columns according to the method of Eiden and Nichols[3]. 0.5 ml of total RNA preparation was applied to the column and fractions of 3 ml were collected throughout. The radioactivity in each fraction was determined by counting a 0.5 ml aliquot in Triton X-100 scintillation mixture (2 volumes of the toluene scintillation mixture and 1 volume of Triton X-100).

Sucrose density gradient centrifugation of RNA fractions bound (poly(A)-containing messenger RNA fraction) and unbound to Sepharose 4B-poly U column was performed as follows: RNA eluted from the column was precipitated by additions of one tenth volume of 20% potassium acetate, 0.5 mg unlabeled yeast RNA, and two volumes of ethanol. The precipitated RNA was collected by centrifugation and dissolved in 0.5 ml of NETS buffer. 0.4 ml of the RNA solution was layered over linear 5-20 % sucrose gradient (in the same buffer) and centrifuged in an SW 25 rotor at 22,500 rpm for 15 hr at 25°C. Fractions of 1.5 ml were collected from the bottom of the tube and the radioactivity in 0.5 ml portions of each fraction determined in the Triton X-100 scintillation mixture.

Results

The RNA preparation extracted from soybean by the phenol: chloroform: isoamyl alcohol method appeared to be free from serious contamination by protein and others as revealed from its absorption spectrum (Fig. 1). Both OD_{260}/OD_{280} and OD_{260}/OD_{230} ratios possessed the values higher than 2.25. The RNA preparation contained no significant level (<5 % of total nucleic acid extracted) of DNA as assessed by the diphenylamine reaction and DNase treatment.

Both Millipore filter and Sepharose 4B-poly U column specifically retained authentic poly A (Sigma) but not unlabeled soybean total RNA to detectable degrees (Fig. 2 and 3). Furthermore, when ^{3}H-uridine labeled RNA from soybean and *P. megasperma* var. *sojae* was separated by Sepharose 4B-poly U columns and followed by sucrose density gradient centrifugation, the RNA unbound to the column consisted of heavy and light ribosomal and transfer RNA, and the bound fraction appeared to be free from such non-messenger RNAs and revealed heterogeneity in size distribution (Fig. 4). The poly(A)-containing RNA sedimented

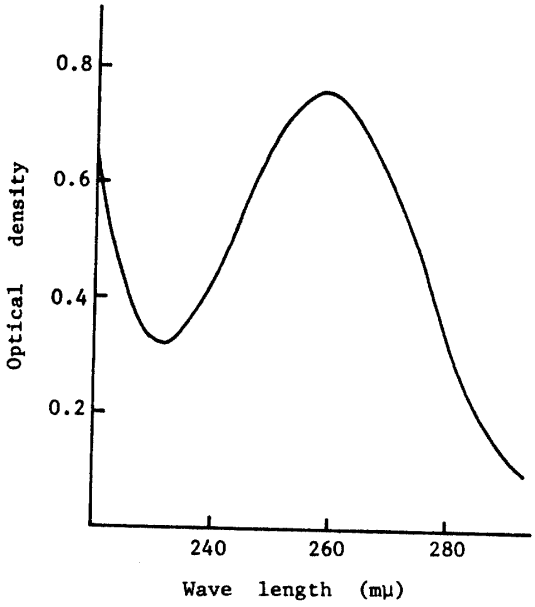

Fig. 1. Absorption spectrum of RNA extracted from soybean by the phenol: chloroform: isoamyl alcohol method.

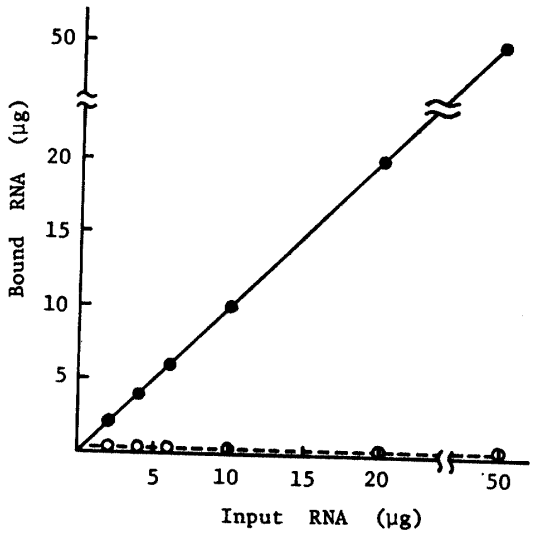

Fig. 2. Specific binding of poly A to Millipore filter.
● — ● : poly A, ○ ··· ○ : soybean total RNA (unlabeled).

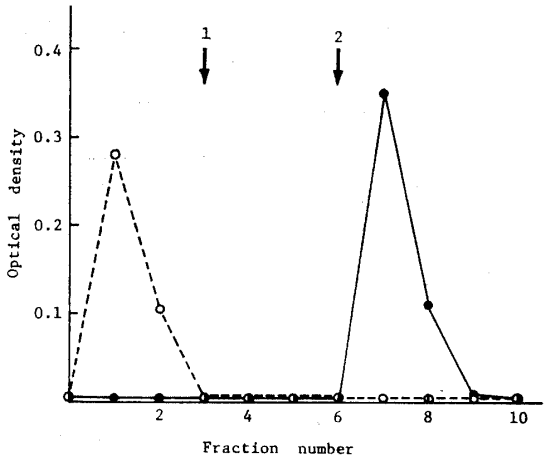

Fig. 3. Affinity chromatography on Sepharose 4B-poly U column of poly A (●—●) and soybean total RNA (unlabeled, ○ ··· ○). The arrows indicate the point of addition of buffer (0.1 M NaCl-0.01 M Tris-HCl, pH 7.4) (1) and water (2).

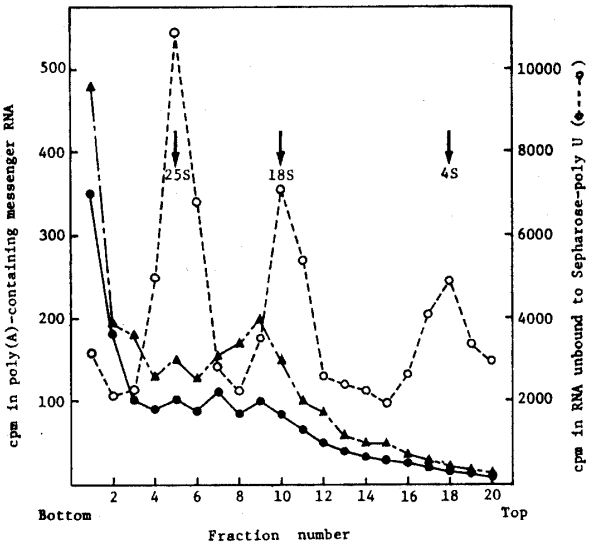

Fig. 4. Sucrose density gradient centrifugation of ^{3}H-uridine labeled RNA unbound (○ ··· ○) and bound (●—●) to Sepharose 4B-poly U column extracted from soybean, and the bound RNA (▲···▲) from *P. megasperma* var. *sojae*.

Table 1. Incorporation of ^{3}H-uridine into poly (A)-containing messenger RNA of soybean and *Phytophthora* spp. as assessed by Millipore and Sepharose 4B-poly U column assays[a]

Material	A Total uptake (cpm×10^{-3})	B Total RNA (cpm×10^{-3})	% of B/A	C Bound to Millipore (cpm×10^{-1})	% of C/B	D Bound to Sepharose-poly U (cpm×10^{-1})	% of D/B
Soybean	3387	189(1.0)[b]	5.6	163(1.0)	0.86	175(1.0)	0.93
Phytophthora capsici	1586	785	49.5	862	1.10	885	1.13
P. megasperma var. *sojae*	976	433	44.4	411	0.95	424	0.98
Soybean treated with culture filtrate of *P. megasperma* var. *sojae*	3615	242(1.3)	6.7	354(2.2)	1.46	388(2.2)	1.60

[a] Incorporation values were corrected for initial total samples from which ^{3}H-uridine pulse-labeled RNA was extracted.
[b] Values in parenthesis are the ratios of culture filtrate-treated over non-treated in ^{3}H-uridine incorporation.

at the bottom of the gradient is presumably heterogenous nuclear RNA[11] as suggested by its large size. Thus, both the methods can well separate poly(A)-containing messenger RNA from the bulk of pulse-labeled RNA.

The RNA preparations from soybean hypocotyl and the two plant pathogenic fungi, *P. capsici* and *P. megasperma* var. *sojae*, did contained poly(A)-containing messenger RNA (Table 1). Both Millipore and Sepharose 4B-poly U assays indicated that approximately 1% of the plant and fungal RNA newly synthesized during 2 hr labeling was poly(A)-containing messenger RNA. It also appeared from Table 1 that the fungi incorporated more rapidly the labeled precursor into RNA than did soybean hypocotyl. Furthermore, fungal product(s) contained in the culture filtrate stimulated the synthesis of poly(A)-containing messenger RNA by soybean, and to lesser degree the synthesis of the non-messenger RNAs.

Discussion

The present study reveaied that soybean hypocotyl as well as its parasites, *P. megasperma* var. *sojae* and *P. capsici*, like other eukaryotic organism, can synthesize poly(A)-containing messenger RNA. This provided the first evidence for the presence of poly(A)-containing messenger RNA in plant pathogenic fungi. The radioactivity incorporated into poly(A)-containing messenger RNA amounted to approximately 1 % of that into total pulse-labeled RNA among the plant and fungi studied, and the amounts were equivalent or slightly less compared to those reported in yeast[10] and other plants[12,17]. The further characterization of poly(A)-containing messenger RNA fraction should be conducted by testing its biological activity to support protein synthesis before identifying it as real messenger RNA, and the experiments are now under the way employing the *in vitro* wheat germ protein synthesis system.

The culture filtrate of *P. megasperma* var. *sojae* stimulated the synthesis of poly(A)-containing messenger RNA by soybean hypocotyl (Table 1). This would be of interest when considering that the infection of soybean hypocotyl by the fungus results in an enhanced synthesis of poly(A)-containing messenger RNA at early stage of the infection[20] when the fungus has not yet invaded inside the host tissue. Therefore, the results may suggest that such enhanced synthesis is elicited by certain product(s) secreted by the parasite.

Literature cited

1) Aviv, H. & P. Leder (1972) : Proc. Nat. Acad. Sci. USA 69 : 1408-1412.
2) Darnell, J. E., L. Philipson, R. Wall, & M. Adesnik (1972) : Science 174 : 507-510.
3) Eiden, J. J. & J. L. Nichols (1973) : Biochemistry 12 : 3951-3955.
4) Flor, H. H. (1971) : Ann. Rev. Phytopathol. 9 : 275-296.
5) Greenberg, J. R. (1975) : J. Cell Biol. 64 : 269-288.
6) Heitefuss, R. & M. I. Bauer (1969) : Phytopath. Z. 66 : 25-37.
7) Klarman, W. L. & J. W. Gerdemann (1963) : Phytopathology 53 : 1317-1320.
8) Lai, M. & P. H. Duesberg (1972) : Nature 235 : 383-386.
9) Lee, S. Y., J. Mendecki, & G. Brawerman (1971) : Proc. Nat. Acad. Sci. USA 69 : 1331-1335.
10) Mchaughlin, C. S., J. R. Warner, M. Edmonds, H. Nakazato, & M. H. Vaughan (1973) : J. Biol. Chem. 248 : 1466-1471.
11) Ruize-Carrillo, A., M. Beato, G. Schutz, P. Feigerlson, & V. G. Allfrey (1973) : Proc. Nat. Acad. Sci. USA 70 : 3641-3645.
12) Sagher, D., M. Edelman, & K. M. Jakob (1974) : Biochim. Biophys. Acta 349 : 32-38.
13) Tani, T., M. Yoshikawa, & N. Naito (1971) : Ann. Phytopath. Soc. Japan 37 : 43-51.
14) ——— (1973) : Phytopathology 63 : 491-494.
15) ——— (1973) : Ann. Phytopath. Soc. Japan 39 : 7-13.
16) ——— (1975) : Physiol. Plant Pathol. 5 : 193-199.
17) Van de Walle, C. (1973) : FEBS Letters 34 : 31-34.
18) Weinberg, R. A., Z. Ben-Ishai, & J. E. Newbold (1972) : Nature New Biol. 238 : 111-113.
19) Wolf, G. (1967) : Phytopath. Z. 59 : 101-104.
20) Yoshikawa, M. & H. Masago. Submitted to Physiol. Plant Pathol.

摘 要 ダイズ胚軸および2種の植物病原菌, *Phytophthora megasperma* var. *sojae* と *P. capsici* を ^{3}H-uridine でパルスラベルし，それらから得た RNA 中に poly(A)-containing messenger RNA が存在するかどうかについて，セファロース 4B-poly U カラムによる affinity クロマトグラフィーまたミリポアフィルターによる binding assay で調べた。その結果，ダイズまた糸状菌いずれの場合もパルスラベルした全 RNA の約1%が poly(A)-containing messenger RNA として存在することが明らかとなった。ショ糖密度勾配遠心法によると，それらの poly(A)-containing messenger RNA は非常に大きい分子量をもつと思われるピークを除いては，そのサイズが neterogenous であった。また，*P. megasperma* var. *sojae* の培養ろ液中にはダイズ胚軸における poly (A)-containing messenger RNA の合成を促進する活性が認められた.