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Cytokinin Activity in the RNA of Tobacco Mosaic Virus

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Enzymatic hydrolysates of highly purified RNA from tobacco mosaic virus (TMV) were analyzed for cytokinin activity by tobacco and soybean callus bioassays after extracting the hydrolysates with water-saturated *n*-butanol and separating the components by column or paper chromatography. The chromatographic properties of the active components suggested that they are the different forms of zeatin and N^6-(Δ^2-isopentenyl)-adenine.

Cytokinins are involved in the regulation of many phases of plant growth and development. The naturally occurring cytokinins, a group of the N^6-substituted adenine derivatives, are present in plants in the free state and as constituents of certain tRNAs (1–4). In addition, cytokinins occur adjacent to the anticodon in specific tRNAs of many other organisms, ranging from mycoplasmas to animals (*5–11*). Until very recently the occurrence of cytokinins in viral RNA had not been investigated, although it had been shown that the RNAs extracted from tobacco mosaic virus (TMV) (*12–15*) and some other plant viruses contain modified nucleotides (*15–18*). Earlier we analyzed a butanol extract of an acid hydrolysate of TMV RNA, and found it to contain cytokinin activity (*19*). However, the methods used in that work could not exclude that possibility that the cytokinin activity detected in hydrolyzed TMV RNA was due to minor impurities in the RNA preparation. In this paper, by a different methodology, we show that the cytokinin activity is associated with full-length RNA from highly purified TMV particles.

The U1 strain of TMV was purified from *Nicotiana tabacum* L. cv. Turkish Samsun by a procedure devised to remove protein contaminants from TMV (*20*). Infected leaves were frozen and homogenized in a Waring Blendor. Two milliliters of 0.5 *M* Na_2HPO_4 containing 0.5% ascorbic acid was used per gram of leaves. After filtering through four layers of gauze, 5 g of Celite was added to each 100 ml of the filtrate, and this fluid was kept on ice for 60 min. The extract was centrifuged at 10,000 rpm for 30 min and the supernatant was filtered. NaCl and PEG 6000 were added to the filtrate to give final concentrations of 1 and 3% (v/v), respectively, and these mixtures were stirred for 10 min and then kept on ice for 20 min. The liquid was centrifuged at 10,000 rpm for 30 min and the pellets were resuspended in 0.01 *M* Na_2HPO_4 containing 0.01% ascorbic acid. After stirring for 10 min, 3 g Celite was added to each 100 ml of the extract and centrifuged again. PEG 6000 and NaCl were added to the filtered supernatant fraction as before. After stirring, the sample was again centrifuged at 10,000 rpm for 30 min and the pellet was resuspended overnight in 200 ml of 0.01 *M* Na_2HPO_4 containing 0.01% ascorbic acid. The liquid was centrifuged at 10,000 rpm for 10 min. Triton X-100 was added to a concentration of 5% and the solution was incubated for 30 min at room temperature and then centrifuged in a Beckman L5-50 centrifuge with a 30 rotor at 27,000 rpm for 2 hr. The pellet was resuspended in 100 ml of 1 m*M* EDTA and an equal volume of 0.5 *M* phosphate buffer, pH 7.0, was added and incubated at 37° for 2 hr and then centrifuged in a Beckman L5-50 as before. The pellet was resuspended in 10 ml of 1 m*M* EDTA and then centrifuged at 9000 rpm for 10 min. The virus concentration in the supernatant

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fluid was estimated spectrophotometrically ($E_{260}^{0.1\%} = 3.0$). To further assure that extraneous RNA was not associated with the surface of the virion, the RNase–Pronase procedure of Siegel (*21*) was used. The virus preparation was incubated with 15 μg/ml ribonuclease A (Worthington) at 37° for 2 hr and then with 0.1 mg/ml Pronase B (Calbiochem) for 2 hr at the same temperature. The mixture was centrifuged again twice in the Beckman ultracentrifuge using the same speed and running time as before. The pelleted virus was resuspended in 1 m*M* EDTA and used for the RNA preparation.

To 15 ml of purified virus in 1 m*M* EDTA was added 300 mg of SDS. The sample was placed at 70° for 50 sec and then transferred to ice water and stirred for 10 min. Three milliliters of 0.4 *M* Tris–0.04 *M* acetic acid, pH 9.0, was added, followed by 17.6 ml liquid phenol (saturated with 0.1 *M* Tris, 0.01 *M* acetic acid, pH 9.0). The mixture was shaken mechanically for 20 min at 20°. The two phases were separated by centrifugation at 10,000 rpm for 20 min and the aqueous phase was saved. The phenol phase was extracted two more times with buffer and all three phases were pooled. The RNA was recovered by precipitation with 2 vol of cold ethanol for 12 hr at −20°. The precipitate was collected by centrifugation and washed two times with 2 *M* sodium acetate, 0.2 *M* acetic acid, pH 9.0, in 67% ethanol and dried under N_2 (*22*).

The viral RNA was further purified by centrifugation in 0–35% linear-log sucrose density gradients in SSC buffer (0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7.0) (*23*). The gradient centrifugation was made in a SW 41 rotor for 6 hr at 38,000 rpm and gradients were fractionated using an ISCO density gradient fractionator. Only the major uv-absorbing peak corresponding to full-length RNA molecules was collected. They were pooled and dialyzed overnight in SSC. The RNA was subjected to another gradient centrifugation under the same conditions and recovered by precipitation with ethanol and freeze-dried. The redissolved TMV RNA at 1 mg/ml was hydrolyzed to nucleosides. The hydrolysis was performed using 10 μg/ml pancreatic ribonuclease (Reanal) in 0.01 *M* Tris–HCl, pH 7.8, for 20 hr. At this point the pH was adjusted to 8.4 and to 0.01 *M* MgCl, and alkaline phosphatase (Sigma) was added at a concentration of 2 μg/ml. After 8 hr snake venom phosphodiesterase (Sigma) was added to 0.15 μg/ml and alkaline phosphatase was added to the solution. The incubation was continued for another 16 hr, and then the solution was heated to 60° for 30 min. The hydrolysate was partitioned three times against an equal volume of water-saturated *n*-butanol at pH 7.8. The butanol phase was evaporated to dryness under vacuum in a rotary evaporator and all the butanol was removed by repeated reevaporation with water. The redissolved material was subjected to column or paper chromatography. The column chromatography was done on a Sephadex LH 20 column using 35% ethanol for elution. The eluate was collected in 60 fractions and suitable aliquots from each fraction were bioassayed by the tobacco stem pith callus (*24*) and soybean callus assays (*25*). Following chromatography of the extract of hydrolyzed TMV RNA, cytokinin standards were chromatographed on the same column to determine their elution volumes. The mixture of standards consisted of zeatin (Z), methylthiozeatin (ms-Z), zeatin riboside (ZR), N^6-(Δ^2-isopentenyl)-adenine (2iP), its nucleoside (IPA), and 2-methylthio derivatives of 2iP and IPA.

Ascending paper chromatography was also employed for separating the different components in a butanol extract of hydrolyzed TMV RNA. The same cytokinin standards were applied for paper chromatography as for the column chromatography. The developed chromatograms were divided into 10 equal strips and assayed for cytokinin activity by the soybean callus bioassay. The strips corresponding to specific R_F regions were cut into small squares and put into basal medium for a soybean callus bioassay (*25*). Three pieces of callus from a 4-week-old stock culture were placed in each of 10 Erlenmeyer flasks containing basal medium. The callus had a fresh weight of about 50 mg at the start of the experiment. The callus cultures were kept in darkness at 27° for 28 days and then weighed.

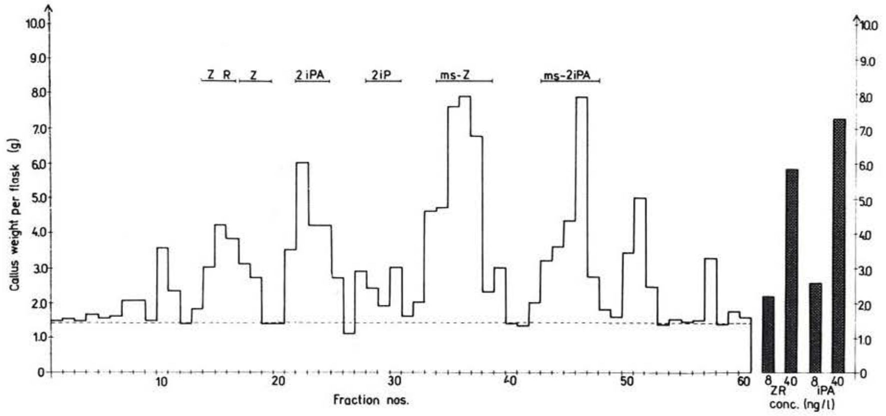

FIG. 1. Tobacco callus bioassay of a column-chromatographed extract of hydrolyzed full-length TMV RNA. A butanol extract of a hydrolysate of 2 mg of TMV RNA was chromatographed on a column of Sephadex LH 20 A (2.5 × 75 cm) and eluted with 35% ethanol at a flow rate of 30 ml/hr. Thirty-milliliter fractions were collected and 3.75 ml was withdrawn for bioassay.

All experiments were done three times with two replications for each fraction and appropriate blank filter paper controls.

Fig. 1 shows the results of the tobacco callus bioassay of the hydrolysate of full-length TMV RNA. The butanol extract of the hydrolysate was chromatographed on a Sephadex LH 20 column. Cytokinin standards eluted from this column in decreasing order of polarity. The extract of the hydrolysate contained several regions with significant cytokinin activity. The first peak of activity eluted before the ZR, which is the most polar standard. The second peak of activity was found in fraction 15 around which the ZR is centered. It cannot be excluded that a part of the activity in this region was due to the presence of a small amount of Z which eluted just after the ZR. The next cytokinin activity coincided with the mobility of IPA. A fourth cytokinin, probably 2iP, may be responsible for the activity found in fractions 26–29. The two major peaks of cytokinin activity eluted at the positions of the 2-methylthio derivatives. Two additional peaks of activity eluted later than any of the standards used. The mobilities of these peaks were less than that of the ms-2iP which eluted on Sephadex LH 20 with 35% ethanol at fractions 60 and 70. A similar relative abundance of different cytokinin activities were observed when the cytokinin activity of the column eluate was measured by the soybean callus bioassay (data not shown).

Figure 2 shows the distribution of the cytokinin activity by paper chromatography of the butanol extract of hydrolyzed full-length TMV RNA and bioassayed by soybean

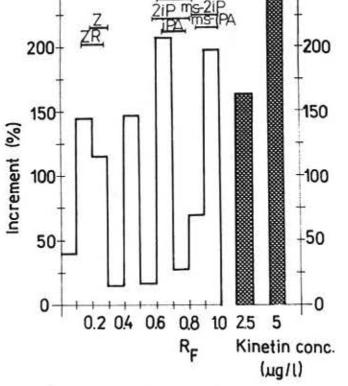

FIG. 2. Distribution of cytokinin activity on a paper chromatogram of a butanol extract of hydrolyzed full-length TMV RNA. A sample extracted from the hydrolysate of 0.125 mg of TMV RNA was chromatographed on Whatman No. 1 paper in ethyl acetate: formic acid:water (3:1:1, v/v). Each of the 10 segments of the chromatogram was incorporated into 10 ml basal medium of soybean callus. The cultures were weighed after 28 days. The increment indicates the increase in weight of callus tissue in comparison to the blank filter paper control, which was equated to zero.

callus. The chromatogram, developed in ethyl acetate:6 *N* HCOOH:water (60:5:35, v/v) revealed four peaks of cytokinin activity. Three peaks (0.1–0.3; 0.6–0.7; and 0.8–1.0) chromatographed to the positions of ZR and Z; ms-Z and IPA; and ms-IPA, respectively. The peak of cytokinin activity at R_F 0.4–0.5 does not correspond to the R_F of any of the standards used. Both column chromatography and paper chromatography show that besides the cytokinin nucleosides there may be free base forms of cytokinins in the extract of the hydrolysate. This conversion may be due to hydrolysis of the glycosidic bonds during the extraction and separation procedures. The chromatographic studies also indicate that the strongest cytokinin activities from hydrolyzed TMV RNA correspond in mobility to the methylthio derivatives.

An extract of enzymically hydrolyzed purified *Escherichia coli* ribosomal RNA (0.5 mg) was analyzed similarly and showed no cytokinin activity in the soybean callus bioassay.

The possibility that the cytokinin activity detected in TMV RNA is due to contamination with host RNA must be discussed. Since the purified TMV was treated with RNase, we conclude that host RNA which may adhere to the surface of virion cannot be a factor in our experiments. However, it could be possible that encapsidated host RNA can account for cytokinin activity. The U1 strain of TMV has an encapsidated host RNA pseudovirion RNA content of less than 2% (*21*). The encapsidated RNA, which can be separated from virus particles by electrophoresis, consists of different amounts of host RNA being of complementary sequence to chloroplast and nuclear DNAs, and little of it is comprised of ribosomal RNA (*21*). Since the cytokinin activity is associated with full-length, density gradient-purified TMV RNA, this reduces the possibility of a tRNA contamination unless the tRNA is bound to the TMV RNA before encapsidation.

These chromatographic studies are inadequate to establish the identities of the cytokinin active compounds, but the data do suggest that the enzymatic hydrolysate of TMV RNA does contain compounds with chromatographic behavior similar to that of cytokinins that have been shown to occur in preparations of tRNA from different sources (*26*).

Only a rough quantitative estimate of the cytokinin residues present in TMV RNA can be obtained using the callus bioassay. We estimated the amount of cytokinin residues in TMV RNA hydrolysates by comparing the data obtained from known cytokinin control flasks. Kinetin is about 10 times less active than the naturally occurring cytokinins (*27*). Assuming full recovery of nucleosides from TMV RNA and an activity corresponding to that of kinetin, TMV RNA would contain 0.08 to 0.26 mol% of total cytokinin activity, corresponding to 5–17 nucleoside residues. The tRNAs from different sources contain cytokinins in similar amounts (*26*).

Although Dunn and Smith (*12*) have shown that a hydrolysate of TMV RNA may contain a trace of 6-methylaminopurine riboside, the TMV RNA has been considered until recently to contain no minor nucleosides (27, 28). However, the lower limit of modified nucleosides detectable by the methods used in these studies cannot exclude the presence of minor nucleosides in a lower quantity in TMV RNA (*13*). Using an entirely different and more sensitive procedure developed especially for the detection of minor nucleosides Niblett *et al.* (*15*) have shown the minor nucleosides N^6-methyladenosine, dihydrouridine, and pseudouridine to be present in the RNA of plant viruses and viroids (C. L. Niblett, personal communication). The RNAs analyzed were purified by density gradient centrifugation or polyacrylamide gel electrophoresis, but this does not exclude the possibility that the minor nucleosides occurred in contaminating host RNAs which copurified with the viral and viroid RNAs. In the minor nucleoside system, ZR or IPA would not be detected because their chromatographic mobilities are much greater than those of the above minor nucleosides (Niblett and Balázs unpublished results).

It is interesting to note that certain fragments of several plant viral RNAs have amino acid acceptor activity at least in *in vitro* systems (*30–35*). However, the 3′-terminal fragment that has the acceptor activity apparently lacks minor bases (*36*). The amino acid accepting fragments of TMV RNA can be esterified with histidine using aminoacyl tRNA synthethase prepared from bean (*37, 38*) or *E. coli* (*39*). We cannot state at present whether cytokinin active nucleosides or other minor nucleosides have a role in the amino acid accepting activity of TMV RNA or in that of other plant viruses.

The results reported here provide evidence for the occurrence of cytokinin active compounds in the RNA of tobacco mosaic virus. The location and the positive identification of the cytokinin active nucleosides and their possible function in virus–host interaction remain to be determined.

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