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SYNTHESIS AND ACCUMULATION OF POLYAMINES AND S-ADENOSYLMETHIONINE IN CHINESE CABBAGE INFECTED BY TURNIP YELLOW MOSAIC VIRUS

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SUMMARY

Infection of Chinese cabbage with turnip yellow mosaic virus caused a significant increase in the levels of S-adenosylmethionine and the polyamines, spermidine, spermine and putrescine in the leaves. A significant portion of the spermidine and spermine in the juice of infected plants is precipitated by antiserum to the virus. The rate of incorporation of radioactivity from [2-^{14}C]methionine into spermidine, spermine, S-adenosylmethionine and protein appeared to be very similar in leaf discs derived from both healthy and infected leaves, suggesting that enzymes for biosynthesis of spermidine and spermine from methionine are not elevated in the infected leaves.

INTRODUCTION

Polyamines are present in turnip yellow mosaic virus (TYMV) in amounts sufficient to neutralize approximately 20% (1) to 36% (2) of the viral RNA-phosphate. Spermidine (spd) is the major polyamine component. TYMV capsids lacking nucleic acid, i.e. "top" component, contain fewer than five molecules of spermidine per particle (A. Protter and S.S. Cohen-unpublished data), suggesting a relation of this polyamine with viral RNA rather than with the protein coat. Indeed spermidine has been found on isolated TYMV RNA (2). One probable role of the polyamines in TYMV is to produce a more compact, stable RNA structure within the virus particle (3,4).

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The stabilization of DNA by spermidine or spermine (sp) appears to involve the interaction of either of the amines with a double-stranded region of the molecule (5). These polyamines also stabilize limited helical regions of single-stranded RNA. It has been shown in this laboratory that both tRNA (6-8) and bacteriophage R17 RNA (9) bind significant amounts of spermidine. Spermidine is known to stabilize the tRNA molecule (10) and condense MS2 RNA (11). It is possible therefore that sites of interaction of spermidine with the single-stranded TYMV-RNA would include both the tRNA-like structure at the 3' end (12) and other loops present in the structure.

If polyamines are essential for virus assembly and stabilization, the control of the levels of the amines and their immediate precursors in the host might ultimately control the rate of virus synthesis. The present study follows the levels of the polyamines in healthy and infected Chinese cabbage plants as a function of time after infection and the relative rates of synthesis of the polyamines in leaf tissue. It is recognized that a kinetic study of these parameters will eventually require systems in which cabbage cells can be infected simultaneously, e.g. as in protoplast suspensions (13).

MATERIALS AND METHODS

Growth of plants and inoculation with TYMV: Chinese cabbage seeds (Brassica pekinensis, Rupr, Var. Wong Bok) were obtained from Burpee Seeds, Clinton, Iowa. TYMV was a gift of Dr. J.M. Kaper, USDA, Beltsville, Maryland. Plants were grown in a Percival incubator (Boone, Iowa) set for 18 hour days at 28°C and 20,000 lux using incandescent and fluorescent lighting and 6 hour dark periods at 22°C. When the plants were about five weeks old, the rosette of each plant as well as the two oldest leaves were removed, leaving only two large leaves to be mechanically inoculated using carborundum and a solution of TYMV (0.1 mg virus/ml in phosphate buffer, 0.02 M, pH 7.0). The rosette of control plants was also removed. Newly emerging deribbed leaves were used. The fresh weight of all newly emerging leaves of the TYMV-infected plants was only slightly less than controls over a two week period.

Isolation and quantitation of the polyamines and S-adenosylmethionine: Spermidine, spermine, putrescine (pu) and S-adenosylmethionine (SAM) were extracted from Chinese cabbage leaves in a glass homogenizer with 4 volumes (v/w) of cold 1.5 M perchloric acid (PCA) (14). The extract was placed on ice for one hour, then centrifuged. The pellet was washed with 1.5 M PCA and following centrifugation the perchlorate ion was removed from the combined supernatants (14). The neutralized solution (pH 6.0), was applied to a column (8 cm x 1 cm) of Dowex-50-H^+ (Bio-Rad, AG 50W-X8, 200-400 mesh) which had been equilibrated with water. The resin had been prepared as described (14) just prior to use. Once the sample was absorbed, the column was washed with 20 ml of water followed by 250 ml of 0.8 N HCl, which eluted interfering salts as well as methionine. A final wash of 80 ml of 4.0 N HCl eluted the polyamines and SAM. The 4 N HCl effluent was taken to dryness at reduced pressure at 40°. The residue containing the polyamines and SAM was redissolved in 100 µl of 10^{-4} N HCl and stored at -20°. The percent recovery of the polyamines and SAM isolated from plant tissues was estimated by adding authentic [^{14}C]spermine, [^{14}C]spermidine, [^{14}C]putrescine and [^{14}C]SAM separately to plant-leaf homogenates and reisolating the four compounds by Dowex-50 chromatography. The reported concentrations of the four compounds were corrected for the following recoveries: spermine 90%; spermidine 96%; S-adenosylmethionine 95% and putrescine 63%.

The polyamines were quantitated by the dansyl method (6). SAM was quantitated by a procedure involving in situ reflectance spectrophotometry. Unknowns along with 1 to 12 x 10^{-9} moles of authentic S-adenosylmethionine chloride were spotted on Avicel-F plates (20 cm x 40 cm, 250 um, Analtech, Inc.) and electrophoresed at 8 v/cm for 3 hours in 0.1 M sodium citrate buffer, pH 3.5 (15), using water to cool the apparatus (10-12°C). SAM, located by UV light (254nm), moved at a rate of 2.7 cm/hour. The plate was then scanned with a Vis-UV chromatogram analyzer (Farrand Optical Co.) at 260nm. All peaks were cut out and the weights of unknown peaks were compared to the weights of standards and plotted against the square root of the SAM concentration. The limit of detection is about 5 x 10^{-10} moles SAM.

When determining conversion of [2-^{14}C]DL-methionine to spermidine, spermine or SAM using leaf discs, a two dimensional system of separation was used. An aliquot of Eluate B was added to 20nmoles each of spermidine trihydrochloride, spermine tetrahydrochloride (Calbiochem) and SAM and subjected to paper electrophoresis on Whatman 3MM in 0.1 M sodium citrate buffer, pH 3.5 applying 8 v/cm for 3 hours with water cooling, (10-12°C). Under these conditions, methionine moved at 2.9cm/hour, with SAM, spermine and spermidine respectively moving at 4.1 cm/hour, 6.0 cm/hour, and 6.7 cm/hour. The paper was then dried at room temperature, subjected to ascending chromatography in the second dimension for 3.5 hours using the solvent system: 2-methoxyethanol, propionic acid, and water saturated with NaCl, (70:15:15) (15). The paper was dried in air and sprayed with ninhydrin. The R_f values for methionine, SAM, spermine, and spermidine are 0.71, 0.16, 0.12, and 0.25, respectively. The ninhydrin-positive spots corresponding to the polyamines and SAM were cut out and placed in 5 ml of Liquifluor (New England Nuclear), radioactivity was determined in a Packard Model 3003 scintillation spectrometer.

Incubation of leaf discs with [2-^{14}C]DL-methionine: All equipment, glassware, and solutions were sterilized. Discs of 0.9 cm diameter were punched out from the leaves with a cork borer, weighed and rinsed with cold distilled water. After drying on paper towels, 100 leaf discs were placed top side up in a plastic petri dish (10 cm x 1.5 cm) containing 12 ml of 0.01 M phosphate buffer, pH 7.0 and 8µci of [2-^{14}C]methionine (5.7 µCi/µmole, New England Nuclear) with lens paper covering the bottom. The discs were then incubated at 28° under 20,000 lux for varying lengths of time. After the desired incubation time, the discs were rinsed 4 times with 150 ml distilled water per rinse, and then homogenized with 1.5 M cold PCA. The acid-soluble fraction was analyzed for polyamine and SAM. Radioactivity in the PCA-insoluble material associated with protein was determined by the procedure of Mans and Novelli (16). Following the ether-ethanol wash, the pellet was suspended in about 3 ml of 5% cold TCA and a 100 µl aliquot was applied to a Whatman 3 MM scintillation pad, dried and counted as described above.

Chlorophyll was determined by homogenizing 0.4 g leaf tissue with 2 ml distilled water followed by the addition of 8 ml of acetone to the homogenate. The chlorophyll concentration was determined after centrifugation and dilution with 80% acetone (17).

Serological estimation of TYMV: Virus concentration was estimated by serological precipitation (18) using rabbit antiserum obtained from Dr. H.E. Waterworth, Dept. of Agriculture, Glendale, Md. Leaves were homogenized using an omni-mixer in 4 volumes (w/v) of cold phosphate buffer (0.1 M, pH 7.0). After filtration through 8 layers of cheese cloth, the sap was centrifuged at 15,000 x g for 15 minutes. To 100µl aliquots of twofold serial dilutions of the supernatant with the homogenizing buffer was added 100 µl of the antiserum (1:100 dilution in 0.28 M NaCl). The tubes were incubated at 37° for 2 hours then placed at 4° overnight. Using twofold serial dilutions of a mixture of TYMV in centrifuged plant sap, the lowest concentration of virus giving a detectable precipitate was determined to be 1.5µg TYMV in 100 µl diluted juice.

RESULTS AND DISCUSSION

Polyamine and SAM levels in healthy and TYMV-infected plants: The polyamines, SAM, chlorophyll, and TYMV were determined in healthy and TYMV-infected Chinese cabbage plants as a function of time after infection (Table 1). The concentrations of the polyamines and SAM in the infected leaves were significantly higher than in the healthy leaves. A significant difference in the polyamine levels of the two tissues was seen on day 6, when mosaic symptoms first began to appear. At this time, the amount of virus in the infected tissue was very low, whereas on days 11 through 18, the amount of virus per gram remained relatively constant

Table 1

POLYAMINE CONTENT OF HEALTHY AND

TYMV-INFECTED CHINESE CABBAGE LEAVES

	n moles/gram					
Healthy leaves* (days)	Pu	Spd	Sp	SAM	mg chlorophyll/gram	mg TYMV/gram
6	36	250	63	11	1.0	0.0
11	26	270	31	5.6	0.91	0.0
14	23	86	23	7.0	1.1	0.0
18	21	77	42	6.0	1.0	0.0
Infected leaves* (days)						
6	41	400	98	14	0.95	$\leq$ 0.15
11	66	480	110	8.3	0.92	1.5
14	120	490	71	9.1	0.97	0.8
18	200	370	67	10	0.71	0.9

*Three plants were used for each time point.

at about 1.0 to 1.5 mg TYMV per gram fresh weight. The TYMV concentration in deribbed cabbage leaves is reported to rise rapidly between 7 and 10 days postinoculation and remain relatively constant through the 24th day (19).

Spermidine and spermine remained high throughout infection, whereas the putrescine content increased to very high levels between 6 and 18 days. On the 18th day of infection the infected leaves had 10 times the putrescine content and nearly five times the spermidine content as the healthy leaves. At this time also, a significant portion of the total spermidine (22% $\pm$ 4%) and spermine (40%) in the infected plant sap coprecipitated with the virus when antiserum was added. Only small amounts of exogenous [^{14}C]spermidine, (up to 2% of viral spermidine under conditions of a twofold excess of exogenous spermidine) bind to virus preparations under conditions of precipitation by TYMV antisera (Cohen

Table 2

TIME-DEPENDENT INCORPORATION OF LABEL FROM [2-^{14}C]DL-METHIONINE INTO LEAF DISCS:

	CPM/gram x 10^{-4}			
	Spd	Sp	SAM	Protein
Healthy - hours				
1	1.3	0.28	1.7	10
3	2.0	0.51	7.3	35
6	3.5	1.2	12.0	81
Infected*-hours				
1	1.8	0.45	2.2	10
3	2.7	0.58	4.7	39
6	5.0	1.1	8.4	72

*14 days after infection

and Heyward-unpublished observations). Larger amounts of exogenous spermine do bind; the phenomena are being explored further.

Utilization of [2-^{14}C]DL-methionine by leaf discs: When leaf discs from 14 day post-innoculated plants, were incubated with [2-^{14}C]DL-methionine, the rates of incorporation of label into protein, SAM, spermidine and spermine were approximately the same for the discs of both healthy and infected leaves (Table 2). After 6 hours, approximately 20% of the available [^{14}C]L-methionine had been incorporated into protein in the discs. Methionine also enlarged the pool of SAM to an extent which can account for the amount of label appearing in spermidine and spermine, on the assumption that SAM is the precursor for spermidine. The transfer of label from methionine to SAM, spermidine and spermine appears to support this proposed pathway of polyamine biosynthesis in plant tissues (20). The similar rates of incorporation of label into spermidine and spermine in both healthy and infected tissues

also appear to suggest that infection by TYMV does not elicit a viral-specific synthesis of the enzymes needed for spermidine and spermine biosynthesis. However spermidine biosynthesis in the healthy and infected cells has not yet been accounted for totally by the condensation of putrescine with an aminopropyl moiety derived via the enzymatic decarboxylation of SAM.

As in the case of R17 infection (2), infected tissues accumulate several fold greater concentrations of spermidine. However, in contrast to R17 infection in which putrescine does not accumulate in the infected cell, putrescine accumulation is a pronounced phenomenon in TYMV infection, and may relate to the pathological yellowing of the plant (21).

It is of interest that a significant portion of the high concentration of spermidine found in infected tissue is precipitable by antiserum to the virus. From the data in Table 1, it can be estimated that 22% of the average spermidine content of infected tissue amounts to approximately 90nmoles/gm. This would correspond to about 400 spermidine molecules per virion in the TYMV present in the sap, and falls in the range reported in the literature (1,2). The serological procedure then may prove to be a simple approach to the estimation of spermidine in viruses for which precipitating antisera are available.

From the data in Table 1, the fraction precipitable by antiserum is about 40% of the excess spermidine over that found in normal tissue. It will be of interest to determine the distribution of non-virion spermidine. In any case these data suggest that the increased level of spermidine in TYMV-infected Chinese cabbage leaves is in part if not completely due to the sequestration of polyamine by viral RNA as a step in the formation of intact virus.

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