

The Relation of Tobacco Mosaic Virus X-Protein to Amorphous Cellular Inclusions (X-Bodies)

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ABSTRACT

Two common (U-1[D] and U-1[SB]) strains of tobacco mosaic virus and U-5 were used to determine whether the frequency of amorphous intracellular inclusions (X-bodies) correlated with extractable X-protein. Although U-1(SB) produced

more X-bodies than U-1(D), no consistent differences were found between these common isolates in X-protein yields. The U-5 strain produced X-bodies only rarely, and yielded no X-protein. *Phytopathology* 60:426-430.

Iwanowski (6) described granular, vacuolated, amoeboidlike inclusions, ranging from 5 to 30 μ , in cells of Turkish tobacco plants infected with tobacco mosaic virus (TMV). These were named X-bodies by Goldstein (3, 4), who conducted detailed cytological studies of them. Sheffield (10, 11) later showed that the X-bodies associated with the aucuba strain of TMV stained strongly for protein, and found by micurgical techniques (12) that their contents were highly infectious.

The fine structure of X-bodies was unknown prior to the development of suitable stains and improved thin-sectioning techniques for electron microscopy. With the electron microscope, Shalla (9) found the cytoplasm of TMV-infected tomato cells to contain heavily staining thick filaments which he suggested were a principal component of X-bodies. These filaments were believed to correspond to those seen in cellular extracts by Zech (15). The portion of the cytoplasm rich in the filaments or tubules also contains virus particles, endoplasmic reticulum, ribosomes, and mitochondria. Kolehmainen et al. (7) also found such areas in the cytoplasm of diseased tobacco cells, and further noted that three or more microtubules commonly aggregated to form the filaments. They speculated that the regions might be involved in virus synthesis or assembly, but did not relate them to X-bodies. Esau & Cronshaw (2) compared thin-sections by both light and electron microscopy, and demonstrated that X-bodies were indeed composed, in part, of the filamentous structures, which they called X-tubules or X-components. Esau (1) later described a step-wise process whereby a granular portion of the cytoplasm develops progressively into the tubule complexes, which finally become aggregates of individual tubules.

Esau (1) proposed that the inclusion-body X-tubules are polymerized X-protein, an anomalous protein that had been found in TMV-infected tissue extracts by Takahashi & Ishii (14). Although the protein extracted from the diseased plant is called X-protein whereas that chemically separated from purified whole virus is called A-protein, the two are biochemically identical with viral coat protein (13).

In our previous studies (5), cells infected with the U-5 strain rarely contained X-bodies, whereas cells infected with either of two common strains did contain X-bodies but to different degrees. X-bodies were found more frequently in U-1(SB)- than in U-1(D)-infected tissue. The present study was undertaken to determine whether X-body presence or absence could be correlated with extractable X-protein, and whether varying levels of X-bodies could be correlated with quantitative differences in X-protein in common TMV-infected tissue.

MATERIALS AND METHODS.—*Virus strains and host plants.*—The two common TMV strains, U-1(D) and U-1(SB), and the U-5 strain were the same as previously described (5). The host plants used for cytology (*Nicotiana tabacum* L. 'Turkish') were usually grown in a growth chamber at 70 C under 18 hr of light (1,800 ft-c) supplied by fluorescent tubes and incandescent lamps.

Light microscopy.—A Model GFL Zeiss microscope with a $\times 100$ Apochromatic objective lens was used to detect X-bodies in cells of epidermal strips randomly removed from the abaxial surface of directly inoculated mature leaves. When the strips were stained with calamine orange and "luxol" brilliant green (5), X-bodies became olive brown, nuclei stained orange, and virus crystals were green (Fig. 1). Thus, X-bodies could be readily distinguished from other cellular components. Their prevalence in diseased tissue was assessed as the per cent of the total number of observed cells containing X-bodies.

Purification of X-protein.—X-protein was extracted using a modification of Takahashi & Ishii's procedure (14). At least 50 g of infected leaf tissue was deribbed and frozen. The brittle leaves were powdered and gradually added to 1 volume of 0.1 M sodium phosphate buffer, pH 7.8, containing 10^{-2} M sodium diethylthiocarbamate (DIECA) and 10^{-2} M L-cysteine in a Waring Blendor operating at low speed. The speed was increased to the maximum for 5 to 8 min as more tissue was added. The resulting pulp was filtered through two layers of cheesecloth and adjusted to pH 7 with a few drops of 1 N sodium hydroxide. Clarification by cen-

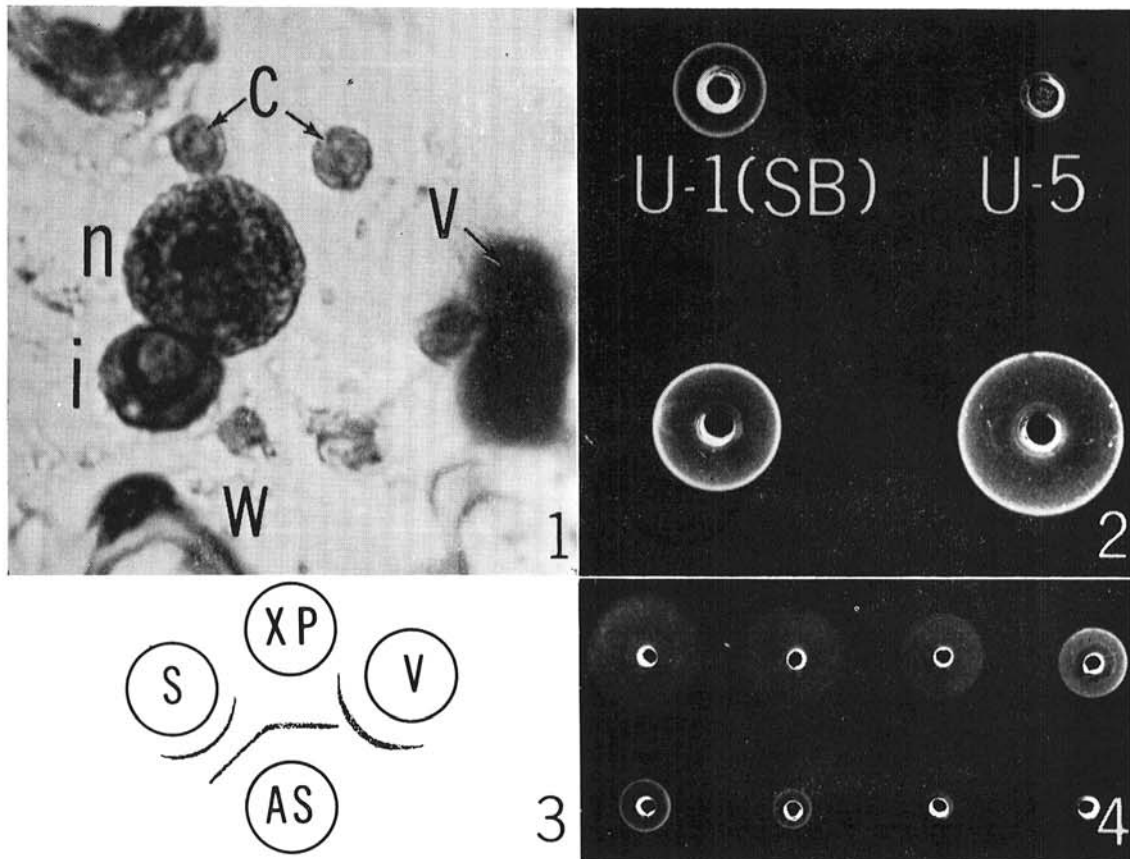


Fig. 1-4. 1) Photomicrograph of an epidermal cell of Turkish tobacco infected with the U-1(SB) strain of tobacco mosaic virus (TMV). Stained with calcomine orange and "luxol" brilliant green; c = chloroplast; i = X-body inclusions; n = nucleus; v = disrupted virus crystal; w = cell wall ($\times 1,080$). 2) Single-diffusion rings in TMV-antiserum-impregnated agar gel. Samples of crude sap from leaf discs taken from plants infected with U-1(SB) or U-5 were placed in the upper wells. Two different concentrations of purified X-protein were placed in the lower wells. Fifteen hr of incubation. 3) Diagram of a double-diffusion serological test in agar gel. Circles represent wells in the gel layer of which were placed crude sap from TMV-infected leaf (S), purified X-protein (XP), purified TMV (V), and TMV antiserum (AS). Shaded lines represent bands of antigen-antibody precipitation. 4) Single-diffusion in antibody impregnated agar gel of twofold dilutions of purified X-protein extracted from U-1(SB)-infected tobacco leaves. Highest concentration is 10 mg/ml as determined by optical density at 280 μ . The precipitin-ring diam decreases with dilution of the X-protein.

trifugation at 10,000 g for 30 min at 5 C was followed by high-speed centrifugation (75,000 g, 90 min, Spinco Model L, No. 30 rotor, 5 C) to remove intact virus particles. The supernatant fluid was agitated with a magnetic stirrer while solid ammonium sulfate (38 g/100 ml) was added. Stirring was continued overnight in a cold room (5 C). The resulting precipitate, separated from the ambient fluid by centrifugation for 15 min at 10,000 g (5 C) was dissolved in about 5 ml 0.1 M sodium phosphate buffer, pH 7.8. This was centrifuged for 90 min at 100,000 to 200,000 g, and the resulting pellets were resuspended in 2 ml 0.1 M phosphate buffer, pH 7. After low-speed clarification (10,000 g, 15 min, 5 C), the protein-containing solution was passed through a 1 \times 25-cm Sephadex column (G-25) eluted with 0.05 M sodium phosphate buffer (pH 7) in order to remove low-molecular-wt material.

The pellet containing virus, obtained from the initial high-speed centrifugation, was resuspended in about 10 ml 0.1 M sodium phosphate buffer, pH 7, was clari-

fied (10,000 g, 15 min), and was assayed by ultraviolet spectrophotometry. Yields of X-protein were expressed as μ g per mg of virus obtained in this fraction.

Assay of X-protein.—The purified X-protein was assayed quantitatively by several methods. Optical density at 280 μ was determined with a Beckman DK-2 spectrophotometer, and the concentration was calculated with an extinction coefficient of 1.25 cm^2/mg .

The X-protein could also be analyzed by serological reaction against TMV antisera. Double-diffusion in 0.75% Ionagar gel (dissolved in saline with 0.1% sodium azide added as a preservative) was used to monitor the extraction procedure and to show relationships between protein and whole virus. The relative concentrations of extracted X-protein were measured with the single-diffusion technique described by Ryan (8), but with some modifications. The same agar gel used in the double-diffusion tests was prepared with the addition of 1% aqueous solution of trypan blue (5 to 8 drops/100 ml) and TMV antiserum (4 ml/100 ml).

The antiserum was added when the heated agar had cooled to 45 to 50 C just prior to pouring it into plastic 15 × 100-mm petri dishes (12 ml/dish). When the gel solidified, 2-mm-diam wells were cut with a syringe cannula, and the agar was removed with gentle vacuum. The wells were filled with antigen solutions through fine capillary tubing (1 mm diam), and the dishes were incubated in a humid atmosphere at room temperature. The antigen diffused into the antibody-impregnated agar, precipitating in radial zones which were measured with a ×7 Bausch and Lomb magnifier containing a mm scale. A dark-field light box illuminated and supported the petri dish for measurements.

Estimation of X-protein in crude sap.—In some experiments, X-protein was measured in extracts of crude sap as follows: leaf discs were cut with a cork borer (16 mm) at various intervals after inoculation. Each disc was triturated in 0.25 ml of distilled water with a ground-glass tissue homogenizer (Ten Broeck type). The resulting extract was analyzed by the single-diffusion technique. After 15 hr of incubation, the precipitin ring was measured, and its diameter was used as a quantitative estimate of X-protein in the sample (Fig. 2). When purified TMV was mixed with sap from healthy plants and similarly placed in wells of single-diffusion gel, no precipitin rings formed after 15 hr of incubation. Thus, the precipitin rings that formed when crude sap from infected leaf discs was introduced (as shown in Fig. 2) indicate X-protein, not protein from whole virus degraded by leaf-disc maceration.

RESULTS.—Characterization of purified X-protein.—When the procedure for extraction of X-protein was followed, the product obtained had the properties described by Takahashi & Ishii (14). These included ultraviolet absorption spectra, lack of infectivity, and ability to polymerize into TMVlike rods. The ultraviolet absorption maxima and minima were at 279 and 250 m μ . The final product of extraction was not infective. When extracts were diluted 1:1,000 in 0.1 M ammonium acetate-acetic acid, pH 5.3, a faint opalescence appeared. When the opalescent solutions were sprayed on collodion-covered carbon-backed electron microscope grids, shadowed with palladium, and viewed with an RCA-EMU-3 electron microscope, rods resembling TMV (though of indefinite lengths) were seen. Only particles less than 20 m μ were observed when the X-protein was diluted with water and similarly prepared for electron microscopy.

The X-protein diffused readily into agar gel, and reacted with antiserum to TMV in the manner depicted in Fig. 3. Two precipitin bands formed between the antibody-containing well and the well containing crude sap from U-1(SB)-infected leaves. The more rapidly diffusing antigen component was serologically indistinguishable from the purified X-protein. When purified virus was placed in a well, the resulting precipitin band corresponded to the slowly diffusing antigen component in the crude sap.

Validity of serological assay.—Several aspects of the single-diffusion technique must be considered before the method can be used for quantitative determinations of protein. It was possible to ascertain that the precipitin

zones had reached maximum diam after 15 hr of incubation by measuring the zones at various times after the wells were filled with different concentrations of extracted protein. Therefore, in all later tests, the ring diam were measured after 15 hr. Whole virus did not cause any precipitin rings in the single-diffusion tests after 40-hr incubation, probably because the larger virions could not readily migrate into the agar gel.

Twofold dilutions of the extracted protein in distilled water were placed in the wells of single-diffusion gel. Decreasing concentrations of X-protein formed progressively smaller precipitin rings, as seen in Fig. 4.

A standard curve was obtained by plotting the log concentration of X-protein (based on ultraviolet absorbance) against the log diam of the precipitin ring (Fig. 5). From this it was concluded that the precipitin ring is proportional to concentration of X-protein placed in the wells, and is a valid quantitative measure of X-protein in the experiments which follow.

X-bodies in relation to yields of purified X-protein.—Our previous studies (5) found X-bodies rarely in U-5 TMV material (1% of infected cells contained them), at an intermediate level in U-1(D)-infected cells (17%), and with greatest frequency in U-1(SB)-infected cells (90%). If the X-bodies are indeed composed of X-protein, then one would expect little or no X-protein in U-5-infected tissue, an intermediate amount in U-1(D) tissue, and the greatest amount in U-1(SB) tissue. As predicted, no detectable X-protein was recovered from U-5-infected plants. With U-1(D)- and U-1(SB)-infected plants, however, X-protein yields were too variable to show any differences (Table 1).

X-protein in crude sap.—Preliminary sampling of

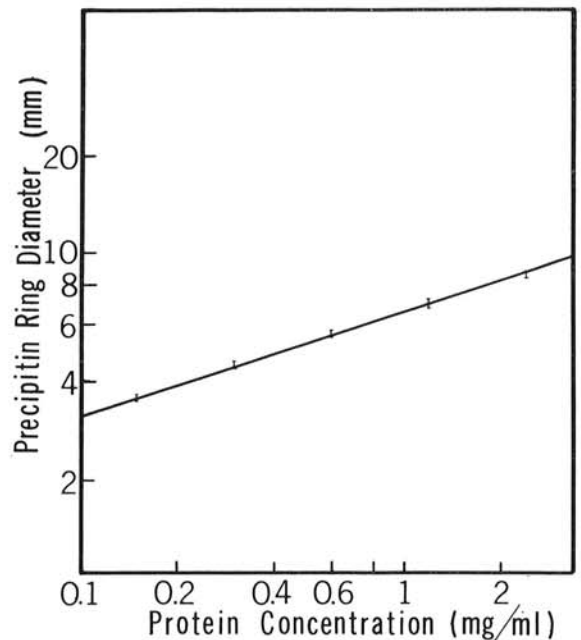


Fig. 5. Relation of the concentration of purified X-protein to diam of precipitin rings formed in single-diffusion serological tests.

TABLE 1. Relative yields of purified X-protein obtained from tobacco plants infected with different strains of tobacco mosaic virus

Experiment	Virus strain		
	U-5	U-1(D)	U-1(SB)
1	0		5.9 ^a
2	0		1.3
3		5.3	11.0
4		7.2	0
5		3.6	3.0

^a µg of X-protein (based on precipitin-ring diam) per mg intact TMV in respective fractions of plant extracts.

trituated leaf discs produced diffusion rings (Fig. 2), indicating that a U-5-infected plant contained little or no X-protein, whereas a U-1(SB)-infected plant contained a measurable quantity. This is consistent with the inability to obtain X-protein from U-5-infected plants by massive extraction and fractionation (Table 1).

The following experiments with the U-1 strains were designed to determine whether X-bodies could be correlated quantitatively with X-protein by the leaf-disc method.

Fully expanded leaves of Turkish tobacco were inoculated with either U-1(D) or U-1(SB). Epidermal strips of the leaves were removed 5, 8, and 10 days after inoculation and stained for light microscopy. Approximately 2,300 cells were examined at each sampling time, and the number of X-bodies recorded in relation to the total number of cells observed. At the same time, two leaf discs were removed from the leaf from which the epidermal strips were taken, and the discs were assayed for X-protein by single diffusion in agar (Fig. 6). X-protein was detected at 6 days, and steadily increased up to 8 days, by which time leaf discs infected with U-1(SB) had significantly more X-protein than leaf discs infected with U-1(D). Also at 8 days, the frequency of X-bodies was very high (83%) in the U-1(SB)-infected plant and low (5%) in the U-1(D)-infected plant. At subsequent sampling times, however, the X-protein yields of the two became irregular, and no definite trends could be shown even though the relative X-body frequency remained consistently higher in the plant infected with U-1(SB).

To overcome possible variations in X-protein levels within different parts of a given leaf, an experiment was conducted involving a larger number of leaf discs. Two sets of plants were inoculated with each U-1 strain. At 3, 7, 10, 14, and 21 days after inoculation, 10 leaf discs were removed randomly from each of the sets and assayed individually for X-protein. On the 14th day after inoculation, epidermal strips were removed from each of the 20 discs prior to their being ground for serological assay. The strips were stained, and X-bodies were counted in approximately 180 cells/leaf disc. The numbers of X-bodies were then compared with the X-protein yield from each disc. The amount of X-protein increased rapidly with each strain, beginning at 4 days postinoculation (Fig. 7). Although the amount of X-protein detected at 7, 10, and 14 days

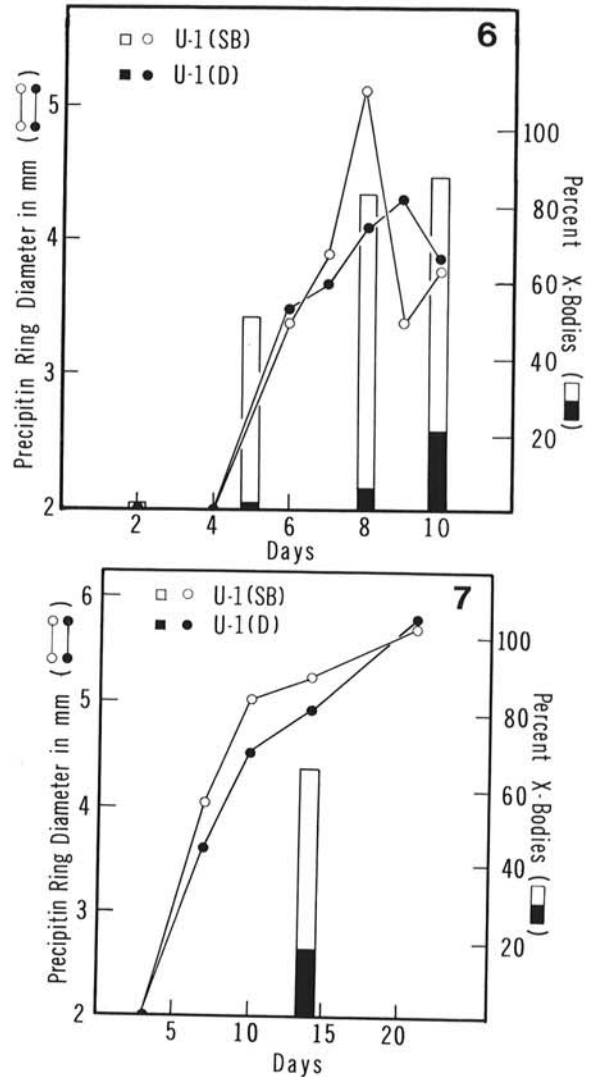


Fig. 6-7. 6) Comparative levels of X-protein and X-body inclusions in tobacco leaves at various intervals following their inoculation with U-1 (D) and U-1(SB) strains of TMV. X-protein concentration is proportional to diam of precipitin rings formed when crude sap was placed in antiserum-impregnated agar. The level of X-bodies is expressed as the per cent of all cells examined that contained them. 7) Comparative levels of X-protein and X-body inclusions in tobacco leaves at various intervals following their inoculation with U-1(D) and U-1(SB) strains of TMV. X-protein concentration is proportional to diam of precipitin rings formed when crude sap was placed in antiserum-impregnated agar. The level of X-bodies is expressed as the per cent of all cells examined which contained them. Epidermal strips used for X-body counts were removed from the same leaf discs used for evaluation of X-protein content.

was slightly higher for U-1(SB) than for U-1(D), the difference was not statistically significant. Again, the average frequency of X-bodies was much higher in the U-1(SB) samples. No correlation existed between X-body frequency and X-protein level when comparisons were made on a disc-by-disc basis at 14 days.

DISCUSSION.—If the tubules within X-bodies are polymerized X-protein, as proposed by Esau (1), then X-protein quantities and X-body level in diseased tissue should vary in the same way. Consistent with this hypothesis is the inability to obtain X-protein from U-5-infected tissue and the rarity of X-bodies in such tissue. In opposition to the hypothesis, however, are the present results with the two common TMV strains. The tissue infected with U-1(D) and U-1(SB) yielded some X-protein, but there was no consistent relation to the gross differences found in X-body frequencies. This is strong evidence against X-bodies having X-protein as their chief component.

Further evidence against co-identity with X-protein has been found in other studies; the apparent inability of the X-tubules to become tagged with ferritin antibodies (Shalla, *unpublished results*). When X-bodies in U-1(SB)-infected cells were exposed to ferritin conjugated with TMV antibodies, virus particles in the immediate vicinity were heavily tagged, whereas the X-tubules never became tagged. Thus, it seems certain that the X-tubules are serologically unrelated to X-protein unless the X-protein arrangement in the tubule prevents interaction with homologous antibodies.

A logical extension of these studies would be to determine the comparative levels of X-bodies and X-protein induced by a much wider range of TMV strains. Still another approach would be to extract X-bodies from infected cells, rinse them, and analyze their contents serologically. This should be possible, for Sheffield removed them intact by micrurgical methods (12). Studies of this nature would provide more direct evidence on the possible relation of X-protein to X-bodies. At present, however, most evidence disavows such a relationship.

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