BARD

FINAL REPORT

PROJECT NO. I-882-85

Further Characterization of IVR, Isolation of its M-RNA, and its Relation to Localization and Necrotization

G. Loebenstein, W. O. Dawson, A. Gera

1989

32133

000000127233

Date: Nov. 1989

BARD
P.O.BOX 6
Bet Dagan, ISRAEL

BARD project NO. I-882-85

Title

Further characterization of IVR, isolation of its m-RNA and it's relation to localization and necrotization

Investigators' Names	Investigators' Institutions
G. Loebenstein	A.R.O The Volcani Center
W.O Dawson	University of California
A. Gera	A.R.O The Volcani Center

Project's staring date: August 10, 1986

Type of Report: 1st annual _____ 2nd Annual _____Final X

G. Loebenstein
Signature
Principal Ivestigator

_____
Signature
Institution's Authorizing Official

Participating Investigators

Dr. Sara Spiegel	-	A.R.O
R. Aly	-	-"-
Dr. R. Salomon	-	-"-
Dr. A. Rosner	-	-"-
A. Lichter	-	-"-
Dr. P. Bubrick	-	Riverside
Dr. J. Culver	-	-"-
Dr. G. Grantham	-	-"-

Table of Contents

c. Abstract

One of the most effective natural resistances of plants to viruses is the local lesion response, where virus remains restricted to several hundred cells around the primary point of entry. We have found that protoplasts from tobacco, carrying the NN gene responsible for resistance (local lesions) to tobacco mosaic virus (TMV), release after their inoculation with TMV a compound - inhibitor of virus replication (IVR) - that inhibits several plant viruses.

During the present grant we succeeded to obtain IVR from the intercellular spaces of TMV-infected tobacco NN plants and from induced resistant tissue. The amount of IVR recovered from 10^8 protoplasts or 100 g of leaf tissue was found to be equivalent to approximatley 1ug of protein; and biological activity could be detected by applying 10-20ng of IVR.

Purified IVR (a protein) gave a single specific band in polyacrylamide gel electrophoresis (PAGE) with a molecular weight around 23K. We have prepared poly- and monoclonal antisera to IVR. We have prepared a cDNA library from resistant plants and selected bacterial clones that produce IVR. In preliminary experiments, this cloned IVR has biological activity.

In respect to induction of necrosis it was demonstrated in Riverside that in NN plants the viral coat protein is not involved in induction of necrosis, while in NN plants the coat protein gene of TMV is an elicitor of the hypersensitive response. TMV mutants that induce necrosis in Xanthi (nn) and N. sylvestris (N') were studied in respect to induction of IVR. One mutant that causes necrotic lesions in N. sylvestris was also found to induce IVR.

d. Objectives of original proposal

The objectives of the original research proposal were:

A. Production of IVR and specific antisera.

1. Improving production of protoplasts and IVR.
2. Additional characterization of IVR - further purification and amino acid sequencing.
3. Prepration of improved specific antisera to IVR - including monoclonals.

B. Isolation and translation of m-RNA to IVR.

C. Construction of c-DNA clones complementary to IVR-m-RNA, and synthetic deoxyoligonucleotide fragments to IVR - optional as project develops.

D. To examine regulation of IVR gene and N gene by:

1. Identifying virus "elicitors" by characterizing non-necrosis inducing mutants and integration of viral cDNA segments into plants containing the N gene.
2. Examining the correlation of IVR production to necrotization.

e. Body of Report

A. Production of IVR and specific antisera (in Israel)

1. Improving production of protoplasts.

Various fertilizing regimes of the source plants, light and temperature conditions in greenhouse, age of leaves, enzyme concentrations and time of incubation with the enzymes were evaluated.

Holding the plants for 7-10 days before sampling them for protoplast production at $22\pm2^{o}C$ after the third nutrient feed, and shortening the time of incubation of peeled leaf pieces in the mixed enzyme solution, gave a regular and stable yield of protoplasts. Using this procedure, production of protoplasts was markedly improved from about $5x10^{6}$ per 1 20 cm petri dish to about $10x10^{6}$, yielding about 10 units of IVR (see below).

2. Recovery of IVR directly from plants (Plant IVR)

Results are summarized in the attached publication: Phytopathology 79:258-262, 1989.

5a

Resistance

Recovery of an Inhibitor of Virus Replication from the Intercellular Fluid of Hypersensitive Tobacco Infected with Tobacco Mosaic Virus and from Uninfected Induced-Resistant Tissue

S. Spiegel, A. Gera, R. Salomon, P. Ahl, S. Harlap, and G. Loebenstein

First, second, third, fifth, and sixth authors: Virus Laboratory, Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel; fourth author: Agricultural Division, CIBA-GEIGY, Basel, Switzerland.
Contribution 2229-E from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel.
This work was supported in part by the United States-Israel Binational Agricultural Research and Development Fund (BARD).
We thank Sharona Hagabi and Judith Tam for technical assistance and J. Cohen for preparing the photographs.
Accepted for publication 19 July 1988 (submitted for electronic processing).

ABSTRACT

Spiegel, S., Gera, A., Salomon, R., Ahl, P., Harlap, S., and Loebenstein, G. 1989. Recovery of an inhibitor of virus replication from the intercellular fluid of hypersensitive tobacco infected with tobacco mosaic virus and from uninfected induced-resistant tissue. Phytopathology 79:258-262.

A compound was obtained from the intercellular fluid of tobacco cultivar Samsun NN infected with tobacco mosaic virus (TMV) which inhibited virus replication when applied to TMV-inoculated protoplasts or leaf disks. Based on the criteria of serology, polyacrylamide gel electrophoresis, and molecular weight estimations, the compound from the intercellular fluid was judged to be identical to the inhibitor of virus replication (IVR) obtained previously from TMV-infected Samsun NN protoplasts. The recovery of IVR is facilitated when leaves are used as a source. A similar compound was obtained from systemic induced-resistant tissue of Samsun NN and cultivar Xanthi-nc. The belief that induced resistance results from an activation of the localizing mechanism is supported by this result.

Additional keywords: induced resistance, inhibitor of virus replication, serology.

Previously, we reported that a substance(s) that is an inhibitor of virus replication (IVR) is released into the medium from tobacco mosaic virus (TMV)-infected protoplasts of a tobacco cultivar in which the infection in the intact plant is localized. IVR inhibited virus replication in protoplasts from both local lesion-responding resistant (cultivar Samsun NN) and systemic-responding susceptible (cultivar Samsun) tobacco plants. IVR was not released from TMV-infected Samsun protoplasts (9). It was suggested that IVR is associated with localization (11). IVR inhibited virus replication in leaf tissue disks and in intact leaves when applied to cut stems or by spray. IVR was partially purified using $ZnAc_2$ precipitation (crude protoplast IVR), and two biologically active principles were obtained with molecular weights of approximately 26,000 (fractionated protoplast IVR-1) and 57,000 (fractionated protoplast IVR-2), as determined by gel filtration. IVR activity is sensitive to the proteolytic enzymes trypsin and chymotrypsin, but not to RNAse, suggesting that IVR is proteinaceous (3). Antisera to fractionated protoplast IVR-1 and IVR-2 are highly cross-reactive (4) which suggests that IVR-2 is a dimer of IVR-1 (9). Polyacrylamide gel electrophoresis (PAGE) of crude protoplast IVR under denaturing conditions identified a 23K protein, which gave a reaction with IVR-1 antiserum. Further purification of IVR yielded a biological active fraction, which contained a single 23K protein (Gera et al, *unpublished*).

Preliminary attempts to recover IVR in reasonable quantities from homogenated leaf tissue were not successful. An objective of the present study was to obtain IVR from the intercellular fluid of TMV-infected leaves of a hypersensitive tobacco. This approach was chosen based on previous findings that IVR is released from infected protoplasts into the incubation medium (9), that pathogenesis-related (PR) proteins accumulate in the intercellular fluid (12), and that the intercellular fluid of TMV-infected Samsun NN leaves has virus-inhibitory activity (14).

The detection of IVR in "induced-resistant" tissue has not been reported previously. Induced resistance is a phenomenon whereby uninoculated parts of hypersensitive hosts become partially and nonspecifically resistant to diverse pathogens (6–8). Virus lesions

developing after challenge inoculation of the resistant tissue are consistently smaller and usually fewer in number than those formed on previously uninoculated control plants. In Samsun NN plants, induced resistance was found to be closely correlated to reduced virus concentration of the challenge virus (13), which indicates that virus replication is suppressed in the resistant tissue. Induced resistance seems to require an active cellular process, depending on the transcription mechanism from DNA to RNA, because its development is markedly inhibited in the presence of actinomycin D (10). It has been suggested that after initial virus inoculation of hypersensitive plants a substance(s) is produced that induces resistance in uninoculated tissue (8).

Here we report that IVR can be obtained from the intercellular fluid of TMV-infected Samsun NN, compare its identity with IVR obtained from protoplast incubation medium, and show its presence in induced-resistant tissue of tobacco cultivars Samsun NN and Xanthi-nc.

MATERIALS AND METHODS

Plants. *Nicotiana tabacum* L. 'Samsun NN,' 'Samsun,' and 'Xanthi-nc' were grown in a greenhouse for 5 to 6 wk after transplanting. A complete nutrient solution was supplied three times at weekly intervals, starting 1 wk after transplanting. One or 2 days before use, plants were transferred to a greenhouse or controlled-temperature chamber maintained at 21–22 C. Each plant was trimmed to four to seven expanded leaves.

Inoculation with TMV. Leaves of Samsun NN used to isolate IVR were inoculated with a solution containing purified TMV (1.5 μg/ml) in one of two ways: inoculation of the entire leaf, giving a lesion density of three or four lesions per square centimeter (treatment a); or inoculation in 10–15-mm strips (25–30 mm apart) on both halves of each leaf parallel to the midvein (treatment b). For systemic induced resistance, Samsun NN plants were trimmed to five leaves. The lower three expanded leaves were inoculated and the upper two leaves (resistant) were used for extraction (treatment c). In addition, the basal halves of two lower leaves of Xanthi-nc plants were inoculated with TMV. The distal uninoculated part of these leaves and the upper five uninoculated leaves were used for extraction (treatment d). Plants inoculated with water were used as controls for all experiments.

Extraction of inhibitory intercellular fluid. Intercellular fluid was extracted as described by Parent and Asselin (12) with the following modifications. Whole leaves (for treatment a) or the tissue between the TMV strips and the TMV strips themselves (for treatment b) were collected 6–7 days after inoculation. Upper leaves were collected 7 and 14 days after inoculating the lower leaves (treatment c). Leaves were cut into 4–6 pieces after removal of the midvein. Leaf pieces were infiltrated in vacuo for two or three periods of 30–50 sec each with a large excess of cold (4 C) 0.05 M phosphate buffer, pH 7.0, containing 0.1% 2-mercaptoethanol. Pieces were gently blotted dry, rolled, and placed in centrifuge tubes containing an inner matching part with a pierced bottom. The intercellular fluid was collected by centrifugation at 2,000 *g* for 10 min. The collected fluid was treated with $ZnAc_2$, as described previously for IVR (9). The term crude tissue IVR will refer to $ZnAc_2$-treated intercellular fluid prepared this way. A preparation obtained from 1 g of leaf tissue will be termed 1 "unit." A unit was found to be roughly equivalent to the amount of crude protoplast IVR obtained from 10^6 protoplasts and is equivalent to approximately 10 ng of protein. This amount was estimated from staining reactions following PAGE and by amino acid analysis after high-pressure liquid chromatography (HPLC) (unpublished results obtained in collaboration with Y. Burstein and V. Buchner, Weizmann Institute of Science, Rehovot, Israel).

An inhibition assay of tissue IVR was done on protoplasts and on leaf disks infected with TMV, either by infectivity assays or by enzyme-linked immunosorbent assay (ELISA), as described previously (2,3,9).

Preparation of protoplasts and crude protoplast IVR. Procedures for maintenance of protoplasts and preparation of IVR from incubation medium were as previously described (9).

Serology. Antisera against fractionated protoplast IVR-1, fractionated protoplast IVR-2, and the 23K band obtained from PAGE ("PAGE" antiserum) were prepared in rabbits (4; Gera et al, *unpublished*). Antisera (1 ml) were absorbed with a lyophilized preparation obtained from the incubation medium of 75×10^6 mock-inoculated protoplasts.

Agar-gel-diffusion tests were done in 55-mm petri dishes containing a 4-mm layer (9 ml) of 0.75% agar (Bacto agar), 0.001 M ethylenediaminetetraacetic acid, 0.85% NaCl, and 0.2% sodium azide at pH 7.8. Agar plates were incubated in a moist chamber at 22 C for 15 hr. Those experiments with intercellular fluids from Xanthi-nc were carried out in agar on glass slides. Intercellular fluids were concentrated 25 times by lyophilization, and 20 μl of each were put into each well. Reference solutions consisted of 1 unit of crude protoplast IVR dissolved in 20 μl of water. After diffusion overnight, slides were washed for 24 hr in 0.8% NaCl, rinsed with water, and dried. They then were stained with a 2% Coomassie blue solution, in 25% ethanol and 10% acetic acid. Plant preparations contained 0.04% sodium dodecyl sulphate (SDS) when PAGE antiserum was used.

Polyacrylamide gel electrophoresis. Ten units of crude protoplast IVR or crude tissue IVR was concentrated to 8 μl, mixed with 4 μl of 0.06 M tris-HCl buffer, pH 6.8, containing 3% SDS, 5% 2-mercaptoethanol, 10% glycerol, and a few grains of bromophenol blue (disruption buffer), and boiled for 2 min. Twelve microliters of the SDS-disrupted IVR then was applied to 5–15% gradient polyacrylamide minigels (10×7.5 cm, 0.45 mm thick) containing 0.1% SDS and separated by electrophoresis using a Bio-Rad Mini Protean II apparatus (Bio-Rad Laboratories, Richmond, CA).

The following marker proteins (Bio-Rad Laboratories) were used: lysozyme (14,400 daltons [Da]), soybean trypsin inhibitor (21,500 Da), carbonic anhydrase (31,000 Da), ovalbumin (45,000 Da), bovine serum albumin (66,200 Da), and phosphorylase B (92,500 Da). The gels were stained with Coomassie blue (1). Extract from the medium of sham-inoculated protoplasts and plant extract from sham-inoculated tissue were prepared similarly.

RESULTS

Extraction of crude tissue IVR from TMV-infected Samsun NN leaves. Intercellular fluid obtained 6–7 days after inoculation of Samsun NN leaves was assayed on TMV-infected Samsun NN protoplasts. Effects of increasing amounts of crude tissue IVR on TMV titers in protoplasts, determined by infectivity assay and ELISA, are summarized in Table 1. Crude tissue IVR from the intercellular fluid of TMV-inoculated Samsun NN leaves consistently inhibited virus replication in protoplasts, and the inhibition was dose responsive. Comparable preparations obtained from the intercellular fluid of Samsun leaves 3 and 7 days after inoculation with TMV were not inhibitory when assayed in the protoplast system.

When crude tissue IVR (3 units) was applied to Samsun leaf disks inoculated with TMV (3), inhibition rates of 60 and 61% were obtained when infectivity was assayed 3 and 4 days after inoculation, respectively.

Partial purification and molecular weight estimation of tissue IVR from TMV-infected Samsun NN leaves. Lyophilized crude tissue IVR obtained from 100 g of TMV-inoculated Samsun NN leaves was dissolved in 1.0 ml of 0.1 M phosphate buffer and passed through a 33×2.3 cm Sephadex G-75 column. One-milliliter fractions were eluted with the same buffer and collected. A preparation from the same amount of sham-inoculated leaves was passed through the same column and collected in a similar manner. The fractions were tested in the protoplast virus-inhibition assay. Activity was detected in two sets of fractions (72 and 73% inhibition, respectively, average of three experiments) expected to contain proteins with molecular weights of 26,000 (fractionated tissue IVR-1) and 57,000 (fractionated tissue IVR-2). These properties are similar to those previously reported for fractionated IVR-1 and fractionated IVR-2 from protoplasts (9).

Serological relationship between tissue IVR and protoplast IVR. The two partially purified active fractions (fractionated tissue IVR-1 and IVR-2), obtained from the Sephadex G-75 column, were compared with fractionated protoplast IVR-1 and IVR-2 in agar-gel-diffusion tests, using antisera against fractionated protoplast IVR-1 and fractionated protoplast IVR-2. Five units of each fraction, dissolved in 0.2 ml of 0.1 M phosphate buffer, pH 7, were applied to each well. Clear precipitation lines were observed between fractionated tissue IVR-1 and the antisera to fractionated protoplast IVR-1 (Fig. 1a) and fractionated protoplast IVR-2 (Fig. 1b). These lines fused completely without spur formation, with the precipitation lines obtained between fractionated protoplast IVR-1 and fractionated protoplast IVR-2 and their respective antisera. Similarly, clear precipitation lines were obtained between fractionated tissue IVR-2 and the antisera against fractionated protoplast IVR-1 (Fig. 1a) and fractionated protoplast IVR-2 (Fig. 1b), indicating that the fractions are serologically similar. Clear precipitation lines also were obtained between the PAGE antiserum and crude tissue IVR (15 units) from the intercellular fluid, fusing without spurs with the precipitation line obtained between crude protoplast IVR (15 units) and this antiserum (Fig. 1c).

PAGE of crude tissue IVR from TMV-infected Samsun NN leaves. A specific band corresponding to a 23K protein was observed regularly in PAGE of crude tissue IVR preparations (Fig. 2, lane 4) and was at the same position as the specific band obtained from crude protoplast IVR (Fig. 2, lane 2).

Extraction of crude tissue IVR from induced-resistant tissue. Preparations purified from the intercellular fluid of induced-resistant tissue were tested for inhibitory activity on TMV-infected protoplasts. Extracts were made from TMV-strips, in between strips, and from systemic induced-resistant leaves 7 days after the inducing inoculation, and they were compared with the respective controls. Results, which are averages from two or three experiments, are summarized in Table 2. Extracts from induced-resistant tissue gave inhibition rates between 46 and 63%; extracts from tissue with local induced resistance between TMV strips had a higher inhibition rate than those from upper leaves with systemic induced resistance. Inhibition rates of 44% were observed when extracts (2 units) from tissue between strips were tested on TMV-infected Samsun leaf disks (averages from two experiments).

Release of IVR from induced-resistant tissue protoplasts. Protoplasts were obtained from the tissue between TMV strips ("resistant protoplasts") 7 days after the inducing inoculation and incubated for 72 hr. The incubation medium then was collected and evaluated in the protoplast virus inhibition assay, as described previously (9). For controls, protoplasts were obtained from tissue between sham-inoculated strips and treated similarly. In addition, resistant protoplasts and protoplasts from tissue between sham-inoculated strips were inoculated with TMV and evaluated for inhibitory activity. Results, which are averages from three or four experiments, are summarized in Table 3.

Protoplasts from the resistant tissue between TMV strips released an inhibitory substance into the incubation medium. Inoculation of these "resistant" protoplasts increased amount of inhibitor in the medium, when compared with that from uninoculated resistant protoplasts and with that from inoculated protoplasts obtained from sham-inoculated control strips.

Serology of tissue IVR from induced-resistant tissue. Crude

TABLE 1. Effect of crude tissue inhibitor of virus replication (IVR) from tobacco cultivar Samsun NN leaves inoculated with tobacco mosaic virus (TMV) on virus replication in Samsun NN protoplasts [a]

Amount of inhibitor added (units)	Infectivity[b] in protoplasts incubated in:		Percent inhibition	Virus yield[d] in protoplasts incubated in:		Percent reduction of virus yield
	Tissue IVR	Sham[c]		Tissue IVR	Sham	
0.5	32.6 ± 3.16	52.6 ± 2.36	38	0.68 ± 0.21	1.2 ± 0.29	43
1.0	28.5 ± 7.36	55.4 ± 12.37	49	0.45 ± 0.14	1.25 ± 0.29	64
3.0	17.3 ± 1.67	54.9 ± 12.81	68	0.33 ± 0.05	1.25 ± 0.35	74
5.0	15.9 ± 0.55	56.4 ± 7.46	72	0.28 ± 0.06	1.8 ± 0.17	84
10.0	12.7 ± 0.70	59.3 ± 8.49	79	0.21 ± 0.04	1.77 ± 0.25	88
Control[e]		55.1 ± 1.60			1.72 ± 0.23	

[a] As determined by local lesion assay and enzyme-linked immunosorbent assay (ELISA). Averages from two experiments.
[b] Average number of local lesions and standard error per 10^6 protoplasts on one half-leaf of *Nicotiana glutinosa* L. calibrated to a standard TMV solution (1.5 μg/ml), which yielded about 70 lesions per half leaf.
[c] Protoplasts incubated in medium with a $ZnAc_2$ preparation from sham-inoculated plants.
[d] Average virus yield (μg/ 10^6 protoplasts) and standard error, as determined by ELISA.
[e] TMV-inoculated protoplasts in incubation medium with no additions.

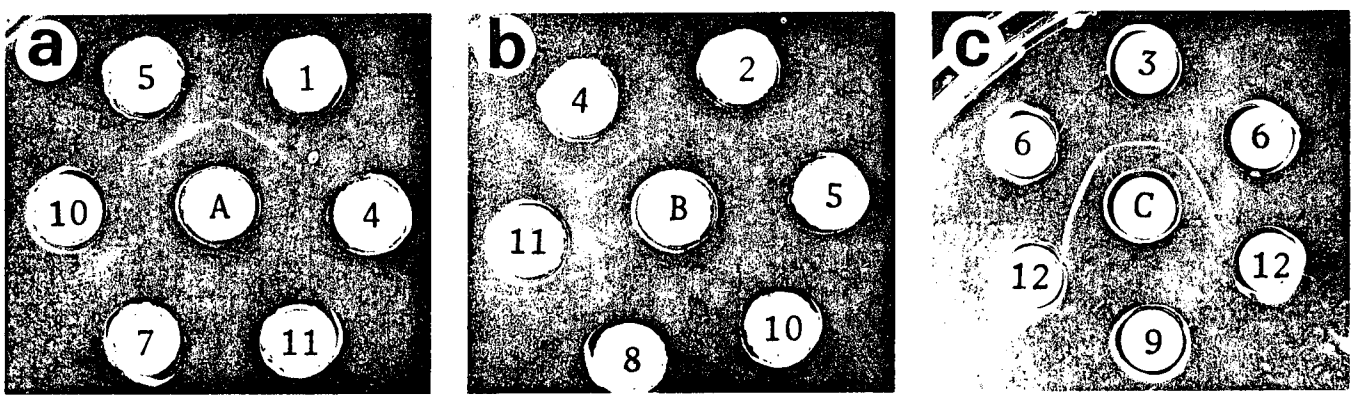


Fig. 1. Agar-gel double-diffusion serology of tissue inhibitor of virus replication (IVR) from the intercellular fluid of tobacco cultivar Samsun NN leaves with antiserum against: **A**, fractionated protoplast IVR-1, **B**, fractionated protoplast IVR-2, and **C**, the 23K protein band (polyacrylamide gel electrophoresis antiserum), compared with fractionated IVR-1, fractionated IVR-2, and crude IVR from protoplasts. 1, fractionated protoplast IVR-1; 2, fractionated protoplast IVR-2; 3, crude protoplast IVR; 4–6, fractionated tissue IVR-1, IVR-2, and crude, respectively, from leaves; 7–12, control preparations for 1, 2, 3, 4, 5, and 6, respectively.

preparations (20 units) obtained from the intercellular fluid from two systemic induced-resistant upper leaves 7 days after inoculation of three lower leaves gave a positive reaction with PAGE antiserum in agar-gel-diffusion tests (Fig. 3). Clear precipitation lines were obtained, which fused without spurs with the precipitation line obtained between crude protoplast IVR and the antiserum. Similar results (not shown) were obtained when the intercellular fluid was recovered 14 days after the inducing inoculation and when tested against fractionated protoplast IVR-1 antiserum.

The intercellular fluid from the resistant tissue of Xanthi-nc also was tested for the presence of IVR in agar-gel-diffusion tests. Clear precipitation lines with fractionated protoplast IVR-1 antiserum were obtained (Fig. 4). IVR was detected in the intercellular fluid from the distal parts of inoculated leaves 7 days after inoculation. It was not detected in the intercellular fluid from upper leaves before 14 days after the inducing inoculation.

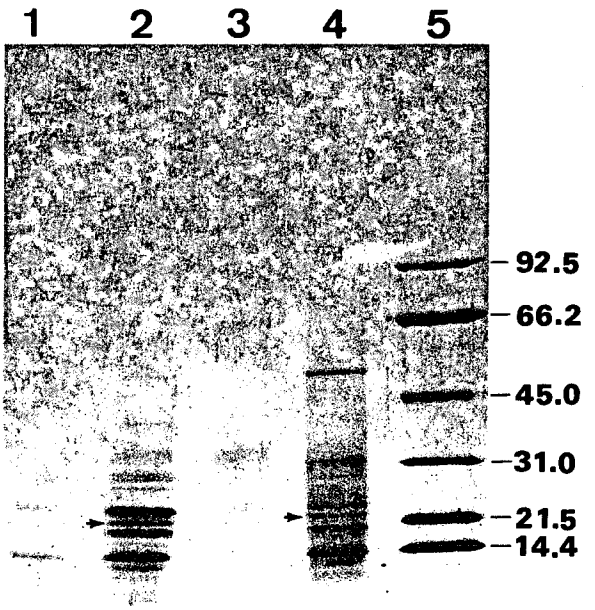


Fig. 2. Polyacrylamide gel electrophoresis of inhibitor of virus replication (IVR) after disruption in sodium dodecyl sulphate. Lane 2, crude IVR after $ZnAc_2$ (10 units from protoplasts); lane 1, control (from protoplasts); lane 4, crude tissue IVR after $ZnAc_2$ (10 units); lane 3, control from plants; and lane 5, molecular weight standards (from top to bottom) phosphorylase B (92.5 kilodaltons [kDa]), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). IVR band indicated by arrow.

TABLE 2. Effect of crude tissue inhibitor of virus replication (IVR)[a] from induced-resistant leaves of tobacco cultivar Samsun NN on tobacco mosaic virus (TMV) replication in Samsun NN protoplasts

Mode of induced resistance	Infectivity[b]	Percent inhibition
Strips, TMV[c]	12.2 ± 0.48	61
Strips, sham[c]	31.4 ± 2.81	
Between strips[c]	12.3 ± 1.4	63
Between strips, sham[c]	33.5 ± 3.62	
Control[d]	30.4 ± 2.19	
Upper leaves	20.5 ± 4.6	46
Control upper leaves[c]	38.0 ± 4.8	

[a]Two "units" of respective extract was added to 10^6 protoplasts.
[b]Average number of local lesions and standard error per 10^6 protoplasts on one half-leaf of *Nicotiana glutinosa* L. calibrated to a standard TMV solution (1.5 μg/ml), which yielded about 70 lesions per half leaf.
[c]Averages from three experiments; intercellular fluid sampled 7 days after the inducing inoculation.
[d]TMV-inoculated protoplasts in incubation medium with no additions.
[e]Averages from two experiments; intercellular fluid sampled 7 days after the inducing inoculation.

TABLE 3. Effect of inhibitor of virus replication (IVR) from protoplasts of induced-resistant tissue on tobacco mosaic virus (TMV) replication in tobacco cultivar Samsun NN protoplasts

Incubation medium[a] from protoplasts from:	Infectivity from test protoplasts[b]	Percent inhibition[c]
1. Between TMV strips, uninoculated	22.9 ± 4.45	44
2. Between TMV strips, inoculated	12.0 ± 4.90	70
3. Between control strips, uninoculated	40.6 ± 4.61	
4. Between control strips, inoculated	17.3 ± 6.66	57
Control protoplasts[d]	37.6 ± 2.19	

[a]Incubation medium (10 ml) from 10^6 protoplasts (1 unit). Protoplasts obtained 7 days after the inducing inoculation.
[b]Average number of local lesions and standard error from three to four experiments. Inoculum prepared from 10^6 protoplasts, suspended in respective incubation medium 4–5 hr after inoculation with TMV, and applied to one half-leaf of *Nicotiana glutinosa* L. Lesion counts were calibrated to a standard TMV solution (1.5 μg/ml), which yielded about 70 lesions per half leaf.
[c]Compared with incubation medium from protoplasts between control uninoculated strips (treatment No. 3).
[d]TMV-inoculated protoplasts in incubation medium with no additions.

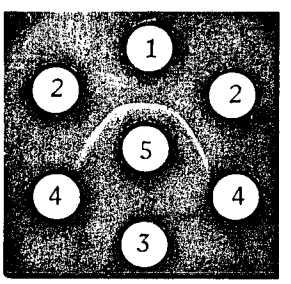


Fig. 3. Agar-gel double-diffusion serology of crude tissue inhibitor of virus replication (IVR) from the intercellular fluid of induced-resistant tissue from the upper two uninoculated leaves of tobacco cultivar Samsun NN plants, 7 days after inoculating three lower leaves with polyacrylamide gel electrophoresis (PAGE) antiserum. 1, crude protoplast IVR; 2, crude tissue IVR from induced-resistant leaves; 3, mock protoplast IVR; 4, intercellular fluid from healthy plant; 5, PAGE antiserum.

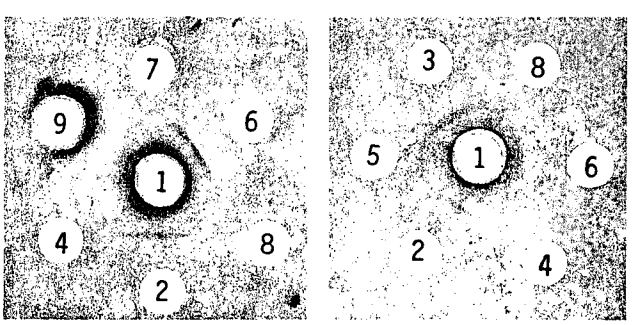


Fig. 4. Agar-gel double-diffusion serology of plant inhibitor of virus replication (IVR) from the intercellular fluid of induced-resistant tissue of tobacco cultivar Xanthi-nc leaves with antiserum against fractionated protoplast IVR-1. 1, fractionated protoplast IVR-1 antiserum; 2 and 3, intercellular fluid from distal part of leaf 7 and 14 days, respectively, after inducing inoculation; 4 and 5, intercellular fluid from upper leaves 7 and 14 days, respectively, after inducing inoculation; 6, crude protoplast IVR; 7, mock protoplast IVR; 8, intercellular fluid from healthy plant; 9, extraction buffer.

DISCUSSION

IVR-like compounds were obtained from the intercellular fluid of Samsun NN leaves infected with TMV and from induced-resistant tissue. Based on serology (using antisera to protoplast IVR), PAGE, and molecular weight estimations by molecular sieving, the compounds from leaf intercellular fluids are indistinguishable from IVR-1 and IVR-2 obtained from TMV-infected Samsun NN protoplasts. The serological data indicate that tissue IVR-1 and IVR-2 obtained from the intercellular fluid have identical serological determinants. This strengthens our previous suggestion (3) that protoplast IVR-2 is a dimer of protoplast IVR-1. The observation that IVR is released from cells into the intercellular fluid of leaf tissue parallels our previous finding that IVR is released from protoplasts into the incubation medium (9). The yield of tissue IVR per cell obtained from the intercellular fluid of leaf tissue was in the same range as that obtained from protoplasts. From 100 g of leaf tissue, which is estimated to contain $1-5\times 10^8$ mesophyll cells, about 100 units of tissue IVR was obtained (equivalent to approximately 1 μg of protein). This is similar to the amount of IVR obtained from 10^8 protoplasts. The procedure for obtaining intercellular fluid is much simpler than the one for preparing protoplasts, which greatly facilitates the recovery of IVR.

Some discrepancies between the molecular weight estimations based on PAGE under denaturing conditions and on gel filtration were noticed. Using SDS-PAGE, a molecular weight of about 23,000 was estimated for IVR-1, from both the intercellular fluid and protoplast media, whereas by gel filtration a value of 26,000 was obtained. Similar observations have been made with other proteins (5). Based on molecular weight estimates, IVR differs from the biological active fractions obtained by Wieringa-Brants and Dekker from the intercellular fluid of tobacco plants with systemic acquired resistance (15).

IVR was detected by serology in induced-resistant tissue as soon as 7 days after the inducing inoculation, in either the distal half of Xanthi-nc leaves or the upper two leaves of Samsun NN plants. In the upper leaves of Xanthi-nc plants, IVR was detected 14 days, but not as soon as 7 days, after the inducing inoculation. In these plants, the basal parts of only two leaves were inoculated and intercellular fluid was extracted from five upper uninoculated leaves. The relatively smaller amount of tissue inoculated may be related to the longer time interval required for the development of IVR in the upper leaves.

The intercellular fluid obtained from the induced-resistant tissue between TMV strips had a higher inhibitory activity than that recovered from upper resistant leaves (Table 2). This parallels observations that the intensity of local induced resistance in the leaf tissue between TMV strips is significantly higher than systemic induced resistance in upper leaves (13).

The presence of IVR in induced-resistant tissue may explain the induced-resistance phenomenon, strengthening the suggestion that in induced-resistant tissue the localizing mechanism is activated before the challenge inoculation (8). The presence of IVR may affect virus replication immediately after challenge inoculation. In noninduced tissue of a hypersensitive plant, because the host genome has first to be activated, IVR production becomes evident only 30–36 hr after inoculation. Whether IVR is transported from the primary infected tissue, or whether there is a signal that moves from the inoculated tissue and activates IVR production in the tissue to become resistant is not yet known and will require further studies.

LITERATURE CITED

1. Alper, M., Salomon, R., and Loebenstein, G. 1984. Gel electrophoresis of virus-associated polypeptides for detecting viruses in bulbeous irises. Phytopathology 74:960-962.
2. Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.
3. Gera, A., and Loebenstein, G. 1983. Further studies of an inhibitor of virus replication from tobacco mosaic virus-infected protoplasts of a local lesion-responding tobacco cultivar. Phytopathology 73:111-115.
4. Gera, A., and Loebenstein, G. 1989. Evaluation of antisera to an inhibitor of virus replication (IVR). J. Phytopathol. (In press).
5. Jamet, E., and Fritig, B. 1986. Purification and characterization of 8 of the pathogenesis-related proteins in tobacco leaves reacting hypersensitively to tobacco mosaic virus. Plant Mol. Biol. 6:69-80.
6. Jenns, A. E., Caruso, F. L., and Kuc, J. 1979. Non-specific resistance to pathogens induced systemically by local infection of cucumber with tobacco necrosis virus, *Colletotrichum lagenarium* or *Pseudomonas lachrymans*. Phytopathol. Mediterr. 18:129-134.
7. Kuc, J. 1983. Induced systemic resistance in plants to diseases caused by fungi and bacteria. Pages 191-221 in: Dynamics of Host Defence. J. A. Bailey and B. J. Deverall, eds. Academic Press, New York.
8. Loebenstein, G. 1972. Localization and induced resistance in virus-infected plants. Annu. Rev. Phytopathol. 10:177-206.
9. Loebenstein, G., and Gera, A. 1981. Inhibitor of virus replication released from tobacco mosaic virus infected protoplasts of a local lesion-responding cultivar. Virology 114:132-139.
10. Loebenstein, G., Rabina, S., and van Praagh, T. 1968. Sensitivity of induced localized acquired resistance to actinomycin D. Virology 34:264-268.
11. Loebenstein, G., and Stein, A. 1985. Plant defense responses to viral infections. Pages 413-433 in: Cellular and Molecular Biology of Plant Stress. J. L. Key and T. Kosuge, eds. Alan R. Liss, New York.
12. Parent, J-G., and Asselin, A. 1984. Detection of pathogenesis-related proteins (PR or b) and of other proteins in the intercellular fluid of hypersensitive plants infected with tobacco mosaic virus. Can. J. Bot. 62:564-569.
13. Stein, A., Spiegel, S., and Loebenstein, G. 1985. Studies on induced resistance to tobacco mosaic virus in Samsun NN tobacco and changes in ribosomal fractions. Phytopathol. Z. 114:295-300.
14. Wieringa-Brants, D. H. 1983. A model to simulate acquired resistance induced by localized virus infections in hypersensitive tobacco. Phytopathol. Z. 106:369-323.
15. Wieringa-Brants, D. H., and Dekker, W. C. 1987. Induced resistance in hypersensitive tobacco against tobacco mosaic virus by injection of intercellular fluid from tobacco plants with systemic acquired resistance. J. Phytopathol. 118:165-170.

3. Further purification of protoplast IVR and preparation of monoclonal antibodies

Results are summarized in the attached publication: J. gen. Virology 70:1293-1296, 1989.

6a

J. gen. Virol. (1989), 70, 1293–1296. Printed in Great Britain

Key words: IVR/hypersensitivity/monoclonal antibodies

Use of Monoclonal Antibodies in the Purification of an Inhibitor of Virus Replication by Affinity Chromatography

By A. GERA,[1]* A. SADKA,[1] S. SPIEGEL,[1] R. SALOMON[1] AND N. I. SMORODINSKY[2]

[1]*Virus Laboratory, Agricultural Research Organization, The Volcani Center, Bet Dagan and* [2]*George S. Wise Faculty of Life Sciences, Tel-Aviv University, Ramat-Aviv, Israel*

(*Accepted 11 January 1989*)

SUMMARY

Mouse monoclonal antibodies (MAbs) were prepared to an inhibitor of virus replication (IVR), released from protoplasts or leaf tissue of hypersensitive tobacco plants infected with tobacco mosaic virus. The MAbs were highly specific for IVR and reduced its antiviral activity. Using these MAbs in affinity chromatography enabled the recovery of purified IVR. SDS–PAGE of the immunoaffinity-purified IVR gave a single M_r 23K band. Immunoblots of IVR from extracts of protoplast or leaf tissue also revealed a single M_r 23K band which suggests that protoplast and tissue IVR are closely related.

In our previous studies we reported that a substance(s) inhibiting virus replication is released into the medium from tobacco mosaic virus (TMV)-infected protoplasts of a hypersensitive tobacco cultivar. The inhibitor of virus replication (IVR) from protoplasts was partially purified using zinc acetate precipitation (crude protoplast IVR) and two biologically active proteins with M_r of approx. 26K and 57K (fractionated protoplast IVR-1 and IVR-2 respectively) were obtained by gel filtration (Loebenstein & Gera, 1981). Recently, IVR was also obtained from the intracellular fluid of hypersensitive tobacco leaves infected with TMV (tissue IVR) (Spiegel *et al.*, 1989). A distinct band (23K) that was not present in control preparations was regularly observed after PAGE of crude protoplast and tissue IVR. Electroelution of the 23K protein band from the gel yielded a biologically active preparation of IVR (A. Gera, G. Loebenstein, A. Frank & S. Harlap, unpublished results). When polyclonal antisera prepared against fractionated IVR-1, IVR-2 and a mixture of IVR-1 and IVR-2 (Gera & Loebenstein, 1989), were used in immunoblot analysis of crude protoplast IVR at least two bands reacted (A. Gera *et al.*, unpublished results).

Here we report the preparation of monoclonal antibodies to protoplast IVR and their use for further purification of IVR by affinity chromatography and for detecting IVR from protoplast and tissue extracts in immunoblots.

Crude protoplast IVR and fractionated protoplast IVR-1 were prepared as described previously (Loebenstein & Gera, 1981). Tissue IVR was obtained from the intercellular fluid of *Nicotiana tabacum* L. cv. Samsun NN inoculated with TMV. A preparation obtained from 1 g leaf tissue will be termed one 'unit'. This was found to be roughly equivalent to the amount of IVR obtained from 10^6 protoplasts. One unit of IVR is equivalent to approx. 10 ng protein (Spiegel *et al.*, 1989). Crude protoplast IVR was further purified by immunoaffinity chromatography, using purified immunoglobulin G from IVR polyclonal serum coupled to CNBr-activated Sepharose 4B (Pharmacia) as described by the manufacturer. Fifty units of lyophilized crude protoplast IVR and an equivalent 'mock' preparation were dissolved in 0·5 ml of 0·1 M-phosphate buffer pH 7, containing 0·15 M-NaCl (starting buffer), placed on a 2 ml column and recirculated for 45 to 60 min. The column was washed with 20 ml of starting buffer, and all the flow through fraction was collected. Elution was done with 0·5 M-glycine buffer pH

Table 1. *Purification of protoplast IVR by polyclonal antibody affinity chromatography**

Sample	Elution buffer	Fraction no.	Infectivity† IVR	Infectivity† Control medium‡	Inhibition of infectivity (%)
Starting material§			10·2 ± 2·8	28·2 ± 5·5	64
Column fractions	0·1 M-Phosphate + 0·15 M-NaCl pH 7	1	32·0 ± 4·1	34·1 ± 6·1	6
	0·5 M-Glycine pH 2·6	2	30·0 ± 5·0	32·0 ± 3·1	0
		3	13·8 ± 3·8	31·4 ± 2·3	56
		4	15·1 ± 1·3	36·7 ± 2·5	59
		5	11·1 ± 2·9	25·2 ± 2·3	56
		6	29·0 ± 4·1	34·0 ± 5·7	15
Control protoplast‖			31·5 ± 2·6		

* Column containing 9·2 mg IgG/g Sepharose.

† Average number of local lesions ± standard error per 10^6 protoplasts on one half leaf of *Nicotiana glutinosa*, with the result normalized such that a standard TMV solution (0·2 μg/ml) yielded approximately 60 to 70 lesions per half leaf.

‡ Control preparation from medium in which uninoculated protoplasts were suspended.

§ Equivalent to 3 units.

‖ Control protoplasts without addition of IVR or control preparation.

2·6. Five 1 ml fractions were collected. All fractions were dialysed three times, against 2 l of double-distilled water, and freeze-dried. The resulting powder was dissolved in 50 to 100 μl of double-distilled water, and the biological activity of each fraction was tested in comparison with the respective control fractions (Gera & Loebenstein, 1983).

Three biologically active fractions were obtained (fractions 3, 4 and 5) (Table 1). The purity and integrity of the biologically active fractions were analysed by SDS–PAGE (Spiegel *et al.*, 1989). A distinct band of 23K and three faint bands between 45K and 82K were visible in fraction 3 and were not present in the control preparation. (Fig. 1*a*). Fractions 4 and 5 also contained two close bands of 25K (not shown). Fraction 3 was freeze-dried and used for immunizing the mice.

A panel of MAbs directed against purified IVR was selected after fusion (Köhler & Milstein, 1975) of NS0 cells with spleen cells of a BALB/c mouse immunized with 5·5 μg of purified IVR (fraction 3). Identification of IVR-specific MAbs was achieved by ELISA (Clark & Adams, 1977) using Costar PVC plates coated with IVR or 'mock' preparations. Of 1300 hybridomas screened for specific antibody secretion nine gave specific ELISA reactions. These nine primary cultures were subcloned by limiting dilution (Moav *et al.*, 1982) and three stable clones (MAbs-1, -2 and -3) were obtained. Subclone 1 gave the highest ELISA values with low non-specific reaction (0·65 compared with 0·06). Hybridoma cells were propagated as ascites tumours in pristane-primed BALB/c mice. Ascitic fluid was clarified by low speed centrifugation and tested against fractionated IVR-1. Again, subclone 1 gave the highest ELISA values with low-specific reactions (1·108 compared with 0·167 at a dilution of 1 : 100). To analyse the specificity of the MAbs further, crude protoplast IVR and 'mock' preparations were subjected to SDS–PAGE and analysed by immunoblotting (Towbin *et al.*, 1979), using MAb-1. An immunoblot of crude IVR showed one 23K protein band that was not present in the control preparation (Fig. 1*c*). Similar results (not shown) were obtained when fractionated IVR-1 and -2 were analysed by immunoblotting. This, as well as data from SDS–PAGE, supports our previous suggestion that the fractionated IVR-2 is a dimer of IVR-1 (Loebenstein & Gera, 1981).

The possibility that reaction with the MAb eliminates the biological activity of crude protoplast IVR was tested. Polystyrene microplates were coated with 200 μl ascitic fluid or

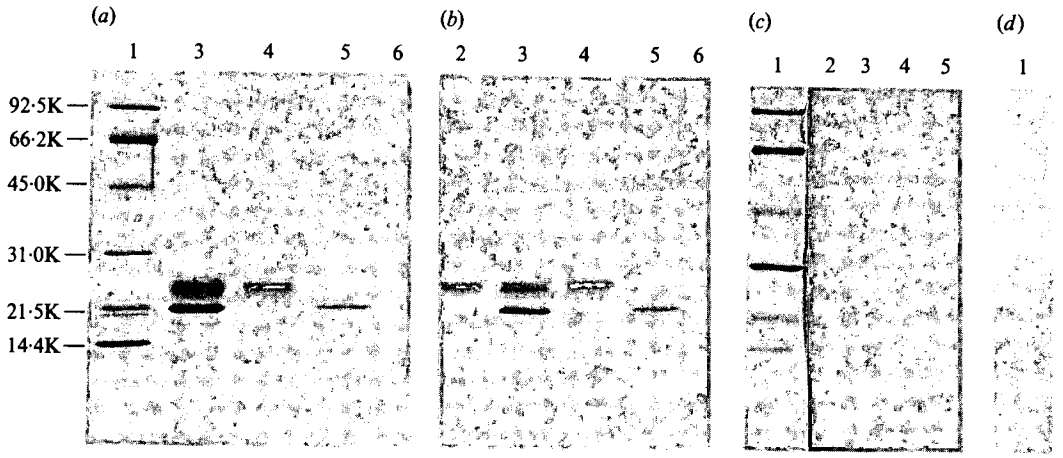


Fig. 1. SDS–PAGE in 5 to 20% gradient gels and immunoblotting of IVR. (*a*, *b*) SDS–PAGE of immunoaffinity-purified protoplast IVR, using polyclonal serum (*a*) or MAb-1 (*b*). Ten units of IVR or equivalent 'mock' preparations are in each lane. Gels were stained with Coomassie Brilliant Blue. Samples were: lane 1, M_r standards phosphorylase B (92·5K), bovine serum albumin (66·2K), ovalbumin (45K), carbonic anhydrase (31K), soybean trypsin inhibitor (21·5K) and lysozyme (14·4K); lane 2, crude control preparation; lanes 3, crude protoplast IVR; lanes 4, effluent; lanes 5 and 6, purified IVR fraction and control, respectively. (*c*) Immunoblot of crude protoplast and tissue IVR. Lane 1, M_r standards subjected to SDS–PAGE and transferred to Immobilon transfer membrane (0·45 μm; Millipore) and stained with Coomassie Brilliant Blue. Lane 2, crude control from tissue; lane 3, crude tissue IVR, when 15 units of each preparation were used. Lanes 4 and 5 as lanes 2 and 3 in (*b*). (*d*) Immunoblot of immunoaffinity-purified IVR (10 units) using MAb-1 (lane 1) as for lane 5 in (*b*).

normal mouse serum diluted 1 : 20 in coating buffer. The plates were incubated for 4 h at 37 °C and washed with phosphate-buffered saline containing 0·05% Tween 20. Fifty μl of crude protoplast IVR (3 units) was then added to triplicate wells. After 3 h incubation, the fluid from the wells was collected and its biological activity was tested on TMV-infected tobacco protoplasts. Crude protoplast IVR placed on wells coated with normal mouse serum was used as a control. MAbs produced by clones 1, 2 and 3 decreased the biological activity of IVR by 77 ± 11% when compared with normal serum. When a further cycle of binding was performed, all inhibitory activity in the crude preparations was removed by binding to the MAbs.

MAb-1 was selected for immunoaffinity purification of crude protoplast IVR, using immunoglobulins from the ascitic fluid. IgGs were precipitated with 40% ammonium sulphate and coupled to CNBr-activated Sepharose 4B (10 mg/ml gel). A column of 2 ml volume was used. Fifty units of lyophilized crude protoplast IVR dissolved in 0·5 ml of starting buffer were applied to the affinity column and recirculated for 30 min. Proteins bound to the immobilized antibodies were eluted with 0·1 M-acetic acid pH 2·5 containing 0·5 M-NaCl, at a flow rate of two drops/min. Four fractions of 1 ml each were collected and neutralized with 30 μl of 2 M-Tris–HCl, dialysed for 48 h against double-distilled water, and treated as described above. Biological activity (> 56% inhibition) was observed in the fractions eluted with 0·1 M-acetic acid, whereas no biological activity could be detected in the flow through fraction. The recovery was almost quantitative (> 90% based on biological activity and PAGE).

All four fractions of the immunoaffinity-purified IVR contained a single 23K protein, not present in the control preparation. This protein migrated to the same position as the specific band observed in PAGE of crude protoplast IVR (Fig. 1*b*) and reacted specifically in immunoblots with MAb-1 (Fig. 1*d*). Tissue IVR also reacted specifically with MAb in immunoblots and the reactive component comigrated with protoplast IVR (Fig. 1*c*). MAb-1 also reacted with tissue IVR isolated from the intercellular fluid of hypersensitive tobacco plants and these results strengthen our previous findings that tissue IVR and protoplast IVR are identical (Spiegel *et al.*, 1989). The sensitivity of the MAbs enabled the detection of 0·1 to 1·0 ng

IVR as measured by ELISA and these MAbs can therefore be used for both improved purification of IVR and its detection.

The authors thank Professor G. Loebenstein for helpful advice and critical reading of the manuscript. This work was supported in part by the United States – Israel (Binational) Agricultural Research and Development Fund (BARD). Contribution No. 2445-E, 1988 series, from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel.

REFERENCES

CLARK, M. F. & ADAMS, A. N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* **34**, 475–483.

GERA, A. & LOEBENSTEIN, G. (1983). Further studies of an inhibitor of virus replication from tobacco mosaic virus-infected protoplasts of a local lesion-responding tobacco cultivar. *Phytopathology* **73**, 111–115.

GERA, A. & LOEBENSTEIN, G. (1989). Evaluation of antisera to an inhibitor of virus replication (IVR). *Journal of Phytopathology* (in press).

KÖHLER, G. & MILSTEIN, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature, London* **256**, 495–497.

LOEBENSTEIN, G. & GERA, A. (1981). Inhibitor of virus replication released from tobacco mosaic virus infected protoplasts of a local lesion-responding cultivar. *Virology* **114**, 132–139.

MOAV, N., SMORODINSKY, N. I., BALIN, B. & WITZ, I. P. (1982). Monoclonal antibodies from mice bearing polyoma virus-induced tumor. *Cancer Immunology and Immunotherapy* **12**, 217–224.

SPIEGEL, S., GERA, A., SALOMON, R., AHL, P., HARLAP, S. & LOEBENSTEIN, G. (1989). Recovery of an inhibitor of virus replication from the intercellular fluid of hypersensitive tobacco infected with tobacco mosaic virus and from non-infected induced resistant tissue. *Phytopathology* **79** (in press).

TOWBIN, H., STAEHELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences, U.S.A.* **76**, 4350–4354.

(*Received 9 August 1988*)

4. Preparation of polycolonal antisera to protoplast IVR

Results are summarized in the attached publication: J. Phytopathology 124:366-371, 1989, and the manuscript (following section, A-5), accepted for publication in Phytopathology.

7a

J. Phytopathology **124**, 366—371 (1989)

ISSN 0931-1785

Department of Virology, Agricultural Research Organization, Volcani Center, Bet Dagan, Israel

Evaluation of Antisera to an Inhibitor of Virus Replication (IVR)

A. GERA and G. LOEBENSTEIN

Authors' address: A. GERA and G. LOEBENSTEIN, Department of Virology, Agricultural Research Organization, Volcani Center, Bet Dagan 50-250, Israel.

With one figure

Received February 8, 1988; accepted May 7, 1988

Abstract

Antisera to an inhibitor of virus replication (IVR) were prepared. The two biologically active fractions — IVR-1 (MW 26,000) and IVR-2 (MW 57,000) were either mixed or used separately for injection into rabbits. The mixed antiserum enabled a clear distinction in ELISA and in agar gel diffusion tests between preparations of IVR from the incubation medium of tobacco mosaic virus (TMV)-infected protoplasts, and the preparation obtained from control protoplasts. Antisera prepared against each of the two fractions were highly cross-reactive in both ELISA and agar-gel diffusion tests. The mixed IVR antiserum totally abolished the inhibitory activity of IVR, whereas normal antiserum had no effect on its biological activity. IVR antiserum did not react with TMV, TMV coat protein, human leukocyte interferon, or the antiviral factor (AVF). These results strengthen our previous suggestion that IVR-2 is a dimer of IVR-1.

Zusammenfassung

Bewertung von Antiseren gegen einen Inhibitor der Virusreplikation (IVR)

Hergestellt wurden Antiseren gegen einen Inhibitor der Virusreplikation (IVR). Die zwei biologisch aktiven Fraktionen — IVR-1 (MGew. 26000) und IVR-2 (MGew. 57000) — wurden entweder zusammen oder allein in Kaninchen injiziert. Das gemischte Antiserum ermöglicht, nach ELISA oder Agargeldiffusionstests, daß IVR-Präparationen aus dem Inkubationsmedium von Tabak Mosaik Virus (TMV)-infizierten Protoplasten und die von Kontrollprotoplasten voneinander deutlich unterschieden werden konnten. Antiseren, die gegen die zwei Fraktionen hergestellt wurden, hatten eine hohe Kreuzreaktion in ELISA und Agargeldiffusionstests. Das gemischte IVR-Antiserum annullierte total die hemmende Aktivität der IVR, normales Antiserum dagegen beeinflußt deren

biologische Aktivität nicht. Das IVR-Antiserum reagierte nicht mit TMV, TMV-Hülleprotein, menschlichem Leukozyteninterferon oder dem Antivirusfaktor. Diese Ergebnisse bestätigen unsere frühere Hypothese, daß IVR-2 ein Dimer des IVR-1 ist.

Previously, we reported that a substance(s) inhibiting virus replication (IVR) is released into the medium from tobacco mosaic virus (TMV)-infected protoplasts of a tobacco cultivar in which the infection in the intact plant is localized. IVR inhibited virus replication in protoplasts from both local lesion-responding resistant (NN) and systemic-responding susceptible (Samsun) tobacco plants (LOEBENSTEIN and GERA 1981). In further studies it was found that IVR inhibited virus replication also in leaf tissue disks and in intact leaves when applied to cut stems or by spray. IVR was partially purified by precipitation with zinc acetate ($ZnAc_2$) and such preparations contained two biologically active principles with molecular weights, as determined by gel filtration, of approximately 26,000 (IVR-1) and 57,000 (IVR-2). IVR was found to be sensitive to the proteolytic enzymes trypsin and chymotrypsin, but not to RNAse, suggesting that it is a protein (GERA and LOEBENSTEIN 1983).

Here we report on the preparation of antisera to IVR and to its two biologically active principles and their evaluation also against Sela's antiviral factor (AVF) (SELA 1981) and interferon.

Material and Methods

Preparation of protoplasts and IVR

Growing of *Nicotiana tabacum* L. cv. Samsun NN plants and the preparation of protoplasts and IVR by precipitation with $ZnAc_2$ were as described previously (LOEBENSTEIN and GERA 1981). Corresponding preparations were obtained similarly from control protoplasts.

A lyophilized $ZnAc_2$ preparation obtained from 3×10^7 inoculated protoplasts was dissolved in 1—2 ml of 0.1 M phosphate buffer, pH 6.0, placed on a Sephadex G-75 (Superfine, Pharmacia) column, and eluted with the same buffer. Fractions of molecular weight equivalent to 26,000 (IVR-1) and 57,000 daltons (IVR-2) were collected (LOEBENSTEIN and GERA 1981), lyophilized, dissolved in 1 ml 0.05 M phosphate buffer, pH 7.0, and dialyzed against the same buffer.

As estimated by amino acid analysis after high pressure liquid chromatography (HPLC) and by staining reactions with Coomassie brilliant blue after polyacrylamide gel electrophoresis, 100 units of IVR (obtained from the incubation medium of 10^8 protoplasts) was equivalent to *c.* 1 μg protein (unpublished results in collaboration with Prof. Y. BURSTEIN and Dr. V. BUCHNER, Weizmann Institute of Science, Rehovot, Israel).

Preparation of antisera

One ml of partially purified IVR (IVR-1, IVR-2, or the mixture of IVR-1 + IVR-2) was emulsified with an equal volume of Freund's incomplete adjuvant. Six intramuscular injections, each of 30 units of IVR (isolated from the incubation medium of 30×10^6 protoplasts), were given to each of three rabbits. The first four injections were given at intervals of 4 days, the fifth 3 weeks later, and the last after 4 days. One month later, one intravenous injection (without adjuvant) was given. The rabbits were bled several days after the last injection and the serum was stored in an equal volume of glycerol at −20 °C. To eliminate non-specific antibodies, 1—2 ml of the antisera was mixed with 50×10^6 control protoplasts which after 6 h were removed by centrifugation.

Enzyme linked immunosorbent assay

(ELISA) and agar-gel diffusion tests

The purification of γ-globulin and its conjugation with alkaline phosphatase were as described by CLARK and ADAMS (1977). The γ-globulins used for ELISA were further absorbed with a lyophilized preparation obtained from the incubation medium of 50×10^6 control protoplasts.

Agar-gel-diffusion tests were done in 55-mm Petri dishes containing a 4-mm layer (9 ml) of 0.75 % agar (Bacto-Agar, Difco), 0.001 M EDTA, 0.85 % NaCl and 0.2 % sodium azide at pH 7.8 (TOMLINSON *et al.* 1973). Agar plates were incubated in a moist chamber at 22 °C for 15 h.

Neutralization of IVR's biological activity by specific antiserum

Polystyrene microplates were coated with γ-globulin specific to IVR from the mixed antiserum (0.02 mg/ml). The plates were incubated for 4 h at 37 °C, and washed with phosphate buffered saline containing 0.05 % Tween 20. Then 0.2 ml of IVR (3 units) was added to triplicate wells. After 3 h of incubation the fluid from the wells was collected and its biological activity tested on TMV-infected tobacco protoplasts (LOEBENSTEIN and GERA 1981). For controls, IVR placed on wells coated with γ-globulin from non-immunized rabbits was used.

Results

Serological evaluation of IVR antisera

The mixed IVR antiserum enabled a clear distinction in ELISA between $ZnAc_2$ preparations from incubation media of TMV-infected protoplasts and those obtained from control protoplasts. Thus, $ZnAc_2$ preparations from 1, 5 and 10 million infected protoplasts per well gave E_{405} values of 0.38, 0.58 and 0.73 (averages from three experiments, each on three wells), compared with 0.035, 0.04 and 0.065 from respective numbers of control protoplasts.

The mixed IVR antiserum did not react in ELISA with purified TMV, TMV-coat protein or TMV-infected Samsun tobacco leaf extracts.

Results on the activity of antisera prepared against the 26,000 (IVR-1) and the 57,000 (IVR-2) fractions separately, are summarized in Table 1. As seen with ELISA, no differences were observed when each of the two antisera was tested against these two IVR fractions, indicating that the two fractions are serologically very similar.

Table 1

ELISA evaluation of antisera against IVR-1 (M.W. 26,000) and IVR-2 (M.W. 57,000)*

Fraction	Absorption (E_{405})** IVR-1 antiserum	IVR-2 antiserum
IVR-1	0.680 ± 0.032	0.665 ± 0.028
IVR-2	0.630 ± 0.051	0.675 ± 0.054
Control***	0.085 ± 0.023	0.063 ± 0.028

* Three units each of IVR-1 and IVR-2 per well.

** Averages from three experiments (three replicates each), ± standard error.

*** Preparations from control protoplasts.

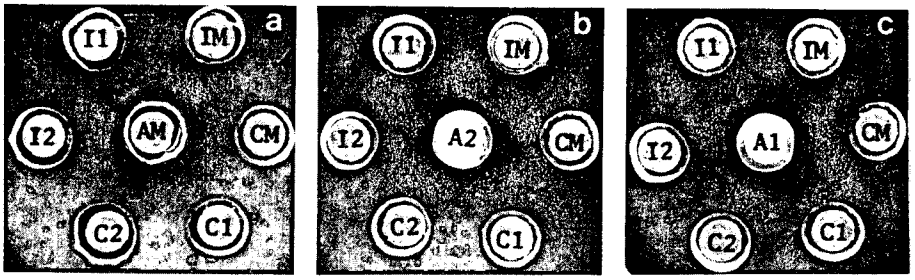


Fig. 1. Agar-gel double diffusion serology showing cross-reactivity between IVR-1 (I 1), IVR-2 (I 2) and mixed IVR 1 + 2 (IM). a, antiserum to mixed IVR (AM). b, antiserum to IVR-2 (A2). c, antiserum to IVR-1 (A1). C1, C2 and CM, parallel control preparations to IVR-1, IVR-2 and mixed IVR 1 + 2

In agar-gel diffusion tests clear precipitation lines were obtained between IVR-1, IVR-2, mixed IVR-1 and 2 (5 units dissolved in 0.1 ml of 0.1 M phosphate buffer, pH 7), and the mixed IVR antiserum (diluted 1 : 5). The precipitation lines fused without spurs (Fig. 1 a). Similar results were obtained when antisera to IVR-1 and IVR-2 were used. The two antisera reacted with both fractions, giving precipitation lines that fused without spurs (Figs. 1 b, c), thus indicating that the fractions are serologically closely related.

Table 2
Neutralization of IVR's biological activity by specific antiserum*

Preparation**	Wells coated with γ-globulin of	Infectivity from test protoplasts treated with the respective preparation***	Percentage inhibition (IVR/control)
IVR	−+	9.9 ± 1.69	
Control	−+	28 ± 2.28	65
IVR	Normal	10.2 ± 1.84	
Control	Normal	29.4 ± 1.77	65
IVR	anti-IVR	25.7 ± 2.76	
Control	anti-IVR	26.2 ± 2.75	2
Control protoplasts++		28 ± 3.25	

* Antiserum prepared by injecting the two IVR fractions (mixed). Averages of two experiments.
** $ZnAc_2$ preparations from incubation medium of 3×10^6 infected or non-infected control protoplasts per well.
*** Average number of local lesions and standard error per 10^6 Samsun NN protoplasts 72 h after inoculation, on one half-leaf of *Nicotiana glutinosa* calibrated to standard TMV (0.2 μg/ml), which yielded ~ 60 lesions per half-leaf.
\+ Non-coated wells.
++ Infectivity from inoculated non-treated protoplasts.

No lines were observed between the antisera and the respective control preparations from the incubation medium of control protoplasts obtained in parallel to IVR-1, IVR-2 and the mixed IVR-1 and 2 (Figs. 1 a, b, c).

Neutralization of IVR's biological activity by specific antiserum

The mixed IVR antiserum totally abolished the inhibitory activity of IVR, while normal antiserum had no effect on the biological activity of IVR (Table 2).

Evaluation of IVR antiserum with AVF and interferon

To determine whether IVR has similarities to AVF (SELA 1981), and to human interferon, the IVR antisera were tested in double-gel-diffusion tests against AVF (obtained from Dr. I. SELA) and human leukocyte interferon (gift from National Institutes of Health, Bethesda, MD, U.S.A.).

No precipitation lines were obtained either with 10^5 units of human leukocyte interferon or with 10 μg AVF per well when the mixed antiserum or the antisera to IVR-1 or IVR-2 were used.

Discussion

Antisera to IVR were obtained by injecting relatively large amounts of IVR (i.e. 210 "units" obtained from the incubation medium of 210×10^6 protoplasts, equivalent to about 2.1 μg protein). This was expected, as both the 26,000 and the 57,000 fractions obtained by Sephadex gel filtration, had biological activity without giving a detectable adsorption at OD_{280}, thus suggesting that IVR is active at extremely low concentrations (LOEBENSTEIN and GERA 1981). Biological activity of IVR is apparently evident at protein concentrations of 1—2 ng/ml.

Antisera prepared against the two main biologically active fractions, IVR-1 (26,000) and IVR-2 (57,000), were highly cross-reactive, and precipitation lines fused completely, indicating the presence of identical determinants in IVR-1 and IVR-2. This supports the previous suggestion (LOEBENSTEIN and GERA 1981) that the 57,000 fraction is a dimer of the 26,000 fraction.

The IVR antiserum abolished IVR's biological activity, while normal antiserum had no effect on the inhibitory activity of IVR. As the antisera were prepared against partially purified IVR and adsorbed with healthy extracts to remove non-specific host components, this neutralization test showed that the antiserum was specific to IVR.

The IVR antiserum gave no reaction with TMV, TMV coat protein, AVF or human leukocyte interferon, results confirming the difference between IVR and these fractions.

Contribution No 1635-E from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel. We thank SHARON HARLAP and REGINE NIDAM for technical assistance. This work was supported in part by the United States—Israel (Binational) Agricultural Research and Development Fund (BARD); the Gesellschaft für Strahlen- und Umweltforschung (GSF), Munich, West Germany; and the National Council for Research and Development (NCRD), Jerusalem, Israel.

Literature

CLARK, M. F., and A. N. ADAMS, 1977: Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. Virology **34**, 475—483.

GERA, A., and G. LOEBENSTEIN, 1983: Further studies of an inhibitor of virus replication from tobacco mosaic virus-infected protoplasts of a local lesion-responding tobacco cultivar. Phytopathology **73**, 111—115.

LOEBENSTEIN, G., and A. GERA, 1981: Inhibitor of virus replication released from tobacco mosaic virus infected protoplasts of a local lesion-responding cultivar. Virology **114**, 132—139.

SELA, I., 1981: Plant-virus interactions related to resistance and localization of viral infections. Adv. Virus Res. **26**, 201—237.

TOMLINSON, J. A., A. L. CARTER, E. M. FAITHFULL, and M. J. W. WEBB, 1973: Purification and serology of the W strain of cucumber mosaic virus. Ann. appl. Biol. **74**, 181—189.

5. Further characterization of IVR-association with a 23K protein species.

Results are summarized in the attached manuscript - accepted for publication in Phytopathology.

Resistance

An Inhibitor of Virus Replication from Protoplast of Hypersensitive Tobacco Cultivar Infected with Tobacco Mosaic Virus is Associated with a 23K Protein Species

A. Gera, G. Loebenstein, R. Salomon and A. Franck

Department of Virology, Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel.

Contribution No. 2458-E, 1989 series, from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel.

This work was supported in part by the United States Israel Binational Agricultural Research and Development Fund (BARD).

ABSTRACT

Gera, A., Loebenstein, G., Salomon, R. and Franck, A. 198 .

An Inhibitor of Virus Replication from Protoplast of Hypersensitive Tobacco Cultivar Infected with Tobacco Mosaic Virus is Associated with a 23K Protein Species. Phytopathology

A specific protein with an approximate molecular weight of 23K was consistently observed by SDS-polyacrylamide gel electrophoresis (PAGE) from crude preparations of an inhibitor of virus replication (IVR) released into the culture medium from tobacco mosaic virus - infected protoplasts of hypersensitive tobacco cultivar. Electroeluted protein from SDS-PAGE gels was biologically active, with about twenty fold increase in specific activity over that of the crude preparation. The active fraction revealed only one protein band at 23K in PAGE,

providing evidence that the 23K protein is IVR purified to a high degree.

A homologous antiserum to the 23K protein was prepared. The antiserum was highly specific for IVR and effeciently eliminates its antiviral activity. Western blots of IVR extracted from protoplasts or leaf tissue of hypersensitive tobacco cultivar revealed a single 23K protein band.

Additional key words: antiviral compound, PAGE, serology.

We have reported that an inhibitor of virus replication (IVR) is released into the medium from tobacco mosaic virus (TMV)-infected protoplasts of a tobacco cultivar in which the infection in the intact plant is localized. IVR inhibited virus replication in protoplasts from both local lesion-responding resistant (Samsun NN) and systemic-responding susceptible (Samsun) tobacco plants. IVR was not released from TMV-infected Samsun protoplasts (9). It was suggested that IVR is associated with localization (10). IVR was partially purified using $ZnAc_2$ precipitation from the culture medium of TMV-inoculated Samsun NN protoplats (crude protoplast IVR). Two biologically active compounds were obtained with molecular weights of approximately 26,000 and 57,000 (fractionated IVR-1 and 2), as determined by gel filtration (9). Antisera were prepared against each of these two fractions which were highly cross-reactive, indicating the presence of identical determinants in both fractions (4).

IVR was also obtained from the intercellular fluid of Samsun NN tobacco infected with TMV (tissue IVR) and from induced resistant tissue (11).

Here we report that the biological activity of IVR is associated with a specific 23K protein, observed in SDS-PAGE of crude protoplast IVR. An antiserum specific to the 23K protein was prepared and used for Western blot analysis.

MATERIALS AND METHODS

Preparation of crude protoplast - and tissue - IVR. Crude protoplast IVR, fractionated protoplast IVR-1 and -2 were prepared from the culture medium of TMV-inoculated Nicotiana tabaccum (cv. Samsun NN) protoplasts

as described previously. Control preparations were obtained from sham-inoculated protoplasts (9).

Tissue IVR was obtained from the intercellular fluid of Samsun NN tobacco leaves inoculated with TMV. A preparation obtained from 1 gr leaf tissue was termed one "unit". This was found roughly equivalent to the amount of IVR obtained from 10^6 protoplast. One unit of IVR is equivalent to approximately 10 ng proteins (12). One unit of IVR applied to protoplast or leaf disks 5 hr after inoculation with TMV, consistantly reduced virus replication by 50-60%, in comparison with the respective control, when determined by local lesion assay or by ELISA (3,9,12)

Polyacrylamide gel electrophoresis of IVR. For PAGE 10-20 units of crude protoplast - IVR were concentrated to 8 ul, mixed with 4 ul of 60 mM tris-HCl buffer, pH 6.8, containing 3% sodium dodecyl sulphate (SDS), 5% 2-mercaptoethanol, 10% glycerol and a few grains of bromophenol blue (disruption buffer), and boiled for 2 min. Twelve ul of the SDS-disrupted IVR was then applied to 6-20% polyacrylamide gradient minigels (10 x 7.5 cm; 0.75 mm thick) containing 0.1% SDS and analyzed by electrophoresis. Gels were stained with Coomassie Brilliant Blue (CBB) (1). An equivalent control preparation obtained from the culture medium of sham-inoculated protoplasts was analyzed.

Electroelution of IVR from SDS-PAGE. 10-20 units of IVR or control preparations per lane were electrophoresed in 6-20% polyacrylamide gradient minigels, containing 0.1% SDS. After electrophoresis, one lane containing IVR and one containing marker proteins were stained with CBB; the other lanes were kept refrigerated until elution. The areas parallel to the stained IVR band were exised and inserted into a dialysis bag containing 1 ml of 50 mM borate buffer pH 8.0, 20% methanol and 0.02%

mercaptoethanol. The sealed dialysis bag was placed in 4 liters of the same solution and protein was eluted from the gel by electrophoresis at 300 mA at 2 C for 6 hr, using a Hoeffer Transphor apparatus, attached to a cooling circulator. Following electroelution, the electrodes were reveresed, and the current applied for another 1 hr to detach the proteins from the dialysis bag (2). Gel pieces were precipitated by low speed centrifugation. Supernatant was dialysed for 24 hr against three changes of 1 liter of distilled water. The eluted protein was freeze dried, dissolved in 30 ul of distilled water and the biological activity assayed with TMV-infected protoplasts, in comparison with an equivalent fraction recovered from the gel used to purify proteins from control preparations. A sample of this preparation was analyzed by SDS-PAGE.

Protein bands corresponding to the M.W of 25.5K and 16K were electroeluted and recovered from the gel. The biological activity of each protein was tested with TMV-infected protoplasts in comparison with the respective control fraction as described above.

Serology. Antisera were prepared by injecting the gel purified 23K protein. 80-120 units of IVR equivalent to 0.8-1.2 ug protein , were recovered from a total of 6 gel channels. Gel pieces containing the 23K protein were washed overnight in 10 ml of distilled water, with three or four changes of water. The washed gel pieces were homogenized in 1 ml PBS and emulsified with an equal volume of Freund's incomplete adjuvant. The emulsion was injected into a rabbit subcutaneously in six places along both sides of the spinal cord. Five additional injections were given similarly at 2-wk intervals, and the rabbit was bled 2 wks after the last injection. The antiserum was absorbed with control preparation isolated

from the culture medium of sham-inoculated protoplasts, to eliminate possible non-specific reactions.

Indirect enzyme-linked immunosorbent assay (ELISA) was performed as described previously (5). Three units of crude IVR or control preparations were dissolved in 50 ul coating buffer and applied to the ELISA wells. After 4 C incubation overnight, attachment sites were saturated with 2% bovine serum albumin (BSA) prior to adding two-fold dilutions of the 23K protein antiserum (1:400-1:1600). Subsequently, goat anti-rabbit conjugate was used to detect and quantify the reaction.

Agar-gel diffusion tests were done in 55 mm petri dishes containing a 4 mm layer of 0.75% agar (Bactro-Agar, Difco), 0.001 M EDTA, 0.85% NaCl and 0.02% sodium azide at pH 7.8 (4,13). Five units of crude protoplast IVR, fractionated IVR-1, IVR-2 and the corresponding control preparations, dissolved in 50 ul of 0.1 M phosphate buffer pH 7, were tested using the 23K protein antiserum (diluted 1:5).

Elimination of IVR's biological activity by the 23K protein antiserum. The possibility that reaction with the 23K protein antiserum eliminates the biological activity of crude protoplst IVR was tested as described previously (4,5). Polystyrene microplates were coated with either the 23K protein antiserum or normal rabbit serum diluted 1:20. The plates were incubated for 4 hr at 37C and washed with PBS containing 0.05% Tween 20. Fifty ul of crude protoplast IVR was added to triplicate wells. After 3 hr incubation, the fluid from the wells was collected and its biological activity was tested on TMV-infected tobacco protoplasts (3,9).

Western blot and protein measurement. Proteins were electrophoretically transferred onto nitrocellulose membranes as described by Towbin et al. (14). Immunoreactive bands were visualized by incubating the membrane in

50 mM sodium acetate, pH 5.0, containing 0.4 mg/ml of 3 amino - 9 - ethylcarbazole and 0.015% [v/v] H_2O_2 until color developed. The reaction was stopped by washing the membrane in deionized water.

Proteins were quantified using the Coomassie blue dye-binding procedure (7) with slight modifications. Briefly, the dye was recovered from the filter paper by shaking instead of ultrasonic elutin. BSA was used as a standard.

RESULTS

PAGE of crude protoplast IVR. A 23K specific protein band that was not present in control preparations was consistently observed in PAGE of crude protoplast IVR disrupted in SDS (Fig. 1a). The calculated M.W of this protein, based on migratory distances relative to the standard protein markers was 23K. As seen in Fig. 1a the amount of protein in the IVR preparation (lane 2) is higher than in the control (lane 3), though both were obtained from the culture media of a similar number of TMV-inoculated- and sham-inoculated protoplats. This is understandable as after infection various pathogenesis-related (PR) proteins are produced (15). Therefore, an additional experiment was done. Fifteen units of IVR were electrophoresed in comparison to 30 units of control preparation. A distinct 23K protein band was observed in the crude protoplast IVR, not observed in the control preparation (data not shown).

Electroelution of IVR after PAGE. The biological activity of IVR was retained after electroelution. When an electroeluted preparation, equivalent to 3 units of IVR, a non saturating concentration (6) was tested in TMV-infected protoplasts, and assayed on N. glutinosa it reduced virus replication by 68%, compared with 71% for the original

crude IVR. The respective control preparations were without inhibitory activity (Table 1).

Electroeluted protein bands corresponding to the M.W of 25.5K and 16K were recovered from the gel. The biological activity of each protein was tested in comparison with the respective control fraction. All four proteins were without inhibitory activity.

Protein contents as determined by the dye method, were 0.0253 and 0.646 ug/unit for electroeluted IVR and crude IVR, respectively (averages from 2 experiments). Apparently, about a twenty fold increase in specific activity was obtained after electroelution.

A single band at 23K was observed when this preparation (20 units) was analyzed by PAGE (Fig. 2a). Similar results were obtained in 3 additional experiments.

Serological evaluation of PAGE antiserum. The 23K protein antiserum enabled a clear distsinction in ELISA between crude protoplast IVR and control preparation. IVR values from indirect ELISA were 1.47, 1.10 and 0.67 (averages from four experiments, two replicates for each sample), compared with 0.214, 0.121 and 0.025 for the control preparation, at serum dilutions of 1:400, 1:800 and 1:1600, respectively.

In agar-gel diffusion tests clear precipitation lines were obtained between crude protoplast IVR, IVR-1 and IVR-2 and the 23K protein antiserum (diluted 1:5). The precipitation lines fused without spurs (Fig. 2), indicating that the fractions were serologically similar.

Elimination of IVR's biological activity by the 23K protein antiserum. The 23K protein antiserum decreased the inhibitory activity of IVR by 85% when compared with normal serum (Table 2). When a further

cycle of binding was performed, all inhibitory activity in the crude preparation was removed by binding to the antiserum.

Western blotting analysis of IVR. The 23K protein band observed in PAGE of crude protoplast IVR reacted specifically in Western blots with the 23K protein antiserum (Fig. 1d). Electroeluted 23K protein which exhibits a single band in SDS-PAGE, reacted specifically in Western blot with the 23K protein antiserum (Fig. 1c). The antiserum reacted specifically also with tissue IVR isolated from the intercellular fluid of hypersensitive tobacco plants, and the active component comigrated with protoplst IVR (Fig. 1d). These results strengthen our previous findings that tissue IVR and protoplast IVR are identical (12).

DISCUSSION

Our main finding in this study is the associaiton of the 23K protein with the biological activity of the inhibitor of virus replication (IVR) isolated from the culture medium of Samsun NN tobacco protoplast infected with TMV. The 23K protein band that was not present in control preparations was consistently observed in SDS-PAGE of crude protoplast IVR. The biological activity could be recovered from the 23K protein band by electroelution. Apparently, after dialysis to remove the SDS, IVR molecule retains its biological activity. This biologically active fraction obtained after electroelution, revealed a 23K protein band when PAGE analyzed. Electroeluted protein fractaions above and below the region of the 23K protein were without inhibitory activity. This together with the increase in specific activity, provides evidence that the 23K fraction is IVR, purified to a high degree.

The amount of protein per unit determined after electroelution is 2-3 times higher than that determined in preliminary experiments for fractions obtained by high pressure liquid chromatography (HPLC) (12). This may be due to contaminating proteins in the electroeluted fraction, or inaccuracy of the Coomassie blue dye-binding procedure (7), or both. It seems therefore reasonable to assume that the content of IVR protein per unit is within the 10-30 ng range.

The antiserum prepared from the 23K band was highly reactive with the two biologically active fractions, IVR-1 and IVR-2, obtained previously from Sephadex G-75 columns (9). Precipitation lines fused completely, indicating that IVR-1 and 2 have identical serological determinants. This strengthens our previous suggestion that IVR-2 is a dimer of IVR-1 (9). Support for this suggestion is given by the fact that in Western blots after SDS-PAGE, only one band around 23K is observed, and under denaturing conditions the dimer IVR-2 is separated to the monomeric form - IVR-1. Similar results were obtained with IVR-1 and 2 obtained from the intercellular fluid of Samsun NN infected with TMV (12).

The 23K protein antiserum described in this study was not only immunoreactive with IVR but also effeciently eliminates its antiviral activity.

The antiserum was further used for the detection of tissue IVR isolated from the intercellular fluid of hypersensitive tobacco. Western blots of both protaplast and tissue IVR revealed a single 23K band, not present in the control preparations. These results are in line with our previous findings that protoplast and tissue IVR are closely related (12).

Some discrepancy between the molecular weight determination based on SDS-PAGE and on gel filtration was noticed. Using SDS-PAGE a molecular weight of approximately 23,000 was estimated for IVR, while by gel filtration a value of 26,000 was obtained. Similar observations have also been made with other proteins (8). The different molecular weight estimation may stem from configuration changes under denaturing conditions.

Many proteins are known to be induced in the leaves of tobacco by pathogen infections that induce the hypersensitive response (15). These PR proteins generally occur at much higher concentrations than IVR and are resistant to proteases. No inhibitory activity has been reported for any of them. One of these proteins, PR-R, with a molecular weight near 23,000, resembles thaumatin (11). Preliminary data (unpublished) indicate a marked difference in the amino acid composition of IVR and PR-R. It seems, therefore, that IVR and PR-R are two different proteins.

LITERATURE CITED

1. Alper, M., Salomon, R. and Loebenstein, G. 1984. Gel electrophoresis of virus-associated polypeptides for detecting viruses in bulbeous irises. Phytopathology 74:960-962.

2. Davidson, I., Malkinson, M. and Becker, Y. 1988. Marek's disease virus serotype-1 antigens A and B and their unglycosylated precursors detected by western blot analysis of infected cells. Virus Genes 24:5-18.

3. Gera, A. and Loebenstein, G. 1983. Further studies of an inhibitor of virus replication from tobacco mosaic virus-infected protoplasts of a local lesion-responding tobacco cultivar. Phytopathology 73:111-115.

4. Gera, A. and Loebenstein, G. 1989. Evaluation of antisera to an inhibitor of virus replication (IVR). J. Phytopathol. 124:366-371.

5. Gera, A., Sadka, A., Spiegel, S., Salomon, R. and Smorodinsky, N.I. 1989. Use of monoclonal antibodies in the purification of an inhibitor of virus replication by affinity chromatography. J. gen. Virol. 70:1293-1296.

6. Gera, A., Spiegel, S. and Loebenstein, G. 1986. Production, preparation and assay of an antiviral substance from plant cells. Meth. Enzymol. 119:829-733.

7. Ghosh, S., Gepstein, S., Heikkila, J.J. and Dumbroff, E.B. 1988. Use of a scanning densitometer or an ELISA plate reader for measurement of nanogram amounts of protein in crude extracts from biological tissue. Anal. Biochem. 169:227-233.

8. Jamet, E. and Fritig, G. 1986. Purification and characterization of 8 of the pathogenesis-related proteins in tobacco leaves reacting hypersensitively to tobacco mosaic virus. Plant Molec. Biol. 6:69-80.

9. Loebenstein, G. and Gera, A. 1981. Inhibitor of virus replication released from tobacco mosaic virus infected protoplasts of a local lesion-responding cultivar. Virology 114:132-139.

10. Loebenstein, G. and Stein, A. 1985. Plant defense responses to viral infections. Pages 413-433 in J.L. Key and T. Kosuge, eds. Cellular and Molecular Biology of Plant Stress. Alan R. Liss, New York.

11. Pierpoint, W.S., Tatham, A.S. and Pappin, D.J.C. 1987. Identification of the virus-induced protein of tobacco leaves that resembles the sweet-protein thaumatin. Phys. Molec. Plant Pathol. 31:291-298.

12. Spiegel, S., Gera, A., Salomon, R., Ahl, P., Harlap, S. and Loebenstein, G. 1989. Recovery of an inhibitor of virus replication from the intercellular fluid of hypersenstive tobacco infected with tobacco mosaic virus and from non-infected induced-resistant tissue. Phytopathology 79:258-262.

13. Tomlinson, J.A., Carter, A.L., Faithfull, E.M. and Webb, M.J.W. 1973. Purification and serology of the W strain of cucumber mosaic virus. Ann. Appl. Biol. 74:181-189.

14. Towbin, H., Staehelin, T. and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. Proc. Nat. Acad. Sci. USA 76:4350-4354.

15. Van Loon, L.C. 1985. Pathogenesis-related proteins. Plant Molec. Biol. 4:111-116.

Fig. 1. SDS-polyacrylamide gel electrophoresis and immunoblotting of protoplast IVR. (a,b) SDS-PAGE of crude protoplast IVR (a) and IVR recovered after electroelution from the 23K band (b). Polypeptide markers phosphorylase B (92.5K), BSA (66.2K), ovalbumin (45K), carbonic anhydrase (31K), Soybean trypsin inhibitor (21.5K) and lysozyme (14.4K), (lane 1); crude IVR (10 units) (lane 2); control preparation (10 units) (lane 3); electroeluted IVR (15 units) (lane 4); electroeluted control preparation (15 units) (lane 5). (c) Immunoblot of electroeluted IVR. Lanes 1 and 2 as lanes 4 and 5 in (b). (d) Immunoblot of crude protoplast and tissue IVR. Crude tissue IVR (10 units) (lane 1); crude control from tissue (lane 2). Lanes 3 and 4 as lanes 2 and 3 in (a). IVR band indicated by arrow. Molecular weight markers are in KDa.

Fig. 2. Agar-gel double diffusion serology of IVR. 1, crude IVR; 2, IVR-1; 3, IVR-2, 4,5,6 parallel control preparations to IVR, IVR-1 and IVR-2, respectively; 7, 23K protein antiserum.

TABLE 1. Effect of the 23K protein electroeluted from SDS-polyacrylamide gel on tobacco mosaic virus (TMV) replication in protoplast from tobacco cultivar Samsun NN.

Sample	Infectivity[a] from test protoplast treated with		Percent inhibition
	Protoplast IVR	Control preparation	
Starting material[b]	7.5±1.5	26±3.8	71
Electroeluted 23K protein	9.0±1.8	28±4.5	68
Control protoplasts[c]	31 ±3.5		

[a]Average number of local lesion and standard error from three to four experiments per 10^6 protoplasts, 72 hr after inoculation on one half leaf of N. glutinosa L. calibrated to a standard TMV (0.5 ug/ml) which yielded about 70 lesions per half leaf.

[b]Equivalent to 3 units.

[c]TMV-inoculated protoplasts with no addition.

TABLE 2. Effect of the 23K protein antiserum on the biological activity of the inhibitor of virus replication (IVR).

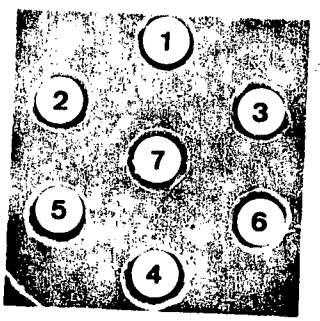
Wells coated with antiserum[a]	Antigen[b]	Infectivity from test protoplast treated with the respective preparation[c]	Percent inhibition
1. -	Protoplast IVR	6.5±2.5	71
-	Control preparation	22.6±5.2	
2. Normal	Protoplast IVR	7.5±2.8	69
	Control preparation	24.5±5.8	
3. Anti-23K protein	Protoplast IVR	18.7±3.5	10
	Control preparation	20.8±4.2	
Control Protoplasts[d]		26.5±4.5	

- [a]Antiserum diluted 1:20 in coating buffer were used to coat ELISA plates.

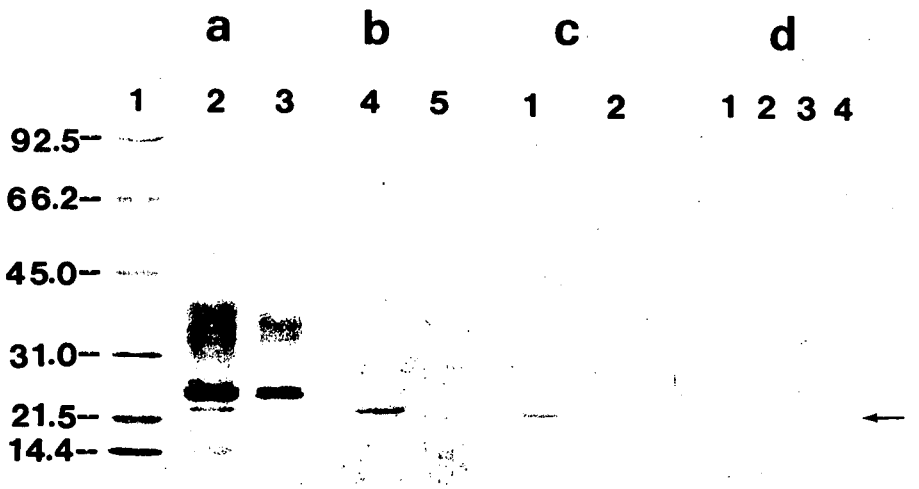
[b]Three units of crude protoplast IVR or control preparation were added.

[c]Average number of local lesion and standard error from three to four experiments per 10^6 Samsun NN tobacco protoplsts 72 hr after inoculation, on one half leave of N. glutinosa L. calibrated to a standard TMV solution (0.5 ug/ml) which yielded about 70 lesions per half leaf.

[d]TMV-inoculated protoplasts in incubation medium with no addition.


1
2
3
7
5
6
4

8q



Similar results were obtained with IVR recovered from the intercellular fluid of TMV-infected leaves (Spiegel, et al., Phytopathology 79:258-262, 89). The electroeluted IVR from the specific 23K band (three units) inhibited TMV replication in protoplasts by 52% (average from three experiments). This provides evidence that the 23K protein is IVR purified to a high degree.

6. Attempts to obtain a partial sequence of the IVR protein

Two attempts were made to obtain a sequence of plant IVR electroblotted onto polyvinylidene difluoride (PVDF, Immobilon) membranes stained with Coomassie Blue, after PAGE. In both cases, crude plant IVR and not electroeluted IVR (see A-5) was electrophoresed, blotted, and used for sequencing. Sequencing was done by Prof. Y. Burstein (Weizman Institute).

In the first experiment, the following sequence of 13 amino acids was obtained: T(P), D, F, R, T, S, E, V, Q, R, G, Y, S.

In the second experiment, the following sequence was obtained: A, V, K, D, F, S(V), V, G, D, L, S(Y), V, P, (D).

As the results of these two experiments were different, it seems that sequencing should be done from IVR purified by electroelution (see A-5) or after two dimensional electrophoresis. Apparently other proteins seem to be close (or overlap) the 23K band obtained in PAGE of crude IVR. These experiments will be done in the future.

7. Miscelaneous studies with IVR

a. Effect of protoplast IVR on replication of virus Y (PVY).

Xanthi-nc tobacco plants were inoculated with necrotic-PVY and kept in the greenhouse. 24 hours after inoculation, disks 11 mm in diameter were cut from inoculated leaves and floated on 5 units of protoplast IVR or control preparation. Disks were incubated at 25°C under continuous illumination. After various times of incubation disks were washed, homogenized and tested by ELISA.

days after inoculation	E_{405} disks floated on	
	IVR	mock
6	0.01	0.18
8	0.002	0.465
9*	0.023	0.216
10	0.043	0.219

*7 days after incubation the medium was removed and fresh IVR and incubation medium were added.

b. Adsorption of IVR to plastic or glass petri dishes.

To see if IVR is lost by adsorption to plastic or glass surfaces, the recovery of IVR by ELISA after different time intervals was evaluated.

Two types of petri dishes (9 cm in diameter) - glass and plastic were tested. 15 units of IVR in 15 ml of 0.05 M sod. phosphate buffer pH 7.0 (1 unit/ml) were placed in each dish. Petris were kept at 25° under about 2000 lux. Samples were taken at various times and assayed by ELISA. 0.2 ml were placed in each well.

Results are averages of three triplicates

E_{405}

	Days after placing IVR in Petri			
	2	3	4	7
Glass	0.566	0.434	0.412	0.295
Plastic	0.243	0.203	0.191	0.194

The original IVR preparation gave E_{405} of 0.511.

Percentage recovery of IVR

	Days			
	2	3	4	7
Glass	100	85	81	58
Plastic	48	40	37	38

As seen under our conditions (15 units/petri) IVR was strongly adsorbed by the plastic and not by the glass. A certain degradation (or adsorption) was observed at day 7.

In small plastic petri dishes (5 cm in diameter) containing 10 units of IVR in 10 ml of sod. phosphate, recoveries of 62 and 54% were observed 3 and 4 days, respectively, after incubation.

B. Preparation of IVR specific m-RNA and immunoprecipitation of it's translation products.

Total mRNA (60 ug) isolated from "induced-resistant" tissue between TMV (I) or control stripes (H) of tobacco Samsun NN, was fractionated on a 5-30% linear sucrose gradient. One ml fractions were collected, and mRNA was recovered from each fraction by adding an equal volume of gradient buffer, 5 ml yeast t-RNA - 9.5 mg/ml and two volumes of absolute ethanol. mRNAs were pelleted and dissolved with 50 ul sterile water. Six 50 ul of the detected mRNA from H_{1-5} and I_{1-5} fractions were translated in a rabbit reticulocyte lysate system in a 50 ul incubation mixture.

The radioactivity incorporated in the translation products was counted after precipitation of 3 ul reaction mixture in 5% trichloroacetic acid on Whatmann 2.5 MM filters. Highest counts were obtained in fractions 2 and 3; ie., 85360 cpm of I-2 compared to 33356 cpm of H-2, and 52760 cpm of I-3 compared to 16042 cpm of H-3. The control retic gave 2012 cpm, and TMV RNA (3 ug) 646486 cpm.

Immunoprecipitation: Immunoprecipitation was carried out in principle as described by Hooft van Huijsduijnen et al., (1985). To 50 ul translation products, sterile H_2O (140 ul) and 25 ul pre-immune serum (to eliminate nonspecific complexes) were added. After five min., 80 ul of a 50% (w/v) protein A-Sepharose (Pharmacia) was added, and the supernatant was treated with anti-IVR serum (100 ul) for 15 min., followed by protein A-Sepharose. The mixture was incubated for 60 min. at room temperature. Samples were centrifuged through a sucrose cushion in immune buffer (0.25 M NaH_2PO_4, 0.25 M NaCl, 5% Triton X-100, 2.5% sodium deoxycholate, and 0.5% SDS, pH 7.3) at 16000 rpm for 25 min. The precipitates were washed twice with immune buffer, resuspended in sample buffer, heated at 100°C for five min., and electrophoresed.

Radiolabelled proteins were visualized by fluorography using Agfa X-ray films.

The poly (A) from infected plants I-2 yielded a translation product which was precipitable with IVR antiserum. The estimated mol. wt. of the specific band corrresponded to that of the IVR; i.e., it was observed in the 20-23,000 region (Fig. 1, lane 8). No such translation products of poly (A) from healthy plants (H-2 and H-3, lanes 5 and 3) were observed.

In another experiment, an attempt to obtain such a specific band from unfractionated poly (A) corresponding to that of the IVR was made. Total mRNA (5 ug) isolated from "induced-resistant" tissue between TMV (Iun) or control stripes (Hun) in Samsun NN was translated in a 50 ul incubation mixture and immunoprecipitated as explained above.

Highest counts again were obtained in the unfractionated-infected treatment (Iun); i.e., 92580 cpm compared to 37050 cpm in the unfractionated-healthy treatment (Hun). The control retic gave 4164 cpm and TMV-RNA (3 ug) 800852 cpm.

After the immunoprecipitation, the poly (A) from infected tissue (Iun) gave a specific band in the 20-30,000 region (Fig. 2, lane 8). This specific band was not observed in the helathy treatment (Hun) lane 7.

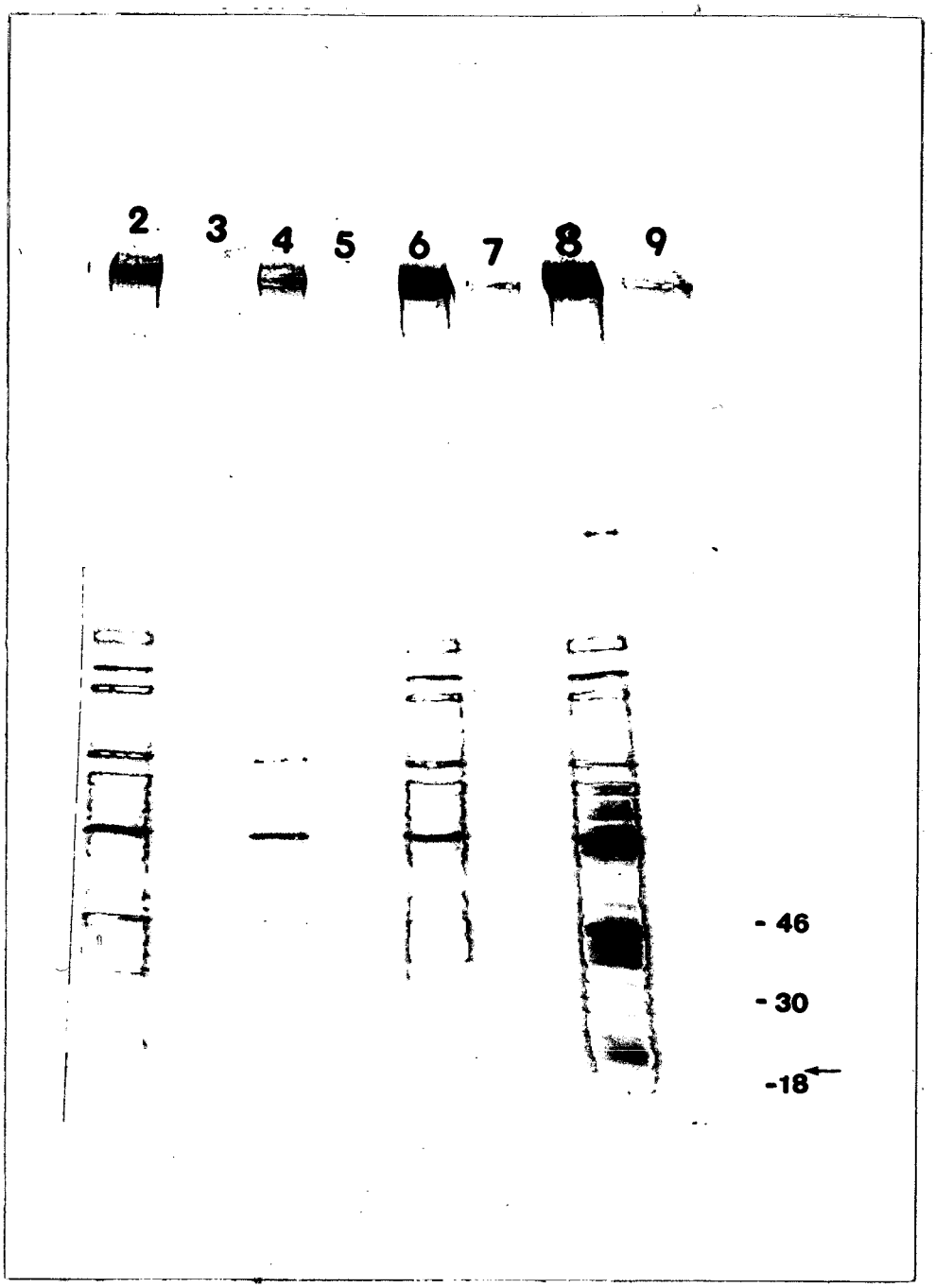


Fig. 1. SDS-PAGE of immunoprecipitated translation products. Translation products of fractionated poly (A) from H-2, H-3 (lanes 4 and 2) and from I-2, I-3 (lanes 8 and 6) immunoprecipitated with normal serum. The same fractions (after precipitation with normal antiserum) were precipitated with IVR antiserum: H-2, H-3 (lanes 5 and 3); I-2, I-3 (lanes 9 and 7). Specfic band is indicated by arrow.

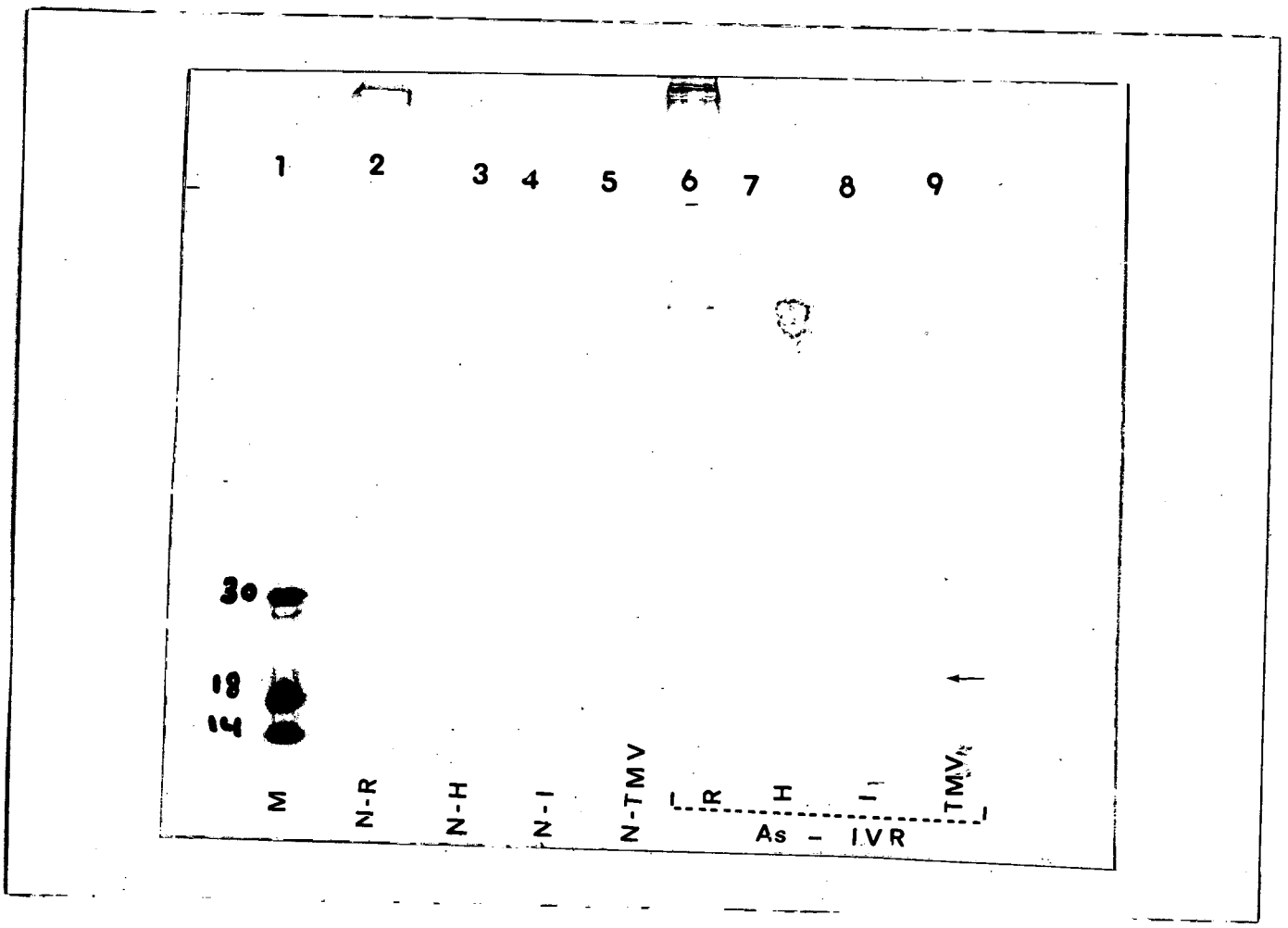


Fig. 2. SDS-PAGE of immunoprecipitated translation products. Radiolabelled products of unfractionated poly (A) from healthy Hun (lane 3), infected plants-Iun (lane 4), retic lysate (lane 2), and TMV-RNA (lane 5) were immunoprecipitated with normal serum; and the supernatant was then precipitated with IVR antiserum, Hun - lane 7, Iun - lane 8, retic - lane 6, and TMV - lane 9. Specfic band is indicated by arrow. The mol. wts of marker proteins (lane 1) are given in Kd.

Poly A translation products form induced resistant tissue

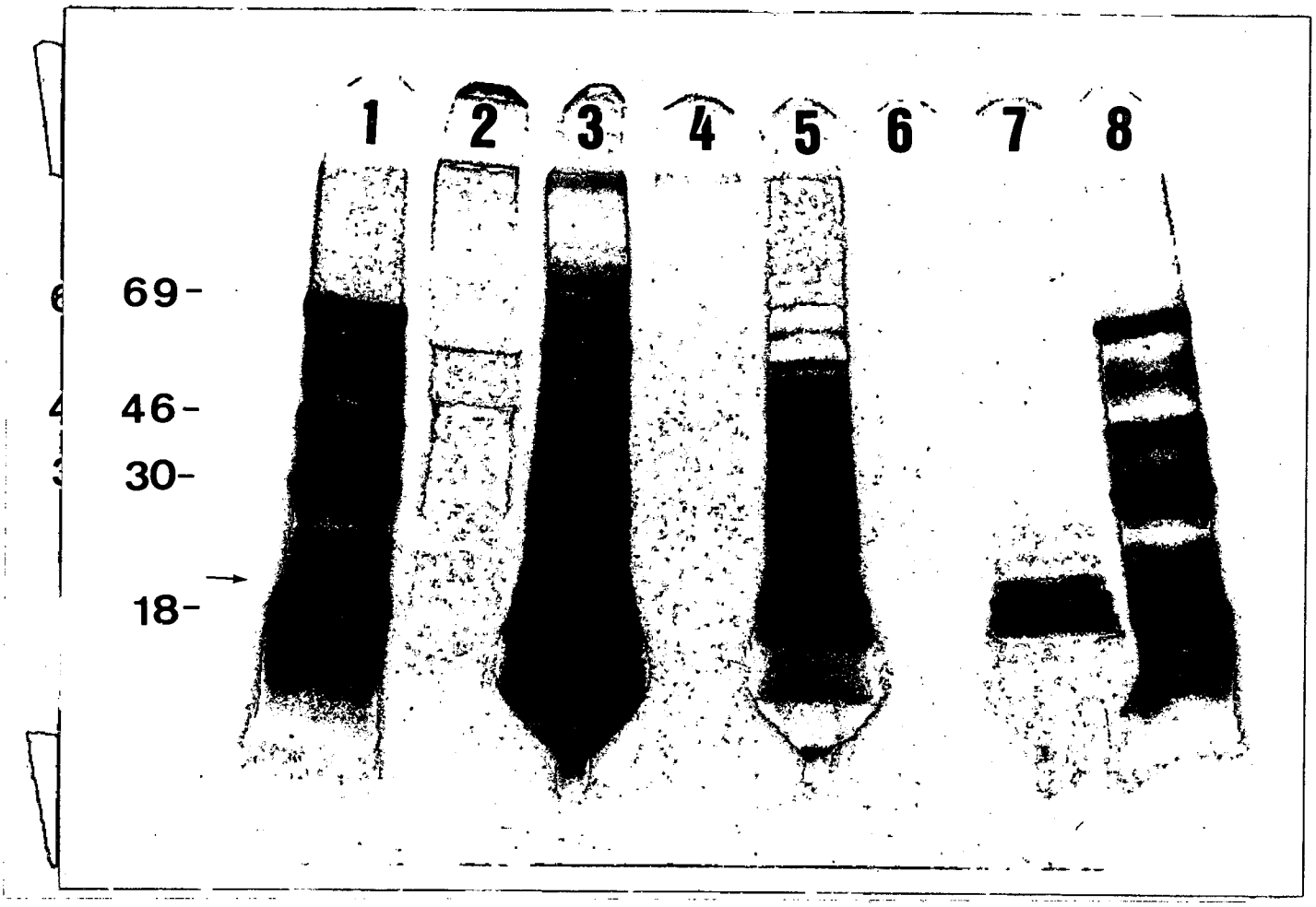


Fig. 2a. SDS-PAGE of immunoprecipitated translation products. Radiolabelled products of unfractionated poly (A) from healthy tobacco NN (lane 5), induced-resistant tissue between stripes (lane 3), and retic lysate (lane 7), were incubated with IVR antiserum and then precipitated; healthy (lane 4), induced-resistant tissue (lane 2), retic (lane 6). Radioactive markers (lanes 1 and 8).

C. Construction and screening of a cDNA library in lambdla gt11 for IVR.

1. Construction and screening

Total RNA was extracted from induced-resistant tobacco Samsun NN tissue between TMV or control strips as described by Hooft Van Huijsduijnen, et al. (1985).

Poly (A) mRNA was obtained by oligo (dT) cellulose chromatography according to Aviv and Leder (1972).

cDNA Synthesis: The poly (A) mRNA was copied into DNA using Amersham cDNA Synthesis System RPN-1256, based on the cDNA synthesis method of Gubler and Hoffman (1983):

The first-strand cDNA was carried out in a reaction volume of 20 ml containing 50 mM Tris-HCl pH 8.3, 10 mM $MgCl_2$, 10 mM DTT, 4 mM Na-pyrophosphate, 1.25 mM De-oxynucleoside triphosphate mix, 15 uCi of ($-^{32}$p)dCTP, 100 u"g/ml of oligo (dT), 1 u"g total poly $(A)^+$ RNA and 20 units reverse transcriptase for 40 min. at 42°C. For second-strand synthesis, 240 n"g of single-stranded cDNA were processed in 20 mM Tris-HCl pH 7.5, 5 mM MgCl2, 10 mM$(NH_4)_2SO_4$, 100 mM KCl, 0.15 mM B-NAD, 50 u"g/ml BSA, 40 mM dNTPs, 0.8 units of E. coli RNase-H, 16 units/ml DNA, polymerase I and 2 units E. coli DNA ligase. Incubations were sequentially 60 min. at 12°C, 60 min at 22°C, and 10 min. at 70°C. The products were purified by phenol/chloroform extraction and ethanol precipitation. The amounts of first and second-strand synthesized were estimated by assaying TCA-insoluble radioactivity:

Treatment	Amount of Orginal poly (A)+ mRNA	First Strand	Second Strand
1. Globin (Control)	0.3 u"g	254 n"g	244 n"g
2. Induced-Resistant Tobacco	1 u"g	482 n"g	568 n"g

Radiolabelled products were visualized by electrophoresis on a 1.4% agarose gel under denaturing conditions (50 mM NaOH) and fluorography using Agfa X-ray films.

Cloning: Double-stranded cDNA was cloned into Lambda gt11 (cloning and expression vector used in constructing cDNA and genomic libraries), Stratagene Cat #234211 - using in principle the Amersham cDNA cloning system RPN 1257: Methylation of cDNA by EcoRI methylase, ligation of EcoRI linkers (GG AA TT CC) onto both ends of the cDNA using T_4 DNA ligase. Digestion of EcoRI linkered cDNA with EcoRI, separation of cDNA from excess linker-molecules using columns. Insertion of EcoRI ended cDNA into Lambda gt11 arms and in vitro packaging of ligation mixtures.

In order to determine the quality, the titer and the packaged ligation products of the library, the final packaged product was incubated with the host strain y 1088 - E. coli for 15 min. at 37°C and 3 ml of top agar (45°C) containing 5 mM ispopropylthiogalactoside (IPTG) were added to (NZY [5gr. NaCl, 2gr. $MgSO_4$, 5gr. yeast extract, and 10gr. NZ amine per 1 liter]/Ampicillin/x-gal [50 ug/ml]) plates. Plaques were visible and counted after incubation for eight hours at 37°C.

Treatment	Total No. of Plaques on 90 mm Plate	Titer *pfu	% Recombinant
1. Induced Resistant insert	1500	1.5×10^7	70%
2. Rheo Test Insert Control of Stratagene	2100	2.1×10^7	76%
3. Lambda gt11 Arms Without Insert	500	0.5×10^7	-

* = pfu = plaque forming unit.

Recombinant (clear) phages were distinguished from non-recombinant (blue) by the ability of the recombinant to form colorless plaques when plated on lac' hosts in the presence of x-Gal.

Screening Lambda gt11 Library with IVR Antibody: Bacterial host strains (y 1088, y 1089, and y 1090) were streaked on LB/ampicillin (50 ug/ml) plates and grown overnight at 37°C. Cultures were started from a single colony and grown overnight with vigourous shaking at 37°C in TB media supplemented with 0.2% maltose and 10 mM $MgSO_4$. The cells were centrifuged at 3000 rpm for 10 min., then gently resuspended in 0.5 volumes of 10 mM $MgSO_4$. The bacterial host strain Y 1088 is restriction negative but methylation positive, so all recominant phages were passed through it before being plated or screened on restriction positive hosts (y 1089 and y 1090).

Screening was carried out on 90 mm plates by mixing 0.1 ml of the y 1090 culuture with 0.1 ml of Lambda buffer (100 mM NaCl, 8 mM $MgSO_4$, 1 M Tris-HCl pH 7.5, and 2% gelatin) containing 1.5 x 10^7 pfu of the Lambda gt11 library for each plate. The phage was absorbed to the bacteria at 37°C for 15 min., then 3 ml LB soft agar (pH 7.5) were added to the mixture and poured onto a pre-warmed LB plate containing 50 ug/ml ampicillin. The plates were incubated at 42°C for three hours, and each plate was overlayed with a dry nitrocellulose filter which had been saturated previously in 10 mM IPTG in H_2O. Plates were incubated three hours longer at 37°C (a second filter was overlaid after the first had been removed) and incubated for an additional three hours.

Nitrocellulose filters were immersed in blocking solution (20 mM Tris HCl pH 7.5, 150 mM NaCl, and 1% BSA) at 4°C for overnight, incubated with polyclonal-IVR antibody with gentle agitation for 1.5 hours at room temperature, transferred to a solution containing goat-anti-rabbit alkaline phosphatase at room temperature for one hour. Finally, the substrate (nitro blue tetrazolium

0.3 mg/ml and 5-Bromo 4-Chloro-3-indolyl-phosphate 0.15 mg/ml) in color development solution (100 mM Tris HCl- pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$) was added in dark for five to ten min.

Positive plaques (one to three per plate) from the induced-resistant insert were clearly visible on some of the plates. These plaques were located, cored out to 1 ml Lambda buffer containing 50 ul chloroform, plated, and rescreened with both polyclonal and monoclonal IVR antibody.

A large number of positive plaques appeared after the amplification of the single-positive plaque when the induced-resistant insert was screened with both monoclonal and polyclonal antibodies but not with the normal serum from mouse and from rabbit (Fig. 3).

Two additional treatments (Rheo test insert - control of the Stratagene company and Lambda gt11 arms without insert) as controls were screened and gave negative results with the polyclonal and monoclonal IVR antibody.

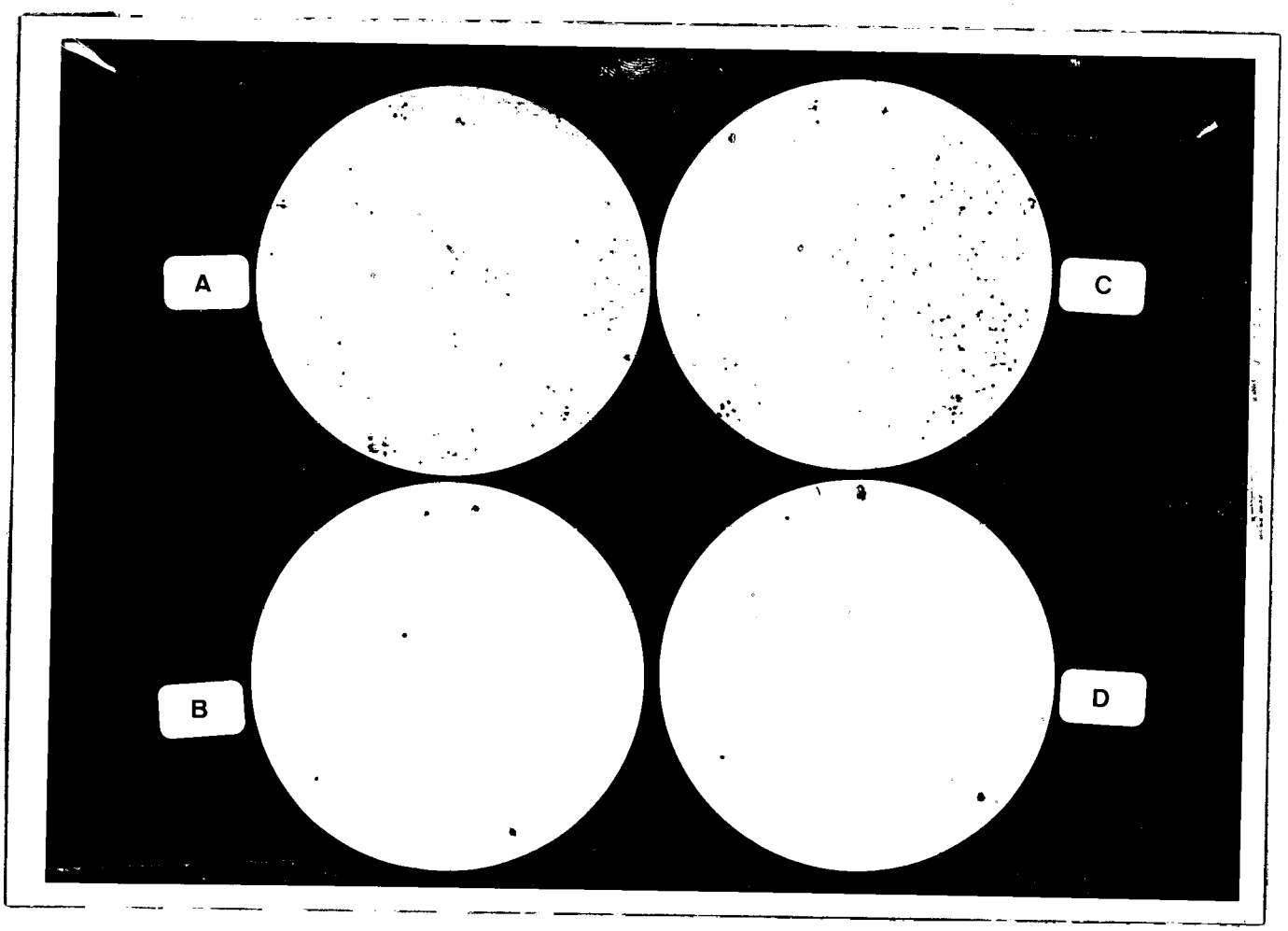


Fig. 3. Immunoscreening (after amplification of positive-reacting plaque) with polyclonal (A) and monoclonal (C) IVR antibodies. B and D normal antisera.

2. Biological activity of cloned IVR

An IVR-like compound was obtained from one of the clones (reacting strongly with the IVR antisera). Crude fusion proteins (I+ = clone strongly positive from the polyclonal and monoclonal screening of the cDNA library in gt11, I- = plant insert from the tobacco NN library, Lambda = phage Lambda gt11 without insert, and C = Rheo test insert control supplied by Stratagene) were precipitated with $ZnAc_2$ as described (Loebenstein & Gera, 1981), and biological activity assayed in the protoplast system, on leaf disks and by spraying.

Protoplast assays were done as described (Loebenstein and Gera, 1981) on tobacco NN protoplasts. Results are summarized in the following table.

Effect of Cloned IVR from Bacteria on Virus Replication in Samsun NN protoplasts[a]

Protein Extract from Bacteria with	Percentage Inhibition Infectivity
IVR clone[b]	75
Plant DNA Insert	0
Control Lysate	0
Control Protoplasts[c]	0

[a]Averages from three to four experiments, tested on 10^6 protoplasts from each treatment (Loebenstein & Gera, 1981). Normalized to a standard TMV solution (2.5 ug/ml) which yielded about 60 lesions.

[b]Approximately one unit.

[c]Inoculated protoplasts without addition of extract.

In the leaf disk assay, the cloned IVR inhibited TMV replication in Samsun tissue. Samsun tobacco plants were inoculated with TMV 2.5 ug/ml and kept at 25°C for five hours. After that, disks, 11 mm in diameter, were collected from the infected leaves and floated on 0.07 M phosphate buffer pH. 7.0, containing the fused proteins (approximately two units from each: I+, I-, C, and Lambda) in 5 cm Petri dishes. The Petri dishes were incubated at 25°C under continuous illumination. After various times of incubation, two disks from each treatment were washed with distilled water and homogenized in 2.5 ml of 0.05 M phosphate buffer pH 7.0. The homogenate was used to inoculate 12 half-leaves of Nicotiana glutinosa plants in comparison with a standard solution of purified TMV (2.5 ug/ml) on the opposite half-leaf. Seventy-two hours after infection, local lesions appeared and were counted. The cloned crude IVR inhibited TMV replication in Samsun leaf disks, the inhibition rate being dose responsive; ie., 0.2, 0.8, and 2 units gave respective inhibition rates of 36%, 68%,a nd 88% assayed 72 hours after inoculation, averages from two experiments.

A similar experiment was done with potato virus X (PVX) infected N. glutinosa leaf disks. When leaf disks were sampled after five days, 83% inhibition was obtained for cloned IVR. No effect was noted when the fusion proteins of the plant insert (I-), control lysate (Lambda), or insert supplied by Stratagene (C) were tested. When approximately two units of cloned IVR were tested, a 76% inhibition (averages from two experiments) was obtained, assayed 72 hours after inoculation.

D. Regulation of the N gene and the IVR gene (U.S.)

1. Induction of necrosis

A major objective of the Riverside laboratory is to understand how viruses induce necrosis. An important extension of that research is to examine how viruses induce IVR and to correlate induction of necrosis, induction of IVR, and confinement of the virus to the initial infection area. We have made progress in understanding how TMV induces IVR in different plants. Objectives of the grant were to define sequences of TMV involved with different plants and to correlate the produciton of IVR to necrotization. In N'N' plants, we have demonstrated that the coat protein gene of TMV is an elicitor for induction of the hypersensitive reaction HR. In NN genotype plants, we have shown that the coat protein gene is not involved in induction of necrosis and that so far induction of IVR is always associated with TMV-induction of necrosis.

In an N'N' genotype plant, N. sylvestris, we demonstrated that alterations in the coat protein gene can be involved in induction of HR (Knorr and Dawson, 1988). A single nucleotide alteration, 6157 (C to U) was shown to be responsible for the change in phenotype from systemic infection to induction of HR. This nucleotide alteration changes coat protein amino acid 148 from serine to phenylalanine.

Results in detail are in the attached publication. Proc. Natl. Acad. Sci. USA 85:170-174, 1988.

Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 170–174, January 1988
Genetics

A point mutation in the tobacco mosaic virus capsid protein gene induces hypersensitivity in *Nicotiana sylvestris*

(*in vitro* transcription/recombinant DNA/genetic mapping/*N'* gene)

DAVID A. KNORR AND WILLIAM O. DAWSON

Department of Plant Pathology, University of California, Riverside, CA 92521

Communicated by George A. Zentmyer, September 8, 1987

ABSTRACT **In *Nicotiana sylvestris*, the *N'* gene confers hypersensitive resistance to some strains of tobacco mosaic virus (TMV) but not to the common strain. TMV sequences responsible for inducing local lesion formation in this host were identified by using cDNA clones to construct genomic recombinants between the common strain genome and a local-lesion-inducing mutant. To assay for sequences conferring the mutant phenotype, *in vitro* transcripts of recombinants were inoculated onto leaves of *N. sylvestris* and observed for the formation of either local lesions or a systemic infection. Sequences from the mutant that converted the hybrid genome to the mutant phenotype were located between nucleotides 5972 and 6206. Sequence analysis of this region revealed point mutations in the mutant at nucleotides 6157 (cytosine to uracil) and 6199 (adenine to guanine). The mutation at 6157 changes the capsid protein gene to specify phenylalanine rather than serine at position 148; nucleotide 6199 occurs in the 3' nontranslated region. When each point mutation was individually substituted into the wild-type background, transcripts containing only the alteration at 6157 produced local lesions on *N. sylvestris*, whereas transcripts containing only the alteration at 6199 produced systemic mosaic symptoms. The frequency of mutation was examined by partially sequencing virion RNA from six additional independent local-lesion mutants. Five mutants had the same alteration at 6157 as the original mutant and none had the alteration at 6199. This work demonstrates that the capsid protein gene of TMV is multifunctional, both encoding the virion structural protein and mediating the outcome of infection in *N. sylvestris*.**

During interactions between plants and parasites, specific incompatibility often is manifested by rapid necrosis of infected and neighboring host cells. Occurrence of this phenomenon, termed the hypersensitive reaction (HR), frequently results in resistance of plants to diseases caused by a variety of pathogens (1–3). Because plant virus diseases are controlled most effectively by host resistance, which often is conferred by single dominant genes, HR-type resistance is useful in plant breeding programs. Unfortunately, sources for HR-type resistance are unavailable for many plant-virus systems, due in many cases to difficulty in transferring plant-virus HR genes by conventional breeding practices (3). Better characterization of host and pathogen genetic elements involved in hypersensitivity has great potential for developing new methods to control losses from plant disease.

Hypersensitivity usually results when host genes for resistance interact with complementary genes for avirulence in the pathogen. Through genetic studies, a number of pathogen-specific host resistance genes and specific avirulence genes in bacterial pathogens have been identified. For instance, avirulence genes have been isolated and characterized by Staskawicz *et al.* (4) and Gabriel *et al.* (5). Both groups isolated avirulence genes by transferring cloned DNA library fragments between different pathogen races, then testing these for incompatibility on host plants with defined race-specific resistance genes. Schoelz *et al.* (6), using engineered recombinations between two strains of cauliflower mosaic virus, showed that gene *VI* determines whether infection will be compatible or result in the HR in *Datura stramonium*.

In RNA plant virus–host systems, many host genes for hypersensitive resistance have been identified. In *Nicotiana* species, the *N* gene, originally from *Nicotiana glutinosa*, confers resistance to all strains of tobacco mosaic virus (TMV). Plants containing the *N* gene respond to TMV by forming small areas of necrosis, termed local lesions, surrounding infection sites (7). The *N'* gene from *Nicotiana sylvestris* confers resistance to some strains of TMV but not to the common strain. However, mutants of the common strain may be isolated that form local lesions on hosts containing the *N'* gene (8). Although there is little understanding of viral genes involved in eliciting this response, some workers have found differences in TMV associated with the ability to induce the HR in *N'* gene hosts (9, 10). However, exact viral sequences involved in conferring the HR phenotype have not been identified.

We have recently generated a system that allows functions of TMV to be precisely mapped. This was accomplished by cloning the entire viral genome as cDNA from which infectious transcripts may be produced *in vitro* (11), thereby extending TMV replication through an artificial DNA phase. This allows the viral genome to be manipulated as DNA, then converted into RNA for observation of biological effects. Using chimeric TMV genomes constructed by exchanging cloned cDNA segments between a local-lesion mutant and its wild-type parent, we have identified a point mutation at nucleotide 6157 that alters the capsid protein gene to specify phenylalanine rather than serine at position 148 and that is responsible for conferring the ability of TMV to elicit the HR in *N. sylvestris*.

MATERIALS AND METHODS

Virus Strains and Mutants. The parent virus for these studies, TMV204, was obtained by infecting Xanthi tobacco (*Nicotiana tabacum* Linnaeus var. Xanthi) with *in vitro* transcripts of plasmid pTMV204 as described previously (11). Purified TMV204 progeny virions were treated with nitrous acid as described by Siegel (12), diluted to 60 ng/μl in 0.1 M potassium phosphate buffer (pH 7.0), then inoculated onto leaves of *N. sylvestris* Spegazzini and Comes. Necrotic local lesions that appeared 3–5 days after inoculation were ground individually between sterilized glass microscope slides in 0.01 M potassium phosphate buffer (pH 7.0) and (1% wt/vol) Celite, then transferred to separate leaves of *N. sylvestris*.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HR, hypersensitive reaction; TMV, tobacco mosaic virus.

After several transfers, mutants producing only local lesions without systemic spread were propagated by systemic infection in tomato or Xanthi tobacco, then purified by differential centrifugation. Care was taken to ensure that mutants derived by nitrous acid treatment were separately maintained, at first on individual leaves and later on separate plants.

cDNA Synthesis and Cloning. Procedures for RNA preparation and cDNA cloning were essentially as described previously (11). Mutant double-stranded cDNA was prepared for cloning by adding synthetic *Xho* I linker sequences, then digesting with *Bam*HI and *Xho* I. To accept mutant cDNAs, plasmid pBR322-XhoI was constructed by removing overhanging nucleotides from *Hin*dIII-digested pBR322 with mung bean nuclease (Pharmacia), then adding *Xho* I linkers. Mutant cDNAs then were inserted between the *Bam*HI and *Xho* I sites of pBR322-XhoI.

Construction of Recombinant Genomes. Subclones and engineered recombinants between mutant and wild-type sequences were generated by ligating specific DNA restriction fragments excised from low melting-point agarose electrophoresis gels according to Crouse *et al.* (13). pDK4 was constructed by ligating the promoter-containing (14) 5′ *Pst* I(−856)–*Bam*HI(3333)* fragment from pTMV204 to the *Bam*HI–*Xho* I(6402) fragment from pDK3, a cDNA clone of the mutant, and inserting both together into *Pst* I–*Xho* I-digested pBR322-XhoI (map in Fig. 1). Two recombinants were generated with mutant sequences between the *Bam*HI-(3333) and *Nco* I(5460) sites (pDK15), and between the *Nco* I(5460) and the *Xho* I(6402) site at the 3′ terminus (pDK14), by exchanging *Nco* I(5460)–*Sal* I fragments between pTMV204 and pDK4 (Fig. 2). To make further recombinations at restriction sites not unique within the TMV sequence, pDK16 was constructed by inserting the 1.8-kb *Hin*dIII-(5081)–*Sau* I fragment from pDK14 into pUC19. Recombinations made with pDK16 as the recipient (Fig. 1) were confirmed by restriction map analysis. In a separate ligation reaction, genomic-length recombinants were regenerated by replacing the *Nco* I(5460)–*Sal* I fragment from pTMV204 with the same fragment from one of the intermediate subclones (Fig. 2). Intermediate pDK17 was constructed by replacing the *Sau* I(5805)–*Sal* I restriction fragment from pDK16 with the same fragment from pTMV204. Intermediate pDK18 was constructed similarly by replacing the *Hin*dIII(5081)–*Sal* I(5805) fragment from pDK16 with the corresponding wild-type fragment. Intermediates pDK21, pDK22, and pDK23 were constructed by replacing the *Asu* II(6364)–*Sal* I, *Nsi* I(6207)–*Sal* I, and *Bsm* I(5971)–*Sal* I fragments from pDK18 with the corresponding wild-type fragments (Fig. 1).

A subclone of pTMV204, p3F1, contains the *Bam*HI-(3333)–*Pst* I(6406) fragment in pUC19. To construct pDK27, the *Acc* I(6056)–*Sfa*NI(6192) fragment from pDK17 and the *Sfa*NI(6192)–*Pst* I(6406) fragment from pDK21 were ligated into p3F1 from which the *Acc* I(6056)–*Pst* I(6406) fragment had been removed. Plasmid pDK28 was constructed similarly, but using the *Acc* I(6056)–*Sfa*NI(6192) fragment from pDK21 and the *Sfa*NI–*Pst* I fragment from pDK17. The *Nco* I(5460)–*Pst* I fragments from pDK27 and pDK28 were separately ligated into pDK17, generating pDK29 and pDK30 (Fig. 1). Recombinations engineered into subclones were incorporated into pTMV204 as illustrated in Fig. 2.

Transcripts of plasmids containing recombinant viral genomes were prepared as described previously (11) and inoculated directly onto *N. sylvestris*.

Nucleotide Sequencing. Nucleotide sequences for cloned TMV cDNAs were determined by using the dideoxy chain-terminating method (15) or by the chemical method (16). Virion

*Numbers in parentheses beside restriction endonucleases indicate positions of sites with respect to the 5′-terminal nucleotide of TMV.

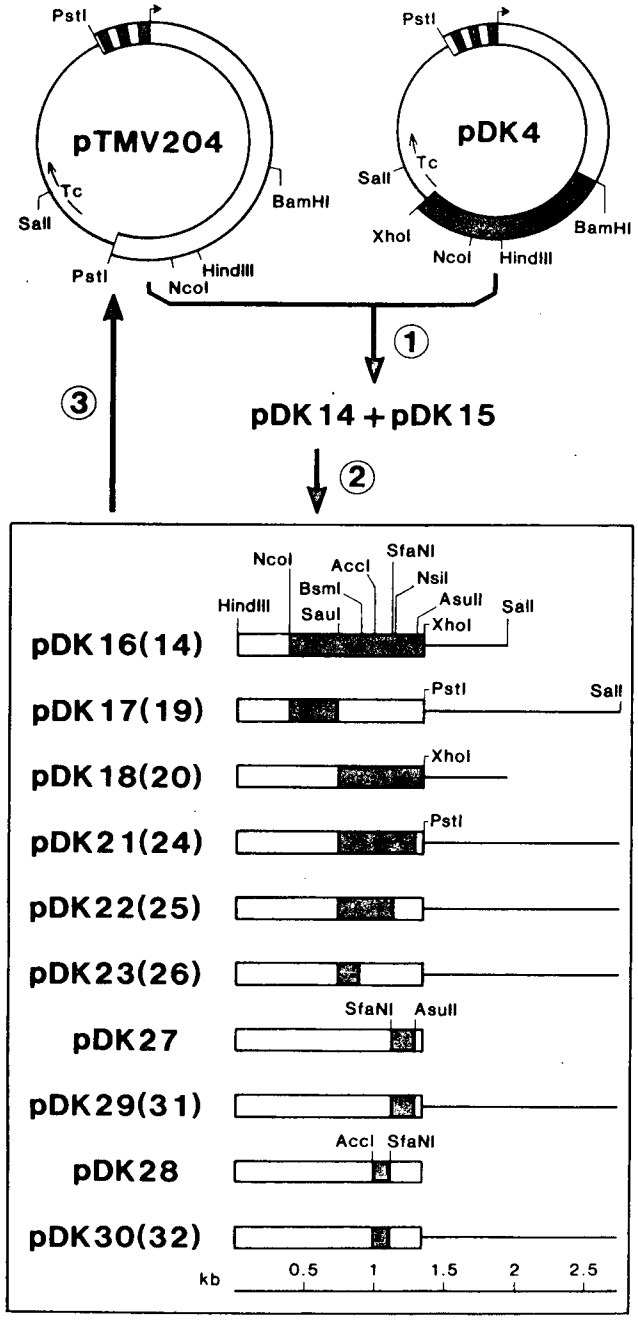


FIG. 1. Intermediate subclones generated for constructing recombinant TMV genomes used to map the mutation responsible for the local-lesion phenotype of mutant TMV204-D1. Circular maps of wild-type pTMV204 and pDK4, a local-lesion-producing recombinant, are represented above. Alternating dark and light bands represent promoter sequences, with transcription starts indicated by bent arrows. Dark areas represent sequences from a mutant cDNA clone (pDK3), open areas represent sequences from pTMV204, and single lines represent vector (pBR322) sequences (Tc, tetracycline resistance gene). Different combinations of restriction fragments from pTMV204 or pDK4 inserted into pUC19, or into p3F1 for pDK27 and pDK28, to form the intermediate subclones are represented in the box below. Step 1, exchange *Nco* I–*Sal* I fragments. Step 2, subclone pDK14 *Hin*dIII–*Sal* I fragment in pUC19. Step 3, replace *Nco* I–*Sal* I fragment from pTMV204 with *Nco* I–*Sal* I fragments from intermediate subclones. Numbers in parentheses indicate the genomic-length recombinant constructed from each subclone. Scale in kilobases (kb) is shown at the bottom.

RNA sequences were obtained essentially as described by Zimmern and Kaesberg (17). Synthetic oligonucleotide primers complementary to specific regions of the genome were annealed with template RNA in water at a primer-to-template molar ratio of 2:1 at 70°C, slowly cooled to 20°C, and then stored on ice. Individual termination reaction mixtures contained 0.2 μg of annealed primer-template, a 5:1 molar ratio of a single deoxynucleoside/dideoxynucleoside triphosphate for each reaction, and 2.0 units of avian myeloblastosis virus reverse transcriptase (Molecular Genetics Resources, Tampa, FL) in a total volume of 6.0 μl.

RESULTS

The initial objective was to obtain TMV mutants that induce the HR in *N. sylvestris* instead of systemically invading this host and causing mosaic symptoms as with the wild-type parent. This mutant phenotype has been previously described (8, 12). Since cDNA clones of these mutants ultimately would be used to construct hybrids with wild type, it was important that they be generated from a uniform population whose cDNA would have common restriction endonuclease sites. Thus, the wild-type parental TMV used in these experiments was obtained from *in vitro* transcripts of pTMV204. This source was expected to provide a near-homogeneous virus population free of contaminating strains that are normally present in naturally derived wild-type virus populations.

Seven nitrous acid mutants of TMV204 that produced local lesions on *N. sylvestris* were isolated, purified, and used in this study. These mutants generally were initially contaminated with wild-type virus that systemically infected the assay host, *N. sylvestris*. Mutants free of the wild-type virus were selected after several single-lesion transfers on the basis of their inability to spread systemically in *N. sylvestris*. Only local-lesion mutants that failed to spread systemically were further analyzed.

Mutant TMV204-D1 was chosen for detailed study to determine which genomic sequences were responsible for conferring the local-lesion phenotype. A library of cDNA clones of mutant TMV204-D1 was made. Restriction map analysis indicated that clone pDK3 contained a cDNA insert corresponding to the 3-terminal 3062 nucleotides of TMV. This cDNA of the mutant TMV204-D1 was used to determine if sequences responsible for altered phenotype mapped in the 3′ half of the TMV genome. The recombinant plasmid pDK4 (Fig. 1) contains viral 5′ sequences from wild-type pTMV204 and viral 3′ sequences from mutant pDK3. Transcripts made *in vitro* from *Xho* I-digested pDK4 produced local lesions on *N. sylvestris*, whereas transcripts of pTMV204 did not, indicating that the local-lesion phenotype of TMV204-D1 maps within the 3′ half of the genome.

To find specific sequences in the 3′ segment of TMV204-D1 responsible for the induction of local lesions in *N. sylvestris*, we constructed a series of recombinants that, in effect, progressively substituted smaller segments of mutant cDNA into the cloned wild-type genome (Figs. 1 and 2). To ensure that changes outside of the segments altered by engineered recombination were not responsible for changes in phenotype, only pTMV204 was used as the genetic background for this study, and *in vitro* transcripts of pTMV204 were inoculated into test plants as a control in each experiment. In no case did transcripts of pTMV204 produce local lesions on *N. sylvestris*. Furthermore, independent transcriptions from each of the engineered genomic-length recombinants were tested several times and in each case the phenotype was consistent.

The 3-kb segment of mutant sequence in pDK4 was split at the *Nco* I(5460) site to give pDK14 and pDK15 (Fig. 2). *In vitro* transcripts from pDK14 produced local lesions on *N. sylvestris*, whereas those from pDK15 produced systemic mosaic symptoms. Because further definition of mutant sequences would require digesting with restriction enzymes recognizing more than one site within the entire TMV cDNA, subclone pDK16 was used as an intermediate for constructing additional changes (Fig. 1). Segments of mutant sequences in pDK16 were progressively replaced by the corresponding wild-type segments from pTMV204. The resulting intermediate constructs then were directionally inserted into pTMV204 in place of the *Nco* I(5460)–*Sal* I restriction fragment (Fig. 1). In the case of pDK19 and pDK20, in which the mutant segment from pDK14 was split at the *Sau* I(5805) restriction site, pDK20 produced local lesions in *N. sylvestris*, whereas pDK19 produced only systemic infection. Three additional plasmids, pDK24, pDK25, and pDK26, were constructed by progressively replacing the mutant sequences in pDK20 with wild-type sequences. Symptoms produced after inoculation with *in vitro* transcripts of the full-length cDNAs indicated that ability to induce local lesions was retained in pDK24 within the 559-base-pair (bp) *Sau* I(5805)–*Asu* II(6364) fragment, and in pDK25, within the 402-bp *Sau* I(5805)–*Nsi* I(6207) fragment. However, pDK26 contains the 166-bp *Sau* I(5805)–*Bsm* I(5971) fragment from the mutant and produces a systemic mosaic rather than local lesions (Fig. 2). Therefore, the difference between ability to induce either local lesions or systemic infection in *N. sylvestris* resided between nucleotides 5971 and 6206.

The nucleotide sequence of the segment that alters phenotype of mutant TMV204-D1 was compared with the same region in the wild-type parent by both chemical and dideoxynucleotide sequencing methods. Two point mutations were found within the *Bsm* I(5971)–*Nsi* I(6207) fragment of pDK21 (Fig. 1). At nucleotide 6157, cytosine found in the wild type was replaced by uracil in the mutant, and at nucleotide 6199, wild-type adenine was replaced by guanine. The same changes within this region were found in TMV204-D1 by sequencing progeny virus RNA. The mutation at nucleotide 6157 occurs within the capsid protein gene (nucleotides 5712–6191) and alters the codon triplet for serine at position 148 to that for phenylalanine. Nucleotide 6199 occurs in the 3′ nontranslated region.

To determine whether both exchanges were required for conferring the mutant phenotype, each point mutation was separately engineered into the wild-type genome (Fig. 1). Sequence analysis of the carboxyl-terminal region of the capsid protein genes showed that pDK31 contains only the point mutation at nucleotide 6199 and pDK32 contains only the mutation at 6157. *In vitro* transcripts of pDK32 produced necrotic local lesions without systemic infection in *N. sylvestris*, whereas those from pDK31 produced only systemic symptoms with no necrosis. Also, the single nucleotide changes in pDK31 and pDK32 were retained by the RNA of progeny virus. Thus, a point mutation at nucleotide 6157 in pDK32 is responsible for conferring the local-lesion phenotype of TMV204-D1.

All TMV mutants with the local-lesion phenotype may not contain the same nucleotide alteration. The frequency of this mutation was examined by determining virion RNA sequences between nucleotides 6115 and 6217 for six other independently derived local-lesion mutants. In five of the mutants, cytosine was replaced by uracil at nucleotide 6157. None of the mutants contained the additional change found in TMV204-D1 at nucleotide 6199, and no other differences from the wild-type sequence were detected. Although sequence data for mutant TMV204-D7 were extended to include nucleotides 5916–6217, no alterations from wild type were detected. A total of six of seven independent mutants that were examined had the same alteration at nucleotide 6157, indicating that this mutation apparently occurred with high frequency. On the other hand, mutant TMV204-D7 did

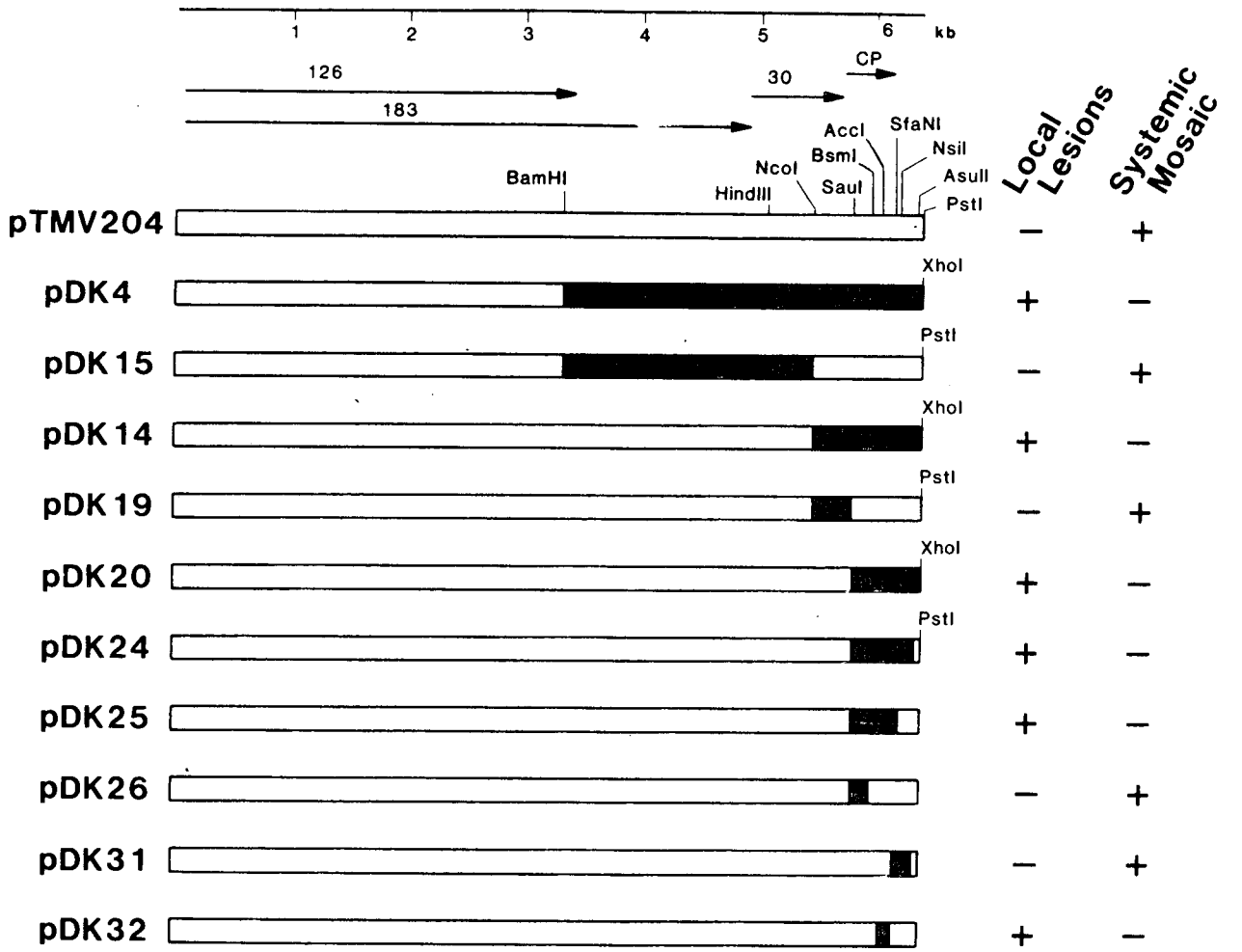


FIG. 2. Composition and symptomatology of wild-type and 10 recombinant TMV genomes. Dark portions of genomes show sequences from TMV204-D1, a mutant that induces local-lesion formation on *N. sylvestris*. Light portions of genomes represent sequences from the wild-type parental strain, TMV204. Relative positions of restriction endonuclease sites used in engineering the constructs are shown for pTMV204. The 3′ termini derived from pDK4 contain a *Xho* I restriction site, whereas all other 3′ termini contain a *Pst* I restriction site. Symptoms produced on *N. sylvestris* plants inoculated with *in vitro* transcripts of the plasmids are indicated beside each genome. The size of the TMV genome in kb is represented above. Arrows represent open reading frames, with the mass of product in kilodaltons; CP, capsid protein.

not contain the same alteration, indicating that changes in another region of the genome could also account for this phenotype.

DISCUSSION

In this study, we identified a point mutation in the coat protein gene of one TMV mutant that is responsible for inducing production of local lesions in *N. sylvestris*. This was accomplished by exchanging cloned cDNA fragments between the mutant and its wild-type parent to engineer a set of defined viral recombinants. The initial construct, pDK4, contained a recombinant TMV genome with a 3-kb cDNA fragment from mutant TMV204-D1. In subsequent constructs, sequences from the mutant cDNA were systematically replaced by corresponding sequences from the wild type. Ultimately, we constructed pDK32, containing only a single nucleotide difference from the wild-type parent virus. The cytosine-to-uracil alteration in this construct changes the capsid protein gene to encode phenylalanine in exchange for serine at position 148. Virus from pDK32 *in vitro* transcripts expressed the mutant phenotype, demonstrating that the single nucleotide substitution was sufficient to induce local lesions in *N. sylvestris*.

This study demonstrates that mutations conferring the ability to induce *N′* gene-associated HR can map within the viral capsid protein gene. This strengthens interpretations of earlier work that associated amino acid exchanges and altered physical properties in the capsid protein with this class of TMV mutants. Amino acid exchanges in the capsid proteins of numerous TMV mutants were identified in the laboratory of Fraenkel-Conrat in early efforts to decipher the genetic code (9). Later, Fraser described differences in the physical properties of capsid proteins of TMV local-lesion mutants (10). Although, several of the mutants studied had phenotypes similar to the ones we have described, the technology available did not allow a positive demonstration that capsid protein changes were related to phenotype changes. It is possible that the ability to induce HR in *N. sylvestris* may be associated with various other amino acid changes. However, because earlier data suggested the likelihood of random changes, we were surprised that six of seven independent mutants had identical changes in the capsid protein gene.

It is possible that the altered capsid protein induces the HR. However, we cannot exclude the possibility that RNA is the active molecule. The exchange in the capsid protein of TMV204-D1 occurs within a loop structure near the protein

surface that is not highly conserved within the tobamovirus group and can undergo rearrangement (18). Other mutants with this same exchange were previously reported by Funatsu and Fraenkel-Conrat (9). Analyzing one of these, van Regenmortel reported it to be distinguishable serologically from wild type (19), suggesting that the mutant capsid protein is structurally different from wild type. However, the mutation probably does not significantly impair the virion assembly function, since normal amounts of stable virions were formed. This implies that the capsid protein gene has a secondary function involved in establishing virus–host compatibility during the infection process. In a number of cellular recognition systems, conformational changes in proteins can alter receptor–target binding characteristics (reviewed in ref. 20). For instance, receptors for fibronectin and vitronectin both depend upon the same tripeptide sequence for binding, yet recognition remains mutually exclusive. Interpretation of analysis of binding with partial peptides indicates that size and conformation of targets may play a role in specific recognition by receptors. With TMV, it is conceivable that altered capsid protein from necrosis-inducing mutants is specifically recognized by cellular receptors involved in induction of the HR. It is not clear whether the mutant, but not the wild-type, capsid protein may act as an elicitor, or if the wild type, but not the mutant, suppresses the plant HR induced by virus-mediated processes.

Sequence alterations outside the capsid protein gene may also induce the *N'* gene HR. Mutant TMV204-D7 produced local lesions on *N. sylvestris* but did not contain the point mutation at nucleotide 6157, nor was a mutation detected in the sequence of approximately two-thirds of the capsid protein gene. Determining which alterations confer phenotype in this mutant would require substitution experiments similar to those used to map TMV204-D1. However, from other experiments, we now know that some viral product other than that related to the capsid protein gene is responsible for induction of hypersensitivity in plants with the *N* gene, because engineered TMV mutants with the entire capsid protein gene deleted induce the HR in Xanthi-nc tobacco (unpublished results). This situation suggests that *N'* gene recognition is triggered by viral products, such as the capsid protein, that may not normally be required for maintaining infection. In contrast, the *N* gene, which no variant of TMV is known to overcome, may recognize a factor essential for virus survival, such as replicase. The data of Kado and Knight (21, 22) suggested that a region in the gene encoding the 30-kDa protein or the carboxyl terminus of the 183-kDa protein might be involved with induction of the HR in *N. sylvestris*. With cauliflower mosaic virus, Schoelz and coworkers have shown that a segment of open reading frame *VI*, which encodes the inclusion body protein, controls the ability to produce necrotic lesions in *Datura stramonium* (6). In addition, mutants of TMV exist that are altered in ability to induce the HR in numerous hosts outside of the genus *Nicotiana*. It will be interesting to determine whether these mutations map within the capsid protein gene or are dispersed throughout the genome.

We thank Dr. Paul Ahlquist and Agrigenetics, Inc., for plasmid pPM1 and Drs. Myron Brakke, William Dougherty, Larry Grill, Andrew Jackson, Noel Keen, T. Jack Morris, and Robert Shepherd for useful comments on the manuscript. This research was funded in part by Grant 8607638 from the National Science Foundation and Grant 85-CRCR-1-1795 from the U.S. Department of Agriculture.

1. Dixon, R. A. (1986) *Biol. Rev.* **61**, 239–291.
2. Sequira, L. (1984) in *Encyclopedia of Plant Physiology*: New Series, eds. Linskens, H. F. & Heslop-Harrison, J. (Springer, Berlin), Vol. 17, pp. 187–211.
3. Russell, G. E. (1978) *Plant Breeding for Pest and Disease Resistance* (Butterworth, Boston), pp. 231–264.
4. Staskawicz, B. J., Dahlbeck, D. & Keen, N. T. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6024–6028.
5. Gabriel, D. W., Burges, A. & Lazo, G. R. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 6415–6419.
6. Schoelz, J., Shepherd, R. J. & Daupert, S. (1986) *Mol. Cell. Biol.* **6**, 2632–2637.
7. Holmes, F. O. (1929) *Bot. Gaz. (Chicago)* **87**, 39–55.
8. Valleau, W. D. (1952) *Phytopathology* **42**, 40–42.
9. Funatsu, G. & Fraenkel-Conrat, H. (1964) *Biochemistry* **3**, 1356–1362.
10. Fraser, R. S. S. (1983) *Physiol. Plant Pathol.* **22**, 109–119.
11. Dawson, W. O., Beck, D. L., Knorr, D. A. & Grantham, G. L. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 1832–1836.
12. Siegel, A. (1960) *Virology* **11**, 156–167.
13. Crouse, G. F., Frischauf, A. & Lehrach, H. (1983) *Methods Enzymol.* **101**, 78–89.
14. Ahlquist, P. & Janda, M. (1984) *Mol. Cell. Biol.* **4**, 2876–2882.
15. Zagursky, R. S., Baumeister, K., Lomax, N. & Berman, M. L. (1985) *Gene Anal. Tech.* **2**, 89–94.
16. Maxam, A. M. & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 560–564.
17. Zimmern, D. & Kaesberg, P. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4257–4261.
18. Altschuh, D., Lesk, A. M., Bloomer, A. C. & Klug, A. (1987) *J. Mol. Biol.* **193**, 693–707.
19. van Regenmortel, M. H. V. (1967) *Virology* **31**, 467–480.
20. Ruoslahti, E. & Pierschbacher, M. D. (1986) *Cell* **44**, 517–518.
21. Kado, C. I. & Knight, C. A. (1966) *Proc. Natl. Acad. Sci. USA* **55**, 1276–1283.
22. Wilson, T. M. A., Perham, R. N., Finch, J. T. & Butler, P. J. G. (1976) *FEBS Lett.* **64**, 285–289.

Results of this last year showed that numerous changes throughout the coat protein gene (amino acids 11, 20, 25, and 46) can induce HR in this plant.

Results in detail are summarized in the attached publication. Plant - Microbe Interactions 2:209-213, 1989.

MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 2, No. 4, pp. 209-213, 1989

Point Mutations in the Coat Protein Gene of Tobacco Mosaic Virus Induce Hypersensitivity in *Nicotiana sylvestris*

James N. Culver and William O. Dawson

Department of Plant Pathology, University of California, Riverside 92521 U.S.A.
Received 6 March 1989. Accepted 5 April 1989.

Four point mutations in different regions of the coat protein gene of tobacco mosaic virus (TMV) were created by site-directed mutagenesis. Mutations were made at nucleotide 5745 (guanine to adenine), 5773 (cytosine to uracil), 5788 (adenine to guanine), and 5850 (adenine to guanine) to alter single amino acids in the coat protein at position 11 (Val to Met), 20 (Pro to Leu), 25 (Asn to Ser), and 46 (Arg to Gly), respectively. Infectious transcripts of the four mutagenized cDNA clones produced phenotypically distinct hypersensitive reactions on *Nicotiana sylvestris* (N′ genotype) and systemic responses on *N. tabacum* 'Xanthi' (n genotype). The parental wild-type virus produced systemic mosaic symptoms on both hosts. Sequence data from virion RNA as well as maintenance of phenotype showed that the introduced mutations were stable. This work demonstrates that a number of sites in the coat protein gene of TMV affect host response.

The N′ gene of *Nicotiana sylvestris* Speg. & Comes confers resistance to some strains of tobacco mosaic virus (TMV) but not others, including the common U1 strain. N′ gene resistance is expressed by the appearance of necrotic lesions on leaves that have been inoculated with the appropriate virus. The production of these lesions, known as the hypersensitive reaction (HR), occurs at the sites of viral infections and results in confinement of the virus to these areas. For this reason, incorporation of the HR resistance trait into plants has been a useful means of controlling virus diseases. However, for many virus-host systems, the HR resistance trait is either unavailable or is difficult to transfer into desirable crop species using existing plant breeding techniques (Russell 1978). Because the gene(s) controlling this resistance mechanism could be mobilized via genetic engineering strategies, it is important to understand the mechanisms involved in HR induction.

Current understanding of the mechanisms of HR is mainly limited to the biochemical processes involved in cellular necrosis (Legrand *et al.* 1976). Little is known about the processes involved in HR induction. However, Flor (1971) demonstrated that HR disease resistance occurs via a specific gene-for-gene recognition between host and pathogen. Work done in both fungal and bacterial systems has shown the presence of specific pathogen avirulence genes responsible for determining race-specific HR (Keen and Staskawicz 1988). For these systems, HR disease resistance is obtained when dominant pathogen avirulence genes interact with dominant host resistance genes (Flor 1971). Unfortunately, plant viral systems have not been as well defined. Earlier work with mutant viruses having amino acid substitutions in their coat proteins indicated a possible correlation between these substitutions and the induction of HR (Funatsu and Fraenkel-Conrat 1964).

Recently, the complete cloning of the TMV genome and the ability to produce infectious RNA transcripts from these cDNA clones have created a unique system in which to study virus-induced HR (Dawson *et al.* 1986; Meshi *et al.* 1986). Work done on this level has mapped the induction of HR in *N. sylvestris* to the coat protein gene of TMV (Saito *et al.* 1987). This was demonstrated through the production of coat protein hybrids of TMV-L, which produces HR in *N. sylvestris*, and TMV-OM, which moves systemically in *N. sylvestris*. When the coat protein gene of TMV-L is replaced by the coat protein gene of TMV-OM, HR is not induced.

More specifically, the cloning of nitrous acid mutants of TMV capable of inducing HR in *N. sylvestris* and the subsequent replacement and sequencing of specific segments of these mutant genomes into the original non-HR-inducing cDNA clone of TMV reveal that a single point mutation in the coat protein gene is responsible for the induction of HR (Knorr and Dawson 1988). The identified point mutation resulted in a single amino acid substitution in the coat protein. By coincidence this amino acid substitution was identical to one of several substitutions previously described (Funatsu and Fraenkel-Conrat 1964). Thus, other point mutations in the coat protein gene of TMV might also lead to the induction of HR in *N. sylvestris*. The discovery of other HR-inducing point mutations should provide a first step toward identifying possible molecular and structural requirements for the induction of HR.

This paper describes the results from the production of specific mutations in the coat protein gene of TMV. Mutations were created by the use of site-directed mutagenesis to specifically change selected nucleotides. Four independent nucleotide changes were selected so as to alter the amino acid sequence at four different places throughout the coat protein. These amino acid substitutions were based on previously described coat protein mutants (Funatsu and Fraenkel-Conrat 1964). Each of the four mutations was found to induce HR in *N. sylvestris*.

MATERIALS AND METHODS

Virus strains and plasmids. pTMV204, a full-length infectious clone of TMV U1, was used as the parental clone for the mutagenesis procedure (Dawson *et al.* 1986). A subclone, pDL3, containing the 3′ end of TMV, including the coat protein gene, was constructed by inserting a *Hin*dIII (5,081 bp) to *Pst*I (6,406 bp) fragment from pTMV204 into the polylinker region of pUC119 (Vieira and Messing 1987).

Site-directed mutagenesis and mutation screening. *In vitro* mutagenesis was accomplished via a modified M13 method previously described (Geisselsoder *et al.* 1987). Second-strand synthesis was accomplished using synthetically created oligomers ranging from 16 to 18 nucleotides in length. Each oligomer contained a single mismatched base from the wild-type coat protein gene sequence of TMV (Goelet *et al.* 1982). Uracil containing single-stranded template of pDL3 was obtained by coinfection of plasmid with phage M13K07 in the *Escherichia coli* strain CJ236 (Vieira and Messing 1987).

Colony hybridization was used to screen mutagenized colonies for the presence of the desired mismatch (Grunstein and Wallis 1979). The same oligomers used for mutagenesis were used in the colony hybridizations. Colonies that tested positive by hybridization were further confirmed for the desired mutation by double-stranded DNA sequencing (Zagursky *et al.* 1985). In addition, to ensure that no other changes had occurred during mutagenesis, the entire *Cla*I (5,664 bp) to *Nsi*I (6,207 bp) fragment, containing the complete coat protein gene, was sequenced for each clone.

Assembly of mutants. Once sequenced, the *Cla*I to *Nsi*I fragments containing each specific mutation were ligated back into previously unmutagenized *Cla*I to *Nsi*I digested pDL3. Appropriate control ligations of vector and insert were performed to ensure that the sequenced, mutagenized fragment was placed back into an unmutagenized pDL3 vector. An *Nco*I (5,460 bp) to *Spl*I (6,245 bp) fragment from each of the reassembled pDL3 vectors was then ligated into similarly cut full-length pTMV204. Each restriction site used in the ligations was recut to ensure maintenance of the sequence.

***In vitro* transcription and RNA sequencing.** *In vitro* transcriptions of full-length clones were performed as previously described (Ahlquist and Janda 1984; Dawson *et al.* 1986). Transcripts were inoculated onto *N. sylvestris* (N′). Single lesion transfers were made from *N. sylvestris* to *N. tabacum* L. 'Xanthi.' After 10 days virions were purified from systemically infected leaves, and virion RNA was extracted as previously described (Knorr and Dawson 1988) and then sequenced (Zimmern and Kaesberg 1978).

RESULTS

Mutagenesis. Second-strand synthesis reactions yielded between 25 and 150 transformants per mutagenesis experiment. Colony hybridizations indicated that the efficiency of the site-directed mutagenesis was between 16 and 44% for the four oligomers used. All of the transformants selected as positive by colony hybridization also sequenced positive for the desired mutation. Sequence analysis of each mutagenized fragment used in the assembly of full-length TMV clones showed no alterations from the original U1 sequence except for the selected mutation. The four full-length TMV coat protein mutant clones and their subsequent amino acid substitutions are listed in Table 1. These changes are located at several positions throughout the coat protein (Fig. 1).

Symptomatology of mutants. Symptomatology was observed under greenhouse conditions, and appearance of symptoms is indicated as days after inoculation. Wild-type TMV 204 produced a systemic mosaic response in 5–7 days when inoculated onto *N. sylvestris*. Transcripts from each of the four full-length mutant clones were infectious and produced local lesions on *N. sylvestris*. However, local lesion phenotypes on *N. sylvestris* varied among the mutants. Mutant TMV 11 (Fig. 2A) induced lesions that characteristically first showed cell collapse at the point of infection on day four, followed by the appearance of necrotic lesions 4–5 mm in diameter on day five. Necrosis induced by this mutant often spread out along leaf veins near the site of infection, leading to the collapse of large portions of the leaf. Mutants TMV 20 (Fig. 2B) and TMV 25 (Fig. 2C) induced phenotypically identical lesions on *N. sylvestris*. These two mutants produced distinct necrotic spots about 1–2 mm in diameter on day three that did not spread out from the point of infection. Mutant TMV 46 (Fig. 2D) produced lesions that showed both cell collapse and necrosis on day four. Lesions produced by TMV 46 were 1–2 mm in diameter and only occasionally moved out from the original point of necrosis. All four mutants produced lesions identical to those of the parental virus,

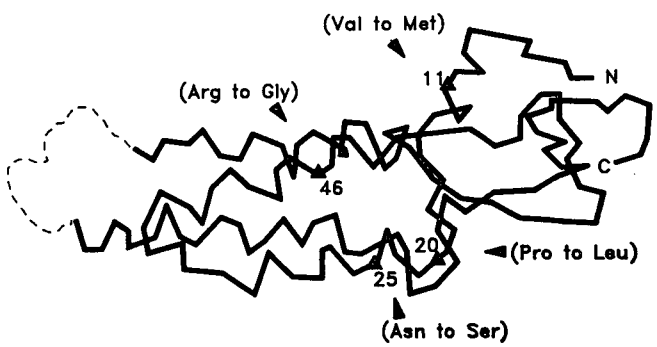


Fig. 1. Diagrammatic representation of the structural configuration of the tobacco mosaic virus coat protein subunit (Bloomer *et al.* 1978), showing the approximate locations of the amino acid substitutions produced by four point mutations.

Table 1. Tobacco mosaic virus coat protein point mutations[a]

Virus	Position[b]	Base change	Amino acid substitution	Symptomatology	
				N. sylvestris	Xanthi
TMV 204	None	None	None	Mosaic	Mosaic
TMV 11	5745	G to A	Val to Met	HR[c]	Mosaic
TMV 20	5773	C to U	Pro to Leu	HR	Mosaic
TMV 25	5788	A to G	Asn to Ser	HR	Mosaic/necrosis
TMV 46	5850	A to G	Arg to Gly	HR	Mosaic/necrosis

[a]The coat protein point mutations were created via site-directed mutagenesis.
[b]Nucleotide number of altered base (Goelet *et al.* 1982).
[c]Hypersensitive reaction.

TMV 204, when inoculated onto the assay host, *N. tabacum* 'Xanthi-nc.'

When infected with the parental virus TMV 204, *N. tabacum* 'Xanthi' characteristically allows the systemic spread of the virus, resulting in a mosaic symptom. The four mutants were also able to move systemically in Xanthi; however, two of the mutants produced distinctly different symptoms. Mutant TMV 25 produced systemic necrosis and leaf deformation on uninoculated systemically infected leaves. Mutant TMV 46 produced limited chlorosis and necrosis in the areas surrounding the veins of systemically infected leaves. Mutants TMV 11 and TMV 20 produced a systemic mosaic similar to that produced by wild-type TMV 204. However, mutant TMV 11 produced some necrosis on inoculated and lower systemically infected leaves. Thus, the coat protein gene mutations also affected the systemic symptoms induced in Xanthi tobacco.

In addition, symptoms were observed in *N. glauca* Graham, *Physalis floridana* Rydb., and *Lycopersicon esculentum* Mill. 'Ace' inoculated with the four mutants. The symptoms observed on these hosts were identical to those produced by the parental virus TMV 204.

Stability of mutants. The stability of the mutants was determined through both maintenance of phenotype and direct sequencing of virion RNA. The distinct phenotype of each mutant was maintained through at least three

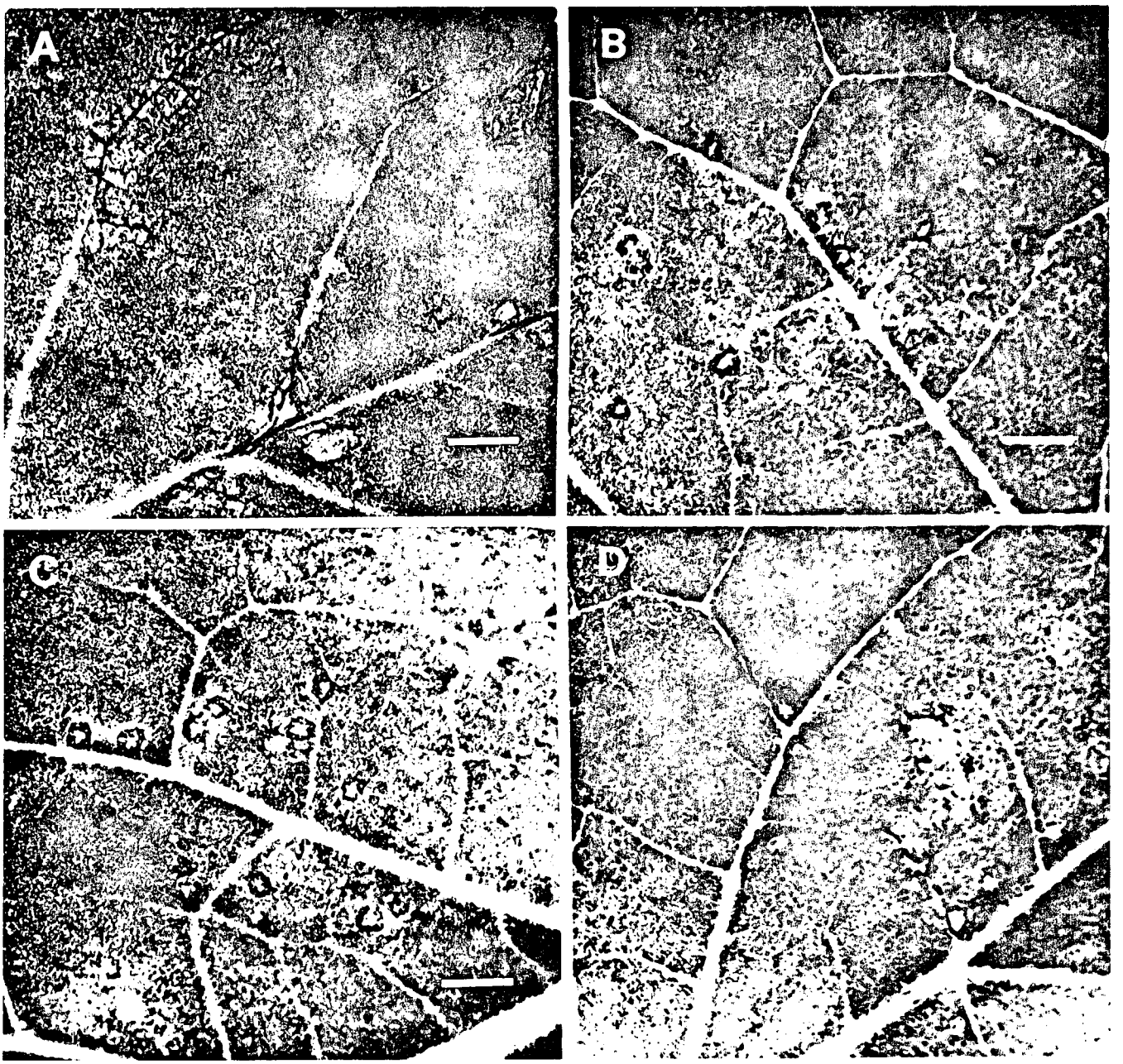


Fig. 2. Local lesions on leaves of *Nicotiana sylvestris* inoculated with tobacco mosaic virus (TMV) coat protein mutants: **A**, TMV 11, 5 days after inoculation; **B**, TMV 20, 3 days after inoculation; **C**, TMV 25, 3 days after inoculation; **D**, TMV 46, 4 days after inoculation. Bars represent 4 mm.

consecutive local lesion passes on *N. sylvestris*. Results of virion RNA sequencing confirmed the stable maintenance of each mutation after a single lesion passage from infectious transcript to systemic host. In addition, the RNA sequence showed no other variations from the wild-type U1 sequence in a 50 base area immediately around each mutation.

DISCUSSION

This study demonstrates that a number of TMV coat protein gene point mutations can alter virus-host interactions. Previous work by Knorr and Dawson (1988) identified an additional coat protein mutation responsible for the induction of HR on *N. sylvestris*. This mutation, at base pair 6,157 (cytosine to uracil), led to the substitution of phenylalanine for serine at amino acid position 148. These point mutations confirm earlier work by Funatsu and Fraenkel-Conrat (1964) and Wittmann and Wittmann-Liebold (1966) that suggested a possible role for the coat protein in the induction of HR on *N. sylvestris*. These earlier studies, directed at deciphering the genetic code, were unable to assign specific coat protein mutations to alterations in host response. However, current advances in technology have allowed us to reexamine this class of mutants and to make precise assignments of sequence to function.

At this time we cannot confirm protein as the active molecule for the induction of HR. It is possible that the altered viral RNA plays this role.

The variations in host responses (local lesions on *N. sylvestris* and systemic symptoms on *N. tabacum* 'Xanthi') observed for the four point mutations were an unexpected result. Similar differences in N′ local lesion phenotypes have previously been observed among various strains of TMV (Fraser 1983). In this study different lesion phenotypes were correlated with the thermal stability of the coat protein subunits. Altered thermal stability indicated potential coat protein structural differences among the strains of TMV. Therefore, one explanation for differences in local lesion phenotypes among mutants is that each mutant has its own structural configuration that has a specific affinity for interacting with a host recognition factor(s). Thus, the ability of a host to mount a resistance response, such as HR, may be directly linked to the efficiency with which the host recognizes a specific virus product.

The point mutations created resulted in widely diverse amino acid substitutions at different positions throughout the coat protein molecule (Fig. 1). However, the point mutations did not prevent the assembly of the virus. Mutant coat protein sequences were subjected to computer analysis for several kinds of protein secondary structure (Devereux *et al.* 1984). Each mutant coat protein showed changes in predicted secondary structure when compared to the wild-type TMV 204 coat protein sequence. However, the predicted changes differed for each of the four mutants. Thus, at this level of computer analysis, no single alteration in coat protein structure explained the ability of these mutations to alter host response. This suggests that several coat protein structural or conformational configurations may result in recognition by host factor(s) and induction of HR. Indeed, previous work in our laboratory (Dawson *et al.* 1988) has shown that large deletions in the TMV coat protein gene can also induce necrosis in *N. sylvestris*. In addition, rates of TMV mutations resulting in mutants that induce HR are significantly higher (10^{-3}) than rates of revertant mutations resulting in mutants that cause systemic symptoms (Aldaoud 1987). This suggests that the TMV wild-type coat protein gene may be a rare sequence responsible for systemic disease in *N. sylvestris*.

Although all four of our mutants induced HR on *N. sylvestris*, Funatsu and Fraenkel-Conrat (1964) listed other coat protein amino acid substitutions that did not induce HR. Therefore, it should be stressed that not every possible nucleotide substitution in the coat protein will lead to the induction of HR. Thus, it seems likely that only specific nucleotide changes that result in specific alterations of the wild-type coat protein sequence will lead to the induction of the host resistance response.

ACKNOWLEDGMENTS

This research was funded in part by grants 87-CRCR-1-12450 from the U.S. Department of Agriculture and I-882-85 from the U.S.-Israel Binational Agricultural Research and Development Fund (BARD).

LITERATURE CITED

Ahlquist, P., and Janda, M. 1984. cDNA cloning and *in vitro* transcription of the complete brome mosaic virus genome. Mol. Cell. Biol. 4:2876-2882.

Aldaoud, R. 1987. Biological characteristics, variations, and factors affecting variation in cloned tobacco mosaic virus. Ph.D. thesis, University of California, Riverside.

Bloomer, A. C., Champness, J. N., Bricogne, G., Staden, R., and Klug, A. 1978. Protein disk of tobacco mosaic virus at 2.8 Å resolution showing the interactions within and between subunits. Nature 276:362-368.

Dawson, W. O., Beck, D. L., Knorr, D. A., and Grantham, G. L. 1986. cDNA cloning of the complete genome of tobacco mosaic virus and production of infectious transcripts. Proc. Natl. Acad. Sci. USA 83:1832-1836.

Dawson, W. O., Bubrick, P., and Grantham, G. L. 1988. Modifications of the tobacco mosaic virus coat protein gene affecting replication, movement, and symptomatology. Phytopathology 78:783-789.

Devereux, J., Haeberli, P., and Smithies, O. 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12:387-395.

Flor, H. H. 1971. Current status of the gene-for-gene concept. Annu. Rev. Phytopathol. 9:275-296.

Fraser, R. S. S. 1983. Varying effectiveness of the N′ gene for resistance to tobacco mosaic virus in tobacco infected with virus strains differing in coat protein properties. Physiol. Plant Pathol. 22:109-119.

Funatsu, G., and Fraenkel-Conrat, H. 1964. Location of amino acid exchanges in chemically evoked mutants of tobacco mosaic virus. Biochemistry 3:1356-1361.

Geisselsoder, J., Witney, F., and Yuckenberg, P. 1987. Efficient site-directed *in vitro* mutagenesis. BioTechniques 5:786-791.

Goelet, P., Lomonossoff, G. P., Butler, P. J. G., Akam, M. E., Gait, M. J., and Karn, J. 1982. Nucleotide sequence of tobacco mosaic virus RNA. Proc. Natl. Acad. Sci. USA 79:5818-5822.

Grunstein, M., and Wallis, J. 1979. Colony hybridization. Methods Enzymol. 68:379-389.

Keen, N. T., and Staskawicz, B. 1988. Host range determinants in plant pathogens and symbionts. Annu. Rev. Microbiol. 42:421-440.

Knorr, D. A., and Dawson, W. O. 1988. A point mutation in the tobacco mosaic virus capsid protein gene induces hypersensitivity in *Nicotiana sylvestris*. Proc. Natl. Acad. Sci. USA 85:170-174.

Legrand, M., Fritig, B., and Hirth, L. 1976. Enzymes of the phenylpropanoid pathway and the necrotic reaction of hypersensitive tobacco to tobacco mosaic virus. Phytochemistry 15:1353-1359.

Meshi, T., Ishikawa, M., Motoyoshi, F., Semba, K., and Okada, Y. 1986. *In vitro* transcription of infectious RNAs from full-length cDNAs of tobacco mosaic virus. Proc. Natl. Acad. Sci. USA 83:5043-5047.

Russell, G. E. 1978. Pages 231-264 in: Plant Breeding for Pest and Disease Resistance. Butterworth Publications, Boston.

Saito, T., Meshi, T., Takamatsu, N., and Okada, Y. 1987. Coat protein

gene sequence of tobacco mosaic virus encodes host response determinant. Proc. Natl. Acad. Sci. USA 84:6074-6077.
Vieira, J., and Messing, J. 1987. Production of single-stranded plasmid DNA. Methods Enzymol. 153:3-11.
Wittmann, H. G., and Wittmann-Liebold, B. 1966. Protein chemical studies of two RNA viruses and their mutants. Cold Spring Harbor Symp. Quant. Biol. 31:163-172.
Zagursky, R. J., Baumeister, K., Lomax, N., and Berman, M. L. 1985. Rapid and easy sequencing of large linear double-stranded DNA and supercoiled plasmid DNA. Gene Anal. Technol. 2:89-94.
Zimmern, D., and Kaesberg, P. 1978. 3'-Terminal nucleotide sequence of encephalomyocarditis virus RNA determined by reverse transcriptase and chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 75:4257-4261.

We also demonstrated that the coat protein itself, not the alterations in the RNA, is responsible for induction of necrosis by removing the start codons of TMV mutants with coat protein gene alterations that induce necrosis (Culver and Dawson, in press). These data suggest that the tertiary configuration of the coat protein is recognized by some component of the plant to inititate processes that lead to virus resistance. By altering the origin of assembly of mutants that induce necrosis, we have found that production of virions is not necessary for eliciting HR (Hilf and Dawson, in preparation). This information suggests that it is viral protein aggregates that are recognized by the host. We are collaborating with Dr. G. Stubbs, Vanderbilt University, who has examined these mutations relative to the structure of the coat protein and found that all of the alterations that induce HR occur in two adjacent "footprints" on the surface of protohelical aggregates of coat protein subunits.

Results in detail are in the attached manuscript accepted for publication. in Virology.

VIROLOGY 173, 000–000 (1989)

28a

Tobacco Mosaic Virus Coat Protein: An Elicitor of the Hypersensitive Reaction but Not Required for the Development of Mosaic Symptoms in *Nicotiana sylvestris*

JAMES N. CULVER[1] AND WILLIAM O. DAWSON

Department of Plant Pathology, University of California, Riverside, California 92521

Received May 23, 1989; accepted August 11, 1989

Specific nucleotide changes in the coat protein gene of tobacco mosaic virus (TMV) have been identified as responsible for the induction of the hypersensitive reaction (HR) in *Nicotiana sylvestris*. Each of these nucleotide changes resulted in amino acid substitutions in the coat protein. To determine if the altered viral RNA or the altered protein acted directly to elicit the HR, the coat protein translational starts were removed from full-length cDNA clones of the HR-inducing mutant TMV 25 and the systemically infecting TMV U1 strain. Infectious transcripts of these altered genomes failed to induce HR in inoculated leaves of *N. sylvestris*. These free-RNA mutants moved poorly out of inoculated leaves and produced a systemic mosaic symptom 9 to 12 weeks after inoculation. Infectious viral RNA, from both mutants, was recovered from inoculated and systemic mosaic leaves. Western blot analysis of both inoculated and noninoculated leaves revealed the presence of TMV-encoded 126-kDa protein and the absence of coat protein for both mutants. This study demonstrates that the coat protein of TMV 25 is an elicitor molecule responsible for the induction of HR in *N. sylvestris* and that the TMV coat protein is not required for the development of systemic mosaic symptoms.

One important method of controlling plant viral diseases is through the incorporation of hypersensitive resistance genes into cultivated crop varieties (*1*). These resistance genes are thought to confer to the plant the ability to recognize an attacking pathogen through interactions with specific pathogen avirulence genes (*2*). This specific interaction then leads to the production of necrotic local lesions and confinement of the pathogen to lesion sites.

The N'N' genotype of *Nicotiana sylvestris* Spegazzini & Comes provides an ideal model for studying the viral-induced hypersensitive reaction (HR). This is because the N'N' genotype confers resistance against some strains of tobacco mosaic virus (TMV) but not others. In addition, TMV mutants capable of inducing HR in *N. sylvestris* can easily be obtained from strains that normally give systemic infections (*3*). Finally, the complete molecular cloning of the TMV genome and the ability to produce infectious RNA from these cDNA clones allows for the use of molecular techniques to study this virus–host interaction (*4, 5*).

Previous work with this system has demonstrated the coat protein gene of TMV to be responsible for the induction of HR in *N. sylvestris* (*6, 7*). Currently, induction of this host response has been precisely mapped to five different point mutations occurring throughout the coat protein gene (*7, 8*). All of these confirmed point mutations result in amino acid substitutions in the coat protein. In addition, a number of other previously reported TMV mutants, selected for their ability to induce HR in *N. sylvestris*, contained other amino acid substitutions in the coat protein (*3, 9*). Therefore, it is evident that a number of different alterations, either in the RNA or the protein, may be responsible for the induction of HR. The identification of which viral molecule, RNA or protein, acts to elicit HR should provide a starting point for determining how this virus–host interaction takes place at the molecular level.

To determine whether RNA or protein was responsible for the induction of HR, the translational start of the coat protein gene was removed from full-length cDNA clones of an HR-inducing mutant (TMV 25) as well as from its systematically infecting parent virus (TMV 204) (*4, 8*). Mutant TMV 25 differs from its parent virus TMV 204 by a single point mutation in the coat protein gene, at nucleotide 5788 (adenine to guanine), that leads to a single amino acid substitution, Asn to Ser, at position 25 in the coat protein. This point mutation has been demonstrated to be responsible for the induction of HR in *N. sylvestris* (*8*). The removal of the coat protein translational start from the TMV 204 clone was performed as a control.

Removal of the translational start was accomplished via site-directed mutagenesis (*10*). Mutagenesis was performed on both the TMV 25 and TMV 204 coat protein genes, *Cla*I (nt 5664) to *Nsi*I (nt 6207) fragments subcloned into pUC128 (*11*), using a synthetic oligomer 21 nucleotides in length and spanning the coat

[1] To whom requests for reprints should be addressed.

0042-6822/89 $3.00

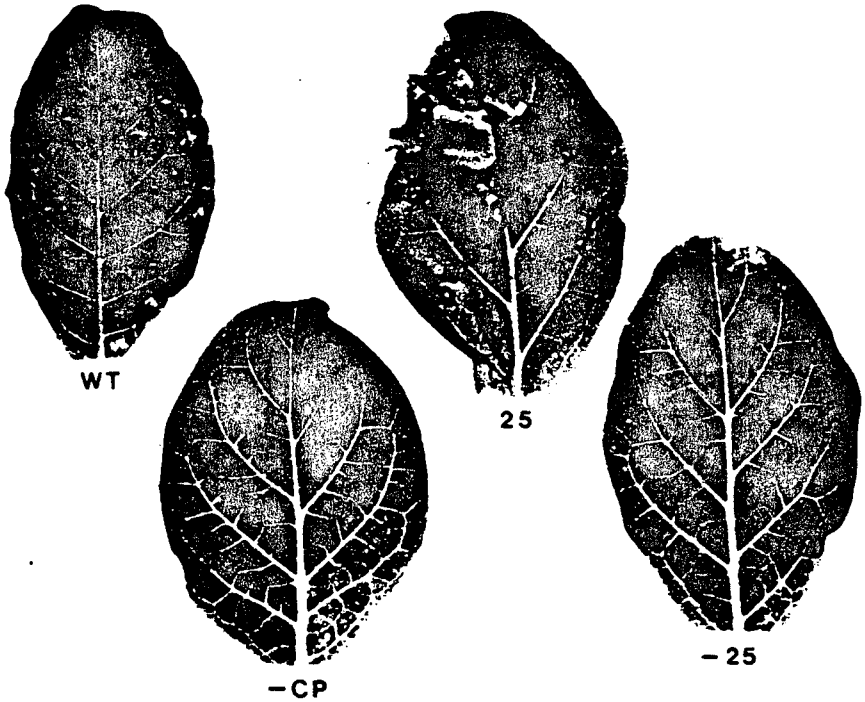


FIG. 1. Symptomatology of TMV 204, TMV 25, TMV [-CP], and TMV [-25] in *Nicotiana sylvestris* at 10 days postinoculation. WT, noninoculated leaf systemically infected with TMV 204 showing systemic mosaic symptoms; −CP, inoculated leaf infected with TMV [-CP] showing no symptoms; 25, inoculated leaf infected with TMV 25 showing local lesions; -25, inoculated leaf infected with TMV [-25] showing no symptoms.

protein translational start. This oligomer contained two mismatched bases from the wild-type TMV sequence that resulted in the alteration of nucleotides 5713 (uracil to guanine) and 5714 (guanine to adenine) (*12*). This alteration changed the coat protein translational start in the viral RNA from AUG to AGA and created a unique *Bgl*II site in the cDNA subclones that was utilized to screen for the desired mutation. To ensure that no other changes had been introduced during the mutagenesis procedure the *Cla*I to *Nsi*I fragments, containing the entire coat protein gene, were sequenced (*13*). Once sequenced, these *Cla*I to *Nsi*I fragments were ligated into subclone pDL3 which contained the remainder of the 3′ end sequences, *Hin*dIII (nt 5081) to *Pst*I (nt 6406), of TMV 204. *Nco*I (nt 5456) to *Spl*I (nt 6245) fragments from the assembled pDL3 clones were then ligated into similarly cut full-length TMV 204. Each restriction site used was recut to ensure maintenance of the sequence.

The two full-length constructs with the coat protein translational starts removed were designated TMV [-25], carrying the HR-inducing sequence, and TMV [-CP], carrying the wild-type sequence. *In vitro* transcriptions from TMV [-25] and TMV [-CP] were inoculated onto expanded leaves of *N. sylvestris*, N′N′ genotype, and *Nicotiana tabacum* L. cultivar Xanthi-nc, NN genotype (*4, 14*). The NN genotype of Xanthi-nc produces distinct local lesions when inoculated with the parental virus TMV 204. Leaves of Xanthi-nc inoculated with either TMV [-25] or TMV [-CP] produced local lesions within 3 days postinoculation. Local lesion phenotypes on Xanthi-nc, for both TMV [-25] and TMV [-CP], were identical to that of the wild-type virus TMV 204. Thus, both mutant TMV [-25] and mutant TMV [-CP] replicated to a level capable of inducing a host response, HR on Xanthi-nc, identical to that of the wild-type virus TMV 204.

Leaves of *N. sylvestris* inoculated with either TMV [-25] or TMV [-CP] did not produce symptoms (Fig. 1). In contrast, leaves of *N. sylvestris* inoculated with TMV 25 produced local lesions within 3 days of inoculation (Fig. 1). Lack of symptom production by TMV [-25] and TMV [-CP] in inoculated leaves was as observed for the parental virus TMV 204. Infectious viral RNA, determined by grinding leaf tissue in cold buffer (*4*) and transferring to the assay host Xanthi-nc, was recovered from inoculated leaves as early as 6 days postinoculation for both TMV [-25] and TMV [-CP].

Proteins extracted from inoculated leaves of *N. sylvestris* at 10 days postinoculation (*15*) were subjected to Western blot analysis (*16, 17*) for the detection of TMV-encoded 126-kDa protein and 17.5-kDa coat pro-

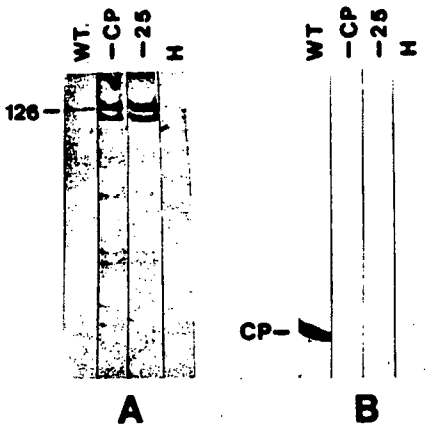


FIG. 2. Western blot analysis of leaf proteins extracted from leaves of *Nicotiana sylvestris* infected with TMV 204, TMV [-CP], and TMV [-25]. Leaf proteins, extracted from 1 g fresh tissue at 10 days postinoculation, were separated by SDS–PAGE and electroblotted to nitrocellulose. Blots were then probed with either (A) antiserum specific to the TMV 126-kDa protein or (B) antiserum specific to the TMV coat protein. WT, proteins extracted from TMV 204 inoculated leaf; -CP, proteins extracted from TMV [-CP] inoculated leaf; -25, proteins extracted from TMV [-25] inoculated leaf; H, proteins extracted from a healthy control.

tein (Lehto *et al.*, manuscript in preparation). Western blot results showed that TMV [-25] and TMV [-CP] produced the 126-kDa protein but did not produce detectable levels of coat protein (Fig. 2). Levels of 126-kDa protein in inoculated leaves of both TMV [-25] and TMV [-CP] were higher than that observed for the wild-type TMV 204 at the 10-day sampling time (Fig. 2). However, the 126-kDa protein levels for TMV [-25] and TMV [-CP] observed at the 10-day sampling time were similar to 126-kDa protein levels observed for the wild-type TMV 204 at earlier sampling times (data not shown). This indicated that both TMV [-25] and TMV [-CP] replicated sufficiently to produce levels of 126-kDa protein near that of wild-type TMV 204 in inoculated leaves of *N. sylvestris* and did not produce coat protein.

To confirm that TMV [-25] maintained the original point mutation of TMV 25, responsible for the induction of HR, total RNA was extracted from TMV [-25] inoculated leaves of *N. sylvestris* at 10 days postinoculation (*18*). Viral RNA was then sequenced using a method previously described (*19*). Analysis of sequencing data showed that TMV [-25] maintained the original point mutation of mutant TMV 25, nucleotide 5788, responsible for the induction of HR. No other variations from the wild-type TMV 204 sequence were observed in a 50 nucleotide region immediately flanking the original mutation.

TMV [-25] and TMV [-CP] constructs were designed to alter as little of the viral RNA as possible and still eliminate the production of coat protein. Development of local lesions on Xanthi-nc and the levels of 126-kDa protein suggested that both TMV [-25] and TMV [-CP] replicated to any threshold level of viral RNA required for the induction of HR. Therefore, the inability of TMV [-25] to induce HR in inoculated leaves of *N. sylvestris* was due to the lack of coat protein and not RNA. This demonstrates that the coat protein of TMV 25 is an elicitor of the HR in *N. sylvestris.*

Additional observations revealed the development of systemic mosaic symptoms in approximately half of the *N. sylvestris* plants inoculated with either TMV [-25] or TMV [-CP]. These systemic mosaic symptoms developed slowly, 9 to 12 weeks postinoculation, when compared to the development of wild-type TMV 204 mosaic symptoms 5 to 7 days postinoculation (Fig. 3). Infectious viral RNA from noninoculated leaves of *N. sylvestris* showing mosaic symptoms was obtained from plants inoculated with TMV [-25] and TMV [-CP]. Repeated attempts to obtain infectious viral RNA from noninoculated leaves before the development of mosaic symptoms or from *N. sylvestris* plants that did not

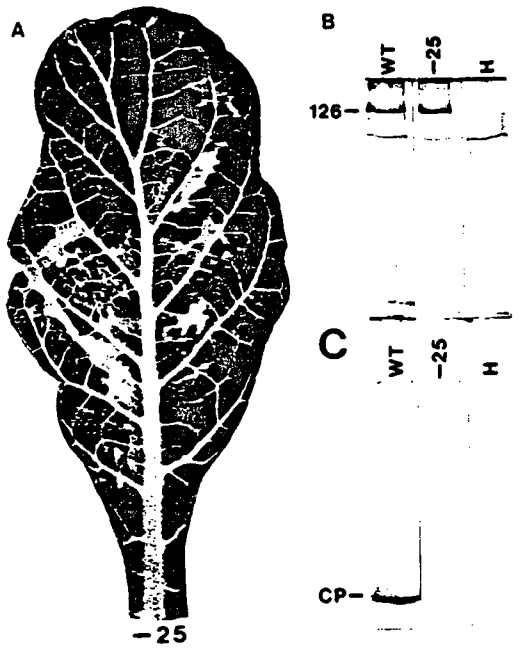


FIG. 3. Symptomatology and Western blot analysis of *Nicotiana sylvestris* leaves systemically infected with TMV [-25] at 14 weeks postinoculation. Similar results were obtained for plants infected with TMV [-CP]. (A) Noninoculated leaf systemically infected with TMV [-25] showing systemic mosaic symptoms; (B) and (C) Western blots of leaf proteins extracted from 1 g fresh tissue and probed with either (B) TMV 126-kDa protein-specific antiserum or (C) TMV coat protein-specific antiserum; WT, proteins extracted from TMV 204 systemically infected leaf; -25, proteins extracted from TMV [-25] systemically infected leaf; H, proteins extracted from healthy control.

develop mosaic symptoms failed. This indicated that TMV [-25] and TMV [-CP] moved poorly out of inoculated leaves.

Western blot analysis of proteins extracted from noninoculated mosaic leaves of *N. sylvestris*, infected with TMV [-25] and TMV [-CP], also showed the presence of TMV 126-kDa protein and the absence of TMV coat protein (Fig. 3). Therefore, the ability of both TMV [-25] and TMV [-CP] to produce systemic mosaic symptoms in *N. sylvestris* indicates that coat protein is not required for development of this disease symptom. The delayed development of mosaic symptoms induced by TMV [-25] and TMV [-CP] and the lack of detectable amounts of infectious viral RNA in noninoculated leaves before the development of mosaic symptoms suggest that coat protein may play a role in the rapid, long distance movement of the virus.

ACKNOWLEDGMENT

We thank Dennis Lewandowski for design of the oligomer used in the mutagenesis. This research was funded in part by grants 87-CRCR-1-12450 from the U.S. Department of Agriculture and I-882-85 from the US–Israel BARD.

REFERENCES

1. RUSSELL, G. E., *In* "Plant Breeding for Pest and Disease Resistance" (G. E. Russell, Ed.), pp. 231–264. Butterworth Publications, Boston.
2. KEEN, N. T., and STASKAWICZ, B., *Annu. Rev. Microbiol.* **42**, 421–440 (1988).
3. FUNATSU, G., and FRAENKEL-CONRAT, H., *Biochemistry* **3**, 1356–1361 (1964).
4. DAWSON, W. O., BECK, D. L., KNORR, D. A., and GRANTHAM, G. L., *Proc. Natl. Acad. Sci. USA* **83**, 1832–1836 (1986).
5. MESHI, T., ISHIKAWA, M., MOTOYOSHI, F., SEMBA, K., and OKADA, Y., *Proc. Natl. Acad. Sci. USA* **83**, 5043–5047 (1986).
6. SAITO, T., MESHI, T., TAKAMATSU, N., and OKADA, Y., *Proc. Natl. Acad. Sci. USA* **84**, 6074–6077 (1987).
7. KNORR, D. A., and DAWSON, W. O., *Proc. Natl. Acad. Sci. USA* **85**, 170–174 (1988).
8. CULVER, J. N., and DAWSON, W. O., *Mol. Plant–Microbe Interact.* **2**, 209–213 (1989).
9. WITTMAN, H. G., and WITTMAN-LIEBOLD, B., *Cold Spring Harbor Symp. Quant. Biol.* **31**, 133–172 (1966).
10. GESSELSODER, J., WITNEY, F., and YUCKENBERG, P., *Biotechniques* **5**, 786–791 (1987).
11. KEEN, N. T., TAMAKI, S., KOBAYASHI, D., and TROLLINGER, D., *Gene* **70**, 191–197 (1988).
12. GOELET, P., LOMONOSSOFF, G. P., BUTLER, P. J. G., AKAM, M. E., GAIT, M. J., and KARN, J., *Proc. Natl. Acad. Sci. USA* **79**, 5818–5822 (1982).
13. ZAGURSKY, R. J., BAUMEISTER, K., LOMAX, N., and BERMAN, M. L., *Gene Anal. Tech.* **2**, 89–94 (1985).
14. AHLQUIST, P., and JANDA, M., *Mol. Cell. Biol.* **4**, 2876–2882 (1984).
15. GODEFROY-COLBURN, T., GRIGERY, M., BERNA, A., and STUSSI-GARAUD, C., *J. Gen. Virol.* **67**, 2233–2239 (1986).
16. LAEMMLI, U. K., *Nature (London)* **227**, 680–685 (1970).
17. TOWBIN, H., STAELIN, T., and GORDON, J., *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354 (1979).
18. LOGEMANN, J., SCHELL, J., and WILLMITZER, L., *Anal. Biochem.* **163**, 16–20 (1987).
19. ZIMMERN, D., and KAESBERG, P., *Proc. Natl. Acad. Sci. USA* **75**, 4257–4261 (1978).

2. IVR gene in different tobacco - TMV mutant interactions

One of the objectives of the grant was to examine how viruses induce IVR and to correlate induction of necrosis to induction of IVR, and confinement of the virus to the initial infection area.

A series of insertion and/or deletions mutants were made in Dr. Dawson's lab. Two of these mutants induce necrotic local lesions in Xanthi tobacco which has the nn genotype. Other mutants which induce local lesions in N. sylvestris were isolated and cloned as cDNA.

During April 1989, Dr. Gera spent 3 weeks in Dr. Dawson's lab. He worked in cooperation with the Riverside group to examine the induction of IVR in correlation with the development of necrosis.

The following combinations were tested:

1) Mutants CP-10 and CP-27 in Xanthi (nn). These two mutants induced distinct necrotic lesions on inoculated leaves of Xanthi. The lesions produced on inoculated leaves were slightly larger and occurred later than lesions produced on Xanthi-nc. Mutants CP-10 and CP-27 are usually restrictive to local lesions on inoculated leaves of Xanthi, but occasionally move systemically (Dawson et al., 1988).

2) Mutants M-11 and M-25 in N. sylvestris. M-11 induced lesions that characteristically first showed cell collapse at the point of infection on day four followed by the appearance of necrotic lesions 4-5 mm in size, on day five. M-25 produced distinct necrotic spots approx. 1-2 mm in size on day three.

Plants were inoculated with TMV-RNA of the mutants and maintained at 25°C. Seven days after inoculation, IVR was extracted as described previously, subjected to SDS-PAGE and analysed by immunoblotting.

To our surprise only Mutant-11 induced both necrosis and IVR. However mutant-25 induced necrosis but no detectable IVR. Mutant-11, which has coat

protein amino acid 11 changed from Val to Met, induces both necrosis and IVR. However, mutant-25, which has coat protein amino acid 25 changed from Asn to Ser, induces necrosis but not detectable IVR.

We have not been able to detect the production of IVR in Xanthi infected with CP-10 and 27.

These results require further studies but may indicate that the IVR gene is not expressed in tobacco without the N or N' gene.

f. Description of Cooperation

The proposal was jointly planned and prepared. Reports and ideas were exchanged by bitnet and during international meetings. Cloned c-DNA mutants prepared by Dr. Dawson were received in Israel and antisera prepared in Israel were sent to Dr. Dawson. Dr. A Gera spent 3 weeks in Dr. Dawson's lab to test various mutant - host reactions for IVR. The project was interactive in many respects, but especially in exchanging materials and joint work on various tobacco - TMV mutant interactions. A joint publication is being planned and we hope to continue this cooperation.

g. Evaluation of Research Achievements

Improved production of protoplasts and IVR was achieved. IVR was further purified and poly- and monoclonal antisera were prepared. IVR was also obtained directly from plants and from induced resistant tissue. IVR is associated with a 23K protein species. A cDNA library from induced resistant tissue was prepared and bacterial clones that produce IVR were selected. This cloned IVR had biological activity. All these achievements were within the aims of the original proposal and should lead now to the preparation of "transgenic" plants producing IVR constitutively. If these plants will be resistant to virus(es) this will be of major importance for agriculture - providing a novel way of obtaining virus resistant plants. Data regarding the amino acid sequence of IVR are so far not conclusive, and this will be done in the future.

It was further shown that the viral coat protein gene is not involved in the induction of necrosis in tobacco NN plants. In N'N' plants the coat protein gene of TMV is an elicitor of HR. TMV mutants that induce necrosis in Xanthi (nn) and N. sylvestris (N') were studied in respect to induction of IVR. One mutant was found to induce IVR in N. sylvestris. These results, within the original aims, are most interesting as they may indicate that IVR is not expressed in the absence of the N or N' gene, and requires an interaction with specific sequences in the viral genome.

h. List of Publications

Culver, J.N. and W.O. Dawson, 1989. Point mutations in the coat protein of tobacco mosaic virus induce hypersensitivity in Nicotiana sylvestris. Molec. Plant-Microbe Interactions 2:209-219.

Culver, J.N. and W.O. Dawson, 1989. Tobacco mosaic virus coat protein: an elicitor of the hypersensitive reaction but not required for the development of mosaic symptoms in Nicotiana sylvestris. Virology 173 (in press).

Knorr, D.A. and W.O. Dawson, 1988. A point mutation in tobacco mosaic virus capsid protein gene induces hypersensitivity in Nicotiana sylvestris. Proc. Natl. Acad. Sci. USA 85:170-174.

Dawson, W.O., P. Bubrick, and G.L. Grantham. 1988. Modification of the tobacco mosaic virus coat protein gene affects replication, movement and symptomatology. Phytopathology. 78:783-789.

Dawson, W.O., Knorr, D.A. and P. Bubrick, 1988. Elicitor active coat protein mutations in cDNA clones of tobacco mosaic virus. pp. 139-147 in: Physiology and Biochemistry of Plant-Microbial Interactions, Proc. 11th Ann. Symp. in Plant Phys.

Gera, A. and Loebenstein, G. 1988. An inhibitor of virus replciation associated with green island tissue of tobacco infected with cucumber mosaic virus. Physiol. Molec. Plant Pathol. 32:373-385.

Gera, A. and Loebenstein, G. 1988. Evaluation of antisera to an inhibitor of virus replication. J. Phytopathol. 124:366-371.

Loebenstein, G. and Gera, A. 1988. Resistance responses of plants to virus infections. Acta Horticulturae 234:403-411.

Spiegel, S., Gera, A., Salomon, R., Ahl, P., Harlap, S. and Loebenstein, G. 1989. Recovery of an inhibitor of virus replication from the intercellular fluid of hypersensitive tobacco infected with tobacco mosaic virus and from uninfected induced-resistant tissue. Phytopathology 79:258-262.

Gera, A., Sadka, A., Spiegel, S., Salmon, R. and Smorodinsky, N.I. 1989. Use of monoclonal antibodies in the purification of an inhibitor of virus replication by affinity chromatography. J. gen. Virol. 70:1293-1296.

Gera, A., Loebenstein, G., Salomon, R. and Franck, A. 1990. An inhibitor of virus replication from protoplasts of hypersensitive tobacco cultivar infected with tobacco mosaic virus is associated with a 23K protein species. Phytopatholgoy 80: (in press).

Loebenstein, G. and Gera, A. 1990. Inhibitor of virus replication associated with resistance responses. NATO Advanced Research Workshop "Recognition and response in plant-virus interactions (in press).