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BARD

FINAL REPORT

PROJECT NO. I-882-85

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

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Title

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

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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 approximately 1 μ g 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. Preparation 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 $22 \pm 2^{\circ}\text{C}$ 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 5×10^6 per 1 20 cm petri dish to about 10×10^6 , 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.

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.

4. Preparation of polyclonal 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.

Resistance

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

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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 protoplasts (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 tabacum (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, consistently 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 excised 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 reversed, 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 protoplast 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 elution. 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 protoplasts. 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 distinction 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 protoplast 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 association 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 fractions 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 efficiently 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 protoplast 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.

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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 $\pm$ 1.5	26 $\pm$ 3.8	71
Electroeluted 23K protein	9.0 $\pm$ 1.8	28 $\pm$ 4.5	68
Control protoplasts ^c	31 $\pm$ 3.5		

^a Average number of local lesion and standard error from three to four experiments per 10⁶ 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	

- ^aAntiserum diluted 1:20 in coating buffer were used to coat ELISA plates.

^bThree units of crude protoplast IVR or control preparation were added.

^cAverage number of local lesion and standard error from three to four experiments per 10⁶ Samsun NN tobacco protoplasts 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.

^dTMV-inoculated protoplasts in incubation medium with no addition.

8p

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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. Miscellaneous 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

	<u>E₄₀₅</u>			
	<hr/>			
	Days after placing IVR in Petri			
	<u>2</u>	<u>3</u>	<u>4</u>	<u>7</u>
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.				

	<u>Percentage recovery of IVR</u>			
	Days			
	<u>2</u>	<u>3</u>	<u>4</u>	<u>7</u>
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 H₁₋₅ and I₁₋₅ 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 H₂O (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 NaH₂PO₄, 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 corresponded 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 healthy 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). Specific 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. Specific band is indicated by arrow. The mol. wts of marker proteins (lane 1) are given in Kd.

Poly A translation products from 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 lambda 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 MgCl₂, 10 mM DTT, 4 mM Na-pyrophosphate, 1.25 mM De-oxynucleoside triphosphate mix, 15 uCi of (-³²p)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(NH₄)₂SO₄, 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 Original 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 T₄ 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 isopropylthiogalactoside (IPTG) were added to (NZY [5gr. NaCl, 2gr. MgSO₄, 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 vigorous shaking at 37°C in TB media supplemented with 0.2% maltose and 10 mM MgSO₄. The cells were centrifuged at 3000 rpm for 10 min., then gently resuspended in 0.5 volumes of 10 mM MgSO₄. The bacterial host strain Y 1088 is restriction negative but methylation positive, so all recombinant 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 culture with 0.1 ml of Lambda buffer (100 mM NaCl, 8 mM MgSO₄, 1 M Tris-HCl pH 7.5, and 2% gelatin) containing 1.5×10^7 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 overlaid with a dry nitrocellulose filter which had been saturated previously in 10 mM IPTG in H₂O. 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

^aAverages 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.

^bApproximately one unit.

^cInoculated 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%, and 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 production 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.

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).

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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.

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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.

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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).