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A COMPARATIVE STUDY OF VIRAL RNA SYNTHESIS IN LEAVES AND PROTOPLASTS

Ph.D. Thesis

of

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S Z E G E D

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C O N T E N T

INTRODUCTION

Plant virology was hampered for several decades by the special drawback of plant material that it is impossible to infect simultaneously all cells of a plant organ. By applying the usual inoculation techniques about one-ten-thousandth cells of a tobacco leaf are infected simultaneously. The virus then moves from cell to cell and during a period of 8 to 14 days practically all cells become infected in a susceptible host /Hagiladi, et al., 1975/. This is a gradual process which lacks the synchrony of various steps of virus multiplication which would be a prerequisite of any reasonable biochemical approach to study the event of host parasite interaction at the biochemical or molecular level as it has been done in bacteriophage/bacterium system /Fraenkal-Conrat, 1969/. As a result plant virology is lagging behind because of the inherente problems of this system.

The advent of plant protoplast research gave a new impetus to plant virology because this system makes

it easier to study events occurring during synchronous multiplication of plant viruses. The study of virus multiplication in protoplast system is a new field since the first successful infection of isolated plant protoplasts with TMV and TMV-RNA in high proportion and synchronous manner was achieved only, as late as 1969 by Aoki and Takebe, even if the possibility of sporadic infection of tomato fruit protoplast has been reported by Cocking, 1966.

Just because relatively little work has been done on, the biochemistry of virus infected and/or healthy protoplasts it is still a problem, whether or not the protoplastssystem is fully comparable to the cells in situ and whether or not the conclusions we draw from the study of the protoplast system can be generalised.

Isolated protoplasts were found to be physiologically normal even insthe highly artificial environments by Sahai and Takebe, 197o in their study on some metabolic functions. - Protoplasts exhibit typical RNA and protein turnover /Watss and King, 1973/.upon virus infection viral RNA and proteins are also synthetised in isolated protoplasts /Takebe and Otsuki, 1969; Sakai and Takebe, 1972; Coutts et al., 1972/. Although, in

the above mentioned features protoplasts seem to resemble the organ from which they derived. There is still one puzzling problem the answer for which is not yet available. The intriguing question is why infected protoplasts from hypersensitive responding host show no hypersensitive reaction /Otsuki et al., 1972/. Also CCMV which induces chlorosis in the primary infection region of tobacco leaves shows no such effect when tobacco protoplasts are infected /Motoyoshi, 1973/. In addition protoplasts had been shown to have much elevated level of a relative purine-specific ribonuclease as compared to the unaffected tissue /Lázár et al., 1973/. Therefore, it is clear that metabolism of the protoplasts at least in some respect differs from that of the tissue they were derived. In spite of the sproradic recognition of this fact except for the study of hypersensitive reaction in tobacco, there is no systematic study which would compare any physiological or biochemical parameter of a protoplast population, with the activity of cell in situ in tissue state. The aim of this work was; /A/ To compare the ability of tobacco leaf protoplast to synthesize

various classes of RNA /rRNA and TMV-RNA/ with the inherent capacity of cells of the same genotype and physiological state *in situ*.

/B/ To study the significance of cell to cell contact in leaf state in the expression of the "N"-gene of tobacco known to be responsible for the hypersensitive necrotic death of TMV-infected cells in leaves of *Xanthi* nc. tobacco, and known to be ineffective in protoplast state /Otsuki et al., 1972/.

MATERIALS AND METHODS

Nicotiana tabacum cv. Xanthi plants were grown under normal greenhouse conditions. Plants were infected mechanically with the OM strain /Nozu and Okada, 1968/ of tobacco mosaic virus /TMV/. Local lesion assays were done on N. tabacum cv. Xanthi nc. plants essentially according to Otsuki et al. 1972. However, instead of half leaves we used for each assay 24 discs of 2.5 cm in diameter cut from different leaves but systematically arranged in such a way that all samples to be compared maximum 8 at a time, had the same number of discs punched out from one and the same leaf /the leaves of standard size used in these experiments yielded 8 discs of the above diameter/.

Protoplasts were isolated under sterile conditions /Laminar flow cabin/ from the leaf tissues, that were surface sterilized by washing in sodium hypochloride for 3 min. and later in alcohol for 30 seconds which were further washed thrice with distilled water, by mixed enzyme method. One step procedure has been deviced by which protoplasts are prepared from the leaves without first harvesting cells. Peeled leaf pieces were

floated on the surface of 0.5 % macerozyme R-10 /Kinki Yakut MFG. Co. Ltd., Nishinomiya, Japan/. 0.5 % potassium dextran sulphate /Meito Sangyo Co., Ltd., Japan/, 2 % Cellulase "Onozuka" R-10 /Kinki Yakut, MFG, Co. Ltd. Nishinomiya, Japan/, in 0.7 M mannitol, pH 5.8 at 35 - 36°C. After digestion for 2-3 hrs the protoplasts were washed in a washing medium deviced by Aoki and Takebe, /1969/, /comprising of 0.7 M mannitol, 10 mM $CaCl_2$ and 1 mM $MgSO_4$/ thrice. The washed protoplasts were incubated for 18 hours at 25°C and 500 lux in TMV incubation medium /Aoki and Takebe, 1969/ from which inorganic phosphorus was omitted.

TMV incubation medium

0.7	M	Mannitol	48.3 ml
0.1	M	KH_2PO_4	0.1 ml
0.1	M	KNO_3	0.5 ml
0.1	M	$MgSO_4$	0.5 ml
1.0	M	$NaCl_2$	0.5 ml
1.0	mM	KJ	0.05 ml
0.01	mM	$CuSO_4$	0.05 ml
100	ug/ml	2,4-D	0.5 mlml

pH = 5.8

Then carrier-free ^{32}P potassium orthophosphate was added to the incubation medium 25 uCi/ml and the proto-

plasts were incubated with isotope for an additional 6 hrs. Attached half leaves or the other half which were removed for preparing the protoplasts to provide fully comparable experimental materials were labelled via a cotton thread through the petioles as described by Hirai and Wildman /1967, 1969/. 0.5 uCi ^{32}P in 0.5 ml of 0.05 Tris-HCl buffer pH 7.2 was taken up by each leaf in 60 min. The labelling of the protoplasts and that of the corresponding half took place at the same time, i.e. starting 18 hrs after the isolation of the protoplasts. This 18 hrs of incubation time was chosen after preliminary experiments in which evidence was obtained that in the isolated protoplasts new viral RNA synthesis sets in and reaches a well detectable level after 16-20 hrs of incubation.

Nucleic acids were extracted by the phenol-cresol method as described by Ingle and Burns /1967/. Before extraction the protoplasts were washed three-times with the washing medium.

Procedure to extract nucleic acids

The solutions and glassware /Chromic acid washed/ were chilled before use. The leaf tissues /appr. half

g/ are homogenized in 2 ml extraction buffer composed of 0.5 M Tris-HCl, pH 7.5, 10 % TNS, 0.3 g PAS, 0.5 M NaCl and 0.1 M $MgCl_2$ in a glass homogenizer for 1.5 minutes. To the homogenate equal volume of 80 % phenol-cresol solution is added. Phenol re-distilled invacuo was used. The deproteinizing effect of phenol was increased by adding M-cresol /10 % to the phenol solution/. Furthermore, 8 -hydroxi-quinoline /0.1 %/ was also added because of the fact that it is useful in inhibiting oxidation of the phenol. The mixture was shaken throughly for 10 min. The two phases were separated from each other by centrifugation at 4000 rpm for 10 min. The whole procedure was carried out at 4°C. The nucleic acid containing aqueous phase was pipetted off and shaken once more for 10 min with an equal amount of water saturated phenol to which was added 0.15 ml/ml of 3 M NaCl. The mixture was centrifuged once more at 4000 rpm for 10 min. The upper phase containing nucleic acids were precipitated with two volumes of ice cold 96 % ethanol overnight at -20°C. The precipitate was collected by centrifugation next day and washed thrice in a washing solution comprising of 73 ml 96 % alcohol 10 ml 20 % sodium-acetate, 17 ml distilled water.

After washing, the pellets were dried completely and dissolved in 0.4 ml Tris-HCl buffer to estimate the O.D. units to be layered over the gels. The above mentioned procedure was followed in the same manner for the preparation of nucleic acids from protoplasts but for protoplasts homogenization, extraction buffer was found to be unnecessary, therefore, they were suspended throughly in the buffer and rest of the procedure was the same.

Since the fractionation of RNA by electrophoresis in supporting gels offers the possibility of more extensive and precise separation, polyacrilamide gel electrophoresis was performed on 2.5 % gels according to Loening /1967/ where gel pore size can be closely controlled. The gels are sufficiently transparent to be scanned in UV light. Recrystalized monomers were used.

Preparation of the gels

For the preparation of dilute gels in which the final acrylamide concentration is 2.4 % a mixture of the solutions with the following components were prepared.

A Acrylamide - 75 g + Bis-acrylamide 0.375 g in 50 ml water;

B Electrophoresis buffer with - 21.8 g; $NaH_2PO_4 \cdot 2\ H_2O$ 23.4 g; $MgCl_2$ 1.01 g in 1 l of distilled water;

C 10 % $/NH_4/_2S_2O_8$ - 50 mg/0.5 ml in distilled water /Catalyst/

D TEMED

The ratio: Solutions - A = 5 ml

- B = 6.25 ml /diluted 5 times before use/

- C = 0.25 ml

- D = 25 ul.

After achieving of the desired concentration of acryl amide and bis-acrylamide, solution was degassed at room temperature invacuo for about 20 seconds. NNNN - tetramethylethylene-deamine /TEMED/ /0.25 ml/ and ammonium persulphate /25 ul/ were added. The solution was mixed, excessive aeration being avoided, and rapidly pipetted into the vertical tubes. Plexiglass tubes 1/4 in internal diameter 5 in long were used. This facilitated later removal of the gels since polyacrylamide does not adhere to perspex. Rubber rings were inserted into the basis of the tubes after polimerization of the gels to prevent the soft gels from slidening out. 150 ml volume of buffer with platinum electrodes was used in each buffer compartment for a set of 6 tubes. The buffer was normally used once

only. Electrophoresis was carried out at about 5°C in a refrigerator. The gels warmed several degrees during the run. A drop of water is placed over the gels before pre-run, in order to prevent them from drying. The pre--run was started after the gels polymerized at room temperature. Up to 5 m A per gel was applied during the run. The current was normally applied for up to half an hour to remove the polymerization catalyst, and other impurities. The RNA sample dissolved /in 25 - 50 ul buffer/ and of 1 O.D. units was then layered over the gels and electrophoresis continued. Suitable amounts of RNase-free sucrose was usually added to the samples before layering, in order to increase the density of the samples. Good separations were obtained in three hours. After the run the rubber rings were removed and the gels were gently blown out of the tubes with a rubber teat into a beaker containing distilled water. The gels were picked up by sucking into tubes of the same diameter for scanning. The gels were scanned by using a Joyce-Loebl Chromoscan microdensitometer UV-light. The gels were held in a parallel sided Quartz container and scanned. After optical scanning, the gels were placed in a horizontal metal trough, with a top end of the gels /the origin/ lay-

Assay of virus concentration in protoplasts

Protoplasts of palisade cells were isolated by the method described previously from infected leaves. These were washed three times with sterile mannitol solution /0.7 M/ containing 0.1 M $CaCl_2$ and were suspended in the TMV incubation medium /Aoki and Takebe, 1969/ at a density of 3 to $4x10^5$ cells/ml. The protoplast suspension was divided into 10 ml portions and these were incubated in cotton pluged 100 ml Erlenmeyer flasks at room temperature under continuous illumination /500 lux/. Incubation was performed without shaking. After 18 hrs of incubation protoplasts were harvested washed once with mannitol solution. The samples were homogenized in a glass homogenizer for 1.5 minutes in 5 ml 0.1 M phosphate buffer pH 7. The homogenates were centrifuged for 10 min at 8000 g and the supernatant solutions were assayed for virus titer. The extract were appropriately diluted with 0.1 M phosphate buffer, pH 7 to give countable lesion numbers. Lesion count on each disc was calibrated against the standard virus solution /0.1 ug/ml/ inoculated on 24 corresponding leaf discs.

Fluorescent antibody staining was used for the de-

termination of the number of infected cells in the experimental material /cf. Otsuki and Takebe, 1969/.

A

Studies on viral and rRNA synthesis

The synthesis of viral RNA was studied in three different situations.

/a/ In primarily infected leaves 8 to 10 days after inoculation, and in protoplasts prepared from them.

/b/ In secondarily infected leaves 2 months after inoculation, and in protoplasts prepared from them.

/c/ In "true dark-green islands" /Atkinson and Matthews, 1970; cf. Footnote/ of systemically infected leaves in which virus replication stops at a low level as compared to yellow areas of the same leaf and protoplastssprepared from them.

Footnote

Tobacco leaves infected systemically with TMV developed various kinds of symptoms depending on the age of the plant, the position of the infected leaves etc. Best known is the mosaic pattern consisting of green and yellow areas which developed exclusively in secondarily infected leaves developing above the insertion level of the primarily infected leaf. As the leaves developed the yellow areas tend to become bigger and the green areas gradually break down./Atkinson and Matthews, termed true dark islands, those areas which are the most stable and contain the lowest amount of virus.

/a/ One fully expanded leaf 5 to 6th from the base of 6 to 7 weeks old tobacco plants was infected with TMV /1 mg/ml 0.01 M phosphate buffer, pH 72./ and after 8 to 10 days assayed for its capacity to synthesize TMV-RNA as described in Materials and Methods. As shown in Figure 1 in the attached hald of the leaf the synthesis of viral RNA was very much limited /Fig. 1A/ in contrast, there was a burst of viral RNA synthesis in the protoplasts prepared from the other half of the same leaf /Fig. 1B/.

/b/ A single secondarily infected leaf 5 to 6th from the base, 2 months after the inoculation of lower leaf was tested for its capacity to synthesize viral RNA by using the techniques described in Materials and Methods. In such leaves by the time of the experiment the virus induced mosaic pattern was almost entirely broken down and the leaves had turned yellow. As shown in Figure 2A in the attached half of the leaf no viral RNA synthesis was detected. In the protoplasts prepared from the other half there was a well defined although not very intensive incorporation of ^{32}P into the viral RNA, Fig. 2B.

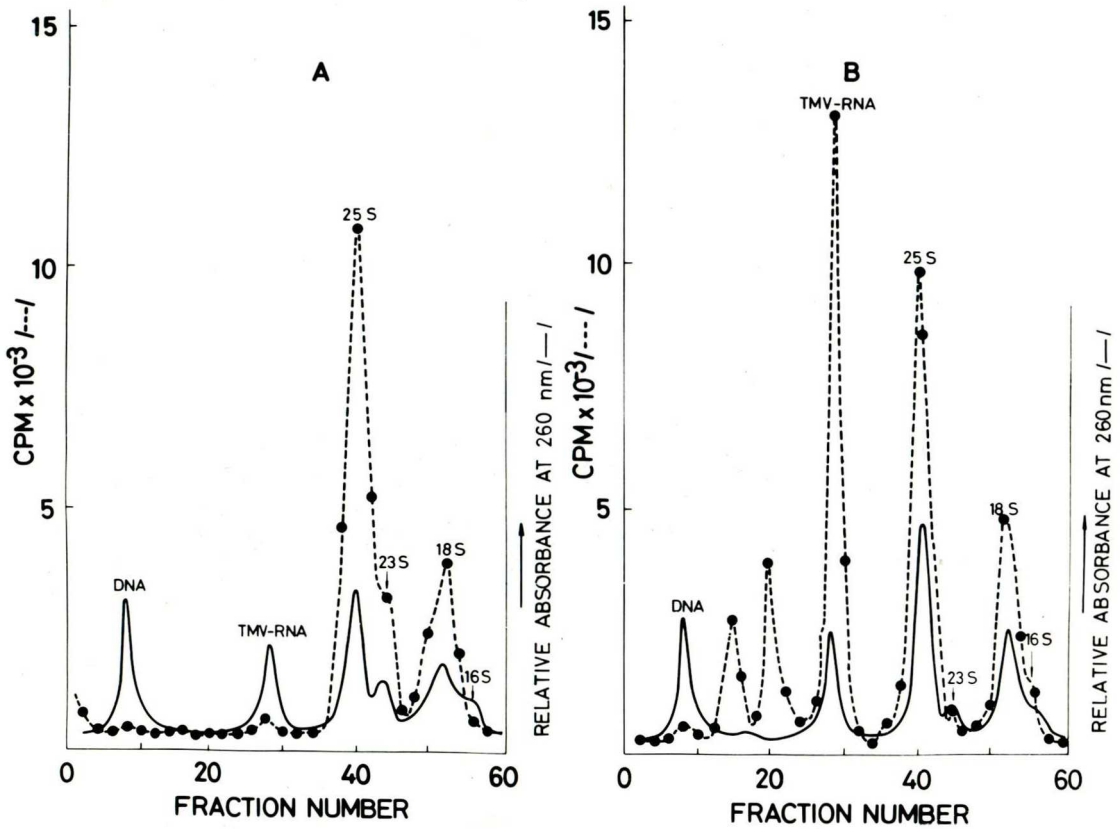

Figure 1

RNA synthesis in an attached half leaf of Xanthi tobacco primarily infected with TMV /A/; and in protoplasts prepared from the other half of the same leaf /B/.
For further details cf. Materials and Methods.

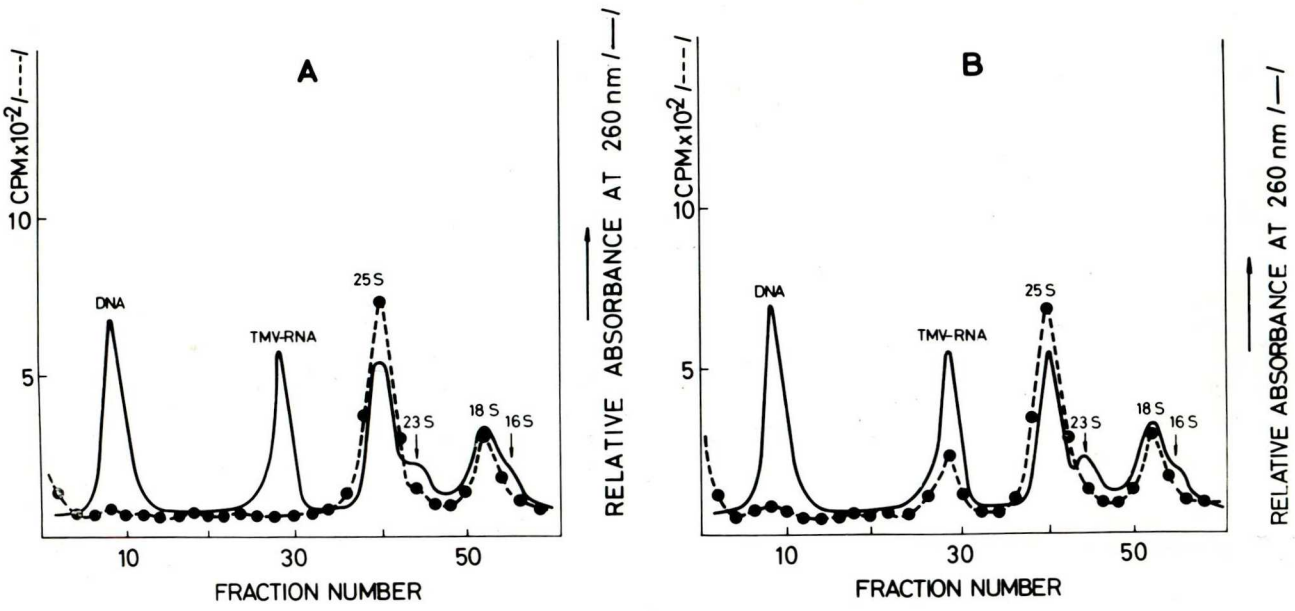

Figure 2.

RNA synthesis in an attached half leaf of Xanthi tobacco secondarily infected with TMV /A/, and in protoplasts prepared from the other half of the same leaf /B/.
For further details cf. Materials and Methods.

/c/ Leaves of approximately 2 to 3 months old plants infected with TMV in the 4 to 5 leaf stage and showing typical mosaic symptoms were used for these experiments. "Trus dark-green islands" as termed by Atkinson and Matthews /1970/ were cut out from halves of a number of leaves for the preparation of protoplasts, isotope feeding and nucleic acid extraction. The other attached halves of the same leaves were also fed with isotope. After the completion of the treatment /6 hrs/ the green islands were cut out from the attached halves leaves for the extraction of nucleic acids. The electrophoretic assay gave results which were similar to those obtained with systems A and B /cf. Figure 1 and 2/ i.e. the viral RNA synthesis was more intensive in the protoplasts preparations than in the "intact tissues" /Fig. 3/. This observation were confirmed by local lesion assay /Fig. 4/.

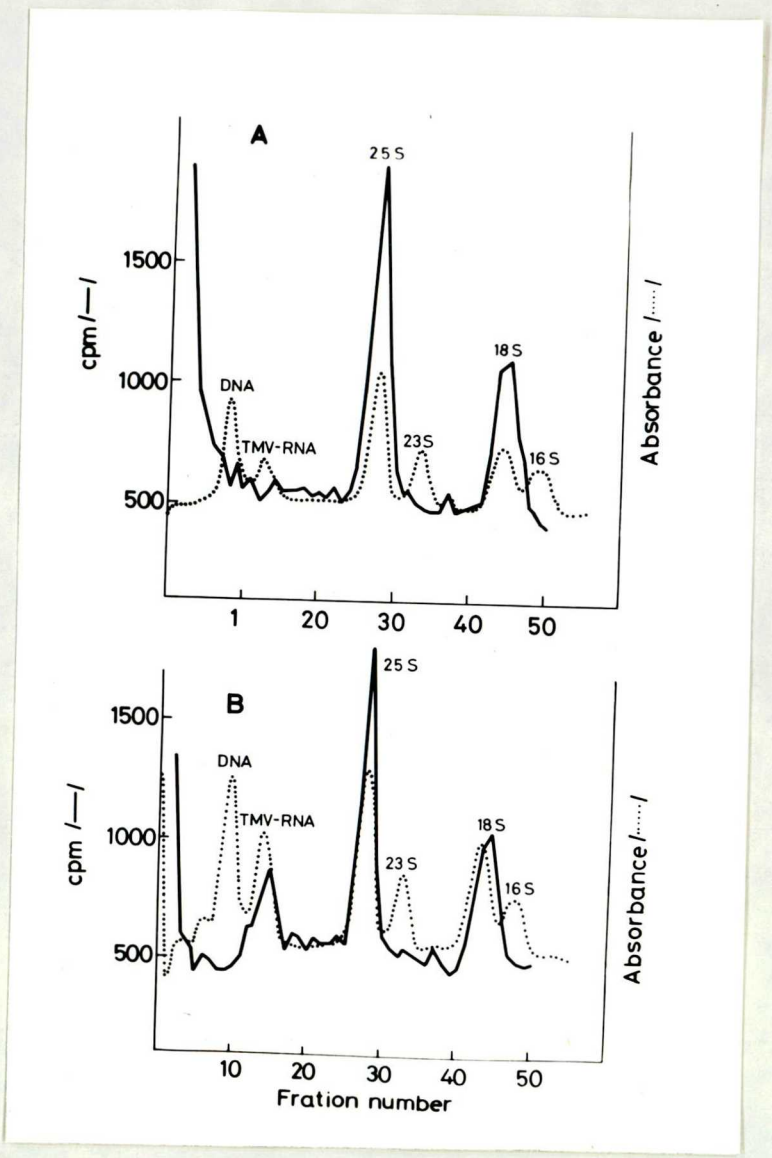

Figure 3

RNA synthesis in green islands ofaattached half leaves of Xanthi tobacco /A/ and in protoplasts prepared from green islands of other halves of the same leaves /B/. Details of the experiment described in Materials and Methods except that non-labelled infected carrier tissue was added to the sample before the extraction of nucleic acid to make reliable nucleic acid extraction possible in spite of the small sample size. This explains the presence of TMV peaks in the O.D. profile.

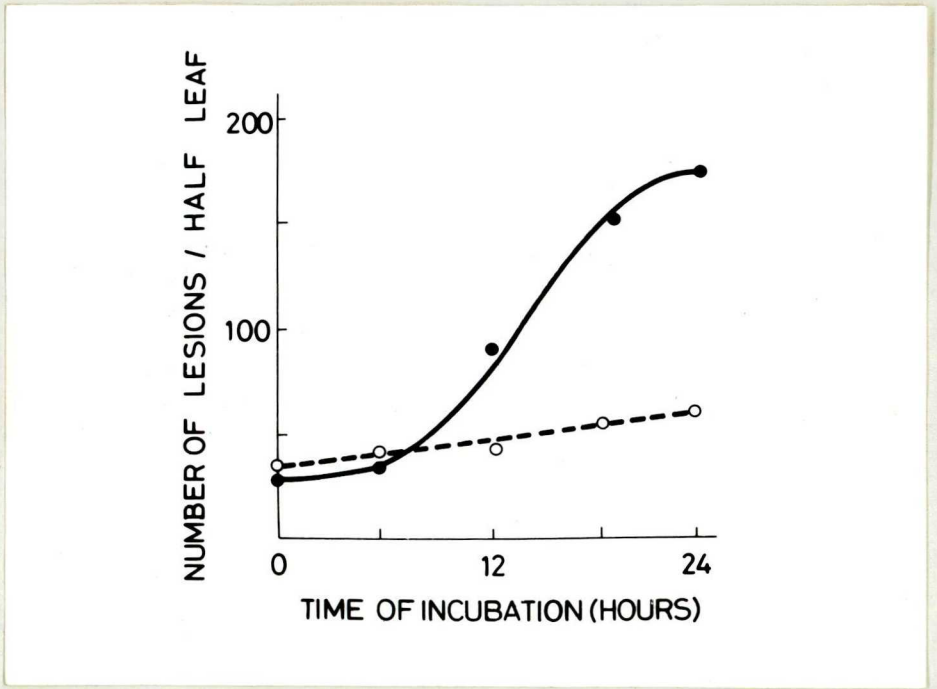

Figure 4

Virus multiplication in green islands of leaves of systemically infected Xanthi tobacco plants and in protoplasts prepared from them. Local lesion assay of virus content as a function of time. Protoplasts were prepared at O-time and their virus-content was followed for 24 h /_____/. Simultaneously the trend of change in virus content was followed in discs punched from comparable intact tissues /------/.

Percentage of infected cells in the various samples was established right after the isolation of protoplasts as well as at the end of 18 hrs incubation period by staining with fluorescent antibody. The leaves used in experiment "A" and "B" were found to be infected up to 90 % except in "C" that was on the average 30. No change in the percentage of protoplasts was experienced during incubation. A typical picture of virus containing protoplast stained with fluorescent antibody is shown in Figure 5.

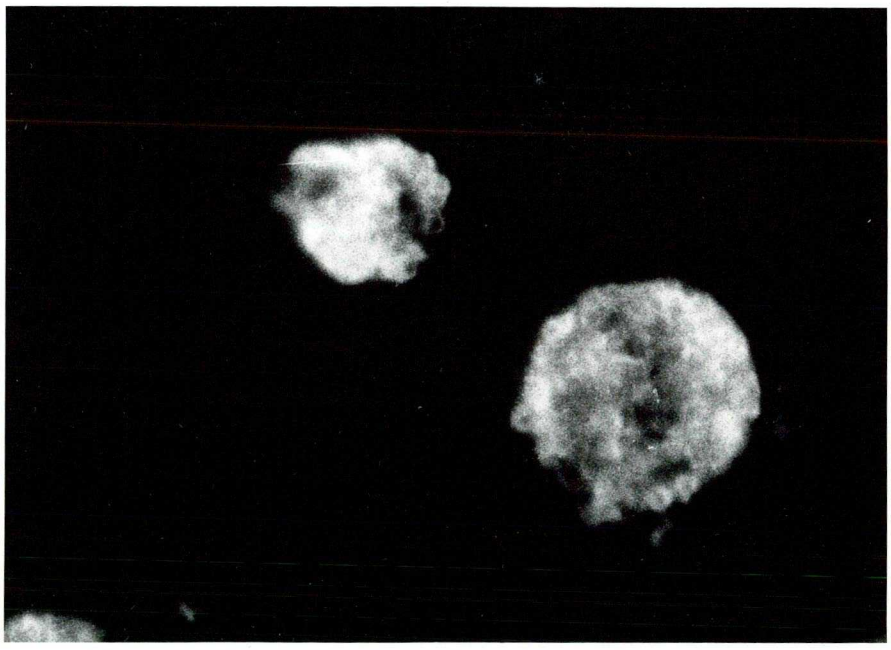

FIGURE 5

Virus containing tobacco leaf protoplast stained with fluorescent antibody. Picture taken in UV light.

The leaves used in experiment "B" almost invariably contained crystaline viral inclusion bodies which shows that the accumulation of TMV particles reached a high level /Figure 6/.

No inclusion bodies were seen in other types of tissues studied.

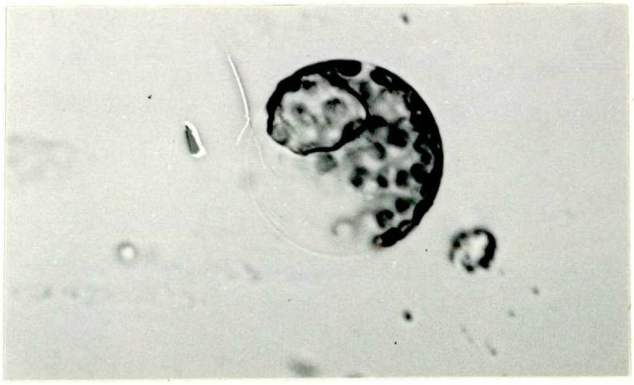

FIGURE 6

Typical inclusion body in protoplast isolated from Xanthi tobacco leaf secondarily infected by TMV.

DISCUSSION OF PART A

Isolated plant protoplasts should permit the direct investigation of many physiological, biochemical and genetic problems in plant biology, which previously have been unapproachable. The conversion of a cell of tissue into protoplast is certainly associated with dramatic changes in many pathways, both biosynthetic and degradative. Some processes have been shown to be stimulated after the isolation of protoplasts. Thus, it has been known since the first observations of Pojnar et al., /1967/ that the isolated protoplast form a new wall in a few hours visible in UV microscope with Calcofluor white as a specific stain. This wall formation has been studied by several workers especially by Roland and Prat /1973/; Nagata and Takebe /1970/. Protoplast isolation had also been accompanied by changes in the ultrastructural morphology of organelles. Thus, in isolated protoplasts there was an increase in the electron opacity of the chloroplast stroma and the mitochondria matrix compared to the same regions of these organelles in the intact leaf cells resulting in the chloroplast grana losing their prominence MacKenzie et al. /1973/ in young

cladodes, Asparagus officinalis L., Pea and broad bean leaf. During plasmolysis important ultrastructural modification of Nicotiana glutinosa L. has been noticed such as highly condensed chromatin within nuclease and mitochondria hypertrophy. Ruesink /1973/ had reported that structural changes in the protoplast membrane take place. In addition, a rapid increase in the level of a ribonuclease in isolated protoplasts has been described /Lázár et al., 1973/. In spite of these changes protoplasts seem to be physiologically normal in many ways due to the capacity to fuse, regenerate, to support virus multiplication etc. There is some evidence available to indicate that protoplasts are metabolically similar to the organ from which they derived /cf. Introduction/.

The present results pinpoint an important difference between the metabolic functions of protoplasts versus cells in situ. It was shown that intensified virus RNA synthesis is resumed in protoplasts prepared from tissues in which virus multiplication already has slowed down or stopped. It is not clear which are the factors that regulate the rate of the virus accumulation in tissues and set an upper limit when at the same time the synthe-

sis of other RNAs continue. Even less is known about the limit of virus multiplication in the green island of mosaic leaves, Atkinson and Matthews /1970/. According to these authors the islands of dark-green tissue containing very low amount of virus may arise from single cell by a process like lysogeny in bacteria, and these islands os cells may have escaped virus infection.

They also add that whatever converts healthy cells into dark-green cells must be able to spread from cell to cell at an early stage after systemic movement of the virus. Such dark-green areas appear to be resistant to TMV multiplication because no increase in concentration of TMV is detectable after super-inoculation with an original infecting strain and because junctions between yellow - green tissue which has a high concentration of TMV and dark-green island remain sharp for many weeks in spite of the fact that the two types of cells are connected by plasmodesmeta. The present experiments show that the "upper limit" of virus synthesis in such systems, is not a fixed one since, the limit is shifted to a much higher level if the cells of a tissue are converted into protoplasts. Therefore, the mechanism in favour of virus RNA synthe-

sis was triggered in these cells when they had been converted into protoplasts. Though no known factor can be attributed to this phenomenon it is due certainly to the removel of some kind of blockage which inhibits viral RNA synthesis. Therefore, the technique applied in the present work /Conversion of the cells of virus infected tissue into protoplasts/ appears to be useful in studies on the factors involved in the regulation of the synthesis of viral RNA. In experiments of these kind the most critical factors is the choice of a proper control, because of inherent difficulties due to the inhomogeneity of the plant material especially in dark-green areas. For a proper comparison of the properties of protoplasts "intact" tissue systems the simultaneous use of attached corresponding half leaves seem to be a prerequisite of obtaining reliable results. Even by using the attached half leaves as control it is not easy to compare the extent of incroporation of ^{32}P into the TMV-RNA peak with that into cytoplasmic ribosomal RNAs. Thus, the results presented are interpreted to mean that after the isolation of protoplasts the ratio of viral RNA/ribosomal RNA synthesis is shifted towards higher values.

A similar approach i.e. to draw conclusions as to the measure of relatively increase viral RNA synthesis by comparing the ratio of viral RNA/ribosomal RNA peaks on gel electrophoretograms was used by Zaitlin and Beachy /1974/ in other types of experiments.

In such type of experiments as described above the problem arises whether or not during the isolation of protoplasts uninfected cells become infected by virus particles released from broken protoplasts and the "new synthesis" described is actually due to new infection process and not to an intensification of viral RNA synthesis which has already slowed down. However, the fact that the rate of viral RNA synthesis is constant during incubation /Jackson et al., 1972/ indicates that viral RNA synthesis occurred only in cells that were infected at the time of isolation and no additional protoplasts became infected during the incubation period and that no new centers of viral RNA synthesis were established within and/or in addition to the cell already synthesizing virus. Moreover in our experiments the percentage of protoplasts staining with fluorescent antibody did not increase during the incubation of the protoplasts. We

obtained additional evidence by the finding that TMV /2 ug/ml/ added to the maceration medium during the preparation of protoplasts from healthy tissue did not result in infected protoplasts as shown by gel assay of their ^{32}P labelled RNAs /results not shown/. Furthermore, protoplasts derived from a cell population obtained by digesting healthy and systemically infected leaf pieces together did not show a higher level ^{32}P incorporation into TMV-RNA as compared to the "control" consisting of protoplasts obtained exclusively from infected tissues /results not shown/.

B

Studies on the hypersensitive reaction

Tobacco varieties carrying "N"-gene deriving from Nicotiana glutinosa L. respond to infection by tobacco mosaic virus by the formation of necrotic lesions, thereby localizing infection Holmes /1938/. Gross virus yield in the leaves of these varieties is consequently much lower than in those of parent tobacco varieties which permit systemic spread of virus. Data so far, available suggest that only a limited virus synthesis takes place in the leaf cells of necrotic varieties although the initial rate of virus replication might be higher at the site of infection. The behavior of necrotic varieties carrying the "N"-gene varies according to temperature. At 22°C the total amount of TMV accumulation in leaves of these varieties was much less as compared with that in a comparable systemic host. No significant difference in TMV multiplication was however found between both the types of host incubated at 28°C or above Shimomura /1971/. Evidences so far obtained indicate that localization of TMV in its local lesion host depends primarily on some virus limiting factor which acts in advance of cell necrosis /i.e. around the lesions/.

These varieties carrying the "N"-gene do not exhibit their characteristic responce to TMV infection under two different conditions. /1/ When infected leaf tissues were kept at high temperature and /2/ when isolated protoplasts were infected. This indicates that the introduction of the "N"-gene has no influence on the inherent capacity of individual tobacco leaf cells to synthesize TMV. It is known that the rate of virus multiplication in the leaf tissue of necrotic and systemic varieties with the same speed at least during the early phase of infection which again indicates that the function of the "N"-gene does not appear to be directly related to the structural or physiological aspects of individual leaf cells which effect initial virus multiplication and/or cell to cell movement of virus. This pioneering works of Otsuki et al. /1972/ suggest that the initially infected cell of these necrotic varieties produced a substancial amount of TMV before they collapse as a result of necrotic reaction which is supported further by the faet that the virus continues to multiply at a considerable rate as the lesions enlarge. A particularly intriguing question is why infected protoplasts from hypersensitive responding host show no hypersensitiv reaction even at 22^{o}C, a

temperature at which necrotic lesion formation is induced in leaf tissues. The prevelant explanation for this phenomenon is that the process of necrotisation is based on interaction of infected cells with the cells of the surrounding tissues, which may or may not be infected Otsuki et al. /1972/.

The present study was undertaken to test this working hypothesis from another angle. Specifically we studied whether or not cell to cell contact is needed for the triggering of "N"-gene action or is it needed also for the phenotypic expression of the gene /necrotisation/ after triggering has already taken place.

Two systems were used to study the above problem.

/a/ On Nicotiana tabacum cv. Xanthi nc. at 20°C when after TMV-infection local lesions develop, and

/b/ leaves of the same variety exposed to 30°C for inducing systemic spread of the virus in practically all cells by turning off the "N"-gene at that temperature and then when all cells were infected, we shifted the temperature to 22°C to turn on the "N"-gene to induce systemic necrosis of the tissue.

In both cases we used the plasmolysis of the tissues as a technique, to disrupt cell to cell connection within a tissue system /a technique not applied so far

in studying this problem/ at different times after infection to differentiate between "initiation" /triggering/ of the action of the "N"-gene and expression of the gene effect depending on whether or not cell to cell contact was or was not present at a particular time /when the gene was active or inactive according to the temperature regime chosen/.

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Nicotiana tabacum cv. Xanthi nc. plants were grown under normal greenhouse conditions. Plants were infected mechanically with the OM strain /Nozu and Okada, 1968/ of tobacco mosaic virus /TMV/.

Protoplasts when required were prepared as mentioned earlier. The main osmoticum to plasmolyse tissues used was mannitol but also allied compounds like sorbitol and glycerol were also tried.

To get systemic infection Xanthi nc. plants were mechanically infected with high inoculum /1 mg/ml/ TMV and kept at least for a week at a high temperature /30^{o}C/ in a phytotron at 10 000 lux, 16hrs per day, in order to accelerate virus multiplication. The plants were transferred to 22^{o}C to observe necrosis whenever needed.

——— • ———

We found that the infected hypersensitive leaf tissues kept at 22°C when exposed to mannitol at 0--tim failed to produce local lesion despite the fact that the viability of the cells remain unaltered. This was shown by their capacity to liberate viable protoplasts when enzymatically isolated after mannitol treatment. However, the same tissues respond normally, viz, by producing lesions, when they had been incubated at least for 24 hrs in water and then transferred to high osmoticum /0.7 M/. The lesions appear normal when the incubation time in water is increased to 48 hrs and later transferred to mannitol. In our system at 22°C lesion after 48 hrs were not yet visible even in the water control. In infected tissues after exposure for 24 to 48 hrs on water before transfer to mannitol the lesions were lesser in number and smaller in size. The summary of these results are given in Table I.

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The other system studied /in which the tissues became systemically infected due to the turning off "N"-gene at 30°C/ had the advantage that majority of the cells contained high amounts of virus, /this was shown by the presence of inclusion bodies in protoplasts

Table 1

Effect of plasmolysis, induced by 0.7 M mannitol, at various intervals after infection on lesion production* in N tabacum cv. Xanthi nc. leaf tissues

The medium tissues were floated on for various periods: Time after inoculation	0-24 h	24 - 48 h	48 - 72 h	72 - 96 h	Average No. of lesions developed
	H_2O	H_2O	H_2O	H_2O	25
	H_2O	Mannitol	Mannitol	Mannitol	5
	H_2O	H_2O	Mannitol	Mannitol	20
	H_2O	Mannitol	H_2O	H_2O	3
	Mannitol	Mannitol	Mannitol	Mannitol	0
	Mannitol	Mannitol	H_2O	H_2O	0
	Mannitol	H_2O	H_2O	H_2O	0

* The appearance of the lesions was detectable 72 hrs after infection

isolated from such tissues and by fluorescence antibody/. Upon transfer of the tissues to 22°C a rapid collapse of the whole tissue takes place within 8 hrs in air and visible necrosis develops on tissues floated on water in about 36 to 48 hrs /the possibility of water loss speeds up the reaction/.

We found that if the tissues are plasmolysed at 30°C and then transferred to 22°C no typical necrosis would develop /occasional appearance of diffuse areas was observed, cf. Figure 7/. But if inoculated and floated for 6 hrs on water and transferred then to 22°C on water, necrosis was visible /cf. Figure 8/. When mannitol treatment was carried out 24 hrs after the transfer of the tissues to 22°C /i.e. the tissues were kept for 24 hrs on water at 22°C/ the necrosis developed /cf. Figure 9./, to the same extent as in tissues kept throughout, on water during the whole period of exposal to 22°C /for 48 hrs/.

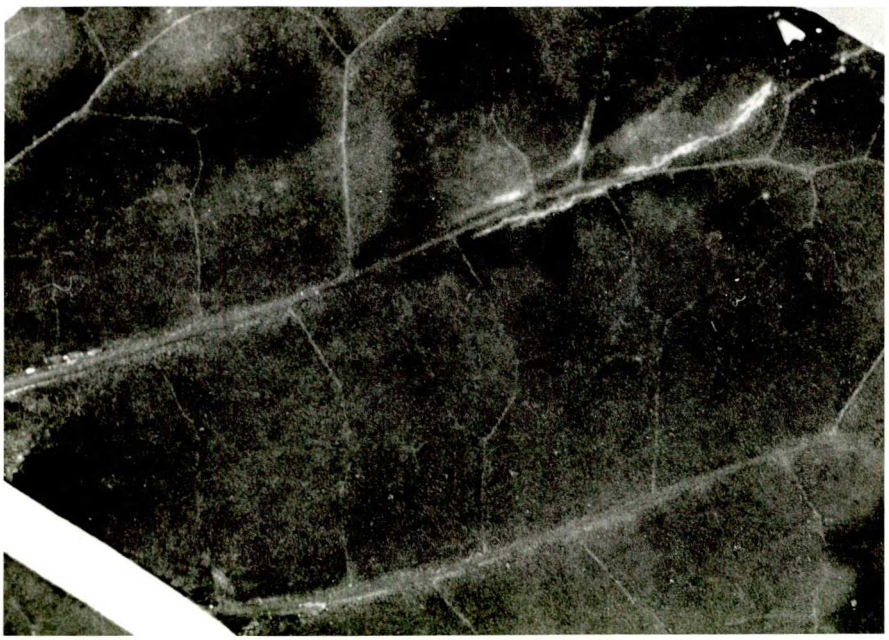

Figure 7

Systemically infected tobacco leaf carrying the "N"-gene plasmolysed for 6 hrs at 30°C before transfer to 22°C. Photograph was taken 48 hrs after incubation of the tissues on 0.7 M mannitol at 22°C.

Figure 9

Necrotisation of systemically infected tobacco tissue carrying the "N"-gene, the tissues were kept at 30°C on water for 6 hrs and then transferred to 22°C for 24 hrs on water and incubation was continued for an additional 24 hrs on 0.7 M mannitol at 22°C.

DISCUSSION OF PART B

The above experiments are preliminary in nature, but they allow the conclusion that cell to cell contact is needed for the initiation /triggering/ of the hypersensitive reaction, but not for the phenotypic expression. Plasmolysis is known to disrupt as a rule, cell to cell connection and plasmodesmata are not re-formed after the plasmolysis. This may be the reason why no necrotic lesions were formed in the local lesion tissues if they were plasmolysed right after infection and de-plasmolysed after 24 hrs /cf. Table I/. Such a de--plasmolysed probably does not represent a tissue in which the cell to cell contact is re-established. However, once in a system in which virus multiplication and/or spread reached a sufficient level on the conditions when cell to cell contact was present and the "N"-gene was turned on /22^{o}C/ the manifestation of the activity of the "N"-gene, the appearance of lesions was possible even if the cell to cell contact was broken by plasmolysis. This result is in line with the hypothesis that cell to cell contact is needed only for the initi-

ation but not for the manifestation of the hypersensitive reaction. The results obtained by the use of system II. /systemically infected "N"-gene carrying leaves/ eliminates the possibility that in system I the cell to cell contact is needed only for the spread has already occured and still there was a need to expose the tissue to inductive conditions /22°C when the "N"-gene is turned on/ in such a state when cell to cell contact was present. Necrosis developed in this system too, when after initiation this cell to cell connection was disrupted by plasmolysis.

SUMMARY

In protoplasts isolated from TMV-infected Xanthi tobacco leaves in an advanced stage of systemic virus infection, in which virus synthesis has already slowed down or stopped, viral RNA synthesis was shown to be renewed. This was demonstrated, by the use of isotope techniques, in three different systems:

/a/ In primarily infected leaves 8 to 10 days after infection;

/b/ In secondarily infected leaves 2 months after infection, and

/c/ In green islands of leaves exhibiting mosaic symptoms. The

The plant material used was assayed for the percentage of virus-containing cells by fluorescent antibody staining technique and by light microscopy to detect inclusion bodies.

The importance of cell to cell contact in hypersensitive reaction in Xanthi nc. plants was also studied.

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