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SEROTYPES OF RED CLOVER NECROTIC MOSAIC VIRUS I. CHARACTERIZATION OF THREE SEROTYPES

M. MUSIL, J. GALLO

Institute of Virology, Slovak Academy of Sciences, 817 03 Bratislava, Czechoslovakia

Summary. — Three serotypes (A, B and C) were distinguished based on serological differences between isolates of red clover necrotic mosaic virus (RCNMV). Isolate TpM34, representative of serotype A, induced the formation of antibody only against homologous antigen. By contrast, isolate TpM48, representative of serotype B, induced the formation of 3 groups of antibody; the group of type-specific antibody was present in a higher titre than the other two antibody groups. Isolate 63/70, representing serotype C, also induced the production of type-specific antibody in a higher titre as compared with antibodies reacting with type A and B antigens. The distinct behaviour of the 3 serotypes was also manifested on immunoelectrophoresis in agarose gel. In anionic barbital buffer, serotypes A and C showed a higher mobility than representatives of serotype B, but in cationic environment serotype A showed a higher mobility than serotypes B and C.

Key words: *red clover necrotic mosaic virus*; *serotypes*; *immunodiffusion*; *immunoelectrophoresis*

Introduction

Red clover necrotic mosaic virus (RCNMV) infects mainly red clover growing as field crops or on meadows and pastures (Musil et al., 1981). The virus was first described from Czechoslovakia (Musil, 1969) and later found in Poland (Kowalska, 1974; Blaszczak and Micinski, 1980), Sweden (Gerhardson and Lindsten, 1973), Great Britain (Hollings and Stone, 1977) and Australia (Gould *et al.*, 1981). Based on antigenic differences among the isolates, distinct antigenic groups (serological types) of RCNMV could be distinguished.

The present paper summarizes data about the antigenic relationship between representatives of 3 serotypes of RCNMV and the possibilities of their differentiation.

Materials and Methods

Viruses. The three RCNMV isolates used, namely TpM34 (serotype A) and TpM48 (serotype B) — see Musil (1969*a*, *b*), and 63/70 (kindly supplied by Dr. B. Gerhardson, Uppsala), were propagated in garden bean (Phaseolus vulgaris L. cv. Saxa) plants. The above-ground parts of the

infected plants were harvested 5—7 days after inoculation and kept frozen at —20 °C until used for virus purification. The frozen plants were homogenized in 0.1 mol/l phosphate buffer, pH 7.0 (1 g plant material per 1 ml buffer). After thawing, the brei was homogenized in a blender for 3 min with an equal volume of chloroform. The aqueous phase obtained after centrifugation at about 4 000 × g for 30 min was subjected to 2 cycles of differential centrifugation. The sediment obtained after centrifugation for 2 hr at about 100 000 × g was resuspended in 0.1 mol/l phosphate buffer, pH 7.0. The suspension was clarified at 4 000 × g for 10 min. The pellet obtained after the second high speed centrifugation was resuspended in phosphate buffer in a 1/20 volume of the starting aqueous phase. The clarified virus suspensions used as antigen were kept at 4 °C with 0.01% sodium azide added as preservative.

Antisera against isolates TpM34 and TpM48 were prepared by repeated intravenous immunization of rabbits with purified virus suspesnions (Musil, 1969b). Antiserum to isolate 63/70 was obtained from Dr. B. Gerhardson, Uppsala.

Titres of precipitation antibody in the antisera were determined in ring precipitin tests and expressed as reciprocals of the highest antiserum dilution that gave a positive reaction. Antisera were diluted with 0.1 mol/l phosphate buffer, pH 7.0, containing 10% glycerol.

The relationships between RCNMV isolates and their antisera were examined in agar gel double diffusion precipitation (further on immunodiffusion — ID) tests (1% Difco Special agar Noble in 0.05 mol/l phosphate buffer, pH 7.0, containing 0.01% sodium azide). Wells 4 mm in diameter and 7 mm apart were cut into a 1 mm thick agar layer in Petri dishes. The arrangement of the wells is evident from the illustrations. The dishes were incubated in a humid chamber at about 22 °C for 4—5 days, after which they were washed for 1 day in physiological saline and 3 days in distilled water. The precipitation lines were then stained with amido black 10 B.

Absorption of antisera. To one volume of antiserum were added 2—5 volumes of heterologous or homologous antigen. After thorough mixing, the mixtures were incubated in tubes overnight at 4 °C. The precipitates formed were removed by centrifugation for 10 min at 4 000 × g and the absorbed antisera were assayed for antibody and antigen. In controls, antisera were absorbed with sap from healthy bean plants.

Immunoelectrophoresis. One per cent agarose (Loba-Chemie, Austria) gels buffered with 0.04 mol/l sodium diethylbarbiturate — HCl, pH 7.2, or 0.025 mol/l Tris — HCl, pH 8.2, were used. Two-mm thick gels were spread on to glass plates on an area of 8 × 5 cm. Ten microliters of purified virus suspension were placed into the wells (for their arrangement see Figs 2 and 3). Electrophoresis lasted for 18 hr at 4 mA and 20 V per gel. After the end of electrophoresis, the longitudinal grooves were filled with antiserum diluted 1 : 5. The precipitation lines were stained for 2 hr with 0.04% blue R (Serva) in a 45 : 45 : 10 mixture of methanol — distilled water — acetic acid. The gels were destained overnight in the same mixture and then kept in 7% acetic acid.

Results

Immunodiffusion

The titres of precipitation antibody to homologous antigens in the antisera used were 640 (TpM34), 1280 (TpM48) and 2560 (63/70). No antibody to heterologous antigens was found in TpM34 antiserum. In addition to homologous antibody, antisera TpM48 and 63/70 contained antibodies reacting with heterologous antigens in titres from 160 to 640 (see Table 1). The antisera used contained low titres (2—4) of antibody to normal plant proteins that could be absorbed with sap from healthy bean plants.

ID tests, in which homologous and heterologous antigens were placed in peripheral wells and antisera in central wells, revealed the following patterns. In TpM34 antiserum, we demonstrated only a group of antibody reacting with homologous antigen by a marked precipitation line; no precipitation lines were formed against heterologous antigens (Fig. 1a). By contrast, antisera TpM48 and 63/70 contained groups of antibody reacting

Table 1. Titre of precipitin antibody in antisera against virus isolates TpM34, TpM48 and 63/70

Antiserum	Antibody titre Ag TpM34	Ag TpM48	Ag 63/70
As TpM34 (nonabsorbed)	640	0	0
As TpM48 (nonabsorbed)	160	1280	640
As 63/70 (nonabsorbed)	320	640	2560
As TpM48 absorbed with	0	640 (×2)	20 (×4)
As TpM48 absorbed with			
Ag TpM34	0	640 (×2)	20 (×4)
Ag 63/70	80 (×2)	320 (×4)	0
As 63/70 absorbed with			
Ag TpM34	0	20 (×4)	640 ×4)
Ag TpM34	0	20 (×4)	640 (×4)
Ag TpM48	0	0	320 (×4)

In parentheses: n-fold dilution of antiserum samples resulting from the addition of antigen for absorption.

by a marked predipitation line with the respective homologous antigens and antibodies reacting by less marked precipitation lines with heterologous antigens. The precipitation line formed by TpM48 antiserum with homologous antigen showed a spur against both 63/70 and TpM34 antigens. The precipitation lines formed by TpM48 antiserum with TpM34 and 63/70 antigens crossed each other (Fig. 1b). The precipitation line formed by 63/70 antiserum with homologous antigen ended by a spur against both TpM34 and TpM48 antigens. A less marked precipitation line against TpM48 antigen surpassed the precipitation line against TpM34 antigen by a little marked spur (Fig. 1c).

Absorption with homologous antigen removed all antibody from the sera used. Absorption with heterologous antigens removed only their respective antibody groups from antiserum TpM48. On absorption of TpM antiserum by TpM34 antigen, antibody to the latter antigen was removed but antibodies to TpM48 and 63/70 antigens remained in the antiserum. Similarly, after absorption of TpM 48 antiserum with 63/70 antigen, the antiserum still contained antibodies to TpM48 and TpM34 antigens. From 63/70 antiserum, the TpM34 antigen removed only antibodies against this antigen, but antibodies against 63/70 and TpM48 antigens were preserved; the titre of TpM48 antibody dropped after absorption. By contrast, absorption of 63/70 antiserum with TpM48 antigen resulted in removal of antibodies reacting with both TpM48 and TpM34 antigens (see Table 1, Fig. 1 d-i).

Immunoelectrophoresis

The three RCNMV isolates showed different mobilities in anionic and cationic buffers. In anionic buffer (0.04 mol/l sodium diethylbarbiturate — — HCl, pH 8.6), isolates TpM34 and 63/70 showed the greatest mobility,

while TpM48 moved only little if at all (Fig. 2). In cationic buffer (0.04 mol/l sodium diethylbarbiturate — HCl, pH 7.2, or 0.025 mol/l Tris — HCl, pH 8.2) the mobility of the TpM34 isolate was preserved, but isolates 63/70 and TpM48 moved only little if at all. The mobility of the isolates was not affected by virus concentration.

The characteristic mobility of the individual isolates was preserved also on electrophoresis of mixtures of antigens belonging to two serotypes (Fig. 3). By an appropriate selection of buffers it was thus possible to differentiate RCNMV serotypes based on the different mobility of the isolates in agarose gels.

Discussion

The properties of the three antisera and antigens examined (TpM34, TpM48 and 63/70) indicate that these isolates represent three distinct antigenic (serological) groups (types) of RCNMV. In addition to two previously (Musil, 1969b) established groups (serotypes), namely serotype A (isolate TpM34) and B (isolate TpM48), isolate 63/70 represents a further serotype, designate C.

Serotype A, represented by isolate TpM34, is characterized by antiserum with a narrow range of antibody that reacts only with homologous antigen (type-specific group of antibody). By contrast, antisera against isolates TpM48 and 63/70 representing serotypes B and C, respectively, displayed a broade range of antibodies. Of these, type-specific antibody was present in a higher titre than antibodies reacting specifically with heterologous antigens. The reaction of these antibody groups indicates the degree of antigenic relatednes of the individual serotypes. Antibodies reacting with heterologous antigens can be removed from the antisera by absorption with the respective antigen, or they can be eliminated by appropriate dilution of antiserum, the activity of the type-specific antibody remaining preserved. Antisera treated in this way react only with antigen of the respective serotype and are suitable for serotyping of RCNMV isolates (Musil *et al.*, 1982).

Differences between the three RCNMV serotypes became manifested also on immunoelectrophoresis in agarose gel. Isolates TpM34 (serotype A) and 63/70 (serotype C) in anionic barbiturate buffere showed a greater mobility than isolate TpM48 (serotype B). But in cationic buffer (Tris — HCl, pH 8.2, or barbiturate, pH 7.2) only isolate TpM34 moved distinctly more rapidly than the two other isolates. Hollings and Stone (1977), however, reported that, in phosphate buffer, isolate TpM48 showed a greater mobility than two other isolates.

Difference in electrophoretic mobilities similar to those found in type representatives of the individual serotypes were also found in other RCNMV isolates, depending on the serotype. Isolates representing mixtured of two serotypes were separated on immunoelectrophoresis according to the mobility in a given environment. Precipitation lines were formed at distances corresponding to those characteristic of the type isolates. The equal electro-

phoretic mobility of RCNMV isolates belonging to a given serotype makes it possible to use immunoelectrophoresis for the classification (differentiation) of RCNMV isolates (Musil *et al.*, 1982).

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Explanation of Figures (Plates L and LI):

Fig. 1. ID tests with three RCNMV isolates and the respective non-absorbed and absorbed antisera.
Central wells: antisera —
a — As TpM34, non-absorbed
b — As TpM48, non-absorbed
c — As 63/70, non-absorbed
d — As TpM48 absorbed with Ag TpM34 and 63/70
e — As TpM48 absorbed with Ag TpM34
f — As 63/70 absorbed with Ag TpM34
g — As 63/70 absorbed with Ag TpM34 and TpM48
h — As TpM48 absorbed with Ag 63/70
i — As 63/70 absorbed with Ag TpM48
Peripheral wells: antigens — A — Ag TpM34, B — Ag TpM48, C — Ag 63/70.

Fig. 2. Immunoelectrophoresis of RCNMV isolates with homologous antisera in agarose gel buffered with 0.04 mol/l sodium diethylbarbiturate — HCl, pH 8.6.
1—3: isolate TpM34, 4—6: isolate TpM48, 7—9: isolate 63/70. Three samples of each isolate, prepared at different periods of time, were tested.

Fig. 3. Immunoelectrophoresis of RCNMV isolates with a mixture of antisera (As to TpM34 + As to TpM48 + to 63/70) in agarose gel buffered with 0.04 mol/l diethylbarbiturate — HCl, pH 8.6.
1 — TpM34, 2 — TpM34 + TpM48, 3 — TpM48, 4 — 63/70, 5 — 63/70 + TpM48, 6 — TpM48, 7 — 63/70, 8 — 63/70 (diluted 1 : 10), 9 — 63/70 + TpM34.