Serological Grouping of Tomato Ringspot Virus Isolates: Implications for Diagnosis and Cross-Protection

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ABSTRACT

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This study was done to determine the serological relationships between a wide range of tomato ringspot virus isolates and to identify a model system for studies of cross-protection. Polyclonal antisera were prepared to the "standard isolates": Chickadee, grape yellow vein, peach yellow bud mosaic, and West Virginia-18. Thirty-three isolates, collected from different areas of North America and Europe, were then compared by serology and a limited host range. The isolates could be classified into five serogroups by direct double-antibody sandwich enzyme-linked immunosorbent assay, using the antisera to the standard isolates. None of the antisera was able to detect all of the isolates. Indirect enzyme-linked immunosorbent assay was less specific but only suitable with purified or partially purified antigen. Agar double-diffusion tests confirmed that the standard isolates were related but not identical. Some isolates could be clearly distinguished by their virulence on *Nicotiana benthamiana*. Chickadee and peach yellow bud mosaic were found to be serologically and biologically distinct isolates that provide an excellent system for studies of cross-protection with tomato ringspot virus.

Tomato ringspot virus (TmRSV), a nepovirus, has a wide host range, including herbaceous and woody plants, and is transmitted by nematodes in the genus *Xiphinema* (21). TmRSV causes severe diseases in fruit and berry crops in North America (21) and has been isolated from ornamental and berry crops in other parts of the world (4,7,11,16,17,21).

The level of severity of TmRSV-incited diseases varies according to geographic location. For example, symptoms of peach stem-pitting disease (1) are much more severe in the Middle Atlantic area of North America than in western New York State. Similarly, the incidence of apple union necrosis and decline disease (25), which presumably is also caused by TmRSV, is far more pronounced in the eastern part of New York State than in the western part, even though surveys of apple orchards in both areas revealed comparable TmRSV incidence (19). Moreover, the hypothesis has recently been advanced that there might be strains of TmRSV on the East Coast of North America that are distinct from strains on the West Coast. However, it was not known whether observed differences in disease severity were due to environmental conditions or to different virus strains.

TmRSV-induced diseases in fruit trees are difficult to control. Current control strategies—such as the use of virus-free planting stocks, fallowing, and the use of pesticides to lower nematode vector populations and weed hosts (virus inocula)—are expensive, often ineffective, and potentially hazardous to the environment. Cross-protection might offer an alternative for controlling diseases caused by TmRSV. It is the phenomenon by which one strain of a virus (the protecting strain) can protect a plant against infection or damage by a second, related strain (the challenge strain).

For carrying out cross-protection studies, a method is needed to distinguish between the protecting and challenging strains. Serology might provide the tool, even though little serological variability among TmRSV isolates was detected in earlier studies (done with limited numbers of isolates) in tube precipitin and agar double-diffusion tests between some isolates (3,5,8,9,11,18,24,26), indicating that these TmRSV isolates contain both similar and different antigenic determinants. More pronounced serological distinctions between TmRSV isolates were expected with enzyme-linked immunosorbent assay (ELISA), since it generally is more powerful than immunodiffusion in differentiating between closely related viruses and virus isolates (10). With ELISA as a diagnostic tool, it was indeed difficult or impossible to detect some TmRSV isolates with certain antisera (2,12,20).

An understanding of serological differences between TmRSV isolates is a prerequisite for efficient use of ELISA in diagnostic work. On the other hand, serological properties might be correlated with the geographic origins of the isolates, and serology might be used in epidemiological studies. Furthermore, serologically (and biologically) distinct isolates would provide a very useful tool for the study of the control of peach stem pitting by cross-protection that is currently under way in our laboratory.

The objectives of this study were to determine the serological relationships between TmRSV isolates from a representative wide range of geographic origins, to investigate a possible correlation between serogroups and the geographic origin of the isolates, and to identify a model system for our cross-protection work as a disease control strategy, in which TmRSV isolates could be independently traced in plant tissue by ELISA.

MATERIALS AND METHODS

Virus isolates. Thirty-three TmRSV isolates were acquired from 13 states of the United States, from Canada, and from Europe (Table 1). They were obtained from woody as well as from herbaceous host plants, and five isolates were collected from soil by baiting with cucumber seedlings. The isolates were biologically purified by at least four consecutive single-lesion passages through cucumber (*Cucumis sativus* L. 'Marketer') or through *Chenopodium quinoa* Willd. and then transferred to *Nicotiana benthamiana* Domin, cucumber, and cowpea (*Vigna unguiculata* (L.) Walp. subsp. *unguiculata* 'California Blackeye') for further evaluation and for storage by freeze-drying. The isolates were also maintained in periwinkle (*Catharanthus roseus* (L.) G. Don) and globe amaranth (*Gomphrena globosa* L.) in the greenhouse. Four TmRSV isolates were selected for purification and antiserum production: Chickadee, introduced into New York State in rooted MM.106 apple layers (22), an isolate that has not caused any symptom of apple union necrosis and decline disease in New York

State since its introduction 12 years ago; grape yellow vein (GYV), isolated from diseased grapevines in California (8); peach yellow bud mosaic-Geneva (PYBM), an isolate originally obtained from California, which systemically infects peach trees in the field in New York State (M. W. Bitterlin, *unpublished*); and West Virginia-18 (WV-18), obtained by soil baiting from a peach orchard affected with peach stem-pitting disease in West Virginia. For convenience, these four isolates are referred to as the *standard isolates.*

Virus propagation and purification. Local-lesion isolates of Chickadee, PYBM, and WV-18 were purified from cowpea, essentially according to a procedure described for cucumber mosaic virus (14). Primary leaves of cowpea plants were inoculated 10–14 days after planting. The plants were maintained in the greenhouse at about 24 C with 13- to 14-hr supplemental fluorescent lighting. Systemically infected leaves were harvested 10–14 days later. The GYV isolate was purified from *N. benthamiana*. These plants were inoculated when 5–8 wk old, and symptomatic leaves were harvested 6–9 days later. Tissue was ground 1:2 (w/v) in a blender in extraction buffer (0.5 M sodium citrate containing 0.1% thioglycolic acid, pH 6.5), to which chloroform was added 1:1 (w/v) during grinding. After low-speed centrifugation, the aqueous phase was poured through glass wool. The virus was then precipitated with polyethylene glycol (MW 6,000 or 8,000 at 10% concentration) and resuspended in 0.05 M sodium citrate containing 2% Triton X-100, pH 7.0. The virus was concentrated by two cycles of differential centrifugation in PEN buffer (0.01 M sodium phosphate, 0.001 M ethylenediaminetetraacetic acid, and 0.001 M sodium azide, pH 7.0), further purified by sucrose density gradient centrifugation (10–40% sucrose, w/v, in PEN buffer), and finally concentrated from the sucrose fractions by high-speed centrifugation.

Production of antiserum. Antisera to Chickadee, PYBM, and WV-18 were produced in New Zealand white rabbits by repeatedly injecting 0.9–1 mg of purified virus in 1 ml of PEN buffer emulsified 1:1 with Freund's adjuvant. Complete adjuvant was used in the initial injection and incomplete adjuvant in subsequent ones. Two intramuscular injections of Chickadee and WV-18 were administered at 7- and 8-day intervals, respectively. With PYBM, part of the emulsion (about 0.2 ml) was injected interdigitally and the remainder intramuscularly during the first two immunizations (6 days apart), and 0.4 mg of virus was administered intramuscularly 23 days after the second injection. The rabbits were bled, starting 13–23 days after the first injection, at 4- to 11-day intervals for a period of 1–4 mo. Antisera titers were determined by Ouchterlony double-diffusion serology. The antiserum to GYV was obtained from G. V. Gooding, Jr. (North Carolina State University, Raleigh).

Agar double-diffusion tests. Serological relationships between the standard isolates (Chickadee, GYV, PYBM, and WV-18) were determined by testing their homologous and heterologous reactions to antisera obtained from bleedings 23–41 days after the first injection of the rabbits. The tests were conducted in polystyrene petri plates containing a 2-mm layer of 0.75% Ionagar, 0.85% sodium chloride, and 0.13% sodium azide. Wells 7 mm in diameter were spaced 5 mm apart in a hexagonal pattern. The center wells were filled with 55 μl of purified γ-globulin (167 μg/ml) or with unfractionated antiserum diluted 1:32 in 0.05 M Tris-Cl buffer, pH 7.2, containing 0.85% sodium chloride, 5% bovine serum albumin, and 0.02% sodium azide. The antigens,

TABLE 1. Origins of tomato ringspot virus isolates, reactions on *Nicotiana benthamiana*, and serological grouping[a]

Isolate	Origin of isolate		Symptoms on *N. benthamiana*[c]	Serogroup[d]
	Host	Location[b]		
Chickadee	Apple MM.106	Oregon	Mild	A
Grape yellow vein	Grape	California	Moderate	B
PYBM-Geneva[e]	Peach	California	Severe	C
West Virginia-18	Cucumber baiting	West Virginia	Severe	D
PYBM-Cal	Peach	California	Severe	C
Oregon	*Rubus* spp.	Oregon	Severe	C
Washington	Raspberry	Washington	Severe	C
Rhubarb	Rhubarb	Wisconsin	Severe	C
Michigan	Raspberry	Michigan	Severe	E
Indiana	Cucumber	Indiana	Severe	D
Amberg	Apple MM.106	New York	Severe	E
Chickadee S	*N. benthamiana*	New York	Severe	A
HV-1	Dandelion	New York	Severe	E
HV-2	Cucumber baiting	New York	Severe	E
HV-Dressel	Apple MM.106	New York	Severe	E
HV-Prune	Peach	New York	Severe	E
Smith L15-2	Cucumber baiting	New York	Severe	E
Smith K18-4L	Cucumber baiting	New York	Severe	E
Smith K18-3R	Cucumber baiting	New York	Severe	D
Smith Temik D	Dandelion	New York	Severe	E
Apricot	Apricot	Maryland	Severe	E
Vermont	Apple MM.106	Vermont	Severe	E
Harrisburg D	Dandelion	Pennsylvania	Severe	E
Harrisburg A	Apple MM.106	Pennsylvania	Severe	D
WV 9-3	Peach	West Virginia	Severe	D
WV 16-3	Peach	West Virginia	Severe	D
Winchester	Apple MM.106	Virginia	Severe	D
Denmark-9717	Geranium	Denmark	Severe	C
Staff	Grape	Canada	Severe	E
PBL-Alonzo	Prune	California	Moderate	C
Black tartarian	Cherry	New York	Moderate	E
New Jersey	Peach	New Jersey	Moderate	E
Mazzard West	Mazzard rootstock	New York	Mild	D

[a] The first four isolates of the table are the standard isolates; antisera to these isolates were used.
[b] State in the United States or country of origin.
[c] Symptoms are illustrated in Figure 5.
[d] Serogroups are defined in Table 2.
[e] PYBM = peach yellow bud mosaic.

applied to peripheral wells, consisted of purified virus at 50 μg/ml in PEN buffer or of undiluted sap prepared from freshly harvested leaves of infected *N. benthamiana* or periwinkle. The plates were observed for precipitin reactions up to 64 hr after incubation at room temperature (Fig. 1).

ELISA. Serological studies were made in two different experiments. In one experiment, the standard isolates (purified antigens) were compared by the direct double-antibody sandwich form (6), hereafter referred to as direct ELISA, as well as by indirect ELISA (13) using γ-globulins purified from antisera of the same bleedings used in the agar double-diffusion tests. In another experiment, crude antigens from 33 isolates were tested by direct ELISA. These antigens consisted of crude sap of *N. benthamiana*, periwinkle, cucumber, and *G. globosa* leaf tissue, diluted 1:22 to 1:25 (w/v) in phosphate-buffered saline containing 0.05% Tween 20 and 2% polyvinylpyrrolidone (6).

For the direct ELISA procedure, plates (Immulon 2, Dynatech Co., Chantilly, VA) were coated with γ-globulin at 1 μg/ml in coating buffer (0.05 M sodium carbonate containing 0.02% sodium azide, pH 9.6). Alkaline phosphatase-labeled γ-globulin was applied at dilutions of 1:2,000, 1:800 to 1:1,000, 1:1,000 to 1:1,250, and 1:1,250 (v/v) for Chickadee, GYV, PYBM, and WV-18, respectively. The substrate *p*-nitrophenyl phosphate was added at 1 mg/ml. The plates were read at 405 nm with a Microelisa Auto Reader MR 580 (Dynatech) three or four times after 10–60 min of hydrolysis reaction.

Extraction buffer, healthy tissue, TmRSV-infected tissue, and/or purified virus were included as controls in each plate. The data in Figures 2 and 3 are averages of 30- or 45-min readings from four replicate wells in two tests, giving values between 0.9 and 1.5 for the optical density (OD) at 450 nm in the homologous reactions (at the lowest dilutions). The data for the standard isolates in Figure 4 represent averages of 14 replicate wells per plant species in seven repeated tests (using different plant species, such as *N. benthamiana*, periwinkle, cucumber, globe amaranth, and *C. quinoa*), and the data for the other isolates are averages of six to 14 wells in at least three repeated tests. The conditions for indirect ELISA were the same as those for direct ELISA, with two exceptions: the antigens were diluted in coating buffer, and goat antirabbit immunoglobulin G (whole-molecule) alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO, product number A 8025) was used at a dilution of 1:1,000.

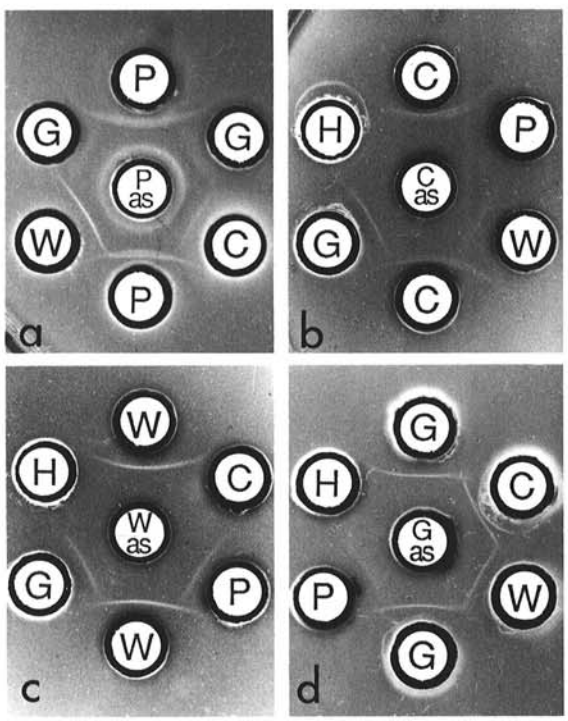

Fig. 1. Serological reactions of the four standard isolates of tomato ringspot virus in agar double-diffusion tests. The center wells contained unfractionated antiserum (as) to peach yellow bud mosaic-Geneva, diluted 1:32 (**A**), and purified γ-globulin at 167 μg/ml to Chickadee (**B**), West Virginia-18 (**C**), and grape yellow vein (**D**). The peripheral wells contained either purified virus at 50 μg/ml or infected or healthy sap of *Nicotiana benthamiana* or periwinkle: P = peach yellow bud mosaic virus; C = Chickadee; W = West Virginia-18; G = grape yellow vein; H = healthy tissue.

Biological properties of isolates. With regard to our cross-protection study, we were interested to find a host plant that would reveal differences in virulence between serologically distinct isolates. At least six plants each of *C. quinoa*, cucumber, cowpea, *N. benthamiana, N. tabacum* L. 'H-423,' and *N. rustica* L. were inoculated with isolates of TmRSV. Virus infection was confirmed by ELISA. The plants were kept in the greenhouse, and symptom severity was assessed.

RESULTS

Virus yield and production of antiserum. Initially, cowpea was used as a virus propagation host in all purifications in order to avoid the host variability factor. However, very different yields were obtained with various isolates: 12.8 mg/kg of tissue for PYBM, 4.0 mg/kg for Chickadee, 0.7 mg/kg for WV-18, and no measurable yield for GYV. Because no measurable yield was obtained from cowpea with GYV, this isolate was purified from *N. benthamiana*, yielding 0.7 mg/kg.

Virus-specific antisera titers of 2,048 (denominator of dilution), determined in agar double-diffusion plates against purified virus at 50 μg/ml or against crude antigens from undiluted tissue, were obtained to all isolates 5–7 wk after the first injection of the rabbits. These antisera reacted with healthy cowpea tissue at dilutions of 1/2 (PYBM), 1/4 (Chickadee), and 1/32 (WV-18). Antisera from these bleedings were used for all subsequent serological studies, except for WV-18 antiserum, for which a bleeding 23 days after the first injection was used. This antiserum had a virus-specific titer of 256 and a nonspecific titer of 8 against healthy cowpea. There was no reaction of any antiserum with healthy *N. benthamiana* or periwinkle, the plant tissues used in the agar double-diffusion tests.

Agar double-diffusion tests. Reciprocal comparisons of the four standard isolates (PYBM, WV-18, Chickadee, and GYV) in agar double-diffusion tests showed that they were serologically related but not identical (Fig. 1). Usually, the homologous precipitin lines were most pronounced, and heterologous reactions were often very weak; spurs (partial fusions) were formed between homologous and heterologous isolates. Spurs also occurred between different isolates when employed against a third antibody, e.g., between GYV and WV-18 isolates against the PYBM antibody but not between Chickadee and GYV (Fig. 1A). No reaction with healthy plant tissues was observed.

ELISA. Reciprocal comparison of TmRSV isolates by direct ELISA revealed that the four standard isolates were serologically distinct (Fig. 2). When purified antigens were diluted serially, the homologous reactions were considerably stronger than the heterologous ones at all antigen dilutions (Fig. 2). No isolate was detected with all antibodies. For example, Chickadee and GYV cross-reacted with GYV and Chickadee antibodies but were not detectable with PYBM and WV-18 antibodies, and the PYBM isolate reacted essentially only with the homologous antibody. Reactions against healthy tissue were negligible (the average OD at 405 nm was less than 0.05) and are not presented in the figures.

By indirect ELISA (Fig. 3), more cross-reactivity among isolates was observed with the antibodies; yet, the trends were similar to those established by direct ELISA. For example, Chickadee and GYV isolates reacted with antibodies to PYBM and WV-18 by indirect ELISA (Fig. 3A and C) but not by direct ELISA (Fig. 2A and C). Similarly, cross-reactions with the Chickadee antibody were much more pronounced by indirect ELISA.

Crude antigens of some isolates were also tested by indirect ELISA (data not presented). The antibodies were generally less specific than in direct ELISA, but the indirect ELISA was not as reliable for comparing crude antigens. Irregular results were

obtained, probably because of a lack of sufficient binding of antigens to the plate in the initial ELISA step.

Since the direct ELISA was able to distinguish among TmRSV isolates, the 33 isolates were compared against the antibodies to the standard isolates, using crude sap from infected tissue of several plant species as antigens. The results are shown in Figure 4. Relative values of the absorbance (OD at 405 nm) are presented as average percentages of the absorbance in homologous reactions obtained with the standard isolates (the top four isolates of Fig. 4) in repeated tests. Each antibody reacted most strongly with the homologous isolate, giving OD values (at 405 nm) between 1.1 and 1.4 after about 30–45 min. As with the standard isolates, no isolate was detectable with all four antibodies. PYBM antibody was able to detect most but not all of the isolates, followed by antibodies to WV-18 and GYV. The Chickadee antibody was unique in that fairly strong heterologous reactions were only obtained with GYV and Chickadee S, an isolate (a mutant or selection) that is biologically distinct from Chickadee (see Biological Properties of TmRSV Isolates, in this section). The results obtained with crude sap from different host plants were very similar, indicating that the degree of detectability was due to the serological properties of the isolates rather than related to the propagation host plant. The uninfected host plants that were included as negative controls gave OD values (at 405 nm) below 0.05 (not presented in the figure).

Serogroups and geographic origin of isolates. The 33 isolates of TmRSV (Table 1) could be classified in five serogroups according to the results obtained by direct ELISA (Table 2). For that purpose, relative values of at least 14% of the values obtained in the homologous reaction were considered ELISA-positive. A relative value of 14% corresponded to an actual OD value (at 405 nm) of at least 0.13, which was significantly above that of the healthy control (0.05 or below). Isolates in different serogroups, typified with the letters A–E in Table 2, were detectable by direct ELISA by different sets of antibodies. For example, the two isolates in serogroup A were detectable only with antibodies to Chickadee (strong reaction) and GYV (medium reaction), whereas the 15 isolates in serogroup E could reliably be detected with the PYBM and WV-18 antibodies.

This serogrouping was used to investigate a possible correlation between serological properties and the origin of isolates. The host

TABLE 2. Serogroups of tomato ringspot virus derived by direct double-antibody sandwich enzyme-linked immunosorbent assay against four antibodies

Antibody[a]				Serogroup		
Ch	GYV	PYBM	WV-18	Group[b]	Type isolate	Number of isolates in group
+++[c]	++	−	−	A	Chickadee	2
++	+++	−	−	B	GYV	1
−	+−	+++	+	C	PYBM	7
−	+	++	+++	D	WV-18	8
−	+−	++	++	E	Staff	15

[a] Ch = Chickadee; GYV = grape yellow vein; PYBM = peach yellow bud mosaic-Geneva; WV-18 = West Virginia-18.
[b] Groups were arbitrarily named A–E. Isolates in different groups are detectable with different sets of antibodies.
[c] +++ = Strong reaction; ++ = medium reaction; + = low positive reaction; +− = reaction at the border line of positive and negative; − = negative.

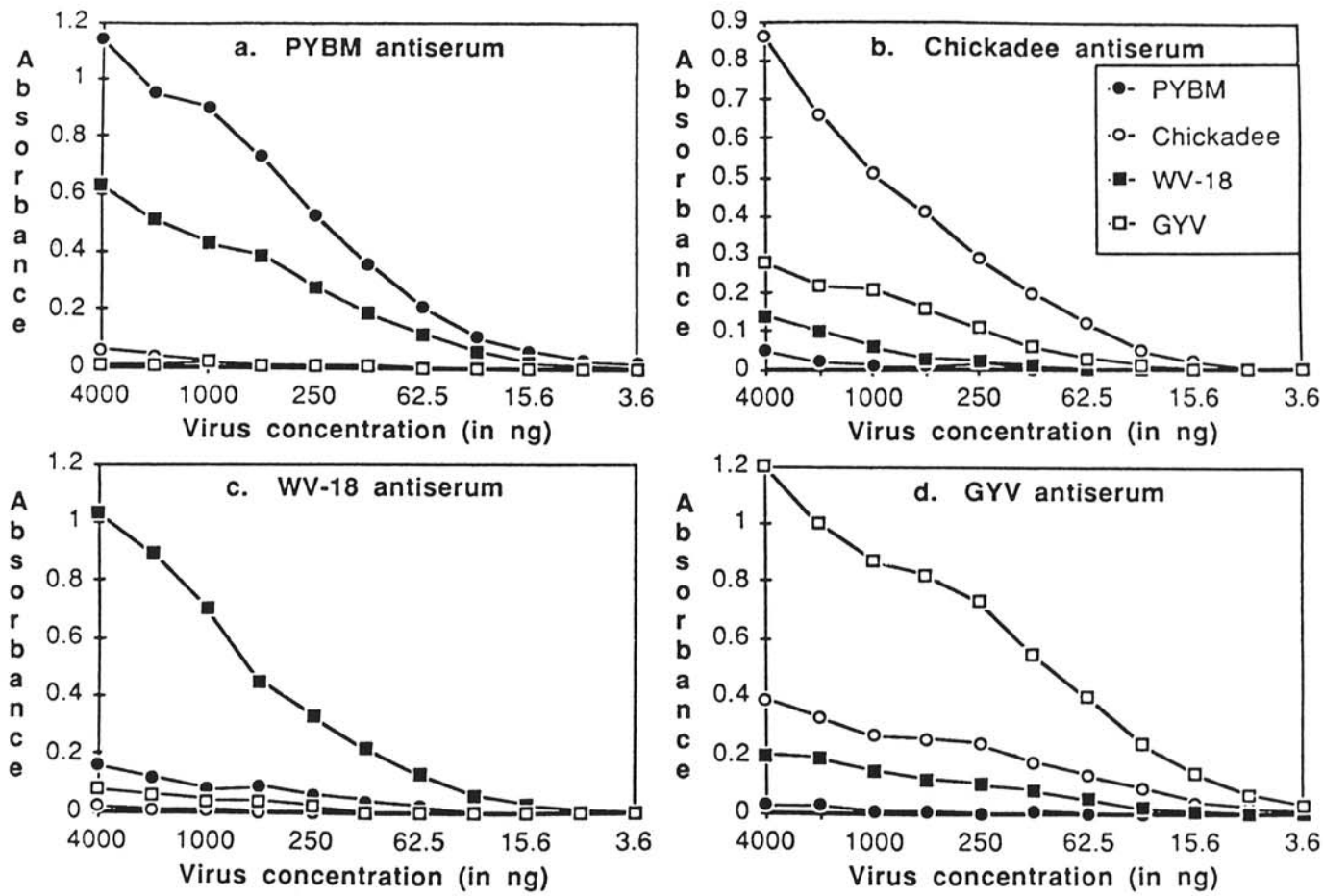

Fig. 2. Reciprocal comparisons of the four standard isolates of tomato ringspot virus by direct double-antibody sandwich enzyme-linked immunosorbent assay. Purified virus antigens of peach yellow bud mosaic-Geneva (PYBM), Chickadee, West Virginia-18 (WV-18), and grape yellow vein (GYV) were serially diluted from 4,000 to 3.6 ng/ml and tested against antisera to PYBM (**A**), Chickadee (**B**), WV-18 (**C**), and GYV (**D**). γ-Globulin was at 1 μg/ml, and the enzyme conjugate was at 1:1,250 for PYBM and WV-18, 1:2,000 for Chickadee, and 1:1,000 for GYV. Reactions against healthy tissue were negligible (the average optical density at 405 nm was less than 0.05).

origins and the location of collections are presented in Table 1. Our data do not support the hypothesis of a serological classification of TmRSV into East Coast versus West Coast isolates, even though more isolates from the West Coast should be investigated before a final conclusion is possible. In the eastern part of the country, however, where more isolates were available, most isolates collected in New York State fit into one serogroup (E), whereas most of the "southern" isolates belonged to serogroup D (Table 1).

Biological properties of TmRSV isolates. Since serologically distinct isolates of TmRSV were detected, the biological characterization mainly concentrated on the identification of a suitable host plant for a cross-protection study. *N. benthamiana* was the only host that clearly distinguished some isolates (Table 1 and Fig. 5). Most isolates incited necrotic lesions and ring spots on inoculated leaves and severe systemic necrosis leading to death of the plant, especially when inoculated at a young age (Fig. 5). Chickadee, however, caused very light chlorotic spots on inoculated leaves and systemic veinclearing about 5–8 days after inoculation. Young leaves appearing afterwards were either completely symptomless or slightly chlorotic (Fig. 5). Virus titers in *N. benthamiana* were as high in plants infected with Chickadee as in plants infected with PYBM, as judged by the amount of antigens detected by ELISA and by bioassay on *C. quinoa*. For example, in samples collected 5 days after inoculation of similar leaf positions of 12 *N. benthamiana* plants, average OD values (at 405 nm) after 30 min were 0.96 (with a range of 0.68–1.22) for Chickadee and 0.93 (with a range of 0.88–0.97) for PYBM. When subsamples of corresponding *N. benthamiana* leaves were mechanically inoculated to *C. quinoa*, the number of local lesions per leaf observed 6–10 days after inoculation was over 400 for Chickadee and 300–400 for PYBM. Apparently, the observed "mildness" reflected a characteristic of the virus isolate rather then the virus titer.

Virulence on *N. benthamiana* was not correlated with serological properties. The two isolates (Chickadee and Mazzard West) that caused similarly mild symptoms on *N. benthamiana* belonged to different serogroups (Table 1). Conversely, isolates that incited moderate symptoms and those that incited severe symptoms belonged to different serogroups. Symptom severity of some TmRSV isolates could also be distinguished to some degree in inoculation of *C. quinoa*. For example, PYBM caused necrotic local lesions (ring spots) with sharp margins, whereas Chickadee incited irregularly shaped lesions with more diffuse margins and chlorosis. In addition, Chickadee generally caused apical necrosis in *C. quinoa*, whereas PYBM often did not. However, these differences were not always reliable enough to serve as biological markers. They were also influenced by plant or leaf age as well as by environmental conditions.

The biological properties of TmRSV isolates as indicated by symptoms on *N. benthamiana* seemed to be quite stable over several years, although symptom expression fluctuated, apparently because of environmental conditions. However, a severe isolate (designated Chickadee S) suddenly appeared after frequent (weekly to biweekly) transfers of the mild isolate Chickadee on *N. benthamiana*. These two isolates were serologically indistinguishable.

DISCUSSION

The results show that the 33 isolates of TmRSV obtained from

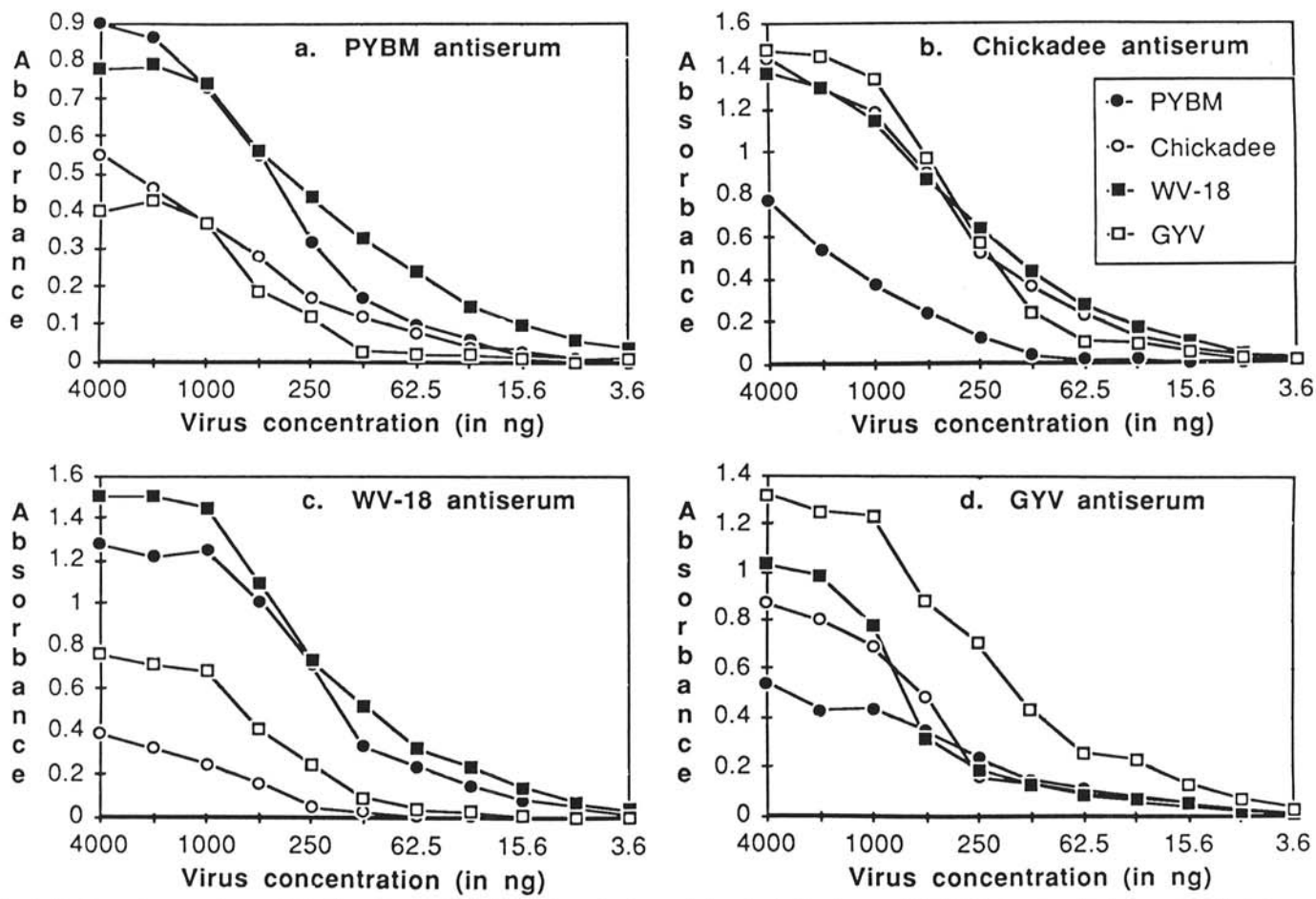

Fig. 3. Reciprocal comparisons of the four standard isolates of tomato ringspot virus by indirect enzyme-linked immunosorbent assay. Purified virus antigens of peach yellow bud mosaic-Geneva (PYBM), Chickadee, West Virginia-18 (WV-18), and grape yellow vein (GYV) were serially diluted from 4,000 to 3.6 ng/ml and tested against antisera to PYBM (**A**), Chickadee (**B**), WV-18 (**C**), and GYV (**D**). γ-Globulin was at 1 μg/ml, and the antirabbit enzyme conjugate was at 1:1,000. Reactions against healthy tissue were negligible (the average optical density at 405 nm was less than 0.05).

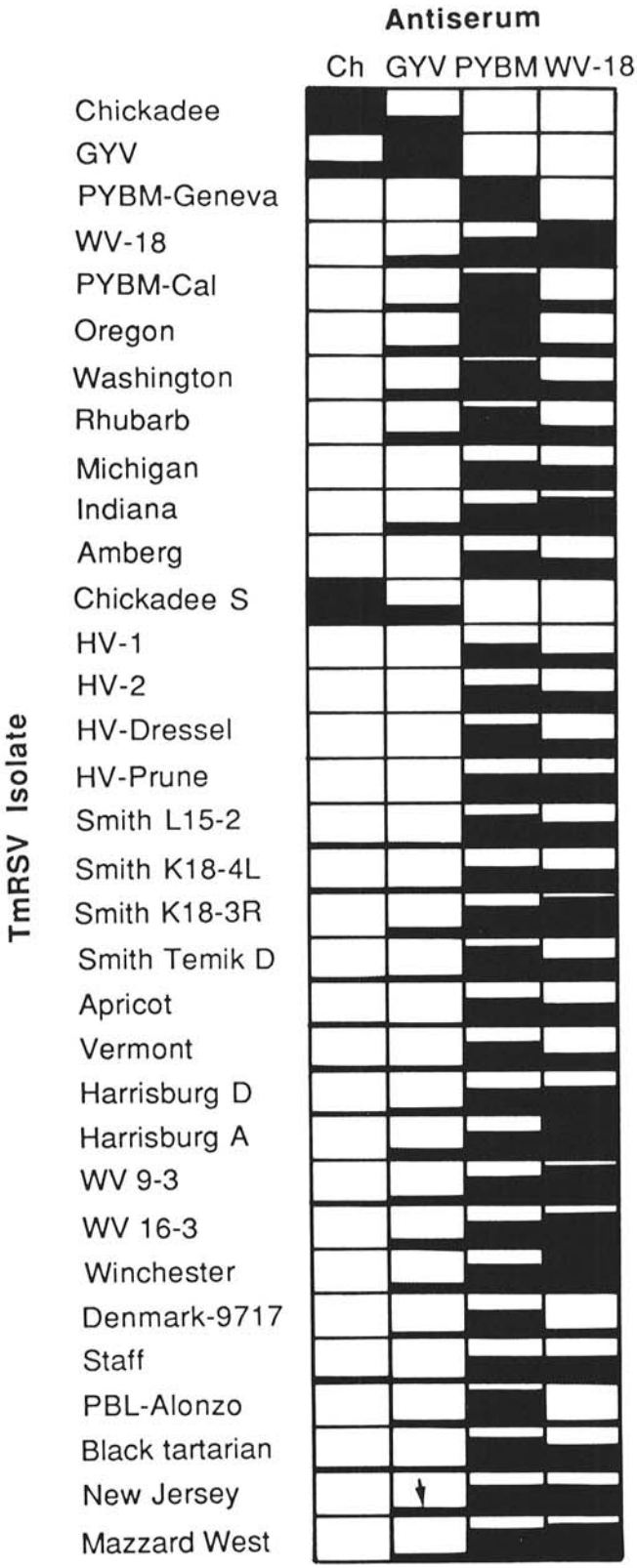

Fig. 4. Relative reactions of 33 isolates of tomato ringspot virus (TmRSV) to antisera of the standard isolates, as determined by direct double-antibody sandwich enzyme-linked immunosorbent assay (ELISA). The isolates are listed as in Table 1, with the four standard isolates—Chickadee (Ch), grape yellow vein (GYV), peach yellow bud mosaic-Geneva (PYBM), and West Virginia-18 (WV-18)—at the top of the figure. The results, calculated as percentages of the absorbance in the the homologous reactions (which were set to 100% for each antiserum), are presented as black fractions of the rectangles. The arrow points to the minimal reaction considered ELISA-positive. The reactions with healthy tissues were negligible (not presented in the figure).

North America and abroad could be separated by ELISA into five serogroups (Fig. 4 and Tables 1 and 2). The assignment of absorbance values at 405 nm to either the ELISA-positive or the ELISA-negative category was clear for most isolates, especially as far as the reactions to the PYBM, WV-18, and Chickadee antibodies were concerned. However, classification according to the reaction with the antibody to GYV was somewhat arbitrary, since for some isolates OD values at 405 nm were close to the border line between positive and negative (indicated by the arrow in Fig. 4). The relative value of 14% of the absorbance in the homologous reaction was chosen as the cutoff point since this value could be visualized by eye and was significantly above the values of the healthy controls.

TmRSV is a very damaging virus in fruit and berry crops and is widespread in North America. In its host range, geographic distribution, and association with its nematode vector, it is very similar to tobacco ringspot virus (15,21), which is not easily distinguished without serology. ELISA provides an important tool for diagnosing TmRSV infections in routine diagnostic work. Our findings demonstrate that care has to be taken in selecting antisera for diagnosis. Figure 4 clearly shows that no antibody was able to detect all isolates, nor was any isolate detectable with all four antibodies by direct ELISA. Thus, knowledge of the existence of the serological differences is essential for an efficient use of ELISA, in order not to miss any isolates (20).

Our study included a significant number of representative TmRSV isolates collected from different geographic areas. For the first time, 33 isolates were compared at one place using the same antisera. We showed that TmRSV can be classified by serology using the direct double-antibody sandwich ELISA. The Chickadee isolate, which has not been used in serological studies before, apparently is a serologically unusual isolate, since its homologous antibody is very specific and does not detect any other isolates except Chickadee S and GYV. Conversely, the antibody to PYBM detects almost any TmRSV isolate except Chickadee.

In order to definitively evaluate the hypothesis that there might be a correlation between the serological properties of TmRSV isolates and their geographic origin, more isolates have to be examined than were available for our study, especially more from the West Coast.

Even though indirect ELISA was less specific than direct ELISA (Figs. 2 and 3), our experiences suggest that the standard indirect ELISA is not an adequate alternative for testing crude plant sap. However, we did not thoroughly investigate the possibilities of improving the indirect system, as has been done with other viruses for the direct ELISA (23). Until these difficulties are overcome, we propose that antibodies to two isolates (e.g., Chickadee and PYBM) be used in direct ELISA for routine detection of TmRSV.

Agar double-diffusion (Fig. 1) showed differences between the standard isolates, but all the standard isolates were detectable. Difficulties in detection did occur (e.g., Chickadee isolate with WV-18 antiserum in Fig. 1C), probably because of insufficient concentrations of the antigen or an inappropriate antigen-antibody ratio rather than the antiserum specificity.

With the Chickadee and PYBM isolates, we have identified serologically distinct isolates (Figs. 2 and 4) and biologically distinct isolates (Fig. 5), which provide an excellent model system for studies of cross-protection. Infection with TmRSV isolates and the distribution and titer of these isolates can now be independently traced by direct ELISA and bioassay on *N. benthamiana*. In preliminary cross-protection studies with this system, we used Chickadee as the protecting isolate and PYBM as the challenging isolate. We found that both the protecting Chickadee and the challenge PYBM replicated in the plant, even though the symptoms of PYBM were not expressed.

The fact that generally no correlation between virulence on *N. benthamiana* and serogroups was observed (Table 1) is not too surprising. Serological properties reflect only portions of the virus genome. Thus, two virus isolates with coat proteins indistinguishable by ELISA can differ in other parts of the genome and induce different symptoms on host plants (as observed, e.g., with Chickadee and Chickadee S). The opposite (isolates having the

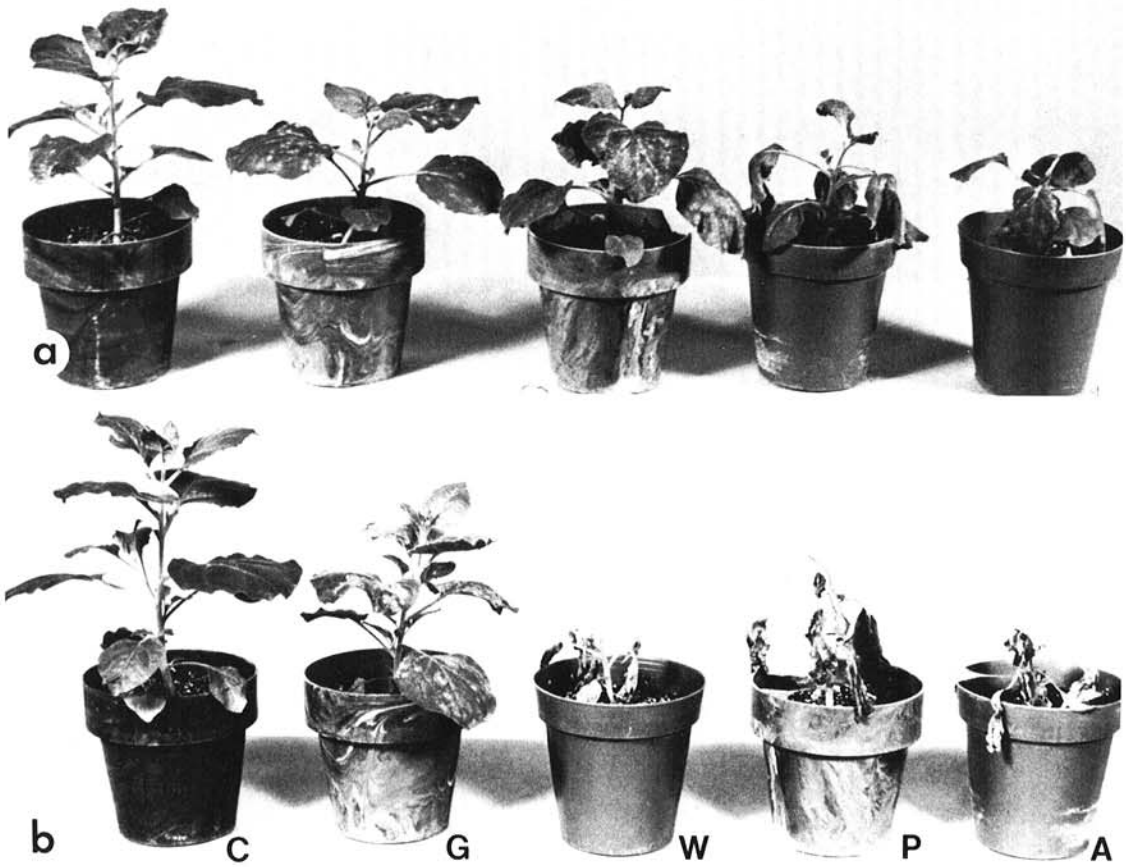

Fig. 5. Distinct symptoms on *Nicotiana benthamiana* caused by different isolates of tomato ringspot virus, 1 wk after inoculation (**A**) and 2 wk after inoculation (**B**). The isolates were (left to right) Chickadee (C), grape yellow vein (G), West Virginia-18 (W), peach yellow bud mosaic-Geneva (P), and apricot (A). Note the mildness of symptoms of the plants infected with Chickadee and the severity of symptoms of the plants on the right.

same genomic part for virulence but differing in coat protein) might apply for Chickadee and Mazzard West (Table 1). Our model system (*N. benthamiana* and TmRSV isolates with biological and serological markers) would be a suitable system for virus-host interaction studies as well as for genetic studies of TmRSV.

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