Detection of Tomato Ringspot Virus with Monoclonal Antibodies

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ABSTRACT

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Monoclonal antibodies were made to an apple isolate of tomato ringspot virus (TmRSV-A) by fusion of splenocytes from immunized mice with NS-1 myeloma cells. Most IgG class monoclonal antibodies reacted strongly with homologous antigen in direct or indirect ELISA. The reactivity of most IgM class monoclonal antibodies was considerably less. Both rabbit and mouse polyclonal antibodies to TmRSV-A generally reacted better than the monoclonal antibodies. Most of the monoclonal antibodies that reacted strongly with TmRSV-A also reacted strongly with isolates of TmRSV from blueberry, geranium, peach, and cymbidium orchid but not with the Chickadee strain of TmRSV. Sensitivity of the monoclonal antibodies for detecting TmRSV-A could be increased by mixing selected antibodies. Optimum sensitivity for detecting TmRSV-A was attained by using a two-animal-species indirect ELISA in which the plate was coated with rabbit antibody and a mixture of two monoclonal antibodies was used as the detecting (second) antibody.

Tomato ringspot virus (TmRSV), a member of the nepovirus group, causes economically important diseases in several woody and ornamental hosts (7). Because many hosts are propagated vegetatively, the virus and its induced symptoms persist in the absence of its daggar nematode (*Xiphinema* spp.) vector. Control strategies for TmRSV-induced disease usually involve certification programs or disease surveys that require assaying large numbers of samples for the virus. Virus detection techniques also are important in epidemiological studies that involve rate of spread, prevalence, vector relations, alternate hosts, strain variability, and cultivar susceptibility. However, detection of TmRSV can be problematic because of low virus titer, uneven virus distribution (2,3), strain variability (1), and false positives in uninfected plants (6). In an attempt to overcome some of these difficulties, monoclonal antibodies have been prepared to TmRSV (8). This report describes the use of these antibodies to detect TmRSV.

MATERIALS AND METHODS

Virus isolates. The TmRSV isolates from apple (TmRSV-A), blueberry (B), geranium (G), and cymbidium orchid (O) have been described and characterized immunologically (10). The TmRSV isolate from peach (TmRSV-P) was mechanically transmitted to *Chenopodium quinoa* Willd. from the bark of a young peach tree from New Jersey with the Prunus stem-pitting disease. The Chickadee isolate of TmRSV was characterized immunologically by Bitterlin and Gonsalves (1). All of the above isolates are closely related to each other serologically except for the Chickadee isolate, which is distinct both immunologically and biologically (1). The tobacco ringspot virus (TbRSV) isolate used as a control was transmitted to cucumber by *Xiphinema* spp. collected from a declining Cascade grapevine vineyard. TmRSV (10) and TbRSV (9) were purified as previously described.

Preparation of hybridomas. Two BALB/c mice were immunized by intraperitoneal injection with 0.5 ml of 1 mg/ml TmRSV-A in PBS (phosphate-buffered saline; 0.15 M sodium chloride, 0.015 M sodium phosphate, pH 7.0) mixed with 0.5 ml of Freund's complete adjuvant on day 1 and again on day 14. On day 21 the mice were boosted with a 0.1-ml intravenous injection containing 0.1 mg/ml TmRSV-A in PBS. On day 24 the mice were sacrificed by decapitation, and the blood was collected and used as the source of mouse polyclonal antibodies in these studies.

The fusion protocol was developed and optimized between 1981 and 1984 for a clone of NS-1 myeloma cells using mock-inoculated mice. The spleen and thymus were harvested from each mouse. The thymuses were passed through a stainless-steel mesh and placed in HB102 media (Hana Biologics) containing 1 mM sodium pyruvate, 500 μg/ml L-glutamine, and 30 μg/ml gentamicin. The two spleens were placed in 10 ml of HB102 media, cut into six to 10 pieces with a scalpel, pressed through a stainless-steel mesh, and placed in a 15-ml plastic centrifuge tube. The particulate material was allowed to settle to the bottom of the tube for 10 min. The cell-containing supernatant was removed and centrifuged at 1,400 rpm for 8 min through a 1-ml fetal calf serum pad. The pellet was resuspended in 2.0 ml of 0.83% ammonium chloride for 1 min to lyse the red blood cells. The ammonium chloride was diluted by adding 10 ml of HB102 media. Particulate material was again allowed to settle for 1 min. The spleen cells in the supernatant were centrifuged at 1,400 rpm for 8 min and resuspended in 10 ml of HB102 media containing a volume of NS-1 myeloma cells equal to that of the spleen cells. Both cell types were centrifuged together at 1,400 rpm for 8 min. Cell fusion was initiated by gently resuspending the cells in 1 ml of 50% polyethylene glycol (PEG, $M_r = 1{,}000$). After 1 min, 1 ml of HB102 media was gently added; after another minute, 2 ml of media was added; and after another 2 min, 5 ml of HB102 media containing 20% fetal calf serum was added. The fused cells were then cen-

Table 1. Reactivity of antibodies with tomato ringspot virus-A in plate-trapped antigen indirect ELISA[a]

Antibody	Number tested[b]	Mean OD	Range[c]
Rabbit polyclonal to TmRSV-A	3	1.102	0.823–1.463
Mouse polyclonal to TmRSV-A	2	0.940	0.816–1.064
Rabbit polyclonal to TbRSV	2	0.054	0.048–0.060
Mouse monoclonal (IgG1) to TmRSV-A	6	0.733	0.354–0.829
Mouse monoclonal (IgG2a) to TmRSV-A	14	0.740	0.309–0.867
Mouse monoclonal (IgG2b) to TmRSV-A	6	0.821	0.630–0.948
Mouse monoclonal (IgM) to TMRSV-A	37	0.161	0.059–0.485
Mouse monoclonal (IgG2b) to TbRSV	5	0.023	0.017–0.054

[a] The test for each antibody consisted of coating 10 wells with 30 μg/ml of TmRSV-A, followed sequentially by 10 μg/ml of test antibody, a 1:10,000 dilution of alkaline phosphatase-conjugated goat antirabbit or antimouse antibody, and substrate. The reaction time was 30 min. Buffer control values for all antibodies were below 0.05.

[b] Number of independent antibody sources tested.

[c] Standard deviations of the 10 replicated wells for each antibody were between 0.004 and 0.101.

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Table 2. Detection of tomato ringspot virus by DAS-ELISA using selected antibodies[a]

Antibody	Mean OD
Rabbit polyclonal to TmRSV-A	0.873 ± 0.015
Mouse polyclonal to TmRSV-A	0.462 ± 0.022
Rabbit polyclonal to TbRSV	0.035 ± 0.017
Mouse monoclonal (IgG1-1) to TmRSV-A	0.193 ± 0.007
Mouse monoclonal (IgG2a-1) to TmRSV-A	0.208 ± 0.008
Mouse monoclonal (IgG2b-1) to TmRSV-A	0.341 ± 0.016
Mouse monoclonal (IgM-1) to TMRSV-A	0.073 ± 0.008
Mouse monoclonal (IgG2b) to TbRSV	0.048 ± 0.010

[a] The test for each antibody consisted of coating 10 wells with 10 μg/ml of the antibody, followed sequentially by 30 μg/ml of purified TmRSV-A, a 1:2,000 dilution of alkaline phosphatase-conjugated homologous antibody, and substrate. The reaction time was 30 min. Buffer control values for all antibodies were below 0.04.

Table 3. Reactivity of selected antibodies with tomato ringspot virus strains in plate-trapped antigen indirect ELISA[a]

Antibody	TmRSV strain[b] A	B	G	P	O	Ch
Rabbit polyclonal to TmRSV-A	1.204	1.105	1.033	1.365	0.960	0.205
Rabbit polyclonal to TmRSV-B	1.135	1.302	1.231	1.003	1.350	0.232
Rabbit polyclonal to TbRSV	0.075	0.082	0.035	0.022	0.037	0.043
Mouse polyclonal to TmRSV-A	0.844	0.735	0.698	0.756	0.536	0.073
Mouse monoclonal (IgG1-1) to TmRSV-A	0.872	0.624	0.731	0.865	0.697	0.031
Mouse monoclonal (IgG2a-1) to TmRSV-A	0.795	0.728	0.704	0.739	0.702	0.023
Mouse monoclonal (IgG2b-1) to TmRSV-A	0.920	0.745	0.791	0.831	0.704	0.097
Mouse monoclonal (IgM-1) to TMRSV-A	0.236	0.193	0.189	0.200	0.111	0.011
Mouse monoclonal (IgG2b) to TbRSV	0.035	0.023	0.031	0.022	0.041	0.015

[a] The test for each antibody consisted of coating 10 wells with 30 μg/ml of purified TmRSV, followed sequentially by 10 μg/ml of test antibody, a 1:10,000 dilution of alkaline phosphatase-conjugated goat antirabbit or antimouse antibody, and substrate. The reaction time was 30 min. Standard deviations ranged from 0.003 to 0.111. OD values of controls with buffer or healthy *Chenopodium quinoa* sap were always below 0.05.

[b] A = apple, B = blueberry, G = geranium, P = peach, O = cymbidium orchid, and Ch = Chickadee isolate. The numbers are the average optical densities of the 10 replicates.

trifuged at 1,200 rpm for 7 min and gently resuspended in 10 ml of HAT media (HB102 media containing 34 μg/ml hypoxanthine, 9.7 μg/ml thymidine, 0.36 μg/ml aminopterin, and 20% fetal calf serum). The fused cells were then placed in an incubator at 37 C, 5% carbon dioxide for 1 hr. After this resting period, the cells were diluted in 500 ml of HAT media and seeded into 96-well microtiter plates. Cell lines secreting antibody to TmRSV were subcloned three times by limiting dilution, weaned from fetal calf serum (HB102 media will readily support this NS-1 cell line and hybridomas derived from it without fetal calf serum), and used to produce at least 100 ml of HB102 antibody-containing culture fluid. Culture fluid was screened for monoclonal antibodies to TmRSV by indirect double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using homologous purified antigen. The monoclonal antibodies were typed using a Bio-Rad (Richmond, CA) kit and protocol.

Enzyme-linked immumosorbent assays. Direct and indirect ELISA and antibody conjugation were performed as previously described (7,10). All antibodies were prepared at the Hershey Medical Center, Hershey, Pennsylvania. Polyclonal and IgG class antibodies were purified by affinity chromatography (Affi-Gel Blue, Bio-Rad). IgM class antibodies were partially purified by ammonium sulfate precipitation.

Conditions for the various ELISA operations were standardized. Unless otherwise stated, the concentration of coating antibody or test antibody was 10 μg/ml, the concentration of antigen was 30 μg/ml, the dilution of alkaline phosphatase-conjugated antibody stock solutions was 1:10,000 (the optimal dilution for each stock solution based on preliminary experiments), and the concentration of the *p*-nitrophenyl phosphate substrate was 1 mg/ml. Plates were read at 405 nm after a 30-min reaction time. Each treatment was replicated 10 times. Plates were coated with either antigen or antibody in 0.05 M sodium carbonate, pH 9.6. The substrate was dissolved in 10% diethanolamine, pH 9.8. All other ELISA operations were performed in PBS containing 0.05% Tween 20 and 2.0% polyvinylpyrrolidone (TPBS).

Plant extracts. Plant extracts were obtained from leaves of *C. quinoa* plants (20 infected with TmRSV-A and 20 uninfected) and from roots of nectarine trees (20 infected with TmRSV and 20 uninfected). The nectarine trees were naturally infected with field isolates of the virus and showed symptoms of the Prunus stem pitting disease. These trees were known to be infected with TmRSV based on preliminary ELISA tests in which many samples were collected from the same tree. Roots were used as the antigen source because previous ELISA tests have shown that roots are the best tissue for detecting TmRSV in peaches or nectarines (*unpublished*). ELISA samples were prepared by triturating approximately 1 g of tissue from a single leaf (*C. quinoa*) or three different roots (nectarine) in 7.5 ml of TPBS using a Tissumizer.

RESULTS

Monoclonal antibody production and typing. Cell fusion resulted in approximately 500 independently derived cell lines. Of these, 88 secreted antibody specific for TmRSV and survived three subcultures. Typing of these antibodies using rabbit antimouse class and subclass specific antibody yielded 6 IgG1, 14 IgG2a, and 6 IgG2b subclass antibodies and 62 IgM class antibodies. There were no IgG3 or IgA antibodies. The reactivity of 26 of the IgM class antibodies was weak and poorly reproducible. These 26 antibodies were not tested further.

Table 4. Lowest concentration of tomato ringspot virus-A detected by selected antibodies in indirect ELISA[a]

Antibody	Lowest concentration of TmRSV-A (ng/ml)
Rabbit polyclonal	3
Mouse polyclonal	10
Mouse monoclonal IgG2a-1	100
Mouse monoclonal IgG2a-2	300
Mouse monoclonal IgG2a-1 + IgG2a-2	10
Mouse monoclonal IgG2a-3	300
Mouse monoclonal IgG2a-1 + IgG2a-3	100
Mouse monoclonal IgM-1	100
Mouse monoclonal IgM-2	10,000

[a] The test for each antibody consisted of coating 10 wells each with 0, 1, 3, 10, 30, 100, 300, 1,000, 3,000, 10,000, or 30,000 ng/ml of TmRSV-A, followed sequentially by 10 μg/ml of test antibody, a 1:10,000 dilution of alkaline phosphate-conjugated goat antirabbit or antimouse antibody, and substrate. A test was considered positive if all the wells containing a particular concentration of TmRSV reached an optical density of at least 0.1 before the buffer control reached 0.05.

Reactivity of antibodies with TmRSV-A. The ability of rabbit polyclonal, mouse polyclonal, and the four classes and subclasses of monoclonal antibody to react with a fixed concentration (30 μg/ml) of homologous antigen (TmRSV-A) by indirect ELISA is compared in Table 1. The homologous antibodies, with the exception of many of the IgM class antibodies, reacted well

with TmRSV-A. The reaction of the polyclonal antibodies was in general stronger than that of the monoclonal antibodies. The two heterologous antibody controls, polyclonal and monoclonal antibody to TbRSV, did not react with TmRSV-A.

Because the method usually employed for routine diagnosis of TmRSV is DAS-ELISA, selected polyclonal and monoclonal antibodies were conjugated with alkaline phosphatase and compared using this assay (Table 2). Differences between the antibodies were similar to those with indirect ELISA but more pronounced. Rabbit polyclonal antibody detected 30 μg/ml of TmRSV-A substantially better than mouse polyclonal antibody, which detected TmRSV better than monoclonal antibody. A selected IgM antibody that had good affinity for TmRSV-A by indirect ELISA did not react well with the virus when conjugated.

The reactivity with six different isolates of TmRSV was determined for the same selected antibodies used in the above experiment plus a rabbit polyclonal antibody to TmRSV-B (Table 3). There was little difference in the reaction of any of the antibodies with TmRSV isolates from apple, blueberry, geranium, peach, or orchid. The reaction of all the antibodies with the Chickadee isolate of TmRSV was considerably weaker. Wells containing rabbit polyclonal antibodies had the highest absorbance, and those with monoclonal IgM antibody had the lowest absorbance.

Selected antibodies also were compared in indirect ELISA by varying the TmRSV-A concentration and holding the test antibody concentration constant at 10 μg/ml (Table 4). The lowest concentration of TmRSV detected by rabbit and mouse polyclonal antibody was 3 and 10 ng/ml, respectively. Three mouse monoclonal IgG2a class antibodies detected between 100 and 300 ng/ml of virus. Mixing two of the IgG2a class antibodies (1 and 2) improved the detection to 10 ng/ml, the same as that of the mouse polyclonal antibody. Mixing two other monoclonal IgG2a class antibodies (1 and 3) did not improve the detection level. There was a wide variety in the lowest concentration of TmRSV-A detected by monoclonal IgM class antibodies. IgM-1 detected 100 ng/ml of virus, whereas IgM-2 detected only 10,000 ng/ml of virus. Results identical to those in Table 4 were obtained when known concentrations of purified virus were diluted in healthy *C. quinoa* sap (*data not shown*).

Several combinations of antibodies were evaluated in order to develop an optimum ELISA for detecting TmRSV (Table 5). Coating with purified antibodies from rabbit and detecting with mouse antibodies gave higher relative absorbancies than coating with mouse antibodies and detecting with rabbit antibodies. Coating with polyclonal rather than monoclonal antibodies was more effective. The optimum ELISA for detecting purified TmRSV-A consisted of coating with rabbit polyclonal antibody and using either mouse polyclonal antibody or a mixture of mouse monoclonal antibodies IgG2a-1 and IgG2a-2 as the second antibody, which is detected by alkaline phosphatase-conjugated goat antimouse antibody.

The same combinations of antibodies were evaluated for their ability to detect TmRSV in sap from infected *C. quinoa*

Table 5. Detection of tomato ringspot virus using various antibody combinations[a]

	Second antibody			
Coating antibody	**Rabbit polyclonal**	**Mouse polyclonal**	**Mouse monoclonal IgG2a-1**	**Mouse monoclonal IgG2a-1 and IgG2a-2**
Rabbit polyclonal	0.712 ± 0.015	1.323 ± 0.034	1.047 ± 0.081	1.375 ± 0.044
Mouse polyclonal	0.955 ± 0.109	0.331 ± 0.022	0.295 ± 0.026	0.200 ± 0.027
Mouse monoclonal IgG2a-1	0.375 ± 0.029	0.283 ± 0.028	0.204 ± 0.026	0.274 ± 0.011
Mouse monoclonal IgG2a-1 and IgG2a-2	0.443 ± 0.020	0.392 ± 0.018	0.214 ± 0.011	0.303 ± 0.016

[a] The test for each antibody combination in which the coating and second antibodies were from the same animal species (DAS-ELISA) consisted of coating 10 wells with 10 μg/ml of the coating antibody, followed sequentially by 30 μg/ml of purified TmRSV-A, a 1:10,000 dilution of alkaline phosphatase-conjugated antibody, and substrate; these values are underlined. In tests in which the coating and second antibodies were from different animal species (indirect DAS-ELISA), the second antibody, which was not conjugated, was followed by a 1:10,000 dilution of alkaline phosphatase-conjugated goat antirabbit or antimouse antibody; these values are not underlined. The numbers represent the mean optical density for 10 wells after 30 min. Controls in which the coating or second antibody was to TbRSV or in which the antigen was TbRSV consistently gave optical densities of less than 0.05.

Table 6. Percentage of plants in which TmRSV was detected using various combinations of antibody to TmRSV[a]

	Second antibody							
	Rabbit polyclonal		**Mouse polyclonal**		**Mouse monoclonal IgG2a-1**		**Mouse monoclonal IgG2a-1 and IgG2a-2**	
Coating antibody	**Cq**	**N**	**Cq**	**N**	**Cq**	**N**	**Cq**	**N**
Rabbit polyclonal	100	65	100	80	100	80	100	85
Mouse polyclonal	100	55	100	25	100	25	100	30
Mouse monoclonal IgG2a-1	100	30	100	55	100	25	100	25
Mouse monoclonal IgG2a-1 and IgG2a-2	100	50	100	35	100	30	100	30

[a] The test for each antibody combination in which the coating and second antibodies were from the same animal species (DAS-ELISA) consisted of coating two wells with 10 μg/ml of the coating antibody, followed sequentially by plant sap, a 1:10,000 dilution of alkaline phosphatase-conjugated antibody, and substrate; these values are underlined. In tests in which the coating and second antibodies were from different animal species (indirect DAS-ELISA), the second antibody, which was not conjugated, was followed by a 1:10,000 dilution of alkaline phosphatase-conjugated goat antirabbit or antimouse antibody; these values are not underlined. The numbers in the Cq and N columns are the percentages of TmRSV-infected *Chenopodium quinoa* and nectarine plants, respectively, that tested positive for TmRSV. An individual plant was judged positive if both test wells reached an optical density of 0.1 before the buffer control reached 0.05. The number of nectarine and *C. quinoa* samples tested with each antibody combination was 20. None of 20 control samples from uninfected *C. quinoa* or nectarine trees tested positive. The mean healthy *C. quinoa* and nectarine values when the buffer reached 0.05 were 0.055 and 0.057, respectively, with no significant difference among treatments. The mean substrate incubation time was 53 min, with a range of 43–68 min.

leaves and nectarine roots (Table 6). Results compare favorably with those using purified virus. All of the antibody combinations detected TmRSV in 100% of the *C. quinoa* plants; none of the antibody combinations detected TmRSV in 100% of the nectarine trees known to be infected with the virus. Coating with rabbit polyclonal antibody and using a mixture of mouse monoclonal antibodies IgG2a-1 and IgG2a-2 as the second antibody, which is detected by alkaline phosphatase-conjugated goat antimouse antibody, improved detection of TmRSV in nectarine trees compared with DAS-ELISA using rabbit polyclonal antibody (85 vs. 65%, respectively).

DISCUSSION

The reactivity of a variety of polyclonal and monoclonal antibodies to TmRSV has been studied using several ELISA protocols, and some general observations can be made for these experiments. First, polyclonal antibodies gave higher absorbance readings than monoclonal antibodies. This is not surprising, since the polyclonal antibody is a mixture of antibodies with different affinities and specificities for different epitopes, whereas the monoclonal antibody does not have this diversity. Second, indirect ELISAs were more sensitive than direct ELISA. This phenomenon has been reported several times (5,7). Third, conjugation had a greater adverse effect on low-affinity antibodies than on high-affinity antibodies. A comparison of the absorbance readings in Tables 1 and 2 shows a reduction for the rabbit polyclonal antibody to TmRSV-A of 1.463 to 0.873 (40%) and for the mouse polyclonal antibody of 1.064 to 0.462 (57%); reductions for the monoclonal antibodies were 77% for IgG1, 76% for IgG2a, 64% for IgG2b, and 85% for IgM. Similar phenomena have been reported by Koenig (4) in which conjugation of low-avidity antibodies made them nonreactive. Fourth, mixing two selected monoclonal antibodies enhanced detection of TmRSV. This indicates that TmRSV-A has at least two epitopes. Preliminary experiments have shown that these two antibodies do not compete with each other, whereas other monoclonal antibodies that do not enhance detection when mixed do compete with each other (*unpublished*). Fifth, there were large, reproducible differences in antibody affinity for TmRSV within a particular class. This was most apparent with the IgM class antibodies. Whether these differences are due to specificity for different epitopes or different affinities for the same epitope, or both, has not yet been resolved.

The reaction of the polyclonal and monoclonal antibodies with various isolates of TmRSV was expected. Double-diffusion and ELISA experiments have demonstrated that TmRSV virus isolates A, B, G, P, and O are closely related (10) and that TmRSV-Ch is in a different serologic subgroup than TmRSV-A (1).

Although TmRSV-A and TmRSV-Ch share at least one common antigenic determinant, none of the monoclonal antibodies tested recognized this common determinant. This is also not surprising, since the mouse polyclonal sera did not react with TmRSV-Ch.

The antigen concentration in the experiments comparing antibodies and ELISA procedures was a relatively high 30 μg/ml. This concentration was selected on the basis of preliminary experiments showing that 30 μg/ml was necessary to obtain above-background readings with the lower affinity antibodies. Consequently, the substrate incubation times were short (30 min) in order to keep absorbance values for the higher affinity antibodies low enough to read accurately. A few experiments in which lower antigen concentrations (1.0 and 0.1 μg/ml) and longer substrate incubation times (2 hr) were used yielded similar results.

A major purpose for producing monoclonal antibody to TmRSV was to improve diagnostic procedures for a virus that causes economically important diseases in many commercially grown plant hosts. ELISA for TmRSV can be improved by using an indirect double-antibody sandwich protocol in which the coating antibody is rabbit polyclonal and the second antibody is a mixture of two monoclonal antibodies that are detected by alkaline phosphatase-conjugated goat antimouse antibody. This procedure increased the efficiency of detecting TmRSV in nectarine trees from 65 to 85% when compared with DAS-ELISA. This should greatly improve the reliability of TmRSV diagnosis for both certification programs and epidemiological studies.

Even with use of this improved ELISA, 15% of infected trees would test negative. Attempts to reduce false negatives using rabbit polyclonal sera from several different sources and varying ELISA incubation times and sera concentrations have thus far failed (*data not shown*). Stace-Smith (11) optimized ELISA for detecting TmRSV in raspberries using polyclonal and monoclonal antibodies. He coated with polyclonal antibody overnight or longer, followed sequentially by a 16-hr antigen incubation, a 6-hr incubation with conjugated monoclonal antibody, and a 24-hr incubation in substrate (all steps at 20 C). Incubation periods of this length have not been tested for detecting TmRSV using the Pennsylvania monoclonal antibodies. Increasing the level of detection of TmRSV in *Prunus* spp. above 85% may be difficult using immunological methods. The use of nucleic acid probes to further improve detection of TmRSV in woody plants is currently under investigation.

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