Serological Comparison of Tospovirus Isolates Using Polyclonal and Monoclonal Antibodies

G. Adam,[1] D. Peters,[2] and R.W. Goldbach[2]

[1]University of Hamburg, Institute of Applied Botany, P.O. Box 302762, Marseillerstr. 7, D-20309, Hamburg, Germany; and [2]Department of Virology, Wageningen Agricultural University, Binnenhaven 11, 6700 EM, Wageningen, The Netherlands

A test was conducted to compare tospovirus isolates using different poly- and monoclonal antibodies. All isolates and antibodies were compared under identical conditions. From 130 tospovirus isolates, which were obtained from all over the world and included well-characterized isolates from all four serogroups, 96 were chosen because they could be recovered after inoculation to Nicotiana benthamiana *and* N. rustica. *Antigen extracts were tested at 1:100 and 1:1000 in parallel in two different enzyme-linked immunosorbent assay (ELISA) protocols: double antibody sandwich ELISA (DAS-ELISA) with 10 polyclonal antisera and triple antibody sandwich ELISA (TAS- ELISA) with 33 monoclonal antibodies (MAbs). According to both tests, 47 isolates belonged to serogroup I (TSWV); 8 belonged to serogroup II (2 TCSV, 2 GRSV, and 4 of uncertain classification); 8 belonged to serogroup III (INSV); and 6 belonged to serogroup IV. Among the remaining 27 isolates, 16 did not react with any of the different antibodies; whereas, 11 were detected by only a few antibodies that related them either to serogroup I or II. It can be assumed that they include hitherto unknown tospovirus isolates. DAS-ELISA revealed that polyclonal antisera, prepared against complete virions, had a much broader reactivity than nucleocapsid (N)-specific antisera; however, in this comparison they were only available for TSWV. TAS-ELISA included both N- and glycoprotein (G)-specific MAbs against TSWV and revealed a high degree of variability, reflecting the presence of several different epitopes on both proteins. In addition, a polymerase chain reaction (PCR) detection method was tested with three different primer sets that were designed to detect tospoviruses in general as well as specifically. Two different primer sets were able to detect tospoviruses from all four serogroups.*

The genus *Tospovirus* contains important species of plant viruses that have been well characterized according to nucleic-acid sequence, serology, and transmission by thrips vectors (Ullman et al. 1989; Palmer et al. 1990; de Ávila et al. 1990, 1993a,b; Law and Moyer 1990; Reddy et al. 1992; Yeh et al. 1992; Adam et al. 1993; Heinze et al. 1995; Satyanarayana et al. 1996). Their classification is predominantly based on serological relations between N proteins and sequence homologies of the S RNA (Adam et al. 1993; de Ávila et al. 1993a,b; Adam and Kegler 1994). This provides the basis for a reliable detection of tospoviruses in infected plants or thrips by ELISA, PCR, or nucleic acid hybridization (Sherwood

et al. 1989; Huguenot et al. 1990; Wang and Gonsalves 1990; Adam et al. 1991, 1995; Mumford et al. 1994).

Among the tospovirus species, the type strain tomato spotted wilt virus (TSWV) is present worldwide; whereas groundnut ringspot virus (GRSV) is restricted to South Africa, tomato chlorotic spot virus (TCSV) to South America, impatiens necrotic spot virus (INSV) to North America and Western Europe, and groundnut bud necrosis virus (GBNV) and watermelon silver mottle virus (WSMV) to India and Far Eastern countries (de Ávila 1992). This situation will probably change because of the intensive transportation of living plant material between countries and continents.

Furthermore our knowledge about the variability of these tospovirus species is based on too few isolates that have been studied in detail. Therefore, a comparison of a large number of tospovirus isolates was carried out using several poly- and monoclonal antibodies obtained worldwide.

In parallel, PCR-detection was also applied. Designed primers were based on the available sequence data of the different tospovirus species. This allows the specific or general amplification of genome parts of different tospovirus species (Mumford et al. 1994).

Material and Methods

Virus isolates

The virus isolates were in most cases inoculated to both *N. benthamiana* and *N. rustica* to serve as fresh leaf material for the preparation of antigens and to compare their symptoms under identical conditions. The isolates that were used in the test are listed in Table 1.

Antibodies

Four laboratories provided polyclonal antisera (Table 2), prepared against different tospovirus isolates, in the form of purified immunoglobulin G (IgG) and alkaline phosphatase-conjugated IgG ready to be used for DAS-ELISA. In addition, several MAbs were provided from six laboratories (Table 3), either in the form of purified antibodies or as culture supernatant. As a third antibody for TAS-ELISA, we used either rabbit anti-mouse or rabbit anti-rat antibodies labelled with alkaline phosphatase (Sigma).

ELISA protocols

In the ELISA-test, the buffer system of Clark and Adams (1977) was used, except that washing tap water was used for two subsequent washes, followed by one step with PBS-Tween. Nunc high-binding microtiter plates were used, which were

Table 1. Virus isolates used in the test.

Isolate	Original code	Host source	Country of origin	Supplier
1	INSV-Glox1	gloxinia	Italy	P. Roggero
2	TSWV-T365	tomato	Italy	P. Roggero
3	INSV-Eu 2	*Euphorbia marginata*	Italy	P. Roggero
4	TSWV-T432	tomato	Italy	P. Roggero
5	L-91672	*Lactuca sativa*	Spain	Paloma Abad
6	T-941114	tomato	Spain	Paloma Abad
7	L-93940	*Lactuca sativa*	Spain	Paloma Abad
8	TSWV-Bas	*Ocimum basilicum*	Germany	U. Oertel
9	TSWV-Imp	*Impatiens* sp.	Germany	U. Oertel
10	TSWV-AK8	tobacco	Bulgaria	S. Winter (DSMZ)
11	VE-225	bell pepper	Brazil	S. Winter (DSMZ)
12	PV-0283	tomato	Taiwan	S. Winter (DSMZ)
13	PV-0281	*Anemone* sp.	Germany	S. Winter (DSMZ)
14	Orient-4	tobacco	Bulgaria	S. Winter (DSMZ)
15	PV-0279	*Impatiens* sp.	USA	S. Winter (DSMZ)
16	Orient-1	tobacco	Bulgaria	S. Winter (DSMZ)
17	Bu-164	tobacco	Bulgaria	S. Winter (DSMZ)
18	PV-0204	*Impatiens* sp.	Germany	S. Winter (DSMZ)
19	PV-0207	*Alstroemeria* sp.	Germany	S. Winter (DSMZ)
20	PV-0205	groundnut	South Africa	S. Winter (DSMZ)
21	RIA	tobacco	Bulgaria	S. Winter (DSMZ)
22	VE-223	tomato	Brazil	S. Winter (DSMZ)
23	PV-0182	tobacco	Bulgaria	S. Winter (DSMZ)
24	INSV-Ole	*Nerium oleander*	France	S. Winter (DSMZ)
25	K207	bell pepper	Greece	N. Katis
26	T5	?	Thailand	D. Peters
27	SIN	*Senecio x hybridus*	Finland	A. Lemmetty
28	EL-7F	?	South Africa	D. Peters
29	EL-1F	?	South Africa	D. Peters
30	JF-3	?	South Africa	D. Peters
31	JF-1	?	South Africa	D. Peters
33	NL-16	?	Netherlands	D. Peters
34	NL-17	?	Netherlands	D. Peters
35	NL-05	?	Netherlands	D. Peters
36	NL-06	?	Netherlands	D. Peters
37	IY1	?	France	G. Selassie

(Continued)

Table 1. Virus isolates used in the test (continued).

Isolate	Original code	Host source	Country of origin	Supplier
38	IY2	?	France	G. Selassie
39	R4	?	France	G. Selassie
40	TT1	?	Thailand	D. Peters
41	TT2	?	Thailand	D. Peters
42	TT3	?	Thailand	D. Peters
43	T3	?	Thailand	D. Peters
44	T1400	?	Thailand	D. Peters
45	T14	?	Thailand	D. Peters
46	T7	?	Thailand	D. Peters
47	CHH-1	?	France	G. Selassie
48	LYE-190	?	France	G. Selassie
49	980	?	France	G. Selassie
50	LA2CM24	?	France	G. Selassie
51	ANC-TOM	?	France	G. Selassie
52	ANC-Xanthi	?	France	G. Selassie
53	RA-2B	?	France	G. Selassie
54	ANC-1968	?	France	G. Selassie
55	LAS-6329	?	France	G. Selassie
56	REN-35	?	France	G. Selassie
57	LA2C-224	?	France	G. Selassie
58	LAS-6329	?	France	G. Selassie
59	CAA-21	?	France	G. Selassie
60	CAA-25	?	France	G. Selassie
61	T8	?	Thailand	D. Peters
62	ZA-01	?	South Africa	D. Peters
63	T1	?	Thailand	D. Peters
64	CZ-7047	chrysanthemum	Czechia	J. Mertelik
65	CZ-7090	*Symphytum officinale*	Czechia	J. Mertelik
66	CZ-7084	*Zantedeschia aetiopica*	Czechia	J. Mertelik
67	CZ-7126	chrysanthemum	Czechia	J. Mertelik
68	CZ-7000	*Capsicum annuum*	Czechhia	J. Mertelik
69	PT-02-Nr	tomato	Portugal	D. Louro
70	PT-01-Nr	tomato	Portugal	D. Louro
71	GB-02	chrysanthemum	UK	J. Barker
72	GB-10	bell pepper	UK	J. Barker

(*Continued*)

Table 1. Virus isolates used in the test (continued).

Isolate	Original code	Host source	Country of origin	Supplier
73	LAS-6329b	?	France	G. Selassie
74	TAO-1	?	France	G. Selassie
75	C-2000	?	France	G. Selassie
76	MA2	bell pepper	Marocco	D. Peters
77	RA-1N	?	France	G. Selassie
78	1117	?	Spain	Paloma Abad
79	Oleander	*Nerium oleander*	France	G. Selassie
80	HA-931100	*Vicia faba*	Spain	Paloma Abad
82	A1-P	*Campanula erinus*	Portugal	I. Cortés
83	G 16	*Gerbera* sp.	Portugal	I. Cortés
84	G 15	*Gerbera* sp.	Portugal	I. Cortés
85	G 21	*Gerbera* sp.	Portugal	I. Cortés
86	GBNV-Ind	groundnut	India	D. Peters
87	TS/95/6-T	tomato	Hungary	E. Kiss Ferencné
88	TS/95/10-T	tomato	Hungary	E. Kiss Ferencné
89	TS/95/13-C	*Capsicum annuum*	Hungary	E. Kiss Ferencné
90	TS/95/12-C	*Capsicum annuum*	Hungary	E. Kiss Ferencné
91	TS/95/11-C	*Capsicum annuum*	Hungary	E. Kiss Ferencné
93	BR-01	tobacco	Brazil	D. Peters
94	BR-03	tomato	Brazil	D. Peters
95	SA-05	groundnut	South Africa	D. Peters
96	NL-07	*Impatiens* sp.	Netherlands	D. Peters

All samples were from fresh leaf material of either *N. rustica* or *N. benthamiana*, except sample 86, which was freeze dried, and sample 67, which was supplied as infected chrysanthemum leaf material.

coated with antibodies and incubated with antigen for 12 h at 4°C. Each antigen was applied in two dilutions, 1:100 and 1:1000 (w/v). For DAS-ELISA, the conjugated antibodies were added for 4 h at room temperature; whereas, for TAS-ELISA, the MAbs and subsequently the third antibody were incubated for 2 h each at room temperature.

Polyclonal antibodies were used in various combinations of first and second antibodies, as listed in Table 2, to evaluate the influence of the coating antibody specificity. For TAS-ELISA a mixture of three different polyclonal IgG-preparations was applied as first antibody (Table 3).

Table 2. Polyclonal antibodies used in DAS-ELISA.[a]

Test code[b]	Coating antibody	Conjugated antibody
1	BR-01	BR-01
2	BR-01	BR-03
3	BR-01	SA-05
4	BR-01	NL-07
5	BR-01	To-293
6	BR-01	To-310[c]
7	BR-01	AS-272
8	BR-01	AS-282
9	BR-01	AS-0115
13	BR-01	AS-0118
15	SA-05	SA-05
16	B R-03	BR-03
17	NL-07	NL-07
21	AS-0118	AS-0118
22	AS-0115	AS-0115
23	To-31 0	To-310
24	To-293	To-293
25	triple To[d]	BR-01
26	triple To	BR-03
28	triple To	SA-05
29	triple To	NL-07
31	AS-282	AS-282

[a]The antisera BR-01, BR-03, and NL-07 were supplied by D. Peters, The Netherlands; AS-0115 and AS-0118 by S. Winter, Germany; To-292, To-293, and To-310 by P. Roggero, Italy; and AS-282 and AS-272 by U. Oertel, Germany.
[b]Numbers refer to DAS-ELISA test numbers in Table 4.
[c]This antiserum has been saturated with healthy *N. benthamiana* sap and TSWV N protein.
[d]A mixture of three different polyclonal antisera: To-310; To-293; and To-292. From each IgG-preparation, 1 µg/mL was used.

For all plates, the first two columns were coated with the BR-01 IgG throughout. All reagents were applied in 100 µL. The first column served as the buffer blank; whereas, each second column contained BR-01 antigen and sap from healthy plants. Both columns were treated with BR-01 conjugate to serve as internal controls for the standardization of the different plates. The remaining wells of each plate were used for the test samples. This necessitated three plates for each antibody combination and the selected 96 test samples. Plates were incubated with substrate (p-nitrophenolphosphate) and read after 15 min, 1 h, and 2 h.

Table 3. Monoclonal antibodies used in the test.

Test no.[a]	MAb code	Antigen/ Test code[b]	Specific reaction against	Reference/ Supplier[c]
100	1B4	DSMZ-283/12	N protein	–/DSMZ
101	2F7	DSMZ-283/12	N protein	–/DSMZ
102	6E4	DSMZ-283/12	N protein	–/DSMZ
103	6F5	DSMZ-283/12	N protein	–/DSMZ
104	1E12	DSMZ-280/-	N protein	1/DSMZ
106	5E4	DSMZ-280/-	N protein	1/DSMZ
107	5E8	DSMZ-280/-	N protein	1/DSMZ
108	5G11	DSMZ-280/-	N protein	1/DSMZ
109	5G4	DSMZ-280/-	N protein	1/DSMZ
110	1B1	DSMZ-182/23	N protein	2/DSMZ
111	1C7	DSMZ-182/23	N protein	2/DSMZ
112	2A8	DSMZ-182/23	G protein	2/DSMZ
113	2B6	DSMZ-182/23	G1 protein	2/DSMZ
114	2D12	DSMZ-182/23	N protein	2/DSMZ
115	3F8	DSMZ-182/23	N protein	2/DSMZ
116	4F2	DSMZ-182/23	N protein	2/DSMZ
117	6G3	DSMZ-182/23	N protein	2/DSMZ
120	67/ 263.22	TSWV-GB 03/-	N protein	–/CSL, I. Barker
121	67/ 430.95	TSWV-GB 03/-	N protein	–/CSL, I. Barker
122	67/ 480.84	TSWV-GB 03/-	N protein	–/CSL, I. Barker
123	MAFF 59	TSWV-GB 03/-	N protein	–/CSL, I. Barker
105	14G9/D4	INSV-HT1/-	N protein	–/USDA-ARS, H.T. Hsu
118	8C4	TSWV/-	G protein	–/USDA-ARS, H.T. Hsu
119	33 F11/1F10	INSV/-	N protein	–/USDA-ARS, H.T. Hsu
125	8H6A7	INSV-HT1/-	N protein	–/USDA-ARS, H.T. Hsu
126	33 E11/2C7	INSV/-	N protein	–/USDA-ARS, H.T. Hsu
131	6G8H11	INSV-HT1/-	N protein	–/USDA-ARS, H.T. Hsu
124	2B 3/2D11	TSWV-G/2	G protein	3/IFA, P. Roggero
127	6F3	TSWV/-	G1 protein	4/Agr. Can.
128	Wag N1	TSWV BR-01/93	N protein	5/D. Peters
129	Wag N2	TSWV BR-01/93	N protein	5/D. Peters
130	Wag G1	TSWV BR-01/93	G protein	5/D. Peters
132	Wag G3	TSWV BR-01/93	G protein	5/D. Peters

[a]Numbers refer to TAS-ELISA test numbers in Table 5.
[b]Numbers after slash refer to the antigen for immunization as listed in Table 1.
[c]References: 1 = Adam and Lesemann 1992; 2 = Adam et al. 1991; 3 = Adam et al. 1995; 4 = MacKenzie and Ellis 1992; 5 = Huguenot et al. 1990.

Table 4. Reaction of tospovirus isolates with monoclonal antibodies in TAS-ELISA.[a]

	Number of polyclonal antibody combinations																						
	TSWV Antibodies							TCSV Antibodies			GRSV Antibodies			INSV Antibodies							SG IV	
	1	25	24	5	31	7	8	16	2	26	15	3	28	17	4	29	22	9	23	6	21	13
39	±	+	–	–	+	+	+	+	–	+	++++	++	++	–	–	–	–	–	–	–	–	–
20	+	+	–	–	+	+	+	+	±	+	++++	+	++	–	–	–	–	–	–	–	–	–
95	+	+	–	–	+	+	+	+	±	+	+++	++	++	–	–	–	–	–	–	–	–	–
11	±	+	–	–	+	+	+	+	+	+	++	+	+	–	–	–	–	–	–	–	–	–
94	+	±	–	–	–	+	+	+	+	+	++	+	+	–	–	–	–	–	–	–	–	–
36	–	–	–	–	+	+	–	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–
31	+	+	–	–	+	+	+	–	–	–	±	–	–	–	–	–	–	–	–	–	–	–
60	+	++	+	+	++	+	++	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–
49	+	++	+	±	++	+	++	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–
54	+	+++	+	+	++	+	++	–	–	–	+	+	+	–	–	–	+	–	–	–	–	–
64	+	++	+	–	++	+	++	–	–	–	+	+	+	–	–	–	–	–	+	–	–	–
59	+	+++	+	+	++	+	++	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–
28	+	+++	+	+	+++	+	++	+	–	–	+	+	+	–	–	–	–	–	–	–	–	–
47	+	++	+	+	++	++	++	–	–	–	+	+	+	–	–	–	–	–	+	–	–	–
16	+	+	–	–	++	+	+	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–
90	+	+	–	–	+	+	+	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–
8	+	+	–	–	+	+	+	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–
68	+	+++	+	+	++	+	+++	–	–	–	+	+	+	–	–	–	+	–	+	–	–	–
66	+	+++	+	+	+++	++	+++	–	–	+	+	+	+	–	–	–	+	–	–	–	–	–
48	+	+++	+	+	+++	+	+++	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–
58	+	+++	+	+	+++	+	+++	–	–	+	+	+	++	–	–	–	–	–	–	–	–	–
50	+	++++	+	+	+++	+	+++	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–
56	+	++++	+	+	++++	+	+++	–	–	+	+	+	++	–	–	–	–	–	–	–	–	–
53	+	+++	+	+	+++	+	+++	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–
52	+	++++	+	+	+++	+	+++	–	–	+	+	+	++	–	–	–	–	–	–	–	–	–
55	+	++++	+	+	+++	+	+++	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–
70	+	+++	+	+	+++	++	+++	–	–	–	+	+	+	–	–	–	–	–	+	–	–	–
10	+	++	+	+	++	+	++	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–
65	+	+++	+	+	+++	++	+++	–	–	+	+	+	+	–	–	–	+	–	–	–	–	–
27	+	+	+	–	++	+	++	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–
51	+	++++	+	+	+++	+	+++	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–
19	+	+++	+	+	+++	+	+++	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–
9	+	++	+	–	+	+	++	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–
69	+	++++	+	+	+++	++	+++	–	–	+	+	+	+	–	–	–	+	–	–	–	–	–
71	+	++++	+	+	+++	++	+++	–	–	–	+	+	+	–	–	–	+	–	–	–	–	–
17	+	++	+	+	+++	+	++	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–
72	+	++++	+	+	+++	++	+++	+	±	+	++	+	++	–	–	–	+	–	–	–	–	–
14	+	++	+	+	+++	+	++	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–

(Continued)

Table 4. Reaction of tospovirus isolates with monoclonal antibodies in TAS-ELISA (continued).[a]

	Number of polyclonal antibody combinations																					
	TSWV Antibodies							TCSV Antibodies			GRSV Antibodies			INSV Antibodies							SG IV	
29	+	+++	+	+	+++	+	+++	+	–	+	++	+	+	–	–	–	–	–	–	–	–	–
57	+	++++	+	+	+++	++	+++	–	–	+	+	+	++	–	–	–	–	–	–	–	–	–
62	+	+++	+	+	+++	++	++	–	–	±	+	+	+	–	–	–	+	–	–	–	–	–
7	++	++	+	+	++	+	++	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–
93	++	++	+	–	+	++	++	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–
6	++	++	+	+	++	+	+++	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–
4	++	++	+	+	+	+	+++	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–
35	++	+++	+	+	++++	++	++	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–
18	++	+++	+	+	+++	+	++	+	–	±	+	+	+	–	–	–	–	–	–	–	–	–
63	++	+++	+	+	+++	++	++++	–	–	+	+	+	+	–	–	–	+	–	–	–	–	–
21	+++	++	+	+	+++	++	+++	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–
22	+++	+++	+	+	+++	+	++	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–
5	+++	++	+	+	++	+	+++	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–
2	+++	++	+	+	++++	+	+++	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–
23	+++	+++	+	+	++++	++	+++	+	±	–	+	+	+	–	–	–	–	–	–	–	–	–
78	–	–	–	–	+	–	–	–	–	+	–	–	–	–	–	–	+	–	–	–	–	–
46	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
43	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
45	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
38	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
42	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
41	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
37	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
40	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
80	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
33	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
34	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
77	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
44	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
30	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
83	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
25	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
87	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
82	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
86	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
75	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
84	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
85	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

(Continued)

Table 4. Reaction of tospovirus isolates with monoclonal antibodies in TAS-ELISA (continued).[a]

	Number of polyclonal antibody combinations																					
	TSWV Antibodies							TCSV Antibodies			GRSV Antibodies			INSV Antibodies							SG IV	
73	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+
74	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
26	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	±	–
88	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
91	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
89	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
67	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
61	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	±	–
1	–	–	–	–	+	–	–	–	–	–	–	–	–	+++	+	+	+	+	+++	+	–	–
79	–	–	–	–	+	–	–	–	–	–	–	–	–	++	–	+	+	+	++	+	–	–
76	–	–	–	–	–	–	–	–	–	–	–	–	–	++	–	+	+	–	++	–	–	–
15	–	–	–	–	–	–	–	–	–	–	–	–	–	++	–	+	+	–	+++	–	–	–
24	–	–	–	–	+	–	–	–	–	–	–	–	–	++	–	+	+	–	++	–	–	–
96	–	–	–	–	–	–	–	–	–	–	–	–	–	++	–	+	+	–	+	–	–	–
3	–	–	–	–	–	–	–	–	–	–	–	–	–	++	–	+	+	–	++	–	–	–
13	–	–	–	–	–	–	–	–	–	–	–	–	–	++	–	+	+	–	++	–	–	–

[a]Numbers in the top row refer to RT No. in Tables 2 and 3, describing the antibody combinations or Mabs used as second antibodies. The plus scheme covers the following ELISA readings: ± < 0.1; + 0.1–0.7; ++ 0.7–1.3; +++ 1.3–1.9; and ++++ > 1.9.

To evaluate the ELISA data and make a comparison of the different plates possible, the corresponding blank values were first subtracted for each plate separately. Thereafter, the ELISA values were analyzed according to Rek (1987) to determine the cut-off values for positive samples, and values below the limits were set to zero. These data were then converted into a plus (+) minus (–) scheme, using the following limits: <0.1 = ±; 0.1–0.7 = +; 0.7–1.3 = ++; 1.3–1.9 = +++; and >1.9 = ++++. All evaluations were carried out using Quattro Pro 6.0 (Borland).

PCR Amplification

RNA extraction

Total plant RNA was extracted according to Logemann et al. (1987) as described by Mumford et al. (1994). Approximately 0.2 g of tissue was frozen in liquid nitrogen, then homogenized in 2 volumes of guanidine buffer (4 M guanidine thiocyanate, 20 mM MES, pH 7.0, 20 mM EDTA, and 50 mM ME) with the addition of 1% PVP (w/v) to prevent inhibitory effects of phenolics (Maliyakal 1992). The homogenate was then transferred to a microfuge tube containing about

Table 5. Reaction of tospovirus isolates with monoclonal antibodies in TAS-ELISA.[a]

	Monoclonal antibody numbers																												
	Serogroup IV-specific						INSV-specific							TSWV N-specific											TSWV G-specific						
	100	102	103	105	125	131	104	106	107	108	109	119	126	110	111	114	115	116	117	120	121	122	123	128	129	112	113	118	124	127	130	132
58	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+++	++++	++++	++	++++	++++	++++	++++	++++	+++	+++	++	+	+++	+	+	++	++
56	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+++	++++	++++	++	++++	++++	++++	++++	++++	+++	+++	++	++	+++	++	+	+++	++
55	–	–	–	–	–	–	–	–	–	–	–	–	–	+	++++	++++	++++	++	++++	+	++	+	++++	+++	++	++	+	++++	–	+	+	+
50	–	–	–	–	–	–	–	–	–	–	–	–	–	+	++++	++++	++++	++	++++	++++	++++	++++	++++	++	+++	++	+	+++	+	+	++	++
57	–	–	–	–	–	–	–	–	–	–	–	–	–	+	++++	++++	++++	++	++++	++++	++++	++++	++++	+++	+++	++	+	+++	+	+	+++	++
59	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+++	++++	++++	++	++++	++++	++++	++++	++++	+++	+++	++	++	+++	+	+	+++	++
51	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	+++	++++	++++	++	++++	++++	++++	++++	++++	+++	+++	++	++	+++	+	+	+++	++
53	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	+++	++++	++++	++	++++	++++	++++	++++	++++	+++	++	++	++	+++	+	+	+++	++
72	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	+++	+++	++++	++	+++	+++	++++	+++	++++	+++	+++	++	+	+++	+	+	++	++
52	–	–	–	–	–	–	–	–	–	–	–	–	–	++++	++++	++++	++++	++	++++	++++	++++	++++	++++	+++	+++	++	+	+++	+	+	+++	++
47	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+++	++++	+++	++	++++	++++	++++	++++	++++	+++	+++	++	+	+++	++	+	++	++
48	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+++	++++	+++	++	+++	++++	++++	++++	++++	++	++	++	+	+++	+	+	++	++
60	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+++	++++	+++	++	++++	+++	+++	++++	++++	++	+++	++	+	+++	+	+	++	++
69	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+++	++++	+++	++	++++	+++	+++	+++	++++	+++	+++	++	+	++	+	+	++	+
54	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	+++	++++	+++	++	++++	+++	++++	+++	++++	+++	++	++	+	+++	+	+	++	++
49	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	++++	+++	++	++++	++	++	++	++++	++	++	++	+	+++	–	+	+	+
62	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	+++	++++	+++	++	+++	+++	+++	+++	++++	+++	++	+	+	++	+	+	++	+
66	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+++	++++	+++	++	+++	++++	++++	+++	++++	+++	++	+	+	++	+	+	++	++
65	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+++	++++	+++	++	++++	++++	++++	++++	++++	+++	++	+	++	++	+	+	++	++
63	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	+++	+++	+++	++	+++	++	++	++	++++	+++	++	++	+	++	+	+	++	+
93	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	+++	+++	+++	++	+++	++	++	++	++++	+++	++	++	+	+++	+	+	+	+
70	–	–	–	–	–	–	–	–	–	–	–	–	–	+	++	+++	+++	++	+++	+++	–	+++	++++	++	++	++	+	+++	+	+	++	+
68	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++	+++	++	+	+++	+	–	±	++++	+++	++	+	–	++	–	+	–	±
64	–	–	–	–	–	–	–	–	–	–	–	–	–	++	++	+++	++	+	+++	++	+++	++	+++	++	++	+	+	++	+	+	++	+

(Continued)

Table 5. Reaction of tospovirus isolates with monoclonal antibodies in TAS-ELISA (continued).[a]

	Monoclonal antibody numbers																													
	Serogroup IV-specific						INSV-specific							TSWV N-specific											TSWV G-specific						
	100	102	103	105	125	131	104	106	107	108	109	119	126	110	111	114	115	116	117	120	121	122	123	128	129	112	113	118	124	127	130	132
90	–	–	–	–	–	–	–	–	–	–	–	–	–	++	+++	+++	++	++	++	+	+	+	+++	++	+	++	–	++	+	+	+	+
23	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	+++	+++	++	++	++	+++	+++	+++	++++	++	+++	+	+	+++	+	+	++	+
18	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	++	+++	++	++	+++	+	+	–	++++	++	+++	+	–	+++	+	±	–	+
71	–	–	–	–	–	–	–	–	–	–	–	–	–	++	++	++	++	++	+++	+	+	+	+++	++	++	+	+	++	–	+	+	+
2	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	++	+++	++	++	+++	++	++	++	++++	++	+++	+	+	+++	+	+	+	+
5	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	++	+	++	++	++	+++	+++	+	++++	++	+++	+	+	+++	+	+	++	+
29	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	++	+++	++	++	++	++	++	+	++++	++	+++	+	+	+++	+	–	+	+
22	–	–	–	–	–	–	–	–	–	–	–	–	–	++	++	+++	++	++	++	+	+	–	++++	++	+++	+	–	+++	–	±	–	+
28	–	–	–	–	–	–	–	–	–	–	–	–	–	++	++	+++	++	++	++	+	+	+	++++	++	+++	+	+	+++	–	–	+	+
6	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	++	+++	++	++	+++	++	++	++	++++	++	+++	+	+	+++	+	+	+	+
19	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	++	+++	++	++	++	+++	+++	+++	++++	++	+++	+	+	++	–	+	++	+
35	–	–	–	–	–	–	–	–	–	–	–	–	–	++	++	+++	++	++	++	+	+	+	++++	++	+++	+	–	+++	–	–	+	+
7	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	++	++	++	++	++	++	–	++	++++	++	++	+	+	++	+	–	+	–
4	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	++	+	++	++	++	++	++	++	++++	++	+++	+	+	+++	–	–	+	+
21	–	–	–	–	–	–	–	–	–	–	–	–	–	++	++	++	++	++	++	++	+++	++	+++	++	+++	+	+	++	+	+	+	+
17	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	++	+++	++	++	++	++	++	++	++++	++	+++	+	+	++	+	+	+	+
14	–	–	–	–	–	–	–	–	–	–	–	–	–	++	++	++	++	++	++	+++	+++	++	++++	++	+++	+	+	++	+	+	+	+
10	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	++	++	++	++	++	+++	+++	+++	++++	++	++	+	+	++	+	+	+	+
27	–	–	–	–	–	–	–	–	–	–	–	–	–	++	++	++	+	+	++	+	+	–	++++	++	++	+	–	++	–	–	–	±
9	–	–	–	–	–	–	–	–	–	–	–	–	–	++	+	++	+	+	++	++	+++	–	++++	+	++	+	+	++	+	±	+	+
8	–	–	–	–	–	–	–	–	–	–	–	–	–	++	+	++	+	+	++	++	++	++	++++	++	++	+	+	++	+	–	+	+
16	–	–	–	–	–	–	–	–	–	–	–	–	–	++	+	++	+	+	+	+	+	+	++	++	++	+	+	++	+	–	+	+
31	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	–	–	–	+	+	+	+	–	+	–	–	–	–
82	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	±

(Continued)

Table 5. Reaction of tospovirus isolates with monoclonal antibodies in TAS-ELISA (continued).[a]

	Monoclonal antibody numbers																												
	Serogroup IV-specific						INSV-specific							TSWV N-specific											TSWV G-specific						
	100	102	103	105	125	131	104	106	107	108	109	119	126	110	111	114	115	116	117	120	121	122	123	128	129	112	113	118	124	127	130	132
73	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
34	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
67	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
33	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
25	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
77	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
37	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
78	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
30	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
38	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
43	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
80	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
74	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
26	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
40	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
42	–	–	–	–	–	±	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
44	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
41	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
83	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–
46	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	±	–	–	–	–	–	–	–	–	–	–	–
81	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–	±	–	–	–	–
87	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	±	–	–	–	–
75	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
84	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–

(Continued)

Table 5. Reaction of tospovirus isolates with monoclonal antibodies in TAS-ELISA (continued).[a]

	Monoclonal antibody numbers																													
	Serogroup IV-specific						INSV-specific							TSWV N-specific											TSWV G-specific						
	100	102	103	105	125	131	104	106	107	108	109	119	126	110	111	114	115	116	117	120	121	122	123	128	129	112	113	118	124	127	130	132
89	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	+	–	–	–	–
91	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	+	+	+	–	±	–	–	–	+	–	–	–	–
39	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	++	–	+	+	–	–	+	–	+	–	–	–	+	–	–	–	–
36	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	+	–
11	–	–	–	–	–	–	–	–	–	–	–	–	–	++	+	++	–	+	–	–	–	++	–	+	–	+	+	+	–	–	+	–
20	–	–	–	–	–	–	–	–	–	–	–	–	–	++	+	++	–	+	+	–	–	++	–	+	–	–	+	++	+	–	++	–
95	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	+++	+++	+	+	++	+	–	++++	–	++	–	+	++	+++	+	–	++	–
94	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	++	+++	–	+	–	–	–	++	–	–	–	–	+	+	+	–	+	–
85	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–
45	+	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–
86	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
61	+	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
12	+	±	±	+	+	+	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
76	–	–	–	–	–	–	++	±	+	–	++	+++	+++	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
79	–	–	–	–	–	–	++	+	+	–	++	+++	+++	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	+	–	–	–
1	–	–	–	–	–	–	+++	+	+	+	+++	+++	+++	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–
3	–	–	–	–	–	–	++	+	++	±	++	++	++	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
13	–	–	–	–	–	–	+++	+	+++	±	+++	+++	+++	–	–	+	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–
24	–	–	–	–	–	–	++	+	+++	±	+++	++	++	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–
15	–	–	–	–	–	–	++	+	+++	+	+++	+++	+++	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
96	–	–	–	–	–	–	+++	++	+++	+	+++	+++	++++	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–

[a]Numbers in the top row refer to RT No. in Tables 2 and 3, describing the antibody combinations or MAbs used as second antibodies. The plus scheme covers the following ELISA readings: ± < 0.1; + 0.1–0.7; ++ 0.7–1.3; +++ 1.3–1.9; and ++++ > 1.9.

30 µL of sterile high-vacuum grease (Dow Corning) to act as a barrier to separate the phases after phenol extraction (Mukhopadhyay and Roth 1993). After adding an equal volume (400 µL) of phenol:chloroform (5:1 v/v), the tube was topped up with DEPC-treated H_2O, vortexed, and centrifuged for 15 min. The aqueous layer was collected and mixed with 2.5 volumes ethanol and 0.1 volumes 3 M sodium acetate to precipitate the RNA. The RNA was pelleted by centrifugation, and the pellet was washed, dried, and resuspended in 50 µL of DePC-treated H_2O.

Oligonucleotide primers

Three different primer pairs synthesized by Alta Bioscience were used. The sequences for the design of primers were based on publications from de Haan et al. (1991, 1992) and de Ávila et al. (1993a). A degenerated primer pair was used for the general tospovirus detection. S1 UNIV and S2 UNIV had the sequences: 5'-TGTA(G/A)TG(T/G)TCCAT(T/A)GCA-3' and 5'-AGAGCAAT(T/C) GTGTCA-3', respectively (flanking a 871 base region in the S RNA). The primers for the specific detection of TSWV were: L1 TSWV 5'-AATTGCCTTG CAACCAATTC-3', L2 TSWV 5'-ATCAGTCGAAATGGTCGGCA-3' (flanking a 276 base portion of the L RNA of TSWV), and for INSV were: S1 INSV 5'-AAGCTTAAATCAATAGTAGCATTA-3', S2 INSV 5'-AAGCTTCCTCAAGAAT AGGCA-3' (flanking a 602 base portion of the S RNA of INSV).

Reverse transcription (RT)

Total plant RNA (1 µL) was added to 9 µL of RT reaction mix [1 µL DTT, 2 µL 5 x M-MLV buffer (BRL), 2 µL 5 mM dNTPs mix, 0.5 µL 5 mM random hexamers (Promega), 0.5 µL (100U) M-MLV reverse transcriptase (BRL), 0.2 µL (0.5U) RNasin RNase inhibitor (Promega), and 3.75 µL DEPC-treated H_2O]. The reaction was mixed and incubated at 37 °C for 1 h.

PCR

After the RT reaction, 40 µL of PCR reaction mix [5 µL 10 x *Taq* buffer (Bioline) containing 15 mM $MgCl_2$, 0.5 µL 20 µM primer 1, 0.5 µL 20 µM primer 2, 0.25 µL (1.25 U) *Taq* DNA polymerase (Bioline), and 33.75 µL of H_2O] was added to the RT mix. The reaction mix was then overlaid with 50 µL of mineral oil and subjected to thermal cycling [1 min denaturation at 94°C, 1 min annealing at 55°C (an annealing temperature of 48 °C was used for the degenerate primers) and 1 min extension at 72°C, for 30 cycles] using a Perkin Elmer thermocycler. The first cycle had an extended 94°C incubation of 5 min, and the last cycle had an extended 72°C incubation of 10 min. Following PCR, 15 µL of product was removed, mixed with loading buffer, electrophoresed in a 1% agarose gel containing 0.5 µg/mL ethidium bromide, and examined under UV light (Sambrook et al. 1989).

Results

DAS-ELISA with polyclonal antibodies

The TSWV-specific antisera can be separated into antisera prepared against purified nucleoprotein complexes, containing only or predominantly N protein, and antisera prepared against complete virions. The antisera should contain antibodies against all structural proteins, especially G proteins. To evaluate the influence of the presence of G-specific antibodies, different combinations of first and second antibodies, as described in Table 2, were used.

The results obtained with DAS-ELISA showed three positively reacting clusters (Table 4). Cluster one in the upper left corner of Table 4 contained isolates of serogroup I and II. Cluster two, in the lower right, contained only isolates of INSV, and the third cluster of serogroup IV isolates in the far right column just above cluster two. This arrangement was achieved by simply sorting the complete spreadsheet according to the values in the columns 1, 15, and 17 because there was an almost complete lack of cross-reactions. Serogroup I and II isolates showed cross-reactions with each other and are therefore grouped into one cluster.

In case of the homologous combination BR-01/BR-01 antibodies for coating and detection, the intensity of the ELISA-readings can at least be divided into two blocks, 35 isolates reacting only with + and 17 isolates reacting with ++ or more. However, when the mixture of three different tospovirus antisera was used for coating, the resulting pattern changed and became similar to those obtained with antisera against complete virions (tests No. 1 versus 25). Interesting is the fact that the use of the highly N-specific antibody BR-01 for coating and antisera against complete virions as second antibodies did not change the pattern as compared with homologous combinations (tests No. 7 and 8 versus 31). Samples reacting low in tests No. 7, 8, 25, and 31, as well as with serogroup II-specific antisera, are probably due to low virus titer. The remaining variability is probably due to intraspecies variability.

The N-specific antisera against the two serogroup II tospoviruses revealed strong cross-reaction with each other and some reactions with TSWV isolates. These cross-reactions were lowest when the BR-01 antiserum was used for coating (tests No. 2 and 3, Table 4); whereas, the broad-spectrum coating with "triple To" (tests No. 26 and 28, Table 4) enhanced the intensity and number of cross-reactions. It is, however, clearly evident that isolates belonging to these two tospoviruses can be differentiated using two antibodies, and that they differ significantly from the TSWV isolates.

Three INSV-specific antibodies were compared. Two (NL-07 and AS-0115) were prepared against nucleoprotein complexes. The third was prepared against complete

virions but had been saturated with TSWV-containing plant sap. If they were used in homologous combinations or with the broad-spectrum coating "triple To" they reacted almost exclusively with INSV isolates and only minor reactions were observed with other isolates. However, if the TSWV N-specific antiserum BR-01 was used for coating, the intensity of the cross-reactions decreased or was even absent and reactions with INSV isolates were drastically reduced. This further corroborated the clear serological difference between serogroup I + II and III isolates.

Only one antiserum against a serogroup IV isolate could be used in this comparison. It had been prepared against nucleoprotein complexes and reacted most strongly with its homologous antigen. When the antiserum was used in homologous combination, two additional weakly positive isolates were observed, of which one turned out to be positive in the more sensitive TAS-ELISA. Cross-reactions with other tospovirus isolates were absent.

In summary, the DAS-ELISA tests clearly showed that three main clusters of tospoviruses exist and that they have almost no serological relations when N-specific antisera are used. However, if antisera also contained G-specific antibodies, no matter if they are used only for coating or as conjugated antibodies, the intensities of reactions increased, and the number of cross-reactions between the groups definitely increased. Intraspecies variability of the isolates of defined species became evident, especially among TSWV where the largest number of isolates was present.

TAS-ELISA with MAbs

The comparison with MAbs comprised antibodies prepared against three different tospovirus species: TSWV, INSV, and WSMV, isolate Tospo-To (Table 3). Only among the TSWV-specific MAbs did antibodies react with G protein(s), either due to the selection process during screening, or due to the use of purified G protein as antigen. All other MAbs were N-specific. When the complete spreadsheet was sorted with converted data using the three columns (125, 107, and 115), the reaction pattern summarized in Table 5 was obtained. It showed clearly the arrangement of positively reacting isolates in four different clusters: clusters one to four from the top right in clockwise direction.

Cluster one contained almost exclusively serogroup I isolates, which were the same 47 isolates identified by the polyclonal antibodies against TSWV. The N-specific MAbs revealed a high degree of intraspecies variability (Table 5), which indicated the modulation of the different epitopes in TSWV N proteins. In addition, interspecies reactions were evident. Three MAbs (test No.115, 123, and 129) reacted almost only with TSWV isolates; test No. 128 reacted also with GRSV but not with TCSV isolates; whereas, five MAbs detected TSWV, GRSV, and TCSV

Table 6. Results of the polymerase chain amplification. Table shows results of RT-PCR reactions carried out on 25 tospovirus isolates using three different primer pairs.[a]

		Primers		
	Isolate[a]	UNIV	TSWV	INSV
1	TSWV (BR 01) [93]	++	+	–
2	GRSV (SA 05) [95]	+	+	–
3	TCSV (BR 03) [94]	+	+	–
4	INSV (NL 07) [96]	++	+	–
5	GBNV [36]	+	++	–
6	TSWV (ZA 1) [62]	–	++	–
7	CONTROL	–	–	–
8	TSWV (182) [23]	++	±	–
9	TSWV (280) [–]	±		–
10	INSV (MA 2) [76]	++	–	–
11	TCSV (VE 225) [11]	+	–	–
12	INSV (OLE) [24]	++	–	++
13	INSV (PV 281) [15]	++	–	++
14	T 1400 [44]	–	–	–
15	IRIS [32]	±	–	–
16	REDDY [86]	–	–	–
17	TSWV (JF 1) [31]	+	+	–
18	TSWV (EL 1F) [29]	±	–	–
19	INSV (EU 2) [3]	+	–	++
20	Chrysanthemum CZ [67]	–	–	–
21	TSWV (VE 223) [22]	–	+	–
22	PYS (TT 1) [40]	–	–	–
23	GBNV (T3) [43]	±	–	–
24	TSWV (G 80) [–]	±	+	–
25	GBNV (PV 283) [12]	missing	+	–

[a] The + and – are used to represent band intensities with ++ representing a very intense band, ± a very faint band, and – no band at all.
[b]Numbers in parentheses refer to the isolate number in Table 1.

isolates. In addition, two MAbs reacted with serogroup III isolates and three even showed slight reactions with some serogroup IV isolates. This indicated the presence of at least four different epitopes to which MAbs were directed, when using TSWV isolates as antigen. TSWV G-specific MAbs also revealed intraspecies variability and interspecies reactions, especially between serogroups I and II. Only two MAbs (test No. 127 and 132) were highly specific for TSWV. The rest reacted variably with TCSV and GRSV, and in one case (test No. 124) also with INSV. Again this indicates at least four different epitopes covered by the MAbs tested. Two of the G-specific MAbs even detected a serogroup IV-suspicious isolate.

In comparison with the polyclonal antibody reaction pattern in Table 4, the sorting of the reactions clearly separated serogroup II isolates from group I. All well-defined serogroup II isolates were found in cluster two, independent of the protein-specificity of the MAb. A tight group of eight isolates contained all four well-characterized serogroup II isolates in the test; the remaining four may not belong to this serogroup because of their reactions with the polyclonal antibodies. Seven additional isolates that did not react with all cross-reacting MAbs might belong to serogroup II.

As observed with the polyclonal antibodies, the INSV MAbs, which all reacted with N protein, detected INSV isolates exclusively. They are grouped in cluster three. Although only eight INSV isolates were included in the test, some intraspecies variability was evident. Six MAbs detected all of the isolates; whereas, one MAb missed two. No interspecies reactions were observed.

Among the seven serogroup IV-specific MAbs, only four (No. 100–103), were prepared against the Tospo-To isolate. The other three were made against an INSV isolate from gloxinia (INSV-HT 1). The positively reacting isolates are grouped in cluster four, in the bottom left of Table 4. Under the test conditions, six MAbs detected the Tospo-To isolate from Taiwan (No. 101 did not react at all and was omitted from Table 5). Two additional isolates from Thailand also reacted with the original Tospo-To and INSV-HT1 derived MAbs. Surprisingly, however, the three INSV-HT 1 MAbs detected two more isolates in the test. This may indicate that serogroup IV contains different virus species.

PCR-amplification of selected isolates

The results of the PCR-amplification are summarized in Table 6. The universal primer pair led to positive results in most cases. However, not in all cases where serological results indicated TSWV, did both the universal and TSWV-specific primers give a positive result. The same held true for the INSV-specific primers. In two samples with evidently INSV isolates, no reactions were obtained. However, it remains to be emphasized that clearly defined isolates, representing all four serogroups or five species, have been successfully amplified by at least one of the two primer pairs, i.e., the universal and TSWV.

Discussion

This is the first report of a broad survey of tospoviruses and related antibodies in which a large number of isolates and antibodies were compared against each other. Comparisons on a smaller scale have been reported before (de Ávila et al. 1990; Law and Moyer 1990; Kitajima et al. 1992) and led to the establishment of serogroups I – III. However, the number of isolates was probably not large enough to illustrate the amount of intraspecies variability. Even with the expected

variability, it was reassuring that the differentiation into the serogroups held true in this larger scale comparison. The collection of representative tospovirus isolates established in this study could serve as a reference for those who need to develop a reliable diagnostic procedure.

The different reaction patterns that were obtained in DAS-ELISA, depending on the specificity of the coating antibodies, probably are due to G-protein specific antibodies and an incomplete denaturation of the virions in the sample extraction buffer. This would explain why even after coating with the highly N-specific BR-01 antibody, the antisera raised against complete virions reacted as broadly as in a homologous combination. Another explanation might be that intensive purification of nucleoprotein complexes by high salt gradients may have caused partial denaturation and loss of epitopes. The intraspecies variability that was observed especially with MAbs against TSWV N protein might be due to small variations reported for the amino-acid sequences of the N proteins that have been observed in as comparisons of sequence data (de Ávila et al. 1993a).

The separation of serogroup II, containing GRSV and TCSV, appears to be justified from both DAS- and TAS-ELISA. However, it becomes evident that serogroup I and II have much more in common to each other than with the other two serogroups (Adam et al. 1993). This is corroborated by the results obtained with TSWV MAbs where a lage number of N-specific MAbs reacted with serogroup II isolates, especially the MAb Wag N1, which has been described to react with TSWV and GRSV (de Ávila et al. 1990).

The interspecies reactions of some MAbs against TSWV N protein is probably not due to an artefact because Hall and Moyer (1993) described the presence of a common epitope for serogroup I and III at the amino-terminus of the N proteins. The eight INSV isolates reacted similarly, especially in DAS-ELISA; whereas, the MAbs revealed some intraspecies variability, but no interspecies reactions.

A significant contribution to interspecies reactions that were observed in DAS- and TAS-ELISA for TSWV antibodies can be clearly attributed to common epitopes in the G proteins, probably G1, because MAb 2B6 has been determined to be G1-specific (Adam et al. 1995). This cross-reaction of G-specific antibodies has already been described (Law and Moyer 1990; Adam et al. 1991, 1995; Adam and Lesemann 1992; Kitajima et al. 1992; Yeh et al. 1992). The G-specific MAbs in this test behaved in most cases as described, for example test No. 132 was clearly TSWV-specific (de Ávila et al. 1990) and test No. 124, an MAb described as having a broad interspecific reaction (Adam et al. 1995) reacted with serogroups I–III.

One advantage of such broad-scale testing may be the discovery of new and unexpected reactions of already available antibodies. In TAS-ELISA, it became

evident that three MAbs reacted specifically with serogroup IV isolates, but had a reactivity that was different from those prepared against the WSMV isolate Tospo-To. They were prepared against the isolate INSV HT-1 (Lawson et al. 1994), which was described to differ serologically from INSV. It was isolated from an INSV sample by high-temperature treatment (Lawson et al. 1993). Whether this isolate is a mutant of INSV or whether the original plant contained different tospoviruses remains unclear. However, the complete lack of interspecies reactions of the INSV MAbs in this test makes the mutant explanation highly unlikely. If INSV-HT-1 is a serogroup IV isolate, the obtained results may reflect the fact that serogroup IV contains at least two species isolates from groundnut in India and WSMV from Far Eastern countries. WSMV isolates are similar, at least their sequence homology is larger than 95% (Heinze et al. 1995; Yeh and Chang 1995). The Indian bud necrosis virus differs from these isolates by host range and serology (Reddy et al. 1992; Yeh et al. 1992; Adam et al. 1993). In addition, sequence data show that they also differ enough to warant their separation into two species (Satyanarayana et al. 1996). At least one of the isolates, that reacted only with the INSV-HT 1-derived MAbs is of Indian origin; whereas, the isolates that reacted with both types of antibodies were of Far Eastern origin. It remains to be determined whether the reaction pattern we observed reflects separation into species.

The most interesting question from this comparison will be: Are there new tospoviruses hidden among the negative or spurious reacting isolates? We cannot exclude that some isolates were recovered poorly during the propagation and this may have led to negative results. It is, however, certain that two tospovirus isolates (confirmed by electronmicroscopy) that reacted negative in all cases are among this group, i.e., test No. 33 and 34. Others, however, gave clear symptoms and did not react or reacted only with a few of the several antibodies. Under the assumption that poor but positive reactions in TAS- or DAS-ELISA may also indicate new tospoviruses, thirteen isolates from this test are likely such cases. Two of them are Far Eastern isolates and one is from South Africa, but at least eight have European origin. It is interesting to note that most of these isolates were associated with cluster two, which contained the serogroup II isolates. The assumption that serogroup II is not present in Europe was abolished by this test because at least one isolate (test No. 39) reacted in DAS-and TAS-ELISA like GRSV.

The results from the PCR tests applied to the broadest available range of known tospoviruses revealed the strength of this method to detect members of each known serogroup by using only two primer pairs. It could be used to detect tospovirus isolates that react poorly or negatively in the serological test.

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