Potato Research 33 (1990) 365–375

Preparation of additional monoclonal antibodies for detection and discrimination of potato virus Y isolates infecting potato

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Accepted for publication: 18 December 1989

Additional key words: ELISA; serotyping, pepper

Summary

Monoclonal antibodies (MAb) specific to potato virus Y (PVY) were obtained from hybrid cells produced by fusion of a non-secreting myeloma cell line with spleen cells from BALB/c mice immunized with isolate Bintje PVY°. Six MAb were characterized. The reactions of the MAb have been assayed by indirect double-antibody sandwich enzyme-linked immunosorbent assay (ELISA-IDAS) against forty-nine PVY isolates and other potyviruses, representative of different potato areas in the world.

All MAb reacted to at least some isolates in group O + C in ELISA-IDAS. One MAb did not react to any group N isolates. MAb 10E3 reacted to all isolates tested. Using standard double-antibody sandwich enzyme-linked immunosorbent assay (ELISA-DAS), two MAbs did not react to any isolates, one reacted to some isolates in group O + C and MAb 10E3 reacted to all isolates but two in group C. A mixture of MAb 10E3 and 1E10 detected all isolates in ELISA-DAS and sensitivity was improved over that obtained with polyclonal antibodies from antisera.

Introduction

Potato virus Y (PVY) is one of the most important infectious agents attacking this and other crops in the world. Virus diagnosis is substantial in seed certification and breeding programmes for resistance. The successful development of the enzyme-linked immunosorbent assay (ELISA; Clark & Adams, 1977) has enabled the routine performance of thousands of highly sensitive analyses needed for selection of disease-free planting stocks. The main problem remaining is the moderate specificity of current antisera. The possibility of producing monoclonal antibodies (MAb) by hybridoma technology (Köhler & Milstein, 1975) opened the feasibility of developing highly specific antibodies for potato virus diagnosis. MAb specific to PVY (Gugerli & Fries, 1983; Sanz et al., 1985b; Yao et al., 1985; Rose & Hubbard, 1986), potato virus X (PVX) (Sanz et al., 1985b; Koenig & Torrance, 1986; Torrance et al., 1986; Sober et al., 1988) and potato leaf roll virus (PLRV) (Martin & Stace-Smith, 1984; Massalski & Harrison, 1987) have been produced. The use of MAb has improved specificity in diagnosis of

potato viruses and greatly increased the sensitivity of ELISA (Fernandez-Northcote & Gugerli, 1987). We describe here the preparation and characterization of additional PVY-specific MAbs which detect the range of existing serotypes, and increase the possibilities of discrimination of serotypes O, C, and N. One MAb capable of recognizing an universal epitope from all PVY strains is also described.

Materials and methods

Virus isolates. Forty-nine characterized PVY isolates, representative of different potato areas in the world were used. Other potyviruses tested included six potato virus A isolates (PVA-C, R, 1, 5, 327, 328), five potato virus V isolates (PVV-Vroege P., Luiker, T., GL, AB, UF), one Peru tomato virus (PTV) isolate, one wilt potato mosaic virus (WPMV) isolate and two plum pox virus (PPV-V, 24-4SE) isolates. The origins of different characterized virus isolates are listed in Table 2. Two hundred unclassified PVY strains from potato and twenty-five unclassified PVY strains from pepper (*Capsicum annuum*) were also evaluated.

Virus purification. An isolate of PVY° from cv. Bintje, provided by Dr. J. A. de Bokx, was purified from *Nicotiana tabacum* var. *Xanthi* according to the method described by Moghal & Francki (1976) with slight modifications. Briefly, leaves of infected plants were homogenized in two volumes of 0.1 M borate buffer, pH 8 containing 0.15 % thioglycollic acid and one-half volume each of chloroform and carbon tetrachloride. Virus was precipitated by the addition of 4 % polyethylene glycol 6000 and 1.75 % NaCl, and incubated for 16 h at 4 °C. The precipitate was resuspended in 0.1 M borate buffer, pH 8 and centrifuged at 78000 *g* for 75 min. This step was repeated once. This semipurified virus stock was resuspended in 0.01 M borate buffer, pH 8 and stored at −70 °C.

Production of hybridomas secreting MAb specific to PVY. Production of hybrid cells secreting MAb specific to PVY was performed by fusion between the non-secreting mouse myeloma X63/Ag 8653 (Kearny et al., 1979) and spleen cells from PVY-immunized mice. BALB/c mice were immunized by intra-peritoneal injection of 0.1 ml PVY preparation (50 μg protein) emulsified in an equal volume of complete Freund's adjuvant (Difco). Fifteen and 30 days later, mice were injected with the same amount of virus emulsified in incomplete Freund's adjuvant. For 4 days before fusion, mice were injected with a daily dose of 50 μg PVY preparation. Hybridization was carried out following the procedure described by Vela et al. (1986). Screening for presence of antibodies against PVY was performed by indirect ELISA (see below). Specific antibody-secreting hybridomas were cloned under conditions of limiting dilution using a feeder layer (Sanz et al., 1985a). Cloning was repeated three times and established hybrids were grown in HT medium (hypoxanthine-thymidine medium).

Determination of MAb isotype, production of ascitic fluids and antibody purification were made following the procedure described by Vela et al. (1986).

Indirect ELISA. Indirect ELISA was used to detect specific antibodies to PVY° strain in supernatant fluids of the cell cultures. Polystyrene plates (Bioreba) were coated with 1 μg/well of purified virus in 100 μl carbonate buffer (0.05 M, pH 9.6), and incubated overnight at 4 °C. Plates were washed with PBS (0.15 M NaCl in 0.1 M sodium phos-

phate pH 7.4) containing 0.02 % Tween 20 (PBS-Tween), and 100 μl/well of undiluted cell-free culture fluid was pipetted into the wells. Peroxidase-labelled rabbit anti-mouse IgG (heavy and light chains) was used as the conjugate. As a control, plates were coated with a filtered extract of healthy tobacco plants made in carbonate buffer (1:10, w/v).

Reactions were considered positive when the absorbance readings were three or more times greater than those obtained when the supernatant fluid from myeloma P3-X63-Ag8 (Köhler & Milstein, 1975) was used in place of the MAb.

Serotyping with MAb. The indirect double-antibody sandwich enzyme-linked immunosorbent assay (ELISA-IDAS) was done as described by Bar-Joseph & Malkinson (1980) and Vela et al. (1986). The plates were coated with 2 μg/ml of polyclonal antibodies (PAb) from Boehringer Mannheim or from the Centro Internacional de la Papa. Plant extracts from leaves were prepared with the aid of a Pollähne press according to Casper (1979). Plant extracts from potato tubers were made with a Tecan 1200 (Bioreba) sap extractor, according to Gugerli (1979). The extraction buffer was 0.01 M sodium phosphate pH 7.2, 0.14 M NaCl, 1 % polyvinylpyrrolidone 10000. The extraction ratio of buffer to plant material was 1:20 (w/v). The assay was completed by adding 1 μg/ml of the different PVY-specific MAb, and goat anti-mouse IgG (Behring Institute) conjugated with alkaline phosphatase. Four replicates were made from each MAb/isolate combination. The results were measured at 405 nm in a Titertek Multiskan (Flow Laboratories) photometer. A parallel standard double-antibody sandwich enzyme-linked immunosorbent assay (ELISA-DAS) was performed with the antisera for presence of antigens in the extracts.

Ability of different MAb to detect PVY by ELISA-DAS. Each MAb, or a mixture of them as shown in Table 3, was assayed as coating (1 μg/ml) and as conjugate (0.1 μg/ml) to form the double sandwich. The conjugates were prepared with alkaline phosphatase following the method described by Cambra et al. (1983). The coating was made as described by Clark & Adams (1977) and the results were measured as in ELISA-IDAS. As control, the ELISA-DAS was performed in parallel with the same extracts, but using a commercial kit from Boehringer Mannheim based on PAb.

Routine detection of PVY with MAb and PAb by ELISA-DAS. Extracts from the unclassified PVY isolates from potato and pepper were assayed by ELISA-DAS using a mixture of the MAb 1E10 and MAb 10E3 at the reported concentrations. In a parallel experiment, the same extracts were tested using the A7 antiserum obtained from rabbits immunized with strain PVY° at the IVIA, Valencia, Spain.

Results

Production of monoclonal antibodies. Three fusion experiments were performed and the fusion products were plated into 1400 culture wells. Twenty supernatant fluids reacted positively with the PVY° preparation by indirect ELISA without reaction with healthy plant extract. Table 1 shows the characteristics of the six hybrid lines secreting PVY-specific antibodies, selected after three cloning steps.

Serotyping with MAb. Table 2 shows the results obtained by ELISA-IDAS with five MAb assayed against forty-nine PVY isolates from a number of countries. All of the

Table 1. Characteristics of hybrid lines secreting PVY-specific antibodies.

Hybrid line	Antibody isotype	Title by indirect ELISA	
		Culture fluid [a]	IgG (ng/ml)
1E10	IgG2b	2375	20
3E9	IgG1	1875	20
7C4	IgM	5	500
10E3	IgG2a	1225	10
12C4	IgG2a	1875	-
12C4-B3	IgM	25	-

[a] Reciprocal of dilution endpoint.

isolates reacted when tested in a polyclonal antiserum (PAb) based DAS-ELISA. MAb 1E10 reacted with all of the PVY^c isolates and 66.6 % of the PVY^o isolates tested. It did not react to any PVY^n isolate of European origin but did detect 5/10 of the PVY^n isolates from America, and gave a strong reaction with isolate 201. The MAb 10E3 reacted with all of the PVY isolates (O, N and C) tested.

The MAb 12C4, 3E9, and 7C4 were assayed only against the isolates of European origin. The 3E9 and 7C4 MAb reacted against all the PVY O, N, and C isolates. The MAb 12C4 reacted to all but two isolates from the Netherlands (Thorbecke and Alcmaria) and these two also gave the lowest A 405 nm values with the other MAbs.

The 10E3 MAb showed the greatest and most homogeneous A 405 nm values. The lowest A 405 nm reading resulted from the reaction of 7C4 MAb against the Gov isolate (PVY °). None of the MAbs reacted with PVA, PVV, PTV, WPMV and PPV isolates. However, the antiserum from Boehringer Mannheim produced a weak reaction against two PVA isolates (data not shown).

Ability of MAb to react in ELISA-DAS. Table 3 shows the results obtained by the ELISA-DAS technique with the five MAbs and with the mixture of two of the MAbs against 29 PVY and 2 PVV isolates. The MAbs 7C4 and 3E9 did not react with any of the isolates assayed with ELISA-DAS. The MAbs 10E3 and 12C4 reacted with all the isolates of PVY^o and PVY^n, but did not react with two of the PVY^c isolates. The MAb 1E10 did not react with any PVY^n isolate and only reacted against five PVY^o isolates of Spanish origin, and against the homologous PVY^o isolate Bintje from the Netherlands. It did react with all the PVY^c isolates, except one.

The DAS-ELISAs incorporating the antiserum or the mixture of MAbs (1E10 plus 10E3) reacted with all of the PVY isolates. In most instances, A 405 nm values were greater with the mixture of the two MAbs than with the polyclonal antiserum. Neither assay reacted with the two isolates of PVV.

Routine detection of PVY by ELISA-DAS. Extracts of two-hundred potato tubers and extracts of twenty-five pepper plants, infected with unclassified PVY isolates, were tested by ELISA-DAS. Duplicate samples of the extracts were tested with the mixture of MAbs (1E10 and 10E3) and with A7 antiserum. All of the samples gave positive reactions in both tests, however, higher A 405 nm values were obtained in the test using

a mixture of the two MAbs. The MAbs mixture also gave fewer nonspecific reactions with healthy material from potato and pepper (data not shown).

Discussion

Six hybridomas secreting PVY-specific antibodies representing four different isotypes were obtained by immunizing mice with semipurified preparations of the Bintje isolate (PVY^o). Five of the six MAbs obtained were tested against several PVY isolates. Three of the MAbs (10E3, 3E9 and 7C4) strongly reacted with all the PVY isolates tested by indirect ELISA (ELISA-IDAS). One MAb (12C4), recognized all PVY isolates except two of PVY^c (Thorbecke and Alcmaria) and one MAb (1E10) showed a different pattern of reactivity. This MAb recognized a highly variable epitope, which is present in PVY^c isolates, some PVY^o and some American PVY^n isolates. However, this epitope is lacking in European PVY^n isolates. The MAb 10E3 probably recognized an universal epitope because it reacted with the 49 PVY isolates from different origins.

When MAbs were used for coating microplates and as enzyme-conjugates in ELISA-DAS to check their ability to detect PVY in routine assays, only three of them (1E10, 10E3 and 12C4) gave a positive reaction. The lack of reaction of the MAb 3C9 and 7C4 in ELISA-DAS could be due to several causes, but probably the capture of the virus by the coating MAb inhibits the binding of the same MAb conjugated to alkaline phosphatase because there are no more identical epitopes available for reaction. The MAb 1E10 can react with the PVY^o and PVY^c isolates, but none of the PVY^n isolates. On the other hand, MAbs 10E3 and 12C4 reacted with all the isolates tested, except for two PVY^c that were detected only by the MAb 1E10. For this reason, the mixture of the MAbs 1E10 and 10E3 was tested and proved to be able to recognize all the isolates tested by ELISA-DAS. The failure in detecting two isolates by MAb 10E3 in ELISA-DAS could be explained, as previously discussed, for MAb 3E9 and 7C4. Addition of 1E10 to MAb 10E3 allowed positive detection by ELISA-DAS for all known isolates.

Our results with ELISA-DAS confirm the existence of serological differences between the groups O, N, and C and within groups (Gugerli & Fries, 1983). The PVY^c isolates Alcmaria and Thorbecke show a different behaviour with all the MAbs. Differences have also been found between European and American isolates of PVY^n, and among the PVY^o isolates, using the MAb 1E10.

MAb selection and utilization must be done very carefully and the method of detection to be used should be considered when screening new hybridomas. The indirect serological techniques, such as ELISA-IDAS can provide further information when utilizing MAb. This supports the experience of Van Regenmortel (1982) and the trend reported by Sanchez-Vizcaíno & Cambra (1987). Selecting MAbs capable of recognizing plant antigens by ELISA-DAS, alone or in mixture, would be most valuable. This technique is simple and very suitable for routine analyses. Our results show that routine and standard detection of PVY is possible on potato and pepper by ELISA-DAS using a mixture of two MAbs. The MAbs combination provides higher sensitivity than existing pAbs because of high specificity for virus antigens and non-reaction to healthy plant antigens. The use of MAbs, in addition, brings about new possibilities for diagnosis of specific serotypes.

The MAbs obtained show a distinct behaviour and complement those obtained by Gugerli & Fries (1983) and Rose & Hubbard (1986) from PVY^n isolates. These authors obtained MAbs specific to PVY^n isolates, and we have produced MAbs specific to

Table 2. Serotyping of PVY-specific monoclonal antibodies by ELISA-IDAS.

Virus and isolates		Origin[a]	Monoclonal antibodies and reaction (A 405)					Antisera a or b reaction (A 405 nm) ELISA-DAS
			1E10	10E3	12C4	3E9	7C4	
PVY°	E	Spain (1)	0.70	0.79	0.79	0.62	0.49	0.62a
	P	Spain (1)	0.63	0.80	0.78	0.71	0.80	0.73a
	RP	Spain (1)	0.72	0.78	0.81	0.71	0.50	0.63a
	Gon	Spain (1)	0.69	0.76	0.76	0.59	0.78	0.70a
	Bintje (Bu)	Holland (2)	0.55	0.81	0.81	0.65	0.61	0.81a
	Kitting	Holland (2)	0.70	0.80	0.66	0.66	0.74	0.71a
	Libertas	Holland (2)	0.61	0.78	0.78	0.54	0.59	0.67a
	Paul Kruger	Holland (2)	0.00	0.78	0.77	0.48	0.80	0.65a
	Record	Holland (2)	0.00	0.75	0.73	0.65	0.84	0.74a
	Bintje	Holland (2)	0.80	0.81	0.82	0.81	0.70	0.82a
	Gov	United Kingdom (3)	0.00	1.40	1.00	0.79	0.34	0.61a
	SF	Perú (4)	0.00	>2.00	nt	nt	nt	1.54b
	255	Perú (4)	0.69	1.43	nt	nt	nt	0.69b
	T	Perú (4)	0.00	1.28	nt	nt	nt	0.78b
	220	Perú (4)	0.51	0.22	nt	nt	nt	0.43b
	224	Perú (4)	0.00	>2.00	nt	nt	nt	1.52b
	H-13	Perú (4)	0.00	>2.00	nt	nt	nt	1.14b
	2	Chile (4)	0.33	>2.00	nt	nt	nt	>2.00b
	171	Chile (4)	1.79	>2.00	nt	nt	nt	0.95b
	189	Chile (4)	1.65	>2.00	nt	nt	nt	>2.00b
	52 N	Eduador (4)	0.00	0.54	nt	nt	nt	0.24b
PVY°	Zeeuwse Blauwe	Holland (2)	0.78	0.75	0.83	0.71	1.25	0.84a
	Thorbecke	Holland (2)	0.48	0.57	0.00	0.49	0.61	0.55a
	Alcmaria	Holland (2)	0.44	0.56	0.00	0.44	0.91	0.70a
	Gelderse R	Holland (2)	0.72	0.71	0.78	0.75	1.30	0.82a
	L.R. Star	Holland (2)	0.77	0.78	0.88	0.87	0.81	0.78a
	R	United Kingdom (3)	0.81	1.41	1.26	0.93	0.45	0.87a
	85 N	Ecuador (4)	0.56	1.44	nt	nt	nt	0.62b

PVY^N	ME1	Spain (1)	0.00	0.89	0.81	0.47	0.77	0.91a
	ME2	Spain (1)	0.00	0.61	0.44	0.49	1.10	0.97a
	RP1	Spain (1)	0.00	1.32	0.88	1.02	0.42	1.04a
	G	Holland (2)	0.00	1.33	0.62	0.65	0.45	0.89a
	Alpha	Holland (2)	0.00	0.75	0.71	0.54	0.62	0.85a
	Bintje	Holland (2)	0.00	0.83	0.76	0.67	0.59	0.72a
	Eersteling	Holland (2)	0.00	0.82	0.69	0.62	0.65	0.81a
	Record	Holland (2)	0.00	0.89	0.79	0.63	0.51	0.92a
	Bintje CH 605	Switzerland (5)	0.00	0.76	0.79	0.65	0.54	0.88a
	MAFF	United Kingdom (3)	0.00	1.46	1.01	0.97	0.53	1.33a
	Hansa L	Germany (6)	0.00	0.85	0.81	0.69	0.58	0.77a
	CC5	Perú (4)	0.41	1.03	nt	nt	nt	0.31b
	140	Perú (4)	0.22	>2.00	nt	nt	nt	1.23b
	201	Perú (4)	1.13	>2.00	nt	nt	nt	1.24b
	240	Perú (4)	0.00	1.23	nt	nt	nt	0.25b
	198	Perú (4)	0.52	1.55	nt	nt	nt	0.76b
	133	Chile (4)	0.00	1.35	nt	nt	nt	1.42b
	8	Argentina (4)	0.35	0.97	nt	nt	nt	0.36b
	36	Argentina (4)	0.00	0.42	nt	nt	nt	0.25b
	38	Argentina (4)	0.00	0.64	nt	nt	nt	0.26b
	48	Argentina (4)	0.00	1.36	nt	nt	nt	0.95b
	Healthy potato		0.00	0.00	0.00	0.00	0.00	0.00a and b
	Healthy tobacco		0.00	0.00	0.00	0.00	0.00	0.00a

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a – Antiserum from Boehringer Mannheim, Germany (commercial kit).
b – Antiserum from Centro Internacional de la Papa, Lima, Perú.
nt – non tested.

Table 3. Ability of different MAb to detect PVY to ELISA-DAS.

Virus and isolates		Origin[a]	Monoclonal antibodies and reaction (A 405)						Antiserum reaction (A 405 nm)
			1E10	10E3	12C4	3E9	7C4	1E10 + 10E3	
PVY[o]	E	Spain (1)	0.74	0.71	0.82	0.00	0.00	0.80	0.65
	P	Spain (1)	0.72	0.73	0.81	0.00	0.00	0.75	0.63
	RP	Spain (1)	0.93	0.72	0.75	0.00	0.00	0.86	0.65
	GoN	Spain (1)	0.66	0.75	0.73	0.00	0.00	0.81	0.56
	Bintje	Holland (2)	0.00	1.44	1.42	0.00	0.00	1.20	0.57
	Kitting	Holland (2)	0.00	0.63	0.92	0.00	0.00	0.58	0.68
	Libertas	Holland (2)	0.00	1.12	0.96	0.00	0.00	1.04	0.28
	Paul Kruger	Holland (2)	0.00	1.33	1.27	0.00	0.00	1.22	0.43
	Record	Holland (2)	0.00	0.73	0.95	0.00	0.00	0.67	0.37
	Bintje	Holland (2)	1.54	1.76	1.74	0.00	0.00	>2.00	0.84
	SF	Perú (3)	0.00	0.77	0.85	0.00	0.00	0.64	0.54
	Go	United Kingdom (4)	0.00	1.46	1.77	0.00	0.00	1.01	0.27
PVY[c]	Zeeuwse B.	Holland (2)	0.71	0.73	0.76	0.00	0.00	0.79	0.64
	Thorbecke	Holland (2)	0.25	0.00	0.00	0.00	0.00	0.23	0.81
	Alcmaria	Holland (2)	0.46	0.00	0.00	0.00	0.00	0.40	1.09
	Gelderse R.	Holland (2)	0.91	>2.00	1.12	0.00	0.00	>2.00	0.45
	L.R. Star	Holland (2)	1.63	1.65	1.67	0.00	0.00	>2.00	0.67
	R	United Kingdom (4)	0.00	0.65	1.03	0.00	0.00	0.57	0.47
PVY[N]	ME1	Spain (1)	0.00	0.53	0.37	0.00	0.00	0.54	0.29
	ME2	Spain (1)	0.00	0.71	0.46	0.00	0.00	0.68	0.27
	RP-I	Spain (1)	0.00	0.74	0.63	0.00	0.00	0.70	0.55
	G	Holland (2)	0.00	0.51	0.74	0.00	0.00	0.61	0.46
	Alpha	Holland (2)	0.00	0.76	1.07	0.00	0.00	0.74	0.88
	Bintje	Holland (2)	0.00	1.23	>2.00	0.00	0.00	1.02	0.96
	Bintje CH605	Switzerland (5)	0.00	1.55	1.78	0.00	0.00	1.62	0.92
	Eersteling	Switzerland (5)	0.00	0.68	1.10	0.00	0.00	0.59	1.24
	Record	Switzerland (5)	0.00	1.63	>2.00	0.00	0.00	1.61	0.97
	MAFF	United Kingdom (4)	0.00	1.22	>2.00	0.00	0.00	1.11	0.93
	Hansa L.	Germany (6)	0.00	0.72	0.74	0.00	0.00	0.70	0.44

PVV	Vroege P.	Holland (2)	0.00	0.00	0.00	0.00	0.00	0.00		0.00
	Luiker T.	Holland (2)	0.00	0.00	0.00	0.00	0.00	0.00		0.00
	Healthy potato		0.00	0.00	0.00	0.00	0.00	0.00		0.00
	Healthy tobacco		0.00	0.00	0.00	0.00	0.00	0.00		0.00

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PVY^{o} + PVY^{c}. In agreement with other workers, we also found MAbs able to recognize a possible universal epitope. The MAbs produced were patented in Spain in 1984 (patent number 536924) and since that year, over two million tests have been done for detecting PVY in potato.

Acknowledgements

The authors would like to thank the International Potato Centre of Lima, Perú, and particularly Dr L. Salazar, Dr E. N. Fernandez-Northcote, and Dr J. Nakashima for their cooperation in the serotyping work that was done in Lima, and Dr J. A. de Bokx for providing the isolate of PVY^{o} from cv. Bintje. Appreciation is expressed to Dr S. M. Garnsey, U.S. Department of Agriculture, Orlando, Flordia, for critical reading and to A. Borras, T. Sebastia and Marion Baker for her assistance in the translation and typing. This work has been carried out thanks to the financial support of CAICYT to the agreement INGENASA-IVIA, Spain.

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