PROPERTIES OF TWO MONOCLONAL ANTIBODIES SPECIFIC TO THE CHERRY STRAIN OF *PLUM POX VIRUS*

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SUMMARY

Two monoclonal antibodies (MAbAC and MAb-TUV) raised against the sweet cherry isolate of *Plum pox virus* (PPV-SwC) were tested against homologous and heterologous PPV strains from different geographical origins. Both MAbs proved to be PPV-C (Cherry) strain-specific. They reacted in DASI-ELISA with PPV-SwC and the sour cherry isolate of PPV (PPV-SoC) from *Nicotiana benthamiana*, but not with any of additional 44 PPV isolates tested belonging to the Dideron, Marcus and El Amar strains. The strain specificity of both MAbs was also assessed by Western blot and immuno electron microscopy. As indicated by competitive ELISA, the two MAbs are elicited by different epitopes.

Key words: cherry, *Plum pox virus*, PPV-C, strain-specific MAbs, ELISA, Western blot.

INTRODUCTION

For many years sweet cherry (*Prunus avium*) has been regarded as resistant or immune to *Plum pox virus* (PPV), the agent of sharka disease of stone fruits (Németh, 1986; Dunez and Sutic, 1988). Experimental transmission of PPV isolates from peach, plum and apricot to sweet cherry and *P. mahaleb* either failed or remained localised at the inoculation site (Dosba *et al.,* 1987). However, reports from Moldova (Kalashyan and Bilkey, 1989) and Italy (Crescenzi *et al.,* 1994) have shown that sour cherry (*P. cerasus*) and sweet cherry can be naturally infected by PPV. These findings were soon confirmed by reports from Bulgaria (Topchiiska, 1997), Syria (Al-Chaabi *et al.,* 1997) and Hungary (Kölber *et al.,* 1998).

The sweet cherry isolate from Southern Italy (PPV-SwC) (Crescenzi *et al.,* 1994), and the sour cherry isolate from Moldova (PPV-SoC) (Kalashyan *et al.,* 1994), did not seem to differ biologically, but were serologically distinct from the conventional strains Marcus (M) and Dideron (D) (Crescenzi *et al.,* 1997a). These isolates were therefore proposed as members of a new PPV serotype, denoted PPV-cherry (PPV-C) (Crescenzi *et al.,* 1996; Nemchinov *et al.,* 1996). This proposal was supported by differences in the RFLP pattern of the 3' terminal region of the coat protein (Nemchinov and Hadidi, 1996; Crescenzi *et al.,* 1997a) and replicase (Hammond *et al.,* 1998) genes, and the generation of PPV-C specific riboprobes (Nemchinov *et al.,* 1996) and PCR primers (Nemchinov and Hadidi, 1998).

There is a risk that PPV-C may spread world-wide, as current certification and quarantine regulations do not require testing for PPV in cherry (Nemchinov *et al.,* 1998). Thus a serological test for specific and reliable PPV-C detection, would be highly desirable (Crescenzi *et al.,* 1997a; Candresse *et al.,* 1998). Recently, an antiserum (PAb-PEP) specific to PPV-SwC was raised to a synthetic peptide corresponding to the first 14 amino acids of the N-terminal region of the coat protein of PPV-SwC (Crescenzi *et al.,* 1997b), and monoclonal antibodies (MAbs) were obtained against PPV-SwC (Boscia *et al.,* 1998). These MAbs were only partially characterised and their strain specificity assessed against a limited number of PPV isolates (Boscia *et al.,* 1998). The investigations were therefore extended, as reported in this paper.

MATERIALS AND METHODS

MAb isotype, titre and conjugation. MAb titre was determined by DASI-ELISA (Cambra *et al.,* 1994) using increasing dilutions from 1:10 to 1:10,240 for cell culture supernatants and from 1:1000 to 1:2,048,000 for ascitic fluids. A mouse antibody isotyping kit (Sigma Immuno Type kit ISO-1) was used for MAb isotyping, according to manufacturer's instructions. IgGs were purified with a protein A-sepharose column (Clark and Bar-Joseph, 1984) and conjugated with alkaline phosphatase as described by Avrameas (1969).

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Virus isolates. PPV isolates, previously characterised, were supplied from different collections: IVIA, Spain; DCBG-PUO, Czech Republic; ARI-C, Yugoslavia; INRA-PV, France and IV-SAS, Slovakia.

PPV antibodies. In our serological tests serotype-specific and universal monoclonal antibodies were used, *i.e.* universal MAb5B and MAb4DG5, specific to PPV-D (Cambra *et al.,* 1994), MAbAL, specific to PPV-M (Boscia *et al.,* 1997), and MAbEA24, specific to PPV-EA (Myrta *et al.,* 1998). The polyclonal antibody used for coating the plates was PabBA/AL (Myrta *et al.,* 1996).

Specificity to PPV-C

DASI-ELISA. Strain specificity was assessed by DASI-ELISA (Cambra *et al.,* 1994) testing 46 PPV isolates from different geographical areas, belonging to the M, D, El Amar (EA), and cherry strains, using the antibodies cited previously.

Western blot. The test was carried out as described by Nemchinov *et al.* (1996). Dissociated coat protein from crude sap of *Nicotiana* plants infected with isolates of PPV-EA, PPV-M, PPV-D, PPV-SoC and PPV-SwC were transferred to three nitrocellulose membranes, then incubated separately with MAbAC, MAbTUV and IgGs from PAbBA/AL, an antiserum previously raised against the isolate PPV-AL (Boscia *et al.,* 1997), in 2% nonfat milk powder-phosphate buffered saline (PBS). The membranes were washed, incubated with antimouse (for MAbs) and goat anti-rabbit (for PAb) IgG alkaline phosphatase conjugates as secondary antibody. Substrate consisted of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma Chemical Co., St. Louis).

Immunoelectron microscopy. MAbs obtained from diluted ascitic fluid were used for decorating (Milne, 1993) particles of the homologous virus and representative isolates of strains PPV-M, PPV-D and PPV-EA.

Competitive binding ELISA. ELISA plates were coated with PAbBA/AL IgGs at 1 µg ml^{-1} in carbonate buffer and incubated 2 h at 37°C. After three washings, plates were loaded with extracts from *N. benthamiana* infected with PPV-SwC and incubated overnight at 4°C. The plates were washed three times and incubated for 2 h at 37°C separately with MAbs AC, TUV and AL (negative control), diluted from 1:25 to 1:12,800 in PBS. After three additional washings, alkaline phosphatase conjugated MAbAC (AC*) was added and incubated for 2 h at 37°C. After washing, substrate was added and incubated for 1 h at room temperature.

To verify possible competition of PAb-PEP, specific to PPV-SwC (Crescenzi *et al.,* 1997b), with MAbAC, ELISA plates were coated with PAbBA/AL IgGs at 1 µg ml^{-1} in carbonate buffer and incubated 2 h at 37°C. After three washings the plates were incubated overnight with PPV-SwC infected *N. benthamiana* extracts. After three washings, the ELISA plates were loaded separately with polyclonal antisera to PEP, PPV-AL and *Grapevine fleck virus* (GFkV) (negative control), diluted in PBS from 1:25 to 1:3200. AC* was added successively and incubated for 2 h at 37°C. After washing, substrate was added and incubated for 1 h at room temperature.

RESULTS

MAb titre and isotyping. The titre of MAbAC was higher than that of MAbTUV, both in culture supernatant and ascitic fluid. Respective values were 1:5120 for supernatant and 1:512,000 for ascitic fluid (MAbAC), versus 1:640 and 1:64,000 (MAbTUV). MAbAC and MAbTUV belonged to IgG subclass 2a and 2b, respectively.

Strain specificity of MAbs. The strain specificity of MAbAC and MAbTUV was confirmed by their behaviour against 46 PPV isolates, tested comparatively with other serotype-specific MAbs. In particular, the universal MAb5B reacted with all PPV isolates (46), while all PPV-M isolates (22), PPV-D isolates (19), a natural mixed infection (PPV-AL) and PPV-EA (2) were recognised only by the respective specific MAbs. MAbTUV and MAbAC reacted only with PPV-C isolates (Table 1).

In Western blots, both MAbs recognised PPV-C isolates (PPV-SwC and PPV-SoC), but did not react with extracts from *N. benthamiana* or peach GF 305 infected with PPV-M, PPV-D and PPV-EA (Fig. 1).

Both MAbs decorated homologous particles (PPV-SwC), but not those of strains PPV-M, PPV-D and PPV-EA (data not shown).

Competitive binding ELISA. Satisfactory IgG purification and conjugation was obtained only with MAbAC, thus limiting competitive ELISA tests to this MAb.

As shown in Fig. 2, the reaction obtained when using MAbTUV and subsequently MAbAC* (conjugated with alkaline phosphatase) was very similar to that obtained with the negative control (MAbAL/MAbAC*) but different to the very poor reaction obtained with MAbAC/MAbAC*. This indicates competition between AC and AC*, but not between TUV and AC*. This is evidence that MAbAC and MAbTUV are determined by two distinct and distant epitopes.

Table 1. Comparative DASI-ELISA analysis of PPV strains using different monoclonal antibodies.

No.	PPV isolate	Laboratory of origin*	PPV strain	Mab					
				5B	4DG5	AL	AC	TUV	EA24
1.	ISPAVE-31	DPPM-UBA	D[1]	+	+	–	–	–	–
2.	Pesco-Piemonte	DPPM-UBA	D[1]	+	+	–	–	–	–
3.	Susino-Trento	DPPM-UBA	D[1]	+	+	–	–	–	–
4.	Canino	IVIA	D[2]	+	+	–	–	–	–
5.	RB 3.3	IVIA	D[2]	+	+	–	–	–	–
6.	R 3	IVIA	D[2]	+	+	–	–	–	–
7.	483	IVIA	D[2]	+	+	–	–	–	–
8.	3.4 RB/RB	IVIA	D[2]	+	+	–	–	–	–
9.	RB Mp/clo 1	IVIA	D[1,2]	+	+	–	–	–	–
10.	Hongria	IVIA	D[1]	+	+	–	–	–	–
11.	5.15	IVIA	D[2]	+	+	–	–	–	–
12.	Plovdiv	IVIA	D[1]	+	+	–	–	–	–
13.	CH 1	DCBG-PUO	D[1,2]	+	+	–	–	–	–
14.	W	DCBG-PUO	D[1,2]	+	+	–	–	–	–
15.	Ag	DCBG-PUO	D[1]	+	+	–	–	–	–
16.	S 10	DCBG-PUO	D[1]	+	+	–	–	–	–
17.	S 3	DCBG-PUO	D[1,2]	+	+	–	–	–	–
18.	BBR 1	IV-SAS	D[1]	+	+	–	–	–	–
19.	BOJ 3	IV-SAS	D[1]	+	+	–	–	–	–
20.	Pesco-EL	DPPM-UBA	M[1]	+	–	+	–	–	–
21.	Albicocco-Gr	DPPM-UBA	M[1]	+	–	+	–	–	–
22.	Impalata	DPPM-UBA	M[1]	+	–	+	–	–	–
23.	E. Crest	DPPM-UBA	M[1]	+	–	+	–	–	–
24.	O. Henry	DPPM-UBA	M[1]	+	–	+	–	–	–
25.	Marcus	DPPM-UBA	M[1]	+	–	+	–	–	–
26.	Quiot Guisante	IVIA	M[1]	+	–	+	–	–	–
27.	M	IVIA	M[1]	+	–	+	–	–	–
28.	Ms 89	IVIA	M[2]	+	–	+	–	–	–
29.	Tarek M	IVIA	M[1]	+	–	+	–	–	–
30.	A1	IVIA	M[2]	+	–	+	–	–	–
31.	Ms 89 Mp2	IVIA	M[2]	+	–	+	–	–	–
32.	SP	IVIA	M[2]	+	–	+	–	–	–
33.	Var	IV-SAS	M[1]	+	–	+	–	–	–
34.	Var-2	IV-SAS	M[1]	+	–	+	–	–	–
35.	CAH 2	IV-SAS	M[1]	+	–	+	–	–	–
36.	BOR 3	IV-SAS	M[1]	+	–	+	–	–	–
37.	302	DCBG-PUO	M[1]	+	–	+	–	–	–
38.	DS	ARI-C	M[1]	+	–	+	–	–	–
39.	Kaldesi	ARI-C	M[1, 2]	+	–	+	–	–	–
40.	SSR	ARI-C	M[1]	+	–	+	–	–	–
41.	PS 3	ARI-C	M[1]	+	–	+	–	–	–
42.	PPV-AL	DPPM-UBA	M+D[2]	+	+	+	–	–	–
43.	PPV-SwC	DBDB-UB	C[3]	+	–	–	+	+	–
44.	PPV-SoC	USDA-NGRL	C[3]	+	–	–	+	+	–
45.	El-Amar	INRA-PV	EA[3]	+	–	–	–	–	+
46.	Egypt 2	INRA-PV	EA[3]	+	–	–	–	–	+

[1] RFLP analysis (*RsaI*) of PCR product from CP gene (Wetzel *et al.,* 1991).
[2] PCR with serotype-specific primers (PD and PM) (Candresse *et al.,* 1995).
[3] Sequence analysis.
* ARI-C: Agricultural Research Institute, Cacak (Yugoslavia).
DBDB-UB: Dipartimento di Biologia, Difesa e Biotecnologie Agro-Forestali, University of Basilicata, Potenza (Italy).
DCBG-PUO: Department of Cell Biology and Genetics, Palacky University Olomouc (Czech Republic).
DPPMA-UBA: Dipartimento di Protezione delle Piante e Microbiologia Applicata, University of Bari (Italy).
INRA-PV: Institut National de la Recherche Agronomique, Station de Pathologie Végétale, Villenave d'Ornon (France).
IV-SAS: Institute of Virology, Slovak Academy of Sciences, Bratislava (Slovak Republic).
IVIA: Instituto Valenciano de Investigaciones Agrarias, Valencia (Spain).
USDA-NGRL: United States Department of Agriculture, National Germplasm Resources Laboratory, Beltsville (USA).

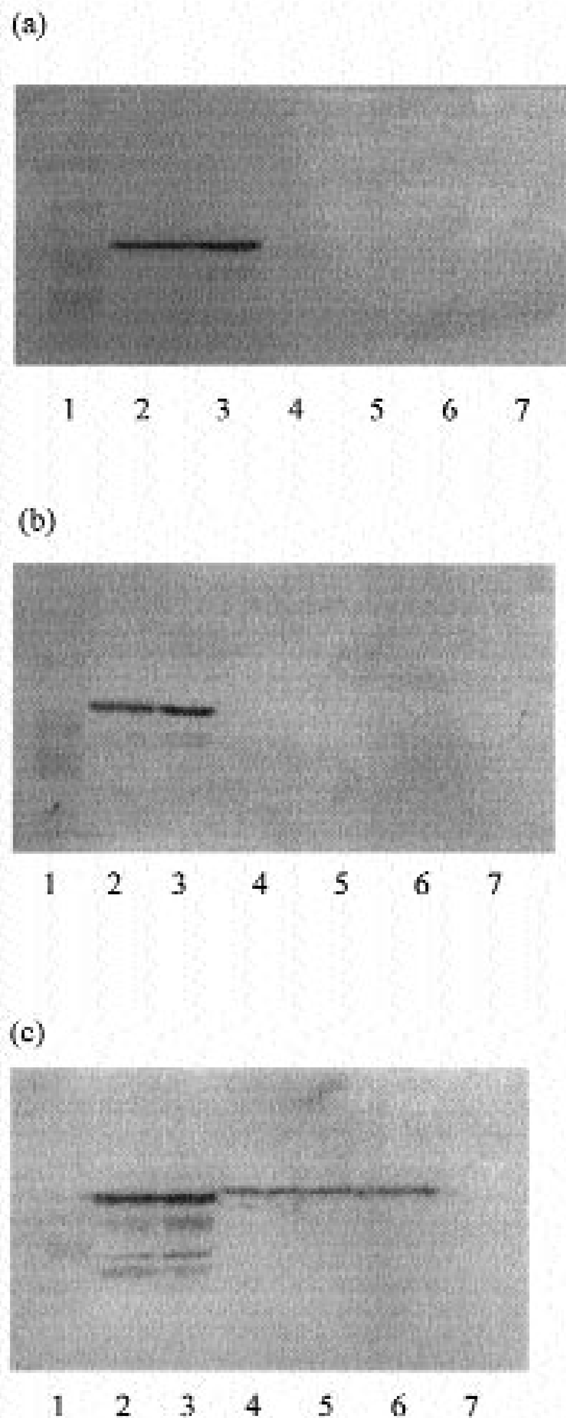

Fig. 1. Western blot of dissociated PPV coat protein preparations from crude sap of infected *N. benthamiana* exposed to MAbAC (panel **a**), MAbTUV (panel **b**) and PAbBA/AL (panel **c**). Lane 1: prestained marker; lane 2: PPV-SwC; lane 3: PPV-SoC; lane 4: PPV-EA; lane 5: PPV-M; lane 6: PPV-D. PAbBA/AL recognised all PPV isolates (panel **c**, lanes 2-6) whereas MAbAC and MAbTUV recognised only the coat protein of PPV-SwC (panel **a** and **b**, lane 2) and PPV-SoC (panel **a** and **b**, lane 3), respectively. Healthy controls in lane 7.

As to PAbPEP, Fig. 3 shows inhibiting activity of MAbAC which disappears at dilutions higher than 1:400. However, this may not derive from saturation of the AC site but rather from steric hindrance probably due to the proximity of the two sites. Likewise, steric hindrance may be responsible for the inhibitory effect of PAbBA/AL, an antiserum raised to a PPV isolate not recognised by MAbAC (PPV-AL, Table 1). As expected, the negative control (PAb-GFkV) caused no inhibition. These findings indicate that PEP is distinct, but probably adjacent to the AC site.

DISCUSSION

Both MAbs confirmed that isolates PPV-SwC and PPV-SoC belong to the same serotype and can be distinguished from the other three PPV serotypes described.

The strain specific MAbAC and MAbTUV complete the set of highly specific monoclonals currently available for the recognition of the four extant PPV serotypes (M, D, EA, C).

The reliability of ELISA for PPV-C detection in field-grown cherry trees remains to be tested because there is very little information about the natural spread of PPV-C and its distribution in infected plants. Preliminary testing of cherry trees adjacent to PPV-infected stone fruit orchards in Hungary (M. Kölber, personal communication, 1998) and Czech Republic (R. Karesova, personal communication, 1999), was negative. However, Kölber *et al.* (1998) reported a low correlation between ELISA and PCR for PPV detection in cherry. By contrast, in a limited number of ELISA tests carried out in our laboratory, PPV-C strain was specifically detected in cherry tissues. Extensive surveys must be done to establish if these ambiguities stem from a low virus concentration in cherry or from serological variability between PPV-C isolates.

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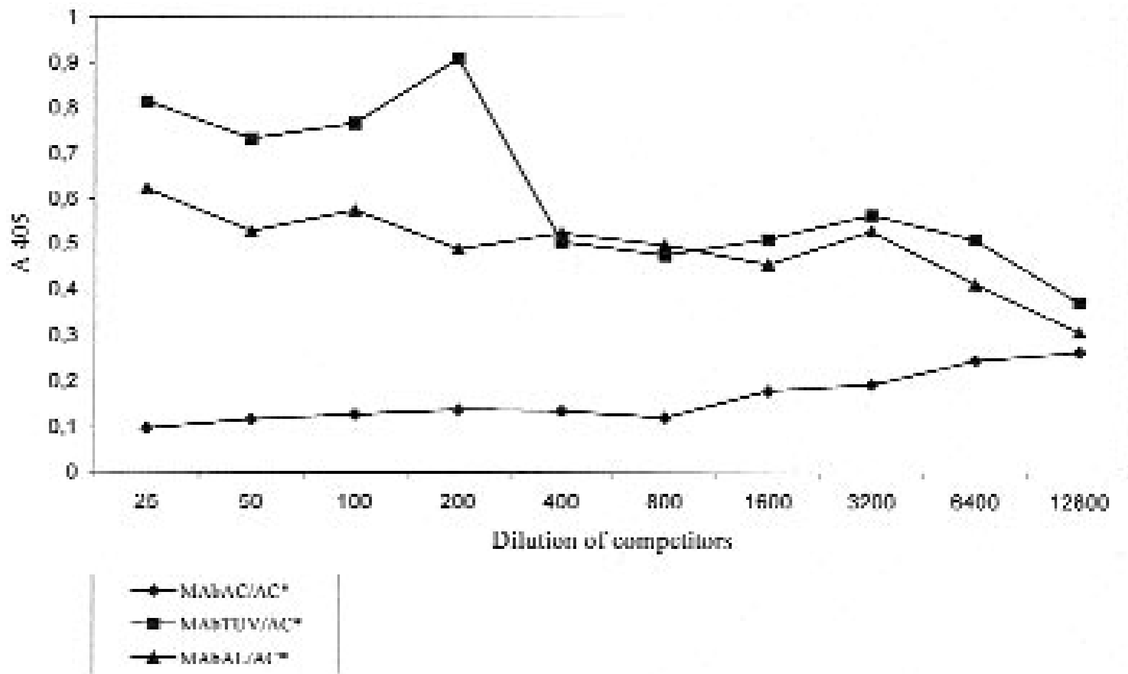

Fig. 2. Competition binding in DASI-ELISA between MAbTUV and enzyme-conjugated MabAC (AC*).

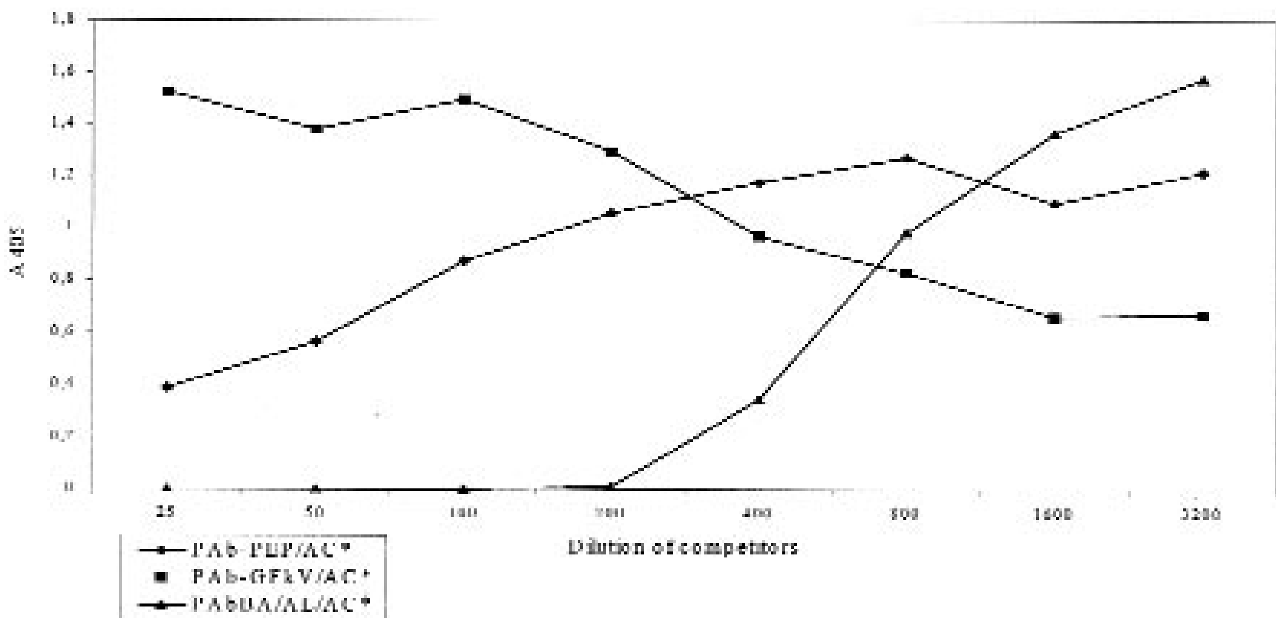

Fig. 3. Competition binding in DASI-ELISA between PAb-PEP and enzyme-conjugated MAbAC (AC*).

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