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Detection of plum pox potyvirus using monoclonal antibodies to structural and non-structural proteins[1]

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Eleven monoclonal antibodies specific to plum pox potyvirus (PPV) coat protein were obtained by hybridoma technology from Spanish PPV isolates. In addition, two monoclonal antibodies specific for PPV cylindrical inclusions (CIP non-structural proteins) were obtained. The monoclonal antibodies specific for PPV coat protein were assayed by DASI ELISA against 81 PPV isolates. At least nine different epitopes were found and 21 distinct serological patterns of reaction (serogroups) were established using nine selected monoclonal antibodies against the collection of PPV isolates, indicating the high variability of coat protein among PPV isolates. Changes in epitope composition were observed after aphid and mechanical transmission, indicating the occurrence of mixtures of isolates in field trees. Monoclonal antibody 5B reacted with all PPV isolates assayed, with very high affinity, using DASI ELISA. This method was compared with immunocapture-PCR on field samples in spring, and showed very good coincidence of results. The efficiency of PPV detection can be slightly increased using monoclonal antibodies specific to cylindrical inclusions mixed with monoclonal antibodies against structural proteins, and using mixtures of monoclonal antibodies against different epitopes of coat protein. ELISA-I and immunoprinting-ELISA were able to detect CIP and PPV in extracts and tissue section, respectively, of woody plants. Two monoclonal antibodies offer the possibility of distinguishing between Marcus and Dideron PPV types (M or D). These D-specific monoclonal antibodies can be used in routine tests with high affinity.

Introduction

Plum pox potyvirus (PPV) was first detected in Spain in 1984 (Llácer *et al.*, 1985a,b). Initially the disease was localized in Sevilla in the south, and in Lérida in the north-east. In the former area, PPV was detected in *Prunus salicina* (Japanese plum) cv. Red Beaut and in apricot cv. Canino. In the latter, PPV was detected in peach cv. Rojo del Rito, very probably through infected rootstocks. Today, plum pox is present in most Spanish regions where stone-fruit cultivation is economically important. The incidence of the disease is serious in regions in which apricot and *P. salicina* coexist. *P. salicina* is a natural host of the disease (Llácer & Cambra, 1986), from which PPV spreads very effectively to apricot (Avinent *et al.*, 1993). This situation has led to the establishment all over Spain of eradication programmes, surveys and large numbers of tests and controls, to reduce the incidence of the disease.

The problems in using diagnostic methods (serological or not) for routine testing of plants for PPV are the irregular distribution and translocation of the virus in the tree and the low titre outside the active growth period (EPPO/CABI, 1992). In addition, the use of polyclonal

[1] Paper presented at the EPPO Conference on Plum Pox, Bordeaux (FR), 1993-10-05/08.

antibodies from antisera (for serological techniques such as ELISA) very often implies problems of specificity and consequently of sensitivity. Nevertheless, ELISA is the most appropriate method for virus detection, when analysis of large numbers of samples is required.

Monoclonal antibodies specific for PPV coat protein (CP-structural protein) have been produced in Spain (Cambra *et al.*, 1986; Vela *et al.*, 1987; López-Moya *et al.*, 1994) to improve PPV detection, to attempt selective diagnosis of PPV isolates differing in virulence, to study the occurrence of mixtures of strains in nature, and to compare ELISA with other techniques. To improve the detection of plum pox, monoclonal antibodies to cytoplasmic inclusions or pinwheel inclusions (CIP non-structural proteins) have been produced. The aim is to detect infections indirectly from the presence of the abundant non-structural proteins which are very typical of potyviruses (Dougherty & Carrington, 1988).

Materials and methods

Virus isolates

Eighty-one PPV isolates representative of different hosts and geographical origins were used. The collection was established under quarantine with 14 Spanish isolates and 67 isolates from Belgium, Bulgaria, Czech Republic, Egypt, France, Germany, Greece, Hungary, Italy, Poland, the former Yugoslavia and the former USSR. Thirty-eight isolates (22 from France and 16 from INRA-Bordeaux Collection) were kindly provided by F. Dosba, and 29 isolates were kindly provided by G. Adam, M. Barba, T. Candresse, J. A. García, M. Kölber, E. Maiss, M. Navratil, L. Palkovics, J.B. Quiot, S. Steyer and C. Varveri.

A substantial part of the collection was graft-inoculated: on GF305 peach seedling (51 isolates), on Japanese plum (1 isolate) and on apricot (1 isolate). The rest (28 isolates) were inoculated on *Nicotiana benthamiana* and/or *N. clevelandii*.

Some of the isolates were transmitted by *Myzus persicae* (Avinent *et al.*, 1994) from GF305 to GF305 and to *N. benthamiana*, and from *N. benthamiana* to the same host. Some isolates were also mechanically transmitted from GF305 to *N. benthamiana*, and cloned by local lesions on *Chenopodium foetidum*.

Antigen purification

PPV was purified from infected *N. benthamiana* plants according to Lain *et al.* (1988) using protease inhibitors (López-Moya *et al.*, 1994). The Spanish PPV isolates 1.20 RR from peach, 5.15 from apricot, 3.3 RB and RB both from Japanese plum were used. All these isolates were of Dideron (D) type (Kerlan & Dunez, 1979). CIP was purified from *N. clevelandii* plants infected with PPV isolate R3 from the former Yugoslavia (D type), following a method based on differential centrifugation.

Production of monoclonal antibodies

Hybrid cells secreting monoclonal antibodies specific to PPV and to CIP were produced by hybridoma technology (Köhler & Milstein, 1975). BALB/c mice were intraperitoneally immunized with a solution of 250 $\mu g\,ml^{-1}$ PPV coat protein or 500 $\mu g\,ml^{-1}$ CIP, using 100 μl per injection following the immunization schedule described by Vela *et al.* (1986). Hybridization, screening for presence of specific antibodies, cell cloning, monoclonal antibody isotype determination and production of ascitic fluid were performed according to Vela *et al.* (1986). The monoclonal antibodies were purified from ascitic fluid by affinity chromatography using protein A (Beckman).

Table 1. Monoclonal antibodie
concentrations used in the assa
Anticorps monoclonaux obtenu
des tests, et références

Antibody designation	Homo anti
3C6	1.20 RR/
XR4	1.20 RR/
4F4	1.20 RR/
9A11	1.20 RR/
4DB12	5.15/PPV
1EB6	5.15/PPV
4DG5	5.15/PPV
4DG11	5.15/PPV
4DB7	5.15/PPV
4CB1	5.15 and
5B	RB/PPV
11E5H	CIP
11F	CIP

* A = Cambra *et al.* (1986); B
preparation).

Double antibody sandwic

DASI ELISA (Cambra *et*
immunoplates with 200 μl
immunoglobulins purified
1976), kindly provided by S
PPV sources were prepar
inoculated GF305 and *N.*
antibodies were added at th
goat anti-mouse immunogl
405 nm at 15-min intervals f
(Flow) automatic reader, ze
negative (above cited) cont
different plates feasible. Fo
for different PPV sources.
PPV isolates was calculated

Indirect ELISA (I)

Nunc Polisorp microplates
sources (Table 2), prepared
PPV coat protein, and 1
concentrations indicated in
for DASI ELISA.

Immunoprinting-ELISA (I

Indirect IP-ELISA was p

Table 1. Monoclonal antibodies obtained for plum pox potyvirus (PPV), homologous antigen, isotypes, concentrations used in the assays, and references
Anticorps monoclonaux obtenus contre le PPV, antigène homologue, isotypes, concentrations utilisées lors des tests, et références

Antibody designation	Homologous antigen	Isotype	Concentration used ($\mu g\ ml^{-1}$)	Reference*
3C6	1.20 RR/PPV	IgG_1, K	0.25	A, B, C
XR4	1.20 RR/PPV	IgG_1, K	0.25	A, B, C
4F4	1.20 RR/PPV	IgG_{2a},K	0.20	A, B, C
9A11	1.20 RR/PPV	IgM	0.50	A, B, C
4DB12	5.15/PPV	IgG_1, K	0.10	C
1EB6	5.15/PPV	IgG_1, K	0.10	C
4DG5	5.15/PPV	IgG_1, K	0.10	C
4DG11	5.15/PPV	IgG_1, K	0.10	C
4DB7	5.15/PPV	IgG_1, K	0.10	C
4CB1	5.15 and 3.3 RB/PPV	IgM, K	0.10	C
5B	RB/PPV	IgG_1, K	0.05	D
11E5H	CIP	IgG_1, K	0.50	D
11F	CIP	IgM	0.50	D

*A = Cambra *et al.* (1986); B = Vela *et al.* (1987). C = López-Moya *et al.* (1994). D = Cambra *et al.* (in preparation).

Double antibody sandwich indirect ELISA (DASI)

DASI ELISA (Cambra *et al.*, 1991) was performed by coating Nunc Maxisorp certified immunoplates with 200 μl per well of a solution of 1 $\mu g\ ml^{-1}$ carbonate buffer of polyclonal immunoglobulins purified (Cambra *et al.*, 1983) from antiserum 206 (Maroquin & Rassel, 1976), kindly provided by S. Steyer (SPE, Gembloux, BE). Extracts 1w:10v from the different PPV sources were prepared in PBS + 0.2% DIECA + 0.2% PVP-10, pH 7.2–7.4. Non-inoculated GF305 and *N. benthamiana* were used as negative controls. Purified monoclonal antibodies were added at the concentration indicated in Table 1. Alkaline phosphatase-linked goat anti-mouse immunoglobulins (Boehringer Mannheim) were used. The plates were read at 405 nm at 15-min intervals for 1 h without stopping reaction, in a Titertek Multiscan Plus MKII (Flow) automatic reader, zeroed on an empty plate. A standard positive (RB/PPV isolate) and negative (above cited) controls were introduced in every plate to make comparisons between different plates feasible. Four wells per plate were used as positive and negative controls, and for different PPV sources. The serological reaction (SR) (Alarcón *et al.*, 1987) of the different PPV isolates was calculated according to Siverio *et al.* (1993).

Indirect ELISA (I)

Nunc Polisorp microplates were coated (100 μl per well) with filtered extracts of different PPV sources (Table 2), prepared in carbonate buffer 1w:10v. Monoclonal antibodies 5B, specific to PPV coat protein, and 11E5H, specific to CIP, were used singly or in mixture at the concentrations indicated in Table 1. The reaction was terminated following the same steps as for DASI ELISA.

Immunoprinting-ELISA (IP)

Indirect IP-ELISA was performed according to Lin *et al.* (1990). Monoclonal antibodies

Table 2. Optical densities (405 nm) obtained by indirect ELISA with crude extracts from healthy and PPV-infected plants, using monoclonal antibodies to PPV coat protein (CP-PPV) and/or to cylindrical inclusions (CIP)
Densités optiques à 405 nm obtenues par ELISA indirecte sur des extraits bruts de plantes saines ou infectées par le PPV, à l'aide d'anticorps monoclonaux contre la protéine capside du PPV (CP-PPV) ou les inclusions cylindriques (CIP)

Plant extract	11E5H (CIP)	5B (CP-PPV)	11E5H + 5B
Non-inoculated *N. benthamiana*	0.163	0.141	0.206
N. benthamiana PPV-3.3 RB	0.465	2.317	2.412
N. benthamiana PPV-Marcus 79	0.235	0.627	0.983
GF305 peach seedling PPV-D	0.125	0.773	0.864
Japanese plum Red Beaut PPV-3.15 RB	0.688	0.797	1.555

specific to PPV or CIP were used as indicated in Table 1, separately or in mixture. Fresh sections of healthy and PPV-infected stems of peach seedling GF305 were gently pressed onto a nitrocellulose 0.45-μm membrane (Millipore). Blotted membranes were blocked with 1% BSA overnight and developed by ELISA-I as above. The localized stained areas of the tissue imprints were observed under a binocular microscope (× 10), after incubation with precipitating substrate (NBT-BCIP) for alkaline phosphatase (Garnsey *et al.*, 1994).

Detection of PPV with mixtures of monoclonal antibodies

Three selected PPV isolates were analysed by DASI ELISA. Monoclonal antibodies specific for PPV (5B, 1EB6 and 4DB12) were used singly and in all different combinations. The concentration of the monoclonal antibodies was 0.1 μg ml^{-1} when used alone and 0.05 μg ml^{-1} when used in mixture. A total of 487 apricot and Japanese plum trees was analysed in May, by DASI ELISA using four young shoots per plant (Cambra *et al.*, 1982). One test was performed with monoclonal antibody 5B and another with a mixture of monoclonal antibodies 1EB6 and 4DB12, using the same extract.

Comparison of ELISA and polymerase chain reaction (PCR)

Nineteen samples of shoots, stems and fruits from apricot, Japanese plum and peach were sampled in May. The samples were symptomless or showing typical PPV symptoms and were collected from PPV-infected trees showing an irregular distribution of symptoms. The samples were simultaneously analysed by DASI ELISA and by PCR with immunocapture (using a mixture of monoclonal antibodies 5B and 4DB12) according to Wetzel *et al.* (1992). The primers were kindly provided by T. Candresse (INRA-Bordeaux, FR).

Results

Eleven hybridoma clones secreting PPV-specific monoclonal antibodies, and two secreting CIP-specific monoclonal antibodies, were obtained. The origin, isotype and ELISA concentration of each monoclonal antibody are indicated in Table 1.

Table 3. PPV serogroups based on DASI ELISA reaction to nine different monoclonal antibodies specific to the coat protein, against 81 PPV isolates
Sérogroupes du PPV en fonction de la réaction en DASI ELISA, de 81 isolats du virus, à 9 anticorps monoclonaux spécifiques de la protéine capside

	Serogroups																				
MCA	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
4CB1	+	+		+																	

SA with crude extracts from healthy and PPV-
otein (CP-PPV) and/or to cylindrical inclusions

e sur des extraits bruts de plantes saines ou
re la protéine capside du PPV (CP-PPV) ou les

5B (CP-PPV)	11E5H + 5B
0.141	0.206
2.317	2.412
0.627	0.983
0.773	0.864
0.797	1.555

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LISA. Monoclonal antibodies specific for
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	Serogroups																				
MCA	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
4CB1	+	+		+																	
4F4	+	+	+	+	+								+								
4DG11	+	+	+	+	+	+	+		+	+	+										
4DG5	+	+	+	+	+	+	+	+	+	+		+									
4BD7	+	+	+	+	+	+	+	+	+			+		+	+						
3C6	+	+	+	+	+	+		+	+	+			+	+		+	+				
1EB6	+	+	+			+	+	+		+	+	+	+	+	+	+		+	+		
4DB12	+		+		+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+
5B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Frequency (%)	2.5	1.25	3.75	1.25	1.25	23.75	11.25	2.5	2.5	3.75	1.25	2.5	1.25	10	7.5	6.25	2.5	6.25	1.25	6.25	1.25

DASI ELISA testing of 81 sources of PPV with 11 monoclonal antibodies to PPV coat protein showed that monoclonal antibody 5B reacted with all PPV isolates with high affinity. Monoclonal antibody 4DB12 was also very reactive but did not recognize five PPV isolates. In contrast, monoclonal antibody 4CB1 only reacted with three isolates. Monoclonal antibodies 4DG5 and 4DG11 seemed to be PPV-D type-specific, because they recognized all D isolates but none of the isolates characterized as belonging to the Marcus (M) type of Kerlan & Dunez (1979).

The serological reaction (SR) was very high for all monoclonal antibodies except 4F4, 3C6, XR4 and 9A11. Twenty-one serogroups were established, on the basis of the different reaction patterns of nine PPV monoclonal antibodies against the 81 isolates tested (Table 3). The most frequent serogroup was number 6 (23.75% of the isolates), representing isolates which reacted with all the monoclonal antibodies except 4CB1 and 4F4. One isolate was only recognized by monoclonal antibody 5B (serogroup 21) and 2.5% of the tested isolates were recognized by all the monoclonal antibodies assayed. The monoclonal antibodies listed in Table 3 represent nine different epitopes on the PPV coat protein.

Changes in epitope composition were observed in some PPV isolates after mechanical transmission, aphid transmission and/or propagation in certain hosts. As an example, the mechanical transmission of PPV-RB isolate from Japanese plum to *N. benthamiana* implied the loss of one epitope. After aphid transmission of some isolates, it was possible to separate other sub-isolates with different epitopic composition and reaction with the monoclonal antibodies.

Mixing the most reactive monoclonal antibodies showed that monoclonal antibody 5B is essential for recognition of all PPV isolates. The most efficient mixture was 5B + 4DB12 + 1EB6. When field samples were tested by DASI ELISA using either 5B monoclonal antibody or 4DB12 + 1EB6 mixture, 109 were positive and 376 were negative by both systems, but 2 apricot samples reacted only with 5B monoclonal antibody.

The analysis of field samples with or without symptoms by DASI ELISA and PCR gave practically the same results. There was a high correlation between the results obtained by the two techniques, except for one symptomless Japanese plum fruit that was ELISA-positive but gave a negative PCR reaction. Symptomless samples from infected apricot trees generally gave negative reactions using both methods, but positive reactions when the symptomless samples were collected from Japanese plum.

Table 2 shows the efficiency of monoclonal antibodies against structural and non-structural proteins in detecting PPV in crude extracts. The use of 5B + 11E5H mixture slightly increased the OD obtained by comparison with single monoclonal antibodies. Immunoprinting confirmed the possibility of detecting CIP or PPV coat protein by ELISA-I. More stained areas were found in GF305 prints when treated with the mixture of monoclonal antibodies against structural and non-structural proteins.

Discussion

The use of monoclonal antibodies improved the specificity of serological techniques for detection and comparison of PPV isolates and provided a tool for characterizing and differentiating PPV isolates.

High variability in coat protein was found among PPV isolates using monoclonal antibodies. At least nine different epitopes are represented by the eleven PPV-specific monoclonal antibodies produced against coat protein. Twenty-one distinct serological patterns were also found using 81 PPV isolates and nine selected monoclonal antibodies. In addition to this, changes in epitope composition were observed after mechanical and aphid transmission and after multiplication in different hosts. All this shows that mixtures of PPV strains occur in field trees.

Monoclonal antibody 5B was able to react with all PPV isolates assayed from our collection or from field samples. This antibody probably represents a well conserved epitope in the coat protein of PPV. Nevertheless, the use of mixtures of monoclonal antibodies representing

selected epitopes, improved
monoclonal antibodies was

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selected epitopes, improved detection of PPV by ELISA. DASI ELISA used with a mixture of monoclonal antibodies was as sensitive as PCR for detection of PPV in spring.

PPV detection can be slightly improved using a mixture of monoclonal antibodies against structural and non-structural proteins. ELISA-I was sensitive enough to detect CIP not only in *N. benthamiana* but also in woody plants infected with PPV. Monoclonal antibody 11E5H recognized the CIP of all PPV isolates assayed.

Immunoprinting-ELISA was able to detect PPV and CIP in tissue sections of woody plants. This technique has some advantages over conventional ELISA because extraction is not necessary and the technique allows localization of CIP or PPV protein very easily and economically.

Most monoclonal antibodies produced against D type also recognized M type. Nevertheless, two monoclonal antibodies (4DG5 and 4DG11) reacted against PPV-D type only, offering the possibility of distinguishing between the two types in field samples analysed by ELISA. To complete the panel of monoclonal antibodies, it would be interesting to produce monoclonal antibodies against the severe PPV type M.

Acknowledgements

Characterization of the monoclonal antibodies produced was possible thanks to international collaboration between participants of the COST 88 project of the European Community. This work was supported by grants from EC contract 8001-CT91-0201, IMPIVA of the Generalidad Valenciana and INIA no. SC94-035.

Détection du plum pox potyvirus à l'aide d'anticorps monoclonaux envers ses protéines structurales et non structurales

Onze anticorps monoclonaux spécifiques de la protéine capside du plum pox potyvirus (PPV) ont été obtenus par la technique des hybridomes à partir d'isolats espagnols du PPV. De plus, deux anticorps monoclonaux spécifiques des inclusions cylindriques du PPV (protéines non structurales) ont été obtenus. Les anticorps spécifiques de la protéine capside du PPV ont été testés sur 81 isolats du PPV, à l'aide de la technique DASI ELISA. A l'aide de neuf anticorps monoclonaux sélectionnés, au moins neuf épitopes différents ont été trouvés, ainsi que 21 types de réaction sérologique différente (sérogroupes), ce qui indique la forte variabilité de la protéine capside chez le PPV. Des modifications de la composition des épitopes ont été observées après transmission mécanique ou par pucerons, indiquant la présence de mélanges d'isolats dans les vergers. L'anticorps monoclonal 5B a réagi en DASI ELISA avec tous les isolats de PPV testés, avec une très forte affinité. Cette méthode a été comparée avec la technique d'immunocapture-PCR sur des échantillons printaniers d'abricotier et les résultats se sont avérés bien corrélés. L'efficacité de la détection du PPV peut être légèrement améliorée en utilisant les anticorps monoclonaux spécifiques des inclusions cylindriques en mélange avec les anticorps monoclonaux spécifiques des protéines structurales, ainsi qu'à l'aide de mélanges d'anticorps monoclonaux envers les différents épitopes de la protéine capside. La technique ELISA conventionnelle et l'immuno-empreintes-ELISA ont permis de détecter les inclusions cylindriques et le virus dans des extraits et des sections, respectivement, de plantes ligneuses. Deux des anticorps monoclonaux permettent de distinguer les types Marcus et Dideron (M et D) du virus. Ces anticorps monoclonaux spécifiques, à forte affinité, de la souche D peuvent s'utiliser dans les analyses de routine.

Детектирование plum pox potyvirus с помощью моноклональных антител к структурным и неструктурным белкам

С помощью метода применения гибридом были получены одиннадцать специфичных антител к испанским штаммам plum pox potyvirus (PPV). Кроме того, были получены два моноклональных антитела, специфичных к цилиндрическим включениям PPV (неструктурные белки). Моноклональные антитела, специфичные к белку капсида PPV, были испытаны методом DASI ELISA против 81 изолятов PPV. Используя девять отобранных антител к набору изолятов PPV, было получено не менее девяти различных антигенных детерминант и 29 отчетливых серологических типов реакции (серогрупп), указывающих на высокую вариабельность белков капсида среди изолятов PPV. Изменения в составе антигенных детерминант наблюдались после механического переноса или передачи тлями, подтверждая существование смешанных изолятов на деревьях плантаций. В опытах с применением метода DASI ELISA моноклональное антитело 5B реагировало со всеми испытанными изолятами, проявляя высокое сродство. Было проведено сравнение этого метода с методом полимеразной цепной реакции синтеза с иммунозахватом на полевых образцах в весенний период и показано хорошее соответствие результатов. Эффективность детектирования PPV может быть несколько увеличена за счет использования моноклинальных антител, специфичных к цилиндрическим включениям, в сочетании с моноклональными антителами к структурным белкам, а также используя совокупности моноклональных антител к различным антигенным детерминантам белков капсида. Методы ELISA-1 и ELISA с иммуно-отпечатками позволяли обнаружить белок цилиндрических включений и PPV, соответственно, в экстрактах и тканевых срезах древесных пород. Два моноклональные антитела дают возможность отличить тип PPV Marcus от PPV Dideron (M или D). Эти D-моноклональные антитела могут быть использованы в рутинных тестах с высоким сродством.

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девяти различных антигенных детерми-
нции (серогрупп), указывающих на высо-
ов PPV. Изменения в составе антигенных
переноса или передачи тлями, подтвер-
евьях плантаций. В опытах с применени-
5B реагировало со всеми испытанными
роведено сравнение этого метода с мето-
ммунозахватом на полевых образцах в
ие результатов. Эффективность детекти-
за счет использования моноклинальных
ениям, в сочетании с моноклональными
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