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Production of strain specific antibodies against a synthetic polypeptide corresponding to the N-terminal region of the plum pox potyvirus coat protein

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Abstract

Comparison of the predicted coat protein amino acid sequence of the 'sweet cherry' strain of plum pox potyvirus (PPV-SwC) with the corresponding regions of several other PPV strains indicated that the main differences are in the N-terminal region. Polyclonal antibodies were produced against a synthetic peptide corresponding to the 1–14 sequence of the N-terminal region of PPV-SwC coat protein. They specifically detected PPV-SwC in different immunochemical tests. © 1997 Elsevier Science B.V.

Keywords: Strain specific polyclonal antiserum; Plum Pox Potyvirus; Synthetic polypeptide; Nucleotide sequence

1. Introduction

Plum pox virus (PPV) is a member of the genus Potyvirus, family Potyviridae (Barnett et al., 1994) and has a monopartite, plus-sense, single stranded RNA genome of approximately 9800 nucleotides, containing a poly(A) tail and a genome-linked protein (VPg) at the 3′ and 5′ ends, respectively. The genome encodes a polyprotein of about 355 kDa processed by virus proteases to give gene products (Riechmann et al., 1992). Virions are flexuous rods of about 750 × 11–13 nm and contain about 2000 copies (Hollings and Brunt, 1981) of a single coat protein (CP) of 36–38 kDa (Pasquini and Barba, 1994). Both CP

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termini are surface located on the virus particles. The N-terminus shows the largest differences in amino acid sequence when CPs of different strains are compared (Shukla et al., 1988a; Van der Vlugt et al., 1993). Serological differentiation of PPV M and D strains is to date made possible by means of strain-specific monoclonal antibodies (Cambra et al., 1994; Boscia et al., 1997). However, no such specific antibodies are available for PPV-El Amar and cherry strains. The specific detection of either serotype is of major importance, because of their different biological, molecular and epidemiological behaviour. The use of antibodies raised against epitopes located at the CP N-terminal region has been found useful for obtaining highly specific diagnostic probes to differentiate virus strains (Shukla and Ward, 1989). Unfortunately, the virus specific immunodominant CP N-terminal region is labile and readily damaged during purification, thus representing a potential problem in raising virus-specific antisera (Shukla et al., 1988a). In this paper the production of strain-specific antibodies to PPV obtained by a synthetic peptide corresponding to the CP N-terminal region is reported.

2. Materials and methods

2.1. Viruses and purification

PPV strains used in this study were PPV-SwC (Crescenzi et al., 1997), an Italian isolate of PPV-D (ISPAVE 31), a Greek isolate of PPV-M, PPV-El Amar (kindly supplied by Dr. Marina Barba, Istituto Sperimentale per la Patologia Vegetale, Roma, Italy). PPV-SwC was purified from *Nicotiana benthamiana* Domin. according to Van Oosten (1972) with some modifications: infected leaves (100 g) were homogenized in 0.1 M sodium citrate buffer pH 8.3 containing 0.02 M NaDIECA and 0.002 M EDTA (1:3 w/v), the homogenate filtered through cheesecloth and then centrifuged at $6000 \times g$ for 10 min. To the supernatant 3% Triton X-100 was added, mixed on ice for 30 min. and centrifuged at $80\,000 \times g$ for 90 min. The pellet was resuspended overnight in 0.01 M sodium citrate buffer pH 8.3 containing 0.5 M urea and 0.1% 2-mercaptoethanol. The suspension was layered onto a 20% sucrose cushion in 0.01 M sodium citrate buffer pH 8.3 and centrifuged at $50\,000 \times g$ for 2 h. The final pellet was resuspended in 0.01 M sodium citrate buffer.

2.2. Cloning and sequencing

Immunocapture (IC) of PPV particles was performed from extracts from naturally infected sweet cherry leaves as described by Minafra and Hadidi (1994), using a commercial antiserum to PPV (Loewe, Munchen, Germany) for virus particle trapping. Since virus concentration in cherry plants is generally low, up to ten IC reaction mixtures were pooled. Trapped virions were digested with proteinase K (50 μg/ml) in 0.01 M Tris–Cl pH 7.8, 0.005 M EDTA, 0.5% SDS. Genomic RNA was phenol-chloroform extracted, alcohol precipitated and resuspended in water. Reverse transcription-polymerase chain reaction (RT-PCR) was carried out as described previously (Crescenzi et al., 1995). Before amplification, cDNA was purified by phenol-chloroform extraction and alcohol precipitated in presence of 2 M ammonium acetate, to increase amplification specificity. Primers for the CP gene and part of the replicase (NIb) gene were 5′-GTTTATGATA-GATACCGAGACCACTACACTC-3′ and 5′-CCAATCCATCGATTGGAGGCAATTTGTGC-3′, as determined on the sequence of PPV-NAT (Maiss et al., 1989). Amplified products were recovered from the gel following the ‘crush and soak method’ (Sambrook et al., 1989) and directly cloned into the pCR™ vector using the TA™ Cloning kit (Invitrogen, San Diego, CA), according to the manufacturer’s instructions. Nucleotide sequence was determined by the dideoxy-sequencing method (Sanger et al., 1977). Alignment of PPV-SwC CP with the corresponding amino acid sequences from PPV strains D (Teycheney et al., 1989), SK68 (belonging to the M strain group) (Palkovics et al., 1993) and El Amar (Wetzel et al., 1991) were carried out using the DNASIS program (Hitachi).

2.3. Peptide synthesis and purification

The peptide H_2N-AKEGNDDDVTLVDA-COOH, corresponding to the 1–14 sequence residues of the N-terminal region of PPV-SwC CP, with an extra cysteine-NH_2 residue on the carboxyl terminus added to allow coupling to the carrier, was synthesized using an Applied Biosystem Peptide Synthesizer mod. 431A (Applied Biosystem, Forest City, CA). Fmoc solid phase chemistry (Atherton and Sheppard, 1989) was used with 1-hydroxybenzotriazole/dicyclohexylcarbodiimide activation. Peptide cleavage from the 4-(2′,4′-dimethoxyphenyl-Fmoc-amino-methyl)-phenoxy resin was carried out with a mixture of trifluoroacetic acid/H_2O/1,2-ethanedithiol/thioanisole/phenol. Peptide purification was performed by reverse phase high performance liquid chromatography (HPLC) and the peptide characterized by automatic sequencing (Applied Biosystem 477A Sequencer).

2.4. Preparation of polyclonal antiserum

The peptide was coupled with bovine serum albumin (BSA) using *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (Scheidtmann, 1989), a heterobifunctional reagent which, under mild conditions, reacts with its *N*-hydroxy-succinimide (NHS) group with the primary amino-groups of proteins.

The peptide was added to the 2-pyridil-disulfide activated BSA. The -SH group of the peptide exchanges with the 2-pyridil sulfide group, forming a disulfide bond between the protein and peptide releasing pyridine 2-thione. Briefly, a 30X molar excess of SPDP in absolute ethanol was added dropwise to BSA (10 mg/ml in 0.1 M phosphate pH 7.5 containg 0.1 M NaCl). The mixture was left at room temperature for 30 min. and dialyzed in 0.01 M phosphate buffer.

Effectiveness of SPDP coupling to BSA was assessed by determining the amount of pyridine-2-thione displaced by 30X molar eccess dithiothreitol (DTT) from an aliquot of the actived BSA 15 mg/ml of the peptide in PBS (Sambrook et al., 1989) was added to 2.56 ml of activated BSA (30X molar excess of peptide to activated groups), mixed and left at room temperature for 2 h. Amount of peptide coupled has been calculated from the OD at 343 nm of the pyridine-2-thione released. The incorporation of peptide ranged usually from 80 to 90%. The peptide-BSA conjugate was dialyzed and lyophilized in aliquots suitable for immunization. Male New Zealand white rabbits were injected subcutaneously with 150 μg antigen in 0.5 ml PBS emulsified with an equal volume of Freund's complete adjuvant. Each rabbit was boosted at four weeks intervals with the same antigen dose in presence of incomplete Freund's adjuvant. Sera were collected from the marginal ear vein two weeks after each boost. The immunization was carried out at IGtech (Salerno, Italy). The titre of the antiserum was determined by indirect ELISA. The IgG fraction was obtained from antisera by DEAE chromatography using Econo-Pac Serum IgG purification kit (BioRad). Biotinylation of purified IgGs was carried out using biotinamidocaproate *N*-hydroxysuccinimide ester (Bayer and Wilchek, 1980).

2.5. Western immunoblot

For Western blot analysis either crude extracts from infected plants or purified virus preparations were used. Leaf discs (15 mg) of *N. benthamiana* infected with PPV-SwC, PPV El Amar, PPV-D and PPV-M were homogenized in 50 mM Tris–Cl pH 6.8, 100 mM DTT, 30% glycerol, 2% sodium dodecyl sulfate, 0.1% bromophenol blue (1:20 w/v). Extracts (20 μl) or purified virions (1 μg) were electrophoresed under denaturing conditions and proteins electroblotted onto a nitrocellulose membrane as described (Crescenzi et al., 1997). The membrane was saturated with gelatin, incubated with antibodies to the synthetic peptide (ASP) diluted 1:1000, then with goat anti-rabbit alkaline phosphatase-conjugated antibodies (Loewe) diluted 1:2000. For antipeptide titration, different dilutions of the antiserum were used. Positive reactions were visualized using the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium Sigma Fast™ kit (Sigma).

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PPV-SwC     AKEGNDDDVT LVDAGKSTVT TAVSTPAVTS SQFPPPPLPN LQSTAPMFDP  50
PPV-ElAmar  *D*KE**EEE -****RPL** *TQQPIVT*T T*QT*ITSTT **A*QA**N*  50
PPV-SK68    *D*EE**EE- -****RP**V **PAATVA*T QPA*VIQPAP QT-*****N*  50
PPV-D       *D*RE*EEE- -*****PI*V **PAATSPIL QPP*VIQPAP RT-****LN*  50

PPV-SwC     IFTPATTQPN VRPIAPVVTS PFSYGVIGNQ SVTPSS-SNA LVTRGKDRDV  100
PPV-ElAmar  *******E*T T*TVPHTT*T TPPSFGVIGN ED*APNA*** V*RT*R****  100
PPV-SK68    *********A ***VP*ISGA KPPSFGVYGN EDASP*T**T **NT*R****  100
PPV-D       *********A TK*VSQ*PGP QLQTFGTYGN EDASP*N*** **NTNR****  100

PPV-SwC     DAGTIGTFSV PRLKSMTSKL SLPKVKGKAI MNLSHLAHYN PAQNKLSNTR  150
PPV-ElAmar  ***S****T* ****A***** ********** ***N***F*S ***VD*****  150
PPV-SK68    ***S****A* ****T***** ********** ***N*****S ***VD*****  150
PPV-D       ***S****T* ****A***** ********** ***N*****S ***VD*****  150

PPV-SwC     APQSCFQTWY EGVKRDYDVS DDDMSIILNG LMVRCIESGT SPNINGMWVM  200
PPV-ElAmar  ********** ***R*****T **E******* ***W***N** **********  200
PPV-SK68    ********** *********T *EE******* ***W***N** **********  200
PPV-D       ********** *********T **E******* ***W***N** **********  200

PPV-SwC     MDGETQVEYP IKPLLDHAKP TFRQIMAHFS NVAEAYIEKR NYEKAYMPRY  250
PPV-ElAmar  ********** ********** ********** ********** **********  250
PPV-SK68    ********** ********** ********** ********** **********  250
PPV-D       ********H* ********** ***R*V*R** D****CV*** **********  250

PPV-SwC     GIQRNLTDYS LARYAFDFYE MTSTTPVRAR EAHIQMKAAA SGNVQNRLFG  300
PPV-ElAmar  ********** ********** ********** ********** LR*A******  300
PPV-SK68    ********** ********** ********** ********** LR********  300
PPV-D       ********** ********** ********** ********** LR********  300

PPV-SwC     LDGNVGTQEE DTERHTAGDV NRNMHNLLGV RGV....... .....       350
PPV-ElAmar  ********** ********** ********** ***....... .....       350
PPV-SK68    ********** ********** ********** ***....... .....       350
PPV-D       ********KQ ******D*** *****TF*** ***....... .....       350
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Fig. 1. Multiple alignment of coat protein amino acid sequences of four plum pox potyvirus (PPV) strains, including PPV sweet cherry (PPV-SwC). Residues that differ from PPV-SwC are indicated.

2.6. ELISA test

For the anti-peptide titration, antigen adsorption indirect ELISA (AAI-ELISA) was used: plates were activated with 5% glutaraldehyde overnight at 4°C, coated with the peptide suspension (0.5 μg/well), saturated with 1% skim milk, exposed to the antipeptide and incubated with goat anti-rabbit peroxidase-conjugated antibodies diluted 1:2000. The reaction was visualized using orthophenylenediamine (OPD) as substrate. For specificity assays, direct double antibody sandwich ELISA (DAS-ELISA) was performed: leaves of *N. benthamiana* infected with PPV-SwC, PPV-El Amar, PPV-D and PPV-M were homogenized in PBS pH 7.4, 2% PVP, 0.05% Tween 20 (1:5 w/v). Plates were coated with commercial anti-PPV 1:500, exposed to plant extracts (100 μl/well), saturated with 1% skim milk and incubated with biotinylated antipeptide IgGs. Reaction was visualized using peroxidase-conjugated streptavidin and OPD as substrate.

2.7. Electron microscopy

Immune electron microscopy (IEM) was carried out according to Milne and Luisoni (1977) with 5 min. absorption of virus particles and 15 min. exposure to ASP diluted 1:256. Immunogold test was carried out as reported by Louro and Lesemann (1984) with some modifications. Briefly, 5 min. absorption of virus particles, 15 min. blocking by incubation with 1% skim milk, 15 min. exposure to antipeptide diluted 1:256, 1 h incubation with goat anti-rabbit gold complexes (10 nm) diluted 1:100. Different antiserum dilutions were also used in both tests for antipeptide titration. Virus decoration was also compared with that obtained using a commercial polyclonal PPV antiserum (Loewe) in both assays. To remove surface

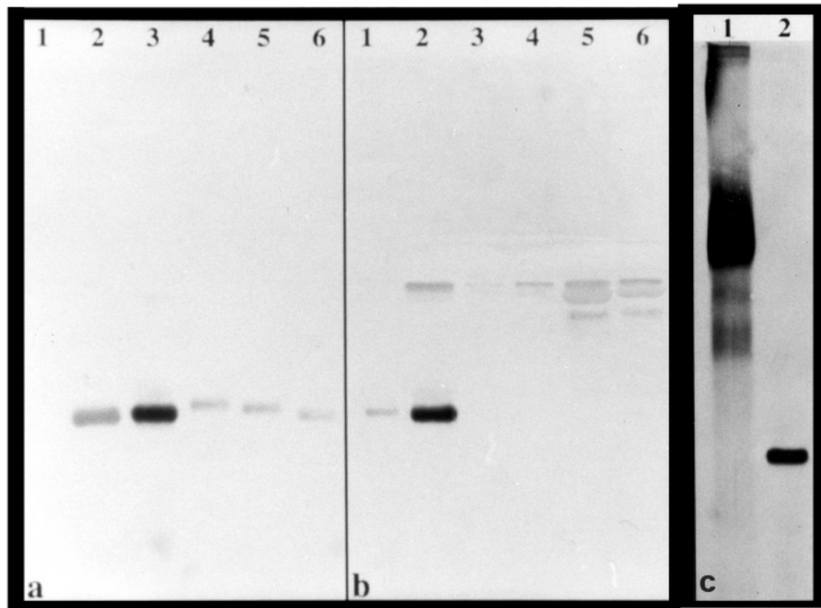

Fig. 2. Western blot analysis of plum pox potyvirus isolates, with: a) polyclonal anti-PPV: 1 = healthy control, 2 = PPV-SwC purified particles, 3 = PPV-SwC, 4 = PPV-M, 5 = PPV-ElAmar and 6 = PPV-D (ISPAVE 31) crude plant extracts from *Nicotiana benthamiana*; b) Polyclonal anti-peptide: 1 = PPV-SwC purified particles, 2 = PPV-SwC, 3 = PPV-M, 4 = PPV-ElAmar, 5 = PPV-D (ISPAVE 31) and 6 = healthy control, crude plant extracts from *N. benthamiana*; c) Polyclonal anti-peptide: 1 = BSA-peptide complex, 2 = PPV-SwC purified particles.

exposed termini from virus particles, freshly purified virions were incubated with bovine type III trypsin (Sigma Chemicals, St. Louis, MO) in the ratio of 1:2 of virus protein, in the same purification buffer for 30 min. at 37°C. After treatment, samples were immediately processed for electron microscopy assays. Untreated purified virions were used as control.

3. Results

3.1. Sequence analysis

The nucleotide sequence of the PPV-SwC 3′ terminal region (EMBL accession No. Y09851) was obtained by amplification from naturally infected cherry tissues to avoid population selection that may occur after mechanical transmission onto herbaceous hosts. The predicted PPV-SwC CP gene is 996 nt in size, starting from GCC (alanine), as obtained by comparison with other PPV sequences, and ending with a TAG stop codon from which the 3′ NCR starts, located 220 nucleotides upstream from the poly(A) tail. The CP gene codes for a 332 amino acid protein of 36.5 kDa. Comparison of PPV-SwC 3′ terminal region with the corresponding sequences of several PPV strains showed similarity ranging from 83.8 to 86.9% (average 86.0%) for the predicted CP amino acid sequence, the N-terminal region being the most divergent (Fig. 1).

3.2. Serological tests

In Western immunoblot the anti-peptide clearly reacted against the BSA-peptide complex, PPV-SwC CP from crude plant extracts and purified PPV-SwC preparations (Fig. 2a, b and c), while no reaction was observed with the other PPV strains. The same result was obtained in DAS-ELISA (Fig. 3). ASP reacted up to 1:10 000 dilutions in AAI-ELISA and 1:256 000 dilution by Western immunoblot. An additional band of higher

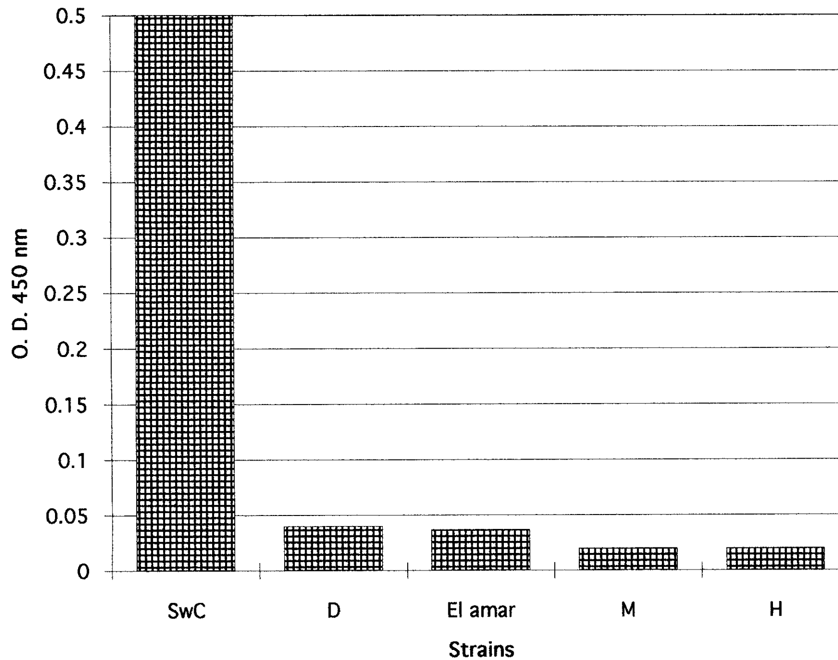

Fig. 3. DAS-ELISA on four isolates of PPV from *Nicotiana benthamiana* with polyclonal anti-peptide; SwC, PPV-SwC; D, PPV-D (ISPAVE 31); El amar, PPV-ElAmar; M, PPV-M and H, healthy controls.

molecular weight was constantly observed in Western immunoblot assays of all crude sap extracts, including the healthy controls. This band was not present in purified virus preparations and disappeared after 1:16 000 dilution of the samples in titration experiments (data not shown). This may be due to the fact that regions present in the synthetic peptide could mimic epitopes of host proteins (Groome, 1994).

3.3. Electron microscopy

In IEM on crude extracts, ASP evenly decorated PPV-SwC virions but not PPV-D, PPV-M and PPV-El Amar (Fig. 4a–f). Decoration along the entire particle length was confirmed in immunogold labelling performed with or without particle staining (Fig. 5a–f). In both tests decoration was less intensive with ASP than polyclonal Anti-PPV. ASP reacted with virus particles up to 1:1024 dilutions in IEM and 1:8192 dilution in immunogold. As expected, trypsinized particles were not decorated (Fig. 4g and Fig. 5g), thus confirming that the peptide corresponds to exposed CP termini, which were removed by the enzymatic treatment.

4. Discussion

The use of antibodies directed towards the CP N-terminal region has proved useful for distinguishing different potyvirus species (Shukla et al., 1988a,b). However, obtaining virus specific polyclonal antisera against potyviruses is not easy since CP termini are degraded during purification and storage, and cross-reactive antibodies may appear in later stages of immunizations (Shukla and Ward, 1989). Our data suggest that this problem may be overcome by the use of a synthetic polypeptide corresponding to the virus specific N-terminal region of the coat protein as an antigen. Van Regenmortel (1989) indicated that about 15 amino acids at the protein surface are involved in the formation of antigenic sites. In the present study the first 14 aminoacids on the N-terminal

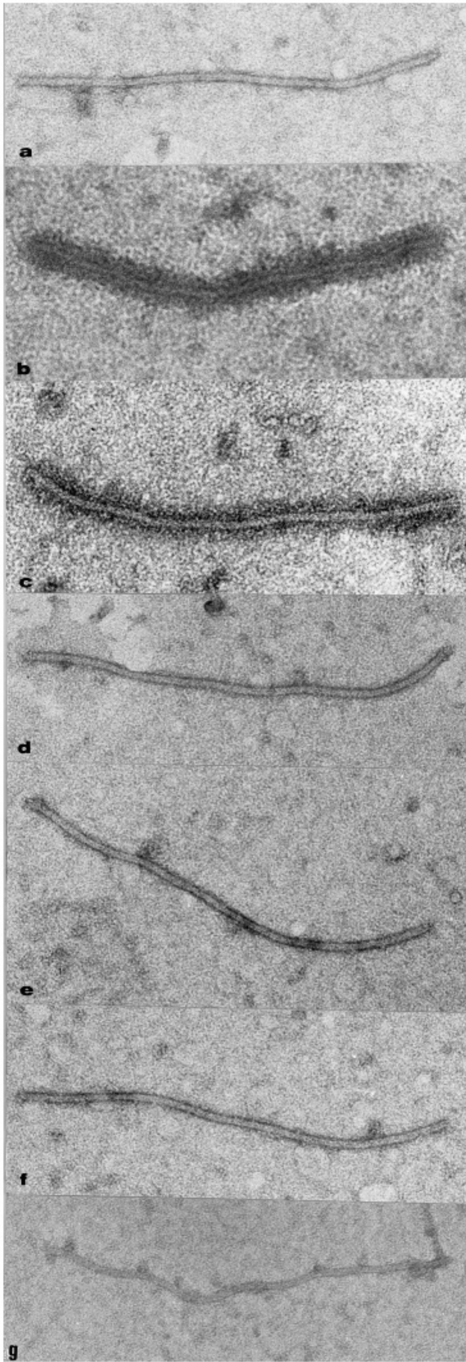

Fig. 4. Particles of PPV in immune electron microscopy: a = PPV-SwC, without decoration, b = PPV-SwC exposed to polyclonal anti-PPV, c = PPV-SwC, d = PPV-M, e = PPV-ElAmar, f = PPV-D (ISPAVE 31) and g = PPV-SwC after treatment with trypsin—exposed to polyclonal anti-peptide (112 000 X).

region of PPV-SwC coat protein were chosen as specific antigenic site.

The importance of correct orientation of a peptide attached to a protein carrier is well known (Tam, 1994). In fact, the best results are obtained when the orientation of the peptide mimics the parent protein (Tam, 1994). Therefore, the peptide was linked via its C-terminal aminoacid to the carrier in order to give more flexibility to the distal N-terminus.

The results clearly indicate that antibodies raised against the synthetic peptide are able to specifically recognize PPV-Swc from infected tissues but not other PPV strains by DAS-ELISA, Western immunoblot and IEM, without differences between the native and denaturated form of the viral protein. In addition, a very satisfactory titre of antibodies was obtained. Production of strain-common antibodies against a synthetic peptide corresponding to the C-terminal but not the N-terminal region of PVY coat protein was reported by Oshima et al. (1992). The final results of this work suggest that the production of specific polyclonal antisera against peptides, corresponding to the N-terminal region of PPV-CP correctely oriented in the peptide carrier conjugates may be a useful and inexpensive tool for obtaining virus- and strain-specific antibodies against virus particles. In addition, the use of synthetic peptides for producing highly specific antisera may help in the detection of poorly immunogenic viruses or viruses which occur in low quantity in plant tissues, as well as viral non-structural proteins in host plants, for which polyclonal or monoclonal antisera are difficult to obtain.

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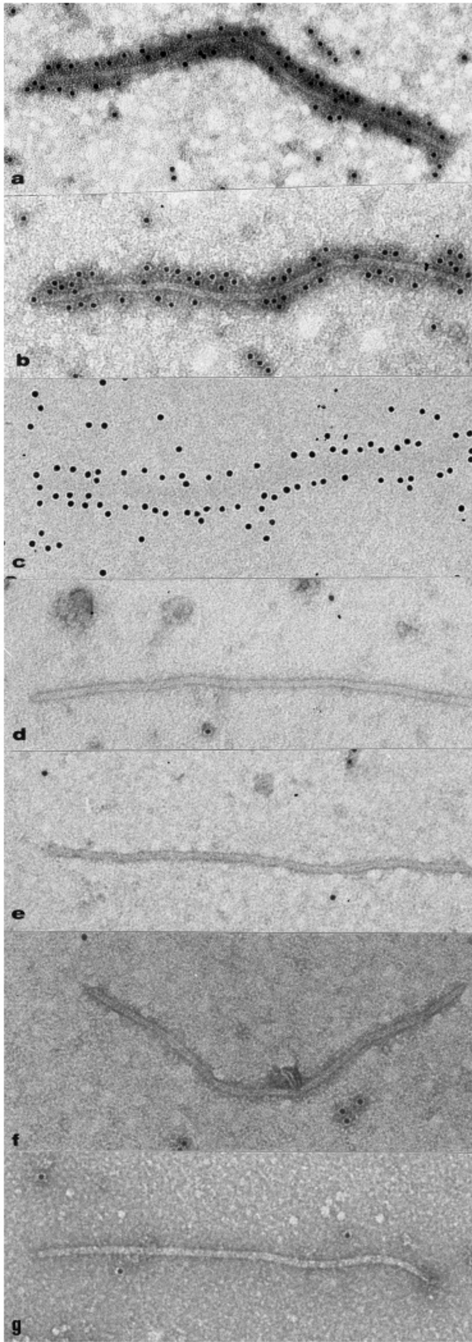

Fig. 5. Complexes of particles of PPV gold-labelled with goat anti-rabbit IgG gold in immune electron microscopy: a = PPV-SwC coated with polyclonal anti-PPV, b = PPV-SwC, c = PPV-SwC unstained, d = PPV-M, e = PPV-ElAmar f = PPV-D (ISPAVE 31) and g = PPV-SwC after treatment with trypsin—coated with polyclonal anti-peptide—(112 000 X).

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