ANALYSIS OF PLUM POX VIRUS VARIABILITY AND DEVELOPMENT OF STRAIN-SPECIFIC PCR ASSAYS

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Abstract

A PCR-based detection technique named immunocapture-PCR (IC-PCR) which allows simple and very sensitive detection of plum pox virus (PPV) the causal agent of sharka disease of stone fruit trees has been developed. In its current version, this assay can detect about 2000 virus particles (10 femtograms of virus) diluted in 100 ul of crude plant sap. This is equivalent to a sensitivity about 2000 times better than that of a standard ELISA assay, the technique most commonly used to detect PPV.

IC-PCR was used, in conjunction with RFLP analysis or sequencing of the amplified material to study the molecular variability of PPV from unpassaged field samples, without the need to transfer the virus to herbaceous hosts or to purify it. In parallel, other techniques have also been used in order to characterise PPV isolates. The results obtained allow the discrimination of three groups of PPV isolates showing limited intra-group variability. Two of these groups correspond to the previously described D and M PPV serotypes, for which recent data indicate quite different epidemiological properties. Typing of PPV isolates belonging to the D or M serotypes can now easily be performed either using an RsaI polymorphism in the amplified cDNA fragment or, alternatively by employing serotype-specific primers developed from the sequence information obtained during this study. These serotype-specific primers should prove extremely useful for the rapid characterisation of the isolates responsible for new PPV outbreaks, a procedure recently rendered compulsory in France in an attempt to improve control of the recently introduced M serotype.

1. Introduction

Plum pox virus is the causal agent of sharka disease of stone fruit trees, the single most important viral disease of these crops in Europe and the Mediterranean (Dunez & Sutic, 1988; Smith et al., 1994). The only effective way to control the disease is through sanitary selection programs coupled, in countries where sharka is still under control, with drastic eradication schemes. The ability to carry out these control measures is, in large part, determined by the availability of reliable detection assays. The diagnosis of plum pox virus infection in fruit trees is complicated by the high variability in virus concentration and by the uneven distribution of the virus in infected plants.

In an effort to obtain more sensitive detection assays, we developed a PCR-based detection technique which is both simple and of high sensitivity. In this technique, named

immunocapture-PCR (IC-PCR), the virus is first trapped from a crude plant homogenate, using anti-PPV specific immunoglobulins, in a fashion reminiscent of the well-known ELISA assay. Using a single set of reagents, the virus is then disrupted, its RNA reverse transcribed and, finally, a short, 243 bp cDNA fragment, is amplified using specific oligonucleotide primers in a PCR reaction (Wetzel et al., 1992). In its current version, this assay can detect about 2000 virus particles (10 femtograms of virus) diluted in 100 ul of crude plant sap. This is equivalent to a sensitivity about 2000 times better than that of a standard ELISA assay, the technique most commonly used to detect PPV. In addition, the IC-PCR assay can be performed in a single day, with approximately the same hands-on time as the current ELISA assays.

In addition to its usefulness as a detection technique, IC-PCR can also be used to rapidly obtain information on the viral genome from unpassaged field samples, without the need to transfer the virus to herbaceous hosts and to purify it. We have used IC-PCR, in conjunction with RFLP analysis or sequencing of the amplified material to study the molecular variability of PPV. In this way, three groups of isolates have been discriminated. Comparison with other techniques potentially useful for the discrimination of PPV serotypes indicate a general agreement between the various techniques, with only a few isolates behaving differently between the different assays. Two of the groups thus delimited correspond to the previously described D and M serotypes, for which recent data seem to indicate quite different epidemiological properties (Adamolle 1993; Adamolle et al., 1994).

Strains belonging to the D or M serotypes can easily be discriminated by an RsaI polymorphism in the amplified cDNA fragment or by direct IC-PCR typing, using serotype-specific synthetic oligonucleotides, allowing specific amplification of PPV strains belonging either to the D or M serotypes. These serotype-specific primers have proven extremely useful for the rapid typing of newly isolated PPV strains, a procedure now compulsory for any new outbreak of sharka in France.

2. Materials and Methods

2.1. Virus isolates

The PPV isolates used in this study were part of the collections of PPV isolates kept at INRA Montpellier, at INRA Bordeaux or, in some instances at IVIA Valencia (Spain). Most isolates were propagated in GF305 peach seedlings or in Pisum sativum var. Colmo.

2.2. Nucleotide sequences

Nucleotide sequences from the following PPV isolates were used : PPV-D (Teycheney et al., 1989), PPV-NAT (Maiss et al., 1989), PPV-Rankovic (Lain et al., 1989), PPV-El Amar (Wetzel et al., 1991b), PPV-o6 and PPV-PS (Cervera et al., 1993), PPV-SK68 (Palkovics et al., 1993). In addition, partial unpublished nucleotide sequences were kindly provided for PPV-AT and PPV-Bulgarie by E. Maiss (BBA, Braunschweig, Germany).

2.3. Primers

The P1 and P2 PCR primers initially described by Wetzel et al., (1991a) were used throughout this study. It is important to note however that their sequence was inadvertently erroneously reproduced in that publication. Their correct sequence therefore is : P1 : 5' ACCGAGACCACTACACTCCC 3' and P2 : 5' CAGACTACAGCCTCGCCAGA 3'. In some assays, the serotype-specific primers PD and PM (Candresse et al., submitted for publication)

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2.4. Sample preparation

The plant samples (leaves, bark...) were ground (1:10, w:v) in a PBS-Tween buffer routinely used for ELISA assays, supplemented with 2% polyvinylpyrrolidone K25 (PVP K25), 20 mM sodium diethyldithiocarbamate. After grinding, the extracts were rapidly clarified by centrifugation (10 min, 13000 rpm, 4^0C).

2.5. IC-PCR assay

The IC-PCR amplification protocol used was derived (with modifications) from the procedure of Wetzel et al., (1992). Briefly, 100 ul of plant extract were submitted to immunocapture in PCR tubes precoated with anti-PPV immunoglobulins. Following one wash with PBS-Tween buffer, the tubes were dried and further processed for amplification. Fifty microliters of RT-PCR mix [Tris-HCl pH 8.8 10 mM; $MgCl_2$ 1.5 mM; KCl 50 mM; Triton X100 0.3%; 5% formamide; 250 uM of each dNTPs; 1 uM of each of the two primers; 0.5 U of AMV Reverse transcriptase (RTase) and 1 U Taq polymerase] were added to each tube. After vortexing and quickly spinning down the droplets, the mix was overlaid with 50 ul of mineral oil. The cycling scheme was the following: 15 min at 42°C (RT reaction), 5 min at 92°C to denature the templates and the RTase followed by 40 cycles of amplification : 20s at 92°C, 20s at 62°C and 40s at 72°C. Ten microliter aliquots were analysed by 1.5% agarose gel electrophoresis in 1XTBE buffer.

For cloning purposes, a two-step RT-PCR assay derived from the procedure of Wetzel et al., (1991a) was used. Briefly, after an immunocapture step, a 10 ul RT reaction was performed (but omitting the decapsidation/denaturation steps) followed by amplification using the Pfu polymerase (Stratagene) in a final volume of 50 ul, in order to minimise potential sequence artifacts. The amplification buffer used was the one provided by the supplier while the cycling conditions were similar to those used with the Taq polymerase.

2.6. RFLP analysis of PCR-amplified cDNAs

For RFLP analysis, the PCR-amplified materials were first purified by phenolchloroform and chloroform extractions followed by ethanol precipitation. An aliquot equivalent to 10 ul of the PCR reactions were then digested for 2 hours by 5 units of RsaI restriction enzyme in the buffer described by the supplier. The digestion products were then analysed either by agarose gel electrophoresis as above or by 8% polyacrylamide gel electrophoresis in 1XTBE buffer.

2.7. Cloning of PCR fragments and sequence analysis

Following amplification with the Pfu polymerase, the PCR fragments were purified by agarose gel electrophoresis in 1XTAE buffer followed by extraction using the Geneclean (BIO 101) kit. The purified fragments were then directly cloned in Sma I cut, nondephosphorylated pUC9 vector (Gora et al., 1994). The ligation mixture was then used for electroporation of E. coli cells (strain JM83). Recombinant plasmids were finally selected and sequenced on both strands using the Sequenase (US Biochemical) kit.

2.8. Sequence comparison and phylogenetic analysis

Sequence acquisition was performed using a digitizing table and the Microgenie

(Beckman) package. Multiple sequence alignments and phylogenetic trees were prepared using the Clustal V (Higgins and Sharp, 1989) suite of programs.

3. Results

3.1. Detection of PPV using immunocapture-PCR

Comparison of the original, more complex version of IC-PCR (Wetzel et al., 1991a) with the simplified version presented here indicated that their sensitivty is in the same range (results not shown). In a series of detailed studies, the sensitivities of several techniques for the detection of PPV in GF305 peach seedlings (Varveri et al., 1987; Varveri et al., 1988; Wetzel et al., 1990; Wetzel et al., 1991a; Wetzel et al., 1992) were compared. The data from these experiments is summarised in Table 1. The sensitivity of an ELISA assay involving a polyclonal antiserum was in the range of 1 ng of virus per assay, which translates in a limit of detection of 4 ng of virus per milliliter of plant extract. Using identical extracts, molecular hybridisation had a sensitivity approximately 10 times greater (limit of detection around 0.5 ng/ml) and direct RT-PCR (Wetzel et al., 1991a) 20 times greater (0.2 ng/ml).

The immunocapture step concentrates and pre-purifies the virus particles, thus a much larger sample volume can be used than for direct RT-PCR. This results in a dramatically improved sensitivity. The current detection levels in crude plant sap are of 200 fg of purified virus (approximately 2000 viral particles), which translates in a detection limit of 2 pg/ml. Thus, immunocapture-PCR is 2000-fold more sensitive than a polyclonal antibodies-based ELISA assay for the detection of plum pox virus.

In addition to increasing dramatically the sensitivity of the PCR reaction, immunocapture is a simple, rapid and inexpensive way to process plant samples prior to PCR amplification. The whole procedure from grinding of the plant samples to the final gel electrophoresis step can now be routinely performed within a single working day with minimal hands on time, features which compare advantageously to those of the ELISA assay.

3.2. Phylogenetic analysis of PPV isolates using various regions of the genome

As a preliminary to the use of PCR-derived sequence information for the analysis of PPV molecular variability, it is necessary to make sure that the phylogenetic grouping of PPV isolates obtained is independent of the region of the genome used. Such is the case if all parts of the genome coevolve as a single unit, even if the divergence rate is not the same for all parts of the genome. Since a number of PPV isolates have already been totally or partially sequenced, it is possible to test this assumption in the case of PPV. Several independent phylogenetic analyses using various parts of the genome (part of the polymerase gene, coat protein gene, 3' non coding region) were therefore performed. In each case, although branch lengths were different (reflecting differences in evolutionary rates), phylogenetic trees of identical topologies were obtained. In addition, an identical grouping of the various isolates used (with one exception) was also observed (Figure 1). The only PPV isolate which does not show a phylogenetic behaviour independent of the region used is PPV-o6, which has been shown to be a recombinant isolate (Cervera et al., 1993).

An identical grouping of isolates was again observed using only the information corresponding to the 203 nucleotides of the PCR fragment amplified using our detection primers (not shown). These results demonstrate that, on a general basis, the various parts of the PPV genome have coevolved, with the apparently rare exception of a possible recombinational event. In addition, these results show that the sequence of the small PCR

fragment, which maps at the 3' end of the coat protein gene (Wetzel et al., 1991a) can be used as a reliable marker of the phylogenetic affinities of PPV isolates.

3.3. Phylogenetic analysis of PPV isolates using the sequence of PCR-amplified material

For this part of the study, PPV isolates were amplified using Pfu polymerase, a thermostable enzyme known to have an error rate 12 times lower than that of Taq polymerase, due to its 3' to 5' exonuclease activity (Lundberg et al., 1991). Following their amplification, the PCR fragments were gel purified, cloned in plasmid pUC9 and their sequence finally determined. In this way, the nucleotide sequence of the fragment from nine isolates was determined. For three isolates, corresponding to recent PPV outbreaks in southern France, two independent clones were sequenced, revealing sequence heterogeneities in each of these isolates. In addition to the sequences from the nine PPV isolates already cloned and sequenced, a total of twelve sequences from nine PPV isolates are thus available. Figures 2 and 3 present the phylogenetic grouping of PPV isolates obtained using this information and the multiple alignment of the 21 nucleotide sequences used in this study.

As can be seen in Figure 2, the inclusion of the data corresponding to the newly sequenced PPV isolates gives a tree with a topology similar to those shown in Figure 1. Three groups of PPV isolates are clearly delineated by this analysis. One group, typified by PPV-D (isolate Dideron from southern France) contains isolates AT and NAT (Germany), Red Beaut (Spain), Rankovic (Yugoslavia), Pillnitz (ex RDA) and Autriche (Austria). Thus, it mostly contains isolates from western Europe. The second group, typified by PPV-M (isolate Markus from Greece) contains in addition the following isolates : Bulgarie (Bulgaria), o6, PS and Belacrvna (Yugoslavia), SK68 (Hungary), Turquie (Turkey), and Valence, Benedicte and Salon (recent outbreaks in southern France). This second group thus contains mostly isolates from southern and eastern Europe. The third group contains only the Egyptian isolate PPV El Amar, confirming its divergent status previously noted by Wetzel et al. (1991b).

As can be seen on the multiple alignments presented in Figure 3, variation between the various PPV isolates was observed at a number of positions in the PCR fragment (28 out of 203, almost 14 % of the positions) and about two-thirds of these mutations are silent (20 out of 28). The two major groups of isolates (D and M) are clearly separated by a total of eight fully conserved mutations, all of which are silent. As one of these mutations overlaps a restriction enzyme site (Rsa I), it is possible to discriminate between the groups by performing a simple Rsa1 digestion of the amplified fragment. The existence of this restriction polymorphism had already been noted by Wetzel et al., (1991a).

3.4. Comparison with other PPV typing techniques

Recent evidence indicates that PPV isolates can be discriminated by the mobility of their coat protein (CP) in denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Two major groups of isolates can thus be discriminated, with estimated CP molecular weights of respectively 36 and 38 kDa (Adamolle, 1993). These two groups of isolates correspond to the two serotypes of PPV initially described by Kerlan and Dunez (1979) as evidenced by differences in the antigenic properties of the amino- and carboxy-terminal parts of their CP (Bousalem et al., 1994a; 1994b). Comparison of the behaviour of 28 PPV isolates using these three typing techniques (RFLP analysis of PCR fragments, CP mobility and CP N-ter serology) showed a perfect correlation between the various techniques (Bousalem et al., 1994a; 1994b). These results indicate that RFLP analysis of PCR fragments can indeed be used to discriminate PPV isolates and allocate them to the D or M serotypes of Kerlan and Dunez (1979).

Comparison of the behaviour of about 40 PPV isolates towards a panel of 26 monoclonal antibodies (COST 88 Plum pox virus workshop, Valencia, Spain) and of their RFLP typing was also performed. Using the reactivities towards the 26 monoclonals, two groups of PPV isolates were discriminated (Mariano Cambra, personal communication). These two groups showed perfect correlation with the two groups defined by our RFLP analysis, again lending support to the validity of the RFLP approach for the serotyping of PPV isolates. However, no perfect correlation was observed when comparing RFLP behaviour of PPV isolates with their reactivities towards individual monoclonal antibodies (T. Candresse and M. Cambra, manuscript in preparation).

3.5. Development of serotype-specific PCR primers

Using the sequence information presented in Figure 3, we have been able to design PCR primers allowing the specific amplification of PPV isolates belonging either to the D or M PPV isolates obtained using this information and the multiple alignment of the 21 nucleotide sequences used in this study.

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Using the sequence information presented in Figure 3, we have been able to design PCR primers allowing the specific amplification of PPV isolates belonging either to the D or M serotypes. These primers were designed to anneal to the most variable region between the two serotypes in the sequenced fragments, the cluster of five silent mutations around the Rsa I site (Candresse et al., submitted for publication). Serotype-specific amplification and detection of PPV isolates can therefore be obtained by using, in conjunction with downstream primer P1, one or the other of these serotype-specific primers (result not shown).

4. Discussion

Our results demonstrate the great potential of immunocapture PCR, both as an extremely sensitive detection technique and as a tool allowing the rapid characterisation of PPV isolates. The phylogenetic analyses show that the sequence of the small, 203 bases PCR fragment is a good predictor of the affinities of PPV isolates, since it allows unambiguous serotype assignment. This information has been put to further use through the development of a new, serotype-specific IC-PCR assay.

Recent field and experimental evidence indicates that the two major PPV serotypes might have quite different epidemiological behaviour (Adamolle 1993, Adamolle et al., 1994; J.B. Quiot, personal communication). Based on this evidence, typing of any new PPV outbreak is now compulsory in France, as the serotype of the PPV isolate present is likely to affect the spread of the disease and therefore the efforts needed to control it. The availability of fast and sensitive detection and typing assays is therefore a key to the control of the sanitary situation.

At the moment, there appears to be no perfect correlation between PCR typing results (obtained either by RFLP or using serotype-specific primers) and the reactivity of individual monoclonal antibodies. This result indicates that full definition of PPV serotypes must rely on the use of a set of monoclonal antibodies defining a set of epitopes. The use of a single monoclonal could clearly provide erroneous results for mutant isolates that may have lost the specific epitope being evaluated. On the other hand, the PCR-based techniques described here rely on the identification of a set of non-coding nucleotide differences that have no influence on the coding properties of the CP gene.

There is, however, a limitation to the approach used in the present study. Given the small size of the PCR fragment, the PCR-based techniques are very unlikely to detect recombination events in PPV isolates. However, such a limitation also applies to most, if not

all, typing approaches other than the clearly unpractical approach of complete sequencing of the viral genome. This is illustrated by the case of PPV o6 which on both PCR and serological bases belongs to serotype M, even though more than 80 % of its genome is representative of a serotype D isolate (Cervera et al., 1993). There is currently no information on the frequency of recombinant PPV isolates and the number of isolates for which significant pieces of the genome have been sequenced is still too low to allow any precise estimation. The current data seem to indicate a fairly clear cut geographical repartition of the PPV serotypes with only a few countries so far (Yugoslavia, France, Greece : M. Tsagris personal communication, Germany : G. Krczal personal communication) where both serotypes have been detected. This has probably limited, thus far, the frequency of co-infection of plants, a clear prerequisite to recombination events.

In conclusion, the advent of rapid and efficient PCR typing and characterisation assays opens new research opportunities in the study of PPV epidemiology and, more broadly, in the study of the virus variability from both the biological and molecular points of view. At the same time, the gradual introduction of these techniques in routine indexing laboratories should further enhance our ability to fight the Sharka disease through sanitary selection and eradication measures.

Acknowledgements

This work was supported in part by grants from the EEC (ECLAIR AGREE CT91-0060, CAMAR no 8001 - CT91-0201). The authors thank Kathryn Mayo-Candresse for correcting the English of the manuscript. Parts of this paper are to appear in the EPPO Bulletin (24/3, in press) as part of the proceedings of the EPPO International Conference on sharka held in Bordeaux in October 1993.

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Table 1 - Limits of detection of PPV using various techniques.

TECHNIQUE	pg virus/ml	particles/ml
ELISA	4 000	40 000 000
MH (cRNA)	500	5 000 000
RT-PCR	200	2 000 000
IC-PCR	2	20 000

ELISA: polyclonal antibodies-based double antibody sandwich ELISA assay (Varveri et al. 1987).
MH (cRNA): molecular hybridization using in vitro transcribed cRNA probes (Varveri et al., 1988; Wetzel et al., 1990).
RT-PCR: reverse transcription PCR assay (Wetzel et al., 1991a).
IC-PCR: immunocapture PCR assay (Wetzel et al., 1992; this study).

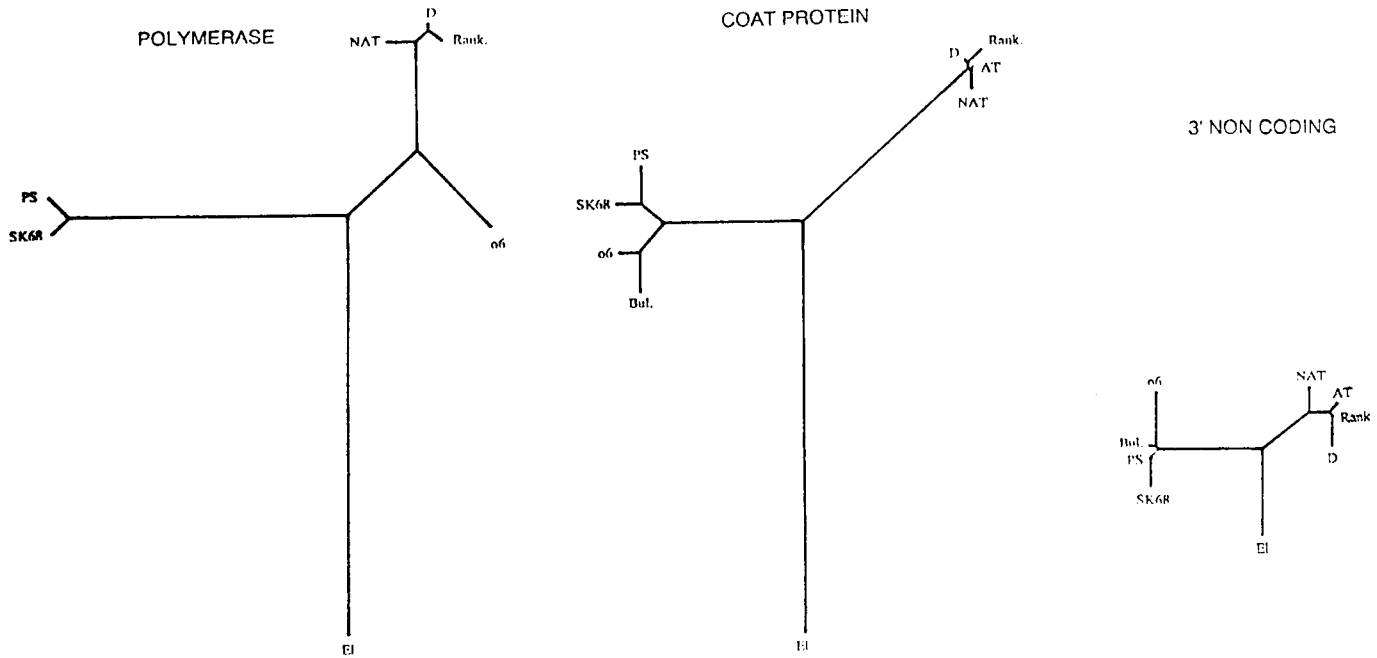


Figure 1 - Phylogenetic grouping of PPV strains using nucleotide sequences from various regions of the genome. The trees were obtained using the Clustal V suite of programs (Higgins and Sharp, 1989) implementing the Saitou and Nei (1987) neighbour-joining method. The following abbreviations are used : o6 : PPV-o6 (Cervera et al., 1993); Bul. : PPV-Bulgarie (Maiss et al., unpublished); PS : PPV-PS (Cervera et al., 1993); SK68 : PPV-SK68 (Palkovics et al., 1993); El : PPV-El Amar (Wetzel et al., 1991b); D : PPV-D (Teycheney et al., 1989); Rank : PPV-Rankovic (Lain et al., 1989); AT: PPV-AT (Maiss et al., unpublished); NAT: PPV-NAT (Maiss et al., 1989).

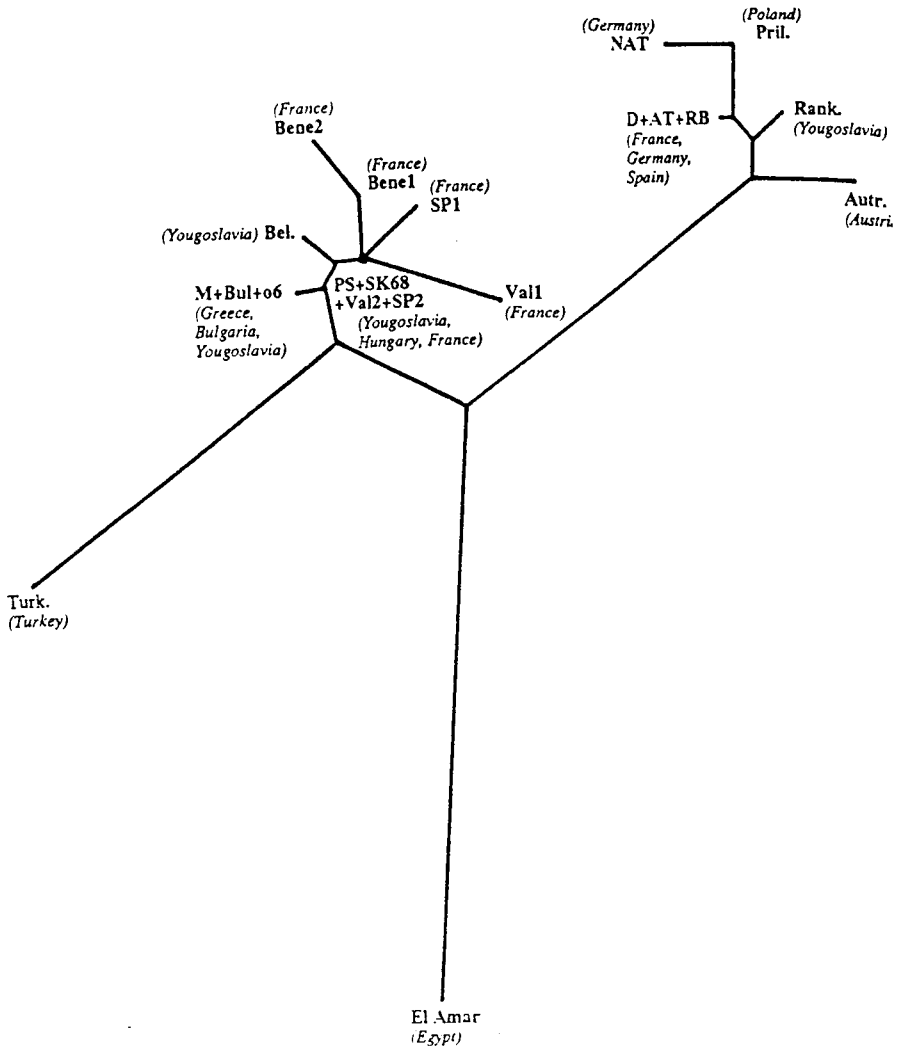
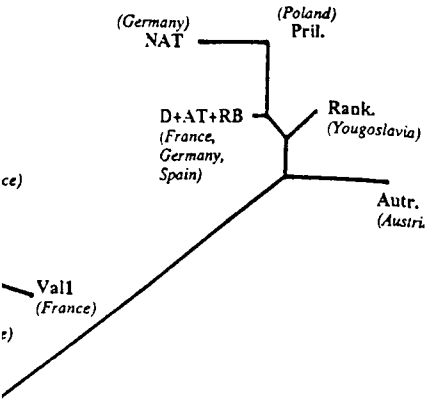


Figure 2 - Phylogenetic grouping of 21 PPV isolates using the 203 nt sequence amplified by immunocapture PCR. Tree construction and abbreviations similar to those of Figure 1. The geographical origin of the various isolates is indicated in italics. In addition to the isolates shown in Figure 1, the following isolates were used : Autr. : PPV-Autriche (Austria); Pril. : PPV-Pillnitz (ex RDA); RB : PPV-Red Beaut (Spa in); Turk. : PPV-Turquie (Turkey); M : PPV-Markus (Greece); Bel. : PPV-Belacrvna (ex Yougoslavia); Bene1 and Bene2: molecular variants of PPV-Benedicte (France); SP1 and SP2 : molecular variants of PPV-Salon de Provence (France); Val1 and Val2 : molecular variants of PPV-Valence (France).



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:ular variants of PPV-Valence (France).

ALIGNMENT OF THE NUCLEOTIDE SEQUENCE OF PCR FRAGMENTS FROM 21 PPV ISOLATES

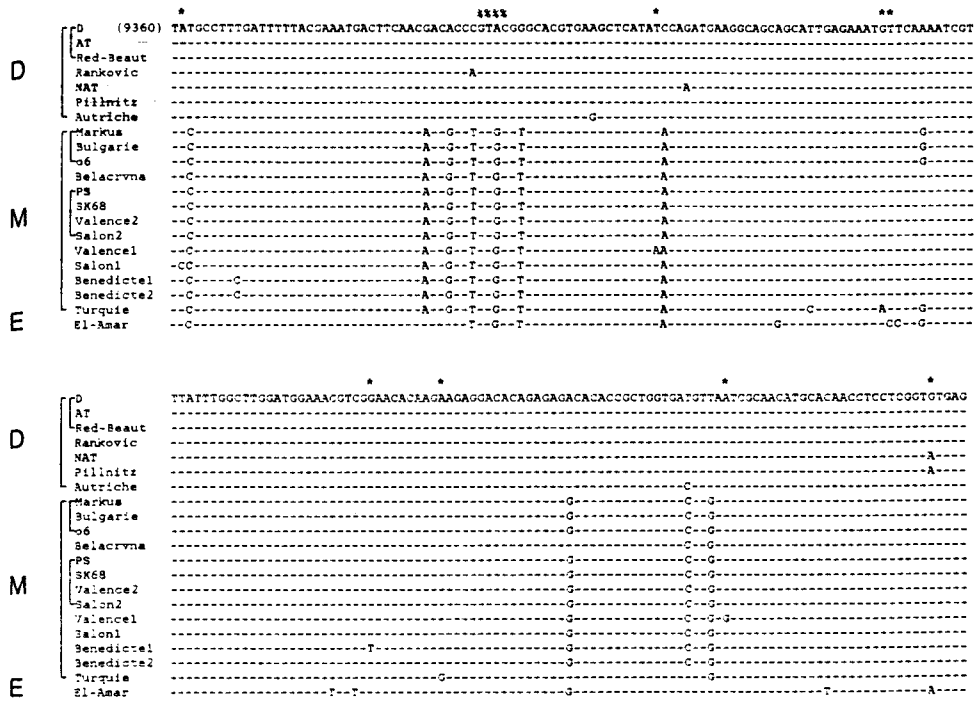

Figure 3 - Alignment of the nucleotide sequence of the PCR fragment from 21 isolates of PPV. PPV isolates are as described in the legend of Figure 2. Only nucleotides differing from those of the master sequence (PPV-D) are indicated. Identical residues are only indicated by a dash. Mutations changing the amino acid sequence of the coat protein are indicated by an asterisk above the master sequence. Variability is observed at 28 positions out of the 203 nucleotides; 20 of these positions yield silent mutations. D and M on left side of the figure indicate that the bracketed isolates belong to the D or M serotypes of PPV, respectively. Second order brackets indicate isolates with identical sequences in the region considered. The residues forming the Rsa1 polymorphic site are indicated by a % sign above the master sequence.