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Detection of plum pox potyvirus and analysis of its molecular variability using immunocapture-PCR[1]

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We have developed an immunocapture-PCR (IC-PCR) detection technique for plum pox potyvirus (PPV) which is both simple and highly sensitive. This single-day assay can detect about 2000 virus particles (200 fg of virus) diluted in 100 µl of crude plant sap, which is equivalent to a sensitivity about 2000 times better than that of a standard ELISA assay. RFLP analysis and sequencing of the amplified cDNA fragment indicate that three groups of strains with limited intra-group variability can be discriminated. Two of these groups correspond to the previously described D and M serotypes of PPV. The third group contains, so far, only the El Amar Egyptian isolate. Strains belonging to the D or M serotypes can easily be discriminated by RsaI polymorphism in the amplified cDNA fragment. Synthetic oligonucleotides allowing specific amplification of PPV strains belonging either to the D or to the M serotypes have also be designed.

Introduction

Plum pox potyvirus (PPV) is the causal agent of sharka disease of stone-fruit trees. It is the single most important viral disease of these crops in Europe and in the Mediterranean region. The only effective way of controlling the disease is through sanitary selection programmes 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. Diagnosis of PPV 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 our efforts to obtain more sensitive detection assays, we have developed a PCR-based detection technique which is both simple and highly sensitive. 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).

Besides its usefulness as a detection technique, IC-PCR can also be used to obtain rapid 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. Two of these groups correspond to the previously described D and M serotypes, for which recent results seem to indicate quite different epidemiological properties. Strains belonging to the D or M serotypes can easily be discriminated by RsaI polymorphism in the amplified cDNA fragment or by direct

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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 proved extremely useful for the rapid typing of newly isolated PPV strains, a procedure now compulsory for any new outbreak of sharka in France.

Materials and methods

Isolates of PPV

The PPV isolates used in this study were part of the collection of PPV isolates kept at INRA Montpellier. They were propagated in GF305 peach seedlings and in *Pisum sativum* cv. Colmo. In order to avoid any cross contaminations, the plants were kept in an insect-proof glasshouse in separate cabinets.

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 (Laín *et al.*, 1989), PPV-El Amar (Wetzel *et al.*, 1991a), PPV-o6 and PPV-PS (Cervera *et al.*, 1993), and 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, DE).

Immunocapture-PCR

The PCR primers used in this study have been described (Wetzel *et al.*, 1991b). The immunocapture-PCR protocol described by Wetzel *et al.* (1992) was used. In order to minimize potential sequence artifacts, the cDNAs used in the cloning experiments were amplified using Pfu polymerase (Stratagene) instead of Taq polymerase. In some experiments, a simplified protocol was used. Briefly, 25 μl of a one-step reverse transcription-PCR (RT-PCR) reaction mix [Tris-HCl pH 8.8, 10 mM; $MgCl_2$, 1.5 mM; KCl, 50 mM; Triton X100 0.3%; 250 μM each of dNTPs; 1 μM each of the two primers; 0.25 U of AMV RTase and 0.5 U Taq polymerase] is directly added to Eppendorf tubes containing immunocaptured virus particles. After vortexing and spinning down the droplets which might have formed, the reaction mix is overlaid by 50 μl of mineral oil. The cycling scheme used is the following: 45 min at 42 °C (RT reaction), 5 min at 92 °C to denature the templates and the RTase followed by 40 cycles of amplification: 20 s at 92 °C, 20 s at 62 °C and 40 s at 72 °C. In every case, 10 μl aliquots of the amplification reactions were analysed by 1.5% agarose gel electrophoresis in 1 × TBE buffer.

RFLP analysis and sequencing of PCR-amplified cDNAs

For RFLP analysis, the PCR-amplified material was first purified with phenol-chloroform and chloroform extractions followed by ethanol precipitation. An aliquot equivalent to 10 μl of the PCR reaction was then digested for 2 h with 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 1 × TBE buffer.

For sequencing of the PCR-amplified cDNAs, the amplified material was prepared as described above and further purified by agarose-gel electrophoresis. The band corresponding to the PCR product was cut and the cDNA extracted using a Gene-Clean kit (Bio-101). The purified material was then ligated in SmaI-cut pUC9 vector. Following transformation in DH5α cells, recombinant plasmids were selected and sequenced using a Sequenase (USB) kit.

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 & Sharp, 1989) suite of programs.

Results

Detection of PPV using immunocapture-PCR

Comparison of the original, more complex version of IC-PCR (Wetzel *et al.*, 1992) with the simplified version presented here indicated that their sensitivity is in the same range. In a series of detailed studies, we have compared the sensitivities of several techniques for the detection of PPV in GF305 peach seedlings (Varveri *et al.*, 1987, 1988; Wetzel *et al.*, 1990, 1991b, 1992). The data from these experiments is summarized in Table 1. The sensitivity of an ELISA assay involving a polyclonal antiserum is in the range of 1 ng of virus per assay, which translates into a detection limit of 4 ng of virus per ml plant extract. Using identical extracts, molecular hybridization has a sensitivity approximately 10 times greater (detection limit around 0.5 ng ml^{-1}) and direct RT-PCR (Wetzel *et al.*, 1991b) 20 times greater (0.2 ng ml^{-1}).

The introduction of an immunocapture step which concentrates and pre-purifies the virus particles allowed use of much larger sample volumes than RT-PCR and resulted in dramatically improved sensitivity. The current detection levels in crude plant sap are 200 fg of purified virus (approximately 2000 viral particles), which translates into a detection limit of 2 pg ml^{-1}. Thus, immunocapture-PCR is 2000-fold more sensitive than a polyclonal-antibody ELISA assay for the detection of PPV. In addition to dramatically increasing the sensitivity of the PCR reaction, immunocapture was found to be a simple, rapid and inexpensive way of processing plant samples prior to PCR amplification.

Phylogenetic analysis of PPV isolates using various regions of the genome

Besides permitting efficient detection of PPV, IC-PCR provided rapid and easy access to the viral genome in the form of amplified cDNAs. The sensitivity of the technique is such that preliminary transfer of the virus to herbaceous hosts or purification was not needed. This technique therefore offers a new and powerful tool for the characterization of PPV isolates and for the determination of relationships between them.

Table 1. Comparison of the detection limit of PPV using various techniques
Comparaison de la limite de détection du PPV par diverses techniques

Technique	pg virus per ml	Particles per 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 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).

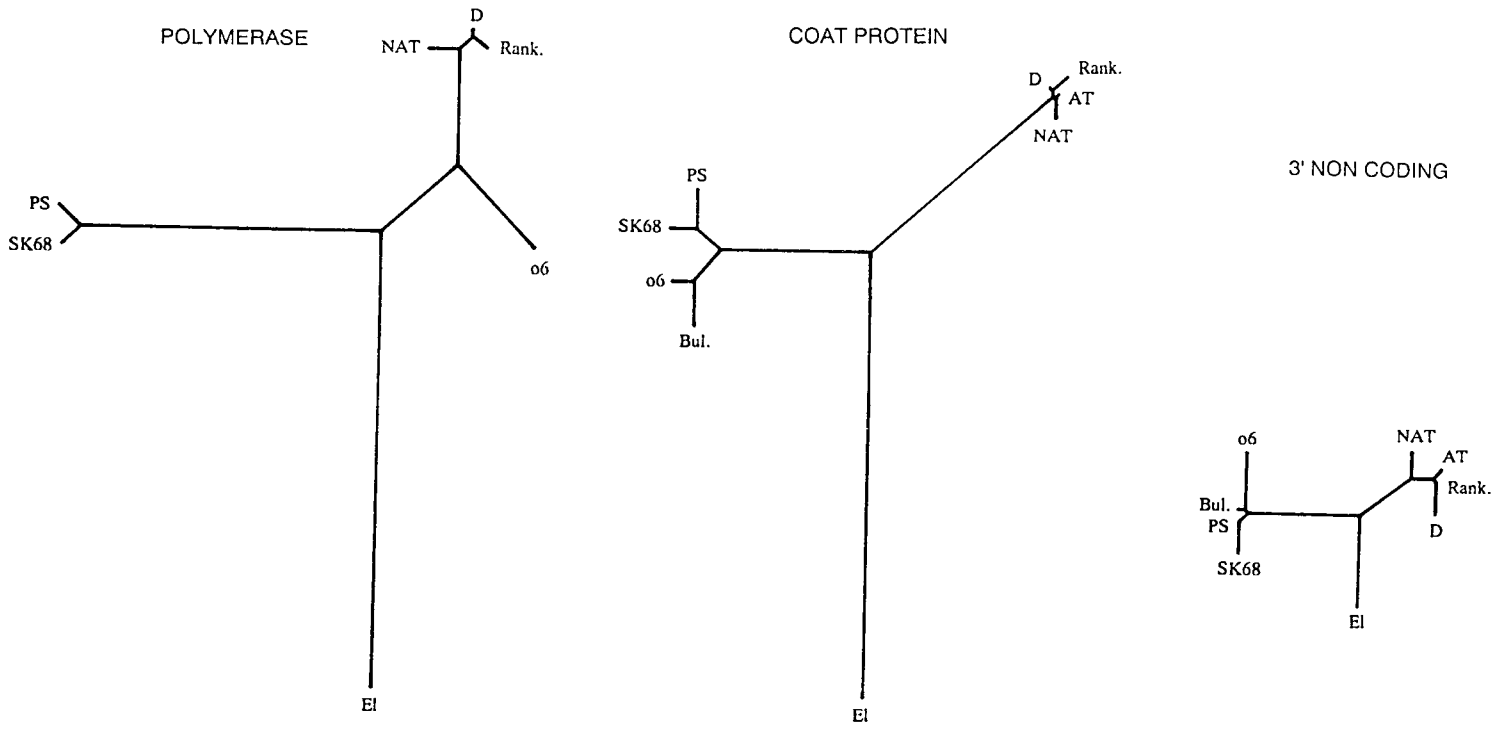


Fig. 1. Phylogenetic grouping of PPV strains using nucleotide sequences from various regions of the genome. The trees were obtained using the Clustal V suite of programs (Higgins & Sharp, 1989) implementing the Saitou & Nei (1987) neighbour-joining method. The following abbreviations are used: o6: PPV-o6 (Cervera *et al.*, 1993); Bul.: PPV-Bulgarie (Maiss *et al.*, pers. comm.); 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 (Laín *et al.*, 1989); AT: PPV-AT (Maiss *et al.*, pers. comm.); NAT: PPV-NAT (Maiss *et al.*, 1989). Groupement phylogénétique des souches de PPV sur la base des séquences de nucléotides correspondant à diverses parties du génome. Les 'arbres' sont construits par le logiciel Clustal V de Higgins & Sharp (1989) à l'aide de la méthode de Saitou & Nei (1987). Les abréviations sont précisées comme indiquées ci-dessus.

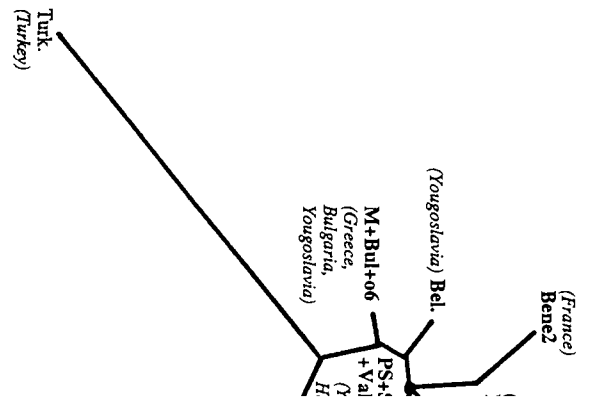


Fig. 2. Phylogenetic grouping of 21 PPV is
PCR. Tree construction and abbreviations
isolates is indicated in italics. In addition t
Autr.: PPV-Autriche (AT); Pril.: PPV-Prilli
M: PPV-Markus (GR); Bel.: PPV-Belcravi
(FR); SP1 & SP2: molecular variants of PF
PPV-Valence (FR).
Groupement phylogénétique de 21 souches
par immunocapture-PCR. Voir Fig. 1 pou
souches ne figurant pas à la figure 1.

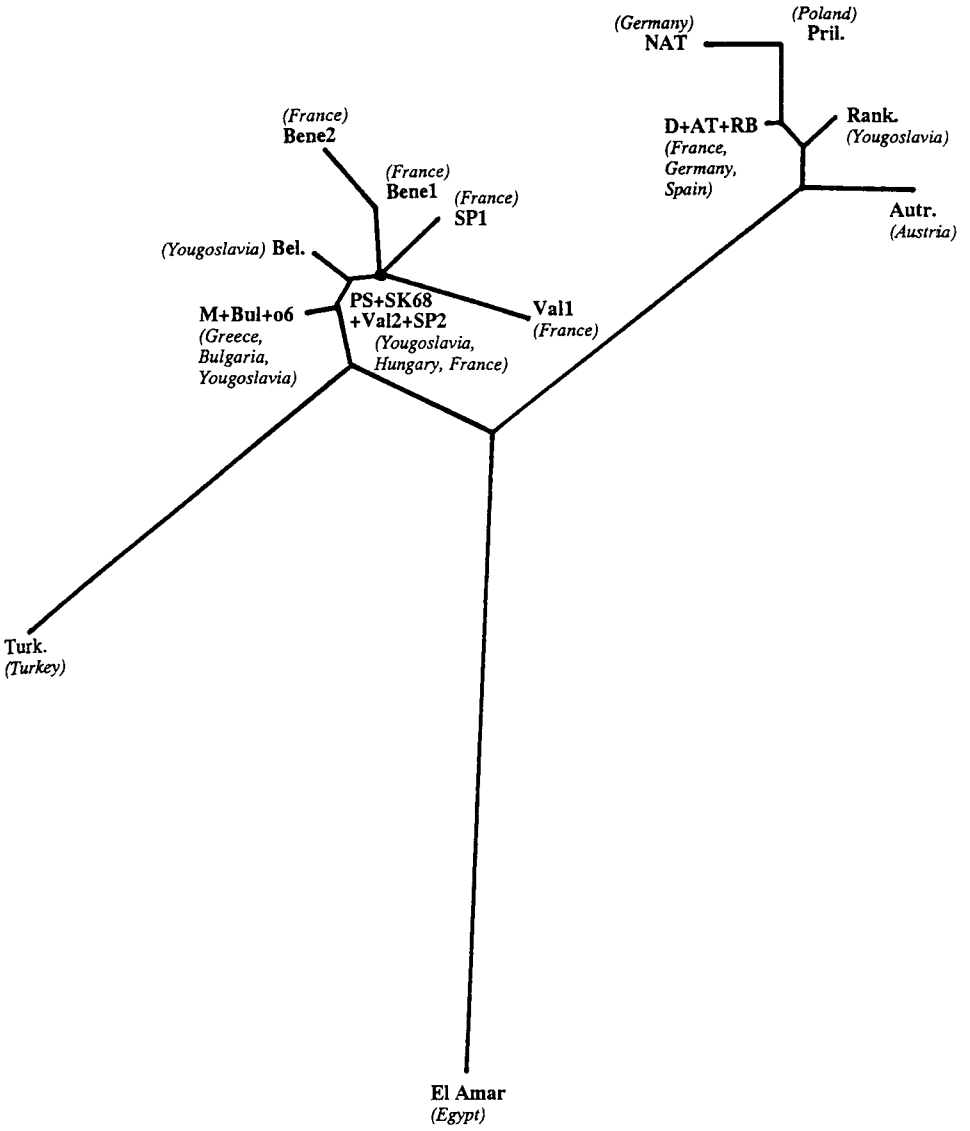
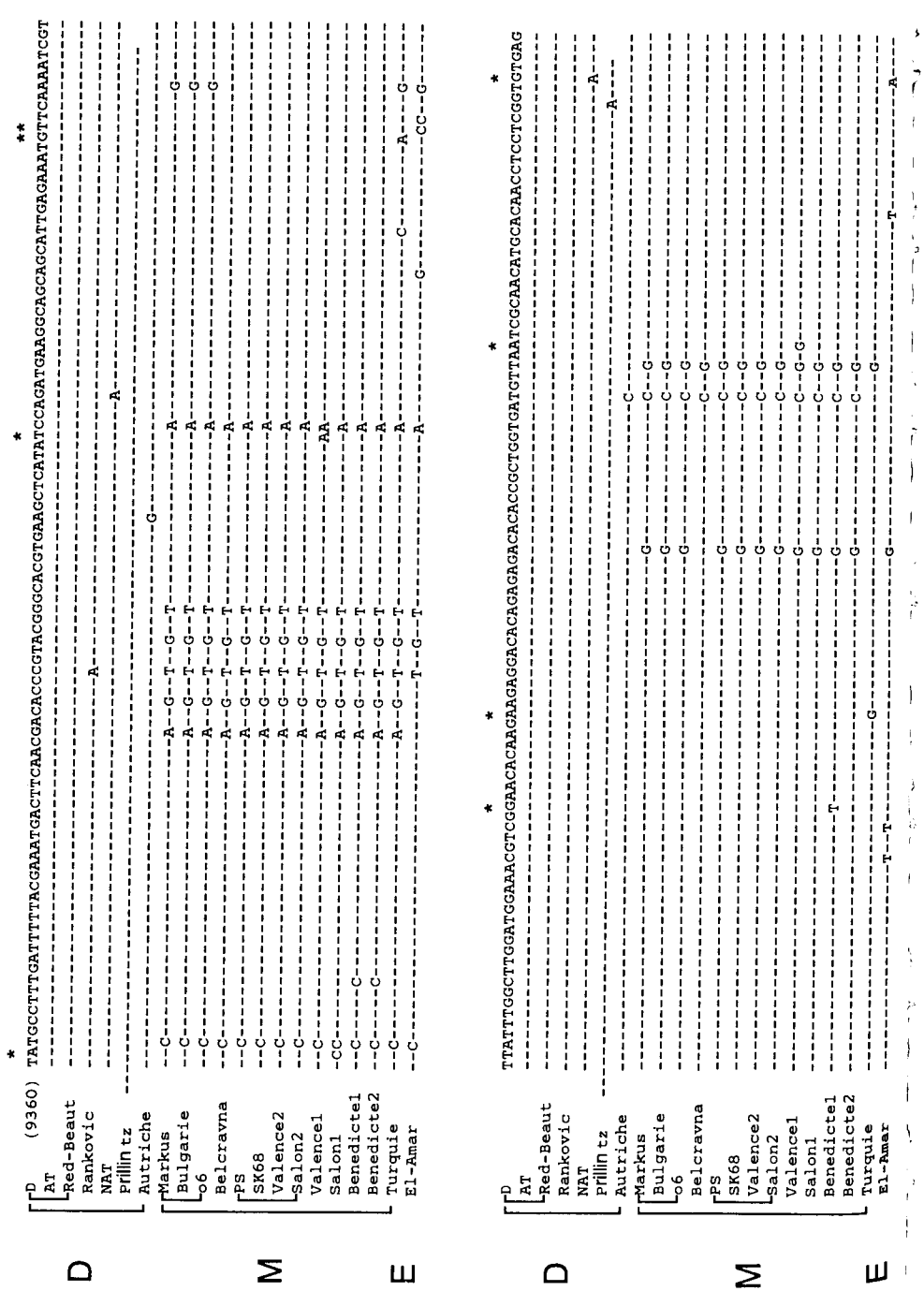


Fig. 1. Phylogenetic grouping of PPV strains using nucleotide sequences from various regions of the genome. The trees were obtained using the Clustal V suite of programs (Higgins & Sharp, 1989) implementing the Saitou & Nei (1987) neighbour-joining method. The following abbreviations are used: o6: PPV-o6 (Cervera *et al.*, 1993); Bul.: PPV-Bulgarie (Maiss *et al.*, pers. comm.); 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 (Laín *et al.*, 1989); AT: PPV-AT (Maiss *et al.*, pers. comm.); NAT: PPV-NAT (Maiss *et al.*, 1989). Groupement phylogénétique des souches de PPV sur la base des séquences de nucléotides correspondant à diverses parties du génome. Les 'arbres' sont construits par le logiciel Clustal V de Higgins & Sharp (1989) à l'aide de la méthode de Saitou & Nei (1987). Les abréviations sont précisées comme indiquées ci-dessus.

Fig. 2. Phylogenetic grouping of 21 PPV isolates using the 203 nt sequence amplified by immunocapture PCR. Tree construction and abbreviations similar to those of Fig. 1. The geographical origin of the various isolates is indicated in italics. In addition to the isolates shown in Fig. 1, the following isolates were used: Autr.: PPV-Autriche (AT); Pril.: PPV-Prillintz (PL); RB: PPV-Red Beaut (ES); Turk.: PPV-Turquie (TR); M: PPV-Markus (GR); Bel.: PPV-Belcravna (YU); Benel & Bene2: molecular variants of PPV-Benedicte (FR); SP1 & SP2: molecular variants of PPV-Salon de Provence (FR); Vall & Val2: molecular variants of PPV-Valence (FR).

Groupement phylogénétique de 21 souches de PPV sur la base d'une séquence de 203 nucléotides amplifiée par immunocapture-PCR. Voir Fig. 1 pour les abréviations, et ci-dessus pour l'origine géographique des souches ne figurant pas à la figure 1.



As a preliminary to the use o
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Phylogenetic analysis of PPV i material

For this part of the work, PPV
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sequences used in this study.

Fig. 3. Alignment of the nucleotide sc
as described in the legend to Fig. 2.
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Une variabilité se manifeste pour 28
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sérotypes D ou M du PPV, respec
identiques dans la zone considérée.

As a preliminary to the use of PCR-derived sequence information for the analysis of PPV molecular variability, we made sure that the phylogenetic grouping of PPV isolates obtained was independent of the region of the genome used. This would be 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 was possible to test this assumption in the case of PPV. We therefore performed several independent phylogenetic analyses using various parts of the genome (part of the polymerase gene, coat-protein gene, 3' non-coding region). In each case, although branch lengths were different (reflecting differences in evolutionary rates), phylogenetic trees of identical topology were obtained. In addition, identical grouping of the various isolates used was also observed (with one exception) (Fig. 1). The only PPV isolate which did not show phylogenetic behaviour independent of the region used was 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 (data not shown). These results demonstrate that, on a general basis, the various parts of the PPV genome are coevolving, 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, can be used as a reliable marker of the phylogenetic affinities of PPV isolates.

Phylogenetic analysis of PPV isolates using sequences derived from PCR-amplified material

For this part of the work, 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 9 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 9 PPV isolates already cloned and sequenced, a total of 21 sequences from 18 PPV isolates were thus available. Figs 2 and 3 respectively present the phylogenetic grouping of PPV isolates obtained using this information and the multiple alignment of the 21 nucleotide sequences used in this study.

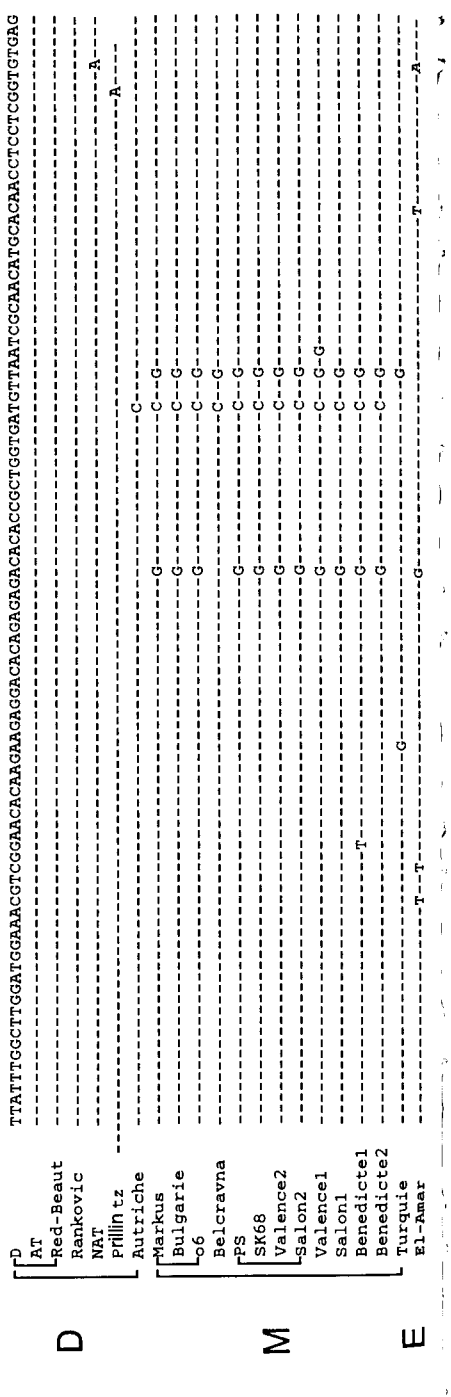

Fig. 3. Alignment of the nucleotide sequence of the PCR fragment from 21 isolates of PPV. PPV isolates are as described in the legend to Fig. 2. Only nucleotides differing from those of the master sequence (PPV-D) are indicated. Identical residues are only indicated by a dash. Mutations changing the 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 respectively to the D or M serotypes of PPV. Second-order brackets indicate isolates with identical sequences in the region considered.

Alignement de la séquence de nucléotides du fragment PCR de 21 souches de PPV. Les isolats sont définis comme à la figure 2. Seuls les nucléotides différents de ceux de la séquence maîtresse (PPV-D) sont indiqués, les nucléotides identiques étant représentés par des tirets. Les mutations qui influent sur la séquence des acides aminés de la protéine capside sont indiquées par un astérisque au-dessus de la séquence maîtresse. Une variabilité se manifeste pour 28 positions sur 203, mais 20 de ces positions sont sans conséquence pour la protéine capside. D et M à la gauche de la figure indiquent l'appartenance des isolats accolés aux sérotypes D ou M du PPV, respectivement. Les crochets indiquent les isolats qui ont des séquences identiques dans la zone considérée.

As can be seen in Fig. 2, the inclusion of the data corresponding to the newly sequenced PPV isolates gives a tree with a topology similar to that shown in Fig. 1. Three groups of PPV isolates were 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), Prillintz (Poland) and Autriche (Austria). It thus 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 Belcravna (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.* (199la).

As can be seen from the multiple alignments presented in Fig. 3, variation between the 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) were clearly separated by a total of 8 fully conserved mutations, all of which are silent. As one of these mutations overlaps a restriction enzyme site (RsaI), it was possible to discriminate between the groups by performing a simple RsaI digestion of the amplified fragment. The existence of this restriction polymorphism had already been noted by Wetzel *et al.* (199lb). Recent evidence (Bousalem *et al.*, 1994a, b) indicates that the two major groups of PPV isolates identified in this study in fact correspond to the two PPV serotypes previously described by Kerlan & Dunez (1979), again typified by PPV-D and PPV-M.

Development of serotype-specific PCR primers

Using the sequence information presented in Fig. 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 RsaI site. Use of one of these serotype-specific primers together with the previously used downstream primer allowed serotype-specific amplification and detection of PPV isolates (data not shown).

Discussion

Our results demonstrate the great potential of immunocapture-PCR, both as an extremely sensitive detection technique and as a tool for rapid characterization of PPV isolates. The phylogenetic analyses showed that the sequence of small, 203-base PCR fragments 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 (J.B. Quiot, pers. comm.). On the basis of 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.

There is, however, a limitation to the approach we have used. Given the small size of the PCR fragment, this technique is very unlikely to detect recombination events in PPV isolates. However, such a limitation also applies to most, if not all, typing approaches besides a clearly impractical approach, the 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

corresponding to the newly sequenced PPV
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bases belongs to serotype M, even though

more than 80% of its genome is representative of a serotype-D isolate. 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. Current data seems to indicate a fairly clear-cut geographical distribution of PPV serotypes, with only few countries (Yugoslavia, France, Germany: G. Krczal, pers. comm.) where both serotypes have been detected so far. This has probably limited the frequency of co-infection of plants, a clear prerequisite for recombination events.

In conclusion, the advent of rapid and efficient PCR typing and characterization 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, gradual introduction of these techniques in routine test laboratories should further enhance our ability to control sharka disease by sanitary selection and eradication measures.

Détection du plum pox potyvirus et analyse de sa variabilité moléculaire à l'aide de l'immunocapture-PCR

Nous avons développé une technique d'immunocapture-PCR (IC-PCR), à la fois simple et très sensible, pour la détection du plum pox potyvirus (PPV). Le test, qui se réalise en une seule journée, est capable de détecter environ 2000 particules virales (200 fg de virus) diluées dans 100 µl de sève brute, ce qui représente une sensibilité environ 2000 fois supérieure à celle d'un test ELISA normal. Par analyse RFLP et séquençage du fragment amplifié d'ADNc, il s'avère que trois groupes de souches peuvent être distingués, compte tenu de la faible variabilité à l'intérieur de chacun. Deux d'entre eux correspondent aux sérotypes D et M du PPV précédemment décrits. Le troisième ne contient pour le moment que l'isolat égyptien El Amar. Les souches des sérotypes D et M se distinguent aisément par le polymorphisme RsaI du fragment d'ADNc amplifié. Il conviendrait aussi de construire des oligonucléotides synthétiques permettant l'amplification spécifique des souches du PPV appartenant aux sérotypes D ou M.

Детектирование plum pox potyvirus и анализ его молекулярной вариабельности с помощью метода полимеразной цепной реакции с иммунозахватом

Нами был разработан метод полимеразной цепной реакции с иммунозахватом, как методики детектирования plum pox potyvirus (PPV), которая отличается простотой и высокой чувствительностью. Однодневный анализ позволяет выявить около 2000 вирусных частиц (200 фг вирусов), находящихся в 100 мкл необработанного сока растений, что соответствует чувствительности, приблизительно в 2000 превышающую стандартную пропись метода ELISA. Анализ полиморфизма рестрикционных фрагментов и секвенирование амплифицированных фрагментов кДНК указывает, что представляется возможным различить три группы штаммов с ограниченной внутригрупповой вариабельностью. Две из них соответствуют ранее описанным серотипам D и M вируса PPV. По данным на сегодняшний день третья группа содержит только египетский изолят Эль Амар. Штаммы, принадлежащие к серотипам D или M, легко различаются по полиморфизму участка Rsa I амплифицированного фрагмента кДНК. Следует также создать синтетические олигонуклеотиды, обеспечивающие специфическую амплификацию штаммов PPV, принадлежащих к серотипу D или M.

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