

SHORT COMMUNICATION

PRESENCE OF A RECOMBINANT ISOLATE OF *PLUM POX VIRUS* IN APULIAA. Myrta¹, M. Al Rwahnih¹ and V. Savino²¹ Istituto Agronomico Mediterraneo, Via Ceglie 9, 70010 Valenzano (BA) Italy² Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Istituto di Virologia Vegetale del CNR, Sezione di Bari, Via Amendola 165/A, 70126 Bari, Italy

SUMMARY

During spring 2003, a small focus of infection by *Plum pox virus* (PPV) that involved four trees was discovered in an apricot orchard in the Province of Brindisi (Apulia, southern Italy). Virus isolates from all these trees were serologically typed by DASI-ELISA using four strain-specific monoclonal antibodies (Mabs). One isolate (PPV-BR) was also characterized by RFLP analysis of PCR products derived from two genome regions, i.e. coat protein (CP) and P3-6K1. PPV-BR proved to be a recombinant as it typed as PPV-M by strain-specific Mabs and RFLP analysis of the CP gene, but as PPV-D by analysis of the P3-6K1 gene. Sequence analysis of the (Cter)NIB-(Nter)CP region of this recombinant showed that the recombination breakpoint was located in the C terminus of the NIB gene, as has been reported for other recognized PPV recombinants. This represents the first record of a recombinant isolate of PPV from Italy.

Key words: apricot, PPV, RT-PCR, RFLP analysis, sequence analysis.

Sharka, the most severe disease of stone fruits in Europe and the Mediterranean region (Roy and Smith, 1994), is caused by *Plum pox virus* (PPV), which occurs in nature as four main strains, Marcus (PPV-M), Dideron (PPV-D), El Amar (PPV-EA), and Cherry (PPV-C) (Pasquini and Barba, 1997; Candresse *et al.*, 1998).

In Apulia (southern Italy), sharka was first recorded in 1988 (Di Terlizzi *et al.*, 1988) but no new *foci* have been detected since 2000, following eradication measures implemented from the beginning of the 1990s. In spring 2003, however, new PPV infections were discovered in an apricot orchard established at Mesagne (Province of Brindisi) with propagating material coming from an extraregional nursery. There were four symptomatic trees, one of cv Ninfa and three of cv San Castrese.

Leaf samples from all sources reacted in ELISA with the universal monoclonal antibody Mab5B (Cambra *et al.*, 1994). All 4 isolates reacted in DASI-ELISA with Mab AL (Boscia *et al.*, 1997), but not with Mabs 4DG5 (Cambra *et al.*, 1994), EA24 (Myrta *et al.*, 1998), and AC (Myrta *et al.*, 2000), which suggests that they belong to PPV-M. Extensive monitoring carried out in spring 2004 failed to identify additional infected apricot trees in the same planting. Likewise, no infections were detected in peach orchards located within a 1 km radius of the PPV focus.

The virus isolate from cv Ninfa, denoted PPV-BR, was kept in tissue grafted on GF305 and maintained in a controlled environment greenhouse. Molecular characterisation was done by RT-PCR for: (i) amplification of the C terminal region of the coat protein (CP) gene using the P1/P2 set of primers (Wetzel *et al.*, 1992); (ii) strain-specific identification according to Candresse *et al.* (1998) using primers P1, PM and PD; (iii) amplification of (Cter)P3-6K1-(Nter)CI using primers PCI and PP3 (Glasa *et al.*, 2002). PCR conditions for all primer sets were: denaturation at 93°C for 2 min, 35 cycles of amplification (94°C for 45 sec, 50°C for 45 sec and 72°C for 1 min), and a final extension for 7 min at 72°C.

PCR using P1 and P2 primers amplified the expected 243 bp fragment from the CP gene and PCR using PP3 and PCI primers amplified the 836 bp fragment from the P3-6K1 gene of PPV-BR. PCR products were subjected to RFLP analysis using *RsaI* for P1/P2 amplicons and *DdeI* for PCI/PP3 amplicons.

PPV-BR was typed as PPV-M based on the *RsaI* polymorphism of the C-terminal part of the CP gene but as PPV-D based on the *DdeI* polymorphism of the P3-6K1 region, suggesting possible recombination between PPV-M and PPV-D. Thus, another primer pair (CP2/NIB1) amplifying a fragment of 829 bp of the (Cter)NIB-(Nter)CP regions was designed (Matic, 2004). The CP2/NIB1-amplified product was purified by using a QIAquick PCR purification Kit (Qiagen, Valencia, Ca, USA), and cloned in pGEM-T Easy® vector (Promega Corp., Madison, Wisconsin, USA) according to the manufacturer's instructions. Two identical cDNA sequences were obtained for the recombinant plasmid by automatic sequencing (MWG Biotech, Ebersberg,

Germany) and were deposited in the GenBank database under the accession number AJ812242.

Visual analysis of the nucleotide sequence alignments suggested a recombination event in the C terminus of the NIB gene around nt 8450. Interestingly, the breakpoint was in the same position as that reported for other PPV recombinant isolates (Cervera *et al.*, 1993; Glasa *et al.*, 2004). This region has a high level of sequence homology among all aligned recombinant PPV isolates. The exact position of recombination breakpoint was determined by PhylPro program (Weiller, 1998) on the PPV-BR sequence and on that of the BOR-3 isolate (Glasa *et al.*, 2002), which was used as a reference for the recombination event. For both sequences, a single peak was observed, indicating a recombination breakpoint at the nucleotide position 8450 (Fig. 1).

Multiple alignments of nucleotide and amino acid sequences of PPV-BR CP and NIB genes were obtained using the default options of Clustal X 1.8, a Windows interface for the Clustal W multiple sequence alignment program. The sequences were compared with previously published sequences overlapping the NIB-CP fragment: BRC-3 (AF421118), LOZ-3 (AF450312), Pd31 (AJ566345), BOR-3 (AY028309), Bt-H2 (AJ566346), MYV-3 (AF450313), BNE-10 (AF420311), KRN-1 (AF421121), SK-68 (M92280), NAT (NC-001445), PENN-1 (AF401295), PS (AJ243957), Dideron (X16415), El Amar (X56258) and SwC (Y09851). Phylogenetic analysis was done using the minimum evolution method of phylogenetic interference (Rzhetsky and Nei, 1993) with 10,000 bootstrap replicates, using the 2.1 version of the Molecular Evolutionary Genetics Analysis software MEGA (Kumar *et al.*, 2001). In phylogenetic trees generated using nucleotide (Fig. 2) or amino acid (not shown) sequences of the fragment corresponding to the C-terminal part of the NIB and N-terminal part of the CP genes, PPV isolates grouped into two main clusters, one comprising all PPV-D isolates and another all

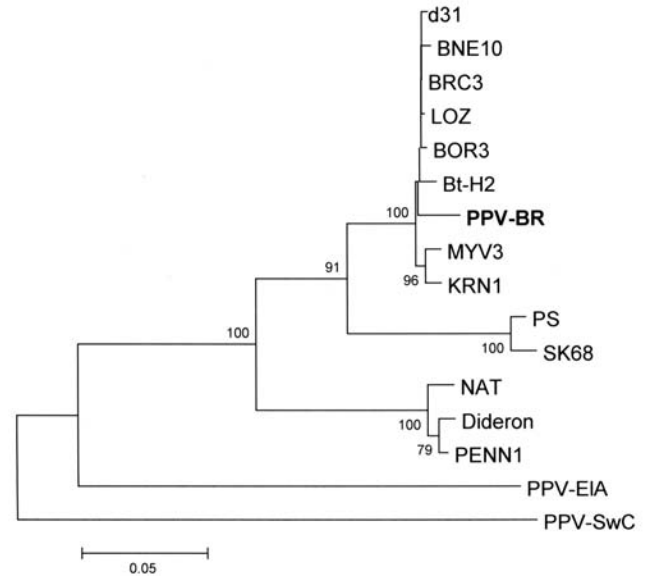


Fig. 2. Phylogenetic position of recombinant isolates based on sequences encoding the c terminus of the NIB and the N terminus of the CP. The scale bar shows a distance of 0,05 distance per site. Only bootstrap values > 70% are shown.

PPV-M isolates. Interestingly, the PPV-M cluster split into two subclusters, comprising typical PPV-M isolates or all recombinant isolates (PPV-Rec). The recombinant isolates clustered in a branch clearly distinct from PPV-M isolates, supporting the proposal by Glasa *et al.* (2004) for the establishment of a new subgroup.

As well as sharing the same recombination breakpoint, the PPV-BR recombinant isolate contained five highly conserved amino acids (K₂₈₁₄, I₂₈₄₈, T₂₈₅₂, I₂₈₆₈, T₂₈₇₈) (Glasa *et al.*, 2004), which were not found in the reference PPV-M and PPV-D isolates. In addition, PPV-BR showed three other changes of amino acids compared with analysed recombinants in the CP (M₂₈₄₃, R₂₈₄₅, and S₂₈₈₂) (not shown).

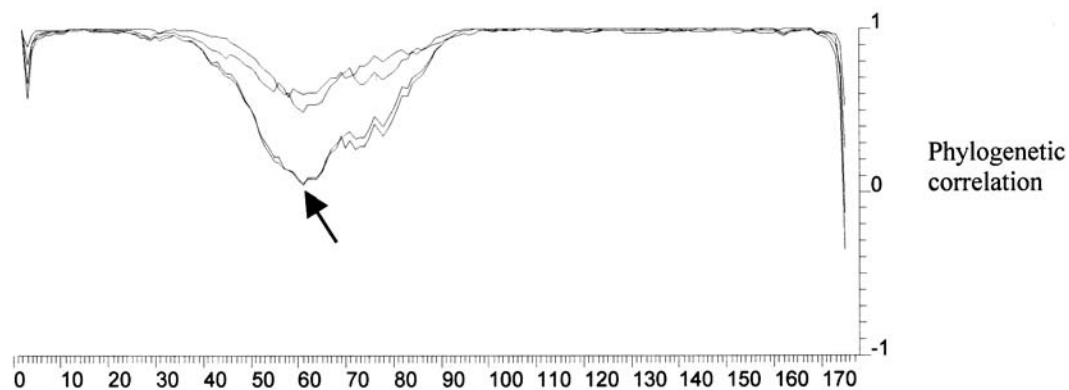


Fig. 1. PhylPro profiles of four PPV isolates. the overlapping lower curves refer to isolates PPV-BR and PPV-BOR3. Upper profiles refer to isolates of PPV-D and PPV-M.

Homologous RNA recombination in PPV was first observed in a virus isolate from Yugoslavia (PPV-o6) (Cervera *et al.*, 1993) and the suggestion was made that cherry isolates may have originated from a heterologous recombination event between a PPV-like virus and another unknown virus RNA, or plant cell nucleic acid (Nemchinov *et al.*, 1998). Sequence analysis of several parts of the virus genome of a number of PPV isolates, has confirmed the presence of a recombination break-point between RNAs of M- and D-strains in the N1b gene (Glasa *et al.*, 2002). Another was located in the P3 gene (Glasa *et al.*, 2004).

The PPV strains frequently found in Italy are PPV-D and PPV-M (Pasquini and Barba, 1994; Frisinghelli *et al.*, 1996; Poggi Pollini *et al.*, 1996; Barba and Pasquini, 1998; Pasquini *et al.*, 1999; Myrta *et al.*, 2001; Bianco *et al.*, 2004), with a single record of PPV-C from sweet cherry (Crescenzi *et al.*, 1994). No recombinant isolates have been detected in Italy; thus this paper is the first such report. The Italian PPV recombinant was genetically very similar to previously reported recombinants, supporting the hypothesis of their having a common origin.

This report also supports the notion that recombinants are more widely spread than currently thought, even in the Mediterranean area.

ACKNOWLEDGEMENTS

Grateful thanks are expressed to prof. G.P. Martelli for his helpful discussion and critical reading of the manuscript. This work was supported by the Apulian regional project "Monitoraggio degli organismi nocivi da quarantena e certificazione del materiale di propagazione vegetale".

REFERENCES

- Barba M., Pasquini G., 1998. Sharka, un pericolo per la peschicoltura italiana. *Informatore agrario* **54** (32): 65-68.
- Bianco P.A., Fanigliulo A., Comes S., Casati P., Crescenzi A., Belli G., 2004. Characterisation of *Plum pox virus* isolates associated to sharka infection in Northern and Southern Italy. In: *Proceeding of the European Meeting 2004 on Plum Pox, Rogow* 2004, 34.
- Boscia D., Zeramdini H., Cambra M., Potere O., Gorriss M.T., Myrta A., Di Terlizzi B., Savino V., 1997. Production and characterization of a monoclonal antibody specific to the M serotype of plum pox potyvirus. *European Journal of Plant Pathology* **103**: 477-480.
- Cambra M., Asensio M., Gorriss M.T., Pèrez E., Camarasa E., Garcia J.A., Moya J.J., Lopez-Abella D., Vela C., Sanz A., 1994. Detection of plum pox potyvirus using monoclonal antibodies to structural and non-structural proteins. *Bulletin OEPP/EPPO Bulletin* **24**: 569-577.
- Candresse T., Cambra M., Dallot S., Lanneau M., Asensio M., Gorriss M.T., Revers F., Macquaire G., Olmos A., Boscia D., Quiot J.B., Dunez J., 1998. Comparison of monoclonal antibodies and PCR assays for the typing of isolates belonging to the D and M serotypes of plum pox virus. *Phytopathology* **88**: 198-204.
- Cervera M.T., Riechmann J.L., Martin M.T., Garcia J.A., 1993. 3'-terminal sequence of the plum pox virus PS and o6 isolates: evidence for RNA recombination within the potyvirus group. *Journal of General Virology* **74**: 329-334.
- Crescenzi A., Nuzzazi M., Levy L., Hadidi A., Piazzolla P., 1994. Infezioni di sharka su ciliegio dolce in Italia Meridionale. *Informatore agrario* **50** (34): 73-75.
- Di Terlizzi B., Savino V., Castellano M.A., Martelli G.P., 1988. Presenza della vaiolatura delle drupacee in Puglia e Basilicata. *Informatore Fitopatologico* **38**: 53-55.
- Frisinghelli C., Grando M.S., Vindimian M.E., 1996. Individuazione di ceppi di plum pox virus D e M in Trentino. *Convegno annuale SIPaV, Udine* 1996, C107.
- Glasa M., Marie-Jeanne V., Labonne G., Subr Z., Kudela O., Quiot J.B., 2002. A natural population of recombinant *Plum pox virus* is viable and competitive under field conditions. *European Journal of Plant Pathology* **108**: 843-853.
- Glasa M., Palkovics L., Komínek P., Labonne G., Pittnerová S., Kúdela O., Candresse T., Subr Z., 2004. Geographically and temporally distant natural recombinant isolates of *Plum pox virus* are genetically very similar and form a unique PPV subgroup. *Journal of General Virology* **85**: 2671-2681.
- Kumar S., Tamura K., Jakobsen I.B., Nei M., 2001. MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**: 1244-1245.
- Matic S., 2004. Sanitary status of stone fruit trees and typing of *Plum pox virus* isolates in Bosnia and Herzegovina. M.S. Thesis Nr. 344. Mediterranean Agronomic Institute of Bari, Italy.
- Myrta A., Potere O., Boscia D., Candresse T., Cambra M., Savino V., 1998. Production of a monoclonal antibody specific to the El Amar strain of plum pox virus. *Acta Virologica* **42**: 248-250.
- Myrta A., Potere O., Crescenzi A., Nuzzazi M., Boscia D., 2000. Properties of two monoclonal antibodies specific to the cherry strain of plum pox virus (PPV-C). *Journal of Plant Pathology* **82**: 95-103.
- Myrta A., Boscia D., Potere O., Kölber M., Nemeth M., Di Terlizzi B., Cambra M., Savino V., 2001. Existence of two serological subclusters of plum pox virus, strain M. *European Journal of Plant Pathology* **107**: 845-848.
- Nemchinov L., Crescenzi A., Hadidi A., Piazzolla P., Verderevskaya T., 1998. Present status of the new cherry subgroup of Plum pox virus (PPV-C). In: Hadidi A., Khetarpal R.H., Koganezawa H. (eds). *Plant Virus Disease Control*, pp. 639-638. APS Press, St. Paul, USA.
- Pasquini G., Barba M., 1994. Serological characterisation of Italian isolates of plum pox potyvirus. *Bulletin OEPP/EPPO Bulletin* **24**: 615-624.

- Pasquini G., Barba M., 1997. Plum pox potyvirus strains: an overview. In: *Proceedings of the Middle European Meeting 1996 on Plum Pox, Budapest 1996*, 168-171.
- Pasquini G., Simeone A.M., Barba M., 1999. Individuazione di PPV ceppo Marcus in piante di albicocco e susino nel Centro Italia. *Informatore Fitopatologico* **49** (5): 33-36.
- Poggi Pollini C., Bissani R., Giunchedi L., Gambin E., Goio P., 1996. Sharka: reperimento di un pericoloso ceppo del virus in coltivazioni di pesco. *Informatore Agrario* **52** (32): 77-79.
- Roy A.S., Smith I.M., 1994. Plum pox situation in Europe. *Bulletin OEPP/EPPO Bulletin* **24**: 515-525.
- Rzhetsky A., Nei M., 1993. Theoretical foundation of the minimum-evolution method of phylogenetic inference. *Molecular Biology and Evolution* **10**: 1073-1095.
- Weiller G.F., 1998. Phylogenetic profiles: a graphical method for detecting genetic recombinations in homologous sequences. *Molecular Biology and Evolution* **15**: 326-335.
- Wetzel T., Candresse T., Macquaire G., Ravelonandro M., Dunez J., 1992. A highly sensitive immunocapture polymerase chain reaction method for plum pox potyvirus detection. *Journal of Virological Methods* **39**: 27-37.

Received 5 December 2004

Accepted 1 April 2005