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Plum Pox Virus and Sharka Disease

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ABSTRACT

Sharka is a disease of fruiting, ornamental and wild *Prunus* species of great socio-economic relevance that has spread to most *Prunus*-growing nations. It is caused by *Plum pox virus* (PPV), a member of the genus *Potyvirus*. In recent years, there have been notable advances in our understanding of genome organization and expression, functions of gene products, and pathogenicity and host range determinants of PPV and other potyviruses. This knowledge is being applied to improve PPV detection and strain differentiation. In addition, the feasibility of engineering the PPV genome through cDNA cloning has opened the possibility of using PPV as a biotechnological tool. The combined application of classical breeding and genetic engineering techniques is yielding first results in the development of *Prunus* cultivars resistant to sharka disease.

Keywords: host range, pathogenicity determinant, plum pox virus, potyviridae, potyvirus, PPV, sharka, virus diagnosis, virus resistance, virus vector
Abbreviations: Co-PCR, co-operational-PCR; **CP**, coat protein; **DAS**, double-antibody sandwich; **DASI**, double-antibody sandwich indirect; **ER**, endoplasmic reticulum; **NASBA**, nucleic acid sequence-based amplification; **NCR**, non-coding region; **PPV**, *Plum pox virus*; **PVA**, *Potato virus A*; **PVX**, *Potato virus X*; **RHDV**, *Rabbit hemorrhagic disease virus*; **RT-LAMP**, reverse transcription loop-mediated isothermal amplification; **TEV**, *Tobacco etch virus*; **TVMV**, *Tobacco vein mottling virus*

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INTRODUCTION

Plum pox virus (PPV) is a member of the genus *Potyvirus* in the family *Potyviridae* that causes sharka, one of the most serious diseases of *Prunus* species (Cambra *et al*. 2006b). Sharka was first detected at 1917-1918 and described as a viral disease in 1932 (Atanasoff 1932). Since then, the virus has progressively spread to a large part of the European continent, around the Mediterranean basin and to the Near and Middle East (Roy and Smith 1994). It has been found in South and North America (Chile, USA, Canada, and Argentina) (Roy and Smith 1994; Levy *et al*. 2000; Thompson *et al*. 2001; Dal Zotto *et al*. 2006) and in Asia (Kazakhstan, China and Pakistan) (Spiegel *et al*. 2004; Navrátil *et al*. 2005; Kollerová *et al*. 2006) (**Fig. 1** shows an overview of the sharka situation worldwide). PPV is transmitted in the field by aphids in a non-persistent manner, but exchanges of infected propagative plant material has probably been the main pathway of spread of sharka over long distances (Cambra *et al*. 2006b). In addition to fruiting *Prunus* species (Llácer and Cambra 2006), several ornamental and wild *Prunus* species have been identified as natural and/or experimental hosts for PPV, although the relevance of these species in the epidemiology of sharka is largely unknown (James and Thompson 2006; Damsteegt *et al*. 2007). PPV is also able to infect some woody species that do not belong to the genus *Prunus*, and a number of herbaceous hosts (Németh 1986; Virscek Marn *et al*. 2004; Llácer 2006; Polák 2006).

The pandemic condition of sharka and the socio-economic importance of losses it produces have provoked intense research focused not only on diagnosis, management and control of the disease but also on basic aspects of PPV molecular biology. This article aims to review recent information on these two areas of PPV research as well as initial attempts to use PPV as a biotechnological tool.

GENOME EXPRESSION, REPLICATION AND PROPAGATION OF PPV

The genome of PPV, like that of the rest of potyviruses, consists of one molecule of positive sense ssRNA, which is encapsidated in flexuous and rod-shaped particles of ~660-750 nm in length and ~12.5-20 nm in width (**Fig. 2A**) (http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB). The PPV genomic RNA, which is 9786 nt in length in most isolates, has a 5′ terminal protein (VPg) and a 3′ poly A tail. It is translated, from the second AUG codon (nt 147-149) of its

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Invited Review

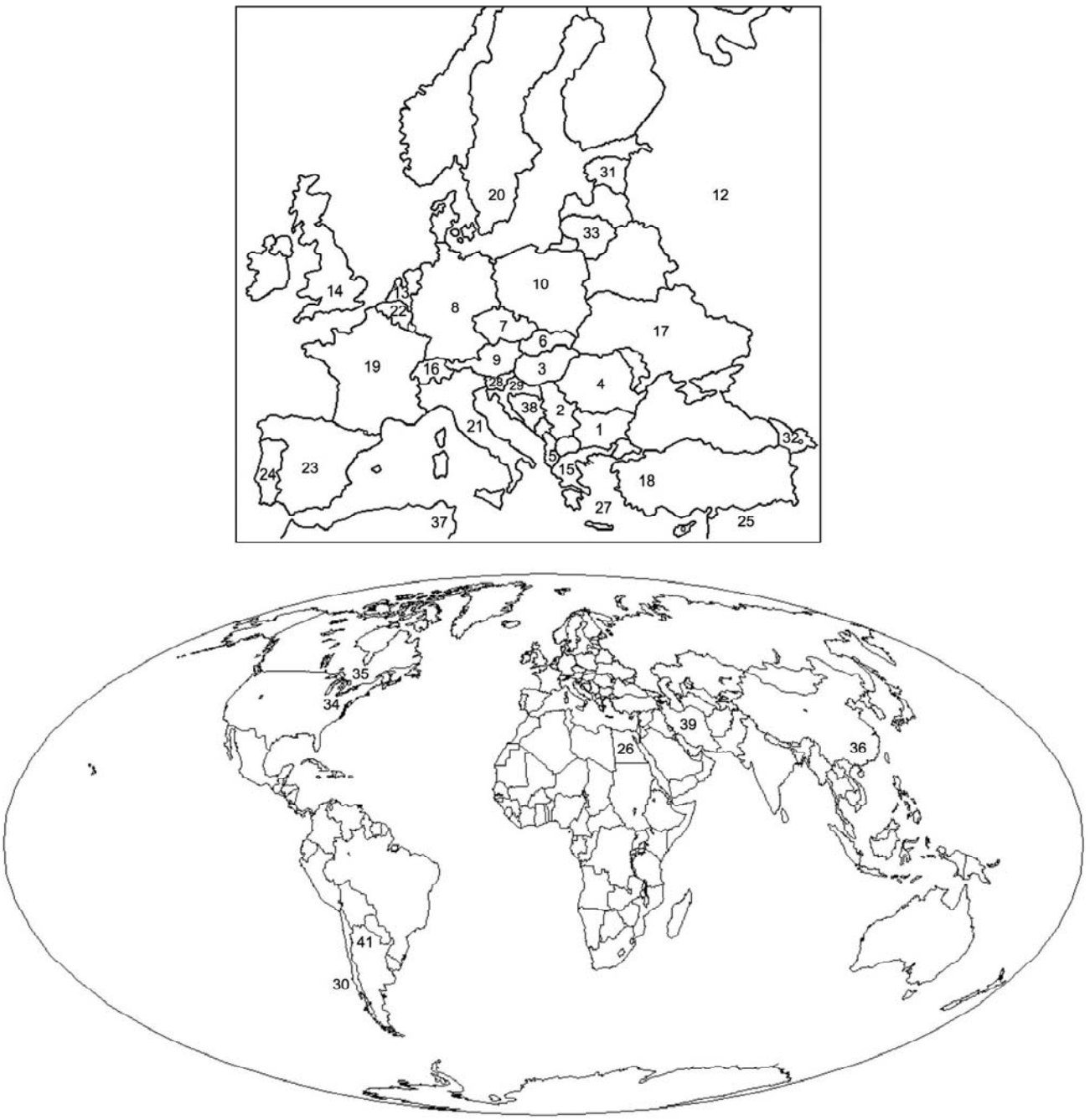

Fig. 1 Worldwide situation of sharka disease and *Plum pox virus*. The year of the first description or detection is shown (References are in EPPO Bulletins vol. 4, nº1, 1974, vol. 24, nº3, 1994, and vol. 36, nº2, 2006).

unique long open reading frame, into a large polyprotein of ~355 kDa, leaving a 3′ non-coding region (NCR) of ~220 nt (Riechmann *et al*. 1991). Translation initiation of PPV RNA probably takes place by a cap-independent leaky-scanning mechanism (Simón-Buela *et al*. 1997), although data from other potyviruses suggest the presence of specific sequences

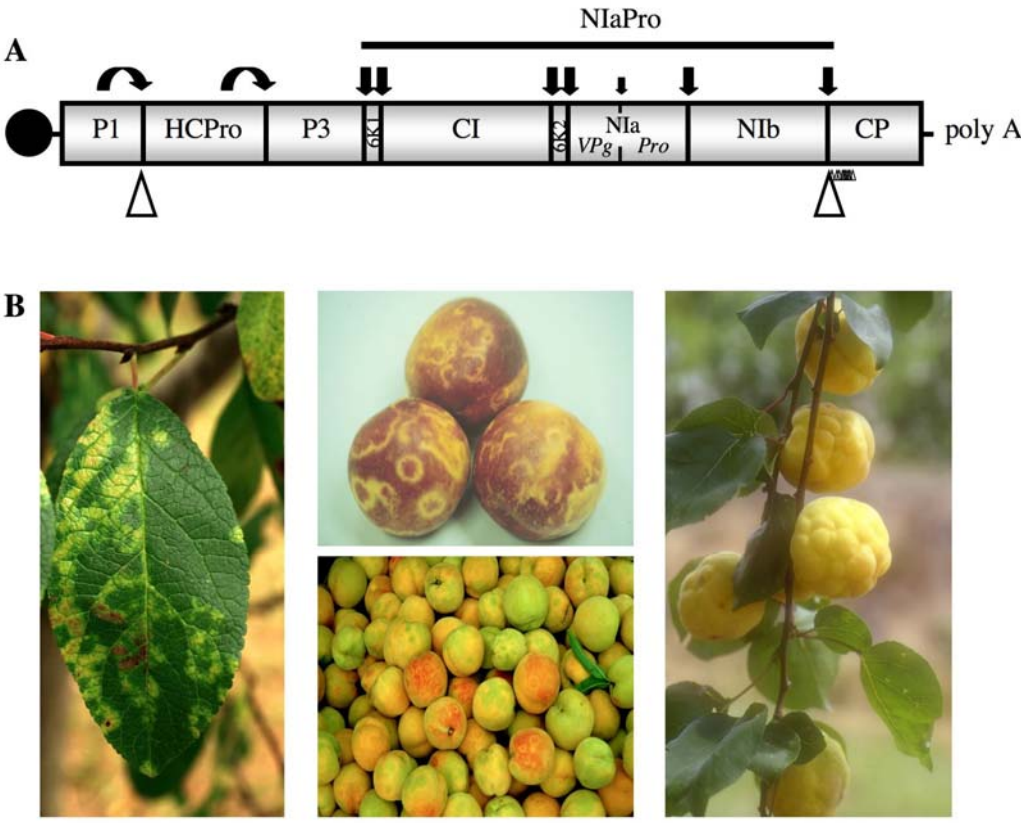

Fig. 2 (**A**) Genomic map of *Plum pox virus*. The open reading frame is represented by a rectangular box and the terminal VPg protein by a black circle. The arrows show cleavage sites recognized by the indicated proteinases. The mature protein products are indicated at their respective locations in the polyprotein. Insertion points for sequences coding small peptides and whole independent proteins in PPV-based vectors are indicated by small and large triangles, respectively. (**B**) Sharka symptoms of leaves and fruits from infected trees.

in the 5′ NCR facilitating an internal translation initiation (Zeenko and Gallie 2005). The PPV polyprotein is proteolytically processed by three virus-encoded proteinases to produce at least 10 mature protein products: P1, HCPro, P3, 6K1, CI, 6K2, VPg, NIaPro, NIb, and the capsid protein (CP) (**Fig. 2A**). P1 and HCPro are a serine and a cysteine proteinase, respectively, that cleave at their own C-termini (García *et al*. 1993; Ravelonandro *et al*. 1993b). The fact that the *in vitro* processing of the P1 of the potyvirus *Tobacco etch virus* (TEV) takes place in a wheat germ system but not in a reticulocyte lysate prompted the suggestion that a plant cofactor is required for P1 activity (Verchot *et al*. 1992). PPV P1 also fails to cleave itself in a reticulocyte lysate (García *et al*. 1993), but a *Tobacco vein mottling virus* (TVMV)-PPV chimeric P1 is active in this system, suggesting that a mammalian protein, possibly a chaperone, can substitute for the plant cofactor depending on the particular structure of the potyviral P1 protein (Sáenz 1998).

Processing of the central and carboxyl regions of the PPV polyprotein is carried out by NIaPro, the C-terminal proteinase domain of the NIa protein (García *et al*. 1989a). NIa, whose N-terminal domain is the genome linked VPg protein, is associated, together with the NIb protein, with crystalline inclusions, mainly detected in the nucleus, but also in the cytoplasm, of PPV infected cells (van Oosten and van Bakel 1970; Martín *et al*. 1992). Target sites of the PPV NIaPro protease have the consensus sequence e/q-x-V-x-H-Q/e↓s and differ in cleavage efficiency and in susceptibility to *in cis* and *in trans* processing (García *et al*. 1990). Studies carried out with NIaPro from TEV showed that the specific behavior of each cleavage site mainly depended on its particular -6 to +1 sequence (Dougherty *et al*. 1989; Dougherty and Parks 1989). However, features modulating the susceptibility to PPV NIaPro processing have also been found outside the conserved heptapeptide (García *et al*. 1989b, 1992). Thus, polyprotein maturing is probably highly regulated and has a relevant role in the control of PPV infection.

In agreement with the fact that RNA replication of plus-strand RNA viruses takes place in association with intracellular membranes (Buck 1996), a membrane extract from PPV-infected leaves enriched in endoplasmic reticulum (ER) and tonoplast vesicles has been shown to be able to synthesize viral RNA from endogenous template (Martín and García 1991; Martin *et al*. 1995). In the case of TEV, targeting of RNA replication complexes to membranous sites of replication has been proposed to involve post-translational interactions between the 6K2 protein and the ER (Schaad *et al*. 1997). NIb is the RNA-dependent RNA polymerase responsible for genome replication of potyviruses (Hong and Hunt 1996), which appears to use as primer VPg uridylylated by NIb (Puustinen and Mäkinen 2004). Mutational analysis has shown that the P3 protein of TVMV is also required for genome amplification (Klein *et al*. 1994). The fact that cleavage between P3 and 6K1 is not essential for PPV viability suggests a functional role for the unprocessed P3+6K1 protein, which might be regulated by processing at the P3/6K1 junction (Riechmann *et al*. 1995). However mature 6K1 has been detected in PPV infected cells, suggesting that this peptide could play a functional role by itself (Waltermann and Maiss 2006). A typical feature of potyviral infections is the accumulation of pinwheel-shaped cytoplasmic inclusions, which are formed by the CI protein. PPV CI has NTPase and RNA helicase activities (Laín *et al*. 1990, 1991; Fernández *et al*. 1995), which are required for viral RNA replication (Fernández *et al*. 1997). Some evidence suggests that PPV CI oligomerization is required for efficient RNA helicase activity, although it appears not to be relevant for NTPase activity (Gómez de Cedrón 2004).

Several studies have shown that the CI protein of PPV (Gómez de Cedrón *et al*. 2006) and other potyviruses (Rodríguez-Cerezo *et al*. 1997; Carrington *et al*. 1998) is also involved in cell-to-cell movement. It is unknown whether the RNA helicase activity of the CI protein is required for its movement function. A self-interaction domain has been mapped to the N-terminal 177 aa of PPV CI (López *et al*. 2001). Point mutations at this domain that disturbed cell-to-cell spread of the virus without noticeable effects on RNA helicase activity or virus replication in protoplasts, caused a notable weakening of CI self interaction, suggesting that CI-CI interactions required for RNA replication and virus movement are to some extent different (Gómez de Cedrón *et al*. 2006).

The CI proteins of PPV and TEV have been found to interact with the photosystem I PSI-K protein and a zinc finger protein from *N. benthaminana* (Jiménez *et al*. 2006), and with the $P58^{IPK}$ protein from tobacco (Bilgin *et al*. 2003), respectively. Although all these interactions appeared to be relevant for virus infection, it is still unknown whether they contribute to CI functions in RNA replication or virus movement, or to other possible CI roles.

The cysteine proteinase HCPro is another potyviral protein that has been implicated in genome amplification and virus movement (Kasschau *et al*. 1997). The functions of TEV HCPro have been shown to correlate with its RNA silencing suppression activity (Kasschau and Carrington 2001). The potyviral HCPro was the first protein shown to interfere with the plant defense mechanism mediated by RNA silencing (Anandalakshmi *et al*. 1998; Brigneti *et al*. 1998; Kasschau and Carrington 1998), and this activity has been confirmed for PPV HCPro (Tenllado *et al*. 2003; Varrelmann *et al*. 2007). HCPro was initially identified as a factor required for aphid transmission of potyviruses, a function that is probably not related to its RNA silencing activity. It has been proposed that HCPro might assist aphid transmission by forming a bridge connecting the aphid stylet and virus particles (Blanc *et al*. 1997, 1998). In agreement with this hypothesis, PPV virions have been found to contain detectable levels of HCPro molecules (Manoussopoulos *et al*. 2000), and interactions between HCPro and CP have been detected in PPV infected plants (Roudet-Tavert *et al*. 2002), although functions for HCPro/CP interactions in processes different from aphid transmission have also been suggested (Roudet-Tavert *et al*. 2002).

The primary function of the PPV CP is encapsidation of the viral genome. Potyviral CP appears to be dispensable for viral RNA replication, although translation to a position between CP codons 138 and 189, and a *cis*-active RNA sequence between CP codons 211 and 246 are essential for TEV RNA replication (Mahajan *et al*. 1996). CP is required for potyvirus movement, although distinct CP determinants are involved in assembly and cell-to-cell and long distance movement of potyviruses in plants (Dolja *et al*. 1994, 1995). Three amino acids from the core region of PPV CP involved in virion assembly have been identified (Varrelmann and Maiss 2000). The N-terminal region of CP is very variable among different potyviruses and is involved in viral long distance movement. It has been shown that an appropriate net charge of the N-terminus of CP rather than a specific amino acid sequence of this region is required for efficient systemic spread of potyviruses (López-Moya and Pirone 1998; Kimalov *et al*. 2004). In addition, a conserved DAG motif in the N-terminal region of CP is required for interaction of this protein with HCPro and for aphid transmission (Atreya *et al*. 1990; Blanc *et al*. 1997), although CP sequence requirements might vary for different potyviruses (López-Moya *et al*. 1995). A natural 15-aa deletion affecting this DAG motif and disturbing aphid transmission has been independently detected in PPV in two instances (Maiss *et al*. 1989; López-Moya *et al*. 1995). This deletion appeared to be associated with PPV propagation in herbaceous plants, suggesting an important role of the N-terminal region of the potyviral CP in host adaptation (López-Moya *et al*. 1995; Asensio 1996).

The PPV CP is phosphorylated and *O*-GlcNAcylated (Fernández-Fernández *et al*. 2002a; Chen *et al*. 2005; Scott *et al*. 2006), and specific sites of *O*-GlcNAc modification have been mapped to the N-terminal region of the protein (Pérez *et al*. 2006). Phosphorylation also affects the CP of another potyvirus, *Potato virus A* (PVA), and this modification down-regulates the RNA binding capacity of PVA CP (Ivanov *et al*. 2001, 2003). Since phosphorylation and *O*-GlcNAcylation can be reciprocal processes, fine tuning of the affinity of CP for RNA by these two post-translation modifications could be an important control element to regulate the amount of genomic RNA allocated for the translation, replication and propagation during potyviral infections (Ivanov *et al*. 2001; Chen *et al*. 2005).

PATHOGENICITY AND HOST RANGE DETERMINANTS

The availability of several full-length genome sequences (Laín *et al*. 1989; Maiss *et al*. 1989; Teycheney *et al*. 1989; Palkovics *et al*. 1993; Sáenz *et al*. 2000; Fanigliulo *et al*. 2003; Glasa *et al*. 2004; James and Varga 2005; Glasa *et al*. 2006; Myrta *et al*. 2006b) and many partial ones has enabled PPV isolates to be classified into six subgroups or strains, D, M, C, EA, W and Rec (Candresse and Cambra 2006). Most PPV isolates belong to the subgroups M and D. PPV M and D strains differ in their ability to infect peach. M isolates appear to cause, in general, faster epidemics and more severe symptoms in peach flowers, leaves and fruits than D isolates (Candresse and Cambra 2006; Llácer and Cambra 2006). However, peach-adapted PPV isolates have also been detected, suggesting that some biological properties of PPV isolates are more dependent on isolate-specific traits than on their taxonomic status (Dallot *et al*. 1998; Levy *et al*. 2000).

PPV-Rec strain consists of a coherent and evolutionary linked group of natural recombinant PPV isolates with a recombination breakpoint in the NIb gene (Cervera *et al*. 1993; Glasa *et al*. 2004). It is generally assumed that PPV-Rec derives from recombination of PPV-D and PPV-M isolates, although current data do not rule out the possibility that PPV-Rec isolates represent an ancestral group, while either PPV-D or PPV-M is the recombinant deriving from the reassortment event at the NIb gene (Glasa *et al*. 2004). PPV-Rec isolates have been mainly detected in plums. Although they can be experimentally transmitted to peach, they appear to resemble PPV-D isolates in being less adapted than PPV-M isolates to transmission to peach hosts (Glasa *et al*. 2004). Since PPV-Rec and PPV-M isolates share the 3′-terminal region of the genome, sequences upstream of the NIb recombination site appear to be especially relevant for peach adaptation (see below). Although all PPV-Rec isolates appear to derive from a single recombination event, other recombination signals have been recognized in different PPV isolates (Glasa *et al*. 2004; Glasa and Candresse 2005), in agreement with the relevant role that has been proposed for RNA recombination in potyviral evolution (Chare and Holmes 2006; Valli *et al*. 2007). This contrasts with the constraints to coinfection by distinct PPV populations that have been shown in both woody (Jridi *et al*. 2006) and herbaceous (Dietrich and Maiss 2003) hosts. Data showing virus spatial separation and frequent recombination could be conciliated if the coexisting constraints observed for closely related PPV populations in the studies cited above are weaker for viruses from different PPV strains. In support of this assumption, although PPV-D and -M isolates were shown to interfere with one another to some extent in Japanese plums (*Prunus salicina*), both PPV isolates coexisted in some trees after several years of infection (Capote *et al*. 2006). It would be interesting to assess whether genomes of both isolates are concurrently present in some cells of these trees.

Cherries were considered non-hosts of PPV for a long time. However, a number of PPV isolates infecting sour (*P. cerasus*) and sweet (*P. avium*) cherry trees have now been identified in several European countries and Turkey (Llácer and Cambra 2006). All these isolates form a distinct monophyletic group, which has been defined as PPV-C strain (Candresse and Cambra 2006). Although PPV-C isolates appear to be specifically adapted to cherry, they are also able to infect other *Prunus* species such as *P. persicae*, *P. marianna*, *P. laurocerasus*, *P. mahaleb* and *P. davidiana* (Crescenzi *et al*. 1997; Desvignes *et al*. 1998; Bodin *et al*. 2003; James and Thompson 2006). PPV El Amar and PPV W317 are atypical PPV isolates that were isolated from apricot in Egypt (Wetzel *et al*. 1991a) and plum in Canada (James *et al*. 2003), respectively. Their genome sequences largely diverge from each other and from isolates of other PPV strains, suggesting that they constitute independent evolutionary lineages. They have, therefore, been proposed

as prototypes of PPV strains EA and W (Candresse and Cambra 2006). Very little information is available about the biological and epidemiological properties of these PPV strains.

Virus virulence depends on the ability of the virus to use specific host factors for its replication and propagation and to escape from innate and virus-induced resistance mechanisms raised by the host. Host-virus interactions required for these duties not always causes deleterious effects in the plant, although they often result in disease symptoms. Extended generation times and tough length and space requirements of phenotypic assays have hampered molecular characterization of the interaction of PPV with its natural woody hosts. However, PPV can infect a number of herbaceous hosts, which are much more affordable for experimental analysis. A recent study of PPV infection in a collection of *Arabidopsis thaliana* accessions has revealed that multiple host factors are involved in the control of PPV infection (Decroocq *et al*. 2006b). Resistance preventing infection of most *Arabidopsis* ecotypes by a PPV-C-type isolate, but not by other PPV isolates, appeared to be controlled by an *R* gene-mediated pathway. Restriction to long-distance movement of PPV-EA and PPV-PS, an M-type isolate, involved the RTM genes, which were previously identified to cooperate in the interference with TEV systemic movement (Whitham *et al*. 2000). Another dominant resistance gene prevents systemic spread of the M-type PPV-PS isolate in *Arabidopsis* Cvi-1, and the ability of the D-type PPV-R isolate to break the resistance conferred by this gene probably depends on the sequence coding for the N-terminal region of CP (Decroocq *et al*. 2006b). Recessive resistance genes affecting long-distance spread of PPV-D type isolates in *Arabidopsis* ecotypes Cvi-1 and L*er* have also been identified. They probably code for host factors involved in virus movement (Decroocq *et al*. 2006b; Sicard O, Loudet O, Candresse T, Keurentjes JJB, Le Gall O, Revers F and Decroocq V, submitted manuscript).

Other host factors required for PPV infection, even at a local level, are the translation initiation factors eIF(iso)4E (Decroocq *et al*. 2006b) and eIF(iso)4G1 (Nicaise *et al*. 2007), in agreement with previous reports linking translation initiation factors with virus infection in various plant species (Robaglia and Caranta 2006). Interestingly, an *eIF(iso)4E* ortholog cosegregates with a major quantitative trait locus of resistance to PPV in peach and apricot, suggesting that translation initiation factors also play an important role in the PPV infection of its natural woody hosts (Decroocq *et al*. 2005). PPV infection of *Arabidopsis* accessions has also shown the existence of host genes specifically involved in the induction of symptoms of different PPV isolates (Decroocq *et al*. 2006b).

Although some PPV isolates can infect both *Prunus* and herbaceous hosts, some others have lost the ability to infect their natural woody hosts after extensive propagation in herbaceous plants (Dallot *et al*. 2001). Through analysis of recombinant hybrid viruses between the D-type PPV-R and PPV-D isolates it has been shown that determinants for host adaptation are largely spread all through the viral genome and that, in the case of this series of viruses, optimal adaptation to *N. clevelandii* and *P. persicae* are mutually exclusive (Salvador B, García JA and Simón-Mateo C, submitted manuscript). In the same study, nucleotide changes in the P1, P3 and 6K1 coding regions were associated with adaptation to *N. clevelandii*. In addition, one nucleotide change in the 6K1 coding sequence appeared to contribute to symptom induction in this herbaceous host. These data are in agreement with previous results localizing pathogenicity determinants for PPV infection in herbaceous (Riechmann *et al*. 1995; Sáenz *et al*. 2000) and woody (Dallot *et al*. 2001) hosts in the P3-6K1 region, and with the relevant role that has been proposed for the potyviral P1 protein in host adaptation (Valli *et al*. 2007). Another protein known to be involved in potyviral pathogenicity, probably as a consequence of its RNA silencing suppression activity, is HCPro (Kasschau *et al*. 2003). In agreement with this, HCPro has been shown to contribute to symptom induction of PPV in *N. clevelandii* (Sáenz *et al*. 2001) and to be a relevant factor for the restriction of PPV systemic spread in *N. tabacum* (Sáenz *et al*. 2002). In addition, synergistic interactions between PPV HCPro and *Potato virus X* (PVX) have also been described (Yang and Ravelonandro 2002; González-Jara *et al*. 2005).

Very little is known about the biochemical basis of physiological disturbances associated with virus infections, in general, or, more specifically, with PPV infection. However, different analyses suggest that an oxidative stress in the apoplastic space produced by imbalance in the antioxidant system of infected leaves of susceptible peach and apricot cultivars may contribute to the deleterious effects caused by PPV infection (Diaz-Vivancos *et al*. 2006).

SHARKA DIAGNOSIS

Sharka symptoms may appear on leaves, petals, fruits and stones (**Fig. 2B**). They are particularly clear on leaves in springtime: mild light green discoloration, chlorotic spots, bands or rings, vein clearing or yellowing, or even leaf deformation. Flower symptoms can occur on the petals (discoloration) of some peach cultivars or in other *Prunus* species. Infected fruits show chlorotic spots or lightly pigmented yellow rings or line patterns. Fruits may become deformed or irregular in shape and develop small brown or necrotic areas. Diseased fruits may show internal browning and gummosis of the flesh and reduced quality. In some cases the diseased fruits drop prematurely from the tree. In general, early cultivars are much more sensitive for symptom expression on fruits than late cultivars. Stones from diseased apricot fruits show typical pale rings or spots.

The virus can be present in symptomless plants and consequently laboratory tests must be performed to assess the sanitary status of a given plant material, especially when this material constitutes the origin of a large vegetative propagation or a nuclear stock. Appropriate sample selection is critical for biological, serological or molecular detection. If typical symptoms are present, flowers, leaves or fruits showing symptoms can be collected. In symptomless plants, the standard sampling involves 5 old growth shoots with mature leaves or 10 fully expanded leaves collected around the canopy of each individual adult tree from the middle of each scaffold branch, until the outcome of high temperatures at the beginning of summer. Preferably, plant material should be selected from the internal structure of the tree. Samples in springtime can be flowers, young shoots with fully expanded leaves or small fruits. Mature leaves can be collected for analysis in autumn. The skin of mature fruits collected from the field or in packinghouses can also be used for analysis.

A European Protocol for detection and characterization of PPV has been developed. The recommended methods include: biological indexing, serological and molecular assays as screening and confirmatory tests as well as sampling, reagents and detailed protocols for each technique (EPPO 2004). An update of this protocol incorporating real-time PCR amplification, sequencing and other novelties is being prepared by the International Plant Protection Convention governed by the Interim Commission on Phytosanitary Measures hosted by the FAO.

Detection of all PPV isolates can be achieved using biological tests based on the graft inoculation of GF305 or Nemaguard peach seedlings, or *P. tomentosa* (Desvignes 1999; Gentit 2006). Universal detection of any PPV isolate can be achieved using either monoclonal antibody 5B-IVIA (Cambra *et al*. 1994) or polyclonal antibodies in double-antibody sandwich indirect (DASI) or in double-antibody sandwich (DAS) ELISA assays (EPPO 2004; Cambra *et al*. 2006a). Molecular techniques based on different PCR assays have been developed for the detection of PPV (Wetzel *et al*. 1991b, 1992; Candresse *et al*. 1994; Levy and Hadidi 1994; Candresse *et al*. 1995; Olmos *et al*. 1996) or for the

simultaneous detection and typing of PPV isolates (Olmos *et al*. 1997). Different systems of viral target preparation prior to PCR have been developed based on immunocapture (Wetzel *et al*. 1992; Candresse *et al*. 1998) or, without the need of extract preparation, on print or squash capture (Olmos *et al*. 1996). The use of immobilised targets on paper (Cambra *et al*. 1997) allowed the detection of PPV in single aphids (Olmos *et al*. 1997) by squash capture-PCR. Nested-PCR in a single closed tube (Olmos *et al*. 1999) has been applied for the sensitive detection of PPV targets. A co-operational-PCR (Co-PCR) system using a universal probe for hybridisation (Olmos *et al*. 2002), has been described, affording a sensitivity similar to that of nested-PCR. All these serological and molecular systems were validated in international ring tests (EPPO 2004). In addition, real-time RT-PCR assays have been developed to detect and quantify PPV targets in plant material and individual aphids (Schneider *et al*. 2004; Olmos *et al*. 2005; Varga and James 2005; Capote *et al*. 2006) with a sensitivity higher than that obtained by the previously described methods and, in some cases, without the need of RNA purification (Olmos *et al*. 2005). Powerful "termocycler-free" techniques, reverse transcription loop-mediated isothermal amplification (RT-LAMP) (Varga and James 2006) and nucleic acid sequence-based amplification (NASBA) coupled with fast flow-through hybridisation (Olmos *et al*. 2007), have demonstrated to be suitable molecular methods for PPV detection. These technologies will be adopted, in the near future, for PPV diagnosis in official protocols.

A number of techniques and reagents are available for PPV identification or characterisation. Given the variability of PPV, all techniques other than sequencing or some PCR-based assays (see below) may provide erroneous answers on the typing of a small percentage of isolates (Candresse *et al*. 1998; Candresse and Cambra 2006). However, discrimination between the main D and M groups of PPV is possible using a variety of techniques (Candresse and Cambra 2006; Olmos *et al*. 2006) that include: (1) different serological patterns or reactions with D- or M-specific monoclonal antibodies (Cambra *et al*. 1994; Boscia *et al*. 1997; Cambra *et al*. 2006a), (2) electrophoretic mobility of the viral CP as assessed by western blot (Bousalem *et al*. 1994; Pasquini and Barba 1994), (3) sequence analysis of PCR fragments corresponding to the C-terminal region of the PPV CP gene and *Rsa*I restriction fragment length polymorphism (Wetzel *et al*. 1991b; Bousalem *et al*. 1994; Candresse *et al*. 1994), and (4) different variants of PCR, heminested-PCR, nested-PCR and Co-PCR using specific primers (Candresse *et al*. 1994; Olmos *et al*. 1997, 1999, 2002, 2003) including colorimetric detection of the amplicons with D- or M-specific probes. In addition, real-time PCR using SYBR green and TaqMan chemistries has recently been applied to discriminate between PPV-D and PPV-M types (Varga and James 2005; Capote *et al*. 2006).

Many of the PPV isolates characterized as D or M type by the serological or molecular techniques based on the detection of CP or the amplification of the CP coding sequence, could actually belong to the PPV-Rec type (James and Glasa 2006). To test whether a putative PPV-D or PPV-M isolate is in fact a PPV-Rec isolate, combined PCR techniques targeting several genome regions must be performed (Glasa *et al*. 2002). A simplified RT-PCR procedure for direct recombinant detection using specific primers bordering the hot spot recombination site has recently been described (Šubr *et al*. 2004).

In addition to D, M and Rec typing, a number of techniques and reagents are available for classifying PPV isolates into the rest of the groups (EA, C and W). These include: ELISA with EA-specific (Myrta *et al*. 1998) and C-specific (Myrta *et al*. 2000) monoclonal antibodies and molecular PCR-based methods with specific primers and probes (Varga and James 2005).

A comparison of the different techniques for PPV detection and identification is given in **Table 1**, based on previous reports (López-Moya *et al*. 2000; Olmos *et al*. 2006).

SHARKA CONTROL

Fruit trees cannot be efficiently protected from sharka infection by using insecticides to control the PPV aphid vectors. Potyviruses are transmitted in a non-persistent manner and, consequently, the virus can be inoculated by a PPV-viruliferous aphid during very short probes before feeding.

In this context, control measures against PPV are basically focused on two strategies: prophylaxis designed to reduce or to eliminate the viral inoculum in the environment (quarantine measures, eradication programs, use of certified virus-tested planting material, etc.) and efforts at breeding for resistance. In countries with endemic infection, a third agronomical strategy, relying on the deployment of varieties with a reduced expression of PPV symptoms on fruits, is widely used, despite the fact that it provides no real disease control.

In countries where infection levels are still moderate or low, strategies based on a strict control of the virus and on prophylaxis are generally used. PPV was recognized early as a major pathogen on stone fruit crops and was, therefore, included on official quarantine lists. Similarly, in many countries PPV is subject to different control measures, including serious monitoring, strict quarantine and eradication schemes (Lebas *et al*. 2006; Levy 2006; Muñoz *et al*. 2006; Rodoni *et al*. 2006; Thompson 2006). To be successful, these strategies must be undertaken early after introduction of the disease in a country or region and must be very vigorously enforced. Generally, eradication schemes are labour intensive and based both on visual inspection of symptoms and on ELISA testing on a large scale. In addition to technical aspects, these approaches also require both political (including economic compensatory measures) and fruit-growers support. Two different programs are being conducted in extensive areas of the USA and Canada, which started in the year 2000, with the aim of eradicating

Table 1 Comparison of different PPV detection and characterization techniques.

Technique	Sensitivity[a]	Specificity[b]	Strain-typing[c]	Rapidity	Feasibility[d]	Capacity for large scale
Biological assays/Indexing	++++[e]	++	-	+	++++	+
DASI-ELISA (monoclonal antibodies)	+++	+++++	++++	++	+++++	+++++
Molecular hybridization	+	+++	+	++	++	+++
Immunocapture-RT-PCR	+++	+++	+++	+++	++	+
Heminested and nested PCR (2 tubes)	++++	++	+++	++	++	+
Nested PCR (single tube)	++++	+++	+	++	+++	++
Cooperative-PCR	++++	+++	+++	++	+++	++
Real-time PCR	+++++	+++++	+++++	+++++	+++	++++
NASBA[f]	++++	+++	+++	++++	+++	+++

[a] Sensitivity: proportion of true positives correctly identified by the test
[b] Specificity: proportion of true negatives correctly identified by the test
[c] Strain-typing: ability to discriminate between viral strains
[d] Feasibility: practicability in routine analysis, execution and interpretation
[e] For each criterion, methods are rated from acceptable (+) to optimum (+++++)
[f] NASBA: Nucleic Acids Sequence Based Amplification

PPV (Gottwald 2006; Thompson 2006). Many countries moved from unsuccessful eradication to containment or management of sharka disease (Mumford 2006; Myrta *et al*. 2006a; Ramel *et al*. 2006; Speich 2006). A complement to quarantine and eradication measures is the wide use of certified, virus-tested or virus-free planting material. Sanitation, detection and sampling techniques, information on the protection of nurseries etc. are available today enabling many countries to develop efficient certification programs.

In parallel with these control measures, important efforts are also focused on the development of PPV-resistant *Prunus* cultivars in many countries. These programs have explored both classical breeding approaches (screening of germplasm to identify resistance sources) and biotechnological approaches. Extensive screening of germplasm has failed to identify sources of resistance within the peach species so current efforts are aimed at the exploitation of resistance identified in the related Chinese wild peach (*P. davidiana*) by introgression of the trait through interspecific hybridization (Bassi 2006). By contrast, in apricot and plum, resistance sources have been identified within the target species so breeding efforts are clearly more advanced (Badenes and Llácer 2006; Decroocq *et al*. 2006a; Hartmann and Neumüler 2006; Karayiannis 2006; Krska *et al*. 2006).

Genetic transformation to create PPV-resistant transgenic plants through the use of different RNA silencing-related strategies was not only successful in experimental herbaceous hosts (Regner *et al*. 1992; Ravelonandro *et al*. 1993a; Guo and García 1997; Guo *et al*. 1998a; Jacquet *et al*. 1998; Barajas *et al*. 2004; Di Nicola-Negri *et al*. 2005; Zhang *et al*. 2006), but also in *Prunus* species (Ravelonandro *et al*. 1997; Scorza and Ravelonandro 2006). Efficient genetic constructs are therefore available, some of which have been successfully validated in field tests (Ravelonandro *et al*. 2000, 2002; Hily *et al*. 2004; Malinowski *et al*. 2006; Capote *et al*. 2007). In addition, novel approaches to interfere with the proteolytic processing of the viral polyprotein (García *et al*. 1993; Gutierrez-Campos *et al*. 1999; Wen *et al*. 2004) and to immunomodulate host-PPV pathogen interactions by expression of antibody genes in plants (Esteban *et al*. 2003) have emerged, but these possibilities have still not been transferred to *Prunus* cultivars. Nevertheless, biotechnology could offer a new and innovative strategy to control PPV and, consequently, sharka disease.

PPV AS A BIOTECHNOLOGY TOOL

Plant viruses have started to be considered not only as targets of biotechnological approaches focused on enhancing crop health, but also as useful tools to be used for the expression of foreign genes with different aims (Burch-Smith *et al*. 2004; Cañizares *et al*. 2005). Although the most developed virus-related plant expression vectors derive from *Tobacco mosaic virus* and PVX, some potyviruses have also been engineered to be used for foreign gene expression (Hsu *et al*. 2004; Beauchemin *et al*. 2005; Kelloniemi *et al*. 2006). The construction of full-length cDNAs of the PPV genome, functional in herbaceous and woody hosts, has allowed the development of PPV-based vectors to express either small peptides fused to the viral capsid protein or independent proteins (García *et al*. 2006).

Several vectors have been developed to express epitopes of foreign infectious agents at the surface-exposed N-terminal domain of PPV CP (Fernández-Fernández *et al*. 1998, 2002b) (**Fig. 2A**). These vectors differed in tolerance to inserted sequences and in the antigenicity and immunogenicity of the expressed epitopes. Small shifts of 1 or 2 aa of the insertion sites notably altered the immunogenicity of foreign sequences presented by PPV capsids (Fernández-Fernández *et al*. 2002b).

PPV-based vectors able to express whole independent proteins in herbaceous and woody plants have also been reported (García *et al*. 2006) (**Fig. 2A**). The GUS gene has been expressed at a modified P1-HCPro junction. The recombinant virus was stably maintained during the first round of infection, and GUS accumulation was estimated in about 20 µg per gram of leaf tissue, but large deletions in the foreign sequence were detected in subsequent plant passages (Guo *et al*. 1998b). Another PPV-based vector, with the insertion site at the NIb-CP junction, allowed the stable expression of about 250 µg of green fluorescent protein per gram of infected tissue (Fernández-Fernández *et al*. 2001). The VP60 protein of *Rabbit hemorrhagic disease virus* (RHDV) has been successfully expressed with this vector, and inoculation of extracts from VP60-expressing plants fully protected rabbits against a lethal challenge with RHDV (Fernández-Fernández *et al*. 2001).

CONCLUDING REMARKS

Sharka (plum pox), the disease caused by PPV, has serious agronomic and sociological consequences because it causes both important direct economic losses and significant indirect losses due to restrictions in the domestic and international trade of plant material.

Tremendous progress has been made on PPV research in the last 15 years, enabling significant improvements in sharka management, but the disease is still spreading to new areas demanding containment efforts.

The only way for a real and durable control of the disease would be the use of PPV-resistant plant material. Limited progress has been made in this direction, with the exceptions of apricot and plum trees, probably due to the difficulties in identification of sources of resistance in compatible species for breeding programs.

Biotechnological approaches could help in solving the problem. RNA silencing-mediated resistance has been proven to be a good method to provide PPV resistance both to herbaceous and woody PPV hosts. Further progress on understanding the interactions between virus and host factors involved in virus replication and propagation, defense responses, and symptom development will facilitate the designing of novel approaches to confer PPV resistance. But improvement on the genetic transformation and especially regeneration systems from mature tissues of commercial *Prunus* cultivars will be also required to develop plants with agronomic applicability.

In addition, a better understanding of the taxonomic relationships among the different PPV isolates and their evolutionary dynamics will contribute to devise strategies to control the spread of sharka and to limit its detrimental effects.

On the other hand, recent information demonstrates that PPV can be engineered to be used as a vector for expression in plants of foreign peptides and proteins. New PPV-based vectors with higher stability and accumulation levels, broader host range and lower deleterious effects on the infected plant are envisaged as the result of a future better knowledge of PPV molecular biology.

In summary, it is expected that in a near future the combined inputs from epidemiology, conventional virology, breeding and genetic engineering-based techniques will offer real solutions for sharka disease control, as well as useful biotechnological tools based on PPV-derived expression vectors.

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